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

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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.

Fish are commonly found living in effluent-dominated environments (34), possibly because they are able to invoke compensatory strategies to offset the potential metabolic costs of wastewater exposure. The purpose of our study was to elucidate the impacts of wastewater exposure on whole-animal metabolism and to understand whether fish exhibit effective respiratory and metabolic plasticity to cope with these greater demands, using bluegill sunfish (*Lepomis macrochirus*). Bluegill and other related centrarchid species have been used in previous ecotoxicological studies (29, 35-39) and are native across a wide range of North America (40). Bluegill were exposed to effluent from a residential WWTP that discharges into Cootes Paradise Marsh (Fig. 1), a protected wetland of western Lake Ontario that serves as an important fish breeding ground but is recognized as an International Area of Concern due to historically heavy nutrient and pollution inputs (41). Given previous work on single compounds, we expect that fish exposed to wastewater would incur a metabolic cost. If fish are able to compensate for these increased metabolic demands, then we should observe changes that improve oxygen uptake, transport, and utilization.

2. Materials and methods

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

2.1 Caged exposures

Bluegill sunfish (collected from Lake Opinicon, Ontario, Canada) were caged for 21 days in summer 2015 at one of three field locations, two of which exposed fish to

effluent from the Dundas WWTP (43º16'2"N 79º56'37"W, Fig. 1). Dundas WWTP is a tertiary treatment plant that serves a population of ~30,000 and treats an average of 15 million litres of wastewater each day (42). The treated effluent is the major source of water flowing into Desjardins Canal, which enters West Pond before joining Cootes Paradise Marsh (Fig. 1). We caged fish at sites 50 m (43º16'0"N 79º56'31"W) or 830 m (43º16'9"N 79º55'59"W) downstream of the outfall pipe ("outfall" and "downstream" experimental groups, respectively) (Fig. 1). We also caged fish at a control reference site in Beverly Swamp (43º21'57"N 80º6'27"W), which is located within the headwaters for Cootes Paradise Marsh (17.4 km upstream from the outfall and the marsh).

Several measures of water quality and contaminant levels were taken during these caged exposures, in conjunction with a parallel study investigating the effects of wastewater exposure on behaviour and physiology of round goby (*Neogobius melanostomus*) (43). We found 17 PPCPs at our wastewater-contaminated sites, including a range of antibiotics, antidepressants, beta-blockers, and hormone medications (Table S1). Only six PPCPs were found at our reference site, all at substantially lower concentrations. Water quality (temperature, dissolved oxygen, pH, conductivity, salinity, total dissolved solids, and flow) was also measured during our exposure period (Table S2). A full description of the methods and analyses of these parameters are described by McCallum et al. (43).

2.3 Respirometry experiments

We used stop-flow intermittent respirometry (Loligo Systems) to measure standard O_2 consumption rates (M_{O_2}) and hypoxia tolerance in resting fish, using well-established

methods (44, 45). Briefly, fish were transferred to respirometry chambers (2.1 l) within 4 h of arrival from the field, and were held there overnight (~18 h) with a continuous flow-through supply of aerated dechlorinated tap water at 20ºC. The next morning, resting M_{O_2} was obtained in normoxia (90-100% air saturation). Hypoxia tolerance (critical P_{O_2} and P_{O_2} at loss of equilibrium) was also measured using a stepwise progressive hypoxia protocol that is common in the literature (46). Fish were then euthanized and sampled ~18 h after respirometry measurements.

2.4 Tissue contaminants

We pooled samples from all fish within each site and sampling time point to have enough tissue to measure contaminant levels in liver (~0.75 g total tissue) and gills (~1 g total tissue). We measured two synthetic musks (Galaxolide and Tonalide; commonly used to add fragrance to cosmetics and detergents) in the fish sampled after respirometry measurements. We also measured four target pharmaceuticals (sertraline and venlafaxine, both antidepressants, O-dm-venlafaxine, a breakdown product of venlafaxine, and metoprolol, a β-blocker) in a separate set of fish sampled immediately upon removal from caged exposures (see Supporting methods). We extracted and identified these compounds following previously described methods (47-49).

2.5 Gill morphometrics

We used stereomicroscopy to analyze gill morphometrics (45, 50). Digital images were taken of all filaments on each of the four arches on one side of the fish, and the lengths and number of filaments on each arch were measured using ImageJ (51). The measured

values of total filament number and total filament length (sum of all filament lengths across all four arches) were multiplied by 2 to account for there being two sides of the fish.

The first gill arch was prepared for histological analyses after stereomicroscopy. Gills were sectioned using a cryostat and then stained with eosin and haematoxylin. Brightfield microscopy images were taken across the entire gill arch from each fish, and we measured total lamellar height, exposed lamellar height, interlamellar cell mass height, and lamellar thickness for ~8 lamellae using ImageJ (51). Lamellar density was also quantified as the number of lamellae per length of filament. Gill surface density was measured using Nikon NIS-Elements D software (Version 4.30) as the length of total surface per length of filament.

2.6 Haemoglobin-O_2 binding

Haemoglobin-O_2 affinity (P_{50}, the P_{O_2} at which haemoglobin is 50% saturated) was determined in lysate of red blood cells using Hemox Analyser and software (TCS Scientific, New Hope, Pennsylvania, USA) at pH 7.0 and 7.4 at a temperature of 25ºC, as recommended by the manufacturer. We calculated pH sensitivity as the difference in P_{50} per unit change in pH.

2.7 Mitochondrial physiology

Mitochondria were isolated from liver using established methods that have been described previously (52, 53), and then used for high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria) at 20°C (Supporting Information; Fig. S1). Mitochondrial respiration

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

by Dunnett's *post-hoc* tests. In the analyses of M_{O_2} and organ masses (Table S3), the absolute values (mmol O_2 h^{-1} and g, respectively) were used in statistical analyses (because body mass was accounted for as a covariate), but are reported normalized to body mass (i.e., mmol O_2 h^{-1} kg^{-1} and % body mass, respectively) to facilitate comparison with the literature. Mitochondrial respiration and ROS emission were analysed with the additional fixed effects of respiratory state and its interaction with exposure site. Haemoglobin P_{50} was analysed with the additional fixed effects of pH and the interaction between exposure site and pH. In each case, interaction terms were dropped from the LMM if they were not significant. Principal component analysis was used to characterize overall physiological variation across exposure sites (Fig. S2, Table S4). Data are reported as means ± standard error mean (s.e.m.) and results with $p < 0.05$ were considered significant.

3. Results

3.1 Survival

Survival remained high at the reference site (97.5 ± 2.5% survival after 21 days) but was significantly lower at the downstream (70.0 ± 10.2%) and outfall sites (43.5 ± 17.5%, Fig. 2). However, body mass of surviving fish was similar across groups (in g: reference, 82.0 ± 8.7; downstream, 81.9 ± 11.7; outfall, 84.6 ± 11.0; LRT_{site} $\chi^2 = 0.027$, $p = 0.99$).

3.2 Markers of contamination

Bluegill exposed to wastewater effluent accumulated the synthetic musks Tonalide and Galaxolide in their tissues, consistent with the overall pattern of waterborne PPCP

exposure (Table S1). Galaxolide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexa-methylcyclopenta[g]-2-benzopyrane, HHCB) was detected at highest levels at the outfall site (4.97 ng g^{-1} fresh weight), followed by the downstream site (4.35), and was undetected at the reference site, and was found at much higher concentrations in the liver than in the gill (outfall, 0.57; downstream, 0.2; reference, not detected). Tonalide (7-acetyl-1,1,3,4,4,6-hexa-methyl-tetra hydronaphthalene, AHTN) exhibited a similar pattern but was only detected in the liver at the outfall (1.49 ng g fresh $weight^{-1}$) and downstream (0.7) sites. None of the four pharmaceuticals assayed (venlafaxine and its metabolite *O*-dm-venlafaxine, sertraline, and metoprolol) were detected in any bluegill from any sites, potentially because the relatively high solubility of these compounds prevented their bioaccumulation (9, 61). EROD activity was similar across fish from the reference (7.45 ± 1.32 pmol resorufin min^{-1} mg $protein^{-1}$, $n = 9$), downstream (4.83 ± 1.66, $n = 8$), and outfall (8.28 ± 2.25, $n = 6$) sites (LRT_{site} $\chi^2 = 3.47$, $p = 0.18$), suggesting that fish were not exposed to aryl hydrocarbons such as polyaromatic hydrocarbons or polychlorinated biphenyls (62).

3.3 Metabolism and hypoxia tolerance

Bluegill caged at the downstream and outfall sites exhibited 30-36% higher standard rates of O_2 consumption (M_{O_2}) than fish caged at the reference site (Fig. 3). However, wastewater exposure did not have a significant effect on hypoxia tolerance. Critical P_{O_2} (P_{crit}) was similar across sites (in kPa: reference, 3.50 ± 0.19, $n = 10$; downstream, 4.33 ± 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 fish lost equilibrium during progressive hypoxia (in kPa: reference, 0.494 ± 0.092, $n = 10$; downstream, 0.412 ± 0.088, $n = 7$; outfall, 0.336 ± 0.027, $n = 6$; LRT_{site} $\chi^2 = 1.89$, $p = 0.39$).

225

226 *3.4 Gill morphometrics and histology*

227 Wastewater exposure increased the respiratory surface area of the gills (Fig. 4). The
228 height of exposed lamellae was 20-45% greater in fish from the downstream and outfall
229 sites than those from the reference site (Fig. 4F), due largely to a 17-29% reduction in the
230 height of interlamellar cell mass (Fig. 4E). Fish from the outfall site also had slightly
231 thinner (Fig. 4H) and longer (Fig. 4D) lamellae, whereas fish from the downstream site had
232 a modest increase in lamellar density (Fig. 4G). Collectively, these changes increased gill
233 surface density (i.e., length of gill surface per length of filament) by 22% in fish from the
234 downstream and outfall sites compared to control fish (Fig. 4I). These changes likely
235 increased the overall surface area of the gills, because there were no differences in average
236 filament length (in mm: reference, 3.69 ± 0.21; downstream, 3.60 ± 0.31; outfall, 3.88 ±
237 0.32; LRT_{site} $\chi^2 = 0.82$, $p = 0.66$), total filament length (in mm: reference, 5179 ± 326;
238 downstream, 4872 ± 523; outfall, 5535 ± 493; LRT_{site} $\chi^2 = 1.34$, $p = 0.51$), and total filament
239 number (reference, 1376 ± 28; downstream, 1335 ± 41; outfall, 1412 ± 38; LRT_{site} $\chi^2 =$
240 1.97, $p = 0.37$) between sites ($n_{reference} = 10$, $n_{downstream} = 9$, $n_{outfall} = 7$).

241

242 *3.5 Haematology*

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

248 (LRT_{site} $\chi^2 = 8.57$, $p = 0.014$), 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

respiration were also reduced from ~0.11% in unexposed fish to 0.08% in fish exposed to wastewater at both the downstream and outfall sites (Fig. 6C).

We found no evidence of mitochondrial oxidative stress with wastewater exposure (LRT_{site} χ^2 = 0.023, p = 0.99). Levels of lipid peroxidation were similar in liver mitochondria among fish from reference (2.98 ± 0.53 nmol cumene hydroperoxide equivalents mg protein^{-1}, n = 9), downstream (3.05 ± 0.33, p = 0.99, n = 10), and outfall (3.05 ± 0.45, p = 0.99, n = 7) sites.

4. Discussion

Here, we show that exposure to wastewater effluent reduces survival of bluegill sunfish (Fig. 2). Exposure also increases standard rates of aerobic metabolism (Fig. 3), which was associated with adjustments across the oxygen transport cascade that expanded the gills' capacity for gas exchange (Fig. 4), facilitated the unloading of O_2 from haemoglobin at the tissues (Fig. 5), and increased the respiratory capacity of liver mitochondria (Fig. 6). There was a significant overall effect of wastewater on physiology when considered using a principal component analysis (Fig. S2, Table S4). These beneficial adjustments in respiratory physiology could help bluegill sunfish cope with the metabolic costs associated with living in polluted environments.

4.1 Metabolic costs of wastewater exposure

Our results contribute to growing evidence that exposure to a range of contaminants can increase metabolic rate, as observed in numerous fish species in response to an 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 O_2 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

(87). The similar increases we observed in this study could increase the liver's capacity for mitochondrial respiration and ATP synthesis, especially when combined with increases in organ size (Table S3), possibly to support the energetic demands of detoxification (88).

Changes in mitochondrial quality in response to wastewater exposure were also associated with reductions in the inherent rate of mitochondrial ROS emission (Fig. 6). Oxidative stress is a common consequence of wastewater exposure in numerous fish species (89-92), and may contribute to the metabolic costs of exposure because energy is required to repair and replace damaged macromolecules (93). Compensatory adjustments to reduce oxidative stress could foreseeably arise by reducing the inherent rate of ROS production in the mitochondria or cytosol, or by increasing the activity of cellular antioxidant systems. Although the latter is a common biomarker of pollutant exposure (94), few studies have examined whether exposure is associated with compensatory reductions in mitochondrial ROS production that minimize oxidative stress. The reductions in mitochondrial ROS emission observed here may have contributed to the low incidence of lipid peroxidation in liver mitochondria of bluegill exposed to wastewater. However, caging itself has been shown to affect cellular ROS production in fish (95), so it will be useful to examine whether similar effects on mitochondrial ROS emission are observed in wild fish exposed to wastewater.

The apparent improvement in mitochondrial quality in bluegill exposed to wastewater stands in contrast to some other studies, in which contaminant exposure impaired mitochondrial respiration. Numerous environmental contaminants, especially metals, are known to disrupt mitochondrial function by impairing activities of respiratory complexes, thereby reducing aerobic capacity (96-101). Alternatively, contaminants can

uncouple oxidative phosphorylation (102), which could increase respiration rates needed to offset proton leak, and thus reduce phosphorylation efficiency. It is worth noting that the vast majority of studies that investigated mitochondrial toxicity applied contaminants directly to mitochondria (rather than exposing the whole animal), so the mitochondria in our study likely encountered much lower and environmentally relevant contaminant concentrations.

4.4 Water quality

Inter-site differences in water quality (Table S2) were unlikely to drive most of the physiological differences we observed. Dissolved O_2 and salinity were in a normal range and the magnitude of variation was modest, so these parameters are not anticipated to induce the observed variation in M_{O_2}, gill structure, or mitochondrial respiratory capacity (45, 52, 103-105). Acclimatization to higher temperatures at the downstream and outfall sites would tend to reduce resting M_{O_2} and mitochondrial respiratory capacity when tested at a common temperature, as they were in this study (106, 107), opposite to the differences observed here. However, as described above, it is possible that these higher temperatures could have contributed to the increase in gill respiratory surface in fish at these sites (104). Otherwise, inter-site variation in water quality is expected to have had little effect, and may have even dampened some the physiological responses to wastewater exposure.

4.5 Metabolism and respiration as ecotoxicological tools

Understanding bioenergetics under contaminant stress can reveal potential trade-offs in allocation of a finite pool of energy, which can have important implications on

organismal and population-level function, giving reason for its application as an ecotoxicological bioindicator over the past three decades (33, 108-111). However, joint consideration of both organismal metabolism and the respiratory physiology that supports this metabolism is infrequent in aquatic toxicology. Consideration of the impacts of contaminants along the oxygen transport cascade and across multiple levels of biological organization helps elucidate mechanistic linkages between subcellular energetics and whole-organismal performance, and thus represents an integrated approach to understanding how fish are coping in modern environments (112-114). Metabolism and respiration are major themes for research into how animals cope with metabolically challenging environmental stressors (e.g., hypoxia, rising temperatures, salinity; reviews by 71, 115), and a similar approach could be used to better understand responses to wastewater stress. We show that metabolism and respiration are indeed sensitive to wastewater exposure in bluegill, invoking a suite of alterations – from biochemistry to whole organism – that improve oxygen uptake, transport, and utilization. Such mechanistic approaches can improve our understanding of and capacity to predict the impacts of aquatic pollution at organismal and population levels, and should thus be considered as an ecologically relevant bioindicator in aquatic toxicology.

Supporting information

S1. Detailed Material and Methods

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.

Fig. S2. The effects of exposure site on the first two principal components from a principal component analysis (PCA).

Table S1. Average estimated time-weighted concentrations of waterborne pharmaceuticals and personal care products at a clean reference site, near the outfall of a tertiary wastewater treatment plant, or further downstream.

Table S2. Water quality measures taken during caged exposures.

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

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

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Tables

Table 1. Properties of mitochondria isolated from the liver of bluegill sunfish. Data are 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)

* represents a significant difference from reference site; SDH, LRT_{site} $\chi^2 = 6.81$, $p = 0.033$ (downstream, $p = 0.066$; outfall, $p = 0.044$); CS, LRT_{site} $\chi^2 = 2.37$, $p = 0.31$; P_{50}, LRT_{site} $\chi^2 = 9.81$, $p = 0.007$ (downstream, $p = 0.012$; outfall, $p = 0.013$); E, LRT_{site} $\chi^2 = 3.34$, $p = 0.19$; LRT_{state} $\chi^2 = 72.9$, $p < 0.0001$ (downstream, $p = 0.98$; outfall, $p = 0.20$); 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 electron transport (E) was assessed with substrates of complex I (E_{PM} with pyruvate, P, and malate, M; E_{PMG} with P, M, and glutamate, G), complex II ($E_{S(Rot)}$ with succinate, S, and complex I inhibitor rotenone, Rot), and both complexes I and II (E_{PMGS} with P, M, G, S).

Figure legends

Fig. 1. Location of study area and sites of caged exposures. Bluegill sunfish were caged for 21 days (1) 50 m from the outfall of a wastewater treatment plant (WWTP) that provides tertiary treatment to the municipality of Dundas, (2) 830 m further downstream in an effluent-dominated canal, or (3) at a clean reference site (17.4 km northwest of the WWTP in Flamborough, ON, Canada).

Fig. 2. Wastewater exposure decreased survival of bluegill sunfish. Bluegill sunfish were caged at the outfall of a wastewater treatment plant, further downstream, or at an uncontaminated reference site for 21 days. * represents significant differences between fish from the reference site (LRT_{site} $\chi^2 = 14.23$, $p = 0.0008$; LRT_{week} $\chi^2 = 3.73$, $p = 0.15$; Dunnett's *post-hoc*: downstream, $p = 0.015$; outfall, $p = 0.002$).

Fig. 3. Standard rates of O_2 consumption increased in resting bluegill sunfish exposed to wastewater effluent. Bluegill caged at the downstream ($n = 10$) and outfall sites ($n = 7$) had significantly higher rates of aerobic metabolism than fish caged at the reference site ($n = 10$). *represents significant differences from the reference site (LRT_{site} $\chi^2 = 8.37$, $p = 0.015$; Dunnett's *post-hoc*: downstream, $p = 0.038$; outfall, $p = 0.012$)

Fig. 4. Bluegill remodelled their gills in response to wastewater exposure. Representative images of gills from bluegill caged at (**A**) reference, (**B**) downstream, and (**C**) outfall sites for 21 days (scale bar represents 1 mm). (**D**) Total lamellar height was

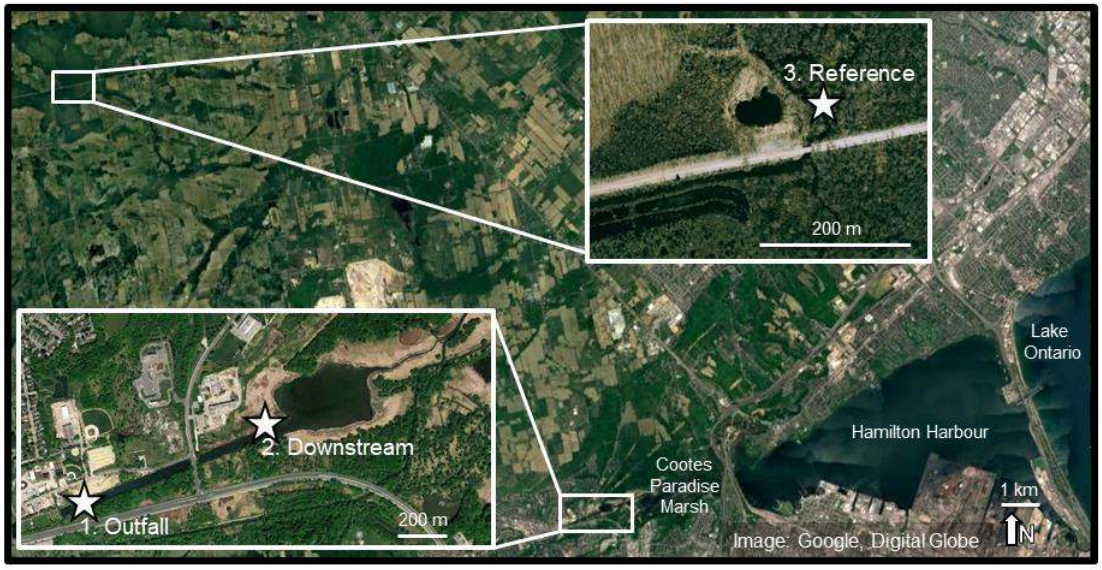
highest in bluegill from the outfall site (LRT_{site} $\chi^2 = 4.18$, $p = 0.12$; Dunnett's *post-hoc*: downstream, $p = 0.62$; outfall, $p = 0.039$). (**E**) Height of interlamellar cell mass (ILCM) was lower after wastewater exposure (LRT_{site} $\chi^2 = 6.63$, $p = 0.036$), and the reductions in the downstream and outfall sites approached statistical significance in Dunnett's *post-hoc* tests (downstream, $p = 0.057$; outfall, $p = 0.053$). (**F**) Exposed lamellar height (the difference between heights of total lamellae and ILCM) increased in bluegill caged at downstream and outfall sites (LRT_{site} $\chi^2 = 22.3$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p = 0.001$; outfall, $p < 0.0001$). (**G**) Lamellar density (LRT_{site} $\chi^2 = 8.50$, $p = 0.014$; Dunnett's *post-hoc*: downstream, $p = 0.006$; outfall, $p = 0.32$) and (**H**) lamellar thickness (LRT_{site} $\chi^2 = 6.34$, $p = 0.04$; Dunnett's *post-hoc*: downstream, $p = 0.94$; outfall, $p = 0.02$) varied with caging exposures. (**I**) Gill surface density increased in bluegill caged at the downstream and outfall sites (LRT_{site} $\chi^2 = 19.1$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p < 0.0001$; outfall, $p < 0.0001$). *represents significant differences from the reference site ($n_{reference} = 9$, $n_{downstream} = 9$, $n_{outfall} = 7$)

Fig. 5. Haemoglobin-oxygen binding affinity was reduced in response to wastewater exposure at the outfall site. (**A**) The P_{50} of haemoglobin (the partial pressure of oxygen at which haemoglobin is 50% saturated) was measured in the lysate of frozen red blood cells and was highest in bluegill caged at the outfall site at pH 7.0 (LRT_{site} $\chi^2 = 20.2$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p = 0.63$; outfall, $p = 0.0003$; $n_{reference} = 7$, $n_{downstream} = 6$, $n_{outfall} = 7$). P_{50} was higher at pH 7.0 compared to pH 7.4 (LRT_{pH} $\chi^2 = 139.9$, $p < 0.0001$). (**B**) pH sensitivity of haemoglobin (measured as the change in P_{50} between pH 7.0 and 7.4 and normalized to 1.0 pH unit) was significantly higher in

bluegill caged at the outfall site (LRT_{site} $\chi^2 = 7.82$, $p = 0.020$; Dunnett's *post-hoc*: downstream, $p = 0.63$; outfall, $p = 0.013$, n the same as above). (**C**) Blood haemoglobin content was similar across all exposure sites (LRT_{site} $\chi^2 = 0.98$, $p = 0.61$); $n_{reference} = 10$, $n_{downstream} = 10$, $n_{outfall} = 6$). *represents significant differences from the reference site

Fig. 6. Wastewater exposure affected the physiology of isolated liver mitochondria. (**A**) Bluegill had higher respiratory capacities for oxidative phosphorylation after 21 days of exposure to wastewater effluent (LRT_{site} $\chi^2 = 7.59$, $p = 0.022$; LRT_{state} $\chi^2 = 83.0$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p = 0.39$; outfall, $p = 0.011$). (**B**) ROS emission rates were reduced in mitochondria from bluegill caged at the downstream and outfall sites (LRT_{site} $\chi^2 = 24.6$, $p < 0.0001$; LRT_{state} $\chi^2 = 35.0$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p < 0.0001$; outfall, $p < 0.0001$). (**C**) ROS emission relative to oxphos respiration were also lower in the downstream and outfall sites (LRT_{site} $\chi^2 = 31.0$, $p < 0.0001$; LRT_{state} $\chi^2 = 49.7$, $p < 0.0001$; Dunnett's *post-hoc*: downstream, $p < 0.0001$; outfall, $p < 0.0001$). Measurements were made during oxidative phosphorylation (oxphos, *P*) with substrates of complex I (CI; P_{PM} with pyruvate, P, and malate, M; P_{PMG} with P, M, and glutamate, G), complex II (CII; $P_{S(Rot)}$ with succinate, S, and complex I inhibitor rotenone, Rot), and both complexes I and II (CI+II; P_{PMGS} with P, M, G, S). *represents significant differences from the reference site ($n_{reference} = 9$, $n_{downstream} = 9$, $n_{outfall} = 7$).

897 Fig. 1


3. Reference
200 m
2. Downstream
1. Outfall
200 m
Lake
Ontario
Hamilton Harbour
Cootes
Paradise
Marsh
1 km
N
Image: Google, Digital Globe

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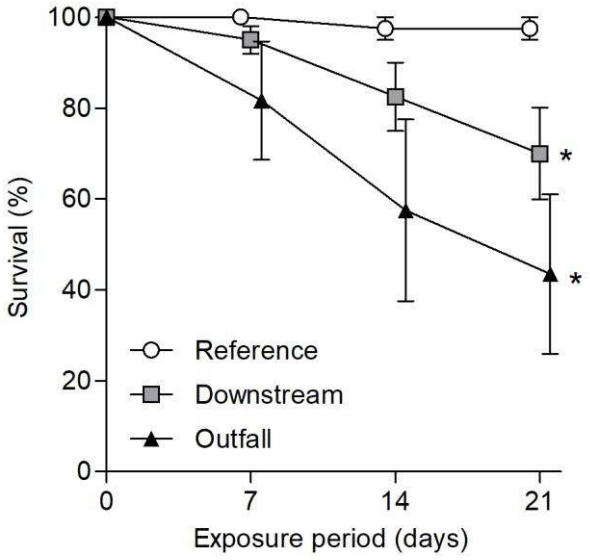
899 Fig. 2



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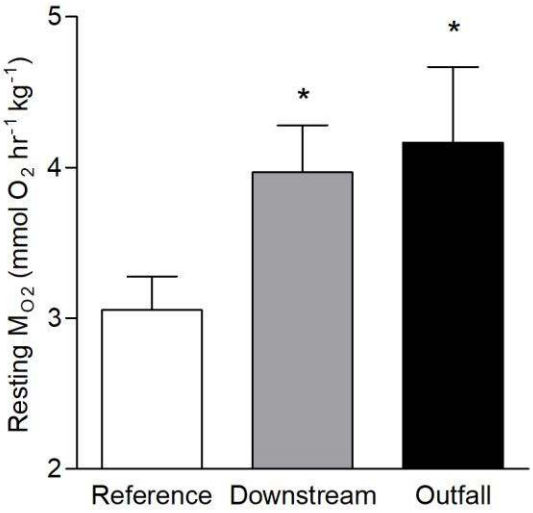
902 Fig. 3



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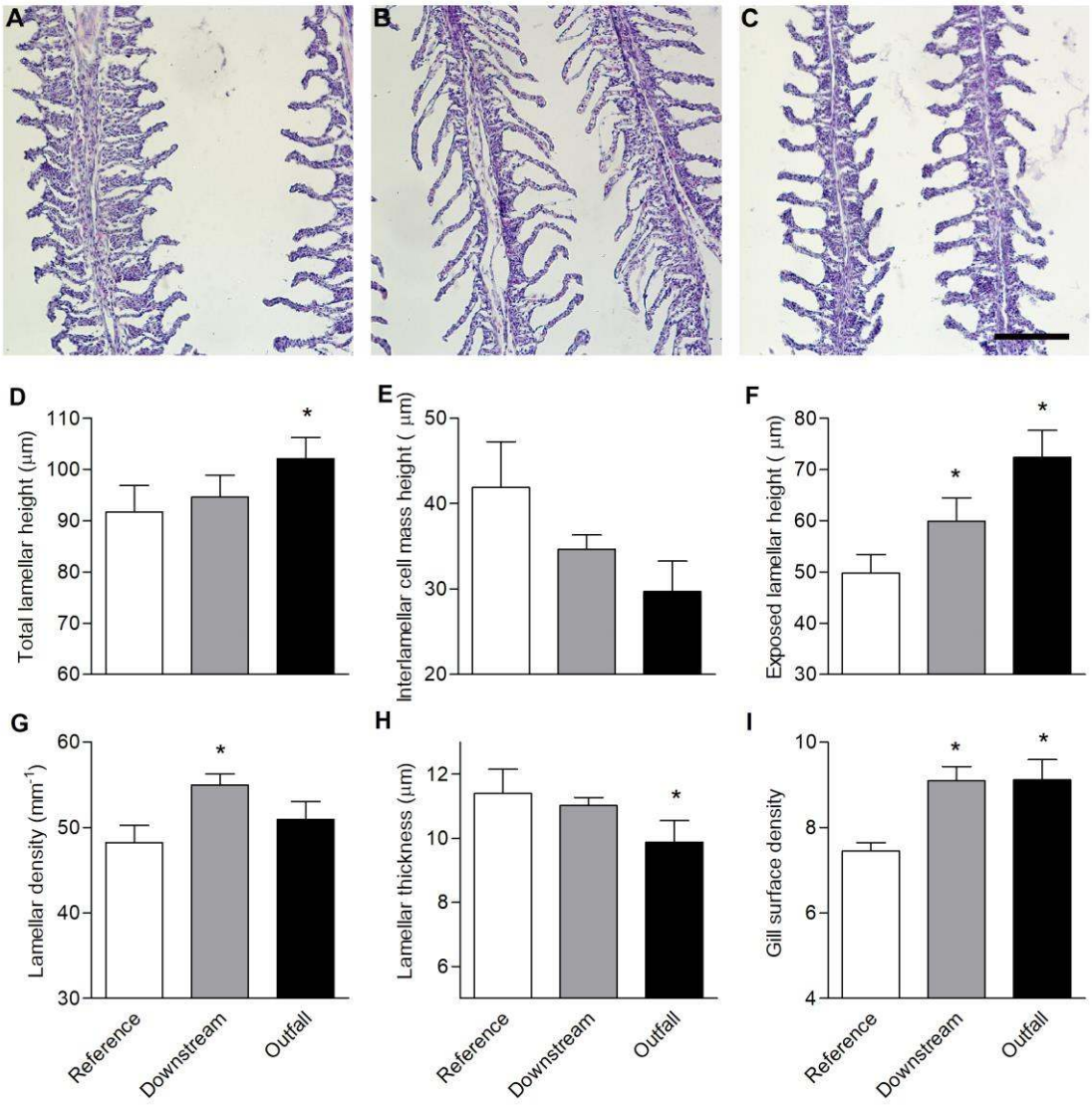
905 Fig. 4



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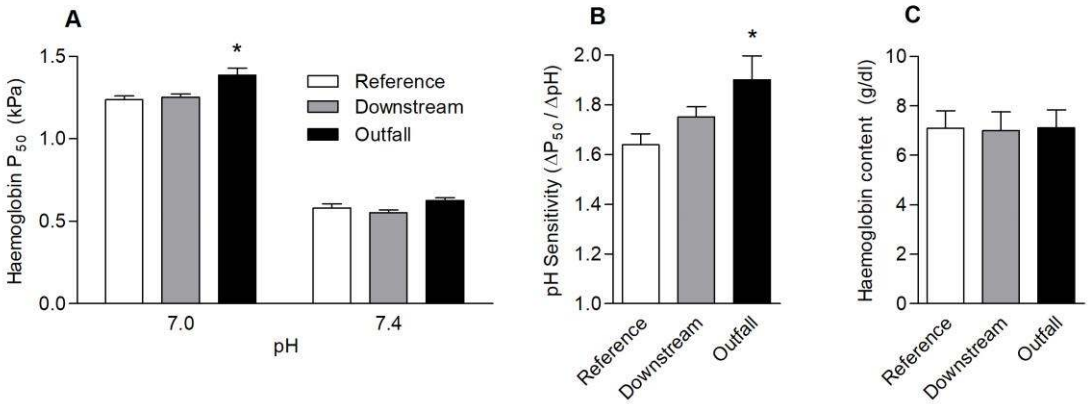
908 Fig. 5



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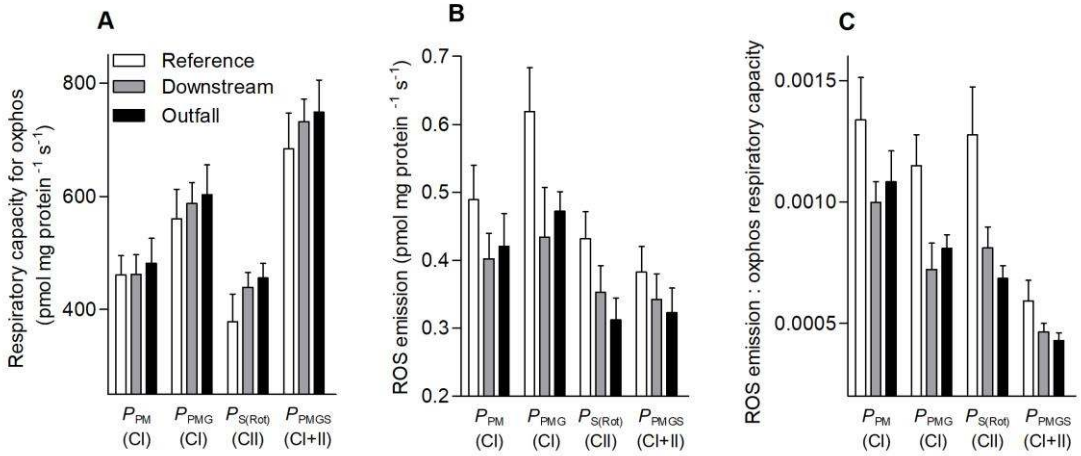
911 Fig. 6



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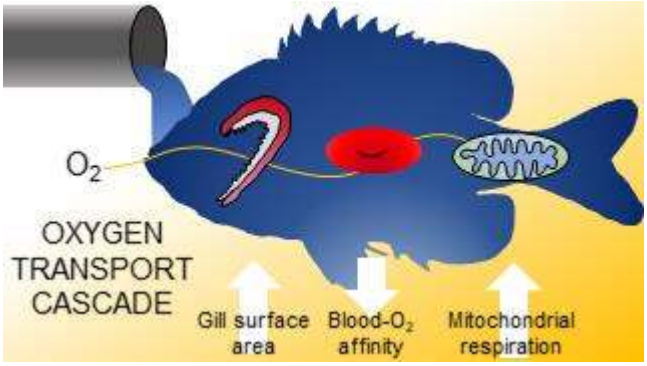

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METABOLIC COSTS OF EXPOSURE TO WASTEWATER EFFLUENT LEAD TO COMPENSATORY ADJUSTMENTS IN RESPIRATORY PHYSIOLOGY IN BLUEGILL SUNFISH

SUPPORTING INFORMATION

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19 PAGES, 2 FIGURES, 4 TABLES

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, -76.328ºW) 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

period lasted from June 15 to August 6, 2015. During this time, we provided food (a mix of squid and cow heart) to the fish once per week, to supplement the food they received from the water column. After the exposure, fish were transported back to McMaster University in dechlorinated tap water for respirometry experiments (see below) or immediate sampling.

S1.3 Hypoxia tolerance

Resting O_2 consumption rates (M_{O_2}) were measured using stop-flow intermittent respirometry at each O_2 tension (P_{O_2}) of a stepwise hypoxia exposure, in which P_{O_2} was reduced every 20 min by 10% air saturation. When O_2 levels reached 10% air saturation, the chambers were closed and fish were allowed to consume the remaining oxygen in the chamber until loss of equilibrium (LOE) was reached (at which point the P_{O_2} at LOE was recorded). Afterwards, the fish was quickly removed from the chamber, allowed to recover, and was maintained in aerated water for ~18 h until they were terminally sampled (see below). The critical P_{O_2} (P_{crit}) was the P_{O_2} at which fish transformed from being an oxyregulator (M_{O_2} is independent of P_{O_2}) to oxyconformer (M_{O_2} is dependent on P_{O_2}), and was calculated using Regress software (3).

S1.4 Sampling

Fish were always sampled ~18 h after completing the respirometry experiment. Fish were euthanized with a cerebral concussion, the tail was severed, and blood was

immediately collected in heparanized capillary tubes. A portion of blood (6 µl) was used to measure haemoglobin content using Drabkin's reagent (Sigma-Aldrich), and the remaining blood was centrifuged for 2.5 min to measure haematocrit. The packed red blood cells were frozen in liquid N_2 and stored at -80ºC for later measurements of Hb-O_2 binding. Brain, spleen, and liver were excised and weighed. Half of the liver was used immediately for mitochondrial isolation, and the other half was immediately frozen in liquid N_2 and then stored at -80ºC for later use in enzymes assays or contaminant analyses (see below). Gills were carefully removed: one side was frozen in liquid N_2 and stored at -80ºC for later use in contaminant analyses, while the other side was stored in fixative (274 mol l^{-1} NaOH, 30 mol l^{-1} Na_2HPO_4, 5.4 mol l^{-1} KCl, 3 mol l^{-1} KH_2PO_4, 2% paraformaldehyde, 2% glutaraldehyde; pH 7.8) for at least 48 h until used for morphometric and histological analyses.

A small subset of fish from each site were not used in metabolic or respiratory analyses and were sampled immediately upon arrival at McMaster University from the field for contaminant analyses (reference, n = 9; downstream, n = 4; outfall, n = 2). We excised liver and gills, immediately froze the tissues in liquid N_2, and then stored them at -80ºC until contaminant analyses were conducted (methods below).

S1.5 Tissue contaminants

Tissues for pharmaceutical and synthetic musk analyses were freeze dried, then homogenized using a mortar and pestle. Pharmaceuticals were extracted from homogenized tissues by pressurized liquid extraction with an ASE 300 instrument (Dionex,

Toronto, Ontario, Canada), diluted with deionized water, and then cleaned using a MCX solid phase extraction cartridge, evaporated, then dissolved in 400 µl of MeOH. A Q-Trap liquid chromatography and tandem mass spectrometry (LC-MS/MS) system with an atmospheric pressure chemical ionization source and a Series 1100 HPLC binary solvent delivery system were used to analyse the sample in positive ion mode. The sample was separated using chromatography (Genesis C18 column). Pharmaceuticals were quantified using an internal standard (based on a nine-point calibration scale).

Synthetic musks were extracted from homogenized tissues using pressurized liquid extraction, filtered through 12 g of sodium sulfate, and concentrated using a rotary evaporator to ~1 ml. Gel permeation chromatography was used to isolate the analyte fraction containing the musks, which was then cleaned with Florisil chromatography. Finally, gas chromatography (Varian 3800, Varian, Palo Alto, California, USA) paired with an ion trap mass selective detector (Varian Saturn 2200) were used with electron impact ionization, and operated in selected ion storage mode. Chromatographic retention time and ion ratios were used to identify musks, and peak areas were quantified using an external standard (four-point calibration). One procedural blank was used for each set of tissues (liver or gill) and for each class of contaminants (pharmaceuticals or musks).

S1.6 Preparation of gills for histological analyses

After images from all gill arches were collected using stereomicroscopy, the first gill arch was submerged in 30% sucrose (in 137 mol l^{-1} NaOH, 15.2 mol l^{-1} Na_2HPO_4, 2.7 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*

Fresh liver tissue (~0.5 g) was finely diced in 10 ml of ice-cold isolation buffer (in mmol l^{-1} unless otherwise stated: 250 sucrose, 50 KCl, 25 KH_2PO_4, 10 hepes, 0.5 EGTA, and 1.5% mass:volume fatty-acid free BSA; pH 7.4). We gently homogenized the tissue on ice with six passes of a loose-fitting Potter-Elvehjem homogenizer at 100 r.p.m. The homogenate was centrifuged at 600*g* for 10 min at 4°C, and the supernatant was filtered through glass wool, and then centrifuged at 6000*g* for 10 min at 4°C (the same conditions for all future centrifugations). We gently rinsed and re-suspended the pellet in 10 ml of fresh isolation buffer, then centrifuged. The pellet was then rinsed and re-suspended in 10 ml ml of storage buffer (same as the isolation buffer, but without BSA and with 2 mmol l^{-1} each of pyruvate and malate), and centrifuged again. The final pellet was re-suspended in 500 µl of storage buffer. Half of the mitochondrial isolate was stored for a short period on ice until respiration and ROS emission experiments (see below), and the other half was frozen at -80ºC for later assays of lipid peroxidation and enzyme activities (see below). Mitochondrial protein content was measured in the isolate using the Bradford assay (Bio-Rad, Mississauga, ON, Canada).

S1.9 Mitochondrial physiology

Respiration (rate of O_2 consumption) and ROS emission rates of mitochondria were measured in 2 ml of respiration buffer (in mmol l^{-1}: 110 sucrose, 60 K-lactobionate, 20 taurine, 20 Hepes, 10 KH_2PO_4, 3 $MgCl_2 \cdot 6H_2O$, 0.5 EGTA, 1.5% mass:volume fatty-acid free BSA; pH 7.4). ROS was detected as the fluorescence of resorufin (excitation wavelength of 525 nm and AmR filter set, Oroboros Instruments), which is produced from hydrogen peroxide (H_2O_2) and Ampliflu Red (Sigma-Aldrich) in a reaction catalysed by 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

mg mitochondrial protein. Mitochondrial O_2 kinetics were analyzed using DatLab 2 software (Oroboros Instruments) to measure maximal mitochondrial respiration (J_{max}), mitochondrial O_2 affinity (P_{50}, the P_{O2} at which respiration is inhibited by 50%).

S1.10 Enzyme assays

Maximal activities (V_{max}) of the metabolic enzymes citrate synthase (CS) and succinate dehydrogenase (SDH) were measured in mitochondrial isolates at 25ºC using a SpectraMax Plus 384 microplate reader. Mitochondria were homogenized and diluted 25-fold in 50 mmol l^{-1} KH_2PO_4 (pH 7.0). We determined CS activity as the reduction of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid; extinction coefficient [ε] of 14.15 l $mmol^{-1}$ cm^{-1} at 412 nm), in an assay mixture containing 0.15 mmol l^{-1} acetyl-coA, 0.15 mmol l^{-1} DTNB, and 0.5 mmol l^{-1} oxaloacetate. SDH activity was determined as the reduction of DCPIP (2,6-dichlorophenolindophenol; ε of 21.9 l $mmol^{-1}$ cm^{-1} at 600 nm), in an assay mixture containing 20 mmol l^{-1} succinate, 0.3 mmol l^{-1} KCN, 0.05 mmol l^{-1} DCPIP, 0.05 mmol l^{-1} decylubiquinone.

EROD (ethoxyresorufin-O-deethylase) activity was measured fluorometrically in liver tissue following previously described methods (4, 5). Liver tissue was homogenized in four volumes of homogenization buffer (50 mmol l^{-1} tris, 0.15 mol l^{-1} KCl, pH 7.4) using an electric homogenizer in pulses totalling 12 s. The S9 fraction (which contains microsomes and cytosol) was isolated by centrifugation at 750*g* for 10 min, and then at 12,000*g* for 10 min. 10 µl of supernatant was loaded into a black 96-well plate and 7-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.

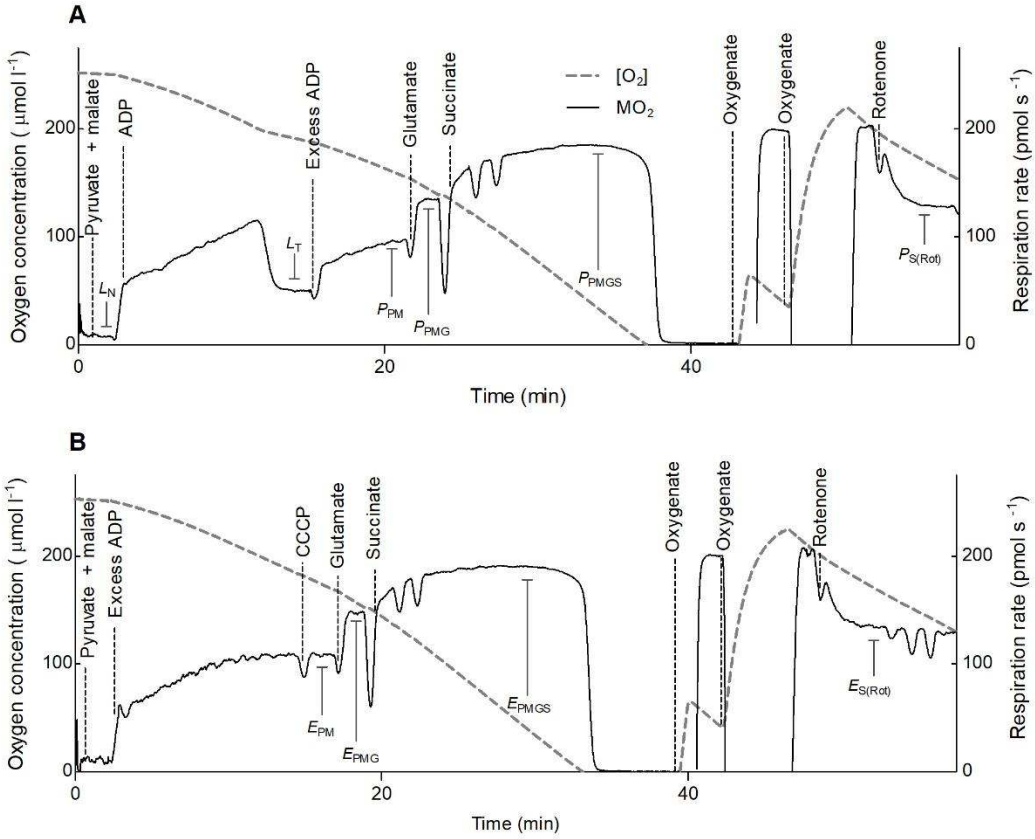
204

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.

S1.12 Supplementary statistical analyses

We conducted a principal component analysis (PCA, "vegan" package, 6) on all response variables except some secondary measures that were used to calculate more important ones that are in the analysis, or those derived from other variables already in the analyses. A full list of the variables included in the PCA is in Table S4. We imputed missing values in the dataset using a single imputation approach with partial mean matching ("mice" package, 7). All response variables were continuous, inspected for linearity and skew, then standardized to unit variance before the PCA analysis. We tested the effect of exposure site on the first two principal components (PC1 and PC2) using a linear mixed effect model, with deployment week as a random effect ("lme4" package, 8).



228

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

239

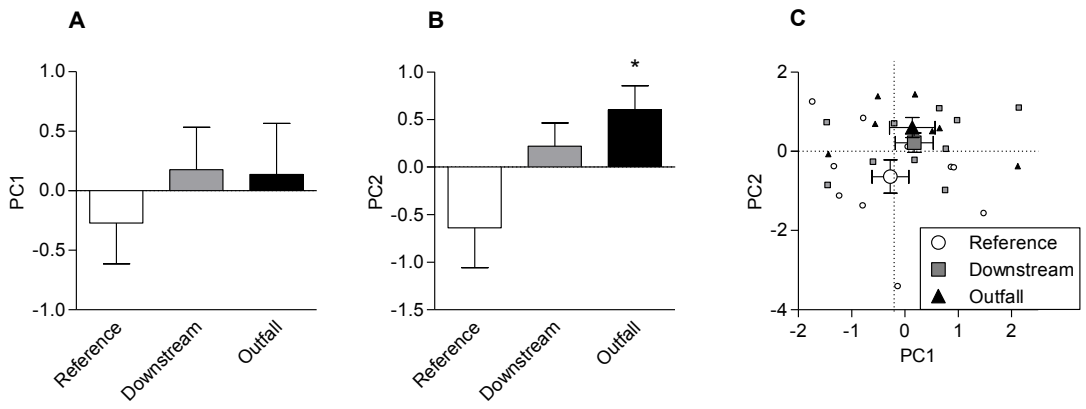


Fig. S2. The effects of exposure site on the first two principal components from a principal component analysis (PCA). (A) Exposure site did not affect PC1 scores (LRT_{site} $\chi^2 = 1.25$, $p = 0.53$), which accounted for 28.0% of the variation. **(B)** Exposure site had a significant effect on PC2 scores (LRT_{site} $\chi^2 = 7.01$, $p = 0.03$) 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.

Table S1. Average estimated time-weighted concentrations (ng l^{-1}) of waterborne pharmaceuticals and personal care products at a clean reference site, near the outfall of a 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

Concentrations were determined using passive polar organic chemical integrative samplers (POCIS) deployed at sites of caged fish (refer to Fig. 1 for map; $n = 3$ replicates per site). ND, not detected; < LOQ, detected, but below limit of quantification; -- excluded from analyses.

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

Table S3. Body and organ mass (% body mass) of bluegill sunfish. Data reported as 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)

Body mass, LRT_{site} $\chi^2 = 0.027$, $p = 0.99$; liver, LRT_{site} $\chi^2 = 6.19$, $p = 0.045$ (downstream, $p = 0.057$; outfall, $p = 0.99$); heart, LRT_{site} $\chi^2 = 4.10$, $p = 0.13$; spleen, LRT_{site} $\chi^2 = 3.81$, $p = 0.15$; brain, LRT_{site} $\chi^2 = 0.78$, $p = 0.68$.

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

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