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

Sherry N. N. Du\textsuperscript{a,*}, Erin S. McCallum\textsuperscript{b}, Maryam Vaseghi-Shanjani\textsuperscript{a}, Jasmine A. Choi\textsuperscript{a}, Theresa R. Warriner\textsuperscript{b}, Sigal Balshine\textsuperscript{b}, and Graham R. Scott\textsuperscript{a}

\textsuperscript{a} Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada
\textsuperscript{b} Department of Psychology, Neuroscience & Behaviour, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada

Keywords: hypoxia tolerance, mitochondrial O\textsubscript{2} kinetics, aerobic metabolism, gill histology, haemoglobin P\textsubscript{50}

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 O₂ 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-O₂ affinity (which likely facilitates O₂ 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 O₂ 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
excess nutrients) into the environment via the treated effluent (1-5). Wastewater effluent is a growing concern because many of these compounds are recognized ecological hazards that may threaten the health of aquatic wildlife (6-10). Exposure to single contaminants can impair performance, reproduction, and behaviour in fish (11-19). However, less is known about how fish physiology is impacted by the complex contaminant mixtures that typify wastewater, which could interact in synergistic ways that are hard to predict, particularly when combined with natural variability in environmental conditions (20).

Metabolism and respiration provide a powerful lens to understand how contaminants influence energy flow within an organism. Metabolism, respiration, and aerobic scope (the difference between maximal and resting rates of O₂ consumption) are linked to growth, reproduction, activity, functional performance, and many important behaviours (21-26). Exposure to aquatic pollution may require that energy be redirected towards detoxification and cellular protection, particularly in tissues that accumulate contaminants and/or play large roles in detoxification (e.g., liver, 27), and may thus impact whole-animal metabolism. Exposure may constrain these processes, because some contaminants cause mitochondrial dysfunction and impair energy production (28-30). Although some studies have investigated the effects of pollution on energy stores (i.e. concentrations of lipid, glycogen, and protein in tissues; 31-33), the mechanisms and functional implications on higher levels of biological organization (i.e. organ systems and whole-organism) remain unclear. This knowledge gap is best-addressed using integrative sets of bioenergetic markers that provide a mechanistic link between cellular changes and 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 (43°16′2″N 79°56′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 (43°16′0″N 79°56′31″W) or 830 m (43°16′9″N 79°55′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 (43°21′57″N 80°6′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 20°C. The next morning, resting $\text{MO}_2$ was obtained in normoxia (90-100% air saturation). Hypoxia tolerance (critical $\text{PO}_2$ and $\text{PO}_2$ 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-O₂ binding

Haemoglobin-O₂ affinity (P₅₀, the Pₐₐ₉ 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 25°C, as recommended by the manufacturer. We calculated pH sensitivity as the difference in P₅₀ 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
(rate of $O_2$ consumption) was measured during oxidative phosphorylation (oxphos, $P$) and during uncoupling to assess electron transport capacity ($E$). We used substrates of complex I ($P_{PM}$ or $E_{PM}$ with pyruvate, $P$, and malate, $M$; $P_{PMG}$ or $E_{PMG}$ with $P$, $M$, and glutamate, $G$), complex II ($P_{S(Rot)}$ or $E_{S(Rot)}$ with succinate, $S$, and complex I inhibitor rotenone, Rot), and both complexes I and II ($P_{PMGS}$ or $E_{PMGS}$ with $P$, $M$, $G$, $S$). Rates of reactive oxygen species (ROS) emission were measured fluorometrically, concurrent with oxphos measurements.

We also measured lipid peroxidation as a marker of oxidative damage (as the formation of Fe(III)-xylenol orange complex) (53, 54), and the maximal activities ($V_{max}$) of metabolic enzymes citrate synthase (CS) and succinate dehydrogenase (SDH) at 25°C (53), in isolated liver mitochondria. EROD (ethoxyresorufin-O-deethylase) activity was measured fluorometrically in liver tissue at 25°C (55, 56).

2.11 Statistical analyses

Data were analysed using R (version 3.2.4; 57). Survival was analysed using a binomial generalized linear mixed effects model (GLMM; glmmadmb package, 58). Site and exposure week were set as fixed effects, and cage ID and deployment date were set as random effects. Likelihood ratio tests (LRTs) were used to test for the main effects of site and duration of exposure, followed by Dunnett’s post-hoc tests (multcomp package, 59) to compare each exposure site to the reference site. All remaining data, unless otherwise noted, were analysed with linear mixed effects model (LMM; lme4 package, 60) using exposure site as a fixed effect, body mass as a covariate, and deployment date as a random 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 Mo₂ and organ masses (Table S3), the absolute values (mmol O₂ 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 O₂ 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 P₅₀ 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; LRTsite χ² = 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 χ² = 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 O₂ consumption (Mo₂) than fish caged at the reference site (Fig. 3). However, wastewater exposure did not have a significant effect on hypoxia tolerance. Critical Po₂ (Pcrit) 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 χ² = 2.45, p = 0.29), as was the Po₂ 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 χ² = 1.89, p = 0.39).
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<sub>site</sub> χ² = 0.82, p = 0.66), total filament length (in mm: reference, 5179 ± 326; downstream, 4872 ± 523; outfall, 5535 ± 493; LRT<sub>site</sub> χ² = 1.34, p = 0.51), and total filament number (reference, 1376 ± 28; downstream, 1335 ± 41; outfall, 1412 ± 38; LRT<sub>site</sub> χ² = 1.97, p = 0.37) between sites (n<sub>reference</sub> = 10, n<sub>downstream</sub> = 9, n<sub>outfall</sub> = 7).

3.5 Haematology

Blood-O₂ binding was altered in response to wastewater exposure (Fig. 5). P<sub>50</sub> (the P<sub>O₂</sub> 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 O₂ 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).
(LRT site $\chi^2 = 8.57, p = 0.014$), but blood haemoglobin content did not vary across sites (Fig. 5C).

3.6 Mitochondrial respiration

Wastewater exposure altered the physiology of liver mitochondria (Fig. 6). Mitochondrial respiratory capacities for oxidative phosphorylation (oxphos, $P$) were ~10% higher in fish caged at the outfall (Fig. 6A). As expected, there was a significant main effect of mitochondrial substrate on oxphos respiration, with respiration rates generally being higher when supported with substrates of complex I ($P_{PM}$ and $P_{PMG}$) compared to complex II ($P_{S(Rot)}$), and the highest respiration rates were observed with convergent inputs to both complexes I and II ($P_{PMGS}$). Associated with the exposure-induced increases in oxphos capacity were increases in succinate dehydrogenase activity, but no change (or a slight non-significant decrease) in citrate synthase activity (Table 1). Wastewater exposure also increased mitochondrial $P_{50}$ (the $P_O$ at which mitochondrial respiration was reduced by 50%) but had no significant effects on respiratory capacities for electron transport (as indicated by respiration in the presence of the uncoupler CCCP) or leak respiration rates with ($L_T$) or without ($L_N$) ATP (Table 1).

3.7 ROS emission rates and oxidative stress

Rates of mitochondrial ROS emission were reduced by 10-30% in fish exposed to wastewater compared to those from the reference site (Fig. 6B), with higher ROS emission when respiration was supported by substrates of complex I than when supported by 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 $\chi^2 = 0.023$, $p = 0.99$). 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
integrated measures of organismal metabolism, as reflected by the rate of O₂ consumption by the animal, are critical to evaluating whether there is a metabolic cost of contaminant exposure. However, although variation in some subordinate indices of metabolism (e.g., metabolite concentrations, metabolic enzyme activities) had previously suggested that this might be the case for wastewater exposure (e.g. 33, 67), the issue had rarely been explored at the organismal level. Our previous work suggested that the metabolic cost we observed in bluegill may not occur in all species, because resting M_O₂ was unaffected in a parallel wastewater exposure study using round goby (Neogobius melanostomus; 43), an invasive species that is now established in many parts of the bluegill’s natural range (55, 68). It is possible that caging (a necessity for assuring that individuals are continuously exposed and cannot leave the effluent stream) was stressful to fish (69), so it will be valuable to examine in future work whether wild un-caged fish exposed to wastewater also exhibit higher metabolic rates.

Increases in metabolism arising from contaminant exposure could impact fitness by reducing aerobic scope (70-72). Aerobic scope, the difference between resting and maximal M_O₂, represents the capacity to increase aerobic metabolism to support functions such as reproduction, growth, and behaviour (24). An increase in resting M_O₂ without a parallel increase in maximal M_O₂ would reduce aerobic scope (71). This has been observed in rainbow trout exposed to copper (73) and killifish (Fundulus heteroclitus) from sites contaminated with polyaromatic hydrocarbons (74). Alternatively, some fish suffer a reduced aerobic scope due to decreases in maximal M_O₂, such as observed in common sole (Solea solea) exposed to petroleum (75) or in rainbow trout exposed to waterborne aluminum (65). However, it is also possible that fish suffering increases in resting M_O₂
could maintain (or even increase) aerobic scope with compensatory increases in maximal \( \text{MO}_2 \) (64, 66). Although we did not measure maximal \( \text{MO}_2 \), the respiratory adjustments of bluegill in response to wastewater exposure (discussed below) suggest that they may be able to increase maximal \( \text{MO}_2 \) and offset reductions in aerobic scope. The changes in metabolism and respiratory physiology that we observed were apparent when fish were tested in clean water. Similarly, rainbow trout exposed to aluminium suffered reduced maximal \( \text{MO}_2 \) and aerobic scope compared to unexposed controls when tested in clean water (65), likely because the persistent physiological effects of exposure were slow to reverse when fish were transferred to clean water for short periods. In our study, testing in clean water was essential for comparing groups in similar conditions to examine the extent to which exposure led to persistent changes in metabolism and physiology. It would be instructive to examine whether the apparent effects of exposure are compounded or otherwise altered if fish are tested in wastewater.

4.2 Wastewater exposure enhanced the capacity for \( O_2 \) uptake and transport

Bluegill caged in wastewater increased the morphological capacity of the gills for gas exchange. This expansion of gill surface area appeared largely as a consequence of reductions in the interlamellar cell masses (ILCM) that increased the length of exposed lamellae (Fig. 4). ILCM remodelling is a highly plastic trait, allowing organisms to respond quickly to environmental stressors that increase the demand for \( O_2 \) uptake (76). It is likely that the combined effects of increases in metabolism and the slightly higher water temperatures near the WWTP contributed to the expansion of gill surface area that we observed (77). The observed increases in gill surface area may come at the expense of
augmented ionoregulatory demands (due to the so called “osmorespiratory compromise”; 78) and greater uptake of environmental contaminants through the gills (79-82). Some fish species instead reduce respiratory surface as a protective mechanism to limit contaminant uptake (83-85), which could reduce maximal O₂ uptake and aerobic scope (65), but that clearly did not occur in the present study.

Bluegill also responded to wastewater exposure by modulating haemoglobin-O₂ binding affinity of the blood. Haemoglobin-O₂ affinity balances the demands of O₂ loading and uptake at the gills (which is facilitated by an increase in affinity) and O₂ unloading at the tissues (which is facilitated by a decrease in affinity) (86). In situations when respiratory O₂ uptake is not compromised, a lower haemoglobin-O₂ affinity is expected to augment O₂ transport to tissues by increasing the P₀₂ of blood passing through the capillaries. Therefore, the increase in haemoglobin P₅₀ at low pH in bluegill exposed to wastewater likely facilitates O₂ transport to respiring tissues (where the blood becomes more acidic), while the expansion of gill surface area helps safeguard branchial O₂ loading into the blood. This appears to be an alternative strategy to improve O₂ transport than increasing haemoglobin content (84).

4.3 Wastewater exposure altered mitochondrial function

Wastewater exposure increased the respiratory capacities for oxidative phosphorylation of liver mitochondria (Fig. 6). The observed increases occurred in concert with a change in the relative activity of succinate dehydrogenase, but not citrate synthase (Table 1). Enhancements in mitochondrial respiratory capacity and enzyme activities are known to contribute to seasonal variation in aerobic capacity in red muscle of rainbow trout...
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 O₂ 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 M₂O₂, 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 M₂O₂ 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.

**Corresponding author**

* dus2@mcmaster.ca

**Funding sources**

This work was supported primarily by a Natural Sciences and Engineering Research Council of Canada (NSERC) Engage Grant to G.R.S. and a Royal Bank of Canada Blue Water Fund Grant to S.B., with additional support from McMaster University, the Canadian Foundation for Innovation, and the Ontario Ministry of Research and Innovation. S.N.N.D. was supported by an Ontario Graduate Scholarship and E.S.M. was supported by a NSERC Post-Graduate Scholarship (Doctoral). G.R.S. and S.B. are supported by the Canada Research Chairs Program.
Acknowledgements

We are grateful for the important contributions of Tys Theýmeër and Jennifer Bowman from Royal Botanical Gardens, and Mark Bainbridge and Bert Posedowski from Hamilton Water. We would also like to thank Nicole Pranckevicius, Neal Dawson, Brittney Borowiec, Jim Sherry, Grant McClelland, Chris Metcalfe, Tamanna Sultana, Brenda Seaborn, and the operators of Dundas Wastewater Treatment Plant for feedback, technical assistance, and analytical support, along with three anonymous referees for their constructive comments on a previous version of this manuscript.

References


27. Chambers, J.E.; Yarbrough, J.D. Xenobiotic biotransformation systems in fishes. 

   testing based on mitochondrial respiratory activity. *Bull. Environ. Contam.*

29. Hiltibran, R.C. Effects of cadmium, zinc, manganese, and calcium on oxygen and 
   phosphate metabolism of bluegill liver mitochondria. *J. Water Pollut. Control 

    down control analysis of the cadmium effects on molluscan mitochondria and the 
    mechanisms of cadmium-induced mitochondrial dysfunction. *Am. J. Physiol. 

31. Cazenave, J.; Bacchetta, C.; Rossi, A.; Ale, A.; Campana, M.; Parma, M.J. 
    Deleterious effects of wastewater on the health status of fish: A field caging study. 

32. Melvin, S.D. Short-term exposure to municipal wastewater influences energy, 
    growth, and swimming performance in juvenile Empire Gudgeons (*Hypseleotris 

33. Smolders, R.; De Boeck, G.; Blust, R. Changes in cellular energy budget as a 
    measure of whole effluent toxicity in zebrafish (*Danio rerio*). *Environ. Toxicol. 


Marentette, J.R.; Gooderham, K.L.; McMaster, M.E.; Ng, T.; Parrott, J.L.; Wilson, J.Y.; Wood, C.M.; Balshine, S. Signatures of contamination in invasive
round gobies (*Neogobius melanostomus*): a double strike for ecosystem health?


56. McCallum, E.S.; Krutzelmann, E.; Brodin, T.; Fick, J.; Sundelin, A.; Balshine, S.


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

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Downstream</th>
<th>Outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.013 ± 0.001 (9)</td>
<td>0.015 ± 0.002 (10)</td>
<td>0.017 ± 0.002* (6)</td>
</tr>
<tr>
<td>(SDH, µmol mg protein⁻¹ min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>0.141 ± 0.015 (8)</td>
<td>0.118 ± 0.008 (10)</td>
<td>0.127 ± 0.014 (6)</td>
</tr>
<tr>
<td>(CS, µmol mg protein⁻¹ min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₅₀ (kPa)</td>
<td>0.033 ± 0.003 (9)</td>
<td>0.046 ± 0.004* (9)</td>
<td>0.047 ± 0.002* (7)</td>
</tr>
</tbody>
</table>

Respiratory capacity for electron transport (E, pmol O₂ mg protein⁻¹ s⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Downstream</th>
<th>Outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eₚₘ (Complex I)</td>
<td>458.5 ± 33.4 (9)</td>
<td>464.7 ± 39.7 (9)</td>
<td>464.2 ± 54.7 (7)</td>
</tr>
<tr>
<td>Eₚₘ₆ (Complex I)</td>
<td>638.9 ± 51.8 (9)</td>
<td>618.8 ± 41.3 (9)</td>
<td>657.5 ± 62.3 (7)</td>
</tr>
<tr>
<td>Eₛ(Rot) (Complex II)</td>
<td>429.1 ± 36.7 (9)</td>
<td>450.7 ± 30.7 (9)</td>
<td>510.2 ± 40.7 (7)</td>
</tr>
<tr>
<td>Eₚₘ₆ₛ (Complex I+II)</td>
<td>754.9 ± 58.0 (9)</td>
<td>740.8 ± 48.7 (9)</td>
<td>797.7 ± 74.7 (7)</td>
</tr>
</tbody>
</table>

Leak respiration with ATP

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Downstream</th>
<th>Outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Lₜ, pmol O₂ mg protein⁻¹ s⁻¹)</td>
<td>310.5 ± 33.7 (9)</td>
<td>278.8 ± 17.2 (9)</td>
<td>259.3 ± 23.9 (7)</td>
</tr>
</tbody>
</table>

Leak respiration without ATP

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Downstream</th>
<th>Outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Lₙ, pmol O₂ mg protein⁻¹ s⁻¹)</td>
<td>38.47 ± 5.25 (9)</td>
<td>38.07 ± 3.81 (9)</td>
<td>40.31 ± 4.81 (7)</td>
</tr>
</tbody>
</table>

* represents a significant difference from reference site; SDH, LRTₚₘₑχ² = 6.81, p =

0.033 (downstream, p = 0.066; outfall, p = 0.044); CS, LRTₚₘₑχ² = 2.37, p = 0.31; P₅₀,

LRTₚₘₑχ² = 9.81, p = 0.007 (downstream, p = 0.012; outfall, p = 0.013); E, LRTₚₘₑχ² =

3.34, p = 0.19; LRTₚₘₑχ² = 72.9, p < 0.0001 (downstream, p = 0.98; outfall, p = 0.20);

Lₜ, LRTₚₘₑχ² = 2.14, p = 0.34; Lₙ, LRTₚₘₑχ² = 1.20, p = 0.55. Respiratory capacity for electron transport (E) was assessed with substrates of complex I (Eₚₘ with pyruvate, P, and malate, M; Eₚₘ₆ with P, M, and glutamate, G), complex II (Eₛ(Rot) with succinate, S, and complex I inhibitor rotenone, Rot), and both complexes I and II (Eₚₘ₆ₛ with P, M, G, and 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 \( \chi^2 = 4.18, p = 0.12 \); Dunnett’s post-hoc: downstream, \( p = 0.62 \); outfall, \( p = 0.039 \). (E) Height of interlamellar cell mass (ILCM) was lower after wastewater exposure \( \chi^2 = 6.63, p = 0.036 \), 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 \( \chi^2 = 22.3, p < 0.0001 \); Dunnett’s post-hoc: downstream, \( p = 0.001 \); outfall, \( p < 0.0001 \). (G) Lamellar density \( \chi^2 = 8.50, p = 0.014 \); Dunnett’s post-hoc: downstream, \( p = 0.006 \); outfall, \( p = 0.32 \) and (H) lamellar thickness \( \chi^2 = 6.34, p = 0.04 \); 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 \( \chi^2 = 19.1, p < 0.0001 \); Dunnett’s post-hoc: downstream, \( p < 0.0001 \); outfall, \( p < 0.0001 \). *represents significant differences from the reference site \( n_{\text{reference}} = 9, n_{\text{downstream}} = 9, n_{\text{outfall}} = 7 \)

**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 \( \chi^2 = 20.2, p < 0.0001 \); Dunnett’s post-hoc: downstream, \( p = 0.63 \); outfall, \( p = 0.0003 \); \( n_{\text{reference}} = 7, n_{\text{downstream}} = 6, n_{\text{outfall}} = 7 \). \( P_{50} \) was higher at pH 7.0 compared to pH 7.4 \( \chi^2 = 139.9, p < 0.0001 \). (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).
Fig. 1
Fig. 2

![Graph showing survival over exposure period](image-url)

- **Reference**
- **Downstream**
- **Outfall**

Survival (%) vs. Exposure period (days)
Fig. 3

- Resting $M_{O_2}$ (mmol O$_2$ hr$^{-1}$ kg$^{-1}$)
- Reference
- Downstream
- Outfall

* indicates statistical significance.
Fig. 4
Fig. 5
Fig. 6
METABOLIC COSTS OF EXPOSURE TO WASTEWATER EFFLUENT LEAD TO COMPENSATORY ADJUSTMENTS IN RESPIRATORY PHYSIOLOGY IN BLUEGILL SUNFISH

SUPPORTING INFORMATION

Sherry N. N. Du\textsuperscript{a,\ast}, Erin S. McCallum\textsuperscript{b}, Maryam Vaseghi-Shanjani\textsuperscript{a}, Jasmine A. Choi\textsuperscript{a}, Theresa R. Warriner\textsuperscript{b}, Sigal Balshine\textsuperscript{b}, and Graham R. Scott\textsuperscript{a}

\textsuperscript{a} Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada

\textsuperscript{b} Department of Psychology, Neuroscience & Behaviour, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada

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, -76.328°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 (~18°C, 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 N₂ and stored at -80°C for later measurements of Hb-O₂ 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 N₂ and then stored at -80°C for later use in enzymes assays or contaminant analyses (see below). Gills were carefully removed: one side was frozen in liquid N₂ and stored at -80°C for later use in contaminant analyses, while the other side was stored in fixative (274 mol l⁻¹ NaOH, 30 mol l⁻¹ Na₂HPO₄, 5.4 mol l⁻¹ KCl, 3 mol l⁻¹ KH₂PO₄, 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 N₂, and then stored them at -80°C 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\(_2\)HPO\(_4\), 2.7 mol l\(^{-1}\) KCl, 1.5 mol l\(^{-1}\) KH\(_2\)PO\(_4\); pH 7.8) for ~24 h and then frozen in embedding medium
Frozen blocks were sectioned (5 µm) at -20°C in a cryostat (Leica CM 1860) and air dried for at least 2 h at room temperature. Sections were stained for hematoxylin and eosin by dehydrating sections in 95% ethanol, incubating in Gills II haematoxylin for ~45 s, and then incubating in eosin for ~15 s, with rinses in distilled water between each step. Sections were then dehydrated in progressively increasing concentrations of ethanol (up to 100%), followed by xylene. Sections were mounted with Permount (Fisher Scientific, Hampton, New Hampshire, USA) and coverslipped. We systematically selected and imaged ~15 sections so as to assure that we analyzed images that were spread throughout and represented the entirety of the first gill arch from each fish. Images were analyzed using a Nikon Eclipse E800 light microscope (Nikon Instruments, Melville, New York, USA). Preliminary measurements verified that this number of images was sufficient to obtain a stable mean value for each trait.

S1.7 Haemoglobin-O₂ binding

Haemoglobin O₂ dissociation curves were determined for the lysate of frozen red blood cells using Hemox Analyser and software (TCS Scientific, New Hope, Pennsylvania, USA). Red blood cell lysate (10 µl) was added to 5 ml of assay buffer solution, which consisted of 50 mmol l⁻¹ tris, 50 mmol l⁻¹ KCl, 5 mmol l⁻¹ EDTA, 0.1% bovine serum albumin (BSA), and 0.2% anti-foaming agent (polydimethylsiloxane emulsion; TCS Scientific).

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\(_2\)PO\(_4\), 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 600g for 10 min at 4°C, and the supernatant was filtered through glass wool, and then centrifuged at 6000g 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 -80°C 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).

1.9 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\(_2\)PO\(_4\), 3 MgCl\(_2\)·6H\(_2\)O, 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\(_2\)O\(_2\)) and Ampliflu Red (Sigma-Aldrich) in a reaction catalysed by horseradish peroxidase. This was accomplished by adding superoxide dismutase (22.5 U
ml⁻¹; to catalyse the formation of H₂O₂ from the superoxide produced by mitochondria), horseradish peroxidase (3 U ml⁻¹), and Ampliflu Red (15 µmol l⁻¹) to the respiration buffer.

We calibrated the resorufin signal (at the beginning and end of the following protocol) with additions of exogenous H₂O₂ to measure ROS emission as the molar rate of H₂O₂ release from mitochondria.

Mitochondrial physiology was measured as follows (Supplementary Fig. 1). Mitochondria (0.6 mg of mitochondrial protein) were added to the respiration buffer, and leak respiration (Lₐ) was measured with complex I substrates pyruvate and malate (2 mmol l⁻¹ each) but without adenylates. We then added 125 µmol l⁻¹ ADP, and measured leak respiration in the presence of ATP (Lₜ) after the mitochondria had converted all of the ADP into ATP. Saturating levels of ADP (1250 µmol l⁻¹) were added to stimulate maximal pyruvate oxidation (Pₚₘ). The capacities for oxphos via complex I (Pₚₘ₉) and complexes I+II (Pₚₘ₉₈) were then determined by adding glutamate (10 mmol l⁻¹) then succinate (10 mmol l⁻¹), respectively. Oxphos respiration via complexes I+II was maintained until all O₂ was consumed (to assess mitochondrial O₂ kinetics), and anoxic conditions were maintained for 5 min. Pₒ₂ was raised slightly to measure respiration immediately after anoxia, and then after a stable reading was achieved, the medium was fully oxygenated.

Rotenone (0.5 µmol l⁻¹), an inhibitor of complex I, was added to measure oxphos capacity via complex II (Pₛ(Rot)). The above mitochondrial physiology experiment was performed twice for each fish, once without and once with the addition of carbonyl cyanide m-chlorophenyl hydrazine (CCCP) after the first addition of maximal ADP. CCCP is used to uncouple respiration (and is added until maximal stimulation, 0.5-2 µmol l⁻¹) so capacities 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 ($J_{\text{max}}$), mitochondrial O$_2$ affinity ($P_{50}$, the P$_{O2}$ at which respiration is inhibited by 50%).

S1.10 Enzyme assays

Maximal activities ($V_{\text{max}}$) of the metabolic enzymes citrate synthase (CS) and succinate dehydrogenase (SDH) were measured in mitochondrial isolates at 25°C using a SpectraMax Plus 384 microplate reader. Mitochondria were homogenized and diluted 25-fold in 50 mmol l$^{-1}$ KH$_2$PO$_4$ (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 750g for 10 min, and then at 12,000g 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
mmol l\textsuperscript{-1} tris, 0.1 mmol l\textsuperscript{-1} NaCl; pH 7.8) was added to a concentration of 2 µmol l\textsuperscript{-1}. The reaction was initiated by adding NADPH (1.33 mmol l\textsuperscript{-1}) and $V_{\text{max}}$ was read over 10 min in a fluorometric microplate reader (SpectraMax Gemini XPS, Molecular Devices, Sunnyvale CA, USA) at excitation and emission wavelengths of 530 and 590 nm, respectively. Protein content was measured on the S9 fraction using the Bradford assay, and EROD activity was expressed as pmol of resorufin formed per min per mg protein.

Activities of CS, SDH, and EROD were assayed in triplicate as the difference in activity between samples with and without a key reagent (oxaloacetate, succinate, and NADPH, respectively). Preliminary experiments confirmed that substrate concentrations were saturating.

### S1.11 Lipid peroxidation

Isolated mitochondria were homogenized for 1 min in methanol (1:5 v:v) in an ice-cold glass tissue grinder, then centrifuged at 1000$g$ for 5 min. The supernatant was stored on ice while a cuvette containing distilled water, 0.25 mmol l\textsuperscript{-1} FeSO\textsubscript{4}, 25 mmol l\textsuperscript{-1} sulfuric acid, and 0.1 mmol l\textsuperscript{-1} xylenol orange (added in this order) was allowed to react for 30 min. After this time period, the homogenate was added and given 2 h to react and an initial absorbance reading was taken at 580 nm using SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, California, USA). Cumene peroxide (0.005 mmol l\textsuperscript{-1}) was then added to the cuvette and allowed to react for 40 min, and a final reading was taken at 580 nm. Lipid peroxidation is standardized to and reported as cumene hydroperoxide 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).
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)
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 (LRTsite χ² = 1.25, p = 0.53), which accounted for 28.0% of the variation. (B) Exposure site had a significant effect on PC2 scores (LRTsite χ² = 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.

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

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 ($n = 7$).

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>Downstream</th>
<th>Outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>17.4 ± 0.70</td>
<td>22.95 ± 0.41</td>
<td>21.73 ± 0.39</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>5.48 ± 0.56</td>
<td>8.84 ± 0.82</td>
<td>11.28 ± 1.15</td>
</tr>
<tr>
<td>pH</td>
<td>8.00 ± 0.16</td>
<td>8.00 ± 0.11</td>
<td>7.95 ± 0.17</td>
</tr>
<tr>
<td>Conductivity (μS)</td>
<td>695.57 ± 30.38</td>
<td>1283.87 ± 40.42</td>
<td>1243.37 ± 41.82</td>
</tr>
<tr>
<td>Salinity (ppm)</td>
<td>315.71 ± 14.06</td>
<td>600.50 ± 19.33</td>
<td>581.38 ± 20.07</td>
</tr>
<tr>
<td>TDS (ppm)</td>
<td>494.71 ± 21.19</td>
<td>910.38 ± 28.72</td>
<td>883.38 ± 30.31</td>
</tr>
<tr>
<td>Flow (m/sec)</td>
<td>0.021 ± 0.0096</td>
<td>0.017 ± 0.0030</td>
<td>0.016 ± 0.0030</td>
</tr>
</tbody>
</table>
Table S3. Body and organ mass (% body mass) of bluegill sunfish. Data reported as mean ± s.e.m. ($n$).

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

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

S16
Table S4. Loadings onto the first two principal components from a principal component analysis.

<table>
<thead>
<tr>
<th>Response</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass</td>
<td>0.67</td>
<td>0.08</td>
</tr>
<tr>
<td>Liver mass</td>
<td>0.59</td>
<td>0.05</td>
</tr>
<tr>
<td>Heart mass</td>
<td>0.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Spleen mass</td>
<td>0.31</td>
<td>-0.17</td>
</tr>
<tr>
<td>Brain mass</td>
<td>0.59</td>
<td>0.24</td>
</tr>
<tr>
<td>Resting O\textsubscript{2} consumption rate</td>
<td>0.70</td>
<td>0.21</td>
</tr>
<tr>
<td>Total filament length</td>
<td>0.52</td>
<td>0.24</td>
</tr>
<tr>
<td>Gill surface density</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.46</td>
<td>0.00</td>
</tr>
<tr>
<td>Haemoglobin content</td>
<td>-0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>Haemoglobin P\textsubscript{50} at pH 7</td>
<td>0.25</td>
<td>0.35</td>
</tr>
<tr>
<td>Haemoglobin P\textsubscript{50} at pH 7.4</td>
<td>-0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Respiratory capacity for oxphos: P\textsubscript{PM}</td>
<td>-0.68</td>
<td>0.24</td>
</tr>
<tr>
<td>Respiratory capacity for oxphos: P\textsubscript{PMG}</td>
<td>-0.64</td>
<td>0.47</td>
</tr>
<tr>
<td>Respiratory capacity for oxphos: P\textsubscript{S(Rot)}</td>
<td>-0.46</td>
<td>0.65</td>
</tr>
<tr>
<td>Respiratory capacity for oxphos: P\textsubscript{PMGS}</td>
<td>-0.66</td>
<td>0.56</td>
</tr>
<tr>
<td>Mitochondrial ROS emission rates: P\textsubscript{PM}</td>
<td>-0.63</td>
<td>-0.39</td>
</tr>
<tr>
<td>Mitochondrial ROS emission rates: P\textsubscript{PMG}</td>
<td>-0.59</td>
<td>-0.43</td>
</tr>
<tr>
<td>Mitochondrial ROS emission rates: P\textsubscript{S(Rot)}</td>
<td>-0.67</td>
<td>-0.43</td>
</tr>
<tr>
<td>Mitochondrial ROS emission rates: P\textsubscript{PMGS}</td>
<td>-0.59</td>
<td>-0.39</td>
</tr>
<tr>
<td>ROS emission : respiratory capacity for oxphos: P\textsubscript{PM}</td>
<td>-0.07</td>
<td>-0.55</td>
</tr>
<tr>
<td>ROS emission : respiratory capacity for oxphos: P\textsubscript{PMG}</td>
<td>-0.19</td>
<td>-0.78</td>
</tr>
<tr>
<td>ROS emission : respiratory capacity for oxphos: P\textsubscript{S(Rot)}</td>
<td>-0.17</td>
<td>-0.80</td>
</tr>
<tr>
<td>ROS emission : respiratory capacity for oxphos: P\textsubscript{PMGS}</td>
<td>-0.11</td>
<td>-0.75</td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>-0.28</td>
<td>0.21</td>
</tr>
<tr>
<td>Succinate dehydrogenase activity</td>
<td>0.50</td>
<td>0.43</td>
</tr>
<tr>
<td>Citrate synthase activity</td>
<td>-0.24</td>
<td>-0.05</td>
</tr>
<tr>
<td>Mitochondrial P\textsubscript{50}</td>
<td>0.21</td>
<td>0.56</td>
</tr>
<tr>
<td>Respiratory capacity for electron transport: E\textsubscript{PM}</td>
<td>-0.62</td>
<td>0.33</td>
</tr>
<tr>
<td>Respiratory capacity for electron transport: E\textsubscript{PMG}</td>
<td>-0.64</td>
<td>0.51</td>
</tr>
<tr>
<td>Respiratory capacity for electron transport: E\textsubscript{S(Rot)}</td>
<td>-0.37</td>
<td>0.60</td>
</tr>
<tr>
<td>Respiratory capacity for electron transport: E\textsubscript{PMGS}</td>
<td>-0.63</td>
<td>0.53</td>
</tr>
<tr>
<td>Leak respiration L\textsubscript{T}</td>
<td>-0.6</td>
<td>0.24</td>
</tr>
<tr>
<td>Leak respiration L\textsubscript{N}</td>
<td>-0.65</td>
<td>0.30</td>
</tr>
<tr>
<td>Critical P\textsubscript{O2}</td>
<td>0.04</td>
<td>0.18</td>
</tr>
<tr>
<td>P\textsubscript{O2} at loss of equilibrium</td>
<td>0.28</td>
<td>0.14</td>
</tr>
<tr>
<td>EROD activity</td>
<td>-0.22</td>
<td>-0.09</td>
</tr>
</tbody>
</table>
References used in Supporting Information


