

**METABOLIC AND RESPIRATORY CONSEQUENCES OF
WASTEWATER EXPOSURE ON FISH**

**METABOLIC AND RESPIRATORY CONSEQUENCES OF
WASTEWATER EXPOSURE ON FISH**

By

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ABSTRACT

Wastewater effluent is a major source of aquatic pollution, discharging a continuous and complex mix of contaminants into the environment. The physiological impacts of exposure to individual contaminants are well characterized in fish, but less is known about the consequences of complex mixtures that typify wastewater effluent in the environment. This thesis explores the metabolic and respiratory effects of *in situ* wastewater exposure on bluegill sunfish (*Lepomis macrochirus*). By assessing multiple levels of biological organization, I provide evidence that wastewater exposure invoked a metabolic cost in bluegill, which was associated with physiological adjustments to enhance oxygen uptake, delivery, and utilization. These findings broaden our understanding of how fish tolerate life in effluent-dominated waters and support the emergence of metabolic and respiratory physiology as biomarkers in ecotoxicology.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a “sandwich” format and consists of three main chapters. Chapter one provides a general introduction and outlines the objectives of my thesis research. Chapter two is a manuscript that is prepared for submission to a peer-reviewed scientific journal. Chapter three discusses these findings and their implications in ecotoxicology. Appendix A is a manuscript of a study I conducted on mitochondrial physiology in hybrid sunfish, which helped develop laboratory techniques necessary for my thesis research.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: METABOLIC AND RESPIRATORY CONSEQUENCES OF EXPOSURE TO WASTEWATER EFFLUENT IN BLUEGILL SUNFISH (*Lepomis macrochirus*)

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CHAPTER 3: GENERAL DISCUSSION

APPENDIX A: HYBRIDIZATION INCREASES MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES IN SUNFISH

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LIST OF ABBREVIATIONS

CS – citrate synthase

E – electron transport

E_{PM} – E with pyruvate and malate

E_{PMG} – E with pyruvate, malate, and glutamate

E_{PMGS} – E with pyruvate, malate, glutamate, and succinate

$E_{S(Rot)}$ – E with succinate and rotenone

EROD – ethoxyresorufin-O-deethylase

L_N – leak respiration in the absence of ATP

L_T – leak respiration in the presence of ATP

LOE – loss of equilibrium

M_{O_2} – rate of oxygen consumption

oxphos or P – oxidative phosphorylation

P_{PM} – P with pyruvate and malate

P_{PMG} – P with pyruvate, malate, and glutamate

P_{PMGS} – P with pyruvate, malate, glutamate, and succinate

$P_{S(Rot)}$ – P with succinate and rotenone

P_{O_2} – partial pressure of oxygen

P_{crit} – critical P_{O_2}

POCIS – polar organic chemical integrative sampler

P_{50} – P_{O_2} at 50% haemoglobin saturation (blood) or maximal respiration (mitochondria)

PPCPs – pharmaceuticals and personal care products

ROS – reactive oxygen species

SDH – succinate dehydrogenase

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Wastewater in the environment

Wastewater is one of the largest point-sources of aquatic pollution, releasing a complex and variable mixture of contaminants into surface waters. In Canada, 6.37 billion tonnes of wastewater were discharged in 2006 (Environment Canada, 2010). As liquid waste from a variety of sources (e.g. residential, commercial, industrial, stormwater; Chambers et al., 1997), wastewater can contain a vast diversity of pollutants including excess nutrients, pesticides, metals, chlorine compounds, pharmaceuticals and personal care products (PPCPs; Brooks et al., 2006; Carey and Migliaccio, 2009; Andreozzi et al., 2003; Gros et al., 2010; Nikolaou et al., 2007; Ternes, 1998; Schultz et al., 2010). Each of these pollutants can individually and synergistically degrade water quality and wildlife living downstream from wastewater outfalls. Excess nutrients (e.g. phosphorus and nitrogen) can cause eutrophication which, along with decaying organic matter in wastewater, deplete dissolved oxygen and constrain available habitat for aquatic wildlife (Carpenter et al., 1998). Many pesticides and pharmaceuticals are endocrine disruptors and affect growth and reproduction in aquatic species (Overturf et al., 2015). Similarly, pharmaceuticals, metals, and several personal care products are biologically active and can induce toxic effects in wildlife (Corcoran et al., 2010; Atchison et al., 1987; Daughton and Ternes, 1999; Brausch and Rand, 2011).

In developed countries, wastewater is often treated at a wastewater treatment plant (WWTP) where excess nutrients and some contaminants are removed to levels suitable for discharge into surface waters as treated effluent. However, WWTPs do not fully remove the vast range of contaminants before the effluent is released, so contaminants are frequently found in environments receiving wastewater (Bolong et al., 2009). The concentrations of each class of contaminants in aquatic ecosystems are highly variable and depend on numerous factors including the season, source of effluent, quality of treatment at the WWTP, and its dilution within the receiving waters. Concern for the health of aquatic ecosystems is magnified as populations grow and concentrate in urban areas served by WWTPs, consequently increasing the amount of effluent discharged to surface waters.

1.2 Tools for studying the ecotoxicology of wastewater

Much of the research conducted to date on wastewater effluent has used controlled exposures with individual contaminants to evaluate impacts on aquatic species. These laboratory studies provide a foundational understanding of the toxicological mechanisms and consequences of individual pollutants, but they do not capture the complexity of exposure to wastewater. In reality, wastewater is highly dynamic and variable, as each WWTP releases a unique contaminant profile depending on the population it serves and the type of treatment it employs, and even effluent from a single WWTP varies daily (Plósz et al., 2010) and seasonally (Tetreault et al., 2013). Coupled with the pressures of other environmental variability encountered in the wild (e.g. dissolved oxygen, rising temperatures), a complete understanding of wastewater would be very challenging to

accomplish in the laboratory. In contrast, studying the species living in environments polluted by wastewater would encapsulate the complexity of environmental variability and dynamic wastewater stress, but would sacrifice control over aspects of the exposure (e.g. parental or developmental exposures, fish's movement along the effluent gradient).

In situ caging integrates the control offered by laboratory experiments with the environmental realism of field studies. This technique involves deploying species in cages at various contaminated sites along a gradient of contamination for a specific duration. Caging offers control in the selection of species (age, size, sex), exposure (location, duration, season), and interspecific interactions (e.g. removal of predation) while maintaining exposure to real combinations of environmental and pollution stresses (Guernic et al., 2016). Similar achievements can be made with laboratory studies of dilutions of whole wastewater effluent, but these experiments often neglect environmental variability (e.g. Melvin, 2016; Aerni et al., 2004; Schmidt et al., 1999; Vajda et al., 2011).

To understand the impacts of wastewater exposure on wildlife, ecotoxicologists use biomarkers, which are measurable indicators or consequences of a given exposure. Biomarkers can be biochemical, physiological, or histological that span multiple levels of biological organization. Traditional biomarkers include activities of detoxification enzymes (e.g. ethoxyresorufin-o-deethylase, EROD) and markers of oxidative stress (i.e. damage to cellular components from excessive production of reactive oxygen species). However, these measurements are often scrutinized for providing ambiguous and inconsistent information on the exposure type, and being poor predictors of broader organismal consequences when used in isolation (Forbes et al., 2006). Current research in

ecotoxicology aims to apply more comprehensive suites of biomarkers that have implications for animal fitness and populations, or to better identify specific contaminants in the water. Biomarkers are important tools in ecotoxicology but require careful selection to meaningfully understand impacts of wastewater exposure at multiple levels of biological organization (Forbes et al., 2006).

In the sections below, I will broadly discuss our current understanding of the biological impacts of wastewater exposure, focusing on commonly used biomarkers. This discussion prioritizes works that test whole wastewater effluent (rather than individual contaminant exposures) using laboratory and field exposures (both *in situ* caging and wild fish).

1.3 Biological responses to wastewater

1.3.1 Reproduction

The reproductive impacts of wastewater effluent exposure have been studied extensively in many fish species across all levels of biological organization (Tyler and Jobling, 2008). Numerous aquatic contaminants found in wastewater effluent have been shown to disrupt reproductive physiology and behaviours including metals, pesticides, and PPCPs. Some of these are endocrine disrupting chemicals (EDC) that often work by agonising or antagonizing hormone receptors (Purdom et al., 2006; Söffker and Tyler, 2012). Of the EDCs, estrogens (e.g. estrone, 17 α -estradiol, 17 β -estradiol, bisphenol-A) have received the most attention due their potency, role in gonadal differentiation,

reproduction, and the conservation of estrogenic receptors among vertebrates (Brown et al., 2014). Other EDCs include progestins, androgens, and aromatase inhibitors, though a suite of non-endocrine contaminants can also impact reproduction (notably selective serotonin reuptake inhibitors, beta-blockers, and blood lipid regulators; reviewed by Overturf et al., 2015).

One of the most well-known reproductive impediments of fish living downstream of WWTPs is feminization of males. Exposure to excess estrogens can cause male fish to develop oocytes within their testis (“testicular oocytes”; Jobling et al., 2005), thus exhibiting both male and female gonads. Intersex fish are common in many effluent-dominated streams (e.g. Jobling et al., 1998; Adeogun et al., 2016; Tetreault et al., 2011; Hinck et al., 2009) and is associated with poor fertilization and reproductive success (Fuzzen et al., 2015). For example, wild intersex roach (*Rutilus rutilus*) suffer delayed spermatogenesis and are less likely to release sperm, and those that do have relatively low sperm quality (Jobling et al., 2002). Nonetheless, not all species appear to be sensitive to estrogenic disruption. In a survey of seven species in Cootes Paradise Marsh, a sensitive marshland of western Lake Ontario that receives treated wastewater, Kavanagh et al. (2004) only found feminization in white perch (*Morone americana*). Even so, effluent-induced feminization is not exclusive to fish: frogs (McDaniel et al., 2008; Abdel-Moneim, 2015) and bivalves (Langston et al., 2007; Chesman and Langston, 2006) are similarly affected. Feminization of males may be due in part to the induction of vitellogenin, a precursor protein of egg yolk that is stimulated in the liver by estrogen and is normally absent in males. However, male rainbow trout (*Oncorhynchus mykiss*) caged downstream

of 15 WWTPs in England have 500 to 100 000-fold higher levels of plasma vitellogenin than male trout from reference sites (Purdom et al., 1994), a pattern commonly observed worldwide (Porter and Janz, 2003; Vajda et al., 2008; Thorpe et al., 2009). Vitellogenin has since become a widely-used biomarker for estrogen exposure (Denslow et al., 2001). These disruptions are likely associated with female-biased populations in municipal effluent-dominated streams, with a proportion of males being replaced by intersex fish (Vajda et al, 2008; Woodling et al., 2006).

Nonetheless, reproductive disruption is not limited to male fish. Laboratory dilutions of wastewater effluent reduced egg production of fathead minnow in proportion to estrogenic content (*Pimephales promelas*; Thorpe et al., 2009), and similar patterns were observed in wild roach downstream of WWTPs (Jobling et al., 2002). Collectively, these disruptions in both male and female reproductive physiology can have deleterious implications on populations, as discussed below.

1.3.2 *Energy metabolism*

Many fish living in effluent-dominated streams have heightened capacity to detoxify and protect cells from contaminant insult (Kosmala et al., 1998; Milla et al., 2011). However, these processes are energetically expensive and could compromise energy budgets for other processes including reproduction and growth. Further, many contaminants of wastewater (e.g. metals and pharmaceuticals) directly target mitochondrial function, which can challenge mitochondrial ability to satisfy higher energetic demands.

Therefore, many studies have investigated the effects of wastewater exposure on bioenergetic markers.

The cellular energy allocation model is used to understand the balance between availability and consumption of energy by comparing cellular energy reserves to activities of mitochondrial enzymes, respectively (De Coen and Janssen, 2003). Wastewater exposure can reduce energy stores in fish, which have been linked to broader changes in body condition (Smolders et al., 2003) and swimming performance (Melvin, 2016). Curimbata fish (*Prochilodus lineatus*) caged near effluent discharge for 96 h reduced muscle and liver glycogen levels without changing lipid or protein stores (Cazenave et al., 2014), though longer exposure (2 weeks) to wastewater in the laboratory reduced lipid and protein stores in empire gudgeons (*Hypseleotris compressa*; Melvin, 2016). Glucose availability increases after 96 h of wastewater exposure in some fishes, as reflected by enhanced activities of glycogen phosphorylase and glucose-6-phosphatase (Rodrigues et al., 2015). Energy demands can gradually deplete energy reserves over the duration of wastewater exposure (Smolders et al., 2004) and this may be associated with changes in AMPK (5'AMP-activated protein kinase), a protein involved in maintaining energy homeostasis. AMPK inhibits ATP-consuming pathways (e.g. synthesis of macromolecules) and instead induces ATP-producing pathways (glycolysis, fatty-acid oxidation, and glucose uptake; Goodchild et al., 2015). AMPK activity has been shown to increase in mussels (*Ellipto complanata*) exposed to wastewater effluent (Goodchild et al., 2015).

The challenges of meeting the energetic demands of wastewater exposure are further exacerbated by mitochondrial disruption. Several contaminants in wastewater specifically target mitochondria (Meyer et al., 2013), especially metals (e.g. copper, cadmium, mercury; Belyaeva et al., 2012) and pharmaceuticals (e.g. psychotropics, analgesics, fibrates; Neustadt and Pieczenik, 2008). Fish living in contaminated waters often suffer reduced aerobic capacities due to low activities of mitochondrial enzymes (e.g. citrate synthase, β -hydroxyacyl coenzyme A; Rajotte and Couture, 2002; Goertzen et al., 2011) which can constrain aerobic swimming and performance, as described in the section below. Reduced aerobic metabolism can foreseeably shift dependence to anaerobic pathways (Castro et al., 2004; Orrego et al., 2011). The use of anaerobic pathways can lead to metabolic acidosis and produce much less ATP than aerobic pathways, so short- and long-term function and performance of fish may suffer during exposure to wastewater effluent.

1.3.3 Performance and behaviour

Alterations in physiology at lower levels of biological organization can impact traits at the organismal level including performance and behaviour. Traits at higher levels of organization tend to be less responsive to stress than those at lower levels (e.g. genetic or biochemical biomarkers) because disruptions at higher levels can be offset by physiological compensations at lower levels (Adams et al., 2000). Nonetheless, performance and behaviours of fish are frequently affected by wastewater exposure though in variable ways.

Swimming abilities in fish are highly dependent on aerobic capacity, which is often constrained by exposure to wastewater. One common measure of swimming performance is critical swimming speed (U_{crit}), the speed at which a fish can no longer maintain swimming in incremental increases in water velocity. U_{crit} has been shown to decrease with both laboratory dilutions and *in situ* exposures of industrial wastewater in fathead minnows (Goertzen et al., 2011; Farrell et al., 2004). Additionally, emperors gudgeons exposed to 100% wastewater for 14 days tended to have poorer swimming complexity in wastewater dilutions as low as 12.5% (Melvin, 2016) and burst speed was significantly lower in wild brown bullhead (*Ameiurus nebulosus*) caught from a contaminated site that receives both industrial and municipal wastewater (Breckels and Neff, 2010). However, swimming performance appears to be highly variable as some studies have found increased endurance, greater maximum velocity, and higher U_{crit} after exposure to municipal (Melvin, 2016) and industrial (Rajotte and Couture, 2002) wastewater.

Swimming capabilities have important implications on other behaviours including predator avoidance and foraging. Though exposures to individual contaminants have been shown to alter fish behaviours (reviewed by Scott and Sloman, 2004), less is known about the impacts of complex mixtures found in wastewater effluent. Generally, behaviour seems to vary widely. Laboratory exposures to municipal wastewater reduced aggression and competitive performance in fathead minnow (Garcia-Reyero et al., 2011; Martinovic et al., 2007), but brown bullhead caught from a site contaminated with industrial and municipal wastewater were more aggressive than those from a clean site (Breckels and Neff, 2010). Reproductive activity also yielded mixed results: wild mosquitofish living near a WWTP

demonstrated greater activity (Saaristo et al., 2014) while goldfish (*Carassius auratus*) demonstrated minor decreases in activity (Schoenfuss et al., 2002). Our research group found that aggression and activity level of round goby (*Neogobius melanostomus*), an invasive and stress-tolerant fish (Marentette et al., 2010), were unaltered by *in-situ* exposure to municipal wastewater (McCallum et al., 2017), but activity was reduced in wild round goby exposed to industrial contaminants (Marentette and Balshine, 2012; Marentette et al., 2012). Thus, the impacts of wastewater effluent on behaviours appear to be specific to effluent type and species (and certainly other parameters too such as time of day, season, and sex; Marentette and Balshine, 2012), making behavioural observations challenging to generalize.

1.3.4 Community

Wastewater effluent has been shown to impact structure and function of fish communities. Fish living near WWTP outfalls tend to be omnivorous and generalists, taking advantage of the increased availability of nutrients and food (Tetreault et al., 2013; Porter and Janz, 2003; but see Grantham et al., 2012). However, living in wastewater-impacted areas comes at the cost of increased contaminant exposure, so only fish with high stress tolerance can exploit this niche, thereby locally reducing species diversity (“species homogenization”; Northington and Hershey, 2006; Tetreault et al., 2013; Porter and Janz, 2003). In our community survey of an effluent-dominated stream in Cootes Paradise Marsh, ON, round goby were highly abundant near the WWTP (<50 m from the outfall), and bluegill sunfish, yellow perch, and brown bullhead (which are more sensitive species)

were more abundant further downstream (550-1000 m) (McCallum et al., in preparation). Populations living closer to WWTP outfalls tend to lack smaller (presumably younger) individuals, possibly indicating that reproductive impediments limit ability to spawn and produce viable offspring (Tetreault et al., 2012; Yeom et al., 2007). However, total fish abundance in effluent-dominated water is highly variable across studies, likely because of the variability in the effluent discharged from WWTPs studied to date, the species present in each region and their individual physiologies, and degree of habitat degradation (Porter and Janz, 2003; Northington and Hershey, 2006; Yeom et al., 2007; Dyer and Wang, 2002; Flinders et al., 2009).

1.4 Oxygen cascade: an integrative biomarker approach

Bioindicators at the organismal, population, and community levels are of special interest to environmental managers and conservation efforts. Regular monitoring at these levels provides crucial information to stakeholders invested in understanding anthropogenic impacts in watersheds. However, community assessments are hard to generalize between watersheds because of differences in “baseline” communities, type and volume of effluent discharged, and differences in aquatic habitat and environmental stressors acting on the ecosystem. It is challenging to disentangle the effects of water quality, environmental stressors (e.g. dissolved oxygen, rising temperatures), habitat, interspecies interactions, and movement of fish, stressing the value of concurrent testing of lower levels of biological organization. However, many lower-level biomarkers that are

frequently used are not predictive of higher levels of functioning and therefore lack ecological relevance (Forbes et al., 2006). One solution could be to apply an integrative set of biomarkers that span multiple levels of biological organization but are interconnected in physiology and function.

The oxygen cascade – the flow of oxygen from its extraction from the environment to its utilization within the animal – is a common toolkit used by experimental biologists to study the impacts of numerous environmental stressors including hypoxia, salinity, and temperature. The oxygen cascade can be understood from the perspective of metabolism and respiration, both of which are linked tightly to organismal fitness and performance (Tierney & Farrell, 2004; McCarthy, 2001; Biro and Stamps, 2010; Chatelier et al., 2006; Metcalfe et al., 1995; Clark et al., 2013). Following the flow of oxygen entails understanding how much oxygen is used by the whole organism (its aerobic metabolism), and how oxygen extraction (*via* gills and skin), transport (*via* blood), and utilization (at the mitochondria) support this demand. Wastewater exposure has already been shown to affect cellular energy reserves (e.g. Smolders et al., 2003; Cazenave et al., 2014; Melvin, 2016), so studying metabolism and respiration provides a lens to approach energetics at multiple levels in a mechanistic and integrative way that becomes directly relevant to the organism.

1.5 Objectives

The primary aim of this thesis was to explore the impacts of wastewater exposure on the oxygen cascade of fish through the lens of metabolism and respiration. The secondary aim was to determine the PPCP profile of Cootes Paradise, a sensitive wetland of western

Lake Ontario, and inform stakeholders that use the watershed. Together, these aims allowed me to develop biomarkers in response to complex effluents.

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

2.1 Abstract

Municipal wastewater effluent is a major worldwide source of aquatic pollution. We sought to determine the metabolic consequences of exposure to wastewater effluent on fish and whether physiological adjustments help fish cope in effluent-dominated water. We caged bluegill sunfish (*Lepomis macrochirus*) at two sites downstream of discharge from a tertiary wastewater treatment plant (WWTP) and at an uncontaminated reference site for three weeks. Survival was reduced in fish caged near the outfall of the WWTP (< 50 m away) and a short distance (830 m) downstream, when compared to the negligible mortality in fish from the reference site. Fish at both wastewater-contaminated sites were exposed to elevated levels of pharmaceuticals and personal care products, as reflected by measurements of a suite of target compounds in the water and by the accumulation of synthetic musks (e.g., Galaxolide® and Tonalide®) in the gills and livers of fish by the end of the exposure period. Resting rates of oxygen consumption increased by 30-36% in fish at contaminated sites, reflecting a metabolic cost of wastewater exposure. Exposed fish expanded the gill surface area available for gas exchange by reducing the interlamellar cell mass and thus exposing more lamellar surface to the water. Blood-O₂ affinity also decreased in exposed fish, facilitating O₂ unloading at respiring tissues. Exposure also

improved the quality of isolated liver mitochondria by increasing respiratory capacities for oxidative phosphorylation (assessed using single and multiple inputs to the electron transport system) and succinate dehydrogenase (but not citrate synthase) activity, while decreasing the 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.

2.2 Introduction

Wastewater effluent contains a dynamic and complex mixture of contaminants that is released into the environment (Environment Canada, 2010; Kolpin et al., 2002; Schwarzenbach et al., 2010). Wastewater treatment plants (WWTP) do not remove all contaminants from wastewater, so receiving waters often contain chemical constituents of wastewater effluent including pesticides, endocrine active substances, metals, and excess nutrients (Brooks et al., 2006; Carey and Migliaccio, 2009). Over the past two decades, attention has been drawn to the presence of pharmaceuticals and personal care products (PPCPs) in wastewater effluent, many of which are recognized as ecological hazards and may pose a risk to aquatic wildlife (Andreozzi et al., 2003; Gros et al., 2010; Nikolaou et al., 2007; Ternes, 1998; Schultz et al., 2010).

Several laboratory studies have shown that controlled exposure to individual chemical constituents of wastewater effluent can impact the physiology and behaviour of fish (reviewed by Scott and Sloman, 2004; Overturf et al., 2015; Heath, 1995; Jezierska et

al., 2009), frogs (Fraker and Smith, 2004; Palenske et al., 2010), and aquatic invertebrates (Lovern et al., 2007; Baldwin et al., 1995). Exposure to single contaminants can reduce growth, survival, and swimming performance in fish and frogs (Little and Finger, 1990; Scott and Sloman, 2004; Bridges, 1997), and low concentrations of individual pharmaceuticals can also disrupt their reproductive physiology and behaviour (Nash et al., 2004; Vajda et al., 2011; Parrott and Blunt, 2005; Bjerselius et al., 2001; Schwendiman and Propper, 2012; Pettersson et al., 2006). For example, exposure to the pesticide dieldrin increased resting rates of oxygen consumption in a dose-dependent fashion in largemouth bass (*Micropterus salmoides*) (Beyers et al., 1999). However, less is known about how fish physiology is impacted by the complex mixtures of contaminants that typify wastewater, which could interact in synergistic ways that are hard to predict, particularly when combined with natural variability in environmental conditions (Mothersill et al., 2007; Hahn, 2011).

Most ecotoxicological studies that have examined the impacts of exposure to the complex mixtures of contaminants found in wastewater effluent have mainly focussed on reproductive endpoints. Aquatic wildlife living downstream of WWTPs can suffer reproductive impairments such as ovarian and testicular histopathology including gonadal intersex (Vajda et al., 2008; Kavanagh et al., 2004; Sowers et al., 2009; Hayes et al., 2002), which may in turn alter the structure and function of aquatic communities (Northington and Hershey, 2006; Birge et al., 1989; Boone et al., 2007; Relyea et al., 2005; Grantham et al., 2012). However, less is known about other physiological endpoints that contribute to fitness and survival. Metabolism and respiration provide a lens to understand energy flow

and utilization in an organism, processes that underlie numerous fitness-related traits and integrate subcellular/cellular processes with whole-animal physiology. Metabolism, respiration, and aerobic scope (the difference between maximal and resting rates of O₂ consumption) are linked to growth, reproduction, activity, social status, functional performance, and many other important behaviours (Tierney & Farrell, 2004; McCarthy, 2001; Biro and Stamps, 2010; Chatelier et al., 2006; Metcalfe et al., 1995; Clark et al., 2013). Aquatic pollution could demand that energy be redirected towards detoxification and cellular protection, with corresponding impacts on whole-animal metabolism and respiration. However, several contaminants also have specific effects on mitochondria that can lead to mitochondrial dysfunction, thereby impairing energy production and compromising ability to satisfy the energetic demands of detoxification and other processes (Kurochkin et al., 2011; Cambier et al., 2009; Hiltibran, 1971; Haubenstricker et al., 1990). Although some studies have investigated the bioenergetic consequences of pollution stress by measuring energy stores (i.e. concentrations of lipid, glycogen, and protein in tissues; Melvin, 2016; Cazenave et al., 2014; Smolders et al., 2003), the mechanisms and functional implications on higher levels of organization (i.e. organ systems and whole-organism) remain unclear.

Despite the potential challenges of wastewater exposure, fish are consistently found living in effluent-dominated environments (e.g. Tetreault et al., 2013). Fish may be able to invoke compensatory strategies to offset the potential metabolic costs of living in polluted environments. It remains unclear whether wastewater exposure impacts whole-animal metabolism and energy demands, and whether fish possess effective respiratory and

metabolic plasticity in order to cope with these greater demands. Using metabolic and respiratory biomarkers allow us to follow the flow of energy from organismal to molecular levels to elucidate whether changes in oxygen extraction, transport, and utilization can support metabolism. This method approaches bioenergetics in a mechanistic and integrative way but has rarely been applied in ecotoxicology. Given previous work on single compounds, we expect that fish exposed to wastewater will incur a metabolic cost. If fish are able to compensate for these increased metabolic demands, then we should see changes at various biological levels that improve oxygen uptake, transport, and utilization.

The purpose of our study was to elucidate the consequences of wastewater exposure on metabolism and respiratory physiology in bluegill sunfish (*Lepomis macrochirus*). Bluegill and other related centrarchid species have been used previously in ecotoxicological studies (Lemley, A.D., 1996; Brooks et al., 2005; Hiltibran, 1971; Theodorakis et al., 1992; Adams et al., 1992; Porter and Janz, 2003) and are native across a broad range of eastern North America (Near and Koppelman, 2009). The WWTP in our study area treats residential wastewater from the community of Dundas in Hamilton, Ontario, Canada. Its effluent is discharged into a canal that eventually flows 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 inputs (International Joint Commission, 1999). Therefore, our study is broadly relevant to understanding the physiological implications of wastewater exposure in fish, but is also of special interest to local community and government agencies that are working to restore this important wetland.

2.3 Materials and methods

2.3.1 Fish collection and housing

We caught bluegill sunfish (mean body mass \pm 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. Bluegill were transported to McMaster University and housed in 500 l flow-through tanks supplied with dechlorinated tap water ($\sim 18^{\circ}\text{C}$) from City of Hamilton 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.

2.3.2 Caged exposures

Bluegill were caged at one of three field locations, two of which exposed fish to effluent from the Dundas Wastewater Treatment Plant ($43^{\circ}16'2''\text{N}$; $79^{\circ}56'37''\text{W}$, Fig. 1). Dundas WWTP is a tertiary treatment plant that serves a population of $\sim 30,000$ and treats an average of 15 million litres of wastewater each day (City of Hamilton, 2011). The treated effluent is the major source of water flowing into Desjardins Canal, which runs into West Pond before joining Cootes Paradise Marsh (Fig. 1). We caged bluegill at sites 50 m ($43^{\circ}16'0''\text{N}$; $79^{\circ}56'31''\text{W}$) or 830 m ($43^{\circ}16'9''\text{N}$; $79^{\circ}55'59''\text{W}$) downstream of the outfall

pipe (“outfall” and “downstream” experimental groups, respectively) within Desjardins Canal (Fig. 1). We also caged bluegill 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) and does not receive wastewater effluent.

Fish were caged over a 21-day exposure period. 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 (4 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 bluegill 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.

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 that have already reported these data (McCallum et al., 2017). Briefly, PPCP concentrations were measured using passive polar organic chemical integrative samplers (POCIS) deployed in empty

cages at each site. We found 17 out of 24 PPCPs that were assayed at both our wastewater-contaminated sites, including a range of antibiotics, antidepressants, beta-blockers, and hormone medications (Table 1). In contrast, we only found six PPCPs at our reference site, each at substantially lower concentrations than quantities detected at the sites near the WWTPs. Water quality parameters (temperature, dissolved oxygen, pH, conductivity, salinity, total dissolved solids, and flow) were also measured during our exposure period (Table 2). A full description of the methods and analyses of these measurements are described by McCallum et al. (2017).

2.3.3 Respirometry experiments

We used stop-flow intermittent respirometry and analysis equipment (Loligo Systems) to measure oxygen consumption rates (M_{O_2}) and hypoxia tolerance, using methods and experimental chambers that have been previously described (Crans et al., 2015). Briefly, bluegill 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 held at 20°C in order to become accustomed to the chambers. The next morning, resting M_{O_2} was obtained in normoxia (90-100% air saturation) and 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 oxygen 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 (Yeager and Ultsch, 1989).

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

A small subset of fish from each site were not used in metabolic or respiratory analyses and were sampled immediately upon arrival at McMaster University from the field for contaminant analyses. 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).

2.3.5 Gill morphometrics

We used stereomicroscopy for morphometrics of the gill filaments on all four arches from one side of the fish (Hughes, 1984; Crans et al., 2015). Digital images were taken of all filaments on all four arches, and the lengths and number of filaments on each arch was measured using ImageJ software (Rasband, 2014). The measured values of total filament length (the sum of the lengths of all filament lengths on all four arches) and total filament number were multiplied by 2 to account for there being two sides to the fish.

After images were collected using stereomicroscopy, the first gill arch was submerged in 30% sucrose (in 137 mol l⁻¹ NaOH, 15.2 mol l⁻¹ Na₂HPO₄, 2.7 mol l⁻¹ KCl, 1.5 mol l⁻¹ KH₂PO₄; pH 7.8) for ~24 h and then frozen in embedding medium (Shandon Cryomatrix, ThermoFisher Scientific). 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 took ~15 images of sections spread throughout the entire tissue from each fish 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. From each image, we measured total lamellar height, exposed lamellar height, interlamellar cell mass height, and lamellar thickness for ~8 lamellae using ImageJ software (Rasband, 2014). Lamellar density was also quantified for each image 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.

2.3.6 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). Haemoglobin-O₂ affinity (P₅₀, the P_{O₂} at which haemoglobin is 50% saturated) was determined at 25°C at pH 7.0 and 7.4, as recommended by the manufacturer, and pH sensitivity was calculated as the difference in P₅₀ per unit change in pH.

2.3.7 *Mitochondrial physiology*

Mitochondria were isolated using standard methods established in previous studies (Du et al., 2016; Fangué et al., 2009). I developed these techniques prior to the start of this study, in a project exploring the mitochondrial physiology of sunfish hybrids (Appendix A). Fresh liver tissue (~0.6 g) was finely diced in 10 ml of ice-cold isolation buffer (in mmol l⁻¹ unless otherwise stated: 250 sucrose, 50 KCl, 25 KH₂PO₄, 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).

We used high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria) to measure the physiology of isolated liver mitochondria at 20°C. Respiration (rate of O₂ 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 KH₂PO₄, 3 MgCl₂·6H₂O, 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 (H₂O₂) 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 (Fig. 6A). Mitochondria (0.6 mg of mitochondrial protein) were added to the respiration buffer, and leak respiration (L_N) 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 (L_T) 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 (P_{PM}). The capacities for oxphos *via* complex I (P_{PMG}) and complexes I+II (P_{PMGS}) 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 O_2 was consumed (to assess mitochondrial O_2 kinetics), and anoxic conditions were maintained for 5 min. P_{O_2} was raised slightly to measure respiration immediately after anoxia, and then after a stable reading was achieved, the medium was fully oxygenated. Rotenone ($0.5 \mu\text{mol } l^{-1}$), an inhibitor of complex I, was added to measure oxphos capacity *via* complex II ($P_{S(\text{Rot})}$). The above mitochondrial physiology experiment was performed twice for each fish, once without and once with the addition of carbonyl cyanide m-chloro phenyl hydrazine (CCCP) after the first addition of maximal ADP. CCCP is used to uncouple respiration (and is added until maximal stimulation, $0.5\text{-}2 \mu\text{mol } l^{-1}$) 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_{max}), mitochondrial O_2 affinity (P_{50} , the P_{O_2} at which respiration is inhibited by 50%), and catalytic efficiency (J_{max}/P_{50}).

2.3.8 Lipid peroxidation

We measured lipid peroxidation (a marker of oxidative damage) in isolated liver mitochondria as the formation of Fe(III)-xylenol orange complex (Hermes-Lima et al., 1995). Isolated mitochondria were homogenized for 1 min in methanol (1:5 v:v) in an ice-cold glass tissue grinder, then centrifuged at 1000g for 5 min. The supernatant was stored on ice while a cuvette containing distilled water, $0.25 \text{ mmol } l^{-1} \text{ FeSO}_4$, $25 \text{ mmol } l^{-1}$ sulfuric

acid, and 0.1 mmol l⁻¹ xylenol orange (added in this order) was allowed to react for 30 min. After this time, 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⁻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.

2.3.9 Enzyme assays

Maximal activities (V_{\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 KH₂PO₄ (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 methods described by Marentette et al. (2010). 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 µmol l⁻¹ in 50 mmol l⁻¹ tris, 0.1 mmol l⁻¹ NaCl; pH 7.8) was added to a concentration of 2 µmol l⁻¹. The reaction was initiated by adding NADPH (1.33 mmol l⁻¹) and V_{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.

2.3.10 Tissue contaminants

We pooled samples from all fish within each site to have enough tissue to measure contaminant levels in liver (~0.75 g total tissue) and gills (~1 g total tissue). We measured four target pharmaceuticals (sertraline and venlafaxine, both antidepressants, O-dm-venlafaxine, a breakdown product of venlafaxine, and metoprolol, a β-blocker) in fish

sampled immediately upon removal from caged exposures, and we measured two synthetic musks (Galaxolide® and Tonalide®; commonly used to add fragrance to cosmetics and detergents) in fish that were sampled ~48 hours later (after respirometry experiments). We extracted and identified these compounds following previously described methods (Chu and Metcalfe, 2007; O’Toole and Metcalfe, 2006; Sultana et al., 2016). Briefly, tissues for all chemical analyses were freeze dried, then homogenized in 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 (nine-point calibration).

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).

2.3.11 Statistical analyses

All data were analysed on R (version 3.2.4; R Core Team, 2016). Survival was analysed using a binomial generalized linear mixed effects model (GLMM; *glmmadmb* package, Fournier et al., 2012). 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 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, Bates et al., 2015) 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 M_{O_2} and organ masses, the absolute values ($\text{mmol O}_2 \text{ hr}^{-1}$ and g, respectively) were used in statistical analyses (because body mass was accounted for as a covariate), but we present the data normalized to body mass (i.e., $\text{mmol O}_2 \text{ hr}^{-1} \text{ kg}^{-1}$ and % body mass, respectively) to facilitate comparison with other studies in the literature. Mitochondrial respiration and ROS emission were analysed similarly, with the additional fixed effects of respiratory state and the interaction across exposure site and respiratory state. Haemoglobin P_{50} was

analysed similarly, with the additional fixed effects of pH and the interaction between exposure site and pH. In each case, if the interactions were not significant ($p > 0.05$) then the interaction term was dropped from the LMM. Data are reported as means \pm s.e.m and a significance level of $p < 0.05$ was considered significant.

2.4 Results

2.4.1 Survival

Wastewater exposure had a significant effect on survival ($LRT_{\text{site}} \chi^2 = 14.23$, $p = 0.0008$, Fig. 2). Survival over the duration of the exposure remained high at the reference site ($97.5 \pm 2.5\%$ survival after 21 days) but was significantly lower at the downstream ($70.0 \pm 10.2\%$) and outfall sites ($43.5 \pm 17.5\%$). Despite these significant differences in survival, body and organ masses of surviving fish were generally similar across groups, with the exception that liver mass was larger in bluegill from the downstream exposure site (Table 3).

2.4.2 Markers of contamination

Bluegill exposed to wastewater effluent accumulated the synthetic musks Tonalide® and Galaxolide® in their tissues (Table 4), consistent with the overall pattern of waterborne PPCP exposure (Table 1). Galaxolide® was detected in higher levels at the outfall site than the downstream site, and at much higher concentrations in the liver than in the gill. Tonalide® exhibited a similar pattern of variation but was only detected in the liver. None

of the four pharmaceuticals we assayed (venlafaxine and its metabolite *O*-dm-venlafaxine, sertraline, and metoprolol) were detected in any bluegill from any sites (Table 4), potentially because the relatively high solubility of these compounds prevents their bioaccumulation (Schultz et al., 2010; Ramirez et al., 2009). Activities of EROD (ethoxyresorufin-O-deethylase) – a protein that is induced by aryl hydrocarbons and is involved in xenobiotic metabolism – were similar among 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 ($\text{LRT}_{\text{site}} \chi^2 = 3.47$, $p = 0.18$), suggesting that fish were not exposed to aryl hydrocarbons such as polyaromatic hydrocarbons (PAH) or polychlorinated biphenyls (PCB) (Bucheli and Fent, 2009).

2.4.3 Metabolism and hypoxia tolerance

Bluegill caged at the downstream and outfall sites had resting rates of aerobic metabolism (O_2 consumption rate, M_{O_2}) that were approximately 30-36% higher than those caged at the reference site ($\text{LRT}_{\text{site}} \chi^2 = 8.37$, $p = 0.015$, Fig. 3). Despite these changes in resting M_{O_2} , wastewater exposure did not have a significant effect on hypoxia tolerance, as reflected by the critical P_{O_2} (P_{crit}) and the P_{O_2} at which fish lost equilibrium during progressively declining P_{O_2} (Table 5).

2.4.4 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 ($LRT_{\text{site}} \chi^2 = 22.3, p < 0.0001$, Fig. 4F), due largely to a 17-29% reduction in the height of interlamellar cell mass ($LRT_{\text{site}} \chi^2 = 6.63, p = 0.036$, Fig. 4E). Fish from the outfall site also had slightly thinner lamellae ($LRT_{\text{site}} \chi^2 = 6.34, p = 0.04$, Fig. 4H) and tended to have longer lamellae ($LRT_{\text{site}} \chi^2 = 4.18, p = 0.12$, Fig. 4D). Fish from the downstream site also had a modest increase in lamellar density ($LRT_{\text{site}} \chi^2 = 8.50, p = 0.014$, 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 fish from the reference site ($LRT_{\text{site}} \chi^2 = 19.1, p < 0.0001$, Fig. 4I). These changes likely increased the overall surface area of the gills, because average filament length, total filament length, and total filament number were all the same across fish from the different exposure sites (summarized in Table 6).

2.4.5 Haematology

Blood-O₂ binding was altered in response to wastewater exposure (Fig. 5). P₅₀ (the P_{O₂} at which haemoglobin was 50% saturated) was generally higher at pH 7.0 compared to pH 7.4 ($LRT_{\text{pH}} \chi^2 = 139.9, p < 0.0001$), and P₅₀ at pH 7.0 increased in bluegill from the outfall site ($LRT_{\text{site}} \chi^2 = 20.2, p < 0.0001$, Fig. 5A). Bluegill from the outfall site also had an enhanced pH sensitivity of O₂ binding ($LRT_{\text{site}} \chi^2 = 7.82, p = 0.020$, Fig. 5B). Haematocrit was higher in bluegill from the outfall site ($38.0 \pm 2.4 \%$, $n = 10, p = 0.023$) than the downstream ($31.2 \pm 1.6, n = 10, p = 0.97$) and reference sites ($31.7 \pm 1.7, n = 7$) (LRT_{site}

$\chi^2 = 8.57, p = 0.014$), but blood haemoglobin content did not vary across sites ($LRT_{\text{site}} \chi^2 = 0.98, p = 0.61$, Fig. 5C).

2.4.6 Mitochondrial respiration and ROS emission rates

Caged exposure to wastewater effluent also affected mitochondrial physiology (Fig. 6). Wastewater exposure increased respiratory capacities for oxidative phosphorylation (oxphos, P) in isolated liver mitochondria (Fig. 6B, $LRT_{\text{site}} \chi^2 = 7.59, p = 0.022$), with ~10% higher respiration levels in bluegill caged at the outfall ($p = 0.011$). As expected, there was a significant main effect of substrate on oxphos respiration ($LRT_{\text{state}} \chi^2 = 83.0, p < 0.0001$), with respiration rates generally being higher when supported with substrates of complex I (P_{PM} and P_{PMG}) compared to complex II ($P_{\text{S(RoI)}}$), and the highest respiration rates were observed with convergent inputs to both complexes I and II (P_{PMGS}). The increases in oxphos respiration appeared to be due to a change in mitochondrial quality, as reflected by an increase in succinate dehydrogenase activity with wastewater exposure but no change (or a slight non-significant decrease) in citrate synthase activity (Table 7). Associated with the exposure-induced increases in oxphos capacity were increases in mitochondrial P_{50} (the P_{O_2} at which mitochondrial respiration was reduced by 50%) (Table 7). However, wastewater exposure 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 7).

Rates of ROS emission from mitochondria were significantly reduced by 10-30% in fish exposed to wastewater compared to those from the reference site (Fig. 6C) ($LRT_{\text{site}} \chi^2$

= 24.6, $p < 0.0001$). There was a significant main effect of respiratory state ($LRT_{\text{state}} \chi^2 = 35.0$, $p < 0.0001$), such that ROS emission rates were higher 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% on average in unexposed fish to 0.08% on average in fish exposed to wastewater at both the downstream ($p < 0.0001$) and outfall ($p < 0.0001$) sites (Fig. 6D, $LRT_{\text{site}} \chi^2 = 31.0$, $p < 0.0001$; $LRT_{\text{state}} \chi^2 = 49.7$, $p < 0.0001$).

2.4.7 Oxidative stress

We found no evidence of mitochondrial oxidative stress with wastewater exposure ($LRT_{\text{site}} \chi^2 = 0.023$, $p = 0.99$). Levels of lipid peroxidation were similar in liver mitochondria among fish from reference (2.98 ± 0.53 pmol resorufin min^{-1} 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.

2.5 Discussion

Wastewater effluent is a significant source of aquatic pollution around the world and contains a complex mixture of contaminants. In this study, we show that exposure to wastewater effluent in the wild reduces bluegill sunfish survival (Fig. 2). Fish that survived the exposure had higher rates of aerobic metabolism (Fig. 3), suggesting that there is a high metabolic cost of maintenance and/or detoxification in fish living in effluent-dominated habitats. The heightened metabolic cost of exposure appears to be associated with adjustments along the oxygen transport cascade that expand the gill's capacity for gas exchange (Fig. 4), facilitate the unloading of O₂ from haemoglobin at the tissues (Fig. 5), and increase mitochondrial respiratory capacity in the liver (Fig. 6). Therefore, bluegill sunfish demonstrated a number of beneficial adjustments in their respiratory physiology to compensate and help cope with the metabolic costs associated with living in polluted environments.

2.5.1 *Metabolic costs of wastewater exposure*

Increases in metabolic rate in response to contaminant exposure, such as those described in our study (Fig. 3), have been observed in numerous fish species in response to a range of other contaminants including dieldrin (Beyers et al., 1999), polychlorinated biphenyl (Cannas et al., 2013), and metals (McGeer et al., 2000; Wilson et al., 1994). To the best of our knowledge, we are the first to demonstrate a metabolic cost of wastewater

exposure in fish. Our previous work suggests that this effect may not occur in all species, because resting M_{O_2} and respiratory physiology were unaffected by similar exposures to wastewater in round goby (McCallum et al., 2017). Bluegill may only be moderately tolerant of contaminants (Brown et al., 2011), in comparison to the highly tolerant round goby (Marentette et al., 2010; McCallum et al., 2014), a pervasive and invasive species that is now established in many parts of the bluegill's natural range.

Increases in metabolic rate arising from contaminant exposure could also impact fitness by reducing aerobic scope (Guderley and Pörtner, 2010; Claireaux and Lefrancois, 2007; Fry, 1947). Aerobic scope is the difference between resting and maximal aerobic rates of metabolism, and represents the capacity to increase metabolism above basal levels to support functions such as reproduction, growth, and behaviour (Clark et al., 2013). An increase in resting M_{O_2} without a parallel increase in maximal M_{O_2} would reduce the aerobic scope available to support these functions (Claireaux and Lefrancois, 2007), and has been observed to result from exposure to waterborne metals (McGeer et al., 2000). Some fish may also suffer a reduced aerobic scope with contaminant exposure due to decreases in maximal M_{O_2} , such as observed after exposure to petroleum (Davoodi and Claireaux, 2007) or waterborne aluminum (Wilson et al., 1994). However, it is possible that fish suffering from exposure-induced increases in resting M_{O_2} could maintain aerobic scope with compensatory increases in maximal M_{O_2} . For example, common sole (*Solea solea*) exhibit increased resting M_{O_2} with exposure to PCBs, but the fish were able to maintain aerobic scope with a concurrent increase in maximal M_{O_2} (Cannas et al., 2012). Although we did not measure maximal M_{O_2} , the respiratory adjustments of bluegill in

response to wastewater exposure suggest that they may be able to increase maximal M_{O_2} and offset reductions in aerobic scope, as described in more detail below.

2.5.2 *Wastewater exposure enhanced the capacity for O_2 transport*

Bluegill responded to wastewater exposure by increasing the morphological capacity of the gills for gas exchange, in association with the increase in resting M_{O_2} . This expansion of gill surface area appeared to result 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 that can respond quickly to environmental stressors that increase the demand for O_2 uptake (Nilsson, 2007), such as warming temperatures and environmental hypoxia (Sollid et al., 2003; Nilsson, 2007). However, there is a trade-off between respiratory gas exchange and ionoregulation, because increases in gill surface area can potentiate ion and water fluxes across the gills (the so called “osmorespiratory compromise”; reviewed by Sardella and Brauner, 2007). Increases in gill surface area (and/or gill ventilation) may also augment the uptake of environmental contaminants (McKim and Goeden, 1982; Hebel et al., 1997; Blewett et al., 2012). In some cases, fish may reduce respiratory surface as a protective mechanism to limit contaminant uptake (Bernet et al., 2004; Farrell et al., 2004; Mallatt, 1985), which can reduce maximal O_2 uptake and aerobic scope (Wilson et al., 1994), but that clearly did not occur in the present study. Therefore, the expansion of gill surface area in bluegill may improve O_2 uptake to support metabolic costs of wastewater exposure, but may come at the expense of

augmented ionoregulatory demands and even greater rates of contaminant uptake through the gills.

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 of O₂ unloading at the tissues (which is facilitated by a decrease in affinity) (Burggren et al., 1991). 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 PO₂ 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 (at which 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 (Farrell et al., 2004). We did not observe any changes in haemoglobin content in this study, but oxygen carrying capacity is generally highly variable across studies (Saleh and Marie, 2016; McCallum et al., 2017; Ruas et al., 2008).

2.5.3 Wastewater exposure altered mitochondrial function

Wastewater exposure increased the respiratory capacities for oxidative phosphorylation of liver mitochondria by changing mitochondrial quality (Fig. 6). The increases in respiratory capacity occurred in concert with a change in the relative activities

of SDH and CS (Table 7). Improvement in mitochondrial quality has previously been observed in fish as a strategy to increase aerobic capacity (St Pierre et al., 1998). These changes could increase metabolic capacity in the liver, especially when combined with increases in organ size (Table 3) to support energetic demands including detoxification (Gagnon, 2002). These findings are in contrast to several other studies, in which tissue aerobic capacities were reduced by pollutant stress (Rajotte and Couture, 2002; Rodrigues Jr. et al., 2015; Goertzen et al., 2011). Bluegill exposed to wastewater also had greater mitochondrial P_{50} (Table 7), which may result from the higher rates of mitochondrial respiration due to higher catalytic turnover rate of cytochrome c oxidase (Gnaiger et al., 1998; Larsen et al., 2011).

Changes in mitochondrial quality in response to wastewater exposure were also associated with reductions in the inherent rate of ROS emission from mitochondria (Fig. 6). Oxidative stress is a common consequence of exposure to wastewater in numerous fish species (Carney Almroth et al., 2008; Cazenave et al., 2014; Avci et al., 2005; Sturve et al., 2008; Lushchak, 2011), likely arising because excess ROS levels lead to damage of cellular components. Oxidative stress may contribute to the metabolic costs of contaminant exposure, because energy is required to repair and replace damaged macromolecules (Metcalf and Alonso-Alvarez, 2010). 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 (Doyotte et al., 1997), few studies have examined whether contaminant exposure is associated with compensatory

reductions in mitochondrial ROS production that minimize oxidative stress. The reductions in mitochondrial ROS emission observed here (Fig. 6) may have contributed to the low incidence of lipid peroxidation in liver mitochondria of bluegill 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 the capacity for oxidative phosphorylation (Adiele et al., 2012; Adiele et al., 2010; Sokol et al., 1993; Cambier et al., 2009; Garceau et al., 2010). In contrast, contaminants can also uncouple oxidative phosphorylation (Bourdineaud et al., 2013; Wallace and Starkov, 2000), which could increase the rates of respiration needed to offset proton leak and thus reduce phosphorylation efficiency. However, leak respiration and phosphorylation efficiency were maintained in bluegill exposed to wastewater (Table 7). 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 may have encountered much lower concentrations of contaminants.

2.5.4 Metabolism and respiration as ecotoxicological tools

The development and application of appropriate bioindicators are critical to our understanding of how fish cope in today's environments (Martin et al., 2013; Forbes et al.,

2006). Energetic markers have been used as bioindicators in ecotoxicology over the past three decades (e.g. Giesy and Graney, 1989; McKenney Jr. and Matthews, 1990; De Coen and Janssen, 1997). Understanding bioenergetics under contaminant stress can reveal the potential trade-offs in allocation of a finite pool of energy, which can have important implications on organismal and population-level function (De Coen and Janssen, 1997; Amiard-Triquet et al., 2012; Melvin, 2016). Metabolism and respiration are relatively new bioindicators that can provide the mechanistic link between subcellular energetics and whole organismal performance. Understanding the impacts of contaminants along the oxygen transport cascade, across multiple levels of biological organization, provides a level of integration, which is often lacking in many studies that apply biomarkers (Forbes et al., 2006; Adams et al., 2000). As shown in our study, respiratory physiology demonstrates compensatory plasticity which enriches our mechanistic understanding of how fish cope with pollutant stress. Respiration and metabolism have long been focal themes for researchers interested in how animals cope with metabolically challenging environmental stressors (e.g. hypoxia, rising temperatures, salinity, and individual metal pollutants; reviews by Claireaux and Lefrancois, 2007; Sokolova et al., 2012), and a similar approach could be used to better understand wastewater stress. Our findings show that metabolism and respiration in bluegill are indeed sensitive to wastewater exposure, invoking a suite of alterations – from organism to protein – that improve oxygen uptake, transport, and utilization, and therefore have strong potential to be ecologically relevant indicators of wastewater exposure that may translate to broader population effects. As wastewater

continues to be a leading source of aquatic pollution, increasing effort will be needed to improve our understanding and ability to predict how wastewater affects our ecosystems.

Table 1. Average estimated time-weighted concentrations of waterborne PPCPs in a canal receiving wastewater input from a tertiary wastewater treatment plant.

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

Concentrations were determined using POCIS samplers and are expressed as ng l⁻¹ (*N* = 3

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

-- excluded from analyses.

Table 2. Water quality measures taken during caged exposures.

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

Table 3. Body and organ mass (% body mass) of bluegill sunfish

	Reference	Downstream	Outfall
Body mass (g)	82.0± 8.7	81.9± 11.7	84.6± 11.0
Liver	0.598± 0.120	1.124 ± 0.122*	0.756 ± 0.047
Heart	0.073± 0.006	0.096 ± 0.007	0.089 ± 0.006
Spleen	0.099± 0.017	0.129 ± 0.018	0.080 ± 0.015
Brain	0.203± 0.018	0.230 ± 0.023	0.210 ± 0.026

* indicates significant difference from reference site; body mass, $LRT_{site} \chi^2 = 0.027, p = 0.99$; liver, $LRT_{site} \chi^2 = 8.05, p = 0.018$ (downstream, $p = 0.013$; outfall, $p = 0.92$); heart, $LRT_{site} \chi^2 = 4.10, p = 0.13$; spleen, $LRT_{site} \chi^2 = 3.81, p = 0.15$; brain, $LRT_{site} \chi^2 = 0.78, p = 0.68$ ($n_{reference} = 10, n_{downstream} = 9-10, n_{outfall} = 7$).

Table 4. Concentrations of pharmaceuticals and personal care products found in gill and liver of bluegill after 21 days of exposure downstream or at the outfall of a tertiary WWTP. Concentrations are expressed as ng/g fresh weight. Organs from multiple fish were pooled ($N = 1$).

Compound	Class	Tissue	Reference	Downstream	Outfall
Venlafaxine	antidepressant	liver	ND	ND	ND
		gill	ND	ND	ND
<i>O</i> -dm-venlafaxine	metabolite	liver	ND	ND	ND
		gill	ND	ND	ND
Sertraline	antidepressant	liver	ND	ND	ND
		gill	ND	ND	ND
Metoprolol	β -blocker	liver	ND	ND	ND
		gill	ND	ND	ND
Galaxolide®	musk	liver	ND	4.35	4.97
		gill	ND	0.2	0.57
Tonalide®	musk	liver	ND	0.7	1.49
		gill	ND	ND	ND

ND, not detected; Galaxolide®, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyrane (HHCB); Tonalide®, 7-acetyl-1,1,3,4,4,6-hexamethyl-tetra hydronaphthalene (AHTN)

Table 5. Hypoxia tolerance by wastewater exposure site.

	Reference	Downstream	Outfall
P_{crit} (kPa)	3.50 ± 0.19	4.33 ± 0.34	4.24 ± 0.81
P_{O_2} at LOE (kPa)	0.494 ± 0.092	0.412 ± 0.088	0.336 ± 0.027

Critical O₂ tension (P_{crit}), $\text{LRT}_{\text{site}} \chi^2 = 2.45$, $p = 0.29$; O₂ tension (P_{O_2}) at loss of equilibrium (LOE), $\text{LRT}_{\text{site}} \chi^2 = 1.89$, $p = 0.39$ ($n_{\text{reference}} = 10$, $n_{\text{downstream}} = 7-10$, $n_{\text{outfall}} = 6-7$).

Table 6. Morphometrics of the gill filaments by wastewater exposure site.

	Reference	Downstream	Outfall
Average filament length (mm)	3.69 ± 0.21	3.60 ± 0.31	3.88 ± 0.32
Total filament length (mm)	5179 ± 326	4872 ± 523	5535 ± 493
Total filament number	1376 ± 28	1335 ± 41	1412 ± 38

Average filament length, $LRT_{\text{site}} \chi^2 = 0.82, p = 0.66$; total filament length, $LRT_{\text{site}} \chi^2 = 1.34, p = 0.51$; total filament number, $LRT_{\text{site}} \chi^2 = 1.97, p = 0.37$ ($n_{\text{reference}} = 10, n_{\text{downstream}} = 9, n_{\text{outfall}} = 7$)

Table 7. Properties of mitochondria isolated from the liver.

	Reference	Downstream	Outfall
Succinate dehydrogenase (SDH, $\mu\text{mol mg protein}^{-1} \text{ min}^{-1}$)	0.013 ± 0.001	0.015 ± 0.002	$0.017 \pm 0.002^*$
Citrate synthase (CS, $\mu\text{mol mg protein}^{-1} \text{ min}^{-1}$)	0.141 ± 0.015	0.118 ± 0.008	0.127 ± 0.014
P_{50} (kPa)	0.033 ± 0.003	$0.046 \pm 0.004^*$	$0.047 \pm 0.002^*$
Respiratory capacity for electron transport (E , $\text{pmol O}_2 \text{ mg protein}^{-1} \text{ s}^{-1}$)			
E_{PM}	458.5 ± 33.4	464.7 ± 39.7	464.2 ± 54.7
E_{PMG}	638.9 ± 51.8	618.8 ± 41.3	657.5 ± 62.3
$E_{\text{S(Rot)}}$	429.1 ± 36.7	450.7 ± 30.7	510.2 ± 40.7
E_{PMGS}	754.9 ± 58.0	740.8 ± 48.7	797.7 ± 74.7
Leak respiration with ATP (L_{T} , $\text{pmol O}_2 \text{ mg protein}^{-1} \text{ s}^{-1}$)	310.5 ± 33.7	278.8 ± 17.2	259.3 ± 23.9
Leak respiration without ATP (L_{N} , $\text{pmol O}_2 \text{ mg protein}^{-1} \text{ s}^{-1}$)	38.47 ± 5.25	38.07 ± 3.81	40.31 ± 4.81

* represents a significant difference from reference site; SDH, $\text{LRT}_{\text{site}} \chi^2 = 6.81$, $p = 0.033$ (downstream, $p = 0.066$; outfall, $p = 0.044$); CS, $\text{LRT}_{\text{site}} \chi^2 = 2.37$, $p = 0.31$; P_{50} , $\text{LRT}_{\text{site}} \chi^2 = 9.81$, $p = 0.007$ (downstream, $p = 0.012$; outfall, $p = 0.013$); E , $\text{LRT}_{\text{site}} \chi^2 = 3.34$, $p = 0.19$; $\text{LRT}_{\text{state}} \chi^2 = 72.9$, $p < 0.0001$ (downstream, $p = 0.98$; outfall, $p = 0.20$); L_{T} , $\text{LRT}_{\text{site}} \chi^2 = 2.14$, $p = 0.34$; L_{N} , $\text{LRT}_{\text{site}} \chi^2 = 1.20$, $p = 0.55$ ($n_{\text{reference}} = 9$, $n_{\text{downstream}} = 9$ -10, $n_{\text{outfall}} = 6$ -7)

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 were caged at the outfall of a wastewater treatment plant, further downstream, or at an uncontaminated reference site for 21 days. * represents significant differences 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, $p = 0.002$).

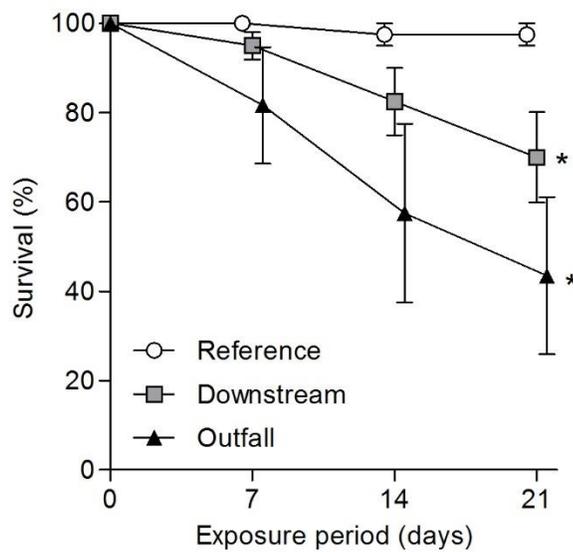


Fig. 3.

Resting rates of oxygen consumption increased in 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 ($LRT_{\text{site}} \chi^2 = 8.37, p = 0.015$; Dunnett's *post-hoc*: downstream, $p = 0.038$; outfall, $p = 0.012$)

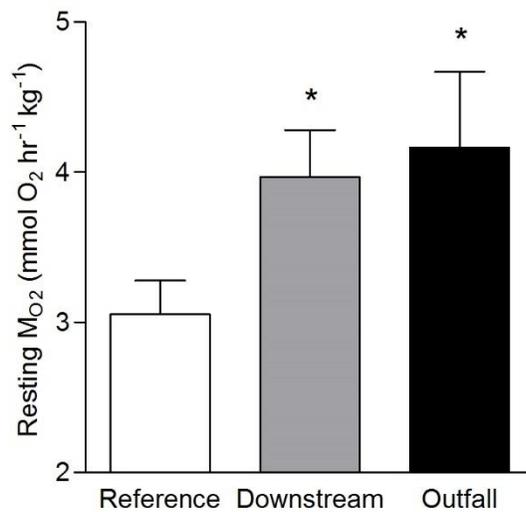


Fig. 4.

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 (Dunnett's *post-hoc*: downstream, $p = 0.62$; outfall, $p = 0.039$). (E) Height of interlamellar cell mass (ILCM) was lower after wastewater exposure, 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 (downstream, $p = 0.001$; outfall, $p < 0.0001$). (G) Lamellar density (downstream, $p = 0.006$; outfall, $p = 0.32$) and (H) lamellar thickness (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 (downstream, $p < 0.0001$; outfall, $p < 0.0001$). *represents significant differences from the reference site ($n_{\text{reference}} = 9$, $n_{\text{downstream}} = 9$, $n_{\text{outfall}} = 7$)

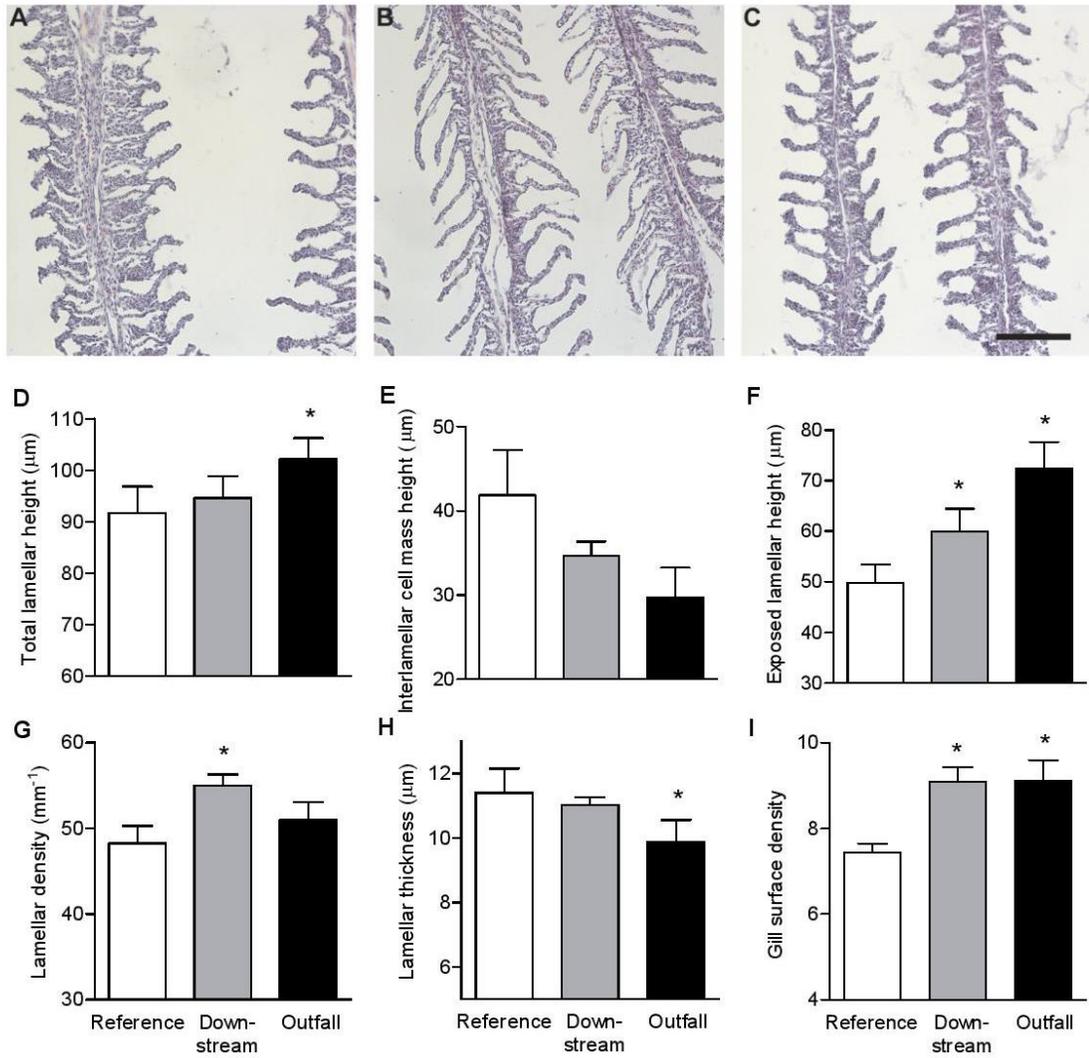


Fig. 5.**Haemoglobin-oxygen binding affinity was reduced in response to wastewater**

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 but not at pH 7.4 (Dunnett's *post-hoc*: downstream, $p = 0.63$; outfall, $p = 0.0003$; $n_{\text{reference}} = 7$, $n_{\text{downstream}} = 6$, $n_{\text{outfall}} = 7$). **(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 (downstream, $p = 0.63$; outfall, $p = 0.013$, n the same as above). **(C)** Blood haemoglobin content was similar across all exposure sites (downstream, $p = 0.87$; outfall, $p = 0.80$; $n_{\text{reference}} = 10$, $n_{\text{downstream}} = 10$, $n_{\text{outfall}} = 6$). *represents significant differences from the reference site

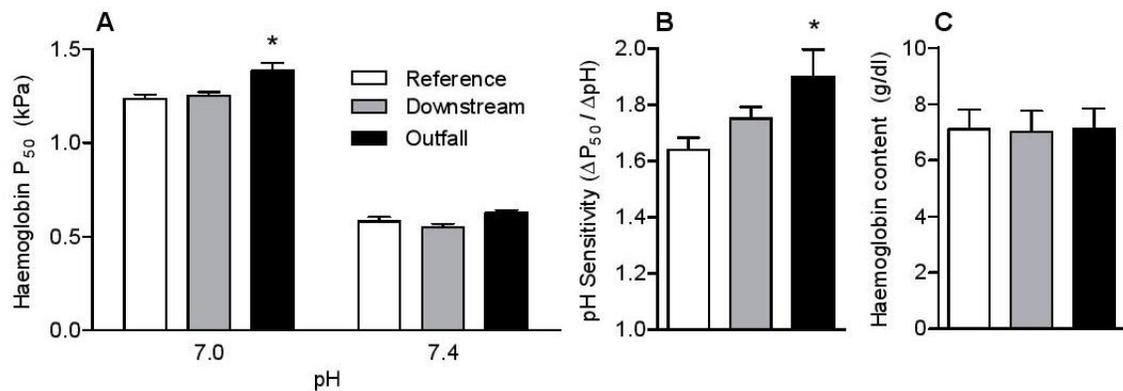


Fig. 6.

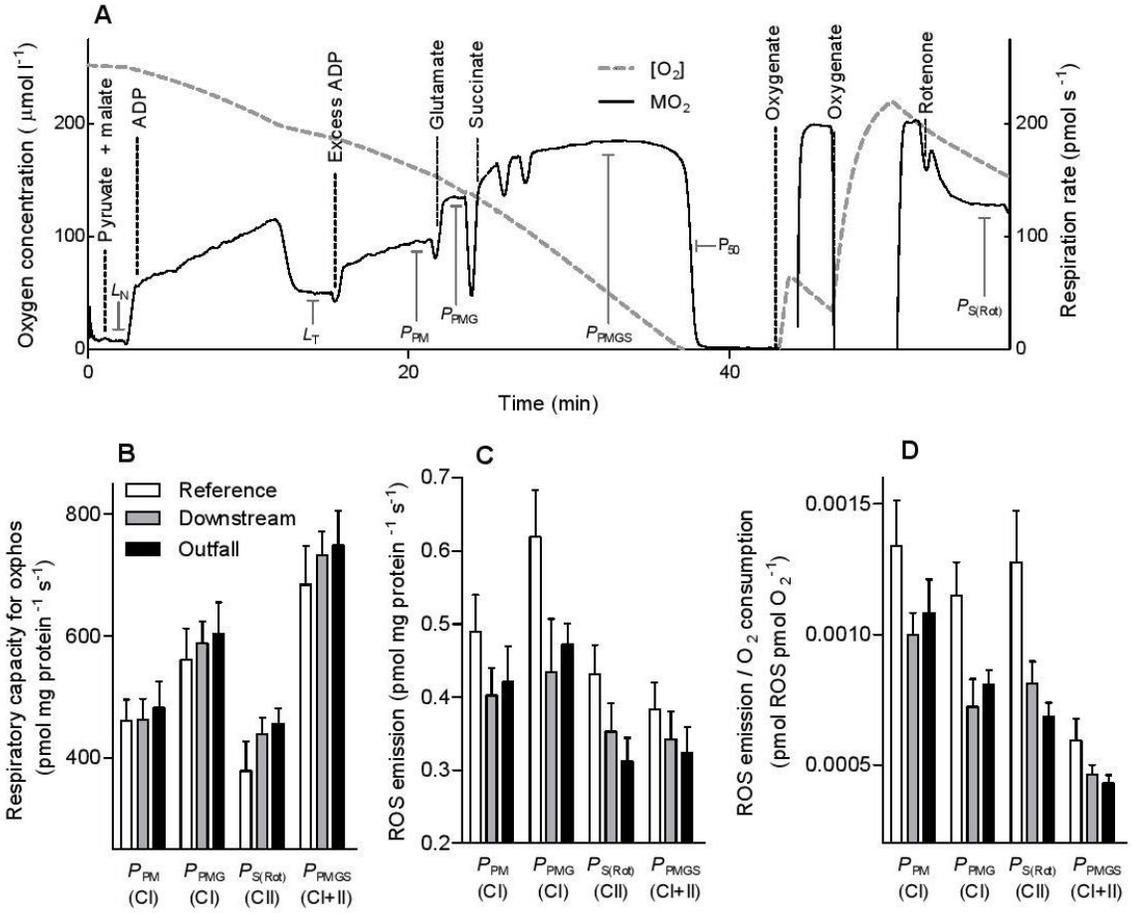
Wastewater exposure affected physiology of isolated liver mitochondria. (A)

Respiration rates of isolated liver mitochondria were measured at 20°C during leak (L) and oxidative phosphorylation (oxphos, P) in response to various manipulations. Reactive oxygen species (ROS) emission rates (not pictured) were measured in parallel by

fluorometry (see Materials and Methods). **(B)** Bluegill had higher respiratory capacities for oxidative phosphorylation after 21 days of exposure to wastewater effluent ($LRT_{\text{site}} \chi^2 = 7.59, p = 0.022$; $LRT_{\text{state}} \chi^2 = 83.0, p < 0.0001$; Dunnett's *post-hoc*: downstream, $p = 0.39$; outfall, $p = 0.011$). **(C)** ROS emission rates were reduced in mitochondria from bluegill caged at the downstream and outfall sites ($LRT_{\text{site}} \chi^2 = 24.6, p < 0.0001$; $LRT_{\text{state}} \chi^2 = 35.0, p < 0.0001$; Dunnett's *post-hoc*: downstream, $p < 0.0001$; outfall, $p < 0.0001$).

(D) ROS emission rates remained lower in the downstream and outfall sites after normalizing for oxphos respiration rates ($LRT_{\text{site}} \chi^2 = 31.0, p < 0.0001$; $LRT_{\text{state}} \chi^2 = 49.7, p < 0.0001$; Dunnett's *post-hoc*: downstream, $p < 0.0001$; outfall, $p < 0.0001$).

*represents significant differences from the reference site ($n_{\text{reference}} = 9, n_{\text{downstream}} = 9, n_{\text{outfall}} = 7$). (L_N and L_T , leak respiration in the absence and presence of ATP, respectively; P_{PM} , P with pyruvate and malate; P_{PMG} , P with pyruvate, malate, and glutamate; P_{PMGS} , P with pyruvate, malate, glutamate, and succinate; P_{50} , P_{O_2} at 50% maximal respiration; and $P_{\text{S(Rot)}}$, P with succinate and rotenone).



CHAPTER THREE: GENERAL DISCUSSION

This thesis demonstrates the physiological plasticity of the respiratory system of bluegill sunfish in the face of aquatic pollution. Bluegill suffered a metabolic cost due to exposure to wastewater effluent, but adjusted with compensatory enhancements in oxygen uptake (by expanding gill surface), delivery (by reducing haemoglobin-oxygen affinity), and utilization (by increasing mitochondrial respiratory capacity). Although these coping strategies may help satisfy increased metabolic demands, wastewater exposure decreased total survival, suggesting overall adverse impacts on fish.

This thesis also demonstrates the utility in applying a suite of metabolic and respiratory biomarkers in ecotoxicological testing. Biomarkers should be integrative and ecologically relevant, but too often, markers used in individual studies are functionally isolated and fail to tell a complete story, making extrapolation to higher levels of organization challenging (Forbes et al., 2006; Triebkorn et al., 1997). Metabolic and respiratory biomarkers trace the path of oxygen flow, which crosses numerous levels of biological organization and can utilize physiological, biochemical, and histological assessments, as achieved in this thesis. Using these markers together encompasses a range of sensitivity (in both toxicity and time), since subcellular responses are usually sensitive and short-term compared to organismal responses (Smolders et al., 2003; Marshall Adams et al., 2000, 2001). Therefore, respiration and metabolism provide a strong framework for ecotoxicological testing and should be incorporated into ecological risk assessments.

The WWTP investigated in this thesis, Dundas WWTP, is expecting upgrades over the next few years with the goal of improving removal rates of phosphorus, nitrogen, and suspended solids. Options for upgrades, which range from \$20.4 million to over \$35 million, are currently under review. Improvements in wastewater treatment could benefit fish and habitat downstream of the outfall. For example, a recent upgrade to a WWTP in Kitchener, Ontario replaced its carbonaceous activated sludge to nitrifying activated sludge, which improved removal of both ammonia and PPCPs. These upgrades were associated with an immediate and striking decrease in incidence of intersex in resident rainbow darter (*Etheostoma caeruleum*; Hicks et al., 2017). Similarly, experimental work on whole ecosystems has revealed that fathead minnow can fully recover from a population crash 3-4 years after the additions of synthetic estrogen were ceased (Blanchfield et al., 2015). The impressive resilience of aquatic ecosystems is encouraging, and highlights the importance of investing in effective wastewater treatment. The potential for recovery of metabolism and respiration are currently unknown, but reductions in contaminant exposures can certainly reduce energetic burdens of detoxification and repairing of the reproductive system. As demonstrated in this thesis, metabolic and respiratory traits are highly plastic and consequently, have potential to respond quickly to improvements in water quality. Therefore, the upcoming upgrades to the Dundas WWTP are promising for the metabolic and respiratory health as well as the survival of fish in Cootes Paradise.

APPENDIX A: HYBRIDIZATION INCREASES MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES IN SUNFISH

A.1 Abstract

Mitochondrial dysfunction and oxidative stress have been suggested to be possible mechanisms underlying hybrid breakdown, as a result of mito-nuclear incompatibilities in respiratory complexes of the electron transport system. However, it remains unclear whether hybridization increases the production of reactive oxygen species (ROS) by mitochondria. We used high-resolution respirometry and fluorometry on isolated liver mitochondria to examine mitochondrial physiology and ROS emission in naturally occurring hybrids of pumpkinseed (*Lepomis gibbosus*) and bluegill (*L. macrochirus*). ROS emission was greater in hybrids than in both parent species when respiration was supported by complex I (but not complex II) substrates, and was associated with increases in lipid peroxidation. However, respiratory capacities for oxidative phosphorylation, phosphorylation efficiency, and O₂ kinetics in hybrids were intermediate between those in parental species. Flux control ratios of capacities for electron transport (measured in uncoupled mitochondria) relative to oxidative phosphorylation suggested that the limiting influence of the phosphorylation system is reduced in hybrids. This likely helped offset impairments in electron transport capacity and complex III activity, but contributed to augmenting ROS production. Therefore, hybridization can increase mitochondrial ROS production, in support of previous

suggestions that mitochondrial dysfunction can induce oxidative stress and thus contribute to hybrid breakdown.

A.2 Introduction

Hybrid breakdown is a post-zygotic isolating barrier in which the hybrid offspring of two distinct species or populations exhibit reduced fitness. The Dobzhansky-Muller model of hybrid incompatibility suggests that hybrid breakdown occurs when parental genes are sufficiently diverged that they are no longer compatible when combined in a hybrid offspring (Orr and Turelli, 2001). Because of the vital importance of mitochondrial energy production for fitness (Brown et al., 2004; Ellison and Burton, 2006; Price et al., 2012), numerous studies have examined the possible effects of Dobzhansky-Muller incompatibilities on mitochondrial function (Ellison and Burton, 2006, 2008; Davies et al., 2012; Bolnick et al., 2008; Liepins and Hennen, 1977).

Previous studies have found evidence for diminished mitochondrial energy production in hybrids as a potential mechanism of hybrid breakdown. The oxidative phosphorylation (oxphos) system of mitochondria involves a series of large, multimeric protein complexes encoded by both nuclear and (with the exception of complex II) mitochondrial genomes. Products of nuclear and mitochondrial genomes must therefore interact to support mitochondrial electron transport and energy production. Although mitochondrial DNA evolves faster than nuclear DNA in most animals (Brown et al., 1979), co-evolution of genes for the interacting subunits in each complex is required to

avoid structural and functional incompatibilities that lead to mitochondrial dysfunction (Burton et al., 2006; Chou and Leu, 2015). Hybridization can combine incompatible combinations of parental genes and result in reduced complex activities (Davies et al., 2012; Ellison and Burton, 2006) and ATP synthesis rates (Ellison and Burton, 2008). The resulting dysfunction of cellular energy production could disrupt a wide range of organismal phenotypes, such as growth rate (Salin et al., 2012) and performance (Conley et al., 2007; Hill, 2014), and ultimately reduce fitness (Ellison and Burton, 2006, 2008; Barreto and Burton, 2013; Speakman, 2005; Selman et al., 2008).

It has been suggested that disruptions in mitochondrial function could also contribute to hybrid breakdown by inducing oxidative stress (Barreto and Burton, 2013; Rand et al., 2006). Mitochondrial electron transport is a major source of reactive oxygen species (ROS), whose production could foreseeably be altered by mito-nuclear incompatibilities in mitochondrial complexes. ROS play important roles in cell signalling, and are usually maintained at relatively low concentrations in the cell by antioxidants and ROS scavengers (Turrens, 2003; Martínez-Álvarez et al., 2005). However, excess ROS emission from mitochondria can overwhelm antioxidant defences and cause oxidative stress (as reflected by oxidative damage to proteins, lipids, and DNA). Some evidence suggests that ROS emission and oxidative stress can be exacerbated by disruptions in the electron transport system (Rand et al., 2006; Gusdon et al., 2007; Szczepanowska et al., 2012). Oxidative stress is believed to be a driving force in life-history trade-offs (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010), and it likely has a strong detrimental effect on fitness, having been associated with

reduced reproductive output, sexual dimorphism, and immunosuppression (Hill, 2014; Alonso-Alvarez et al., 2006, 2007). For example, fitness breakdown (as reflected by reduced fecundity) has been associated with an increased abundance of oxidative stress markers in inter-population hybrids of the marine copepod *Tigriopus californicus* (Barreto and Burton, 2013). Nevertheless, although the prevailing evidence suggests that oxidative stress can reduce fitness, it remains unclear whether increases in mitochondrial ROS emission contribute to oxidative stress in hybrids.

We sought to examine whether hybridization in sunfish (Centrarchidae: *Lepomis*) disrupts mitochondrial function by increasing mitochondrial ROS emission. *Lepomis* species last shared a common ancestor >14.6 million years ago (Near et al., 2005), but many species in this genus can still hybridize naturally and produce viable offspring (Bolnick, 2009; Scribner et al., 2001). *Lepomis* sunfish are therefore a compelling natural system in which to understand the physiological consequences of hybridization. Here, we study naturally occurring hybrids between *L. macrochirus* (bluegill) and *L. gibbosus* (pumpkinseed), species that have overlapping niches and naturally hybridize across North America. Previous surveys conducted in the lake from which we caught these fish (Lake Opinicon, Ontario, Canada) suggest that most hybrids are unidirectional F1 progeny produced from male bluegill and female pumpkinseed ($\geq 95\%$ of all hybrids), and that there is a much lower than expected abundance of F2 hybrids in the wild (Colgan et al., 1975; Konkle and Philipp, 1992; Garner and Neff, 2013). Asymmetric hybridization likely arises from the sneaker male strategy employed by some male bluegill, who may fertilize eggs in the nests of female pumpkinseed (Konkle and Philipp, 1992). F1 hybrid

females may subsequently mate with either bluegill or pumpkinseed males (Garner and Neff, 2013), but in general, post-mating isolation barriers appear to limit the abundance of F2 hybrids (Immler et al., 2011). Because pre-zygotic isolation barriers do not completely prevent the creation of F2 hybrids (Childers and Bennett, 1961; West, 1970), post-zygotic mechanisms likely also contribute to the low abundance of F2 hybrids in the wild (Konkle and Philipp, 1992; Garner and Neff, 2013).

A.3 Materials and methods

A.3.1 Study Animals

Pumpkinseed (body mass range of 68.0 to 145.1 g, mean \pm s.e.m. of 91.2 ± 9.6 g), bluegill (54.4 to 158.8 g, 101.8 ± 12.2 g), and hybrids (63.5 to 154.2 g, 100.6 ± 10.8 g) were collected from Lake Opinicon, Ontario, Canada (44.559°N , -76.328°W) by angling in June 2014. Fish were then brought back to McMaster University and held in 500 L recirculating tanks equipped with charcoal filters at room temperature ($\sim 20^{\circ}\text{C}$) and at a photoperiod of 12 h: 12 h light:dark. Fish were held in these conditions for several months before experimentation, and were fed four times each week with a mix of commercially purchased squid and beef heart. Water quality (pH, ammonia, etc.) was monitored weekly, and water changes were performed as needed. All procedures were carried out in accordance with guidelines set out by the Canadian Council on Animal Care, and were approved by the McMaster University Animal Research Ethics Board. We used a group of nine fish from each species/hybrid for all experiments, and the

number of individuals for each measurement is shown in the Results (as technical issues or sample availability sometimes prevented us from making measurements in all individuals).

We used a previously described PCR approach to distinguish hybrids from parental species (Crans et al., 2015; Davies et al., 2012). Briefly, DNA was extracted from fin clips using a REExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Oakville, ON, Canada). Fragments of the nuclear *S7* gene (forward primer, TGTAACGGGGAGCAGTTAGC; reverse primer, ACAGCCGATGTAGGAAACAG) and the mitochondrial ND2 gene (forward primer, GTCGAAGCCACCACCAAATA; reverse primer CAGGGCCAGGATAATGATTG) were amplified in Ready Mix Taq PCR Reaction Mix (Sigma-Alrich), and the products were electrophoresed on a 3% agarose gel. For the nuclear *S7* fragment, pumpkinseed exhibit a single band at 385 bp and bluegill exhibit a band at 363 bp. For mitochondrial ND2, the primers were designed to amplify a 175 bp fragment of the gene from pumpkinseed but not from bluegill. We identified hybrids as individuals that exhibited two bands for *S7*, one at 363 and one at 385 bp ($n = 9$ for each species). We also found that all hybrids exhibited a 175 bp ND2 band, demonstrating that all hybrids contained mitochondrial DNA from pumpkinseed ($n = 9$ for each species).

A.3.2 Mitochondrial Isolation

Mitochondria were isolated using standard methods (Du et al., 2016; Fangué et al., 2009). Fish were euthanized with a lethal dose of buffered MS-222, and liver tissue (~1 g) was excised and finely diced in 10 ml of ice-cold isolation buffer (in mmol l⁻¹ unless otherwise stated: 250 sucrose, 50 KCl, 25 KH₂PO₄, 10 HEPES, 0.5 EGTA, and 1.5% mass:volume fatty-acid free bovine serum albumin (BSA); pH 7.4 when measured at 20°C). The liver tissue was gently homogenized on ice with six passes of a loose-fitting Potter-Elvehjem homogenizer at 100 r.p.m., then the homogenate was centrifuged at 600g for 10 min at 4°C. The supernatant was poured over glass wool, and centrifuged at 6000g for 10 min at 4°C (the same conditions for all future centrifugation). The pellet was gently rinsed with isolation buffer to remove excess fat, re-suspended in 10 ml of fresh isolation buffer, and centrifuged. The pellet was rinsed and then re-suspended in 10 ml of storage buffer (same as isolation buffer, but without BSA and with 2 mmol l⁻¹ each of pyruvate and malate), and centrifuged one final time. The final pellet was resuspended in 500 µL of storage buffer. The mitochondrial suspension was then split, and one part was stored at -80°C for later assays of complex activities and lipid peroxidation (see below), and the other part was stored on ice until used in physiology experiments. The Bradford assay was used to measure protein content of the mitochondrial suspension, following recommendations from the manufacturer (Bio-Rad).

A.3.3 Mitochondrial physiology

We used high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria) to measure the physiology of isolated liver mitochondria (Appendix Fig. 1). Mitochondria (~0.6 mg of mitochondrial protein) were added to 2 ml of respiration buffer (in mmol l⁻¹: 110 sucrose, 60 K-lactobionate, 20 taurine, 20 Hepes, 10 KH₂PO₄, 3 mM MgCl₂·6H₂O, 0.5 EGTA, 1.5% mass:volume fatty-acid free BSA; pH 7.4) at 25°C. A first experiment measured respiration (rate of O₂ consumption) and ROS emission rates in coupled mitochondria. ROS was measured by fluorescent detection of resorufin (excitation wavelength of 525 nm and AmR filter set, Oroboros Instruments), which is produced from hydrogen peroxide (H₂O₂) and Ampliflu Red (Sigma-Aldrich) in a reaction catalysed by horseradish peroxidase. Mitochondrial superoxide and H₂O₂ were thus detected together by adding superoxide dismutase (22.5 U ml⁻¹; which catalyzes the production of H₂O₂ from superoxide), horseradish peroxidase (3 U ml⁻¹), and Ampliflu Red (15 μmol l⁻¹) to the respiration buffer. ROS emission was thus measured during the following manipulations as the molar rate of H₂O₂ release from mitochondria, by calibrating the fluorescent resorufin signal with the addition of exogenous H₂O₂. We first measured leak respiration (L_N) with complex I substrates pyruvate (2 mmol l⁻¹) and malate (2 mmol l⁻¹). Phosphorylation efficiency was assessed by measuring the P/O ratio with two additions of 125 μmol l⁻¹ ADP (Gnaiger et al., 2000). After ADP was depleted and leak respiration (L_T) was established, saturating levels of ADP (1250 μmol l⁻¹) was added to stimulate maximal pyruvate oxidation (P_{PM}). The capacities for oxphos *via* complex I (P_{PMG}) and complexes I+II (P_{PMGS}) 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 O_2 was consumed (to assess mitochondrial O_2 kinetics), and anoxia was maintained for 5 min. O_2 tension (P_{O_2}) was then raised slightly, mitochondria were allowed to consume all ADP to reach leak state respiration, and all O_2 was again consumed (to assess O_2 kinetics in leak state). After 10 min in anoxia, P_{O_2} was raised slightly to measure leak state respiration immediately after anoxia (data not shown). The medium was then fully oxygenated, and a saturating concentration of ADP was added to again stimulate oxphos *via* complexes I+II. Rotenone ($0.5 \text{ } \mu\text{mol l}^{-1}$), an inhibitor of complex I, was added to measure oxphos capacity *via* complex II ($P_{\text{S(Rot)}}$). Antimycin A ($2.5 \text{ } \mu\text{mol l}^{-1}$), a complex III inhibitor, was then added to measure residual O_2 consumption, which was subtracted from all respiration measurements. A second experiment followed the protocol that is described above, except that CCCP (carbonyl cyanide m-chloro phenyl hydrazone) was titrated after the first addition of maximal ADP to uncouple respiration (added until maximal stimulation, $0.5\text{-}2 \text{ } \mu\text{mol l}^{-1}$) so capacities for electron transport (rather than for oxphos) could be measured.

We also conducted experiments at 15°C and 28°C that were similar but shorter to those carried out on coupled mitochondria at 25°C (Appendix Supplementary Fig. 1). These experiments were performed because pumpkinseed and bluegill have previously been shown to have different thermal sensitivities at the whole-animal level (Borowiec et al., 2016), so we wanted to assure that any differences we observed in hybrids were not restricted to a single narrow range of test temperatures. The sole difference in methodology in these shorter experiments was that we proceeded to add ADP, rotenone,

and antimycin A immediately after the first anoxia bout (effectively skipping only the second anoxia bout in the leak state). Mitochondrial physiology data are expressed per mg mitochondrial protein. O₂ kinetics were analyzed using DatLab 2 software (Oroboros Instruments) to measure mitochondrial O₂ affinity (P₅₀, the P_{O₂} at which respiration is inhibited by 50%). We only included P₅₀ values for individuals in which the model could converge upon an accurate curve fit to the data.

A.3.4 Enzyme Activities

The maximal activities of citrate synthase (CS) and mitochondrial complexes I, II, III, IV, and V in mitochondrial isolates were assayed at 25°C using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Mitochondrial suspensions were homogenized in a glass tissue grinder, and were then kept on ice until assayed in triplicate for all enzymes except CS. The remaining homogenate was frozen and stored at -80°C, and later thawed to assay CS. Enzyme activities were calculated as the reaction rate using all assay components minus the background reaction rate (rate in the presence of inhibitor, -, or without a key substrate, +), and are expressed per mg mitochondrial protein. Measurements were carried out in 50 mmol l⁻¹ KH₂PO₄ (pH 7.2) under the following assay conditions (mmol l⁻¹ unless otherwise stated): CS, 0.5 oxaloacetate⁺ (prepared fresh), 0.15 acetyl-coA, 0.15 5,5'-dithiobis-(2-nitrobenzoic acid) (prepared fresh) (extinction coefficient, ϵ , of 14.15 l mmol⁻¹ cm⁻¹ at 412 nm); complex I, 0.15 NADH (ϵ of 6.22 at 340 nm), 0.3 KCN, 0.06 coenzyme Q10, 0.01 rotenone⁻, and 3

mg ml⁻¹ BSA; complex II, 20 succinate⁺, 0.3 KCN, 0.05 dichlorophenolindophenol (DCPIP; prepared fresh), and 0.05 decylubiquinone (prepared in ethanol) (ϵ of 21.9 at 600 nm); complex III, 0.075 oxidized cytochrome c, 0.05 KCN, 0.1 EDTA, 0.1 decylubiquinol (prepare fresh) (ϵ of 28.5 at 550 nm); complex IV, 0.2 reduced cytochrome c⁺ (ϵ of 28.5 at 550 nm); complex V, 3 Mg·ADP, 10 MgCl₂, 10 glucose, 1.5 NADP⁺, 1 U hexokinase, 1 U glucose-6-phosphate dehydrogenase, 0.005 oligomycin⁻ (prepared in ethanol) (ϵ of 6.22 at 340 nm).

A.3.5 Lipid Peroxidation

Lipid peroxidation was measured in mitochondrial isolates as the formation of Fe(III)-xylenol orange complex, using methods established by Hermes-Lima et al. (1995). Mitochondria were homogenized in methanol (1:5 v:v) in an ice-cold glass tissue grinder for 1 min, then centrifuged at room temperature for 5 min at 1000g. Reagents were added to a 1 ml cuvette containing distilled water, 0.25 mmol l⁻¹ FeSO₄, 25 mmol l⁻¹ sulfuric acid, and 0.1 mmol l⁻¹ xylenol orange (added in order) and allowed 30 min to react without mitochondrial homogenate. Homogenate was then added to the cuvette and allowed to react and produce Fe(III)-xylenol orange for 2 h, and an absorbance measurement was made at 580 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cumene peroxide (0.005 mmol l⁻¹) was then added as a standard and was given 40 min to react, and a final absorbance reading was made at 580 nm. Lipid peroxidation is reported as cumene hydroperoxide equivalents, expressed per mg of mitochondrial protein.

A.3.6 Statistical Analyses

Data are reported as means \pm s.e.m. One- or two-factor ANOVA were used as appropriate to test for the main effects of species/hybrid and (in the case of mitochondrial respirometry and fluorometry experiments) mitochondrial respiratory state, followed by Bonferroni post-tests. Statistics were performed using Prism 5 software (Graphpad Software Inc., La Jolla, CA, USA). A significance level of $p < 0.05$ was considered significant.

A.4 Results

A.4.1 Oxidative Phosphorylation and ROS Emission Rates

The respiratory capacities for oxphos in hybrids were intermediate between the higher rates in pumpkinseed and the lower rates in bluegill when measured at 25°C (Appendix Fig. 2A). Oxphos respiration (P) differed between respiratory substrates ($F_{[3,89]}=21.74$, $p < 0.0001$), as respiration rates were generally higher with substrates of complex I (P_{PM} and P_{PMG}) than complex II ($P_{S(Rot)}$), and the rates measured with substrates of both complexes I and II (P_{PMGS}) were only slightly higher than the P_{PMG} rates. There was a significant main effect of species on oxphos respiration ($F_{[2,89]}=11.80$, $p < 0.0001$) that was caused by pumpkinseed having significantly greater oxphos respiration than bluegill when supported by complex I substrates, but respiration supported by complex II substrates

was similar across all three groups. Similar variation in oxphos respiration was observed at 15°C and 28°C (Appendix Supplementary Fig. 2). Leak state respiration, phosphorylation efficiency (P/O ratio; the amount of ATP produced from a single oxygen atom, which we measured when respiration was supported with pyruvate and malate), and mitochondrial P_{50} (the low P_{O_2} at which maximal respiration is inhibited by 50%) were similar between groups (Appendix Table 1, Appendix Supplementary Table 1).

There was a pronounced increase in ROS emission during oxphos respiration in hybrids (Appendix Fig. 2B). ROS emission rates varied from a main effect of hybridization ($F_{[2,86]}=11.07$, $p<0.0001$) as well as substrate ($F_{[3,86]}=7.419$, $p=0.0002$). The elevated ROS emission in hybrids was only observed with substrates of complex I (P_{PMG}) and complex I+II (P_{PMGS}), ranging from 1.5 to 1.7-fold higher in hybrids than parent species. ROS emission rates were similar between groups when complex I was inhibited with rotenone and respiration was supported exclusively with complex II substrates ($P_{S(Rot)}$). A similar (albeit less striking) pattern of variation in ROS emission rates were observed when measurements were made at 15 or 28°C (Appendix Supplementary Fig. 2). Measurements at each substrate combination were made at similar P_{O_2} between groups, so the observed variation in ROS emission was not caused by biased differences in O_2 availability. The lack of concordance between the variation in oxphos respiration and ROS emission also suggests that the observed variation was not caused by differences in mitochondrial respiration rate.

A.4.2 Electron Transport Capacity

In contrast to oxphos, mitochondrial capacities for electron transport (assessed by measuring respiration in mitochondria that were uncoupled with CCCP; see Materials and methods) were lower in hybrids than in pumpkinseed (Appendix Fig. 3A). In addition to an expected effect of substrate ($F_{[3,83]}=35.53$, $p<0.0001$), there was a significant main effect of species/hybridization ($F_{[2,83]}=41.55$, $p<0.0001$) on respiratory capacities for electron transport (E) that were associated with higher respiration rates *via* complex I (E_{PM} , E_{PMG}) and complexes I+II (E_{PMGS}) in pumpkinseed than in hybrids and bluegill.

Flux control ratios of the respiratory capacities for electron transport (E) relative to oxphos (P) indicated a marked change in mitochondrial quality in hybrids. E/P values were near 1 in hybrids, compared to ~ 1.3 in pumpkinseed and bluegill (Appendix Fig. 3B), and there was a strong main effect of hybridization on E/P values ($F_{[2,82]}=7.441$, $p=0.0011$) but no effect of substrate ($F_{[3,82]}=0.042$, $p=0.988$).

The low capacities for electron transport in hybrids compared to pumpkinseed appeared to be associated with significant reductions in the activity of complex III in mitochondria (Appendix Fig. 4) ($F_{[2,21]}=8.704$, $p=0.0018$). In contrast, the activities of citrate synthase ($F_{[2,24]}=1.305$, $p=0.2898$) and complex IV ($F_{[2,21]}=1.597$, $p=0.2262$) were similar among groups. Hybrids had similar complex V activity compared to pumpkinseed, both of which were higher than bluegill ($F_{[2,21]}=12.42$, $p=0.0003$). Complex I ($F_{[2,22]}=4.409$, $p=0.0245$) and complex II ($F_{[2,21]}=3.234$, $p=0.0597$) activities were intermediate in hybrids between the higher activity in pumpkinseed and the lower activity in bluegill.

A.4.3 Oxidative Stress

Lipid peroxidation was elevated in isolated mitochondria from hybrids ($F_{[2,21]}=3.749$, $p=0.0405$), to levels that were ~2-fold higher than those in isolated mitochondria from pumpkinseed or bluegill (Appendix Fig. 5).

A.5 Discussion

Hybrid breakdown has been suggested to occur from mitochondrial dysfunction, arising when hybridization combines incompatible subunits in the protein complexes involved in oxidative phosphorylation (which contain multiple subunits encoded by both mitochondrial and nuclear genomes) (Wolff et al., 2014; Burton and Barreto, 2012). Mito-nuclear incompatibilities in hybrids have been shown to impair complex activities, diminish ATP synthesis rates, and reduce hybrid fitness (Ellison and Burton, 2006, 2008; Davies et al., 2012). Here, we show that mitochondrial ROS emission and lipid peroxidation are elevated by hybridization in sunfish, but that oxidative phosphorylation is maintained by compensatory reductions in control by the mitochondrial phosphorylation system.

A.5.1 Emission of Reactive Oxygen Species is Elevated from Mitochondria of Hybrids

Our results suggest that hybridization in sunfish increases ROS production (Appendix Fig. 2B) and lipid peroxidation (Appendix Fig. 5) in mitochondria. It has been suggested that mito-nuclear incompatibilities arising from hybridization could affect fitness and longevity by increasing mitochondrial ROS production and oxidative stress (Ellison et al., 2008; Rand et al., 2006). However, although hybrid breakdown has been associated with increases in oxidative stress (Barreto and Burton, 2013), there was little direct evidence until now that hybridization increases mitochondrial ROS emission. Although our ROS measurements reflected the ROS that was released from mitochondria, the observed increase in lipid peroxidation suggested that increases in ROS production damaged the mitochondria themselves. The activities of antioxidant enzymes in liver and other tissues are not elevated in hybrids (Borowiec et al., 2016), so they have no additional capacity to scavenge the excess ROS released by mitochondria *in vivo*, and may therefore suffer from oxidative damage of cell components outside of the mitochondria as well. The corresponding disruptions in cellular function that are expected to result from oxidative stress could increase the metabolic costs of maintenance and repair, a possibility that is supported by previous observations that hybrid sunfish exhibit higher whole-animal metabolic rates (Borowiec et al., 2016), which could reduce the aerobic scope available for activity and behaviour. It is also possible that increases in ROS production and oxidative stress contribute to the relatively poor competitive ability of sperm from F1 hybrids compared to that from parental bluegill or pumpkinseed (Immler et al., 2011), which could provide a mechanistic link between mitochondrial dysfunction and fitness breakdown and thus help explain the relatively low abundance of

F2 hybrids in the wild (Konkle and Philipp, 1992; Garner and Neff, 2013). Nevertheless, future studies are needed to establish the potential relationship between mitochondrial ROS emission, oxidative stress, and fitness breakdown in hybrid sunfish.

The potential involvement of mito-nuclear incompatibilities is supported by our observation that hybrids had elevated ROS emission rates when using substrates of complex I, but not complex II. Mitochondrial complexes I, III, IV, and V are large proteins composed of subunits encoded by both mitochondrial and nuclear genomes. Mitochondrial complex II, by contrast, is entirely encoded by the nuclear genome. Therefore, poor structural or functional relationships between incompatible subunits may have increased electron leak and exacerbated ROS production in hybrids (Lane, 2011; Sharpley et al., 2012).

Elevated ROS emission in hybrids was not associated with any overt reduction in the capacity for oxidative phosphorylation. The respiratory capacities for oxidative phosphorylation in isolated liver mitochondria of hybrids were generally intermediate to those of the parental pumpkinseed and bluegill, consistent with previous observations in sunfish for mitochondria isolated from white muscle (Davies et al., 2012). F2 inter-population hybrids, in which the effects of mito-nuclear incompatibilities are expected to be even greater, have been shown to have a diminished capacity for mitochondrial ATP synthesis in the intertidal copepod *Tigriopus californicus* (Ellison and Burton, 2006, 2008). Our observation that this did not occur in hybrid sunfish may be explained by a compensatory reduction in the restraining influence of the phosphorylation system over electron transport, as described in the next section.

A.5.2 Diminished Control by the Phosphorylation System May Offset Mitochondrial Impairment in Hybrids

Flux control ratios of the respiratory capacities for electron transport (E) relative to oxphos (P) indicated that the phosphorylation system (i.e. complex V, adenine nucleotide translocase, and inorganic phosphate transporter) may have had a diminished influence on oxidative phosphorylation in hybrids. As described by Gnaiger and colleagues (Gnaiger, 2009; Pesta et al., 2012), uncoupling removes the restraining influence of the phosphorylation system over electron transport, such that the E/P flux control ratio can be used as an index of control by the phosphorylation system. Furthermore, E/P is known to vary between species and can offset deficiencies in mitochondrial function (Gnaiger, 2009; Porter et al., 2015). The diminished control by the phosphorylation system that we observed in hybrids may have similarly offset any potential impairment in mitochondrial electron transport that arose from mito-nuclear incompatibilities, and thus help maintain ATP synthesis capacity. This likely explains why mitochondria from hybrids had respiratory capacities for oxidative phosphorylation that were similar to pumpkinseed, even though their electron transport capacities and their activity of complex III were reduced.

The nearly absent influence of the phosphorylation system over oxphos respiration in the mitochondria of hybrids may also contribute to the observed increases in ROS emission. Support for a potentially causal relationship between these observations comes

from previous studies in cardiomyocytes, in which ROS emission was enhanced by overexpression of adenine nucleotide translocase, which likely reduces control by the phosphorylation system by decreasing the restraining influence of this transporter on oxphos (Baines and Molkenin, 2009). This suggests that although the reduction in control by the phosphorylation system in hybrids may compensate for decreases in electron transport capacity and complex III activity, it may come at the expense of elevated rates of ROS emission.

Mitochondrial complex III activity was reduced in hybrids compared to pumpkinseed. This pattern of variation could result from an apparent dominant-recessive relationship for this trait, but it is somewhat consistent with previous findings for mitochondria from white muscle, in which the activity of complex III, but not complexes I, II, IV, or V, was reduced in hybrids (Davies et al., 2012). There are eight non-synonymous sequence differences between pumpkinseed and bluegill in the cytochrome b gene (the lone subunit of complex III that is encoded by the mitochondrial genome), some of which could influence subunit structure or alter its interaction with cytochrome c1, iron-sulfur protein, and subunit 8 (Davies et al., 2012). Some complex III enzymes in hybrids could therefore be composed of incompatible combinations of a cytochrome b subunit from pumpkinseed and nuclear subunits from bluegill that reduce the overall complex III activity that was measured. In contrast, sequences of the mitochondria-encoded subunits of complex IV are relatively invariant between species, consistent with the lack of variation in complex IV activity (Appendix Fig. 4) (Davies et al., 2012). Complex III is a major site of superoxide generation in mitochondria (Murphy, 2009; Jastroch et al., 2010), so it is

possible that dysfunction of this enzyme itself could also contribute to the high rates of ROS emission in hybrids.

A.5.3 Identification of Hybrids

Although the preponderance of data suggest that mitochondrial ROS emission is elevated and leads to oxidative stress in F1 hybrids, some additional possibilities should be considered. Previous work suggests that most ($\geq 95\%$) of the hybrids in the lake from which we caught fish (and thus that are represented in our data set) are F1 hybrids (Colgan et al., 1975; Konkle and Philipp, 1992; Garner and Neff, 2013), possibly because post-mating reproductive barriers limit the abundance of F2 hybrids (Immler et al. 2011). Our genotyping strategy also showed that all hybrids identified as possessing both pumpkinseed and bluegill copies of the nuclear *S7* gene also had the mitochondrial ND2 gene from pumpkinseed (see Materials and methods). Therefore, the available evidence from the literature and shown here suggests that identified hybrids were unidirectional F1 hybrids produced from male bluegill and female pumpkinseed. It is possible that a small minority of fish might have been misclassified, and that our data set may have also included a small number of F2 or backcross hybrids (Boecklen and Howard, 1997). However, in the relatively unlikely event that a F2 or backcross hybrid happened to be present among our fish, our data suggest that it exhibited rates of ROS emission and oxidative stress that were within the range of values exhibited by other individuals with the same presumed identity based on the *S7* gene (which encodes a ribosomal protein that

is not itself expected to have a direct mechanistic role in ROS production or oxidative stress). Nevertheless, our work shows that fish that were identified as hybrids, possessing both pumpkinseed and bluegill copies of the *S7* gene, exhibited elevated rates of mitochondrial ROS emission and oxidative stress. It will be valuable to determine in future studies whether mitochondrial ROS emission and oxidative stress are further accentuated in F2 hybrids, and might thus act as a post-zygotic reproductive barrier that reduces hybrid fitness and contributes to the low abundance of F2 hybrids in the wild (Konkle and Philipp, 1992; Garner and Neff, 2013).

Appendix Table 1. Properties of mitochondria isolated from liver of pumpkinseed, bluegill, and their hybrids (mean \pm s.e.m. (*n*)).

	pumpkinseed	hybrid	bluegill
leak respiration with ATP (L_T , pmol mg protein ⁻¹ s ⁻¹)	277.6 \pm 46.2 (9)	209.0 \pm 17.2 (7)	245.6 \pm 24.2 (9)
leak respiration without ATP (L_N , pmol mg protein ⁻¹ s ⁻¹)	24.6 \pm 4.1 (9)	23.6 \pm 4.2 (6)	24.0 \pm 7.8 (5)
P/O ratio	2.75 \pm 0.26 (9)	2.59 \pm 0.19 (8)	2.47 \pm 0.24 (9)
P_{50} (kPa)			
oxphos respiration	0.137 \pm 0.034 (8)	0.132 \pm 0.0312 (8)	0.080 \pm 0.013 (8)
leak respiration	0.051 \pm 0.007 (9)	0.050 \pm 0.004 (8)	0.050 \pm 0.006 (6)

Appendix Fig. 1.

Representative experiments at 25°C on isolated liver mitochondria to measure

respiration rate (MO_2) during oxidative phosphorylation (oxphos). O_2 concentration

($[\text{O}_2]$) was held at zero for 5 min during the first anoxic period in the oxphos state,

whereas the second anoxic period in the leak state was 10 min in duration. ROS emission

rate was measured concurrently by the fluorescent detection of resorufin at an excitation

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

from mitochondrial superoxide in the presence of superoxide dismutase (22.5 U ml^{-1}),

horseradish peroxidase (3 U ml^{-1}), and Ampliflu Red ($15 \mu\text{mol l}^{-1}$). The same protocol

was used to measure electron transport capacity, except that mitochondria were

uncoupled with titrations of CCCP (carbonyl cyanide m-chloro phenyl hydrazone; titrated

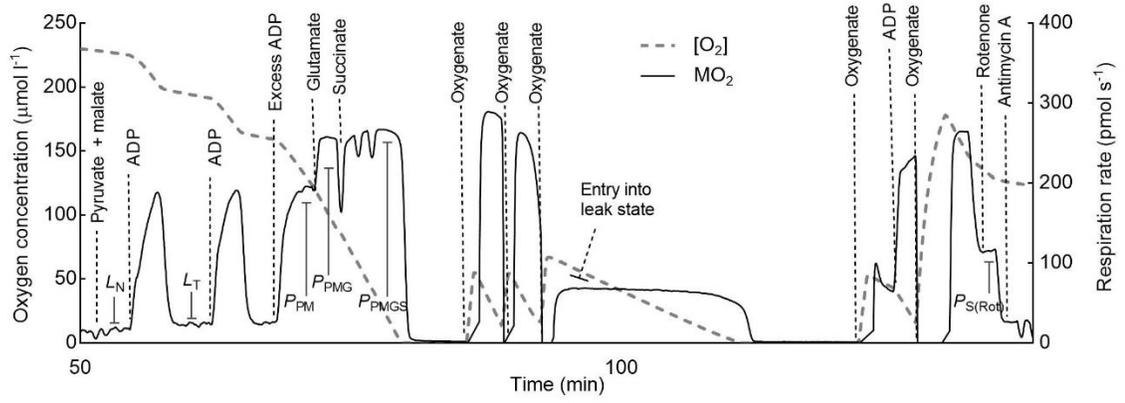
to $0.5\text{-}2 \mu\text{mol l}^{-1}$) prior to the addition of glutamate (see Materials and Methods). (L_N and

L_T , leak respiration in the absence and presence of ATP, respectively; P_{PM} , oxidative

phosphorylation (P) with pyruvate and malate; P_{PMG} , P with pyruvate, malate, and

glutamate; P_{PMGS} , P with pyruvate, malate, glutamate, and succinate; and $P_{S(\text{Rot})}$, P with

succinate and rotenone).



Appendix Fig. 2.

Mitochondrial ROS emission was elevated in hybrids compared to pumpkinseed

and bluegill parents. (A) Mitochondrial respiration during oxidative phosphorylation

(oxphos, P) was measured with maximal ADP along with substrates of complex I (CI;

P_{PM} with pyruvate, P, and malate, M; P_{PMG} with P, M, and glutamate, G), complex II

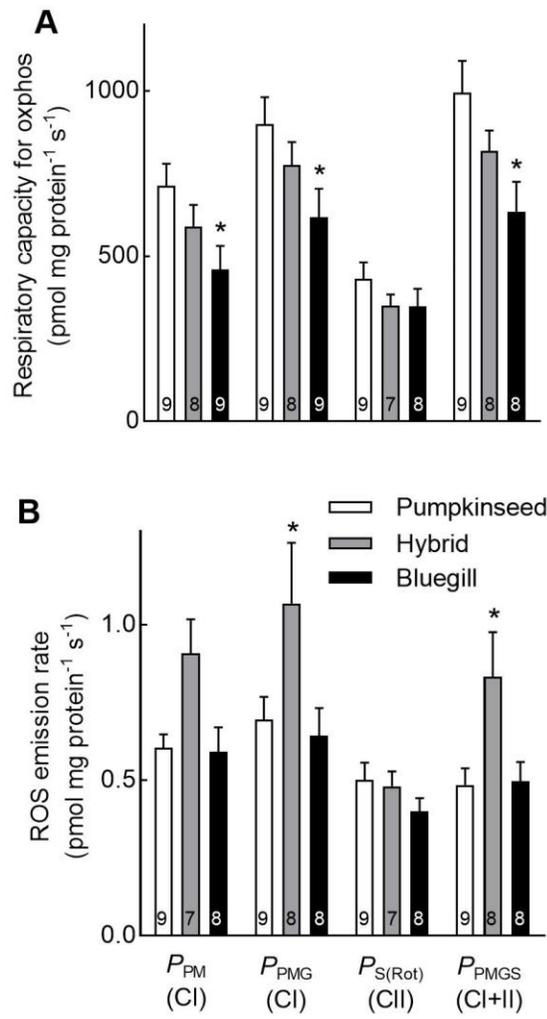
($P_{S(Rot)}$ with succinate, S, and the complex I inhibitor rotenone, Rot), and both complexes

I and II (P_{PMGS} with P, M, G, and S). (B) Mitochondrial ROS emission rate was measured

fluorometrically as the emission of hydrogen peroxide in the presence of superoxide

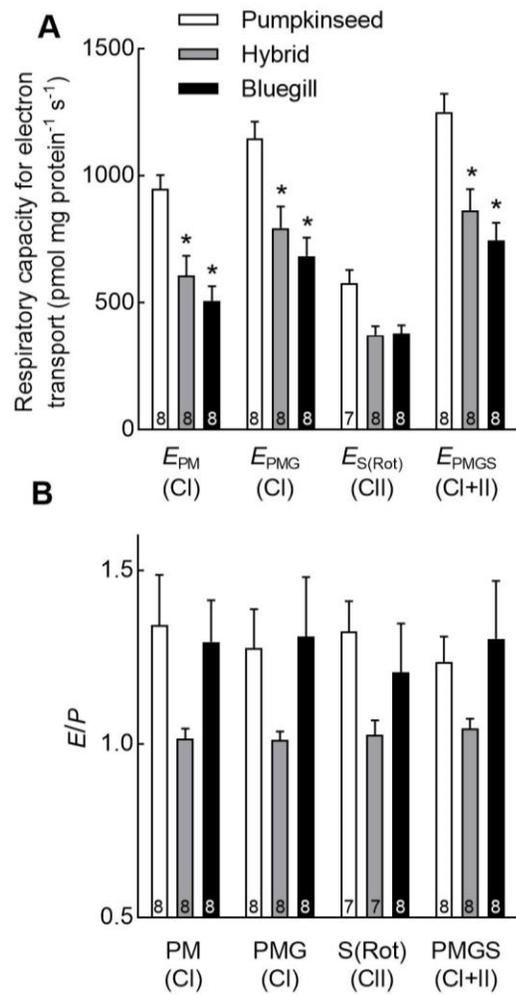
dismutase, horseradish peroxidase, and Ampliflu Red. Numbers in bars represent sample

sizes; * represents significant pairwise differences from pumpkinseed.



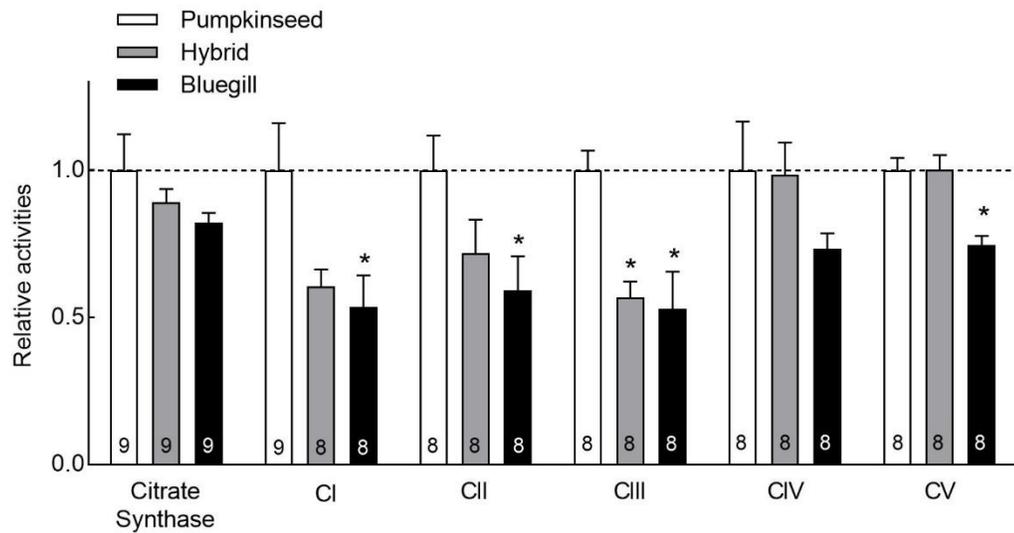
Appendix Fig. 3.

Control over oxidative phosphorylation by the phosphorylation system was reduced in the mitochondria of hybrids. (A) Electron transport capacity was reduced in hybrids compared to pumpkinseed, the maternal parent species, to levels similar to bluegill. The respiratory capacity for electron transport (E) was measured in mitochondria uncoupled with CCCP using substrates of complex I (E_{PM} , E_{PMG}), complex II ($E_{S(Rot)}$) and complex I and II (E_{PMGS}) (symbols as in Appendix Fig. 1). (B) The ratio of the respiratory capacities for electron transport to oxidative phosphorylation (E/P) was used as an index of the control of respiration by the phosphorylation system. Numbers in bars represent sample sizes; * represents significant pairwise differences from pumpkinseed.



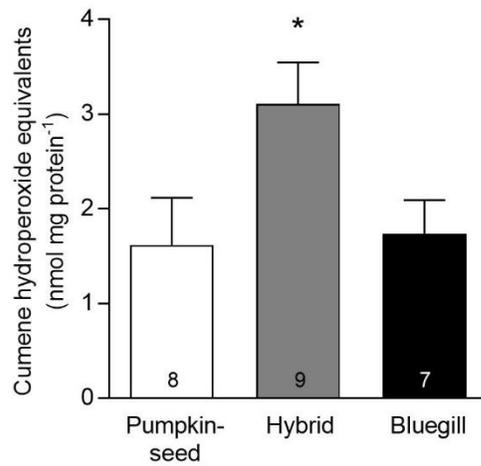
Appendix Fig. 4.

Complex III activity was reduced in mitochondria of hybrids compared to pumpkinseed, the maternal parent species, to levels similar to bluegill. For ease of comparison, the activities of each enzyme are shown normalized to pumpkinseed, whose actual activities were as follows (mU mg protein⁻¹): citrate synthase, 525.0; complex I, 44.26; complex II, 48.29; complex III, 255.44; complex IV, 2020; complex V, 987.19. Numbers in bars represent sample sizes; * represents significant pairwise differences from pumpkinseed.



Appendix Fig. 5.

Hybridization increased oxidative stress in isolated mitochondria. Lipid peroxidation was measured as the formation of Fe(III)-xylenol orange complex (see Materials and Methods), and is expressed as equivalents of cumene hydroperoxide. Numbers in bars represent sample sizes; * represents significant pairwise differences from pumpkinseed.



Appendix Supplementary Table 1

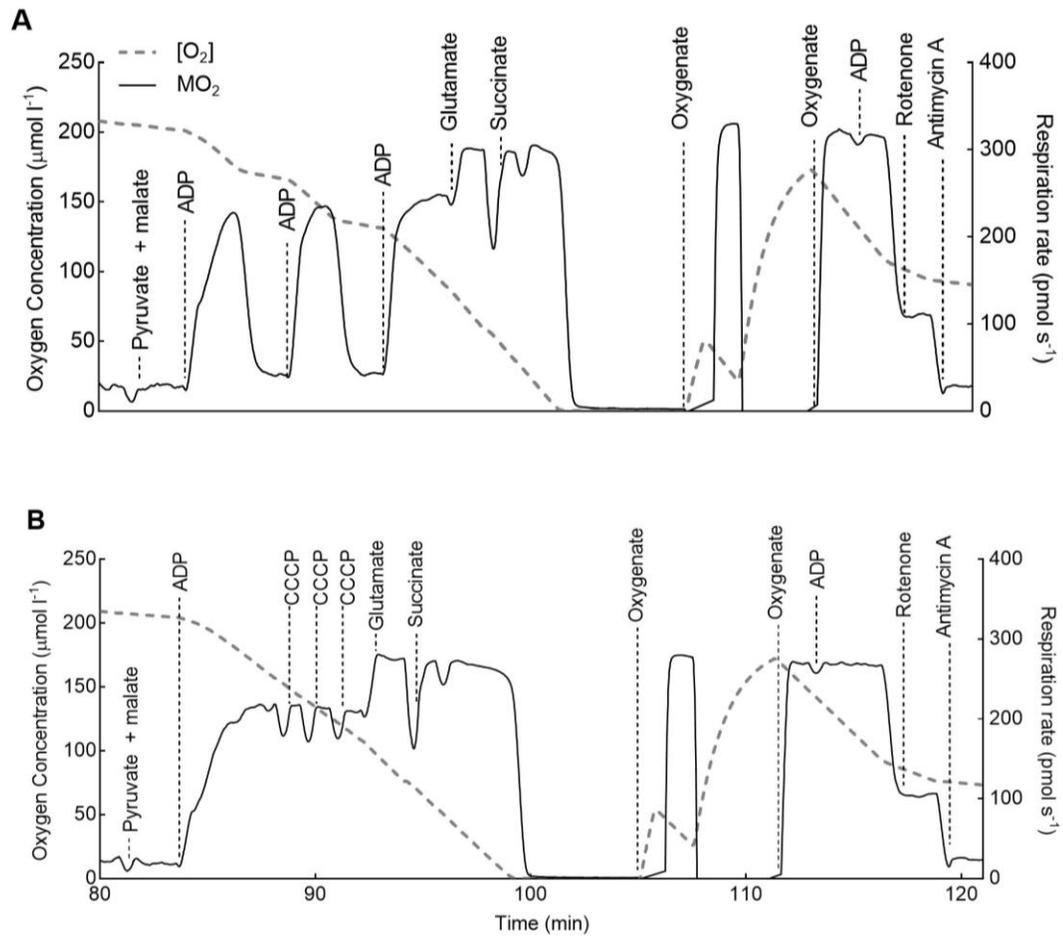
Properties of mitochondria isolated from liver of pumpkinseed, bluegill, and their hybrids at 15 and 28°C (mean \pm s.e.m. (*n*)).

	temperature (°C)	pumpkinseed	hybrid	bluegill
leak respiration with ATP	15	144.1 \pm 24.1 (8)	116.9 \pm 16.8 (9)	133.2 \pm 16.1 (8)
(L_T , pmol mg protein ⁻¹)	28	319.3 \pm 71.7 (8)	208.5 \pm 34.9 (7)	269.5 \pm 39.5 (8)
leak respiration without ATP	15	11.1 \pm 3.1 (8)	7.6 \pm 2.9 (9)	8.9 \pm 2.7 (9)
(L_N , pmol mg protein ⁻¹)	28	26.5 \pm 4.8 (8)	16.8 \pm 7.1 (8)	16.8 \pm 5.2 (9)
P/O ratio	15	2.46 \pm 0.35 (9)	2.72 \pm 0.20 (9)	2.87 \pm 0.26 (8)
	28	2.75 \pm 0.29 (8)	2.82 \pm 0.26 (7)	2.43 \pm 0.25 (8)
mitochondrial P ₅₀ during	15	0.149 \pm 0.024 (9)	0.136 \pm 0.017 (9)	0.109 \pm 0.014 (9)
oxphos respiration (kPa)	28	0.091 \pm 0.014 (8)	0.119 \pm 0.024 (8)	0.087 \pm 0.025 (8)

Appendix Supplementary Fig. 1.

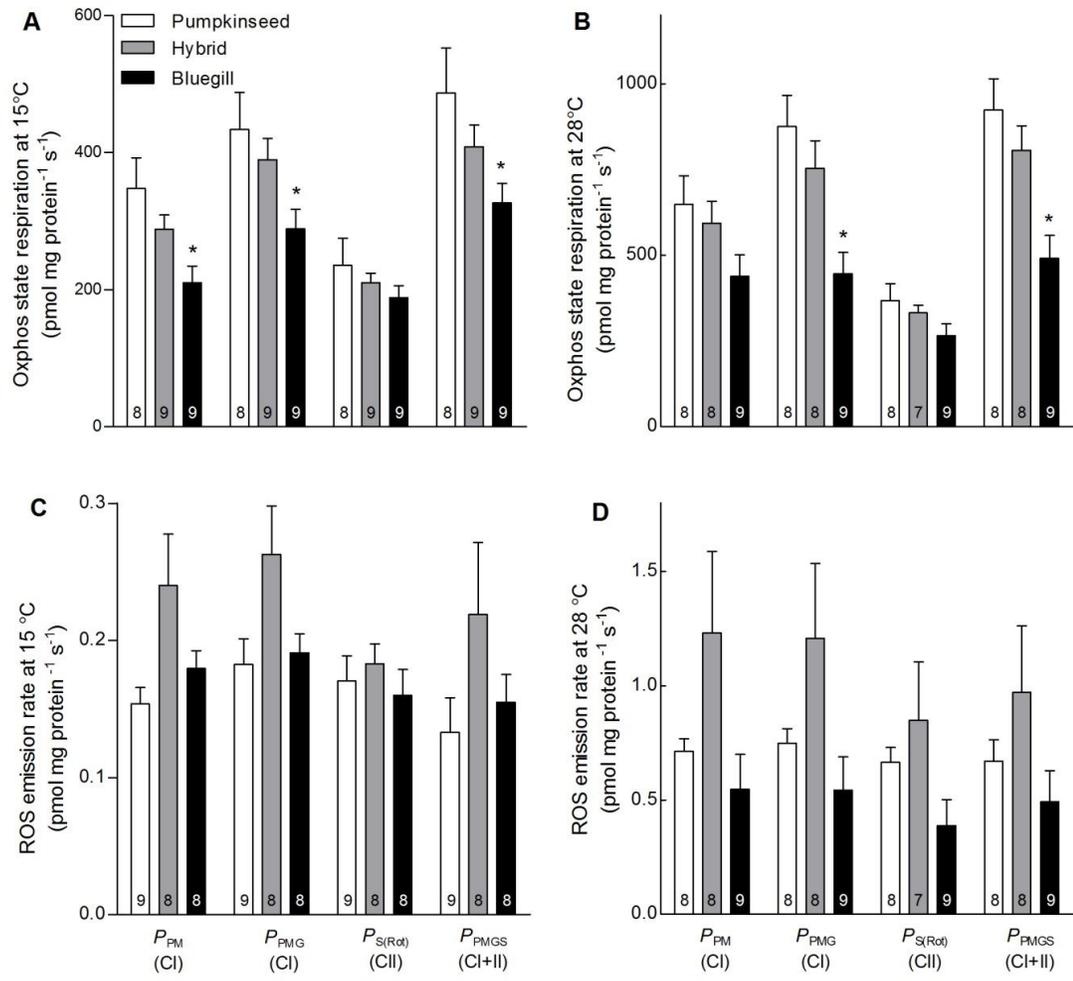
Representative experiments at 28°C on isolated liver mitochondria to measure (A) respiration during oxidative phosphorylation and (B) electron transport capacity during uncoupled respiration.

(A) ROS emission rate was measured concurrently by the fluorescent detection of resorufin at an excitation wavelength of 525 nm and AmR filter set (Oroboros Instruments), which is produced from mitochondrial superoxide in the presence of superoxide dismutase (22.5 U ml⁻¹), horseradish peroxidase (3 U ml⁻¹), and Ampliflu Red (15 µmol L⁻¹). (B) Mitochondria were uncoupled with titrations of CCCP (carbonyl cyanide m-chloro phenyl hydrazone; titrated to 0.5-2 µmol L⁻¹) to determine the maximal capacities for electron transport by the electron transport system. The same protocol was used at 15°C, but at 25°C, there was an additional anoxic bout in the leak state (proceeding the first anoxic bout), and both anoxic periods were held for 10 min (see Materials and Methods).



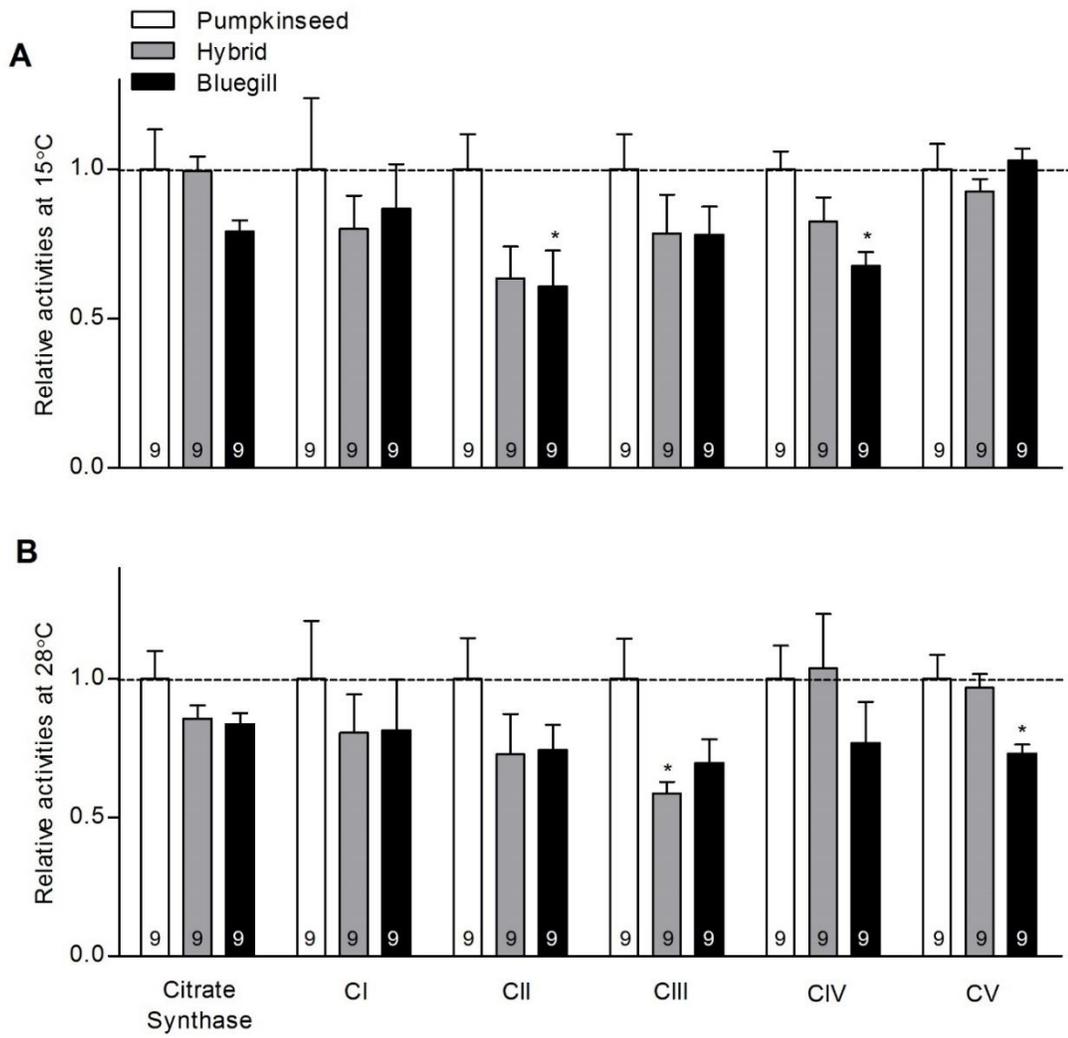
Appendix Supplementary Fig. 2.

Consistent with measurements obtained at 25°C, hybrids have respiration rates during oxidative phosphorylation (oxphos; *P*) similar to or intermediate between the paternal bluegill and maternal pumpkinseed at (A) 15°C and (B) 28°C. Oxphos state respiration was measured with maximal ADP along with substrates of complex I (CI; P_{PM} with pyruvate, P, and malate, M; P_{PMG} with P, M, and glutamate, G), complex II ($P_{S(Rot)}$ with succinate, S, and the complex I inhibitor rotenone, Rot), and both complexes I and II (P_{PMGS} with P, M, G, and S). There was a significant main effect of substrate as well as species on oxphos state respiration at both 15°C (substrate, $F_{[3,92]}=19.25$, $p<0.0001$; species, $F_{[2,92]}=12.29$, $p<0.0001$) and 28°C (substrate, $F_{[3,87]}=22.29$, $p<0.0001$; species, $F_{[2,87]}=20.70$, $p<0.0001$). Despite the lack of variation in oxphos state respiration, hybrids had higher rates of ROS emission than bluegill and pumpkinseed. This main effect of hybridization was evidenced at both 15°C ($F_{[2,88]}=7.553$, $p=0.0009$) and 28°C ($F_{[2,83]}=8.411$, $p=0.0005$). Substrate did not have an effect on ROS emission at both temperatures (15°C, $F_{[3,88]}=1.845$, $p=0.1448$; 28°C, $F_{[3,83]}=0.7125$, $p=0.5472$). Numbers in bars represent sample sizes; * represents significant pairwise differences from pumpkinseed.



Appendix Supplementary Fig. 3.

Mitochondrial complex activity was measured at (A) 15°C and (B) 28°C in pumpkinseed, bluegill, and hybrids. Consistent with measurements at 25°C, complex III activity of hybrids was reduced in hybrids assayed at 28°C ($F_{[2,24]}=4.513$, $p=0.0217$), but not at 15°C ($F_{[2,24]}=1.190$, $p=0.3216$). At 15°C, hybrids exhibited intermediate activities of complex II and IV ($F_{[2,24]}=3.622$, $p=0.0422$; $F_{[2,24]}=6.345$, $p=0.0061$, respectively), but citrate synthase ($F_{[2,24]}=1.985$, $p=0.1593$), complex I ($F_{[2,24]}=0.3381$, $p=0.7165$), III ($F_{[2,24]}=1.190$, $p=0.3216$), and V ($F_{[2,24]}=0.805$, $p=0.4588$) were similar between species. At 28°C, citrate synthase ($F_{[2,24]}=0.2051$, $p=1.694$), complex I ($F_{[2,24]}=0.3734$, $p=0.6923$), II ($F_{[2,24]}=1.365$, $p=0.2745$), and IV ($F_{[2,24]}=0.8455$, $p=0.4417$) were similar between species, and bluegill have reduced complex V activity relative to pumpkinseed ($F_{[2,24]}=5.824$, $p=0.0087$). The activities of each enzyme are shown normalized to pumpkinseed, whose actual activity at 15°C for citrate synthase and the five mitochondrial complexes are (in mU mg protein⁻¹): 343.5, 33.94, 21.14, 137.8, 1190.6, and 659.9, respectively. At 28°C, activities were (in mU mg protein⁻¹): 569.3, 42.88, 57.07, 349.9, 2048.7, and 1123.5, respectively. Numbers in bars represent sample sizes; * represents significant pairwise differences from pumpkinseed.



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