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Temperature modulates the impacts of wastewater exposure on the physiology and behaviour of fathead minnow

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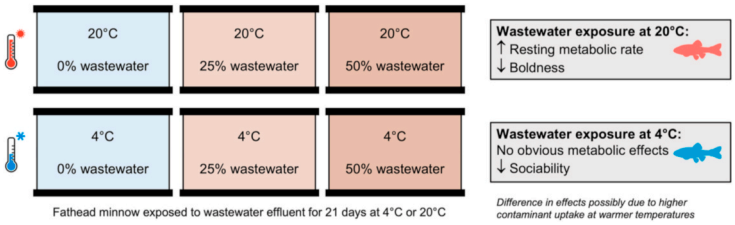
HIGHLIGHTS

- Fathead minnow were exposed for 21 days to 0, 25, or 50% wastewater at 4 °C and 20 °C.
- Exposure to wastewater at 20 °C increased standard metabolic rate and haematocrit.
- Fish exposed to wastewater at 20 °C were less bold.
- Fish exposed to wastewater at 4 °C were less socially-interactive.

GRAPHICAL ABSTRACT



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ABSTRACT

Municipal wastewater treatment plant (WWTP) effluent is a substantial source of pollution in aquatic habitats that can impact organisms across multiple levels of biological organization. Even though wastewater effluent is discharged continuously all year long, its impacts across seasons, specifically during winter, have largely been neglected in ecotoxicological research. Seasonal differences are of particular interest, as temperature-driven metabolic changes in aquatic organisms can significantly alter their ability to respond to chemical stressors. In this study, we examined the effects of multiple levels of wastewater effluent exposure (0, 25, or 50% treated effluent) on the physiological and behavioural responses of adult fathead minnow (*Pimephales promelas*) at temperatures simulating either summer (20 °C) or winter (4 °C) conditions. At 20 °C, wastewater exposure posed a metabolic cost to fish, demonstrated by higher standard metabolic rate and was associated with increased haematocrit and a reduction in boldness. In contrast, fish exposed to wastewater at 4 °C experienced no change in metabolic rate but performed fewer social interactions with their conspecifics. Taken together, our results demonstrate that wastewater exposure can lead to metabolic and behavioural disruptions, and such disruptions vary in magnitude and direction depending on temperature. Our findings highlight the importance of studying the interactions between stressors, while also underscoring the importance of research during colder periods of the year to broaden and deepen our understanding of the impacts of wastewater contamination in aquatic ecosystems.

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1. Introduction

Municipal wastewater treatment plant (WWTP) effluents are one of the largest and most ubiquitous sources of aquatic contamination around the world (Environment Canada, 2001; Strayer and Dudgeon, 2010). WWTPs are not capable of removing all contaminants from wastewater, and as a result, treated effluent released into watersheds still contains a complex mixture of contaminants of emerging concern (CECs), including pesticides, metals, micro- and macroplastics, ammonia, pharmaceuticals and personal care products (PPCPs), and natural and synthetic hormones (Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; Blair et al., 2013; McCormick et al., 2016; Jorgenson et al., 2018). The concentration of such contaminants is often relatively low (in the ng/L - μg/L range); however, due to the continuous discharge of wastewater effluent into receiving waterbodies, fish and other aquatic organisms residing near effluent outfalls can be subjected to chronic exposure of contaminants (Kolpin et al., 2002; Jelić et al., 2012; Jones et al., 2005; Blair et al., 2013). In addition to contaminant exposure, aquatic organisms residing near effluent outfalls are also subjected to poor habitat conditions as a result of excess nutrient loading, eutrophication, oxygen depletion, increased conductivity, and changes in temperature (Odjadjare and Okoh, 2010; Holeton et al., 2011; Tetreault et al., 2012; Melvin, 2016; Hamdhani et al., 2020; Mehdi et al., 2021). The impacts of wastewater effluent exposure have been a subject of growing research and concern, especially since our reliance on WWTPs continues to increase as urban populations grow (Grimm et al., 2008; Holeton et al., 2011; Boxall et al., 2012; Rudd et al., 2014; Hamdhani et al., 2020).

Prior ecotoxicological research on the impacts of wastewater effluent exposure has largely been focused on reproductive endpoints (i.e., endocrine disruption represented in reproductive physiology and behaviour). This has mainly been driven by the abundance of endocrine-active compounds found in wastewater effluent (e.g., 17α-ethinylestradiol) motivating a growing area of research focused on the endocrine-related impacts of wastewater effluent exposure (Kidd et al., 2007; Harris et al., 2012; Tetreault et al., 2011; Bahamonde et al., 2015; Fuzzen et al., 2015). Such impacts include disruption of endogenous hormone levels, an increase in male feminization rates, and a reduction in fertilization success (Bahamonde et al., 2015; Fuzzen et al., 2015). The endocrinological and reproductive impacts of wastewater effluent exposure are commonly studied, as these parameters have direct implications on population growth and sustainability (European Chemicals Agency, 2011; Ågerstrand et al., 2020). However, our understanding of how other fitness-linked facets of biological organization (e.g., metabolic physiology and behaviour) may be impacted by wastewater effluent exposure is still in its infancy. Behaviours such as foraging, predator avoidance, sociability, exploration, and aggression are all essential to fitness and survival (Scott and Sloman, 2004; Brodin et al., 2014; Saaristo et al., 2018). Similarly, metabolic physiological endpoints such as standard metabolic rate, maximal metabolic rate, and aerobic scope (the difference between standard metabolic rate and maximal metabolic rate) provide an excellent insight on how contaminants influence energy transfer within an organism and are tightly linked to growth, reproduction, and many behaviours that are important for survival (Brown et al., 2004; Scott and Sloman, 2004; Biro and Stamps, 2010; Clark et al., 2013). Despite the overall scarcity of research on the physiological and behavioural impacts of wastewater exposure, a handful of studies have recently emerged demonstrating that exposure to wastewater effluent can inflict metabolic costs in both wild-caught and lab-reared fishes, manifesting as increases in whole animal metabolic rate (O_2 consumption rate; Du et al., 2018; Mehdi et al., 2017; Du et al., 2019). Such metabolic costs can be associated with increases in the activities of various metabolic enzymes, changes in metabolic substrate levels (e.g., glycogen), and even tissue- and whole-body morphological abnormalities (Ings et al., 2012; Du et al. 2018, 2019; Mehdi et al., 2017; Nikel et al., 2021). Furthermore, an even smaller but growing number of studies have shown that exposure to wastewater effluent affects various non-reproductive behaviours in fishes, including altered aggression, dampened anti-predator responses, and reduced swimming performance (Saaristo et al., 2014; Melvin, 2016; McCallum et al., 2017a; McLean et al., 2019).

The paucity of research focusing on non-reproductive physiological and behavioural endpoints of wastewater effluent exposure is further exacerbated by the fact that the majority of our current knowledge comes from research conducted during warmer periods of the year and/or under warm lab-rearing temperatures (Lemly, 1993, 1996; Bennett and Janz, 2007; Driedger et al., 2009; Mehdi et al., 2021). The synergistic effects of temperature on the toxicity of wastewater effluent, particularly at the colder end of the spectrum, have largely been ignored in ecotoxicological research. Knowledge of potential interactions between temperature and contaminant exposure is of vital importance for a number of reasons. First, in many temperate and polar regions of the world, effluent from WWTPs is released into cold, near-freezing environments during colder periods of the year, sometimes lasting between 4 and 8 months. Therefore, understanding how effects of wastewater differ across temperatures is critical, particularly, at the colder end of the spectrum. Second, temperature has an overarching influence on numerous biological functions, especially in ectotherms (e.g., fishes; Hochachka and Somero, 2002). Colder temperatures during the winter could reduce whole-animal metabolism and gill ventilation, thereby reducing contaminant uptake and lessening the impacts of wastewater exposure. However, colder temperatures could also reduce the rates of contaminant elimination and remobilization or limit the aerobic supply of energy needed to fuel detoxification, thereby accentuating the impacts of wastewater exposure (Lemly, 1993, 1996; Capkin et al., 2006; Buckman et al., 2007; Noyes et al., 2009; Mehdi et al., 2019). Furthermore, fish during the winter may suffer from endogenous exposure of lipophilic contaminants stored in adipose tissue, as their reliance on lipid energy stores increases when food is scarce (Paterson et al., 2007; Treberg et al., 2016). Even though research on this topic is currently limited, a study by Lemly (1993) found that selenium exposure under simulated cold winter conditions caused reductions in activity, feeding, and lipid stores, as well as significant increases in mortality in juvenile bluegill sunfish (*Lepomis macrochirus*). Whereas under warmer conditions, fish continued to actively feed and lipid depletion did not occur, despite an increase in oxygen consumption. Otherwise, the question of whether colder temperatures reduce or accentuate the impacts of wastewater exposure remains largely unanswered. Studies considering realistic seasonal changes in temperature are much needed when evaluating the toxicity of wastewater effluent as well as other contaminants.

In this study, we examined the influence of temperature on the physiological and behavioural effects of wastewater effluent exposure in fathead minnow (*Pimephales promelas*). Adult fish were exposed to one of three wastewater treatments differing in the proportion of treated effluent (Control 0%; Low 25%; High 50%) for 21-days at temperatures typical of summer (20 °C) or winter (4 °C). We measured various facets of metabolism (standard metabolic rate, maximal metabolic rate, and aerobic scope) in addition to various behavioural endpoints (boldness, sociability, foraging, and response to predator). Based on previous studies, we hypothesized that wastewater effluent exposure would pose metabolic costs, represented by an increase in standard metabolic rate that would lead to a reduction in aerobic scope (Du et al. 2018, 2019; Mehdi et al., 2017). Such metabolic costs would further be manifested in dampening behaviour, with reductions in boldness, sociability, foraging, and anti-predator responses. We further hypothesized that these effects would be less pronounced at 4 °C than at 20 °C, as metabolism, respiration rates, and therefore contaminant uptake, are subdued at lower temperatures.

2. Materials and methods

2.1. Study organism and housing

We used fathead minnow in this study, as they are a well-established laboratory toxicological model organism and are commonly found year-long in effluent-receiving environments across North America (Ankley and Villeneuve, 2006; Mehdi et al., 2021). Adult fathead minnow of both sexes were acquired from lab-reared stocks and maintained in 38 L glass tanks (density of 20 fish/tank; 18 tanks). Each tank was equipped with a sponge filter and an aerator and supplied with Hamilton tap water that underwent reverse osmosis and UV sterilization. Fish were held under 16L:8D light schedule and fed until satiation with Nutrafin Basix Staple Food once daily. The tanks were held within two large environmental chambers at the Canada Centre for Inland Waters, Burlington, ON, Canada. Both environmental chambers were maintained at 20 °C before the start of the experiment, but the temperature in one of the environmental chambers was then incrementally reduced from 20 °C to 4 °C over a period of 12 days. Once the desired temperature was reached, fish were held at that temperature for another two weeks prior to the start of wastewater effluent exposures. All the procedures employed were approved by the animal utilization protocols from the McMaster University Animal Research Ethics Board (# 17-12-45) and the Department of Fisheries and Oceans/Environment Canada Joint Animal Care Committee for the Canada Centre for Inland Waters (# 1956; Burlington, ON, Canada) in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Wastewater effluent exposure

Following the initial two-week period of adjustment to the target temperature, fish were exposed to treated wastewater effluent collected from the Woodward WWTP in Hamilton, ON, Canada. The Woodward WWTP is a secondary conventional activated sludge plant that serves the majority of the Hamilton population (~480,000 people) and has an average daily treatment capacity of 409 million litres (City of Hamilton, 2019). Effluent from this facility flows directly into Red Hill Creek, which empties into Hamilton Harbour, one of 43 locations around the Great Lakes designated as an Area of Concern (AOC) by the International Joint Commission (Great Lakes Water Quality Agreement, 2012). Collections of effluent were conducted after the final stage of treatment, immediately prior to being discharged into Red Hill Creek. Fresh effluent was collected in opaque plastic carboys twice every week (between 0900 and 1130 h) from March to May of 2019, and stored for a maximum of 4 days at 4 °C in the dark to slow down degradation.

Fathead minnow were exposed to one of six treatments for 21 days: (i) 0% wastewater effluent at 20 °C (*warm control*); (ii) 25% wastewater effluent at 20 °C (*warm low*); (iii) 50% wastewater effluent at 20 °C (*warm high*); (iv) 0% wastewater effluent at 4 °C (*cold control*); (v) 25% wastewater effluent at 4 °C (*cold low*); (vi) 50% wastewater effluent at 4 °C (*cold high*). The concentrations of wastewater chosen are representative of effluent-receiving environments in Hamilton Harbour (e.g., downstream of the Dundas and Woodward WWTPs). Additionally, the concentrations of wastewater used did not reduce survival (Table S4) and thus any observed effects can be considered sub-lethal effects of exposure. Three tank replicates were used for each exposure treatment, with 20 adult fish per tank. Water changes were made every fourth day of the exposure with 75% of the water being replaced 1 h after feeding. During water changes in the wastewater exposure treatment tanks, the tanks were re-dosed with newly collected wastewater effluent. Wastewater effluent was brought to the appropriate exposure temperatures overnight before water changes were made, while also being continuously aerated to ensure sufficient dissolved oxygen levels. Wastewater effluent was diluted and well-mixed with clean fresh water to match the exposure conditions prior to dosing.

2.3. Water quality and effluent characterization

In the exposure tanks, temperature was continuously monitored using temperature loggers (HOBO Pendant Temperature Data Logger) placed in a randomly selected tank of each treatment. Dissolved oxygen (YSI Pro DO), pH, total dissolved solids, conductivity, and salinity (Oakton Multiparameter PCS Testr 35) were measured once a week (Supplementary Table 1). A number of water quality parameters were measured on composite final effluent samples that were collected over a period of 24 h at the Woodward WWTP: total suspended solids, carbonaceous biochemical oxygen demand, total phosphorus, total Kjeldahl nitrogen, ammonia + ammonium, nitrate, nitrite, *E. coli*, conductivity, and chemical oxygen demand (measurements provided by City of Hamilton, 2019; Supplementary Table 2). Furthermore, during each effluent collection, a 500 mL sample of freshly collected wastewater effluent was preserved and analyzed for a wide range of PPCPs and other CECs using already established methods described by Arlos et al. (2015) and Mehdi et al. (2021). Throughout the exposure, 125 mL samples of tank water were collected from each tank two times — once ~1 h post-dosing and another prior to the next water change. This sampling regimen allowed us to compare the potency of effluent immediately after collection, during dosage, and after a 4-day period in the exposure tanks. Similar to freshly collected wastewater effluent, tank water was also analyzed for PPCPs and other CECs. Briefly, wastewater and tank water samples were concentrated using solid phase extraction. Extracted samples were then analyzed using an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet Stream (AJS) electrospray ionization in both positive and negative modes. Nine different classes of compounds were analyzed: lipid regulators, antiepileptics, analgesics, stimulants, antibacterials, antibiotics, antidepressants, non-steroidal anti-inflammatory agents (NSAIDs), and herbicides.

2.4. Respirometry

At the end of the exposure period (day 21), a subset of fish (n = 12) from each treatment was used to measure standard and maximal metabolic rates. Metabolic rate measurements were performed at the appropriate exposure temperature for each treatment in clean water (same source water that was used for exposures) following previously described methods in Borowiec et al. (2015). Fish were placed in 90 mL respirometry chambers situated in a darkened, temperature-controlled, and well-aerated buffer tank. Respirometers were equipped with flush and recirculation pumps. Recirculation pumps were connected in circuit to fibre-optic oxygen sensors (OXROB10 PyroScience) and were continuously turned on to ensure water in the chamber was well mixed and continuously flowing past the oxygen sensors. Oxygen sensors were connected to an optical oxygen meter (FireStingO_2 PyroScience) for continuous oxygen concentration measurements. Flush pumps were turned on (7 min) and off (7 min) intermittently to expel the chambers of residual water and supply them with well oxygenated water. Fish were held in respirometry chambers overnight where standard metabolic rate measurements were continuously measured. Standard metabolic rate was determined by calculating the mean of the lowest five metabolic rate measurements. The next day, maximal metabolic rate was determined by transferring fish to a cylindrical tank (diameter = 46.0 cm; height = 20.0 cm) and being chased for 3 min and then air-exposed for 30 s, this method has been demonstrated to elicit maximal metabolic rates in a variety of fish species (Clark et al., 2012; Roche et al., 2013; Norin et al., 2014). Fish were then placed immediately back into their chambers to measure maximal metabolic rate; oxygen consumption rate was continuously measured for another ~60 min. Maximal metabolic rate was determined to be the highest metabolic rate measurement taken during this hour. Absolute aerobic scope was determined by calculating the difference between maximal and standard metabolic rates. Note; fish underwent respirometry experiments after completing the behavioural trials (see below).

2.5. Behavioural assays

To assess the effects of wastewater and temperature on fathead minnow behaviour, four key behavioural traits were assessed in a multi-step behavioural assay: *(i) boldness*, *(ii) sociability*, *(iii) foraging*, and *(iv) response to predator*. These behavioural tests are widely used in behavioural ecology (Bell, 2004; Brodin et al., 2013; Saaristo et al., 2018; McLean et al., 2019; Ågerstrand et al., 2020) and were adapted and validated for fathead minnow using pilot studies. Behavioural trials were also conducted following the 21-day exposure period in a 38L behavioural arena (50.5 × 25.7 × 30 cm) divided into three compartments (all filled with 10 cm of water). Focal fish were placed in the middle compartment (26 × 25.7 × 30 cm) during all assays. Twenty equal-sized grids (5.25 × 5.25 cm) were drawn on the bottom of the focal compartment to facilitate positional scoring of focal fish. The focal compartment was flanked with two equally-divided sides (11.7 × 25.7 × 30 cm), one housing three unexposed conspecific shoal fish, while the other was empty. The two flanked compartments were divided from the focal compartment via permanent transparent barriers that were water-impermeable and two removable black opaque barriers. All behavioural assays were recorded using an overhead camera (GoPro Hero 5); recorded videos were later analyzed using a behavioural annotation software (BORIS v.7.9.4). Behavioural arena setups can be found in Fig. 2 – 5.

Before the start of behavioural trials, three size-matched unexposed and unfamiliar conspecific fish were added to one of the side compartments. These stimuli or "shoal" fish were given an hour to adjust to the arena before a focal fish was introduced into a refuge PVC tube (diameter = 5.0 cm; length = 10.0 cm) placed in the middle of the focal arena. For the first behavioural assay, *boldness* (Fig. 2A), focal fish were allowed to adjust to the refuge for 10 min while being closed off from the rest of the arena by a removable door. Following the adjustment period, the refuge door was remotely lifted, and the focal fish were given 10 min to exit the refuge. We recorded the time at which at least half the body of the fish exited the refuge. If the focal fish did not exit during the allotted 10 min (600 s) period, it was assigned a maximum refuge exit time of 600 s. At 10 min, the entire refuge was remotely lifted from the back; this gently forced any remaining focal fish to swim down and exit and prevented the fish from re-entering the refuge during subsequent assays.

Fish were then given 5 additional min to acclimate following the refuge removal and before the start of the second behavioural assay, *sociability* (Fig. 3A). In the *sociability* assay, the two removable black opaque barriers on either side of the focal central arena were remotely lifted, revealing the two side compartments. One of these side compartments contained a social stimulus (with three shoal fish) and the other side compartment was empty. Using the grids on the bottom of the tank, the focal arena was divided into 5 equally-sized columnar-zones, and the time spent in each zone was recorded for a 10-min period. A sociality index was calculated by multiplying the total time spent in each zone by a zone-specific factor (−2, −1, 0, 1, 2), where the zone closest to the shoal was given a factor of 2 and the zone furthest from the shoal was given a factor of −2. Additionally, the time the focal fish spent interacting with shoal was recorded.

In the third behavioural assay, *foraging* (Fig. 4A), a mesh-lined cassette (2.9 × 4.0 × 0.7 cm) containing ~0.3 g of blood worms (Hikari Bio-Pure), a type of food that our fish were familiar with prior to the exposure period, was remotely dropped into the centre of the focal area. The latency to approach the food cassette by the focal fish as well as the number of interactions with the food cassette were recorded for a 5 min period.

In the fourth and final behavioural assay, *response to predator* (Fig. 5A), a rubber fish model predator (total length = 30 cm) attached to a pole was used to strike the centre of the focal area five times from above. The immediate response to the simulated predator attack as well as the time the focal fish spent being active post-predator attack were recorded for a 5 min period (binned in 1-min increments).

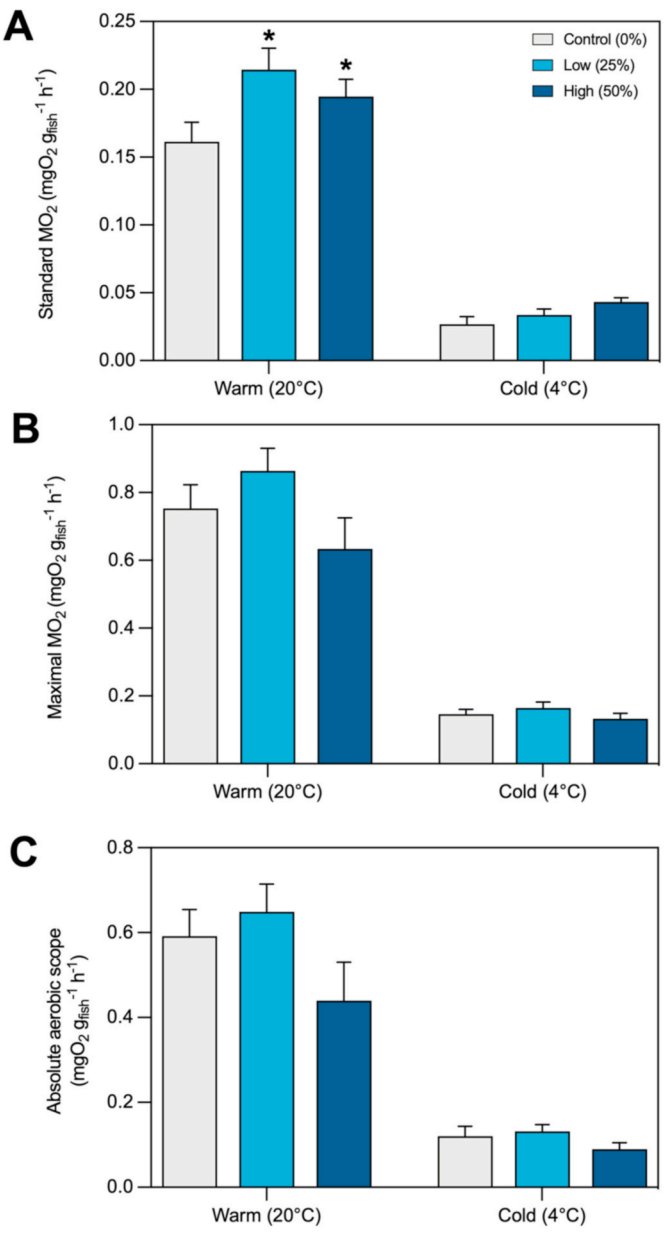

Fig. 1. Mean ± SEM (**A**) Standard metabolic rate, (**B**) maximal metabolic rate, and (**C**) absolute aerobic scope at 20 °C and 4 °C. *represents significant pairwise differences between exposed and control fish within each temperature.

2.6. Fish sampling

Following the post exposure physiological and behavioural assays, fish were euthanized by cerebral percussion; the standard length, total length, and body mass were recorded (Ohaus, Scout Pro SP202, accuracy to 0.01 g). Blood was collected in heparinized capillary tubes via caudal severance, centrifuged at 4750 g for 3 min in a Readacrit centrifuge (Clay Adams) for haematocrit measurement (% of packed red blood cells in the sample).

2.7. Statistical analysis

All statistical analyses were conducted using R (version 4.0.4, R Core Team, 2021). The impacts of wastewater exposure treatment, exposure temperature, and their interaction were analyzed using (i) linear models, (ii) beta regressions, (iii) negative binomial general linear models (GLMs), and (iv) binomial GLMs; depending on the response

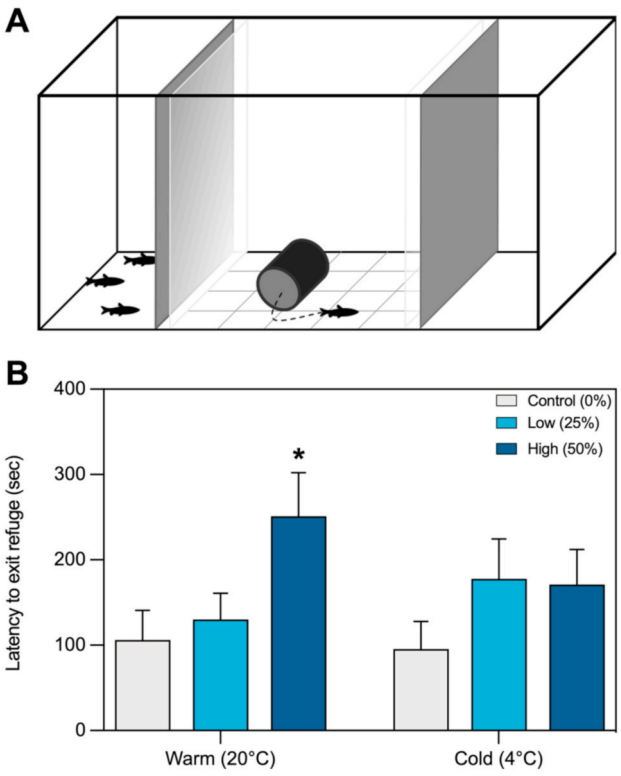

Fig. 2. (**A**) Boldness assay showing fish exiting refuge and (**B**) mean ± SEM boldness, measured by latency to exit refuge (in seconds) at 20 °C and 4 °C. *represents significant pairwise differences between exposed and control fish within each temperature.

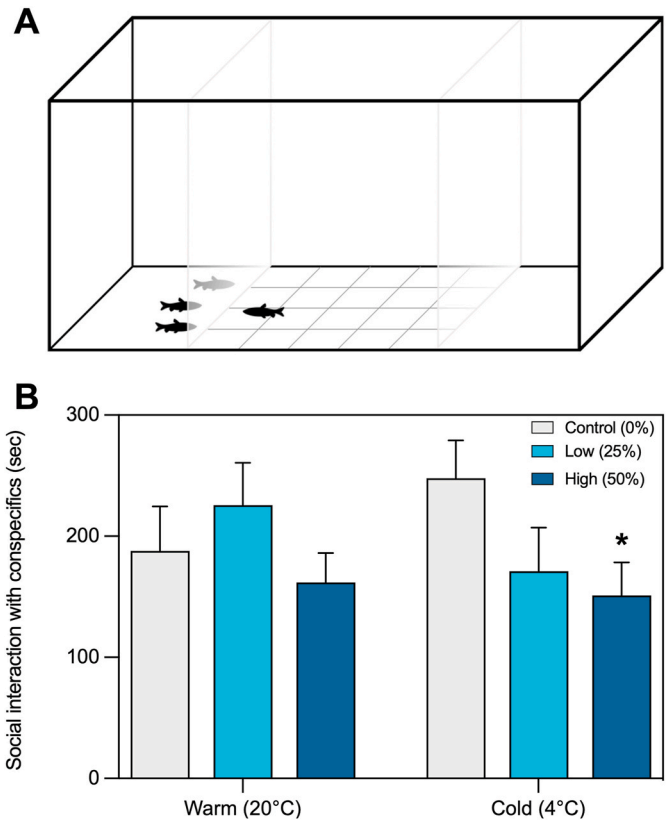

Fig. 3. (**A**) Sociability assay showing fish interacting with shoal and (**B**) mean ± SEM time focal fish spent interacting with shoal at 20 °C and 4 °C. *represents significant pairwise differences between exposed and control fish within each temperature.

variable (see Supplementary Materials for details). Because the conditions of wastewater are dynamic and concentrations of some chemicals can vary between collection dates, we randomly staggered the start date of our tank replicates to help minimize undesired variation between replicates and treatment groups and to ensure sufficient time for all fish to be tested. To account for potential differences in the potency of wastewater effluent across the exposure period, we included the start date of each tank replicate as a covariate. (i) Metabolic rate, haematocrit, boldness, sociability (as measured by zonal scoring), and foraging (as measured by latency to interact with food item) were all analyzed using linear models with sex included as a fixed independent variable and body mass as a covariate. Absolute metabolic rate data was statistically analyzed with body mass as a covariate; however, data is graphically reported as mass-specific metabolic rate ($mgO_2\ g_{fish}^{-1}\ h^{-1}$) to facilitate comparison with previous literature values. (ii) We fit a beta regression (betareg package, Cribari-Neto and Zeileis, 2010) to analyze the proportion of time the focal fish spent socially interacting with their shoal in the sociability assay. (iii) We used negative binomial GLMs for fitting over-dispersed count data to analyze the number of times the focal fish interacted with the food item. (iv) Binomial GLMs were used to analyze the type of response (i.e., dart or no response) focal fish exhibited when presented with the model predator. Data were log transformed when necessary to meet assumptions of normality and homogeneity of variance. Tukey's HSD post-hoc tests were then used to identify significant pairwise differences between each treatment and control within each exposure temperature (emmeans package; Lenth et al., 2018). Data are reported as means ± standard error (SE) unless otherwise stated, and in all analyses, statistical differences were deemed significant at $\alpha = 0.05$.

3. Results

3.1. Characterization of the effluent

We detected nine different classes of chemicals in the final effluent: lipid regulators (gemfibrozil, atorvastatin, p-hydroxy atorvastatin, o-hydroxy atorvastatin), anti-epileptic (carbamazepine), analgesic (acetaminophen), stimulant (caffeine), antibacterials (triclosan, sulfamethazine), antibiotics (trimethoprim, lincomycin, sulfamethoxazole), antidepressants (fluoxetine, norfluoxetine, venlafaxine, desvenlafaxine), non-steroid anti-inflammatory agents (ibuprofen, naproxen, diclofenac), and herbicide (atrazine); see Table 1 for concentrations of each chemical. Most of these compounds (19/20) were also detected in the exposure tanks, although at lower concentrations compared to the concentrations measured in freshly collected effluent. Lower concentrations in the exposure tanks suggest that our dilution regimen was effective and that some degradation of the effluent continued during storage. Some compounds sharply declined between the dosing and water changing periods, while others were more stable. See Supplementary Table 3 for all compound concentrations in exposure tanks.

3.2. Metabolic rate

Overall, standard metabolic rate (SMR) was greatly influenced by temperature; SMR was on average ~5.3 times higher for fish held at 20 °C compared to fish held at 4 °C (LM, $t_{(1,54)} = 211.86$, $p < 0.001$; Fig. 1A). At 20 °C, fish exposed to low and high levels of wastewater effluent demonstrated ~33% ($t = 3.75$, $p = 0.001$) and ~21% ($t = 2.41$, $p = 0.05$) respective increases in SMR relative to control fish. However, fish exposed to wastewater at 4 °C did not exhibit significant changes in SMR relative to control fish ($t_{(Low\ -\ Control)} = 0.17$, $p = 0.99$); ($t_{(High\ -}$

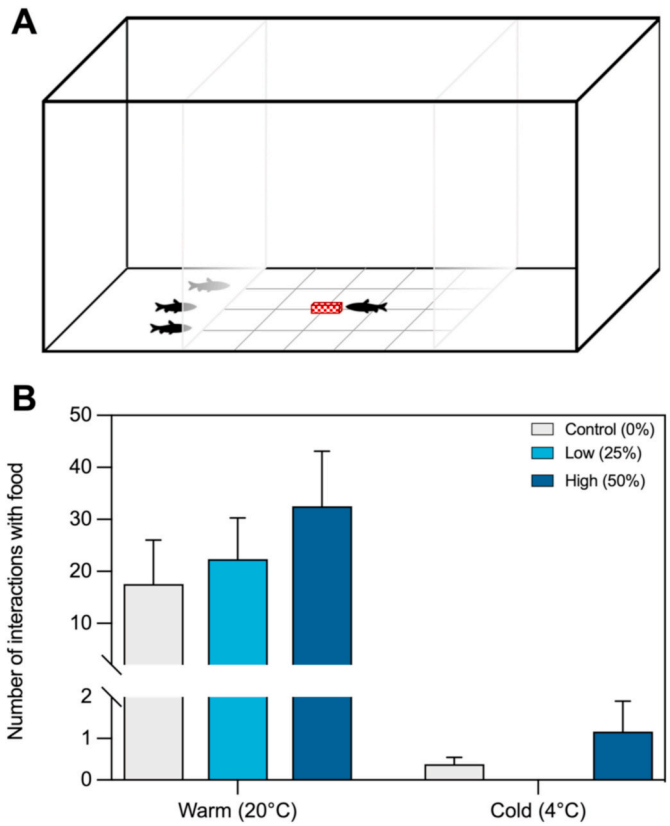

Fig. 4. (A) Foraging assay showing fish interacting with food item and (**B**) mean ± SEM (**A**) number of times focal fish interacted with food item at 20 °C and 4 °C.

$_{Control)} = 0.79$, $p = 0.71$). Similarly, maximal metabolic rate (MMR) was significantly influenced by temperature; MMR was on average ~5.1 times higher at 20 °C than at 4 °C (LM, $t_{(1,54)} = 101.90$, $p < 0.001$; Fig. 1B). Exposure to wastewater had a modest but significant effect on MMR (LM, $F_{(2,54)} = 3.43$, $p = 0.04$; Fig. 1B), but the direction of the effect was inconsistent between wastewater doses, and we did not detect any significant pairwise differences between fish exposed to wastewater effluent and control fish at either temperature ($t_{(20°C: Low - Control)} = 2.09$, $p = 0.10$; $t_{(20°C: High - Control)} = 0.98$, $p = 0.59$; $t_{(4°C: Low - Control)} = 0.30$, $p = 0.95$; $t_{(4°C: High - Control)} = 0.81$, $p = 0.70$).

Similar to SMR and MMR, absolute aerobic scope (AAS), the difference between MMR and SMR, was on average ~5.0 times higher at 20 °C than at 4 °C (LM, $t_{(1,50)} = 48.57$, $p < 0.001$; Fig. 1C). Exposure to wastewater effluent had a marginal, albeit non-significant effect on AAS (LM, $F_{(2,50)} = 2.97$, $p = 0.06$; Fig. 1C); no significant pairwise differences between exposed and unexposed fish at either temperature were detected ($t_{(20°C: Low - Control)} = 1.28$, $p = 0.41$; $t_{(20°C: High - Control)} = 1.47$, $p = 0.32$; $t_{(4°C: Low - Control)} = 0.14$, $p = 0.99$; $t_{(4°C: High - Control)} = 0.67$, $p = 0.78$).

3.3. Behaviour

3.3.1. Boldness

Boldness, measured as the latency to exit refuge in seconds, was not affected by temperature (LM, $t_{(1,134)} = 0.01$, $p = 0.94$; Fig. 2). At 20 °C, fish exposed to high concentrations of wastewater took on average ~2.4 times longer to emerge from refuge compared to control fish ($t = 2.49$, $p = 0.04$), while fish exposed to the low effluent concentrations were similar in their exit times to control fish ($t = 0.76$, $p = 0.73$). At 4 °C, exit times were not significantly different between fish from either exposure treatment and control fish ($t_{(Low - Control)} = 1.56$, $p = 0.27$; $t_{(High - Control)}$

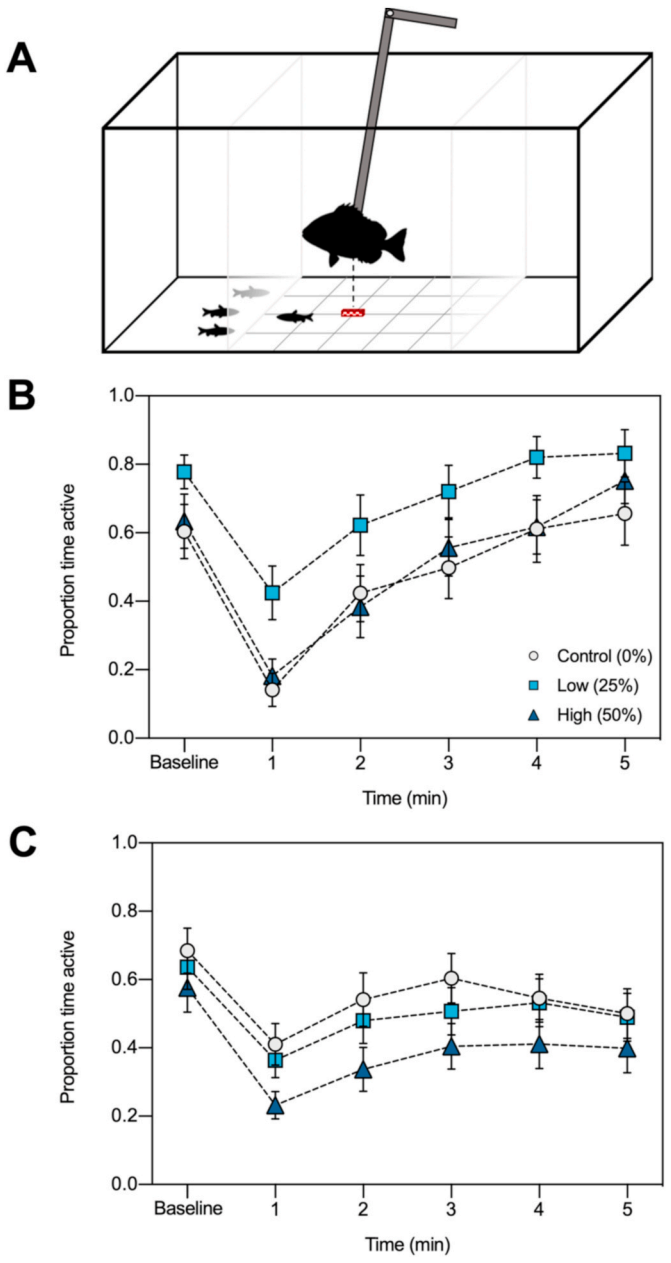

Fig. 5. (**A**) Predator response assay showing model predator striking centre of arena and mean ± SEM proportion of time active post-predator attack at **(B)** 20 °C and (**C**) 4 °C. Baseline indicates activity prior to predator attack (during foraging assay). Activity post-predator attack is presented in 1-min bins.

$= 1.55$, $p = 0.27$).

3.3.2. Sociability

Overall, fish spent the majority of their time in the social zone of the behavioural arena (68%). However, fish held at 20 °C spent ~12% more time on the social side of the tank compared to fish held at 4 °C (LM, $t_{(1,127)} = 5.45$, $t = 0.02$). Wastewater effluent exposure did not affect the amount of time fish spent in the social zone relative to the other zones (LM, $F_{(2,127)} = 0.002$, $p = 0.99$). When we examined how fish spent their time in the social zone, we found that those held at 20 °C and exposed to either concentration of wastewater effluent interacted with a shoal to the same extent as fish in the control group ($Z_{(Low - Control)} = 0.74$, $p = 0.74$; $Z_{(High - Control)} = 0.09$, $p = 0.99$). Whereas at 4 °C, fish exposed to high concentrations of wastewater effluent spent on average ~70% less time interacting with their shoal compared to fish in the control group

Table 1
Mean (±SE) concentration in [ng/L] of chemicals measured in the final effluent upon collection (n = 20). <DL indicates below detection limit. See Mehdi et al. (2021) for detection limits.

Class	Chemical	Concentration (ng/L)
Lipid regulator	Gemfibrozil	73.8 ± 6.83
	Atorvastatin	197 ± 15.1
	p-hydroxy Atorvastatin	339 ± 28.4
	o-hydroxy Atorvastatin	324 ± 27.1
Anti-epileptic	Carbamazepine	249 ± 21.0
Analgesic	Acetaminophen	775 ± 424
Stimulant	Caffeine	4320 ± 1670
Antibacterial	Triclosan	191 ± 15.0
	Sulfamethazine	90.7 ± 10.2
Antibiotic	Monensin	<DL
	Trimethoprim	178 ± 15.5
	Lincomycin	28.0 ± 6.62
	Sulfamethoxazole	341 ± 26.0
Antidepressant	Fluoxetine	24.6 ± 1.30
	Norfluoxetine	18.8 ± 1.42
	Venlafaxine	611 ± 45.0
	Desvenlafaxine	847 ± 65.1
NSAID	Ibuprofen	2290 ± 301
	Naproxen	2130 ± 190.
	Diclofenac	727 ± 32.5
Herbicide	Atrazine	29.3 ± 1.70

($Z_{(High - Control)} = -2.84$, p = 0.01), no such differences were observed in fish exposed to the low concentration of effluent ($Z_{(Low - Control)} = 1.00$, $p = 0.58$). Furthermore, temperature did not affect the overall time spent by the focal fish interacting with a shoal of conspecifics (Beta Regression: N = 137, $\chi^2 = 0.43$, $p = 0.51$; Fig. 3).

3.3.3. Foraging

Fish held at 20 °C interacted ~46 times more frequently with the food (Negative binomial GLM: N = 137, $\chi^2 = 65.98$, $p < 0.001$; Fig. 4A) and were also ~1.7 times quicker to approach the food compared to fish held to 4 °C (LM, $t_{(1,127)} = 33.83$, $p < 0.001$; 4B). Wastewater effluent exposure did not affect the fish's latency to first approach the food (LM, $F_{(2,127)} = 0.76$, $p = 0.47$) nor did it influence the number of times the fish interacted with the food (Negative binomial GLM: N = 137, $\chi^2 = 2.65$, $p = 0.27$). Although we did detect a significant interaction between temperature and wastewater effluent exposure on the number of times a fish engaged with the food (Negative binomial GLM: N = 137, $\chi^2 = 8.52$, $p = 0.01$; Fig. 4A), all pairwise contrasts between treatment groups and control within each temperature were non-significant ($Z_{(20°C: Low - Control)} = 0.44$, $p = 0.90$; $Z_{(20°C: High - Control)} = 0.61$, $p = 0.81$; $Z_{(4°C: High - Control)} = 0.76$, $p = 0.73$; fish exposed to low concentrations of wastewater at 4 °C did not interact with the food).

3.3.4. Response to predator

After the simulated predator attack, 56% of fish darted away while 44% did not respond at all. Fish held at 20 °C were more likely (~71%) to dart away from the predator than fish held at 4 °C (~41%; Binomial GLM, N = 133, $\chi^2 = 4.43$, $p = 0.04$). Wastewater effluent exposure did not have an impact on the type of behavioural response observed (Binomial GLM, N = 133, $\chi^2 = 2.31$, $p = 0.31$). At both exposure temperatures, fish from all treatments responded to the model predator attack with a sharp decline in activity from baseline (Fig. 5). This change in activity was not affected by wastewater effluent exposure (LM, $F_{(2,117)} = 0.45$, $p = 0.64$; Fig. 5). At 4 °C, fish appeared to respond less sharply to the model predator attack, but not significantly so (LM, $t_{(1,117)} = -1.85$, $p = 0.07$; Fig. 5). On average, fish returned to their baseline activity between 2 and 5 min post-model predator attack; time to return to baseline activity levels was not affected by wastewater effluent exposure (LM, $F_{(2,117)} = 0.09$, $p = 0.91$) or by temperature ($t_{(1,117)} = 0.43$, $p = 0.67$).

3.4. Morphology and survival

Total length (TL), standard length (SL), body mass (BM), and body condition (K) were unaltered by wastewater effluent exposure (LM, $F_{(TL; 2,210)} = 0.50$, $p = 0.61$; $F_{(SL; 2,210)} = 0.55$, $p = 0.58$; $F_{(BM; 2,210)} = 0.86$, $p = 0.42$; $F_{(K; 2,210)} = 0.63$, $p = 0.53$). However, fish held at 4 °C appeared to be larger and in better body condition than fish held at 20 °C post exposure (LM, $t_{(TL; 1,210)} = 3.99$, $p = 0.047$; $t_{(SL; 1,210)} = 4.76$, $p = 0.03$; $t_{(BM; 1,210)} = 20.28$, $p < 0.001$; $t_{(K; 1,210)} = 57.36$, $p < 0.001$). Haematocrit was also significantly higher in fish held at 4 °C than in fish held at 20 °C (LM, $t_{(1,205)} = 38.17$, $p < 0.001$). Furthermore, fish exposed to low and high concentrations of wastewater effluent had higher haematocrit than unexposed control fish; however, this was only the case in fish exposed at 20 °C ($t_{(20°C: Low - Control)} = 2.37$, $p = 0.049$; $t_{(20°C: High - Control)} = 2.67$, $p = 0.02$; $t_{(4°C: Low - Control)} = 0.16$, $p = 0.99$; $t_{(4°C: High - Control)} = 0.03$, $p = 0.99$; See Supplementary Table 5). Wastewater exposure did not increase mortality relative to clean water control (see Supplementary Table 4 for survival information and sample sizes for each assay performed).

4. Discussion

In this study, we exposed fathead minnow to different levels of wastewater effluent at 20 °C and 4 °C. We demonstrate that wastewater effluent exposure can have multiple physiological and behavioural impacts on fish, and that these impacts can vary depending on the exposure temperature. We show that exposure to wastewater effluent has metabolic costs in fathead minnow, demonstrated by increases in standard metabolic rate, but only at 20 °C. We also found that wastewater effluent exposure resulted in fish taking longer to leave the safety of a shelter (a boldness measure) at 20 °C and that fish performed fewer social interactions at 4 °C. Additionally, we confirmed the presence of several contaminants of emerging concern that are often detected in wastewater effluents (e.g., venlafaxine, fluoxetine, caffeine, carbamazepine, triclosan, and diclofenac) and are associated with significant impairments in various physiological and behavioural endpoints (Nassef et al., 2010; Martin et al., 2017; McCallum et al., 2017b; Mehdi et al., 2019; Parrott and Metcalfe, 2018; Li et al., 2020; Thompson and Vijayan, 2021).

4.1. Metabolic costs of wastewater exposure

Exposure to wastewater effluent posed a metabolic cost on fish, demonstrated by higher standard metabolic rates, but only at 20 °C. The observed increase in metabolic demands in response to wastewater effluent exposure supports a number of previous studies across multiple species, including bluegill sunfish (Du et al. 2018, 2019) and rainbow darter (*Etheostoma caeruleum*; Mehdi et al., 2017). Increases in metabolic demands in response to contaminant exposure can create metabolic tradeoffs between detoxification and basal processes (e.g., growth and reproduction; Handy et al., 1999; Scott and Sloman, 2004). Such energetic tradeoffs can potentially be detrimental to the health and fitness of exposed fish, especially if energetic demands are not met by sufficient energetic supply (i.e., increased food consumption). It is important to note that we did not observe an increase in foraging rate in fish exposed to wastewater effluent. This may possibly be because the food provided in our foraging assay did not sufficiently mimic a natural food source or was not sufficiently attractive to the fish. Moreover, fish may have perceived the food item presented (in a cassette) as a novel object rather than food. Previous studies have suggested that food consumption is reduced in fish exposed to contaminants (e.g., dieldrin; Beyers et al., 1999 and fluoxetine; Mennigen et al., 2010). Additionally, we observed that fish exposed to wastewater at 20 °C were less inclined to emerge from their refuge. This is of particular concern as fish require additional energetic supply to sustain their increased energetic demands; however, if wastewater exposure is limiting their propensity to forage or their boldness, then fish exposed in the wild may suffer significant fitness

costs.

The observation that standard metabolic rates were not increased by wastewater exposure at 4 °C does not suggest that fish will necessarily suffer fewer detrimental effects in the wild during colder periods of the year. Interestingly, our previous work has demonstrated that fish often congregate near wastewater plumes during the winter, presumably seeking warmer environments as temperatures downstream of WWTPs can be as much 9 °C warmer than in upstream sites during the winter (Mehdi et al., 2021). The increased likelihood of contaminant exposure when residing in wastewater plumes, the greater metabolic costs of wastewater exposure in thermally-enhanced environments, and the fact that the quality of the effluent is often poorer in the winter due to poorer degradation and increased human consumption of PPCPs (Vieno et al., 2005; Gardarsdottir et al., 2010; Ter Laak et al., 2010; Sui et al., 2011; Yu et al., 2013; Suda et al., 2014; Mehdi et al., 2021) may collectively lead to higher metabolic costs of exposure during the winter than in the summer.

4.2. Behavioural effects of wastewater exposure

Fish exposed to wastewater effluent showed modest behavioural effects. At 20 °C, exposed fish were less bold, as reflected by slower emergence from their refuge during behavioural assays. This could indicate that wastewater effluent exposure reduces an individual's tendency to take risks and explore novel environments (Wilson et al., 1994; Wilson and Stevens, 2005; Wilson and Godin, 2009). In the presence of predators, dampened boldness may decrease predation risk and therefore mortality (Dugatkin, 1992). However, in predator-free or low predator abundance systems, dampened boldness may prevent fish from exploiting novel environments, food sources, and mating opportunities, potentially reducing growth and reproduction (Persson and Greenberg, 1992; Brodin et al., 2013). Although boldness is often associated with many other behaviours (e.g., exploration, dispersal, foraging; Fraser et al., 2001; Rehage and Sih, 2004; Wilson and Stevens, 2005), in our study we did not see any clear effects of wastewater effluent exposure on foraging or anti-predator responses.

At 4 °C, we observed that fish exposed to high concentrations of wastewater effluent were less socially interactive with a shoal compared to those that were not exposed. Reduced sociability may increase predation risk, as vigilance against predators decreases when in insolation and an individual's likelihood of being preyed upon increases (*dilution effect*, Magurran, 1990). Additionally, if fish follow each other to good foraging areas, then reduced sociability may make it more difficult for fish to locate food sources (Pitcher et al., 1982), which may be especially concerning for fish exposed to wastewater during the winter – when food is scarce. Interestingly, previous studies that have examined the exposure effects of a contaminant commonly found in wastewater effluents, fluoxetine, have not found any detectable effects on sociability (McCallum et al., 2017b; Meijide et al., 2018; Martin et al., 2019). This, however, is the first study to examine how sociability is affected by whole wastewater effluent, which better reflects the realistic exposures to chemical mixtures that animals are likely to experience in the wild. Although we did detect some wastewater effluent exposure effects on behaviour in the cold, we had initially predicted that the effects would be stronger than those observed. The mild behavioural impacts observed in the cold could partly be due to lowered contaminant uptake linked to metabolic depression and decreased gill ventilation (Capkin et al., 2006; Buckman et al., 2007; Noyes et al., 2009).

4.3. Conclusions

Our study took a unique approach of examining the impacts of wastewater effluent, by focusing on non-reproductive endpoints and by exploring these endpoints at two different temperatures, 4 °C and 20 °C. Our findings suggest that the impacts of wastewater effluent exposure are dependent on temperature. It would be imperative to validate these findings in the field in future studies, and to consider the potential interactive effects of seasonal differences in temperature and effluent quality, as wastewater in the winter is often of poorer quality than in the summer (Mehdi et al., 2021). We believe that our findings will strengthen our understanding of ecotoxicology during the winter, a season that is rarely studied in ecotoxicological research (Larocque et al., 2020; McMeans et al., 2020; Mehdi et al., 2021).

Credit author statement

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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.

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Appendix A. Supplementary data

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