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Metabolic Costs of Exposure to Wastewater Effluent Lead to Compensatory Adjustments in Respiratory Physiology in Bluegill Sunfish

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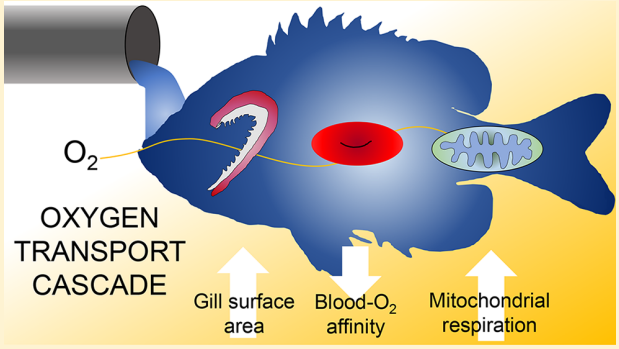
Supporting Information

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 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 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 behavior 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 O_2 consumption) are linked to growth, reproduction, activity, functional performance, and many important behaviors.[21−26] Exposure to aquatic pollution may require that energy be redirected toward 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

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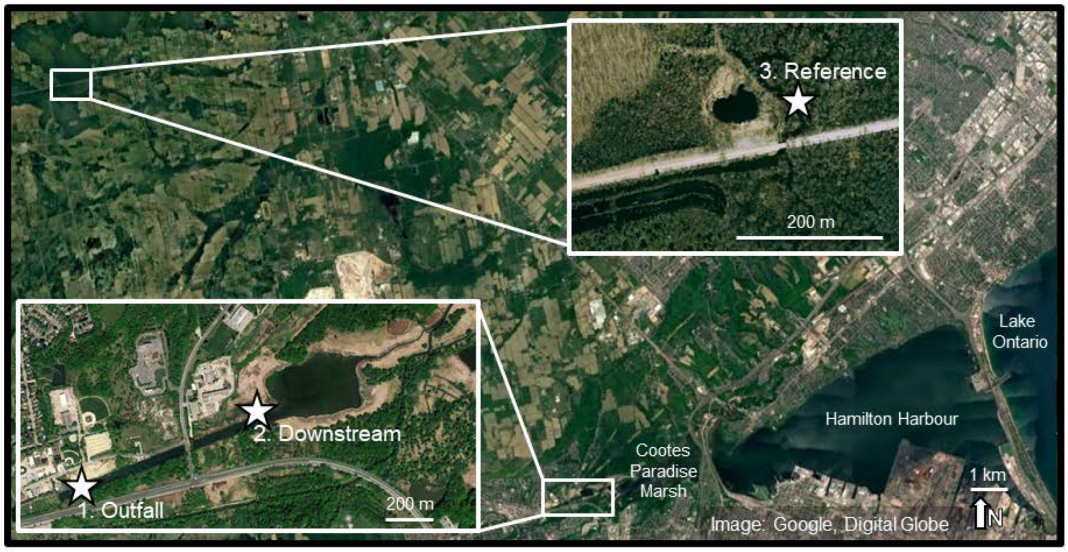

Figure 1. Location of study area and sites of caged exposures. Bluegill sunfish were caged for 21 days (1) 50 m from the outfall of a wastewater treatment plant (WWTP) that provides tertiary treatment to the municipality of Dundas, (2) 830 m further downstream in an effluent-dominated canal, or (3) at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

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 (Figure 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 (SI).

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, Figure 1). Dundas WWTP is a tertiary treatment plant that serves a population of ∼30 000 and treats an average of 15 million liters 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 (Figure 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) (Figure 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 behavior 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 (SI 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 (SI 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_{O2}) 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 M_{O2} was obtained in normoxia (90−100% air saturation). Hypoxia tolerance (critical P_{O2} and P_{O2} 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 SI). We extracted and identified these compounds following previously described methods.[47−49]

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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 hematoxylin. 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. Hemoglobin-O_2 Binding. Hemoglobin-O_2 affinity (P_{50}, the P_{O2} at which hemoglobin is 50% saturated) was determined in lysate of red blood cells using Hemox Analyzer and software (TCS Scientific, New Hope, PA) 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 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 (SI Figure 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 analyzed using R (version 3.2.4;[57]). Survival was analyzed 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 posthoc tests (multcomp package,[59]) to compare each exposure site to the reference site. All remaining data, unless otherwise noted, were analyzed 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 posthoc tests. In the analyses of M_{O2} and organ masses (SI 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 analyzed with the additional fixed effects of respiratory state and its interaction with exposure site. Hemoglobin P_{50} was analyzed 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 (SI Figure 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%, Figure 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} $\chi^2 = 0.027$, $p = 0.99$).

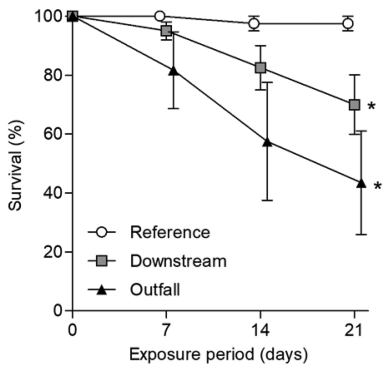

Figure 2. Wastewater exposure decreased survival of bluegill sunfish. Bluegill sunfish were caged at the outfall of a wastewater treatment plant, further downstream, or at an uncontaminated reference site for 21 days. * represents significant differences between fish from the reference site (LRT_{site}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

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 (SI Table S1). Galaxolide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[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-hexamethyl-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 bioaccumula-

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tion.[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} χ^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_{O2}) than fish caged at the reference site (Figure 3). However, wastewater exposure did not have a significant effect on hypoxia tolerance. Critical P_{O2} (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} χ^2 = 2.45, p = 0.29), as was the P_{O2} 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} χ^2 = 1.89, p = 0.39).

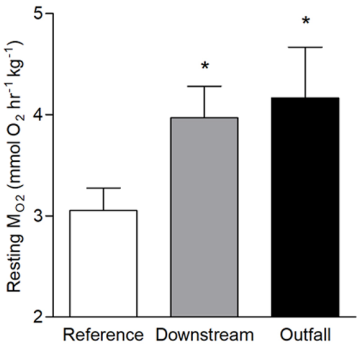

Figure 3. Standard rates of O_2 consumption increased in resting bluegill sunfish exposed to wastewater effluent. Bluegill caged at the downstream (n = 10) and outfall sites (n = 7) had significantly higher rates of aerobic metabolism than fish caged at the reference site (n = 10). *represents significant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

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

3.5. Haematology. Blood-O_2 binding was altered in response to wastewater exposure (Figure 5). P_{50} (the P_{O2} at which hemoglobin was 50% saturated) at pH 7.0 was higher in bluegill from the outfall site compared to other groups (Figure 5A), as was the pH sensitivity of O_2 binding (Figure 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} χ^2 = 8.57, p = 0.014), but blood hemoglobin content did not vary across sites (Figure 5C).

3.6. Mitochondrial Respiration. Wastewater exposure altered the physiology of liver mitochondria (Figure 6). Mitochondrial respiratory capacities for oxidative phosphorylation (oxphos, P) were ∼10% higher in fish caged at the outfall (Figure 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 nonsignificant decrease) in citrate synthase activity (Table 1). Wastewater exposure also increased mitochondrial P_{50} (the P_{O2} 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 (Figure 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 (Figure 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 (Figure 2). Exposure also increases standard rates of aerobic metabolism (Figure 3), which was associated with adjustments across the oxygen transport cascade that expanded the gills' capacity for gas exchange (Figure 4), facilitated the unloading of O_2 from hemoglobin at the tissues (Figure 5), and increased the respiratory capacity of liver mitochondria (Figure 6). There was a significant overall effect of wastewater on physiology when considered using a principal component analysis (SI Figure S2 and 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

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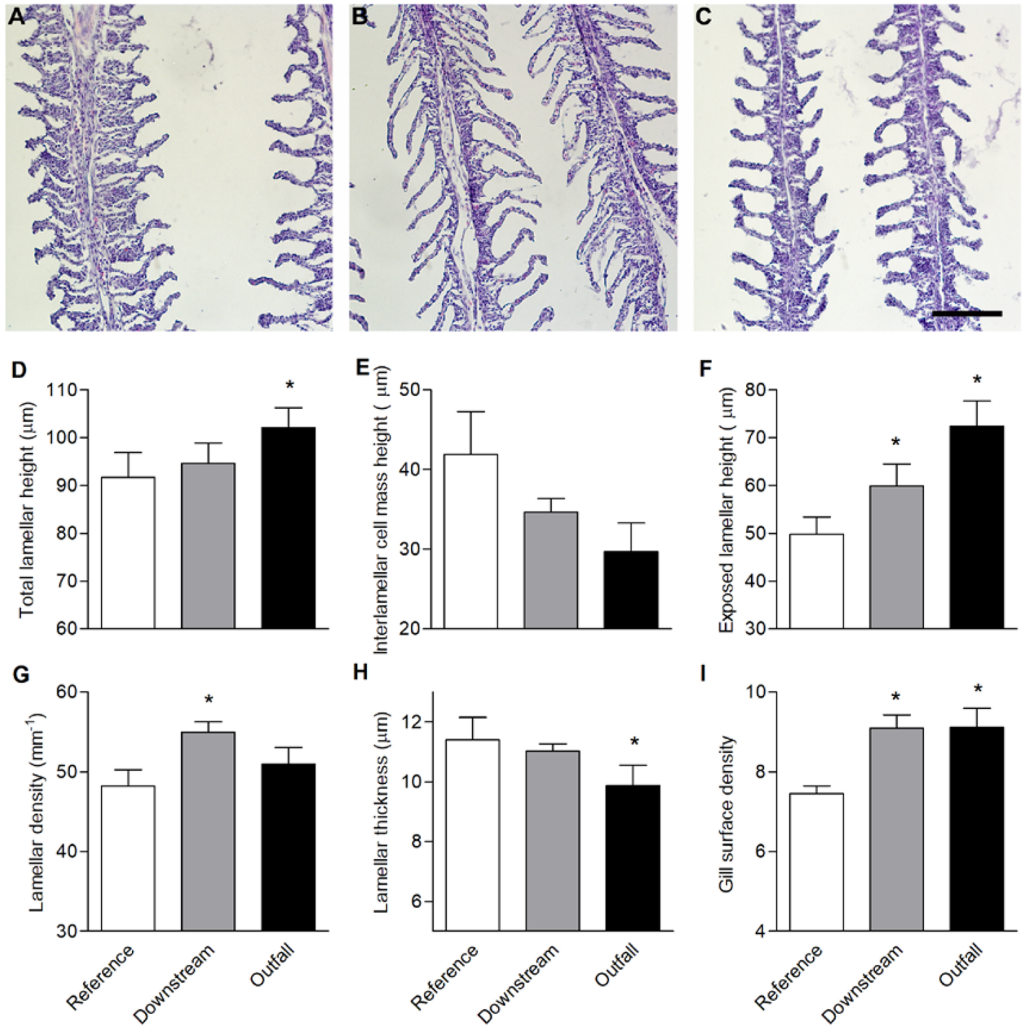

Figure 4. Bluegill remodelled their gills in response to wastewater exposure. Representative images of gills from bluegill caged at (A) reference, (B) downstream, and (C) outfall sites for 21 days (scale bar represents 1 mm). (D) Total lamellar height was highest in bluegill from the outfall site (LRT_{site} χ^2 = 4.18, p = 0.12; Dunnett's posthoc: downstream, p = 0.62; outfall, p = 0.039). (E) Height of interlamellar cell mass (ILCM) was lower after wastewater exposure (LRT_{site} χ^2 = 6.63, p = 0.036), and the reductions in the downstream and outfall sites approached statistical significance in Dunnett's posthoc 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} χ^2 = 22.3, p < 0.0001; Dunnett's posthoc: downstream, p = 0.001; outfall, p < 0.0001). (G) Lamellar density (LRT_{site} χ^2 = 8.50, p = 0.014; Dunnett's posthoc: downstream, p = 0.006; outfall, p = 0.32) and (H) lamellar thickness (LRT_{site} χ^2 = 6.34, p = 0.04; Dunnett's posthoc: 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} χ^2 = 19.1, p < 0.0001; Dunnett's posthoc: downstream, p < 0.0001; outfall, p < 0.0001). *represents significant differences from the reference site ($n_{reference}$ = 9, $n_{downstream}$ = 9, $n_{outfall}$ = 7).

integrated measures of organismal metabolism, as reflected by the rate of O_2 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., ref 33 and 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 uncaged 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 behavior.[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

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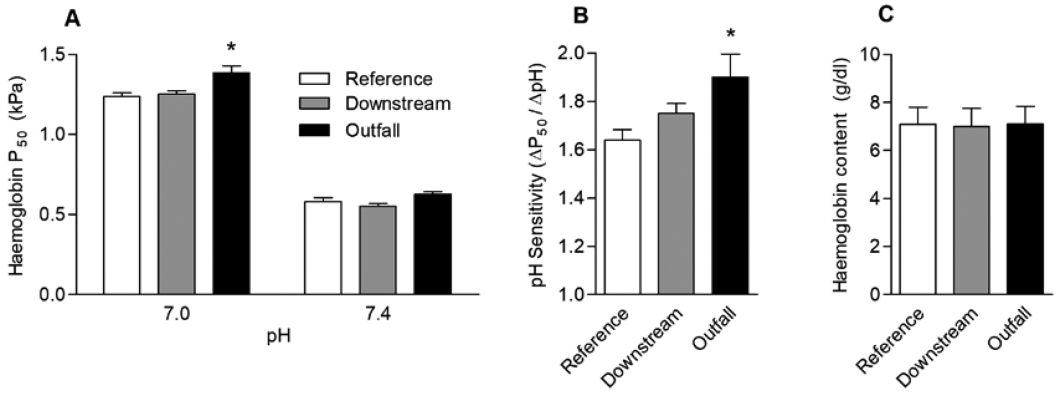

Figure 5. Hemoglobin-oxygen binding affinity was reduced in response to wastewater exposure at the outfall site. (**A**) The P_{50} of hemoglobin (the partial pressure of oxygen at which hemoglobin is 50% saturated) was measured in the lysate of frozen red blood cells and was highest in bluegill caged at the outfall site at pH 7.0 (LRT_{site} χ^2 = 20.2, p < 0.0001; Dunnett's posthoc: 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} χ^2 = 139.9, p < 0.0001). (B) pH sensitivity of hemoglobin (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} χ^2 = 7.82, p = 0.020; Dunnett's posthoc: downstream, p = 0.63; outfall, p = 0.013, n the same as above). (C) Blood hemoglobin content was similar across all exposure sites (LRT_{site} χ^2 = 0.98, p = 0.61); $n_{reference}$ = 10, $n_{downstream}$ = 10, $n_{outfall}$ = 6). *represents significant differences from the reference site.

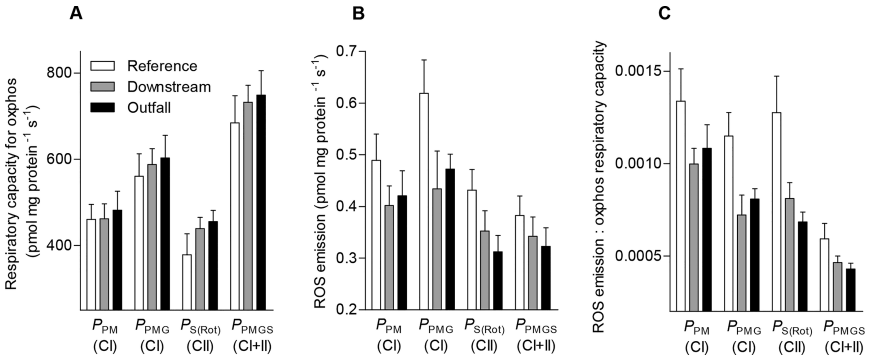

Figure 6. Wastewater exposure affected the physiology of isolated liver mitochondria. (A) Bluegill had higher respiratory capacities for oxidative phosphorylation after 21 days of exposure to wastewater effluent (LRT_{site} χ^2 = 7.59, p = 0.022; LRT_{state} χ^2 = 83.0, p < 0.0001; Dunnett's posthoc: 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} χ^2 = 24.6, p < 0.0001; LRT_{state} χ^2 = 35.0, p < 0.0001; Dunnett's posthoc: 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} χ^2 = 31.0, p < 0.0001; LRT_{state} χ^2 = 49.7, p < 0.0001; Dunnett's posthoc: 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). ($n_{reference}$ = 9, $n_{downstream}$ = 9, $n_{outfall}$ = 7).

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)[a] 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)[a] 9	(0.047 ± 0.002)[a] 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

[a]Represents a significant difference from reference site; SDH, LRT_{site} χ^2 = 6.81, p = 0.033 (downstream, p = 0.066; outfall, p = 0.044); CS, LRT_{site} χ^2 = 2.37, p = 0.31; P_{50}, LRT_{site} χ^2 = 9.81, p = 0.007 (downstream, p = 0.012; outfall, p = 0.013); E, LRT_{site} χ^2 = 3.34, p = 0.19; LRT_{state} χ^2 = 72.9, p < 0.0001 (downstream, p = 0.98; outfall, p = 0.20); L_T, LRT_{site} χ^2 = 2.14, p = 0.34; L_N, LRT_{site} χ^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).

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increases in resting M_{O2} could maintain (or even increase) aerobic scope with compensatory increases in maximal M_{O2}.[64,66] Although we did not measure maximal M_{O2}, the respiratory adjustments of bluegill in response to wastewater exposure (discussed below) suggest that they may be able to increase maximal M_{O2} 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 aluminum suffered reduced maximal M_{O2} 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 (Figure 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 O_2 uptake and aerobic scope,[65] but that clearly did not occur in the present study.

Bluegill also responded to wastewater exposure by modulating hemoglobin-O_2 binding affinity of the blood. Hemoglobin-O_2 affinity balances the demands of O_2 loading and uptake at the gills (which is facilitated by an increase in affinity) and O_2 unloading at the tissues (which is facilitated by a decrease in affinity).[86] In situations when respiratory O_2 uptake is not compromised, a lower hemoglobin-O_2 affinity is expected to augment O_2 transport to tissues by increasing the P_{O2} of blood passing through the capillaries. Therefore, the increase in hemoglobin P_{50} at low pH in bluegill exposed to wastewater likely facilitates O_2 transport to respiring tissues (where the blood becomes more acidic), while the expansion of gill surface area helps safeguard branchial O_2 loading into the blood. This appears to be an alternative strategy to improve O_2 transport than increasing hemoglobin content.[84]

4.3. Wastewater Exposure Altered Mitochondrial Function. Wastewater exposure increased the respiratory capacities for oxidative phosphorylation of liver mitochondria (Figure 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.[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 (SI 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 (Figure 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. Intersite differences in water quality (SI 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_{O2}, 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_{O2} 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, intersite 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

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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 from refs 71 and 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.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b03745.

S1. Detailed Material and Methods. Figure 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. Figure 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 (PDF)

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