Exposure to Wastewater Effluent Disrupts Hypoxia Responses in Killifish (*Fundulus heteroclitus*)

Samantha C. Lau[a], Hossein Mehdi[b], Leslie M. Bragg[c], Mark R. Servos[c], Sigal Balshine[b], and Graham R. Scott[a]

[a]Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada

[b]Department of Psychology, Neuroscience, and Behaviour, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada

[c]Department of Biology, University of Waterloo, 200 University Ave. W, Waterloo, ON, N2L 3G1, Canada

Corresponding author: scottg2@mcmaster.ca

Abstract

Hypoxia (low oxygen) often occurs in aquatic ecosystems that receive effluent from municipal wastewater treatment plants (WWTP). The combination of hypoxia and WWTP effluent could impair fish health, because WWTP effluent contains multiple contaminants that could disrupt the physiological pathways fish use to cope with hypoxia, but the interactive effects of these stressors on fish physiology are poorly understood. We have examined this issue by exposing mummichog killifish (*Fundulus heteroclitus*) to hypoxia (5 and 2 kPa O_2) and/or 100% WWTP effluent for 21 days in a full factorial design. We then measured hypoxia tolerance, whole-animal metabolism, gill morphology, haematology, and tissue metabolites. In clean water, killifish responded to chronic hypoxia with improvements in hypoxia tolerance, as reflected by increases in time to loss of equilibrium at 0.5 kPa (t_{LOE}). These improvements occurred in association with increases in the exposed surface of gill lamellae that resulted from a regression of interlamellar cell mass (ILCM). Concurrent exposure to wastewater attenuated the increases in t_{LOE} and gill remodeling in chronic hypoxia, and nearly depleted brain glycogen stores. Therefore, exposure to WWTP effluent can disrupt the physiological mechanisms fish use to cope with chronic hypoxia and impair hypoxia tolerance. Our research suggests that the combination of stressors near WWTPs can have interactive effects on the physiology and health of fish.

Keywords: Pollution; Respirometry; Pharmaceuticals and personal care products; Aerobic metabolism; Metabolic depression

Capsule

- Combinations of stressors near wastewater treatment plants can have interactive effects on metabolism, low oxygen tolerance, and health of fish.

1. Introduction

Environmental hypoxia (low oxygen availability), is a common feature of many aquatic ecosystems, and it can occur as a result of natural causes as well as from anthropogenic pollution (Diaz, 2001; Friedrich et al., 2014). Many tide pools, estuarine habitats, stratified or ice-covered lakes and ponds are characterized by naturally low or fluctuating oxygen levels (Diaz and Rosenberg, 2008). In some other cases, the depletion of dissolved oxygen can be linked to eutrophication events due to nutrient loading from fisheries, agricultural run-off, or discharge from wastewater treatment plants that leads to the proliferation of primary producers (Breitburg et al., 2009; Brooks et al., 2006; Carey and Migliaccio, 2009; Diaz, 2001; Diaz and Rosenberg, 2008; Friedrich et al., 2014). The occurrence of such hypoxic events is predicted to increase with further progression of climate change, urbanization, and pollution (Friedrich et al., 2014).

Fish exhibit a suite of behavioural, physiological, and biochemical responses to hypoxia that contribute to maintaining the balance between oxygen supply and demand and to thus cope with low oxygen conditions (Bickler and Buck, 2007; Perry et al., 2009; Pollock et al., 2007; Richards, 2011). Such responses have been well described in the literature and often act to improve O_2 extraction from the environment (e.g., by increasing gill ventilation and surface area), reduce activity and metabolism, and/or increase anaerobic energy production (Borowiec et al., 2015; Kramer and McClure, 1982; Perry and Tzaneva, 2016; Regan et al., 2017; Sollid et al., 2003). However, hypoxia often occurs concurrent with other environmental stressors, including fluctuations in temperature and/or salinity or pollution (Friedrich et al., 2014). The potential interactions between hypoxia and many other types of stressors are poorly understood, and whether anthropogenic stressors can disrupt the natural adaptive responses to hypoxia remains unresolved.

Effluents discharged from municipal wastewater treatment plants (WWTP) are complex contaminant mixtures that can contain excess nutrients, pharmaceuticals and personal care products (PPCPs), and industrial chemicals (Brooks et al., 2006). The investigation of these contaminants of emerging concern (CECs) are necessary due to their increasing usage and potential for having significant effects on fish and other aquatic life (Corcoran et al., 2010; Kolpin et al., 2002; Metcalfe et al., 2003; Zenker et al., 2014). Exposure to WWTP effluent can have a number of physiological effects on fish, having been shown to disrupt reproductive function (Bahamonde et al., 2015) and metabolic rate (Du et al., 2019, 2018; Mehdi et al., 2018). Hypoxia exposure is also known to affect metabolic rate and its underlying physiological determinants (Fu

et al., 2011; Hochachka et al., 1996; Richards et al., 2009). The eutrophication of aquatic environments, including wastewater dominated ecosystems, can reduce oxygen levels and potentially lead to hypoxic conditions, but the effects of combined exposure to wastewater effluent and hypoxia are largely unknown.

Given the molecular targets of many pharmaceuticals and other contaminants found in WWTP effluents, exposure to these effluents could disrupt the physiological pathways fish use to cope with hypoxia. For example, the pharmaceuticals in wastewater have molecular targets involved in oxygen sensing (e.g., serotonin reuptake inhibitors), homeostatic regulation by the sympathetic nervous system (e.g., beta blockers), and control of metabolic pathways (e.g., anti-diabetics, lipid lowering drugs) (Arlos et al., 2015; Corcoran et al., 2010; Kolpin et al., 2002; Metcalfe, 2013; Metcalfe et al., 2003). Indeed, exposure to the serotonin reuptake inhibitor, fluoxetine, has been shown to attenuate the cardiovascular and ventilatory responses to hypoxia in Gulf toadfish (*Opsanus beta*) (Panlilio et al., 2016). Several industrial contaminants (e.g., polychlorinated biphenyls, polycyclic aromatic hydrocarbons) may also disrupt hypoxia signaling pathways that underlie the physiological adjustments to chronic hypoxia, such as the hypoxia inducible factor (HIF) pathway (Chan et al., 1999; Kraemer and Schulte, 2004; Vorrink and Domann, 2014; Silva et al., 2017). However, it is largely unknown whether the concentrations of these contaminants in wastewater effluent is sufficient to disrupt the ability of fish to respond and cope with hypoxia.

The objective of this study was to help address these knowledge gaps in the literature by determining whether exposure to WWTP effluent disrupts the adaptive physiological responses of mummichog killifish (*Fundulus heteroclitus*) to hypoxia. We examined the effects of chronic exposure to hypoxia and/or municipal wastewater effluent in a full factorial design on metabolism, hypoxia tolerance, and several underlying respiratory and metabolic traits. Killifish are an ideal model organism for this study as they naturally experience hypoxia and fluctuations in various other environmental conditions in their estuarine habitat (Burnett et al., 2007). This species can also be found in heavily polluted estuarine environments containing high levels of persistent organic pollutants, including dioxins, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals, and some wild populations from such environments have evolved exceptional tolerance of pollution exposure (Bugel et al., 2010; Reid et al., 2016; Weis

and Weis, 1989). We hypothesized that the response of killifish to chronic hypoxia would be disrupted by exposure to municipal wastewater, such that fish would be less tolerant of hypoxia.

2. Materials and methods

2.1 Study animals and housing

Adult mummichog killifish (*Fundulus heteroclitus*) were wild caught by a commercial supplier (Aquatic Research Organisms, Hampton, New Hampshire, USA) and shipped to McMaster University in Hamilton, Ontario, Canada in the autumn of 2018. Killifish were kept in well-aerated brackish water (4 ppt), produced by mixing artificial sea salt (Reef Pro Mix Redline Complete Sea Salt, Fritz Aquatics, Mesquite, Texas) with dechlorinated City of Hamilton tap water. Holding tanks were kept at room temperature (~18°C), and killifish were fed 5 days a week to satiation with commercial pellets (AgloNorse Complete Fish Feed, 0.6-0.9 mm, Tromsø, Norway). The photoperiod was 12 h:12 h light:dark between 0700 and 1900 local time. All animal protocols were developed in accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

2.2 Wastewater effluent collection

Wastewater effluent was collected from Woodward Avenue Wastewater Treatment Plant (43°15'15.6"N; 79°46'25.4"W) in Hamilton, Ontario in March-May of 2019. Woodward WWTP is a secondary conventional activated sludge treatment plant with sludge dewatering and digestion and has a maximum daily capacity of 409 million litres (City of Hamilton, 2019). Effluent was collected every Tuesday and Saturday during the exposure period between the hours of 0930 and 1130 local time. Using a submersible sump-pump, wastewater effluent from the final clarifier chimney was collected into 20 L carboys (Reliance Rectangular AquaPak Containers, BPA Free) for transportation to McMaster University. Effluent was stored in a dark and refrigerated cold room at 4°C for at most three days before use in chronic exposures (see below). We obtained water quality data of 24 h composite samples from each day of effluent collection from the City of Hamilton Environmental Laboratory (Figs. S1-3). For each effluent collection, we also preserved a sample of effluent (500 mL) in pre-cleaned amber glass bottles containing sodium azide (1 g/L) and ascorbic acid (50 mg/L) and stored these samples at 4°C for up to 2 days until extractions for

analytical chemistry, and there were no systematic differences between treatment groups in the duration of storage before extraction.

2.3 Chronic exposures of fish

Killifish were exposed to wastewater and/or hypoxia in a full factorial design in 40 L aquaria for 21 days at room temperature (~18°C). Conditions of normoxia (well aerated to ~20 kPa O_2), moderate hypoxia (5.0 ± 0.1 kPa), or severe hypoxia (2.0 ± 0.1 kPa) were maintained in clean water (Hamilton dechlorinated tap water adjusted to 4 ppt with artificial sea salt) or in 100% wastewater effluent. Each of these 6 chronic exposure groups was replicated 2-3 times over the period of wastewater collection (24 fish per tank). Wastewater effluent was brought up to room temperature and 4 ppt salinity immediately before use. For hypoxic treatments, oxygen tension (PO_2) was reduced from 20 kPa to the treatment setpoint over the first three days of exposure, and was maintained by injecting N_2 gas using O_2 controllers that we have previously described (Borowiec et al., 2015). Bubble wrap was held on the water surface in the hypoxic treatments to reduce O_2 diffusion from the air and to prevent aquatic surface respiration. Water was gently mixed with a submersible aquarium pump and filtered through a sponge filter (but not a charcoal filter). Every 72 hours, faeces and other debris were removed, physicochemical parameters were measured (pH, conductivity, total dissolved solids, salinity, temperature, and NH_3, NO_2, NO_3 levels; Table S1), and the appropriate tank water was renewed (clean water or wastewater). Because effluent was stored for ~3 days after collection before use, which we anticipated would lead to the degradation of some contaminants found in the water, we took water samples (125 mL) for analytical chemistry from each tank replicate in each wastewater treatment group to provide an indication of the level of exposure. We also anticipated that the concentrations of some contaminants may change over the 3 days between water changes, so paired water samples (125 mL) were performed once for each wastewater tank replicate – one immediately after a water change and the second immediately prior to the next water change. Water samples were preserved using sodium azide and ascorbic acid as described above. After completing the 21-day exposure, one subset of fish in each tank was immediately euthanized for tissue sampling, and two other subsets of fish were used for respirometry and hypoxia tolerance measurements (see below). We were not able to make all measurements in all individuals, so the number of individuals used for

each measurement is clearly indicated in each figure and table. In all cases, we used individuals from every tank replicate for every measurement.

2.4 Analytical chemistry of water samples

Preserved water samples were analyzed for twenty-two PPCPs and other CECs using previously established methods (Arlos et al., 2015; Mehdi et al. 2021). The analyzed chemicals included lipid regulators and metabolites, anti-epileptics, analgesics, antibacterials, antibiotics, antidepressants, non-steroidal anti-inflammatory agents, herbicides, and industrial chemicals (Table 1). Wastewater effluent samples were first divided into 100 mL aliquots, and tank samples were divided into 50 mL aliquots. Each sample was individually spiked with 100 µL of 100 µg/L of isotopically labelled standards prior to extraction. Solid phase extraction (SPE) was performed using Agilent Bond Elute Plexa cartridges (6cc, 500 mg) on samples adjusted to pH 2, with final extracts reconstituted in 500 µL methanol with internal standards. These extracts were then stored at -20°C until analysis 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 (Mehdi et al. 2021). Of all the preserved water samples, technical issues precluded the analysis of three samples of wastewater tank replicates. Some individual data points for acetaminophen and caffeine were excluded due to problems with matrix effects for these particular compounds.

2.5 Sampling of fish tissues

The sampled fish were first euthanized with a sharp blow to the head followed by spinal transection, and the tail was severed for blood collection. A portion of blood (5 µL) was stored in Drabkin's reagent for determining haemoglobin concentration according to product instructions (Sigma-Aldrich, St. Louis, MO, USA). The remaining blood was collected in a heparinized capillary tube and centrifuged at 12,700 g for 5 min to determine haematocrit. Brain, liver, and axial white muscle were excised, weighed, freeze-clamped in liquid nitrogen, and then stored at -80°C for later analysis of metabolites. Gills were dissected, placed in 0.2 M PBS (274 mmol/L NaCl, 30.4 mmol/L Na_2HPO_4, 5.4 mmol/L KCl, 3.0 mmol/L KH_2PO_4; pH 7.8) containing fixative (2% paraformaldehyde and, 2% glutaraldehyde) for at least 48 h, then stored in PBS at 4°C for later analysis of gill morphometrics.

177

178 *2.6 Respirometry and hypoxia tolerance*

179 Killifish were subjected to two series of measurements of metabolism and hypoxia
180 tolerance. In one subset of fish from each chronic exposure group, we used stop-flow respirometry
181 in clean water at 4 ppt to measure standard metabolic rate (SMR) and critical oxygen tension (P_{crit}).
182 Fish were fasted for the last 48 h of chronic exposures and were then transferred at ~1700-1900
183 local time to 90 mL cylindrical acrylic chambers containing normoxic water, which were covered
184 in dark plastic to minimize visual disturbance. Measurements of O_2 consumption rate (MO_2) were
185 made overnight in normoxia using an automated respirometry system (AutoResp, Loligo Systems,
186 Viborg, Denmark) that has been previously described in detail (Borowiec et al., 2015, 2020). MO_2
187 was determined from the depletion of water oxygen content over time, and is expressed relative to
188 body mass (μmol/g/h). SMR was calculated as the average of the lowest 10 MO_2 measurements
189 that were made overnight during the inactive phase. Starting at ~1100-1200 the following day,
190 resting metabolic rate (RMR) was calculated as the average MO_2 measured at 100% air saturation.
191 MO_2 was continuously measured throughout a stepwise hypoxia protocol, during which the water
192 O_2 level was reduced by 10% air saturation every 10 min, as previously described (Borowiec et
193 al., 2015, 2020). Critical PO_2 (P_{crit}) was calculated using the 'Respirometry' package in R. In a
194 second subset of fish from each chronic exposure group, we measured time to loss of equilibrium
195 (t_{LOE}) by exposing fish to an O_2 tension of 0.5 kPa in 5 L aquaria. Fish were monitored
196 continuously, and t_{LOE} was determined as the duration of time at 0.5 kPa until the fish was unable
197 to maintain an upright position in the water column and was unresponsive to a gentle tail pinch.
198

199 *2.7 Gill histology*

200 Gill histology was performed for the first gill arch for the normoxic and severe hypoxic
201 exposure groups in both clean water and wastewater. We followed methods that have been
202 previously described in detail for cryoprotection, embedding, sectioning at 5 μm in a cryostat, and
203 haematoxylin and eosin staining (Du et al., 2018). For each individual, 10-15 brightfield images
204 were taken of sections throughout the whole gill arch tissue using a Nikon Eclipse E8000
205 microscope (Nikon Instruments, Melville, New York, USA). Gill morphometrics were analyzed
206 using ImageJ (v1.52a) software (Rasband, 2008).
207

208 *2.8 Tissue metabolite assays*

209 Lactate, free-glucose, and glycogen levels were determined in brain, muscle, and liver. For
210 muscle and liver samples, tissues were first ground into fine powder using insulated mortar and
211 pestle that were pre-cooled using liquid nitrogen and were then stored at -80°C. Approximately 20
212 mg of these powdered tissues and whole brain samples were homogenized in ~300 µL of ice-cold
213 6% $HClO_4$. Homogenate was then vortexed, an aliquot of 100 µL was immediately frozen in liquid
214 nitrogen and stored at -80°C for later analysis of glucose and glycogen, and the remaining unfrozen
215 homogenate was used for analysis of lactate. Assays were carried out using standard protocols that
216 have been previously described in detail (Borowiec et al., 2018).

217

218 *2.9 Statistical analysis*

219 All statistical analyses were performed using the lme4 package (Bates et al., 2015) in R
220 (version 3.6.2, R Core Team, 2019). We tested for the main and interactive effects of chronic
221 hypoxia and chronic wastewater exposure using linear mixed effects models. We included body
222 mass and sex as additional fixed independent variables. Furthermore, to account for any
223 differences in the potency of wastewater as the exposures proceeded, start date of each tank
224 replicate was included in the model as a covariate. Although absolute values of metabolic rate
225 (SMR and RMR in µmol/h) was analyzed statistically with body mass as a covariate, these data
226 are reported normalized to body mass (µmol/g/h) to facilitate comparison with previous literature.
227 Data were log or square root transformed if necessary, to meet the assumptions of the model.
228 Tukey HSD post-hoc tests were used to identify significant pairwise differences (a) between clean
229 water and wastewater within each chronic oxygen treatment, (b) between normoxia and hypoxia
230 within the clean water groups, and (c) between normoxia and hypoxia within the wastewater
231 groups. Data are reported as means ± standard error (SEM) unless otherwise stated, and in all
232 analyses, α was set to 0.05.

233

234 **3. Results**

235 *3.1 Water quality and contaminant concentrations*

236 Several indices of water quality during the exposures were comparable between clean and
237 wastewater treatments (Table S1). Although ammonia and nitrate levels were high in the collected
238 wastewater effluent (Fig. S1), nitrogenous wastes were reduced by the in-tank filters during

exposures and did not vary between treatments or as a function of time. However, killifish were exposed to the complex mixture of contaminants present in the wastewater effluent. Of the twenty-two contaminants that were measured, all but one (monensin) were detected in tank water from the wastewater treatment groups (Table 1). Over the course of our chronic exposures, the quality of water collected from the treatment plant seemed to improve and the concentrations of target chemical contaminants declined or remained stable, and the magnitude of changes for some contaminants appeared to vary between normoxia and hypoxia. This was supported by measurements of several water quality parameters in 24-hour composite samples of final effluent that were taken on the dates of collection (Figs. S1-S3), and from measurements of target chemical contaminants in the wastewater collected for exposures (Figs. S4-S8). Nevertheless, we used a staggered tank replicate design to help minimize undesired systematic variation between treatment groups, and we accounted for any such variation that did exist by including start date of each tank replicate as a covariate in our statistical models. However, the concentrations of target contaminants were higher in wastewater at the time of collection (Figs. S4-S8) compared to those measured after the three-day storage period when wastewater was added to tanks after water changes (Table 1). Degradation of some (but not all) contaminants continued during the tank exposures (Table S2). Therefore, the data in Table 1 provides a relative indication of the exposure conditions in wastewater, but only approximate the variable concentrations that were likely experienced during chronic exposures.

3.2 Metabolic rate

Concurrent exposure to wastewater effluent and chronic hypoxia reduced standard metabolic rate (SMR) (Fig. 1; Table S3). There was a significant interaction between chronic hypoxia and wastewater (p = 0.02) across all oxygen treatments in clean water and wastewater. Hypoxia acclimation did not affect SMR among fish held in clean water, but concurrent exposure to hypoxia and wastewater reduced SMR by 37% and 25% in the moderate and severe hypoxic groups, respectively (Fig. 1A) and likely drove the significant hypoxia effect (p = 0.03). Resting metabolic rate (RMR), which was measured during the daytime in normoxia, did not show the same patterns observed in SMR. There were no statistically significant effects of chronic hypoxia or wastewater on RMR across treatment groups (Hypoxia effect, p = 0.42; Wastewater effect, p = 0.96; Fig. 1B), suggesting that the relationship between SMR and RMR may be altered by chronic

hypoxia and/or wastewater exposure. The data did not appear to result from variation in body mass, as there were no significant differences in body mass across groups, nor were there any differences in indices of condition (Fulton's K, hepatosomatic index; Table S4).

3.3 Hypoxia tolerance

In general, killifish regulated MO_2 across a range of higher PO_2, and MO_2 declined at lower PO_2 (Fig. S9). The data for MO_2 as a function of PO_2 for each individual were used to calculate the critical O_2 tension (P_{crit}). There was a reduction in critical oxygen tension (P_{crit}) after chronic exposure to severe hypoxia, as reflected by a significant main effect of hypoxia (p =0.048), but this trait was unaffected by wastewater exposure (p = 0.84; Fig. 2A; Table S3).

Wastewater exposure impeded the improvement with hypoxia acclimation in a key metric of hypoxia tolerance, the time to loss of equilibrium (t_{LOE}) in near anoxia (0.5 kPa) (Fig. 2B; Table S3). There was a significant main effect of chronic hypoxia on t_{LOE} (p<0.001), driven largely by fish in clean water, which increased t_{LOE} by 1.79-fold on average after acclimation to moderate hypoxia and 3.56-fold after acclimation to severe hypoxia. There was also a significant effect of wastewater on t_{LOE} (p<0.01), which completely eliminated improvements in t_{LOE} in fish acclimated to moderate hypoxia and appeared to reduce the improvements in those acclimated to severe hypoxia.

3.4 Gill morphology

Wastewater exposure disrupted changes in gill morphology that were associated with hypoxia acclimation (Fig. 3; Table S3). The total height, width, and density of lamellae did not vary significantly across treatment groups (Fig. 3B-D), but there was a significant interaction (p = 0.03) between hypoxia and wastewater on the proportion of lamellae that were covered by interlamellar cell mass (ILCM; Fig. 3E). Chronic exposure to severe hypoxia in clean water led to a large reduction in the proportional height of the ILCM, which appeared to drive the significant main effect of hypoxia on this trait (p<0.01). In contrast, among wastewater exposed fish, the proportional ILCM height was unchanged by chronic hypoxia.

3.5 Haematology

Wastewater exposure also altered haematology (Tables 2, S3). Fish in clean water responded to chronic hypoxia with increases in haematocrit (hypoxia effect, $p<0.001$) and decreases in mean corpuscular haemoglobin concentration (MCHC; hypoxia effect, $p = 0.01$), such that there was no significant change in blood haemoglobin content (hypoxia effect, $p = 0.88$). Wastewater affected these haematological responses, increasing haematocrit in normoxia and/or moderate hypoxia and increasing blood haemoglobin in moderate hypoxia (hypoxia×wastewater, $p = 0.01$).

3.6 Tissue metabolites

Exposure to wastewater affected glycogen stores in normoxia and hypoxia (Fig. 4; Table S3). Wastewater had a particularly detrimental effect on glycogen stores in brain tissue, for which there was significant hypoxia×wastewater interaction ($p = 0.04$) and concurrent exposure to wastewater and severe hypoxia led to nearly complete glycogen depletion (Fig. 4A). There was also a significant hypoxia×wastewater interaction on glycogen content in liver ($p = 0.03$), but in this tissue, wastewater exposure appeared to minimize glycogen depletion in severe hypoxia (Fig. 4B), although the decrease in liver glycogen in severe hypoxia compared to normoxia in clean water was not quite significant ($p = 0.075$). In contrast, there was no significant variation in muscle glycogen content, nor were there significant variations in concentrations of glucose or lactate in any tissue (Tables S3, S5).

4. Discussion

Aquatic hypoxia is prevalent in many ecosystems that are heavily burdened by contaminants, including those that receive effluent discharge from municipal wastewater treatment plants, but the interactive effects of hypoxia and wastewater effluent on fish physiology and health are poorly understood. This study helps address this knowledge gap, showing that exposure to wastewater effluent can disrupt many key physiological adjustments for coping with chronic hypoxia. Wastewater exposure had relatively modest effects on killifish in normoxia, but it impeded the improvements in hypoxia tolerance in response to chronic hypoxia and disrupted the plasticity of several associated physiological traits. This suggests that the ability of fish to cope with other natural or anthropogenic stressors may be impaired in environments contaminated with wastewater effluent.

4.1 Wastewater exposure had modest physiological effects in normoxia

The evidence emerging from our findings here in killifish and those from previous studies suggest that the metabolic impacts of wastewater exposure may differ between species. The lack of any effect on metabolic rate in killifish from exposure to wastewater in normoxia is similar to previous findings in round goby that were cage exposed to the receiving waters near a WWTP (McCallum et al., 2017). In contrast, some other species exhibit a prominent increase in metabolic rate in response to wastewater exposure, including bluegill sunfish (*Lepomis macrochirus*) (Du et al., 2019, 2018) and rainbow darter (*Etheostoma caeruleum*) (Mehdi et al., 2018). These differences between studies are unlikely to have been caused by differences in exposure conditions, because the approximate range of contaminant concentrations observed here are generally comparable to those detected in wastewater effluents and surface waters near municipal WWTPs in previous studies (McCallum et al., 2017; Du et al., 2019; Fick et al., 2017; Metcalfe et al., 2010; Verlicchi et al., 2012). This could indicate that some species are more sensitive and experience a greater metabolic cost of wastewater exposure than others. Killifish are often found in contaminated sites and are considered to be a relatively tolerant species, and several populations have evolved especially high resistance to persistent organic pollutants (Crawford et al., 2020; Reid et al., 2016; Weis, 2002). However, it is possible that degradation of some contaminants within exposure tanks weakened the magnitude of exposure here, but exposure had several physiological effects in hypoxia and there did not appear to be any overall difference between normoxia and hypoxia in the level of degradation (Table S2). Killifish may therefore experience less metabolic stress than other less tolerant species when exposed to wastewater effluent, and thus avoid increases in whole-animal energy demands.

4.2 Wastewater exposure disrupts responses to chronic hypoxia

One of the most significant impacts of wastewater exposure was the attenuation of plastic improvements in hypoxia tolerance, as reflect by time to loss of equilibrium (t_{LOE}) in near anoxia (0.5 kPa), which normally occur in response to chronic hypoxia (Fig. 2B). Killifish in clean water responded to chronic hypoxia with increases in t_{LOE}, consistent with previous findings in several killifish species (Borowiec et al., 2020, 2015), which could reflect an improved ability to match O_2 supply and O_2 demand during hypoxia. The effect of wastewater that reduced t_{LOE} in chronic

hypoxia could not be explained by a metabolic cost of exposure that increased O_2 demands, because combined exposure to wastewater and hypoxia reduced rather than increased SMR (Fig. 1). Wastewater exposure could have instead reduced t_{LOE} by impairing tissue O_2 supply. This possibility is supported by our observation that wastewater exposure blunted the plastic responses of gill morphology to chronic hypoxia, eliminating the regression of the ILCM that occurred in response to chronic hypoxia in clean water (Fig. 3). The regression of the ILCM is considered advantageous in hypoxia by increasing the surface area of the gills for O_2 diffusion (Sollid et al., 2003). Wastewater exposure could have also reduced t_{LOE} by reducing the ability of killifish to use anaerobic metabolism. Glycogen stores in the brain are a key fuel for anaerobic metabolism that help determine the ability to maintain ATP levels and avoid losing equilibrium in severe hypoxia (Speers-Roesch et al., 2013). Therefore, the low brain glycogen reserves in killifish that were chronically exposed to wastewater and hypoxia (Fig. 4A) likely reduced the ability to fuel anaerobic metabolism and thus constrained t_{LOE}.

Exposure to wastewater effluent had no effects on P_{crit}. Killifish responded to chronic hypoxia with decreases in P_{crit}, consistent with previous findings in killifish and other species (Borowiec et al., 2020, 2015; Fu et al., 2011), but this plastic response was unaffected by wastewater exposure (Fig. 2A). This contrasts recent findings in three-spined stickleback (*Gasterosteus aculeatus*), in which waterborne copper exposure (20 μg/l) impaired improvements in P_{crit} that result from hypoxia acclimation (Fitzgerald et al., 2019). P_{crit} is often considered to reflect the ability to extract oxygen from the environment during hypoxia, and is considered to be a useful indicator of hypoxia tolerance in many species (Regan et al., 2019; Rogers et al., 2016; Speers-Roesch et al., 2013). However, in hypoxia-tolerant species such as killifish that readily depress metabolic rate (Borowiec et al., 2018), the reductions in MO_2 below P_{crit} (Fig. S9) could reflect a facultative reduction in O_2 demands rather than a hypoxia-induced constraint on tissue O_2 supply. This may explain why wastewater exposure impaired gill plasticity but had no effect on the decreases in P_{crit} in response to chronic hypoxia. Furthermore, this leads to the possibility that effects of contaminant exposure on gill morphology will only affect P_{crit} for species in which MO_2 depression below P_{crit} results from O_2 supply limitation. For example, many populations of threespine stickleback do not employ metabolic depression in hypoxia (Regan et al., 2017), which may make P_{crit} more susceptible to contaminant exposure in stickleback than it is in killifish.

The disruption of hypoxia responses could be attributed to several of the chemical contaminants in wastewater effluent. Selective serotonin reuptake inhibitors (SSRIs) like fluoxetine could inhibit serotonin signaling by oxygen sensing neuroepithelial cells in the gills, which may have disrupted key cardiorespiratory adjustments to hypoxia exposure. Indeed, in gulf toadfish, hypoxia exposure during acute intraperitoneal treatment of 20 or 50 μg/g of fluoxetine have been shown to accentuate oxyconformation (i.e., decreased regulation index) and reduce the ventilatory response, respectively (Amador et al., 2018; Panlilio et al., 2016). Serotonin also regulates glycogenolysis in the brain of rainbow trout (Pérez-Maceira et al., 2012), so SSRI exposure could have also contributed to the effects of hypoxia and wastewater exposure on glycogen levels that were observed here. β-blockers such as propranolol can affect heart rate in zebrafish larvae at waterborne exposure concentrations of 0.05 mM (Fraysse et al., 2006), can disrupt oxygen chemoreception by neuroepithelial cells in the gills of rainbow trout at concentrations of 1-2 mM in the gill perfusate (Burleson and Milsom, 1990), and can disrupt blood-flow distribution through the gills of rainbow trout at 0.1 mM in gill perfusate (Payan and Girard, 1977). Ligands of the aryl hydrocarbon receptor (AHR), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other persistent organic pollutants, could also disrupt cellular responses to hypoxia (Fleming et al., 2009; Kraemer and Schulte, 2004). This is because many of the biochemical and physiological responses to hypoxia result from activation of the hypoxia-inducible factor (HIF) pathway (Pelster and Egg, 2018), and there can be cross-talk between HIF-α and AHR signaling as they require the same dimerization partner (HIF-1β, also called the AHR Nuclear Translocator or ARNT) to drive the expression of target genes. Given that many of the cellular responses to hypoxia lead to adjustments in flux through metabolic pathways, it is also possible that such changes could be disrupted by the various pharmaceuticals that target metabolic pathways (e.g., statins such as atorvastatin, diabetes medications such as metformin, etc.). Although exposure to wastewater makes it difficult to disentangle the relative effects of each of these contaminants, it is nevertheless important to understand the emergent effects of the complex real-world contaminant mixtures that enter the environment and how they might interact with other abiotic stressors like hypoxia.

4.3 Conclusions

Our findings suggest that the interaction between the chemical contaminants in wastewater and the other abiotic stressors near WWTPs may have significant detrimental effects on the physiology and health of fish. Wastewater exposure disrupted the physiological adjustments employed by killifish to cope with chronic hypoxia and thus impaired hypoxia tolerance. Chronic exposure to the combination of wastewater and hypoxia also led to the near depletion of brain glycogen reserves, which could put animals at extreme risk if faced with an additional metabolic stress (e.g., heat waves). Our results thus demonstrate that the real-world complex mixtures of contaminants in wastewater can have significant emergent effects on health and the ability to cope with environmental variability, at least in part by disrupting respiratory and metabolic processes. Further studies should prioritize the elucidation of mechanisms underlying the interactive effects of wastewater and other abiotic stressors, including hypoxia and other stressors that are projected to become more prevalent with climate change (warming temperature, reduced pH). Such mechanistic approaches can improve our capacity to predict the impacts of aquatic pollution at organismal and population levels.

Credit Author Statement

Samantha C. Lau: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Visualization. **Hossein Mehdi:** Conceptualization, Methodology, Formal Analysis. **Leslie M. Bragg:** Investigation. **Mark R. Servos:** Resources. **Sigal Balshine:** Conceptualization, Funding Acquisition. **Graham R. Scott:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, and Funding acquisition.

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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460 **References**

461 Amador, M.H.B., Schauer, K.L., McDonald, M.D., 2018. Does fluoxetine exposure affect
462 hypoxia tolerance in the Gulf toadfish, Opsanus beta? Aquat. Toxicol. 199, 55–64.
463 https://doi.org/10.1016/j.aquatox.2018.03.023

464 Arlos, M.J., Bragg, L.M., Parker, W.J., Servos, M.R., 2015. Distribution of selected
465 antiandrogens and pharmaceuticals in a highly impacted watershed. Water Res. 72, 40–50.
466 https://doi.org/10.1016/j.watres.2014.11.008

467 Bahamonde, P.A., Fuzzen, M.L., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R.,
468 Martyniuk, C.J., Munkittrick, K.R., 2015. Whole organism responses and intersex severity
469 in rainbow darter (Etheostoma caeruleum) following exposures to municipal wastewater in
470 the Grand River basin, ON, Canada. Part A. Aquat. Toxicol. 159, 290–301.
471 https://doi.org/10.1016/j.aquatox.2014.11.023

472 Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models
473 using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01

474 Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: Life with
475 variable oxygen availability. Annu. Rev. Physiol. 69, 145–170.
476 https://doi.org/10.1146/annurev.physiol.69.031905.162529

477 Borowiec, B.G., Darcy, K.L., Gillette, D.M., Scott, G.R., 2015. Distinct physiological strategies
478 are used to cope with constant hypoxia and intermittent hypoxia in killifish (Fundulus
479 heteroclitus). J. Exp. Biol. 218, 1198–1211. https://doi.org/10.1242/jeb.114579

480 Borowiec, B.G., Hoffman, R.D., Hess, C.D., Galvez, F., Scott, G.R., 2020. Interspecific variation
481 in hypoxia tolerance and hypoxia acclimation responses in killifish from the family
482 Fundulidae. J. Exp. Biol. 223, jeb209692. https://doi.org/10.1242/jeb.209692

483 Borowiec, B.G., McClelland, G.B., Rees, B.B., Scott, G.R., 2018. Distinct metabolic adjustments
484 arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish
485 (Fundulus heteroclitus). J. Exp. Biol. 221, jeb.190900. https://doi.org/10.1242/jeb.190900

486 Breitburg, D.L., Hondorp, D.W., Davias, L.A., Diaz, R.J., 2009. Hypoxia, Nitrogen, and
487 Fisheries: Integrating effects across local and global landscapes. Ann. Rev. Mar. Sci. 1,
488 329–349. https://doi.org/10.1146/annurev.marine.010908.163754

489 Brooks, B.W., Riley, T.M., Taylor, R.D., 2006. Water quality of effluent-dominated ecosystems:
490 ecotoxicological, hydrological, and management considerations. Hydrobiologia. 556, 365–
491 379. https://doi.org/10.1007/s10750-004-0189-7

492 Bugel, S.M., White, L.A., Cooper, K.R., 2010. Impaired reproductive health of killifish
493 (Fundulus heteroclitus) inhabiting Newark Bay, NJ, a chronically contaminated estuary.
494 Aquat. Toxicol. 96, 182–193. https://doi.org/10.1016/j.aquatox.2009.10.016

495 Burleson, M.L., Milsom, W.K., 1990. Propranolol inhibits O_2-sensitive chemoreceptor activity in
496 trout gills. Am. J. Physiol. Regul. Integr. Comp. Physiol. 258, R1089–R1091.
497 https://doi.org/10.1152/ajpregu.1990.258.4.r1089

498 Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G. V., Cohen, S., Di Giulio, R.T., Evans, D.H.,
499 Gómez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L.,
500 Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D.,
501 Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn,
502 R.N., Crawford, D.L., 2007. Fundulus as the premier teleost model in environmental
503 biology: Opportunities for new insights using genomics. Comp. Biochem. Physiol. D
504 Genomics Proteom. 2, 257–286. https://doi.org/10.1016/j.cbd.2007.09.001

505 Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to
506 nutrient dynamics in aquatic systems. Environ. Manage. 44, 205–217.
507 https://doi.org/10.1007/s00267-009-9309-5

508 Cazenave, J., Bacchetta, C., Rossi, A., Ale, A., Campana, M., Parma, M.J., 2014. Deleterious
509 effects of wastewater on the health status of fish: A field caging study. Ecol. Indic. 38, 104–
510 112. https://doi.org/10.1016/j.ecolind.2013.10.029

511 Chan, W.K., Yao, G., Gu, Y.Z., Bradfield, C.A., 1999. Cross-talk between the aryl hydrocarbon
512 receptor and hypoxia inducible factor signaling pathways. J. Biol. Chem. 274, 12115–
513 12123. https://doi.org/10.1074/jbc.274.17.12115

514 City of Hamilton, 2019. Wastewater Collection and Treatment Profile.
515 https://www.hamilton.ca/home-property-and-development/water-sewer/wastewater-
516 collection-treatment (accessed 4.9.20).

517 Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: A

518 critical review of the evidence for health effects in fish. Crit. Rev. Toxicol. 40, 287–304.
519 https://doi.org/10.3109/10408440903373590

520 Crawford, D.L., Schulte, P.M., Whitehead, A., Oleksiak, M.F., 2020. Evolutionary physiology
521 and genomics in the highly adaptable killifish (Fundulus heteroclitus). Compr. Physiol. 10,
522 637–671. https://doi.org/10.1002/cphy.c190004

523 Diaz, R.J., 2001. Overview of hypoxia around the world. J. Environ. Qual. 30, 275–281.
524 https://doi.org/10.2134/jeq2001.302275x

525 Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems.
526 Science. 321, 926–929. https://doi.org/10.1126/science.1156401

527 Du, S.N.N., Choi, J.A., McCallum, E.S., McLean, A.R., Borowiec, B.G., Balshine, S., Scott,
528 G.R., 2019. Metabolic implications of exposure to wastewater effluent in bluegill sunfish.
529 Comp. Biochem. Physiol. C Toxicol. Pharmacol. 224, 108562.
530 https://doi.org/10.1016/j.cbpc.2019.108562

531 Du, S.N.N., McCallum, E.S., Vaseghi-Shanjani, M., Choi, J.A., Warriner, T.R., Balshine, S.,
532 Scott, G.R., 2018. Metabolic costs of exposure to wastewater effluent lead to compensatory
533 adjustments in respiratory physiology in bluegill sunfish. Environ. Sci. Technol. 52, 801–
534 811. https://doi.org/10.1021/acs.est.7b03745

535 Fick, J., Lindberg, R.H., Tysklind, M., Larsson, D.G.G.J., 2010. Predicted critical environmental
536 concentrations for 500 pharmaceuticals. Regul. Toxicol. Pharmacol. 58, 516–523.
537 https://doi.org/10.1016/j.yrtph.2010.08.025

538 Fitzgerald, J.A., Urbina, M.G., Rogers, N.J., Bury, N.R., Katsiadaki, I., Wilson, R.W., Santos,
539 E.M., 2019. Sublethal exposure to copper supresses the ability to acclimate to hypoxia in a
540 model fish species. Aquat. Toxicol. 217, 105325.
541 https://doi.org/10.1016/j.aquatox.2019.105325

542 Fleming, C.R., Billiard, S.M., Di Giulio, R.T., 2009. Hypoxia inhibits induction of aryl
543 hydrocarbon receptor activity in topminnow hepatocarcinoma cells in an ARNT-dependent
544 manner. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 150, 383–389.
545 https://doi.org/10.1016/j.cbpc.2009.06.003

546 Fontaínhas-Fernandes, A., Luzio, A., Garcia-Santos, S., Carrola, J., Monteiro, S., 2008. Gill

547 histopathological alterations in Nile tilapia, oreochromis niloticus exposed to treated sewage
548 water. Brazilian Arch. Biol. Technol. 51, 1057–1063. https://doi.org/10.1590/S1516-
549 89132008000500023

550 Fraysse, B., Mons, R., Garric, J., 2006. Development of a zebrafish 4-day embryo-larval
551 bioassay to assess toxicity of chemicals. Ecotoxicol. Environ. Saf. 63, 253–267.
552 https://doi.org/10.1016/j.ecoenv.2004.10.015

553 Friedrich, J., Janssen, F., Aleynik, D., Bange, H.W., Boltacheva, N.A., Cağatay, M.N., Dale,
554 A.W., Etiope, G., Erdem, Z., Geraga, M., Gilli, A., Gomoiu, M.T., Hall, P.O.J., Hansson,
555 D., He, Y., Holtappels, M., Kirf, M.K., Kononets, M.Y., Konovalov, S.K., Lichtschlag, A.,
556 Livingstone, D.M., Marinaro, G., Mazlumyan, S.A., Naeher, S., North, R.P.,
557 Papatheodorou, G., Pfannkuche, O., Prien, R.D., Rehder, G., Schubert, C.J., Soltwedel, T.,
558 Sommer, S., Stahl, H., Stanev, E.V., Teaca, A., Tengberg, A., Waldmann, C., Wehrli,
559 B.,Wenzhöfer, F., 2014. Investigating hypoxia in aquatic environments: diverse approaches
560 to addressing a complex phenomenon. Biogeosciences. 11, 1215–1259.
561 https://doi.org/10.5194/bg-11-1215-2014

562 Fu, S.J., Brauner, C.J., Cao, Z.D., Richards, J.G., Peng, J.L., Dhillon, R., Wang, Y.X., 2011. The
563 effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and
564 swimming performance in goldfish (Carassius auratus). J. Exp. Biol. 214, 2080–2088.
565 https://doi.org/10.1242/jeb.053132

566 Hochachka, P.W., Buck, L.T., Doll, C.J., Land, S.C., 1996. Unifying theory of hypoxia
567 tolerance : Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack.
568 Proc. Natl. Acad. Sci. 93, 9493–9498. https://doi.org/10.1073/pnas.93.18.9493

569 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton,
570 H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S.
571 streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.
572 https://doi.org/10.1021/es011055j

573 Kraemer, L.D., Schulte, P.M., 2004. Prior PCB exposure suppresses hypoxia-induced up-
574 regulation of glycolytic enzymes in Fundulus heteroclitus. Comp. Biochem. Physiol. C
575 Toxicol. Pharmacol. 139, 23–29. https://doi.org/10.1016/j.cca.2004.08.015

576 Kramer, D.L., McClure, M., 1982. Aquatic surface respiration, a widespread adaptation to
577 hypoxia in tropical freshwater fishes. Environ. Biol. Fishes. 7, 47–55.
578 https://doi.org/10.1007/BF00011822

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

583 Mehdi, H., Lau, S.C., Synyshyn, C., Salena, M.G., McCallum, E.S., Muzzatti, M.M, Bowman,
584 J.E., Mataya, K., Bragg, L.M., Servos, M.R., Kidd, K.K., Scott, G.R. Balshine, S., 2021.
585 Municipal wastewater as an ecological trap: Effects on fish communities across seasons.
586 Sci. Total Environ. 759, 143430. https://doi.org/10.1016/j.scitotenv.2020.143430

587 Mehdi, H., Dickson, F.H., Bragg, L.M., Servos, M.R., Craig, P.M., 2018. Impacts of wastewater
588 treatment plant effluent on energetics and stress response of rainbow darter (Etheostoma
589 caeruleum) in the Grand River watershed. Comp. Biochem. Physiol. B Biochem. Mol. Biol.
590 224, 270–279. https://doi.org/10.1016/j.cbpb.2017.11.011

591 Metcalfe, C.D., 2013. Pharmaceutical contaminants of emerging concern in the environment.
592 Environ. Toxicol. Chem. 32, 1683–1684. https://doi.org/10.1002/etc.2293

593 Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010.
594 Antidepressants and their metabolites in municipal wastewater, and downstream exposure
595 in an urban watershed. Environ. Toxicol. Chem. 29, 79–89. https://doi.org/10.1002/etc.27

596 Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral
597 drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada.
598 Environ. Toxicol. Chem. 22, 2881–2889. https://doi.org/10.1897/02-627

599 Panlilio, J.M., Marin, S., Lobl, M.B., McDonald, M.D., 2016. Treatment with the selective
600 serotonin reuptake inhibitor, fluoxetine, attenuates the fish hypoxia response. Sci. Rep. 6,
601 31148. https://doi.org/10.1038/srep31148

602 Payan, P., Girard, J.P., 1977. Adrenergic receptors regulating patterns of blood flow through the
603 gills of trout. Am. J. Physiol. 232, H18-H23.
604 https://doi.org/10.1152/ajpheart.1977.232.1.H18

Pelster, B., Egg, M., 2018. Hypoxia-inducible transcription factors in fish: expression, function and interconnection with the circadian clock. J. Exp. Biol. 221, jeb163709. https://doi.org/10.1242/jeb.163709

Pérez-Maceira, J.J., Mancebo, M.J., Aldegunde, M., 2012. Serotonin-induced brain glycogenolysis in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 215, 2969-2979. https://doi.org/10.1242/jeb.070649

Perry, S.F., Jonz, M.G., Gilmour, K.M., 2009. Chapter 5 Oxygen sensing and the hypoxic ventilatory response, in: Fish Physiology. Academic Press, pp. 193–253. https://doi.org/10.1016/S1546-5098(08)00005-8

Perry, S.F., Tzaneva, V., 2016. The sensing of respiratory gases in fish: Mechanisms and signalling pathways. Respir. Physiol. Neurobiol. 224, 71–79. https://doi.org/10.1016/j.resp.2015.06.007

Pollock, M.S., Clarke, L.M.J., Dubé, M.G., 2007. The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environ. Rev. 15, 1–14. https://doi.org/10.1139/a06-006

Rasband, W.S., 2008. Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nij.gov/ij/

R Core Team, 2019. R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Regan, M.D., Gill, I.S., Richards, J.G., 2017. Metabolic depression and the evolution of hypoxia tolerance in threespine stickleback, Gasterosteus aculeatus. Biol. Lett. 13, 20170392. https://doi.org/10.1098/rsbl.2017.0392

Regan, M.D., Mandic, M., Dhillon, R.S., Lau, G.Y., Farrell, A.P., Schulte, P.M., Seibel, B.A., Speers-Roesch, B., Ultsch, G.R., Richards, J.G., 2019. Don't throw the fish out with the respirometry water. J. Exp. Biol. 222, jeb200253. https://doi.org/10.1242/jeb.200253

Reid, N.M., Proestou, D.A., Clark, B.W., Warren, W.C., Colbourne, J.K., Shaw, J.R., Karchner, S.I., Hahn, M.E., Nacci, D., Oleksiak, M.F., Crawford, D.L., Whitehead, A., 2016. The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. Science. 354, 1305–1308. https://doi.org/10.1126/science.aah4993

Richards, J.G., 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. J. Exp. Biol. 214, 191–199. https://doi.org/10.1242/jeb.047951

Richards, J.G., Farrell, A.P., Brauner, C.J., 2009. Metabolic and molecular responses of fish to hypoxia, in: Fish Physiology: Hypoxia, Volume 27, pp. 426–428. https://doi.org/10.1016/S1546-5098(08)00010-1

Rogers, N.J., Urbina, M.A., Reardon, E.E., McKenzie, D.J., Wilson, R.W., 2016. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). Conserv. Physiol. 4, cow012. https://doi.org/10.1093/conphys/cow012

Silva, G.S. da, Fé, L.M.L., Silva, M. de N.P. da, Val, V.M.F. de A. e, 2017. Ras oncogene and Hypoxia-inducible factor-1 alpha (Hif-1α) expression in the Amazon fish Colossoma macropomum (Cuvier, 1818) exposed to benzo[a]pyrene. Genet. Mol. Biol. 40, 491–501. https://doi.org/10.1590/1678-4685-GMB-2016-0066

Sollid, J., De Angelis, P., Gundersen, K., Nilsson, G.E., 2003. Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. J. Exp. Biol. 206, 3667–3673. https://doi.org/10.1242/jeb.00594

Speers-Roesch, B., Mandic, M., Groom, D.J.E., Richards, J.G., 2013. Critical oxygen tensions as predictors of hypoxia tolerance and tissue metabolic responses during hypoxia exposure in fishes. J. Exp. Mar. Bio. Ecol. 449, 239–249. https://doi.org/10.1016/j.jembe.2013.10.006

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2012.04.028

Vorrink, S.U., Domann, F.E., 2014. Regulatory crosstalk and interference between the xenobiotic and hypoxia sensing pathways at the AhR-ARNT-HIF1α signaling node. Chem. Biol. Interact. 218, 82–88. https://doi.org/10.1016/j.cbi.2014.05.001

Weis, J.S., 2002. Tolerance to environmental contaminants in the mummichog, *Fundulus heteroclitus*. Hum. Ecol. Risk Assess. 8, 933–953. https://doi.org/10.1080/1080-700291905756

Weis, J.S., Weis, P., 1989. Tolerance and Stress in a Polluted Environment. Bioscience. 39, 89–95. https://doi.org/10.2307/1310907

663 Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and
664 biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J.
665 Environ. Manage. 133, 378–387. https://doi.org/10.1016/j.jenvman.2013.12.017

Table 1: Average concentrations of various classes of contaminants and some associated metabolites measured in tank water during wastewater exposures.

Class	Chemical	Concentration (ng/L)
Lipid Regulator	Gemfibrozil	10.0 (±1.4)
	Atorvastatin	10.5 (±3.4)
	p-hydroxy Atorvastatin	26.6(±5.9)
	o-hydroxy Atorvastatin	20.5 (±6.1)
Antiepileptic	Carbamazepine	44.4 (±3.0)
Analgesic	Acetaminophen	27.2 (±26.2)
Stimulant	Caffeine	266.1 (±150.8)
Antibacterial	Triclosan	12.6 (±2.8)
	Sulfamethazine	16.0 (±1.2)
Antibiotic	Monensin	<DL
	Trimethoprim	22.6 (±4.1)
	Lincomycin	7.0 (±1.1)
	Sulfamethoxazole	63.9 (±5.5)
Antidepressant	Fluoxetine	2.2 (±0.6)
	Norfluoxetine	2.2 (±0.6)
	Venlafaxine	89.2 (±6.8)
	Desvenlafaxine	155.3 (±12.9)
NSAID	Ibuprofen	152.5 (±51.1)
	Naproxen	131.2 (±41.5)
	Diclofenac	154.4 (±9.3)
Herbicide	Atrazine	4.6 (±0.2)
Industrial	Bisphenol A	138.3 (±48.0)

Data above represent measurements of samples taken once from each tank replicate in each wastewater treatment group immediately after the renewal of tank water with fresh effluent (see Materials and Methods), shown as means (± SEM) of all measurements made in wastewater (n = 13-14). <DL indicates measurement that was below the detection limit. NSAID: Nonsteroidal anti-inflammatory drug.

Table 2: The effects of hypoxia and/or wastewater exposure on haematological traits.

	Normoxia (20 kPa)		Moderate hypoxia (5 kPa)		Severe hypoxia (2 kPa)	
	Clean	**WW**	**Clean**	**WW**	**Clean**	**WW**
Hct (%)	24.32 ± 0.98 (7)	30.51 ± 3.70* (11)	26.55 ± 1.90 (11)	34.91 ± 1.73* (15)	35.00 ± 1.24† (13)	37.50 ± 1.74 (12)
[Hb] (g/dl)	9.09 ± 0.64 (7)	9.39 ± 0.67 (13)	7.78 ± 0.46 (11)	10.08 ± 0.53* (19)	10.46 ± 0.31 (12)	9.89 ± 0.60 (13)
MCHC (g/dl)	37.48 ± 2.46 (7)	43.33 ± 11.99 (11)	29.73 ± 1.04 (11)	29.93 ± 2.24 (15)	29.41 ± 0.72 (12)	25.98 ± 1.21† (12)

Clean, clean water; WW, wastewater; Hct, haematocrit; [Hb], blood haemoglobin content; MCHC, mean corpuscular haemoglobin concentration. Data are expressed as means ± SEM (*n*). Statistical results of linear models are as follows. Hct: hypoxia effect, $p<0.001$; wastewater effect, $p=0.063$; hypoxia×wastewater, $p=0.279$. [Hb]: hypoxia effect, $p=0.881$; wastewater effect, $p=0.664$; hypoxia×wastewater, $p=0.013$. MCHC: hypoxia effect, $p=0.010$; wastewater effect, $p=0.754$; hypoxia×wastewater, $p=0.230$. Statistical symbols as in Fig. 1.

Figure 1: Chronic exposure to the combination of hypoxia and wastewater reduced standard metabolic rate (MO_2) but not resting MO_2. (**A**) Statistical results of linear models: hypoxia effect, p=0.030; wastewater effect, p=0.066; hypoxia×wastewater, p=0.021. (**B**) Hypoxia effect, p=0.424; wastewater effect, p=0.960; hypoxia×wastewater, p=0.166. *****Significant pairwise difference from clean water controls within an oxygen treatment (p<0.05). † Significant pairwise difference from normoxic controls within clean water or wastewater groups (p<0.05). Data are presented as means ± SEM, with sample sizes indicated within each bar.

Figure 2: Wastewater exposure attenuated the improvement in hypoxia tolerance (as reflected by the time to loss of equilibrium during acute exposure to 0.5 kPa O_2) in response to chronic hypoxia without affecting critical O_2 tension. (**A**) Statistical results of linear models: hypoxia effect, p=0.048; wastewater effect, p=0.836; hypoxia×wastewater, p=0.561. (**B**) Hypoxia effect, p<0.001; wastewater effect, p=0.002; hypoxia×wastewater, p=0.072. Other statistical information as in Figure 1.

Figure 3: Morphological changes in the gills of killifish in response to exposure to hypoxia and/or wastewater. (**A**) Representative images of the gills of killifish exposed to normoxia in clean water, severe hypoxia in clean water, normoxia in wastewater, and severe hypoxia in wastewater. Slides are stained with haematoxylin and eosin. Scale bar represents 50 μm and all images are shown at the same scale. (**B**) Statistical results of linear models: hypoxia effect, p=0.165; wastewater effect, p=0.847; hypoxia×wastewater, p=0.236. (**C**) Hypoxia effect, p=0.451; wastewater effect, p=0.502; hypoxia×wastewater, p=0.392. (**D**) Hypoxia effect, p=0.269; wastewater effect, p=0.719; hypoxia×wastewater, p=0.244. (**E**) Hypoxia effect, p=0.001; wastewater effect, p=0.617; hypoxia×wastewater, p=0.027. Other statistical information as in Figure 1.

Figure 4: The effects of hypoxia and/or wastewater exposure on glycogen content in brain and liver. (**A**) Statistical results of linear models: hypoxia effect, p=0.149; wastewater effect, p=0.706; hypoxia×wastewater, p=0.038. (**B**) Hypoxia effect, p=0.417; wastewater effect, p=0.270; hypoxia×wastewater, p=0.028. Other statistical information as in Figure 1.

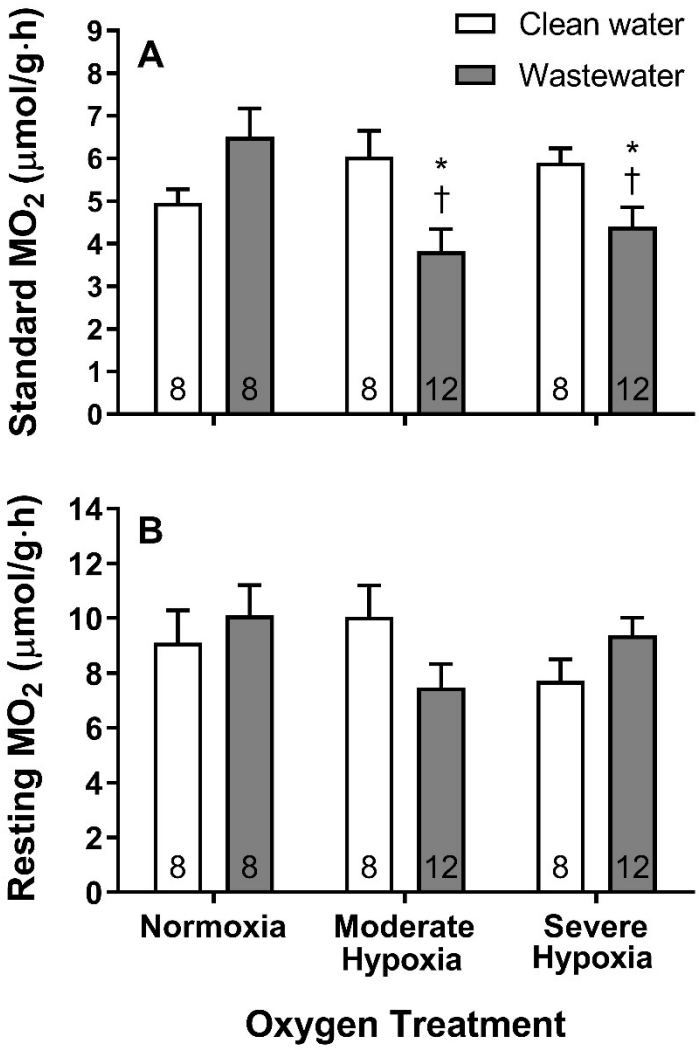
A
Clean water
Wastewater
Standard MO_2 (μmol/g·h)
9
8
7
6
5
4
3
2
1
0
8
8
8
12
8
12
*
†
*
†
B
Resting MO_2 (μmol/g·h)
14
12
10
8
6
4
2
0
8
8
8
12
8
12
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 1.

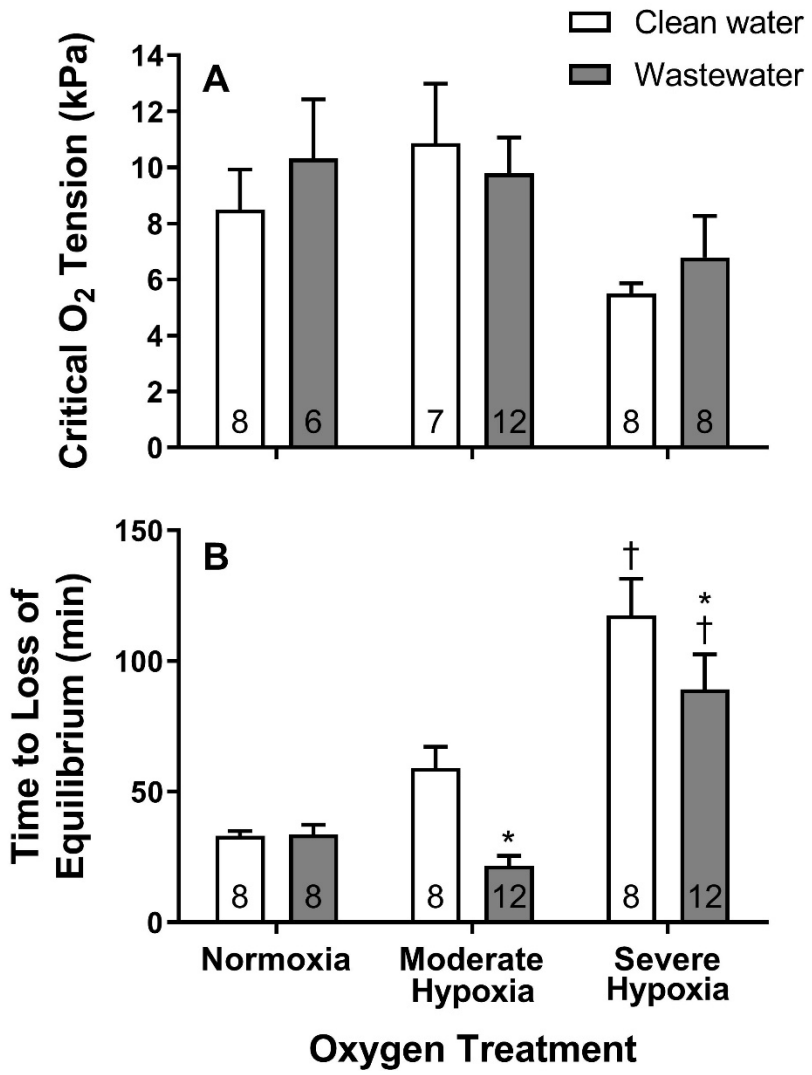
Clean water
Wastewater
A
Critical O_2 Tension (kPa)
14
12
10
8
6
4
2
0
8
6
7
12
8
8
B
Time to Loss of Equilibrium (min)
150
100
50
0
†
*
†
*
8
8
8
12
8
12
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 2.

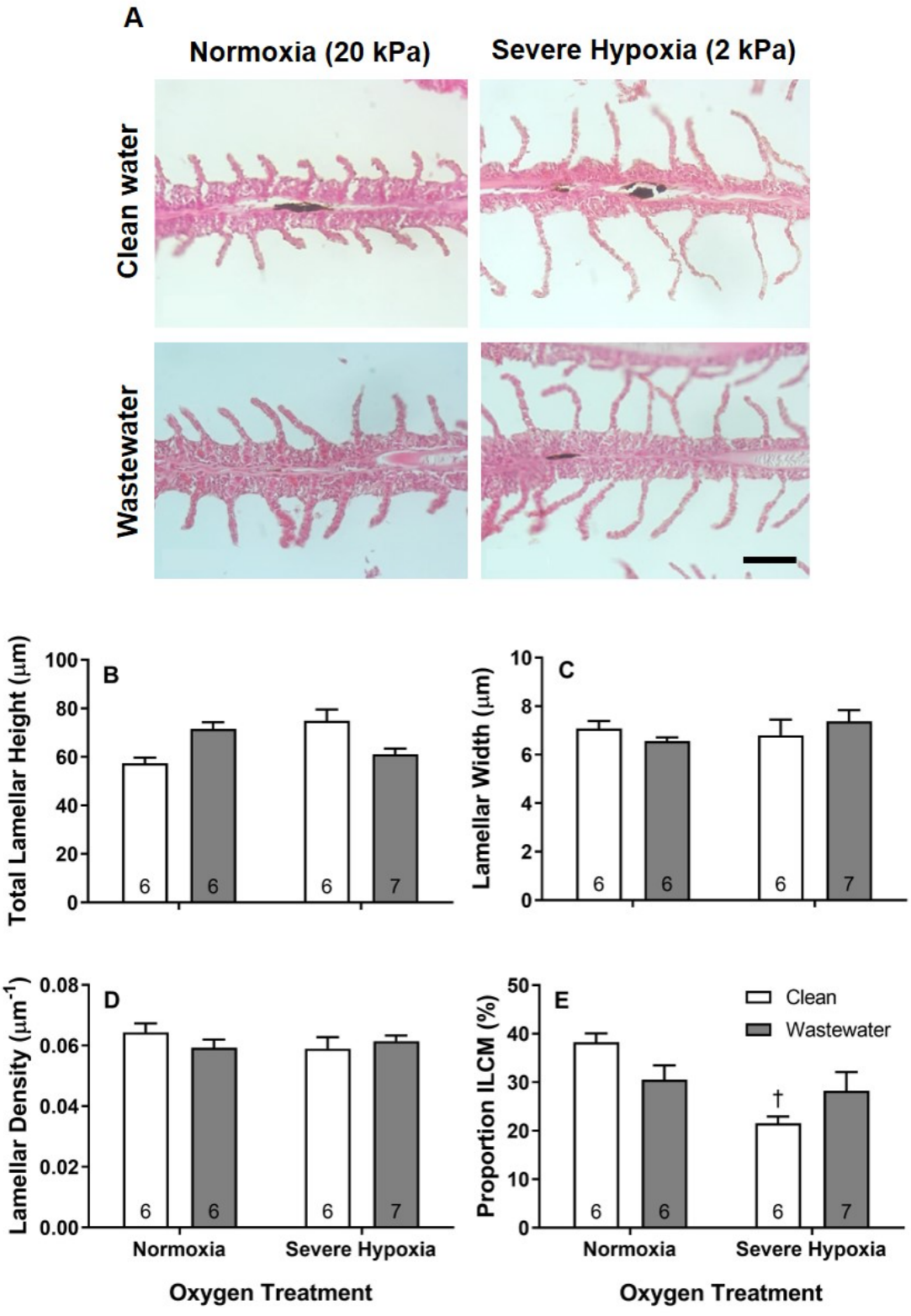
A
Normoxia (20 kPa)
Severe Hypoxia (2 kPa)
Clean water
Wastewater
B
Total Lamellar Height (μm)
C
Lamellar Width (μm)
D
Lamellar Density (μm-1)
E
Proportion ILCM (%)
Clean
Wastewater
†
Normoxia
Severe Hypoxia
Oxygen Treatment

Figure 3.

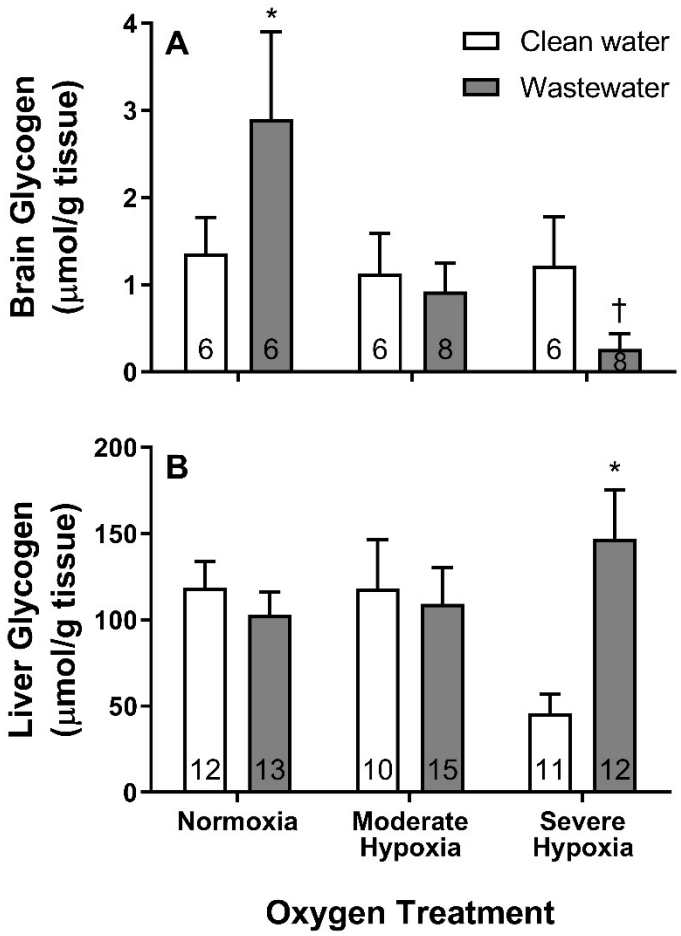
A
Clean water
Wastewater
Brain Glycogen (μmol/g tissue)
4
3
2
1
0
*
†
6
6
6
8
6
8
B
Liver Glycogen (μmol/g tissue)
200
150
100
50
0
*
12
13
10
15
11
12
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 4.