METABOLIC COSTS OF EXPOSURE TO WASTEWATER EFFLUENT LEAD TO COMPENSATORY ADJUSTMENTS IN RESPIRATORY PHYSIOLOGY IN BLUEGILL SUNFISH

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Running title: Wastewater affects fish metabolism and respiration

Abstract

Municipal wastewater effluent is a major source of aquatic pollution and has potential to impact cellular energy metabolism. However, it is poorly understood whether wastewater exposure impacts whole-animal metabolism and whether this can be accommodated with adjustments in respiratory physiology. We caged bluegill sunfish (*Lepomis macrochirus*) for 21 days at two sites downstream (either 50 m or 830 m) from a wastewater treatment plant (WWTP). Survival was reduced in fish caged at both downstream sites compared to an uncontaminated reference site. Standard rates of O_2 consumption increased in fish at contaminated sites, reflecting a metabolic cost of wastewater exposure. Several physiological adjustments accompanied this metabolic cost, including an expansion of the gill surface area available for gas exchange (reduced interlamellar cell mass), a decreased blood-O_2 affinity (which likely facilitates O_2 unloading at respiring tissues), increased respiratory capacities for oxidative phosphorylation in isolated liver mitochondria (supported by increased succinate dehydrogenase, but not citrate synthase, activity), and decreased mitochondrial emission of reactive oxygen species (ROS). We conclude that exposure to wastewater effluent invokes a metabolic cost that leads to compensatory respiratory improvements in O_2 uptake, delivery, and utilization.

1. Introduction

Wastewater treatment plants (WWTP) do not remove all contaminants from wastewater, which leads to the release of a dynamic and complex mixture of contaminants (including pharmaceuticals and personal care products (PPCPs), pesticides, metals, and

42   excess nutrients) into the environment *via* the treated effluent (1-5). Wastewater effluent is

43   a growing concern because many of these compounds are recognized ecological hazards

44   that may threaten the health of aquatic wildlife (6-10). Exposure to single contaminants can

45   impair performance, reproduction, and behaviour in fish (11-19). However, less is known

46   about how fish physiology is impacted by the complex contaminant mixtures that typify

47   wastewater, which could interact in synergistic ways that are hard to predict, particularly

48   when combined with natural variability in environmental conditions (20).

49   Metabolism and respiration provide a powerful lens to understand how

50   contaminants influence energy flow within an organism. Metabolism, respiration, and

51   aerobic scope (the difference between maximal and resting rates of O_2 consumption) are

52   linked to growth, reproduction, activity, functional performance, and many important

53   behaviours (21-26). Exposure to aquatic pollution may require that energy be redirected

54   towards detoxification and cellular protection, particularly in tissues that accumulate

55   contaminants and/or play large roles in detoxification (e.g., liver, 27), and may thus impact

56   whole-animal metabolism. Exposure may constrain these processes, because some

57   contaminants cause mitochondrial dysfunction and impair energy production (28-30).

58   Although some studies have investigated the effects of pollution on energy stores (i.e.

59   concentrations of lipid, glycogen, and protein in tissues; 31-33), the mechanisms and

60   functional implications on higher levels of biological organization (i.e. organ systems and

61   whole-organism) remain unclear. This knowledge gap is best-addressed using integrative

62   sets of bioenergetic markers that provide a mechanistic link between cellular changes and

63   organismal metabolism.

64   Fish are commonly found living in effluent-dominated environments (34), possibly

65   because they are able to invoke compensatory strategies to offset the potential metabolic

66   costs of wastewater exposure. The purpose of our study was to elucidate the impacts of

67   wastewater exposure on whole-animal metabolism and to understand whether fish exhibit

68   effective respiratory and metabolic plasticity to cope with these greater demands, using

69   bluegill sunfish (*Lepomis macrochirus*). Bluegill and other related centrarchid species have

70   been used in previous ecotoxicological studies (29, 35-39) and are native across a wide

71   range of North America (40). Bluegill were exposed to effluent from a residential WWTP

72   that discharges into Cootes Paradise Marsh (Fig. 1), a protected wetland of western Lake

73   Ontario that serves as an important fish breeding ground but is recognized as an

74   International Area of Concern due to historically heavy nutrient and pollution inputs (41).

75   Given previous work on single compounds, we expect that fish exposed to wastewater

76   would incur a metabolic cost. If fish are able to compensate for these increased metabolic

77   demands, then we should observe changes that improve oxygen uptake, transport, and

78   utilization.

79

80   **2. Materials and methods**

81   Methods described here are an abridged version. Additional details are available in the

82   Supporting Information.

83

84   *2.1 Caged exposures*

85   Bluegill sunfish (collected from Lake Opinicon, Ontario, Canada) were caged for

86   21 days in summer 2015 at one of three field locations, two of which exposed fish to

87   effluent from the Dundas WWTP (43º16'2"N 79º56'37"W, Fig. 1). Dundas WWTP is a

88   tertiary treatment plant that serves a population of ~30,000 and treats an average of 15

89   million litres of wastewater each day (42). The treated effluent is the major source of water

90   flowing into Desjardins Canal, which enters West Pond before joining Cootes Paradise

91   Marsh (Fig. 1). We caged fish at sites 50 m (43º16'0"N 79º56'31"W) or 830 m (43º16'9"N

92   79º55'59"W) downstream of the outfall pipe ("outfall" and "downstream" experimental

93   groups, respectively) (Fig. 1). We also caged fish at a control reference site in Beverly

94   Swamp (43º21'57"N 80º6'27"W), which is located within the headwaters for Cootes

95   Paradise Marsh (17.4 km upstream from the outfall and the marsh).

96   Several measures of water quality and contaminant levels were taken during these

97   caged exposures, in conjunction with a parallel study investigating the effects of

98   wastewater exposure on behaviour and physiology of round goby (*Neogobius*

99   *melanostomus*) (43). We found 17 PPCPs at our wastewater-contaminated sites, including

100   a range of antibiotics, antidepressants, beta-blockers, and hormone medications (Table S1).

101   Only six PPCPs were found at our reference site, all at substantially lower concentrations.

102   Water quality (temperature, dissolved oxygen, pH, conductivity, salinity, total dissolved

103   solids, and flow) was also measured during our exposure period (Table S2). A full

104   description of the methods and analyses of these parameters are described by McCallum et

105   al. (43).

106

107   *2.3 Respirometry experiments*

108   We used stop-flow intermittent respirometry (Loligo Systems) to measure standard

109   O_2 consumption rates (M_{O_2}) and hypoxia tolerance in resting fish, using well-established

110   methods (44, 45). Briefly, fish were transferred to respirometry chambers (2.1 l) within 4

111   h of arrival from the field, and were held there overnight (~18 h) with a continuous flow-

112   through supply of aerated dechlorinated tap water at 20ºC. The next morning, resting M_{O_2}

113   was obtained in normoxia (90-100% air saturation). Hypoxia tolerance (critical P_{O_2} and P_{O_2}

114   at loss of equilibrium) was also measured using a stepwise progressive hypoxia protocol

115   that is common in the literature (46). Fish were then euthanized and sampled ~18 h after

116   respirometry measurements.

117

118   *2.4 Tissue contaminants*

119   We pooled samples from all fish within each site and sampling time point to have

120   enough tissue to measure contaminant levels in liver (~0.75 g total tissue) and gills (~1 g

121   total tissue). We measured two synthetic musks (Galaxolide and Tonalide; commonly used

122   to add fragrance to cosmetics and detergents) in the fish sampled after respirometry

123   measurements. We also measured four target pharmaceuticals (sertraline and venlafaxine,

124   both antidepressants, O-dm-venlafaxine, a breakdown product of venlafaxine, and

125   metoprolol, a β-blocker) in a separate set of fish sampled immediately upon removal from

126   caged exposures (see Supporting methods). We extracted and identified these compounds

127   following previously described methods (47-49).

128

129   *2.5 Gill morphometrics*

130   We used stereomicroscopy to analyze gill morphometrics (45, 50). Digital images

131   were taken of all filaments on each of the four arches on one side of the fish, and the lengths

132   and number of filaments on each arch were measured using ImageJ (51). The measured

133 values of total filament number and total filament length (sum of all filament lengths across

134 all four arches) were multiplied by 2 to account for there being two sides of the fish.

135   The first gill arch was prepared for histological analyses after stereomicroscopy.

136 Gills were sectioned using a cryostat and then stained with eosin and haematoxylin.

137 Brightfield microscopy images were taken across the entire gill arch from each fish, and

138 we measured total lamellar height, exposed lamellar height, interlamellar cell mass height,

139 and lamellar thickness for ~8 lamellae using ImageJ (51). Lamellar density was also

140 quantified as the number of lamellae per length of filament. Gill surface density was

141 measured using Nikon NIS-Elements D software (Version 4.30) as the length of total

142 surface per length of filament.

143

144 *2.6 Haemoglobin-O_2 binding*

145   Haemoglobin-O_2 affinity (P_{50}, the P_{O_2} at which haemoglobin is 50% saturated) was

146 determined in lysate of red blood cells using Hemox Analyser and software (TCS

147 Scientific, New Hope, Pennsylvania, USA) at pH 7.0 and 7.4 at a temperature of 25ºC, as

148 recommended by the manufacturer. We calculated pH sensitivity as the difference in P_{50}

149 per unit change in pH.

150

151 *2.7 Mitochondrial physiology*

152   Mitochondria were isolated from liver using established methods that have been

153 described previously (52, 53), and then used for high-resolution respirometry and

154 fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments,

155 Innsbruck, Austria) at 20°C (Supporting Information; Fig. S1). Mitochondrial respiration

7

156   (rate of O_2 consumption) was measured during oxidative phosphorylation (oxphos, P) and

157   during uncoupling to assess electron transport capacity (E). We used substrates of complex

158   I (P_{PM} or E_{PM} with pyruvate, P, and malate, M; P_{PMG} or E_{PMG} with P, M, and glutamate,

159   G), complex II ($P_{S(Rot)}$ or $E_{S(Rot)}$ with succinate, S, and complex I inhibitor rotenone, Rot),

160   and both complexes I and II (P_{PMGS} or E_{PMGS} with P, M, G, S). Rates of reactive oxygen

161   species (ROS) emission were measured fluorometrically, concurrent with oxphos

162   measurements.

163   We also measured lipid peroxidation as a marker of oxidative damage (as the

164   formation of Fe(III)-xylenol orange complex) (53, 54), and the maximal activities (V_{max})

165   of metabolic enzymes citrate synthase (CS) and succinate dehydrogenase (SDH) at 25ºC

166   (53), in isolated liver mitochondria. EROD (ethoxyresorufin-O-deethylase) activity was

167   measured fluorometrically in liver tissue at 25ºC (55, 56).

168

169   *2.11 Statistical analyses*

170   Data were analysed using R (version 3.2.4; 57). Survival was analysed using a

171   binomial generalized linear mixed effects model (GLMM; glmmadmb package, 58). Site

172   and exposure week were set as fixed effects, and cage ID and deployment date were set as

173   random effects. Likelihood ratio tests (LRTs) were used to test for the main effects of site

174   and duration of exposure, followed by Dunnett's *post-hoc* tests (multcomp package, 59) to

175   compare each exposure site to the reference site. All remaining data, unless otherwise

176   noted, were analysed with linear mixed effects model (LMM; lme4 package, 60) using

177   exposure site as a fixed effect, body mass as a covariate, and deployment date as a random

178   effect. LRTs were used to test for the main effects of exposure site and body mass, followed

179   by Dunnett's *post-hoc* tests. In the analyses of M_{O_2} and organ masses (Table S3), the

180   absolute values (mmol O_2 h^{-1} and g, respectively) were used in statistical analyses (because

181   body mass was accounted for as a covariate), but are reported normalized to body mass

182   (i.e., mmol O_2 h^{-1} kg^{-1} and % body mass, respectively) to facilitate comparison with the

183   literature. Mitochondrial respiration and ROS emission were analysed with the additional

184   fixed effects of respiratory state and its interaction with exposure site. Haemoglobin P_{50}

185   was analysed with the additional fixed effects of pH and the interaction between exposure

186   site and pH. In each case, interaction terms were dropped from the LMM if they were not

187   significant. Principal component analysis was used to characterize overall physiological

188   variation across exposure sites (Fig. S2, Table S4). Data are reported as means ± standard

189   error mean (s.e.m.) and results with $p < 0.05$ were considered significant.

190

191   **3. Results**

192   *3.1 Survival*

193   Survival remained high at the reference site (97.5 ± 2.5% survival after 21 days)

194   but was significantly lower at the downstream (70.0 ± 10.2%) and outfall sites (43.5 ±

195   17.5%, Fig. 2). However, body mass of surviving fish was similar across groups (in g:

196   reference, 82.0 ± 8.7; downstream, 81.9 ± 11.7; outfall, 84.6 ± 11.0; LRT_{site} $\chi^2 = 0.027$, p

197   = 0.99).

198

199   *3.2 Markers of contamination*

200   Bluegill exposed to wastewater effluent accumulated the synthetic musks Tonalide

201   and Galaxolide in their tissues, consistent with the overall pattern of waterborne PPCP

202 exposure   (Table   S1).   Galaxolide   (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexa-

203 methylcyclopenta[g]-2-benzopyrane, HHCB) was detected at highest levels at the outfall

204 site (4.97 ng g^{-1} fresh weight), followed by the downstream site (4.35), and was undetected

205 at the reference site, and was found at much higher concentrations in the liver than in the

206 gill (outfall, 0.57; downstream, 0.2; reference, not detected). Tonalide (7-acetyl-

207 1,1,3,4,4,6-hexa-methyl-tetra hydronaphthalene, AHTN) exhibited a similar pattern but

208 was only detected in the liver at the outfall (1.49 ng g fresh weight^{-1}) and downstream (0.7)

209 sites. None of the four pharmaceuticals assayed (venlafaxine and its metabolite O-dm-

210 venlafaxine, sertraline, and metoprolol) were detected in any bluegill from any sites,

211 potentially because the relatively high solubility of these compounds prevented their

212 bioaccumulation (9, 61). EROD activity was similar across fish from the reference (7.45 ±

213 1.32 pmol resorufin min^{-1} mg protein^{-1}, $n = 9$), downstream (4.83 ± 1.66, $n = 8$), and outfall

214 (8.28 ± 2.25, $n = 6$) sites (LRT$_{site}$ $\chi^2 = 3.47$, $p = 0.18$), suggesting that fish were not exposed

215 to aryl hydrocarbons such as polyaromatic hydrocarbons or polychlorinated biphenyls (62).

216

217 *3.3 Metabolism and hypoxia tolerance*

218   Bluegill caged at the downstream and outfall sites exhibited 30-36% higher standard

219 rates of O$_2$ consumption (M$_{O_2}$) than fish caged at the reference site (Fig. 3). However,

220 wastewater exposure did not have a significant effect on hypoxia tolerance. Critical P$_{O_2}$

221 (P$_{crit}$) was similar across sites (in kPa: reference, 3.50 ± 0.19, $n = 10$; downstream, 4.33 ±

222 0.34, $n = 10$; outfall, 4.24 ± 0.81, $n = 7$; LRT$_{site}$ $\chi^2 = 2.45$, $p = 0.29$), as was the P$_{O_2}$ at which

223 fish lost equilibrium during progressive hypoxia (in kPa: reference, 0.494 ± 0.092, $n = 10$;

224 downstream, 0.412 ± 0.088, $n = 7$; outfall, 0.336 ± 0.027, $n = 6$; LRT$_{site}$ $\chi^2 = 1.89$, $p = 0.39$).

225

3.4 Gill morphometrics and histology

Wastewater exposure increased the respiratory surface area of the gills (Fig. 4). The height of exposed lamellae was 20-45% greater in fish from the downstream and outfall sites than those from the reference site (Fig. 4F), due largely to a 17-29% reduction in the height of interlamellar cell mass (Fig. 4E). Fish from the outfall site also had slightly thinner (Fig. 4H) and longer (Fig. 4D) lamellae, whereas fish from the downstream site had a modest increase in lamellar density (Fig. 4G). Collectively, these changes increased gill surface density (i.e., length of gill surface per length of filament) by 22% in fish from the downstream and outfall sites compared to control fish (Fig. 4I). These changes likely increased the overall surface area of the gills, because there were no differences in average filament length (in mm: reference, 3.69 ± 0.21; downstream, 3.60 ± 0.31; outfall, 3.88 ± 0.32; $LRT_{site} \chi^2 = 0.82$, $p = 0.66$), total filament length (in mm: reference, 5179 ± 326; downstream, 4872 ± 523; outfall, 5535 ± 493; $LRT_{site} \chi^2 = 1.34$, $p = 0.51$), and total filament number (reference, 1376 ± 28; downstream, 1335 ± 41; outfall, 1412 ± 38; $LRT_{site} \chi^2 = 1.97$, $p = 0.37$) between sites ($n_{reference} = 10$, $n_{downstream} = 9$, $n_{outfall} = 7$).

3.5 Haematology

Blood-O_2 binding was altered in response to wastewater exposure (Fig. 5). P_{50} (the P_{O_2} at which haemoglobin was 50% saturated) at pH 7.0 was higher in bluegill from the outfall site compared to other groups (Fig. 5A), as was the pH sensitivity of O_2 binding (Fig. 5B). Haematocrit was higher in bluegill from the outfall site (38.0 ± 2.4 %, $n = 7$, $p = 0.023$) than the downstream (31.2 ± 1.6, $n = 10$, $p = 0.97$) and reference sites (31.7 ± 1.7, $n = 10$)

248   (LRT$_{site}$ χ^2 = 8.57, p = 0.014), but blood haemoglobin content did not vary across sites (Fig.

249   5C).

250

251   *3.6 Mitochondrial respiration*

252   Wastewater exposure altered the physiology of liver mitochondria (Fig. 6).

253   Mitochondrial respiratory capacities for oxidative phosphorylation (oxphos, P) were ~10%

254   higher in fish caged at the outfall (Fig. 6A). As expected, there was a significant main effect

255   of mitochondrial substrate on oxphos respiration, with respiration rates generally being

256   higher when supported with substrates of complex I (P_{PM} and P_{PMG}) compared to complex

257   II ($P_{S(Rot)}$), and the highest respiration rates were observed with convergent inputs to both

258   complexes I and II (P_{PMGS}). Associated with the exposure-induced increases in oxphos

259   capacity were increases in succinate dehydrogenase activity, but no change (or a slight non-

260   significant decrease) in citrate synthase activity (Table 1). Wastewater exposure also

261   increased mitochondrial P$_{50}$ (the P$_{O_2}$ at which mitochondrial respiration was reduced by

262   50%) but had no significant effects on respiratory capacities for electron transport (as

263   indicated by respiration in the presence of the uncoupler CCCP) or leak respiration rates

264   with (L_T) or without (L_N) ATP (Table 1).

265

266   *3.7 ROS emission rates and oxidative stress*

267   Rates of mitochondrial ROS emission were reduced by 10-30% in fish exposed to

268   wastewater compared to those from the reference site (Fig. 6B), with higher ROS emission

269   when respiration was supported by substrates of complex I than when supported by

270   substrates of complex II or complexes I and II. The ratios of ROS emission to oxphos

271   respiration were also reduced from ~0.11% in unexposed fish to 0.08% in fish exposed to

272   wastewater at both the downstream and outfall sites (Fig. 6C).

273   We found no evidence of mitochondrial oxidative stress with wastewater exposure

274   (LRT$_{site}$ χ^2 = 0.023, p = 0.99). Levels of lipid peroxidation were similar in liver

275   mitochondria among fish from reference (2.98 ± 0.53 nmol cumene hydroperoxide

276   equivalents mg protein^{-1}, n = 9), downstream (3.05 ± 0.33, p = 0.99, n = 10), and outfall

277   (3.05 ± 0.45, p = 0.99, n = 7) sites.

278

279   **4. Discussion**

280   Here, we show that exposure to wastewater effluent reduces survival of bluegill

281   sunfish (Fig. 2). Exposure also increases standard rates of aerobic metabolism (Fig. 3),

282   which was associated with adjustments across the oxygen transport cascade that expanded

283   the gills' capacity for gas exchange (Fig. 4), facilitated the unloading of O_2 from

284   haemoglobin at the tissues (Fig. 5), and increased the respiratory capacity of liver

285   mitochondria (Fig. 6). There was a significant overall effect of wastewater on physiology

286   when considered using a principal component analysis (Fig. S2, Table S4). These

287   beneficial adjustments in respiratory physiology could help bluegill sunfish cope with the

288   metabolic costs associated with living in polluted environments.

289

290   *4.1 Metabolic costs of wastewater exposure*

291   Our results contribute to growing evidence that exposure to a range of contaminants

292   can increase metabolic rate, as observed in numerous fish species in response to an

293   organochloride pesticide (63), polychlorinated biphenyls (64), and metals (65, 66). Such

294   integrated measures of organismal metabolism, as reflected by the rate of O_2 consumption

295   by the animal, are critical to evaluating whether there is a metabolic cost of contaminant

296   exposure. However, although variation in some subordinate indices of metabolism (e.g.,

297   metabolite concentrations, metabolic enzyme activities) had previously suggested that this

298   might be the case for wastewater exposure (e.g. 33, 67), the issue had rarely been explored

299   at the organismal level. Our previous work suggested that the metabolic cost we observed

300   in bluegill may not occur in all species, because resting M_{O_2} was unaffected in a parallel

301   wastewater exposure study using round goby (*Neogobius melanostomus*; 43), an invasive

302   species that is now established in many parts of the bluegill's natural range (55, 68). It is

303   possible that caging (a necessity for assuring that individuals are continuously exposed and

304   cannot leave the effluent stream) was stressful to fish (69), so it will be valuable to examine

305   in future work whether wild un-caged fish exposed to wastewater also exhibit higher

306   metabolic rates.

307   Increases in metabolism arising from contaminant exposure could impact fitness by

308   reducing aerobic scope (70-72). Aerobic scope, the difference between resting and

309   maximal M_{O_2}, represents the capacity to increase aerobic metabolism to support functions

310   such as reproduction, growth, and behaviour (24). An increase in resting M_{O_2} without a

311   parallel increase in maximal M_{O_2} would reduce aerobic scope (71). This has been observed

312   in rainbow trout exposed to copper (73) and killifish (*Fundulus heteroclitus*) from sites

313   contaminated with polyaromatic hydrocarbons (74). Alternatively, some fish suffer a

314   reduced aerobic scope due to decreases in maximal M_{O_2}, such as observed in common sole

315   (*Solea solea*) exposed to petroleum (75) or in rainbow trout exposed to waterborne

316   aluminum (65). However, it is also possible that fish suffering increases in resting M_{O_2}

317   could maintain (or even increase) aerobic scope with compensatory increases in maximal

318   M_{O_2} (64, 66). Although we did not measure maximal M_{O_2}, the respiratory adjustments of

319   bluegill in response to wastewater exposure (discussed below) suggest that they may be

320   able to increase maximal M_{O_2} and offset reductions in aerobic scope.

321   The changes in metabolism and respiratory physiology that we observed were

322   apparent when fish were tested in clean water. Similarly, rainbow trout exposed to

323   aluminium suffered reduced maximal M_{O_2} and aerobic scope compared to unexposed

324   controls when tested in clean water (65), likely because the persistent physiological effects

325   of exposure were slow to reverse when fish were transferred to clean water for short

326   periods. In our study, testing in clean water was essential for comparing groups in similar

327   conditions to examine the extent to which exposure led to persistent changes in metabolism

328   and physiology. It would be instructive to examine whether the apparent effects of exposure

329   are compounded or otherwise altered if fish are tested in wastewater.

330

331   *4.2 Wastewater exposure enhanced the capacity for O₂ uptake and transport*

332   Bluegill caged in wastewater increased the morphological capacity of the gills for

333   gas exchange. This expansion of gill surface area appeared largely as a consequence of

334   reductions in the interlamellar cell masses (ILCM) that increased the length of exposed

335   lamellae (Fig. 4). ILCM remodelling is a highly plastic trait, allowing organisms to respond

336   quickly to environmental stressors that increase the demand for O_2 uptake (76). It is likely

337   that the combined effects of increases in metabolism and the slightly higher water

338   temperatures near the WWTP contributed to the expansion of gill surface area that we

339   observed (77). The observed increases in gill surface area may come at the expense of

340   augmented ionoregulatory demands (due to the so called "osmorespiratory compromise";

341   78) and greater uptake of environmental contaminants through the gills (79-82). Some fish

342   species instead reduce respiratory surface as a protective mechanism to limit contaminant

343   uptake (83-85), which could reduce maximal O_2 uptake and aerobic scope (65), but that

344   clearly did not occur in the present study.

345   Bluegill also responded to wastewater exposure by modulating haemoglobin-O_2

346   binding affinity of the blood. Haemoglobin-O_2 affinity balances the demands of O_2 loading

347   and uptake at the gills (which is facilitated by an increase in affinity) and O_2 unloading at

348   the tissues (which is facilitated by a decrease in affinity) (86). In situations when respiratory

349   O_2 uptake is not compromised, a lower haemoglobin-O_2 affinity is expected to augment O_2

350   transport to tissues by increasing the P_{O_2} of blood passing through the capillaries.

351   Therefore, the increase in haemoglobin P_{50} at low pH in bluegill exposed to wastewater

352   likely facilitates O_2 transport to respiring tissues (where the blood becomes more acidic),

353   while the expansion of gill surface area helps safeguard branchial O_2 loading into the blood.

354   This appears to be an alternative strategy to improve O_2 transport than increasing

355   haemoglobin content (84).

356

357   *4.3 Wastewater exposure altered mitochondrial function*

358   Wastewater exposure increased the respiratory capacities for oxidative

359   phosphorylation of liver mitochondria (Fig. 6). The observed increases occurred in concert

360   with a change in the relative activity of succinate dehydrogenase, but not citrate synthase

361   (Table 1). Enhancements in mitochondrial respiratory capacity and enzyme activities are

362   known to contribute to seasonal variation in aerobic capacity in red muscle of rainbow trout

363   (87). The similar increases we observed in this study could increase the liver's capacity for

364   mitochondrial respiration and ATP synthesis, especially when combined with increases in

365   organ size (Table S3), possibly to support the energetic demands of detoxification (88).

366   Changes in mitochondrial quality in response to wastewater exposure were also

367   associated with reductions in the inherent rate of mitochondrial ROS emission (Fig. 6).

368   Oxidative stress is a common consequence of wastewater exposure in numerous fish

369   species (89-92), and may contribute to the metabolic costs of exposure because energy is

370   required to repair and replace damaged macromolecules (93). Compensatory adjustments

371   to reduce oxidative stress could foreseeably arise by reducing the inherent rate of ROS

372   production in the mitochondria or cytosol, or by increasing the activity of cellular

373   antioxidant systems. Although the latter is a common biomarker of pollutant exposure (94),

374   few studies have examined whether exposure is associated with compensatory reductions

375   in mitochondrial ROS production that minimize oxidative stress. The reductions in

376   mitochondrial ROS emission observed here may have contributed to the low incidence of

377   lipid peroxidation in liver mitochondria of bluegill exposed to wastewater. However,

378   caging itself has been shown to affect cellular ROS production in fish (95), so it will be

379   useful to examine whether similar effects on mitochondrial ROS emission are observed in

380   wild fish exposed to wastewater.

381   The apparent improvement in mitochondrial quality in bluegill exposed to

382   wastewater stands in contrast to some other studies, in which contaminant exposure

383   impaired mitochondrial respiration. Numerous environmental contaminants, especially

384   metals, are known to disrupt mitochondrial function by impairing activities of respiratory

385   complexes, thereby reducing aerobic capacity (96-101). Alternatively, contaminants can

386 uncouple oxidative phosphorylation (102), which could increase respiration rates needed

387 to offset proton leak, and thus reduce phosphorylation efficiency. It is worth noting that the

388 vast majority of studies that investigated mitochondrial toxicity applied contaminants

389 directly to mitochondria (rather than exposing the whole animal), so the mitochondria in

390 our study likely encountered much lower and environmentally relevant contaminant

391 concentrations.

392

393 *4.4 Water quality*

394 Inter-site differences in water quality (Table S2) were unlikely to drive most of the

395 physiological differences we observed. Dissolved O_2 and salinity were in a normal range

396 and the magnitude of variation was modest, so these parameters are not anticipated to

397 induce the observed variation in M_{O_2}, gill structure, or mitochondrial respiratory capacity

398 (45, 52, 103-105). Acclimatization to higher temperatures at the downstream and outfall

399 sites would tend to reduce resting M_{O_2} and mitochondrial respiratory capacity when tested

400 at a common temperature, as they were in this study (106, 107), opposite to the differences

401 observed here. However, as described above, it is possible that these higher temperatures

402 could have contributed to the increase in gill respiratory surface in fish at these sites (104).

403 Otherwise, inter-site variation in water quality is expected to have had little effect, and may

404 have even dampened some the physiological responses to wastewater exposure.

405

406 *4.5 Metabolism and respiration as ecotoxicological tools*

407   Understanding bioenergetics under contaminant stress can reveal potential trade-

408 offs in allocation of a finite pool of energy, which can have important implications on

409   organismal and population-level function, giving reason for its application as an

410   ecotoxicological bioindicator over the past three decades (33, 108-111). However, joint

411   consideration of both organismal metabolism and the respiratory physiology that supports

412   this metabolism is infrequent in aquatic toxicology. Consideration of the impacts of

413   contaminants along the oxygen transport cascade and across multiple levels of biological

414   organization helps elucidate mechanistic linkages between subcellular energetics and

415   whole-organismal performance, and thus represents an integrated approach to

416   understanding how fish are coping in modern environments (112-114). Metabolism and

417   respiration are major themes for research into how animals cope with metabolically

418   challenging environmental stressors (e.g., hypoxia, rising temperatures, salinity; reviews

419   by 71, 115), and a similar approach could be used to better understand responses to

420   wastewater stress. We show that metabolism and respiration are indeed sensitive to

421   wastewater exposure in bluegill, invoking a suite of alterations – from biochemistry to

422   whole organism – that improve oxygen uptake, transport, and utilization. Such mechanistic

423   approaches can improve our understanding of and capacity to predict the impacts of aquatic

424   pollution at organismal and population levels, and should thus be considered as an

425   ecologically relevant bioindicator in aquatic toxicology.

426

427   **Supporting information**

428   **S1.** Detailed Material and Methods

429   **Fig. S1.** Representative experiments of mitochondria isolated from liver of bluegill

430   sunfish to measure (A) respiration during oxidative phosphorylation and (B) electron

431   transport capacity during uncoupled respiration.

432 **Fig. S2.** The effects of exposure site on the first two principal components from a

433 principal component analysis (PCA).

434 **Table S1.** Average estimated time-weighted concentrations of waterborne

435 pharmaceuticals and personal care products at a clean reference site, near the outfall of a

436 tertiary wastewater treatment plant, or further downstream.

437 **Table S2.** Water quality measures taken during caged exposures.

438 **Table S3.** Body and organ mass (% body mass) of bluegill sunfish

439 **Table S4.** Loadings onto the first two principal components from a principal component

440 analysis.

441

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453

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817 **Tables**

818
819 **Table 1.** Properties of mitochondria isolated from the liver of bluegill sunfish. Data are
820 presented as mean ± s.e.m. (*n*).

	Reference	Downstream	Outfall
Succinate dehydrogenase (SDH, μmol mg protein^{-1} min^{-1})	0.013 ± 0.001 (9)	0.015 ± 0.002 (10)	0.017 ± 0.002* (6)
Citrate synthase (CS, μmol mg protein^{-1} min^{-1})	0.141 ± 0.015 (8)	0.118 ± 0.008 (10)	0.127 ± 0.014 (6)
P_{50} (kPa)	0.033 ± 0.003 (9)	0.046 ± 0.004* (9)	0.047 ± 0.002* (7)
Respiratory capacity for electron transport (E, pmol O_2 mg protein^{-1} s^{-1})			
E_{PM} (Complex I)	458.5 ± 33.4 (9)	464.7 ± 39.7 (9)	464.2 ± 54.7 (7)
E_{PMG} (Complex I)	638.9 ± 51.8 (9)	618.8 ± 41.3 (9)	657.5 ± 62.3 (7)
$E_{S(Rot)}$ (Complex II)	429.1 ± 36.7 (9)	450.7 ± 30.7 (9)	510.2 ± 40.7 (7)
E_{PMGS} (Complex I+II)	754.9 ± 58.0 (9)	740.8 ± 48.7 (9)	797.7 ± 74.7 (7)
Leak respiration with ATP (L_T, pmol O_2 mg protein^{-1} s^{-1})	310.5 ± 33.7 (9)	278.8 ± 17.2 (9)	259.3 ± 23.9 (7)
Leak respiration without ATP (L_N, pmol O_2 mg protein^{-1} s^{-1})	38.47 ± 5.25 (9)	38.07 ± 3.81 (9)	40.31 ± 4.81 (7)

821 * represents a significant difference from reference site; SDH, LRT$_{site}$ $\chi^2 = 6.81$, $p =$

822 0.033 (downstream, $p = 0.066$; outfall, $p = 0.044$); CS, LRT$_{site}$ $\chi^2 = 2.37$, $p = 0.31$; P_{50},

823 LRT$_{site}$ $\chi^2 = 9.81$, $p = 0.007$ (downstream, $p = 0.012$; outfall, $p = 0.013$); E, LRT$_{site}$ $\chi^2 =$

824 3.34, $p = 0.19$; LRT$_{state}$ $\chi^2 = 72.9$, $p < 0.0001$ (downstream, $p = 0.98$; outfall, $p = 0.20$);

825 L_T, LRT$_{site}$ $\chi^2 = 2.14$, $p = 0.34$; L_N, LRT$_{site}$ $\chi^2 = 1.20$, $p = 0.55$. Respiratory capacity for

826 electron transport (E) was assessed with substrates of complex I (E_{PM} with pyruvate, P,

827 and malate, M; E_{PMG} with P, M, and glutamate, G), complex II ($E_{S(Rot)}$ with succinate, S,

828 and complex I inhibitor rotenone, Rot), and both complexes I and II (E_{PMGS} with P, M, G,

829 S).

830

831 **Figure legends**

832

833 **Fig. 1. Location of study area and sites of caged exposures.** Bluegill sunfish were

834 caged for 21 days (1) 50 m from the outfall of a wastewater treatment plant (WWTP) that

835 provides tertiary treatment to the municipality of Dundas, (2) 830 m further downstream

836 in an effluent-dominated canal, or (3) at a clean reference site (17.4 km northwest of the

837 WWTP in Flamborough, ON, Canada).

838

839 **Fig. 2. Wastewater exposure decreased survival of bluegill sunfish.** Bluegill sunfish

840 were caged at the outfall of a wastewater treatment plant, further downstream, or at an

841 uncontaminated reference site for 21 days. * represents significant differences between

842 fish from the reference site (LRT_{site} χ^2 = 14.23, p = 0.0008; LRT_{week} χ^2 = 3.73, p = 0.15;

843 Dunnett's *post-hoc*: downstream, p = 0.015; outfall, p = 0.002).

844

845 **Fig. 3. Standard rates of O₂ consumption increased in resting bluegill sunfish**

846 **exposed to wastewater effluent.** Bluegill caged at the downstream (n = 10) and outfall

847 sites (n = 7) had significantly higher rates of aerobic metabolism than fish caged at the

848 reference site (n = 10). *represents significant differences from the reference site (LRT_{site}

849 χ^2 = 8.37, p = 0.015; Dunnett's *post-hoc*: downstream, p = 0.038; outfall, p = 0.012)

850

851 **Fig. 4. Bluegill remodelled their gills in response to wastewater exposure.**

852 Representative images of gills from bluegill caged at (**A**) reference, (**B**) downstream, and

853 (**C**) outfall sites for 21 days (scale bar represents 1 mm). (**D**) Total lamellar height was

854 highest in bluegill from the outfall site (LRT$_{site}$ χ^2 = 4.18, p = 0.12; Dunnett's *post-hoc*:

855 downstream, p = 0.62; outfall, p = 0.039). (**E**) Height of interlamellar cell mass (ILCM)

856 was lower after wastewater exposure (LRT$_{site}$ χ^2 = 6.63, p = 0.036), and the reductions in

857 the downstream and outfall sites approached statistical significance in Dunnett's *post-hoc*

858 tests (downstream, p = 0.057; outfall, p = 0.053). (**F**) Exposed lamellar height (the

859 difference between heights of total lamellae and ILCM) increased in bluegill caged at

860 downstream and outfall sites (LRT$_{site}$ χ^2 = 22.3, p < 0.0001; Dunnett's *post-hoc*:

861 downstream, p = 0.001; outfall, p < 0.0001). (**G**) Lamellar density (LRT$_{site}$ χ^2 = 8.50, p =

862 0.014; Dunnett's *post-hoc*: downstream, p = 0.006; outfall, p = 0.32) and (**H**) lamellar

863 thickness (LRT$_{site}$ χ^2 = 6.34, p = 0.04; Dunnett's *post-hoc*: downstream, p = 0.94; outfall,

864 p = 0.02) varied with caging exposures. (**I**) Gill surface density increased in bluegill

865 caged at the downstream and outfall sites (LRT$_{site}$ χ^2 = 19.1, p < 0.0001; Dunnett's *post-*

866 *hoc*: downstream, p < 0.0001; outfall, p < 0.0001). *represents significant differences

867 from the reference site ($n_{reference}$ = 9, $n_{downstream}$ = 9, $n_{outfall}$ = 7)

868

869 **Fig. 5. Haemoglobin-oxygen binding affinity was reduced in response to wastewater**

870 **exposure at the outfall site.** (**A**) The P$_{50}$ of haemoglobin (the partial pressure of oxygen

871 at which haemoglobin is 50% saturated) was measured in the lysate of frozen red blood

872 cells and was highest in bluegill caged at the outfall site at pH 7.0 (LRT$_{site}$ χ^2 = 20.2, p <

873 0.0001; Dunnett's *post-hoc*: downstream, p = 0.63; outfall, p = 0.0003; $n_{reference}$ = 7,

874 $n_{downstream}$ = 6, $n_{outfall}$ = 7). P$_{50}$ was higher at pH 7.0 compared to pH 7.4 (LRT$_{pH}$ χ^2 =

875 139.9, p < 0.0001). (**B**) pH sensitivity of haemoglobin (measured as the change in P$_{50}$

876 between pH 7.0 and 7.4 and normalized to 1.0 pH unit) was significantly higher in

877   bluegill caged at the outfall site (LRT$_{site}$ χ^2 = 7.82, p = 0.020; Dunnett's *post-hoc*:

878   downstream, p = 0.63; outfall, p = 0.013, n the same as above). (**C**) Blood haemoglobin

879   content was similar across all exposure sites (LRT$_{site}$ χ^2 = 0.98, p = 0.61); $n_{reference}$ = 10,

880    $n_{downstream}$ = 10, $n_{outfall}$ = 6). *represents significant differences from the reference site

881

882   **Fig. 6. Wastewater exposure affected the physiology of isolated liver mitochondria.**

883   (**A**) Bluegill had higher respiratory capacities for oxidative phosphorylation after 21 days

884   of exposure to wastewater effluent (LRT$_{site}$ χ^2 = 7.59, p = 0.022; LRT$_{state}$ χ^2 = 83.0, p <

885   0.0001; Dunnett's *post-hoc*: downstream, p = 0.39; outfall, p = 0.011). (**B**) ROS emission

886   rates were reduced in mitochondria from bluegill caged at the downstream and outfall

887   sites (LRT$_{site}$ χ^2 = 24.6, p < 0.0001; LRT$_{state}$ χ^2 = 35.0, p < 0.0001; Dunnett's *post-hoc*:

888   downstream, p < 0.0001; outfall, p < 0.0001). (**C**) ROS emission relative to oxphos

889   respiration were also lower in the downstream and outfall sites (LRT$_{site}$ χ^2 = 31.0, p <

890   0.0001; LRT$_{state}$ χ^2 = 49.7, p < 0.0001; Dunnett's *post-hoc*: downstream, p < 0.0001;

891   outfall, p < 0.0001). Measurements were made during oxidative phosphorylation (oxphos,

892   *P*) with substrates of complex I (CI; P_{PM} with pyruvate, P, and malate, M; P_{PMG} with P,

893   M, and glutamate, G), complex II (CII; $P_{S(Rot)}$ with succinate, S, and complex I inhibitor

894   rotenone, Rot), and both complexes I and II (CI+II; P_{PMGS} with P, M, G, S). *represents

895   significant differences from the reference site ($n_{reference}$ = 9, $n_{downstream}$ = 9, $n_{outfall}$ = 7).

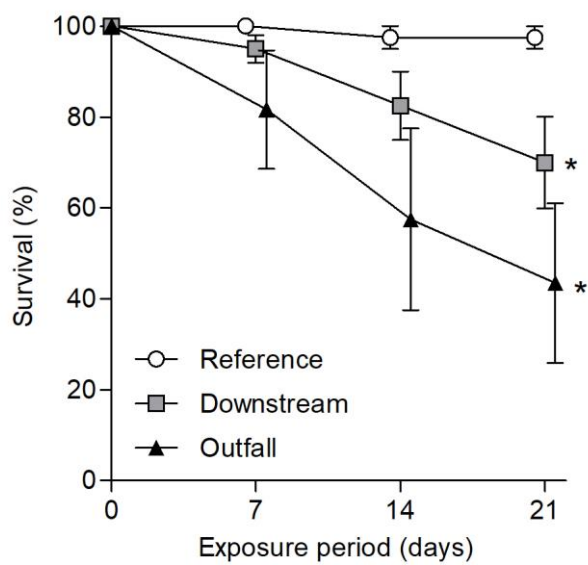896

897   Fig. 1

898

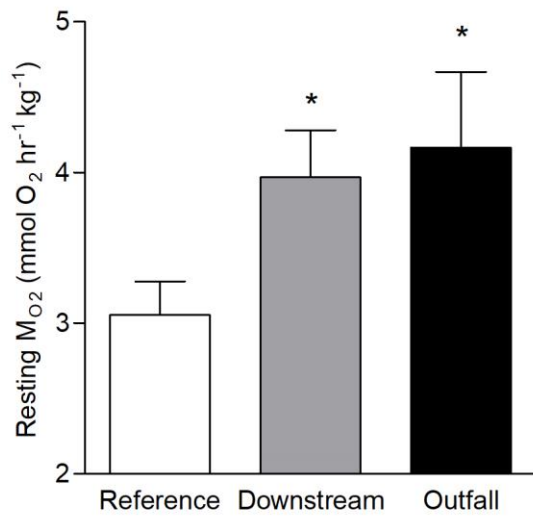899   Fig. 2

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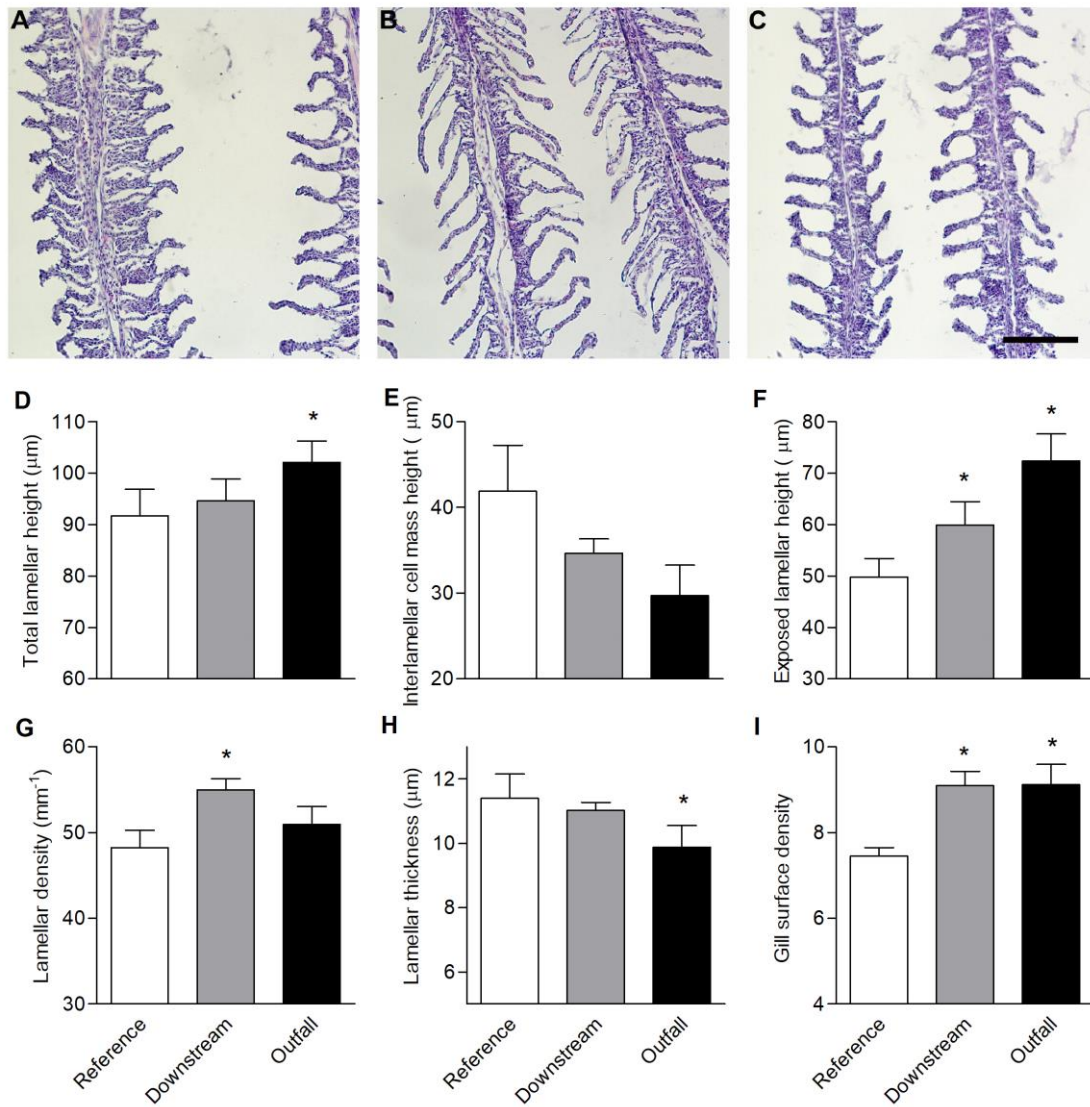
902   Fig. 3

Resting M_{O2} (mmol O_2 hr^{-1} kg^{-1})

Reference   Downstream   Outfall
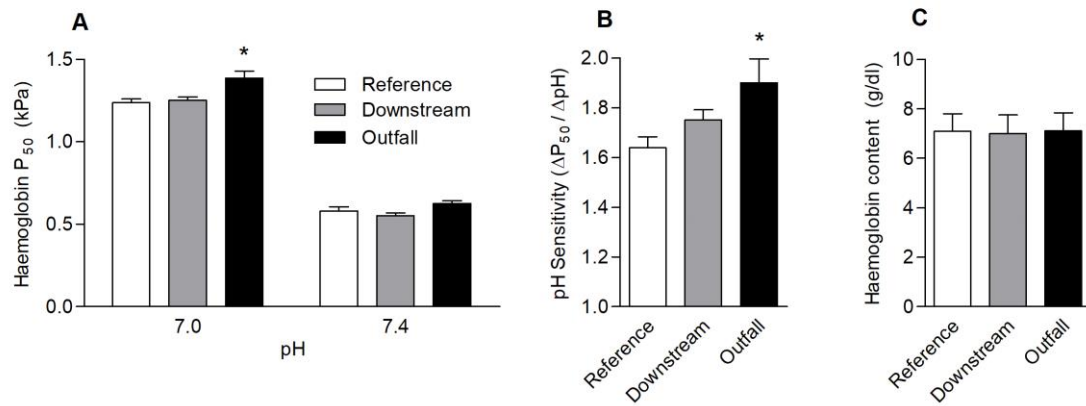
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905   Fig. 4
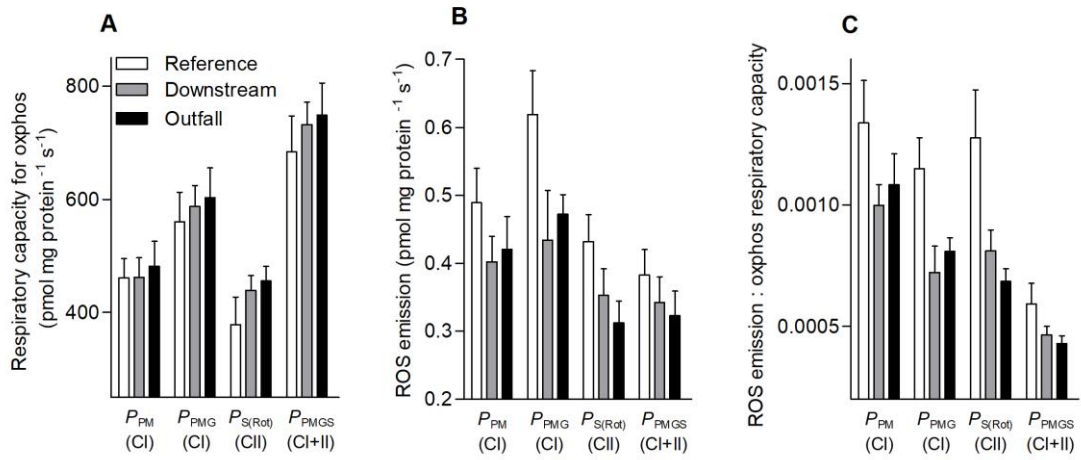

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908   Fig. 5
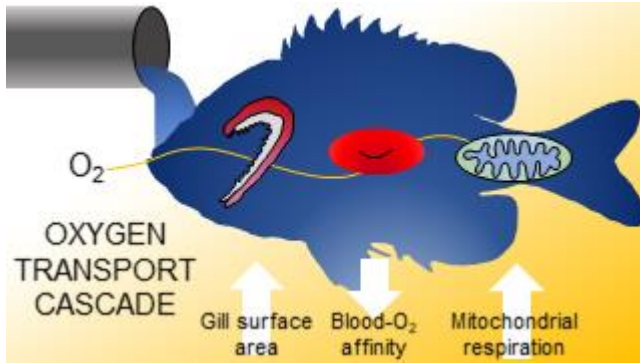

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911   Fig. 6

912

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914   Abstract Art

915

1 METABOLIC COSTS OF EXPOSURE TO

2 WASTEWATER EFFLUENT LEAD TO

3 COMPENSATORY ADJUSTMENTS IN

4 RESPIRATORY PHYSIOLOGY IN

5 BLUEGILL SUNFISH

6 **SUPPORTING INFORMATION**

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13

14 **19 PAGES, 2 FIGURES, 4 TABLES**

15
16

S1. Detailed Material and Methods

S1.1 Fish collection and housing

We caught bluegill sunfish (mean body mass ± s.e.m.: 82.6 ± 5.9 g, range: 34.4-140.3 g) by angling at Lake Opinicon, Ontario, Canada (44.559°N, -76.328°W) in May 2015. Fish were then transported in aerated bins to McMaster University and housed in 500 l flow-through tanks supplied with dechlorinated tap water from City of Hamilton (~18ºC, water quality previously reported (1, 2) with a photoperiod of 12 h:12 h light:dark. Fish were fed four times each week with a mix of commercially purchased beef heart and squid and were held in these conditions for at least two weeks before being caged in the field (see below). All procedures were conducted in accordance with guidelines set out by the Canadian Council on Animal Care, and were approved by the McMaster University Animal Research Ethics Board.

S1.2 Caging exposures

The cages we used to hold the fish were 114 l plastic containers (Rubbermaid®; 81 cm wide, 44.5 cm deep, 51 cm high), drilled with approximately 100 large holes (3 cm diameter) to permit water flow. Each cage was chained to a concrete block to secure the cage in the water column and floats were attached to the lid to help maintain a small (~5 cm) air space. We had five replicate cages per site (starting with 8 fish per cage) and the start date of exposure for each replicate was staggered over five weeks, such that fish from one cage from each site was deployed/tested each week. Therefore, our total exposure

39 period lasted from June 15 to August 6, 2015. During this time, we provided food (a mix

40 of squid and cow heart) to the fish once per week, to supplement the food they received

41 from the water column. After the exposure, fish were transported back to McMaster

42 University in dechlorinated tap water for respirometry experiments (see below) or

43 immediate sampling.

44

45 *S1.3 Hypoxia tolerance*

46   Resting O_2 consumption rates (M_{O_2}) were measured using stop-flow intermittent

47 respirometry at each O_2 tension (P_{O_2}) of a stepwise hypoxia exposure, in which P_{O_2} was

48 reduced every 20 min by 10% air saturation. When O_2 levels reached 10% air saturation,

49 the chambers were closed and fish were allowed to consume the remaining oxygen in the

50 chamber until loss of equilibrium (LOE) was reached (at which point the P_{O_2} at LOE was

51 recorded). Afterwards, the fish was quickly removed from the chamber, allowed to recover,

52 and was maintained in aerated water for ~18 h until they were terminally sampled (see

53 below). The critical P_{O_2} (P_{crit}) was the P_{O_2} at which fish transformed from being an

54 oxyregulator (M_{O_2} is independent of P_{O_2}) to oxyconformer (M_{O_2} is dependent on P_{O_2}), and

55 was calculated using Regress software (3).

56

57 *S1.4 Sampling*

58   Fish were always sampled ~18 h after completing the respirometry experiment.

59 Fish were euthanized with a cerebral concussion, the tail was severed, and blood was

60   immediately collected in heparanized capillary tubes. A portion of blood (6 µl) was used

61   to measure haemoglobin content using Drabkin's reagent (Sigma-Aldrich), and the

62   remaining blood was centrifuged for 2.5 min to measure haematocrit. The packed red blood

63   cells were frozen in liquid N_2 and stored at -80ºC for later measurements of Hb-O_2 binding.

64   Brain, spleen, and liver were excised and weighed. Half of the liver was used immediately

65   for mitochondrial isolation, and the other half was immediately frozen in liquid N_2 and then

66   stored at -80ºC for later use in enzymes assays or contaminant analyses (see below). Gills

67   were carefully removed: one side was frozen in liquid N_2 and stored at -80ºC for later use

68   in contaminant analyses, while the other side was stored in fixative (274 mol l^{-1} NaOH, 30

69   mol l^{-1} Na_2HPO_4, 5.4 mol l^{-1} KCl, 3 mol l^{-1} KH_2PO_4, 2% paraformaldehyde, 2%

70   glutaraldehyde; pH 7.8) for at least 48 h until used for morphometric and histological

71   analyses.

72   A small subset of fish from each site were not used in metabolic or respiratory

73   analyses and were sampled immediately upon arrival at McMaster University from the

74   field for contaminant analyses (reference, $n = 9$; downstream, $n = 4$; outfall, $n = 2$). We

75   excised liver and gills, immediately froze the tissues in liquid N_2, and then stored them at

76   -80ºC until contaminant analyses were conducted (methods below).

77

78   *S1.5 Tissue contaminants*

79   Tissues for pharmaceutical and synthetic musk analyses were freeze dried, then

80   homogenized using a mortar and pestle. Pharmaceuticals were extracted from

81   homogenized tissues by pressurized liquid extraction with an ASE 300 instrument (Dionex,

82   Toronto, Ontario, Canada), diluted with deionized water, and then cleaned using a MCX

83   solid phase extraction cartridge, evaporated, then dissolved in 400 µl of MeOH. A Q-Trap

84   liquid chromatography and tandem mass spectrometry (LC-MS/MS) system with an

85   atmospheric pressure chemical ionization source and a Series 1100 HPLC binary solvent

86   delivery system were used to analyse the sample in positive ion mode. The sample was

87   separated using chromatography (Genesis C18 column). Pharmaceuticals were quantified

88   using an internal standard (based on a nine-point calibration scale).

89   Synthetic musks were extracted from homogenized tissues using pressurized liquid

90   extraction, filtered through 12 g of sodium sulfate, and concentrated using a rotary

91   evaporator to ~1 ml. Gel permeation chromatography was used to isolate the analyte

92   fraction containing the musks, which was then cleaned with Florisil chromatography.

93   Finally, gas chromatography (Varian 3800, Varian, Palo Alto, California, USA) paired

94   with an ion trap mass selective detector (Varian Saturn 2200) were used with electron

95   impact ionization, and operated in selected ion storage mode. Chromatographic retention

96   time and ion ratios were used to identify musks, and peak areas were quantified using an

97   external standard (four-point calibration). One procedural blank was used for each set of

98   tissues (liver or gill) and for each class of contaminants (pharmaceuticals or musks).

99

100   *S1.6 Preparation of gills for histological analyses*

101   After images from all gill arches were collected using stereomicroscopy, the first

102   gill arch was submerged in 30% sucrose (in 137 mol l^{-1} NaOH, 15.2 mol l^{-1} Na_2HPO_4, 2.7

103   mol l^{-1} KCl, 1.5 mol l^{-1} KH_2PO_4; pH 7.8) for ~24 h and then frozen in embedding medium

104 (Shandon Cryomatrix, ThermoFisher Scientific). Frozen blocks were sectioned (5 μm) at -

105 20ºC in a cryostat (Leica CM 1860) and air dried for at least 2 h at room temperature.

106 Sections were stained for hematoxylin and eosin by dehydrating sections in 95% ethanol,

107 incubating in Gills II haematoxylin for ~45 s, and then incubating in eosin for ~15 s, with

108 rinses in distilled water between each step. Sections were then dehydrated in progressively

109 increasing concentrations of ethanol (up to 100%), followed by xylene. Sections were

110 mounted with Permount (Fisher Scientific, Hampton, New Hampshire, USA) and

111 coverslipped. We systematically selected and imaged ~15 sections so as to assure that we

112 analyzed images that were spread throughout and represented the entirety of the first gill

113 arch from each fish. Images were analyzed using a Nikon Eclipse E800 light microscope

114 (Nikon Instruments, Melville, New York, USA). Preliminary measurements verified that

115 this number of images was sufficient to obtain a stable mean value for each trait.

116

117 *S1.7 Haemoglobin-O_2 binding*

118 Haemoglobin O_2 dissociation curves were determined for the lysate of frozen red blood

119 cells using Hemox Analyser and software (TCS Scientific, New Hope, Pennsylvania, USA).

120 Red blood cell lysate (10 μl) was added to 5 ml of assay buffer solution, which consisted

121 of 50 mmol l^{-1} tris, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 0.1% bovine serum albumin

122 (BSA), and 0.2% anti-foaming agent (polydimethylsiloxane emulsion; TCS Scientific).

123

124 *S1.8 Mitochondrial isolation*

125   Fresh liver tissue (~0.5 g) was finely diced in 10 ml of ice-cold isolation buffer (in

126   mmol l^{-1} unless otherwise stated: 250 sucrose, 50 KCl, 25 KH$_2$PO$_4$, 10 hepes, 0.5 EGTA,

127   and 1.5% mass:volume fatty-acid free BSA; pH 7.4). We gently homogenized the tissue

128   on ice with six passes of a loose-fitting Potter-Elvehjem homogenizer at 100 r.p.m. The

129   homogenate was centrifuged at 600g for 10 min at 4°C, and the supernatant was filtered

130   through glass wool, and then centrifuged at 6000g for 10 min at 4°C (the same conditions

131   for all future centrifugations). We gently rinsed and re-suspended the pellet in 10 ml of

132   fresh isolation buffer, then centrifuged. The pellet was then rinsed and re-suspended in 10

133   ml ml of storage buffer (same as the isolation buffer, but without BSA and with 2 mmol l$^-$

134   ^{1} each of pyruvate and malate), and centrifuged again. The final pellet was re-suspended in

135   500 μl of storage buffer. Half of the mitochondrial isolate was stored for a short period on

136   ice until respiration and ROS emission experiments (see below), and the other half was

137   frozen at -80ºC for later assays of lipid peroxidation and enzyme activities (see below).

138   Mitochondrial protein content was measured in the isolate using the Bradford assay (Bio-

139   Rad, Mississauga, ON, Canada).

140

141   *S1.9 Mitochondrial physiology*

142   Respiration (rate of O$_2$ consumption) and ROS emission rates of mitochondria were

143   measured in 2 ml of respiration buffer (in mmol l^{-1}: 110 sucrose, 60 K-lactobionate, 20

144   taurine, 20 Hepes, 10 KH$_2$PO$_4$, 3 MgCl$_2$·6H$_2$O, 0.5 EGTA, 1.5% mass:volume fatty-acid

145   free BSA; pH 7.4). ROS was detected as the fluorescence of resorufin (excitation

146   wavelength of 525 nm and AmR filter set, Oroboros Instruments), which is produced from

147   hydrogen peroxide (H$_2$O$_2$) and Ampliflu Red (Sigma-Aldrich) in a reaction catalysed by

148   horseradish peroxidase. This was accomplished by adding superoxide dismutase (22.5 U

149   ml^{-1}; to catalyse the formation of H_2O_2 from the superoxide produced by mitochondria),

150   horseradish peroxidase (3 U ml^{-1}), and Ampliflu Red (15 µmol l^{-1}) to the respiration buffer.

151   We calibrated the resorufin signal (at the beginning and end of the following protocol) with

152   additions of exogenous H_2O_2 to measure ROS emission as the molar rate of H_2O_2 release

153   from mitochondria.

154   Mitochondrial physiology was measured as follows (Supplementary Fig. 1).

155   Mitochondria (0.6 mg of mitochondrial protein) were added to the respiration buffer, and

156   leak respiration (L_N) was measured with complex I substrates pyruvate and malate (2 mmol

157   l^{-1} each) but without adenylates. We then added 125 µmol l^{-1} ADP, and measured leak

158   respiration in the presence of ATP (L_T) after the mitochondria had converted all of the ADP

159   into ATP. Saturating levels of ADP (1250 µmol l^{-1}) were added to stimulate maximal

160   pyruvate oxidation (P_{PM}). The capacities for oxphos *via* complex I (P_{PMG}) and complexes

161   I+II (P_{PMGS}) were then determined by adding glutamate (10 mmol l^{-1}) then succinate (10

162   mmol l^{-1}), respectively. Oxphos respiration *via* complexes I+II was maintained until all O_2

163   was consumed (to assess mitochondrial O_2 kinetics), and anoxic conditions were

164   maintained for 5 min. P_{O_2} was raised slightly to measure respiration immediately after

165   anoxia, and then after a stable reading was achieved, the medium was fully oxygenated.

166   Rotenone (0.5 µmol l^{-1}), an inhibitor of complex I, was added to measure oxphos capacity

167   *via* complex II ($P_{S(Rot)}$). The above mitochondrial physiology experiment was performed

168   twice for each fish, once without and once with the addition of carbonyl cyanide m-chloro

169   phenyl hydrazine (CCCP) after the first addition of maximal ADP. CCCP is used to

170   uncouple respiration (and is added until maximal stimulation, 0.5-2 µmol l^{-1}) so capacities

171   for electron transport (rather than for oxphos) could be measured. Data are expressed per

172   mg mitochondrial protein. Mitochondrial O_2 kinetics were analyzed using DatLab 2

173   software (Oroboros Instruments) to measure maximal mitochondrial respiration (J_{max}),

174   mitochondrial O_2 affinity (P_{50}, the P_{O2} at which respiration is inhibited by 50%).

175

176   *S1.10 Enzyme assays*

177   Maximal activities (V_{max}) of the metabolic enzymes citrate synthase (CS) and

178   succinate dehydrogenase (SDH) were measured in mitochondrial isolates at 25ºC using a

179   SpectraMax Plus 384 microplate reader. Mitochondria were homogenized and diluted 25-

180   fold in 50 mmol l^{-1} KH_2PO_4 (pH 7.0). We determined CS activity as the reduction of DTNB

181   (5,5'-dithiobis-(2-nitrobenzoic acid; extinction coefficient [ϵ] of 14.15 l mmol^{-1} cm^{-1} at 412

182   nm), in an assay mixture containing 0.15 mmol l^{-1} acetyl-coA, 0.15 mmol l^{-1} DTNB, and

183   0.5 mmol l^{-1} oxaloacetate. SDH activity was determined as the reduction of DCPIP (2,6-

184   dichlorophenolindophenol; ϵ of 21.9 l mmol^{-1} cm^{-1} at 600 nm), in an assay mixture

185   containing 20 mmol l^{-1} succinate, 0.3 mmol l^{-1} KCN, 0.05 mmol l^{-1} DCPIP, 0.05 mmol l^{-1}

186   decylubiquinone.

187   EROD (ethoxyresorufin-O-deethylase) activity was measured fluorometrically in

188   liver tissue following previously described methods (4, 5). Liver tissue was homogenized

189   in four volumes of homogenization buffer (50 mmol l^{-1} tris, 0.15 mol l^{-1} KCl, pH 7.4) using

190   an electric homogenizer in pulses totalling 12 s. The S9 fraction (which contains

191   microsomes and cytosol) was isolated by centrifugation at 750*g* for 10 min, and then at

192   12,000*g* for 10 min. 10 µl of supernatant was loaded into a black 96-well plate and 7-

193   ethoxyresorufin (dissolved in methanol (400 mmol l^{-1}) and diluted to 2.67 umol l^{-1} in 50

194   mmol l^{-1} tris, 0.1 mmol l^{-1} NaCl; pH 7.8) was added to a concentration of 2 µmol l^{-1}. The

195   reaction was initiated by adding NADPH (1.33 mmol l^{-1}) and V_{max} was read over 10 min

196   in a fluorometric microplate reader (SpectraMax Gemini XPS, Molecular Devices,

197   Sunnyvale CA, USA) at excitation and emission wavelengths of 530 and 590 nm,

198   respectively. Protein content was measured on the S9 fraction using the Bradford assay,

199   and EROD activity was expressed as pmol of resorufin formed per min per mg protein.

200   Activities of CS, SDH, and EROD were assayed in triplicate as the difference in

201   activity between samples with and without a key reagent (oxaloacetate, succinate, and

202   NADPH, respectively). Preliminary experiments confirmed that substrate concentrations

203   were saturating.

204

205   *S1.11 Lipid peroxidation*

206   Isolated mitochondria were homogenized for 1 min in methanol (1:5 v:v) in an ice-

207   cold glass tissue grinder, then centrifuged at 1000*g* for 5 min. The supernatant was stored

208   on ice while a cuvette containing distilled water, 0.25 mmol l^{-1} $FeSO_4$, 25 mmol l^{-1} sulfuric

209   acid, and 0.1 mmol l^{-1} xylenol orange (added in this order) was allowed to react for 30 min.

210   After this time period, the homogenate was added and given 2 h to react and an initial

211   absorbance reading was taken at 580 nm using SpectraMax Plus 384 microplate reader

212   (Molecular Devices, Sunnyvale, California, USA). Cumene peroxide (0.005 mmol l^{-1}) was

213   then added to the cuvette and allowed to react for 40 min, and a final reading was taken at

214   580 nm. Lipid peroxidation is standardized to and reported as cumene hydroperoxide

215   equivalents, and is expressed per mg of mitochondrial protein.

216

217   *S1.12 Supplementary statistical analyses*

218   We conducted a principal component analysis (PCA, "vegan" package, 6) on all response

219   variables except some secondary measures that were used to calculate more important

220   ones that are in the analysis, or those derived from other variables already in the analyses.

221   A full list of the variables included in the PCA is in Table S4. We imputed missing values

222   in the dataset using a single imputation approach with partial mean matching ("mice"

223   package, 7). All response variables were continuous, inspected for linearity and skew,

224   then standardized to unit variance before the PCA analysis. We tested the effect of

225   exposure site on the first two principal components (PC1 and PC2) using a linear mixed

226   effect model, with deployment week as a random effect ("lme4" package, 8).

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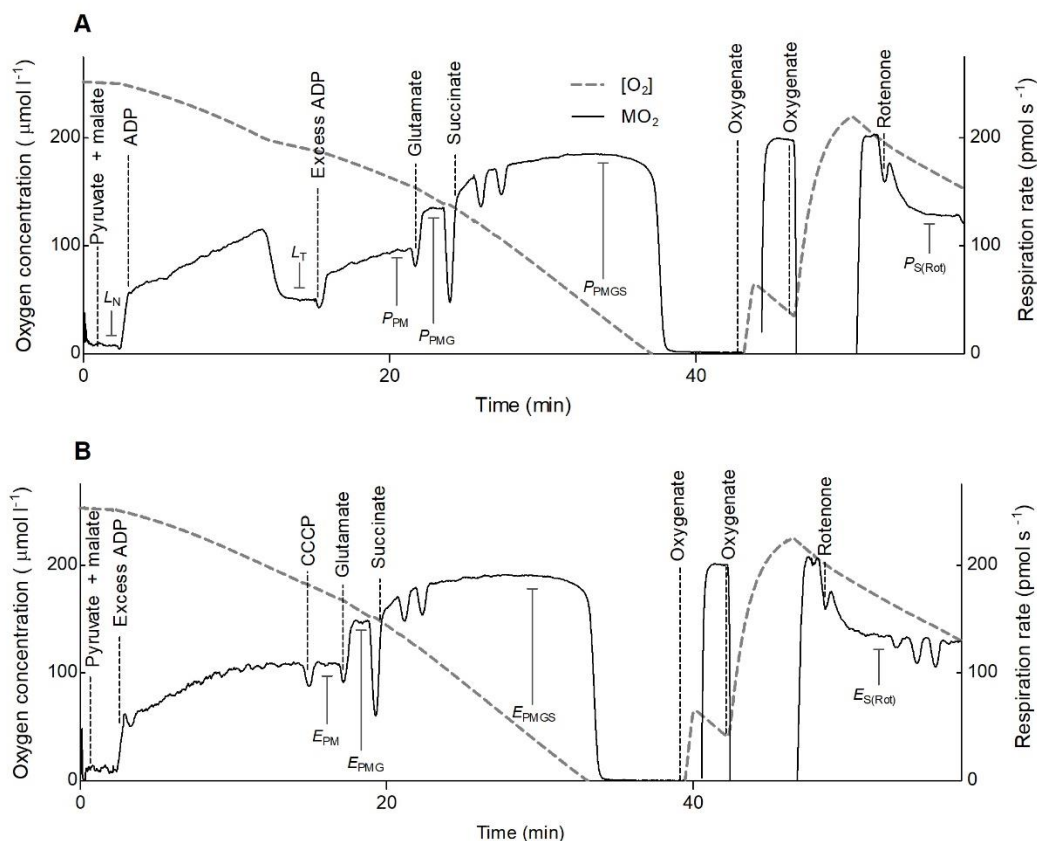
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Fig. S1. Representative experiments of mitochondria isolated from liver of bluegill sunfish to measure (A) respiration during oxidative phosphorylation and (B) electron transport capacity during uncoupled respiration. (A) Reactive oxygen species (ROS) emission rates (not pictured) were measured in parallel by fluorometry (see Materials and Methods above). Measurements were taken at 20°C. (L_N and L_T, leak respiration in the absence and presence of ATP, respectively; P_{PM} or E_{PM}, oxidative phosphorylation (P) or electron transport capacity (E, induced by uncoupling mitochondria with CCCP) with pyruvate and malate; P_{PMG} or E_{PMG}, P or E with pyruvate, malate, and glutamate; P_{PMGS} or E_{PMGS}, P or E with pyruvate, malate, glutamate, and succinate; and $P_{S(Rot)}$ or $E_{S(Rot)}$, P or E with succinate and rotenone)

239
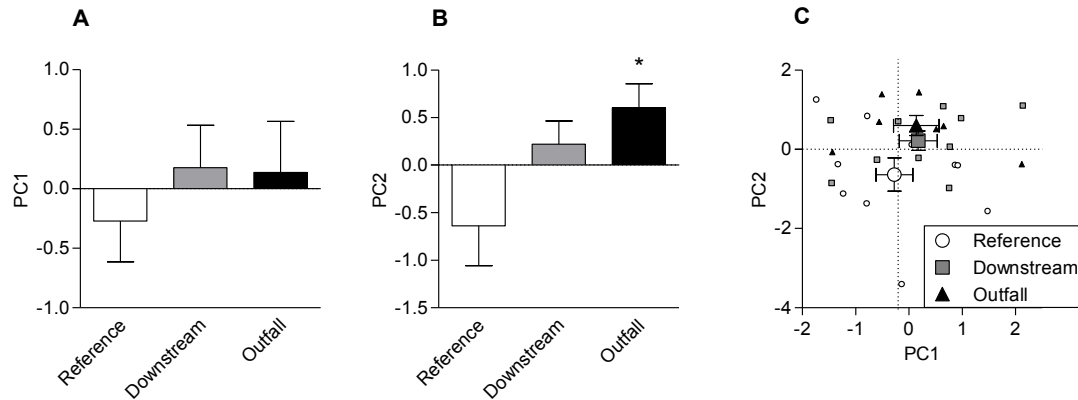
240

Fig. S2. The effects of exposure site on the first two principal components from a principal component analysis (PCA). (A) Exposure site did not affect PC1 scores (LRT$_{site}$ χ^2 = 1.25, p = 0.53), which accounted for 28.0% of the variation. **(B)** Exposure site had a significant effect on PC2 scores (LRT$_{site}$ χ^2 = 7.01, p = 0.03) with fish from the outfall having higher PC2 scores than fish from the reference site (Dunnet's *post-hoc*, p = 0.025). Fish from the downstream site also tended to have higher PC2 scores than fish from the reference site, but this was not statistically significant (Dunnet's *post-hoc*, p = 0.10). PC2 accounted for 19.7% of the variation. **(C)** Biplot of PC2 versus PC1, in which small individual symbols represent individual data, and larger symbols represent means ± s.e.m.

252 **Table S1.** Average estimated time-weighted concentrations (ng l^{-1}) of waterborne
253 pharmaceuticals and personal care products at a clean reference site, near the outfall of a
254 tertiary wastewater treatment plant, or further downstream.

Compound	Class	Reference	Downstream	Outfall
Caffeine	food	73.8	742.5	752.4
Sucralose	food	9.9	2996.0	3130.6
Trimethoprim	anti-biotic	ND	4.7	8.03
Sulfamethoxazole	anti-biotic	0.3	2.5	3.5
Carbamazepine	anti-seizure	<LOQ	54.9	55.1
Acetaminophen	analgesic	0.7	4.5	7.6
Ibuprofen	anti-inflammatory	6.0	20.3	31.0
Gemfibrozil	lipid regulator	ND	1.3	2.9
Naproxen	anti-inflammatory	1.1	30.2	27.9
Triclosan	antibacterial	ND	--	ND
Estrone (E1)	hormone	ND	<LOQ	<LOQ
Estradiol (E2)	hormone	ND	ND	N
Androstenedione	hormone	<LOQ	2.0	2.32
Testosterone	hormone	ND	<LOQ	<LOQ
Venlafaxine	antidepressant	<LOQ	50.7	59.3
O-dm-venlafaxine	metabolite	<LOQ	18.3	36.4
N-dm-venlafaxine	metabolite	ND	4.3	6.9
Sertraline	antidepressant	ND	0.4	1.9
dm-sertrailne	metabolite	ND	ND	ND
Citalopram	antidepressant	ND	ND	ND
Fluoxetine	antidepressant	ND	ND	ND
Atenolol	beta-blocker	ND	10.9	21.5
Metoprolol	beta-blocker	ND	5.7	6.7
Propanolol	beta-blocker	ND	4.7	3.3

255 Concentrations were determined using passive polar organic chemical integrative

256 samplers (POCIS) deployed at sites of caged fish (refer to Fig. 1 for map; *n* = 3 replicates

257 per site). ND, not detected; < LOQ, detected, but below limit of quantification; --

258 excluded from analyses.

259 **Table S2.** Water quality measures taken weekly over 21 d of caged exposures ($n = 7$).

	Reference	Downstream	Outfall
Temperature (°C)	17.4 ± 0.70	22.95 ± 0.41	21.73 ± 0.39
Dissolved oxygen (mg/L)	5.48 ± 0.56	8.84 ± 0.82	11.28 ± 1.15
pH	8.00 ± 0.16	8.00 ± 0.11	7.95 ± 0.17
Conductivity (µS)	695.57 ± 30.38	1283.87 ± 40.42	1243.37 ± 41.82
Salinity (ppm)	315.71 ± 14.06	600.50 ± 19.33	581.38 ± 20.07
TDS (ppm)	494.71 ± 21.19	910.38 ± 28.72	883.38 ± 30.31
Flow (m/sec)	0.021 ± 0.0096	0.017 ± 0.0030	0.016 ± 0.0030

260

261 **Table S3.** Body and organ mass (% body mass) of bluegill sunfish. Data reported as
262 mean ± s.e.m. (*n*).

	Reference	Downstream	Outfall
Body mass (g)	82.0 ± 8.7 (10)	81.9 ± 11.7 (10)	84.6 ± 11.0 (7)
Liver	0.722 ± 0.086 (10)	1.124 ± 0.122 (9)	0.756 ± 0.047 (7)
Heart	0.073± 0.006 (10)	0.096 ± 0.007 (9)	0.089 ± 0.006 (7)
Spleen	0.099± 0.017 (10)	0.129 ± 0.018 (10)	0.080 ± 0.015 (7)
Brain	0.203± 0.018 (10)	0.230 ± 0.023 (10)	0.210 ± 0.026 (7)

263 Body mass, $\text{LRT}_{site}\ \chi^2 = 0.027$, $p = 0.99$; liver, $\text{LRT}_{site}\ \chi^2 = 6.19$, $p = 0.045$ (downstream,

264 $p = 0.057$; outfall, $p = 0.99$); heart, $\text{LRT}_{site}\ \chi^2 = 4.10$, $p = 0.13$; spleen, $\text{LRT}_{site}\ \chi^2 = 3.81$, p

265 $= 0.15$; brain, $\text{LRT}_{site}\ \chi^2 = 0.78$, $p = 0.68$.

266

267 **Table S4.** Loadings onto the first two principal components from a principal component
268 analysis.

Response	PC1	PC2
Body mass	0.67	0.08
Liver mass	0.59	0.05
Heart mass	0.62	0.22
Spleen mass	0.31	-0.17
Brain mass	0.59	0.24
Resting O_2 consumption rate	0.70	0.21
Total filament length	0.52	0.24
Gill surface density	0.41	0.38
Haematocrit	0.46	0.00
Haemoglobin content	-0.12	0.04
Haemoglobin P_{50} at pH 7	0.25	0.35
Haemoglobin P_{50} at pH 7.4	-0.06	0.10
Respiratory capacity for oxphos: P_{PM}	-0.68	0.24
Respiratory capacity for oxphos: P_{PMG}	-0.64	0.47
Respiratory capacity for oxphos: $P_{S(Rot)}$	-0.46	0.65
Respiratory capacity for oxphos: P_{PMGS}	-0.66	0.56
Mitochondrial ROS emission rates: P_{PM}	-0.63	-0.39
Mitochondrial ROS emission rates: P_{PMG}	-0.59	-0.43
Mitochondrial ROS emission rates: $P_{S(Rot)}$	-0.67	-0.43
Mitochondrial ROS emission rates: P_{PMGS}	-0.59	-0.39
ROS emission : respiratory capacity for oxphos: P_{PM}	-0.07	-0.55
ROS emission : respiratory capacity for oxphos: P_{PMG}	-0.19	-0.78
ROS emission : respiratory capacity for oxphos: $P_{S(Rot)}$	-0.17	-0.80
ROS emission : respiratory capacity for oxphos: P_{PMGS}	-0.11	-0.75
Lipid peroxidation	-0.28	0.21
Succinate dehydrogenase activity	0.50	0.43
Citrate synthase activity	-0.24	-0.05
Mitochondrial P_{50}	0.21	0.56
Respiratory capacity for electron transport: E_{PM}	-0.62	0.33
Respiratory capacity for electron transport: E_{PMG}	-0.64	0.51
Respiratory capacity for electron transport: $E_{S(Rot)}$	-0.37	0.60
Respiratory capacity for electron transport: E_{PMGS}	-0.63	0.53
Leak respiration L_T	-0.6	0.24
Leak respiration L_N	-0.65	0.30
Critical P_{O2}	0.04	0.18
P_{O2} at loss of equilibrium	0.28	0.14
EROD activity	-0.22	-0.09

269

270

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