



Fish living near two wastewater treatment plants have unaltered thermal tolerance but show changes in organ and tissue traits

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ABSTRACT

Municipal wastewater treatment plants (WWTPs) are a significant source of anthropogenic pollutants and are a serious environmental stressor in Laurentian Great Lakes ecosystems. In this study, we examined whether three freshwater fish species (bluegill sunfish *Lepomis macrochirus*, green sunfish *Lepomis cyanellus*, and round goby *Neogobius melanostomus*) collected near two wastewater effluent outflows in Lake Ontario showed altered measures of somatic investment and thermal tolerance. Fish of all three species collected near the WWTPs were larger with 50–60% heavier body masses compared to those collected at reference sites. Green sunfish had higher body condition and increased haematocrit at wastewater-contaminated sites, and both round goby and bluegill sunfish had larger livers (controlling for body mass) at wastewater-contaminated sites. Thermal tolerance (critical thermal maximum, CT_{max}) differed between species (green sunfish > bluegill sunfish > round goby), but was similar in fish collected at wastewater-contaminated sites compared to cleaner reference sites. Wastewater-contaminated sites had poorer water quality, higher nutrient loadings, and higher concentrations of anthropogenic contaminants (measured via polar organic chemical integrative samplers, POCIS) than reference sites. Our results suggest that fish in the wild may have some capacity to cope with WWTP effluent and avoid any potential impairments in thermal tolerance. Our findings also suggest that treated wastewater is changing water quality locally in Great Lakes watersheds, and that many fish species may be able to access extra nutrients provided by such effluent outflows. However, if outflow areas become preferred foraging areas this will inadvertently increase exposure to anthropogenic stressors and pollutants.

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Introduction

Across the Laurentian Great Lakes watersheds, aquatic ecosystems face many stressors including habitat loss and modification, invasive species, and chemical inputs from municipal and industrial wastewater treatment plants (WWTPs; Boston et al., 2016; Reid et al., 2018; Schoenfuss et al., 2020; Smith et al., 2019). WWTPs are a major point source of aquatic pollution and have been shown to affect water quality downstream by adding

nutrients (often resulting in eutrophication and reduced oxygen; Hamdhani et al., 2020; Holeton et al., 2011; Tetreault et al., 2011), increasing conductivity (Melvin, 2016; Odjadjare and Okoh, 2010) and changing temperature (Hamdhani et al., 2020; Kaushal et al., 2010; Odjadjare and Okoh, 2010). In addition, WWTPs are not fully capable of removing all compounds, and many chemicals including pesticides, detergents, plastic by-products, and pharmaceuticals and personal care products (PPCPs), are discharged into surface waters (Blair et al., 2013; Jorgenson et al., 2018; Loos et al., 2013). Although these chemicals are often present at low concentrations in WWTP effluent and many do not persist in the environment long-term, they are constantly reintroduced and thus subject the ecosystem to chronic “pseudo-persist

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ent” exposure (Jelić et al., 2012; Jones et al., 2005). Reliance on WWTPs will only continue rising as urban populations grow (Grimm et al., 2008); therefore, there is concern over the impacts that effluents, and the mixtures of pollutants they discharge, have on receiving environments and the wildlife that reside in these habitats (Hamdhani et al., 2020; Holetton et al., 2011).

Prior research has shown that fish exposed to treated wastewater effluent can be affected across multiple levels of biological organization, including altered mRNA transcription (Bahamonde et al., 2014; Garcia-Reyero et al., 2011), changes in endogenous hormone levels (Pottinger et al., 2013), increased metabolism and demands on oxygen transport (Du et al., 2018, 2019; Mehdi et al., 2018), abnormal aggressive and courtship behaviour (McCallum et al., 2017b; Saaristo et al., 2014), and even altered fish community structure (Brown et al., 2011; McCallum et al., 2019; Mehdi et al., 2021; Tetreault et al., 2013). Somatic investment is a commonly measured endpoint in response to effluent exposure, and previous studies have found that fish exposed to effluent or collected near WWTPs tend to be larger and/or in better condition (Melvin, 2016; Porter and Janz, 2003; Pottinger et al., 2013; Reinling et al., 2017; Tetreault et al., 2011; but see also Hemming et al., 2002 for a reduction in condition), likely as a result of increased nutrients, warmer temperatures, and/or reduced population sizes (i.e., less competition) at outfall sites. Haematocrit (% of packed red blood cells in a plasma sample) is another commonly measured indicator for how fish respond to stressors (Sopinka et al., 2016). Haematocrit is a coarse measure of haemoglobin content and oxygen carrying capacity, and it generally increases in response to stressors and pollutants (Sopinka et al., 2016). Together, the somatic measures of wildlife health (i.e., body length/mass, non-eviscerated condition factor) and haematocrit can be especially useful for largescale field sampling or environmental monitoring because they can be measured quickly, with minimal equipment, and without terminally sampling the fish (Dale and Beyeler, 2001).

An organism's capacity to tolerate environmental challenges can be an essential performance trait that is critical to fitness (Madliger et al., 2018; Schulte, 2014). For example, upper temperature tolerance, measured as critical thermal maximum (CT_{max}) is a common indicator of tolerance to a thermal challenge (Madliger et al., 2018). CT_{max} is the temperature at which loss of equilibrium occurs after incremental temperature increases (Beitinger, 1990). Previous work has shown that physiological stress and exposure to metals and pesticides in the laboratory can reduce CT_{max} (Carrier and Beitinger, 1988; Kumar et al., 2016; LeBlanc et al., 2011; Op de Beeck et al., 2017; Patra et al., 2007, reviewed in Beitinger, 1990), indicating that sublethal chemical exposure may limit an organism's scope or ability to tolerate environmental challenges such as high temperatures. Additionally, animals residing at wastewater outfalls may be acclimated to the more stable temperatures characteristic of outfall environments, which might further limit their capacity to tolerate temperature extremes (Schaefer and Ryan, 2006; Strange et al., 2002). However, higher body condition and/or dietary enrichment are associated with increased thermal tolerance in several fish species (Robinson et al., 2008; Tejpal et al., 2014; Turko et al., 2020). This is thought to be driven by the fish's increased energy stores supporting the energy demands needed to face a physiological stressor like high temperature. For example, redbreasted dace (*Clinostomus elongatus*) fed a high ration diet had higher CT_{max} values (Turko et al., 2020). In another example, Kumar et al. (2016) found that Milkfish (*Chanos chanos*) that received dietary enrichment with vitamin B6 were protected against the heat tolerance-limiting effects of exposure to the organochlorine pesticide endosulfan. Taken together, these results suggest that the effects of wastewater, which contains a mixture of pollutants, on thermal tolerance may be

condition dependent, i.e. exposure to the chemical contaminants in wastewater effluent could reduce CT_{max} ; but, if the elevated nutrient levels at wastewater outfalls lead to fish being bigger and/or in better condition, fish may instead have higher CT_{max} .

Here we explored how wastewater effluent affected somatic investment, haematocrit, CT_{max} , and the relationship between these factors in several wild-collected fish species. We first characterized the wastewater receiving environments compared to reference sites by measuring a suite of water quality parameters, including concentrations of nutrients (nitrogen, phosphorus) and anthropogenic contaminants commonly detected in wastewater effluents using polar organic chemical integrative samplers (POCIS). We selected somatic investment and haematocrit as indicators of condition because they are easy to measure onsite or during largescale, multi-species, multi-site sampling, and they are known to be affected by stressors like wastewater effluents (Dale and Beyeler, 2001; Kilgour et al., 2005). We measured CT_{max} as a novel test of whole-organism performance in response to wastewater that may be increasingly important for fitness as waters warm and extreme heat events become more frequent with climate change.

We collected fish at varying distances from the outflow of two WWTPs that discharge their effluent into waters that flow into Hamilton Harbour, at the western edge of Lake Ontario. We measured our endpoints in three common, local species of fish: native bluegill sunfish (*Lepomis macrochirus*) and green sunfish (*Lepomis cyanellus*), and invasive round goby (*Neogobius melanostomus*). We tested two contrasting predictions. First, we predicted that the mixture of contaminants present in wastewater would reduce CT_{max} . Alternatively, if fish living near WWTPs have larger body size and better condition (because of the extra nutrients), then they should have higher thermal tolerance (Kumar et al., 2016; Melvin, 2016; Tetreault et al., 2011; Turko et al., 2020). As a final addition to our main study, we also piloted the feasibility of onsite, high-throughput behavioural assays to measure how wastewater exposure affects fish activity, exploration, and boldness in the field. This behavioural analysis was motivated by the knowledge that exposure-induced increases in metabolism may dampen an animal's aerobic scope for normal behaviours, and also that certain pollutants found in wastewater effluent are designed to modulate human behaviours and may also influence fish that share conserved drug targets (e.g., antidepressants, anxiolytics; Cunha et al., 2017).

Methods

Study sites

In June, August, and October of 2017 we sampled 9 sites of varying distances from two wastewater treatment plants whose effluent eventually flows into Hamilton Harbour, a large bay at the western tip of Lake Ontario, Canada (Fig. 1). Hamilton Harbour is one of 43 locations around the Great Lakes that has been identified as an Area of Concern (AOC) by the International Joint Commission due to environmental degradation (Hamilton Harbour Remedial Action Plan, 1992). Treated wastewater effluent is a significant stressor to this habitat, as 50% of the water flowing into Hamilton Harbour is from WWTPs (Environment and Climate Change Canada, 2017).

The first WWTP studied was the Dundas WWTP. Its effluent is released into Desjardins Canal at the west end of Cootes Paradise Marsh (43°15'59.36"N, 79°56'33.13"W) which is at the westernmost edge of Hamilton Harbour. The Dundas WWTP is a conventional activated sludge plant with nitrification and tertiary treatment that serves approximately 30,000 people and treats 18.2 million litres of wastewater per day (City of Hamilton, 2020). Five

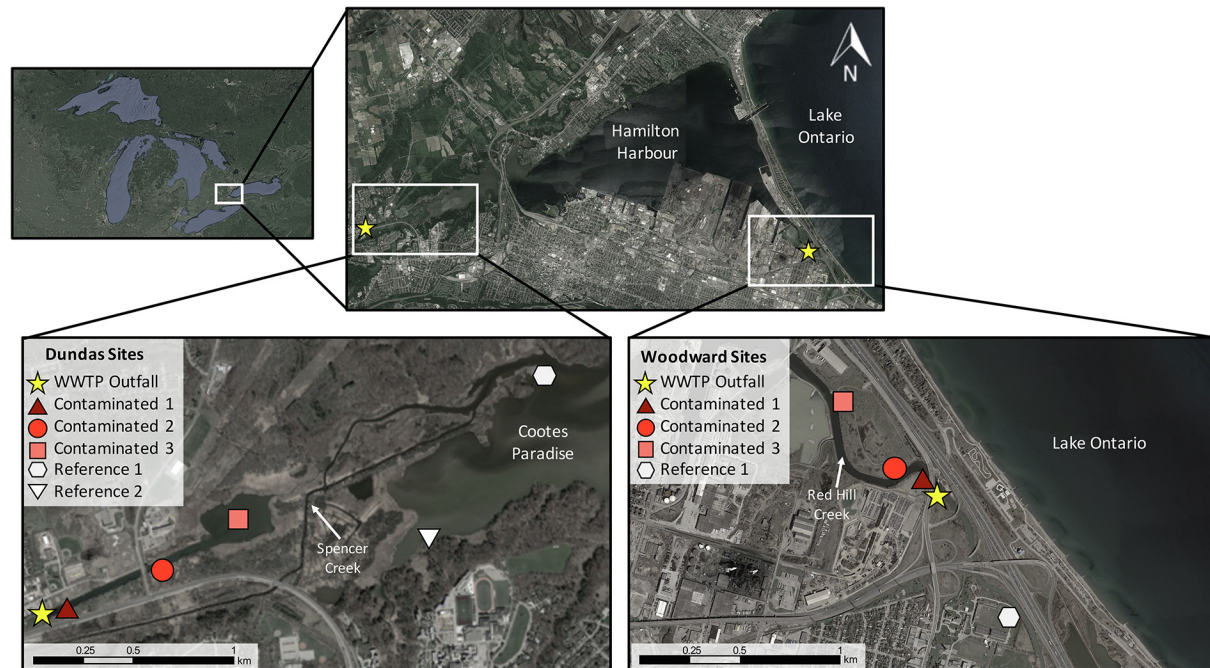


Fig. 1. Fish were collected from sites downstream of the Dundas (bottom left) and Woodward (bottom right) WWTPs, located on the western and eastern edge of Hamilton Harbour, respectively. Hamilton Harbour constitutes the western edge of Lake Ontario. Satellite imagery provided by Google Earth (2020).

locations were sampled at increasing distances from the Dundas WWTP (Fig. 1). The closest sampling sites, Dundas Contaminated 1 (at the WWTP outflow pipe, 0 m downstream) and Dundas Contaminated 2 (550 m downstream) are along the channelized and artificially straightened Desjardins Canal and are exposed to the effluent from the plant. The other downstream sites: Dundas Contaminated 3 (also known as West Pond, 1000 m downstream), Dundas Reference 1 (at the mouth of Spencer Creek, 2800 m downstream) and Dundas Reference 2 (at McMaster Landing, 3750 m downstream, not in the direct path of the effluent) have been less modified by human activity, and have natural shorelines and vegetation and softer substrate; all three of these sites are located within Cootes Paradise Marsh, one of the most degraded coastal wetlands in the Great Lakes Basin (Thomasen and Chow-Fraser, 2012).

The second WWTP, the Woodward WWTP, discharges effluent into Red Hill Creek which flows into the southeast corner of Hamilton Harbour (43°15'44.12"N, 79°46'20.80"W). The Woodward WWTP is a secondary conventional activated sludge plant that serves approximately 480,000 people and treats 409 million litres of water per day (City of Hamilton, 2020). Four sites were sampled near this WWTP, all of which are heavily impacted by human activity and are within an urbanized watershed (Fig. 1). The closest site, Woodward Contaminated 1, is 40 m downstream from the WWTP outfall pipe and is in a channelized stream with vertical cement walls. The next sites downstream are Woodward Contaminated 2 (350 m downstream from the outfall pipe) and Woodward Contaminated 3 (850 m downstream from this outfall pipe); both sites' shores were modified with cobble and boulder. We also sampled an upstream site referred to as Woodward Reference 1 (which is 1000 m upstream of the WWTP outfall pipe). The Woodward Contaminated 2 and 3 sites and the upstream Reference 1 site all have modified shorelines consisting of cobble riprap.

Water and habitat quality, nutrient and contaminant analyses

At each site on each sampling day, we assessed water quality by measuring pH, salinity, conductivity, and total dissolved solids

(TDS; all using PSCTestr 35 Multi-Parameter probe), as well as dissolved oxygen and temperature (YSI Digital Professional Series Pro ODO). Water samples for nutrient analysis were collected using a horizontal Van Dorn sampler (volume 2.2 L) at mid-water depth. Each water sample was stored in snap-seal containers (acid washed and rinsed in distilled water) and transported on ice to the laboratory for analysis (Table 1). At each site we measured total nitrogen (TN: nitrite + nitrate + TKN [Total Kjeldahl Nitrogen]), total ammonia nitrogen (TAN: ammonia + ammonium), total nitrate as N (TNN), soluble reactive phosphorus (SRP: o-Phosphate), and total phosphorus (TP). All samples were analyzed at the City of Hamilton Environmental Laboratory, using a San++ Continuous Flow Analyzer (Skalar) to measure TAN, Anion Chromatography to measure TNN, and colourimetric methodology to measure TN and TP and SRP (see Electronic Supplementary Materials [ESM] Appendix S1 for further analytic details). To measure habitat quality, we measured water clarity (using Secchi depth), flow rate (Höntzsch Instruments flowmeter), and total water depth (using a depth meter). All water and habitat quality data are provided as context of the ecosystem conditions in each of the areas studied.

Concentrations of select anthropogenic contaminants commonly detected in wastewater discharges were determined using polar organic chemical integrative samplers (POCIS) following methods from (Du et al., 2018; Li et al., 2010; McCallum et al., 2017a; Metcalfe et al., 2014). Briefly, POCIS were deployed for two weeks in triplicate at each site following the manufacturer's instructions. They were deployed in stainless steel mesh cages that were anchored and protected at each site by being suspended in a perforated plastic tote held vertically in the water column with plastic floats (see McCallum et al., 2017a). Field blanks were used during deployment and collection at each site to account for any air contamination (no compounds were detected in any field blanks). All POCIS were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) with an electrospray ionization (ESI) source. All target compounds were analyzed by AB Sciex Q-Trap 5500 (Concord, ON, Canada) instrument operated either in positive or negative ion mode. The system was equipped with an Agilent 1100 series (Mississauga, ON, Canada) HPLC

Table 1

Water quality data collected in June, August, and October 2017. Data are presented as mean \pm SEM and the range is given in brackets underneath ($n = 3$). TN = total nitrogen (nitrite + nitrate + TKN (Total Kjeldahl Nitrogen)), TAN = total ammonia nitrogen (ammonia + ammonium), TNN = total nitrate as N, SRP = soluble reactive phosphorus (o-Phosphate), TP = total phosphorus, DO = dissolved oxygen, TDS = total dissolved solids. Some samples were measured lower than the detection limit, and in these cases the measure was taken to be 0. Detection limit for Nitrite 0.1 mg/L, Nitrate 0.01 mg/L, TP 0.010 mg/L, TKN 0.2 mg/L, NH_3 & NH_4 + 0.01 mg/L, o-Phosphate 0.05 mg/L).

	Dundas WWTP					Woodward WWTP				
	Contaminated 1	Contaminated 2	Contaminated 3	Reference 1	Reference 2	Contaminated 1	Contaminated 2	Contaminated 3	Reference 1	Reference 2
TN mg/L	17.8 \pm 0.6 (17.1–18.2)	13.4 \pm 4.1 (9.5–17.6)	6.1 \pm 2.7 (3.1–8.2)	1.3 \pm 0.4 (1–1.7)	1.1 \pm 0.7 (0.5–1.8)	10.4 \pm 1.7 (8.6–11.9)	9.8 \pm 1.5 (8–10.8)	8.9 \pm 1.6 (7.6–10.7)	1.4 \pm 0.2 (1.2–1.5)	
TAN mg/L	0.07 \pm 0.04 (0.03–0.1)	0.15 \pm 0.13 (0.02–0.27)	0.12 \pm 0.16 (0.02–0.3)	0.09 \pm 0.02 (0.07–0.11)	0.01 \pm 0.01 (0–0.01)	0.8 \pm 0.25 (0.61–1.08)	1.08 \pm 0.78 (0.42–1.94)	1.31 \pm 0.54 (0.79–1.87)	0.14 \pm 0.08 (0.09–0.23)	
TNN mg/L	17.13 \pm 0.15 (17–17.3)	12.65 \pm 4.83 (7.95–17.6)	4.04 \pm 3.2 (0.57–6.87)	0.48 \pm 0.14 (0.39–0.64)	0 \pm 0 (0–0)	8.12 \pm 1.53 (6.87–9.83)	7.36 \pm 1.37 (6.19–8.87)	6.53 \pm 1.13 (5.8–7.83)	0.6 \pm 0.29 (0.27–0.82)	
SRP mg/L	0.03 \pm 0.05 (0–0.08)	0.02 \pm 0.04 (0–0.07)	0.02 \pm 0.03 (0–0.06)	0 \pm 0 (0–0)	0.07 \pm 0.07 (0–0.13)	0.22 \pm 0.13 (0.11–0.36)	0.23 \pm 0.1 (0.16–0.34)	0.24 \pm 0.08 (0.17–0.32)	0.05 \pm 0.04 (0–0.08)	
TP mg/L	0.125 \pm 0.014 (0.113–0.141)	0.125 \pm 0.008 (0.119–0.134)	0.232 \pm 0.095 (0.14–0.329)	0.1 \pm 0.02 (0.081–0.121)	0.205 \pm 0.081 (0.135–0.293)	0.379 \pm 0.177 (0.211–0.564)	0.348 \pm 0.109 (0.277–0.474)	0.341 \pm 0.094 (0.263–0.446)	0.113 \pm 0.025 (0.09–0.14)	
Temp °C	19.8 \pm 2.8 (17.1–22.6)	21.1 \pm 4.6 (15.9–24.9)	19.8 \pm 5.8 (13.1–23.2)	18 \pm 4.4 (13–20.9)	19 \pm 5.5 (12.8–23.2)	19.9 \pm 0.7 (19.3–20.6)	20.1 \pm 0.7 (19.3–20.5)	20.4 \pm 1.3 (18.9–21.5)	20.7 \pm 3.1 (17.2–23)	
DO mg/L	10.5 \pm 3.1 (8.13–13.96)	9.1 \pm 4 (6.17–13.71)	6.8 \pm 1.1 (5.9–7.94)	5.9 \pm 1.6 (4.63–7.64)	5.2 \pm 3.4 (2.81–9.18)	5.5 \pm 0.7 (4.95–6.23)	4.5 \pm 1.5 (2.87–5.61)	3.6 \pm 0.9 (2.52–4.14)	5.3 \pm 2.1 (3.53–7.58)	
pH	7.78 \pm 0.48 (7.23–8.15)	7.72 \pm 0.28 (7.42–7.97)	7.79 \pm 0.16 (7.65–7.96)	8.02 \pm 0.09 (7.94–8.12)	7.86 \pm 0.27 (7.66–8.16)	6.97 \pm 0.07 (6.91–7.04)	7.07 \pm 0.09 (6.96–7.14)	7.1 \pm 0.19 (6.92–7.3)	7.74 \pm 0.23 (7.48–7.92)	
TDS ppm	777 \pm 101 (685–885)	759 \pm 78 (670–816)	759 \pm 50 (710–810)	584 \pm 16 (569–600)	591 \pm 24 (563–608)	740 \pm 90 (637–794)	724 \pm 84 (628–781)	704 \pm 98 (596–786)	655 \pm 127 (509–745)	
Cond μS	1093 \pm 146 (958–1248)	1069 \pm 109 (945–1150)	1071 \pm 72 (999–1143)	825 \pm 22 (801–844)	831 \pm 33 (794–855)	1037 \pm 135 (882–1117)	1019 \pm 117 (885–1100)	990 \pm 128 (846–1092)	922 \pm 180 (716–1049)	
Salinity ppm	506 \pm 69 (442–580)	494 \pm 56 (430–535)	493 \pm 35 (463–532)	374 \pm 13 (361–387)	379 \pm 20 (356–391)	480 \pm 64 (406–519)	471 \pm 58 (405–512)	458 \pm 64 (387–510)	425 \pm 87 (326–487)	
Secchi cm	55 \pm 22 (30–70)	50 \pm 10 (40–60)	53 \pm 28 (30–85)	38 \pm 24 (20–65)	36 \pm 31 (10–70)	103 \pm 21 (80–120)	112 \pm 60 (70–180)	93 \pm 40 (50–130)	57 \pm 31 (30–90)	
Depth cm	195 \pm 25 (170–220)	203 \pm 33 (175–240)	102 \pm 18 (85–120)	116 \pm 44 (72–160)	105 \pm 18 (85–120)	212 \pm 38 (170–242)	221 \pm 32 (185–245)	237 \pm 19 (215–250)	143 \pm 43 (100–185)	
Flow m/s	0.01 \pm 0.02 (0–0.03)	0.16 \pm 0.14 (0.05–0.32)	0.03 \pm 0.03 (0.005–0.06)	0.08 \pm 0.11 (0–0.2)	0.05 \pm 0.07 (0–0.13)	0.14 \pm 0.12 (0.03–0.27)	0.1 \pm 0.12 (0.01–0.24)	0.11 \pm 0.04 (0.07–0.14)	0.08 \pm 0.08 (0.03–0.17)	

system. Following procedures of Alvarez (2010), we estimated the time-weighted concentrations (C_w) of each compound in the water column using the following equation:

$$C_w = \frac{N}{R_s T}$$

where N is the amount of compound accumulated by each POCIS in ng/g, R_s is the sampling rate of each compound by the POCIS, and T is the duration POCIS were deployed in the field (14 days). R_s values were provided from multiple sources (Bartelt-Hunt et al., 2011; Gautam et al., 2014; Godlewska et al., 2020; Li et al., 2010; Metcalfe et al., 2014; Morin et al., 2013; Sultana et al., 2016).

Fish collection

We sampled three species of fish (round goby, bluegill sunfish, and green sunfish) in June, August, and October of 2017 using minnow traps, Windermere traps (baited with corn), and fyke nets (unbaited), all deployed over 24 h. We also conducted two 50-m electrofishing transects (Smith-Root 1.5kVA electrofisher operated from a flat-bottomed aluminum jon boat) at each site. Once captured, adult species of interest (round goby, $n = 90$; green sunfish $n = 153$; and bluegill sunfish, $n = 66$; see Table 2 for size ranges of the fish used in our analyses) were housed temporarily in 81 L marine coolers containing aerated site-specific water separated by species. Fish were used for on-site, high-throughput short term behavioural assays (see below and ESM Appendix S2) and were then transported live in coolers to the laboratory at McMaster University for immediate dissection or for next day thermal tolerance tests (see below) after which they were dissected. All other

non-target species caught were returned to their collection site if they were native or euthanized if they were invasive.

Morphological measurements, tissue collection, and haematocrit

Fish were euthanized by ice-bath followed by cerebral concussion and spinal severance. Body mass and length (standard and total or fork length, depending on the species) were then measured, and blood was collected in heparinized capillary tubes by caudal severance. The blood was spun for 2 min at room temperature in a Readacrit centrifuge (Clay Adams) for haematocrit measurement (% of packed red blood cells in the sample). The mass of the liver was measured, the gonads were removed and weighed, and then eviscerated Fulton's body condition (K) ($\text{body mass (g)} - \text{gonad mass (g)} / \text{standard length (mm)}^3 \times 10^5$), gonadosomatic index (GSI , $\text{gonad mass (g)} / [\text{body mass (g)} - \text{gonad mass (g)}] \times 100$) and hepatosomatic index (HSI , $\text{liver mass (g)} / [\text{body mass (g)} - \text{liver mass (g)}] \times 100$) were calculated. If the gonads were too regressed to measure their mass, they were given a default value of 0.001 g corresponding to the lowest measure possible on our scale. See ESM Table S3 for a summary of fish GSI values.

High-throughput behavioural assays

Our behavioural tests quantified boldness, exploration, predator responses, and activity on-site in the field. Prior to testing, fish underwent a 10-min habituation period in small ($15 \times 15 \times 8$ cm) covered opaque bins filled with site-specific water. The habituation period was to allow fish to recover from capture and handling while also ensuring a high-throughput design. However, this habituation period may have been too short

Table 2

Sample sizes of fish used for morphological and physiological analyses (brackets indicate the number of fish used for CT_{max} analysis). Note that sample sizes differed slightly across analyses because sometimes we were not able to take all measures for all fish.

WWTP	Species	Contaminated sites		Reference sites	
		n	Size range (mm)	n	Size range (mm)
Woodward	Green sunfish	87 (36)	46.5–133.0	24 (12)	47.0–111.5
Dundas	Bluegill sunfish	41 (22)	46.1–173.6	25 (13)	46.7–153.0
	Round goby	48 (16)	50.4–105.6	13 (5)	51.7–109.8

to allow fish to fully recover from capturing and handling stress, and we are therefore limited in our ability to draw conclusions of the effects of wastewater on fish behaviour. See full methodological details and results [ESM Appendix S2; Tables S1 and S2 and Figs. S1, S2 and S3](#).

Critical thermal tolerance (CT_{max})

After behavioural tests in the field (see [ESM Appendix S2](#)), these fish were transported to and housed overnight in the laboratory in marine coolers containing aerated site-specific water, allowing them to acclimate at room temperature ($\sim 21^\circ\text{C}$). These fish were tested the next morning for their CT_{max} . We measured CT_{max} in a subset of randomly selected fish (approximately half of the fish collected at each site) caught in August (27 green sunfish, 12 bluegill sunfish, and 10 round goby) and in October (21 green sunfish, 23 bluegill sunfish, and 11 round goby) to establish how fish from the different sites tolerated increases in temperature. During these tests, fish were held individually in containers ($15 \times 15 \times 8$ cm) filled with continuously aerated, dechlorinated tap water. These containers were placed in a large water bath and water temperature was increased at a constant rate of $0.3^\circ\text{C}/\text{min}$ from an initial temperature of $22.5 \pm 1.6^\circ\text{C}$ until loss of equilibrium (LOE), i.e. when the fish was no longer able to maintain a normal vertical orientation. As soon as LOE was reached, the temperature was recorded (to the nearest 0.5°C) and the fish were removed from their container and placed in a recovery tank with aerated dechlorinated water ($\sim 21^\circ\text{C}$). Although most round goby exhibited a LOE, because they lack a swim bladder and are benthic (i.e., resting on the substrate), we anticipated that some individuals might not flip over or exhibit a stereotypical LOE response when they reached their critical temperature. Therefore, when round goby stopped moving we gently prodded the fish with a glass rod; if the fish remained still with no response to prodding, we considered this temperature to be their CT_{max} (adapted from [Cross and Rawding, 2009](#)). Three fish were prematurely removed from the CT_{max} experiment and excluded from CT_{max} (two bluegill sunfish and one round goby) because during the thermal testing we noticed small body lesions on their dorsal surface.

Ethics and data availability

All research protocols were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Research Ethics Board at McMaster University (AUP 17-12-45). All data files are available in the [ESM, Table S4](#) for fish data and [Table S5](#) for environmental data.

Statistical analyses

We did not catch all three of our target species (bluegill sunfish, green sunfish, round goby) at all sampling sites, so we took two steps in the analysis and reporting of our data. First, because sites downstream from the WWTP outfalls had similar water quality and nutrient loadings (see [Table 1](#)), we combined the data for fish

collected at sites close to each WWTP (Dundas Contaminated 1, 2, and 3; Woodward Contaminated 1, 2, and 3) and combined the data for fish from reference sites that received no flow from each WWTP (Dundas Reference 1 and 2; Woodward Reference 1), and refer to these sites as “Dundas Contaminated”, “Woodward Contaminated”, “Dundas Reference”, and “Woodward Reference” sites, respectively. Second, we only report and compared data between contaminated and reference sites when a species was captured at both. This was the case for green sunfish at the Woodward sites, and for bluegill sunfish and round goby at the Dundas sites. We did catch some green sunfish in Dundas contaminated sites ($n = 41$) and some round goby in Woodward contaminated sites ($n = 29$), but we do not report the data for these particular fish because we lacked comparative data for these species from their respective reference sites.

Statistical analyses and graphs were performed using R version 3.5.3 ([R Core Team, 2019; Wickham et al., 2019](#)) and findings were deemed statistically significant at $\alpha = 0.05$. All error bars on graphs are ± 1 standard error of the mean. We log transformed data when necessary to meet assumptions of normality and homogeneity of variance.

All water quality parameters measured were analyzed using principal component analysis (PCA; note that Secchi depth, total depth, and flow were not included as these were habitat measures). To test for overall differences between contaminated and reference sites at each WWTP, the first two components, responsible for the majority of the variation, were further analyzed using a permutation ANOVA with 5,000 permutations (adonis2, vegan package; [Oksanen et al., 2019](#)). Individual water quality parameters (e.g., pH, dissolved oxygen) were further analyzed using a permutation MANOVA with 5,000 iterations (lmPerm; [Wheeler and Torchiano, 2016](#)).

We used linear models to test for effects of exposure (reference vs. contaminated) on all endpoints measured on the fish, unless otherwise specified. Site water temperature was included as a covariate in our CT_{max} analysis, to account for the effects of acclimation temperature on this trait. In all analyses, each WWTP and species was analyzed separately, with the exception of CT_{max} , where we explored species-specific differences in thermal tolerance using a Tukey's HSD post-hoc test. Body mass was used as a covariate for haematocrit analyses. Because haematocrit for both sunfish species was significantly higher in fish that had undergone the CT_{max} test compared to the ones that did not, haematocrit was analyzed and reported separately for those two groups. We compared body condition using an analysis of covariance on the eviscerated measure of body mass against standard length (this method has been proposed by [Jakob et al., 1996](#) as a more reliable index of body condition). We investigated the relationship between CT_{max} and body condition (residuals of eviscerated body mass against standard length) using Pearson's R correlations. For all body condition graphs, we have plotted body condition as eviscerated Fulton's condition factor (body mass (g) – gonad mass (g)) / standard length (mm)³ $\times 10^5$) for ease of interpretation and comparison with other studies (see [Fig. 3b; Fig. 5a](#)). Non-significant interactions in initial models were removed from final

models that are reported here. Sample sizes and sizes of fish used in morphology and CT_{max} analyses are shown in Table 2.

Results

Water and habitat quality, nutrient and contaminant analyses

At both WWTPs, water quality parameters differed significantly between contaminated and reference sites (Permutation ANOVA, $F_{(1, 13)}$ Dundas = 7.39, $p < 0.001$; $F_{(1, 10)}$ Woodward = 7.00, $p < 0.001$; Fig. 2, Table 1). At the Dundas WWTP, contaminated sites had higher conductivity (MANOVA, $p < 0.001$), TDS ($p < 0.001$), salinity ($p < 0.001$), TNN ($p < 0.001$), and TN ($p < 0.01$). At the Woodward WWTP, contaminated sites were significantly higher in TAN ($p = 0.02$), TNN ($p < 0.01$), TN ($p < 0.01$), SRP ($p = 0.02$), and TP ($p < 0.01$) and had lower pH ($p < 0.01$). Concentrations of 14 anthropogenic contaminants at each site from the POCIS samplers are summarized in Table 3, and show that wastewater-contaminated sites have higher concentrations of these pharmaceuticals, food-additives, and personal care products than their respective reference sites.

Somatic and organ/tissue traits

Fish from the more contaminated sites were heavier than fish from reference sites (Fig. 3A; green sunfish, $t_{108} = 4.38$, $p < 0.001$; bluegill sunfish, $t_{64} = 2.69$, $p = 0.009$; round goby, $t_{58} = 2.52$, $p = 0.015$). On average, green sunfish were 58% heavier, bluegill sunfish were 49% heavier, and round goby were 58% heavier at wastewater-contaminated sites. Green sunfish and round goby from contaminated areas also had higher body condition than those from the respective reference sites (Fig. 3B; green sunfish, $t_{108} = 2.49$, $p = 0.01$; round goby, $t_{58} = 2.02$, $p = 0.048$), but there was no difference in body condition in bluegill sunfish from the contaminated sites compared to the reference sites ($t_{63} = 1.70$, $p = 0.09$). Both bluegill sunfish and round goby from the contaminated sites near the Dundas WWTP had larger livers relative to their body mass (HSI; Fig. 3C; bluegill sunfish, $t_{64} = 2.47$, $p = 0.016$; round goby, $t_{58} = 2.04$, $p = 0.046$); however, green sunfish did not show this pattern ($t_{108} = 1.11$, $p = 0.27$). There was a visual

trend for haematocrit to be higher in individuals at more contaminated sites (Fig. 3D). For fish dissected immediately, this was only statistically significant in bluegill sunfish (Fig. 3D; green sunfish, $t_{53} = 0.49$, $p = 0.62$; bluegill sunfish, $t_{22} = 2.11$, $p = 0.047$; round goby, $t_{30} = 1.32$, $p = 0.20$). For fish that underwent a CT_{max} test, the difference was only statistically significant among green sunfish (green sunfish, $t_{41} = 2.75$, $p = 0.009$; bluegill sunfish, $t_{27} = 0.65$, $p = 0.52$; round goby, $t_{13} = 0.49$, $p = 0.63$).

Thermal tolerance (CT_{max})

CT_{max} did not vary between fish collected from the contaminated and reference sites in any of the three species (Fig. 4; green sunfish, $t_{44} = 0.81$, $p = 0.42$; bluegill sunfish, $t_{31} = 1.34$, $p = 0.19$; round goby, $t_{17} = 1.22$, $p = 0.24$). Green sunfish and bluegill sunfish both had higher CT_{max} values when water temperature at the collection sites was warmer (green sunfish, $t_{44} = 2.90$, $p = 0.006$; bluegill sunfish, $t_{31} = 3.80$, $p = 0.0006$); this was not the case for round goby ($t_{17} = 1.64$, $p = 0.12$). We found distinct differences between species in CT_{max} (Fig. 4) regardless of the water temperature differences across sampling periods: green sunfish had the highest CT_{max} (37.5 ± 0.13 °C), followed by bluegill sunfish (35.7 ± 0.22 °C), and then round goby had the lowest CT_{max} (34.2 ± 0.21 °C; all Tukey's HSD post-hoc pairwise t-tests between species were significant; $p < 0.0001$). CT_{max} was not correlated with body condition in any of the three species studied (Fig. 5; green sunfish $r = -0.03$, $p = 0.82$, bluegill sunfish $r = 0.18$, $p = 0.30$, round goby $r = 0.10$, $p = 0.67$).

Behavioural differences

There were no behavioural differences detected in fish collected across the gradient of contaminant exposure; at both WWTPs fish from contaminated sites were equally active and bold in response to a simulated predation attack compared to fish from reference sites. However, the relatively short habituation time used to facilitate a high-throughput approach may not have been long enough to ensure recovery from handling and normal behaviour. Therefore, it is difficult to draw clear conclusions about the effects (or lack thereof) of wastewater on fish behaviour in this study. See

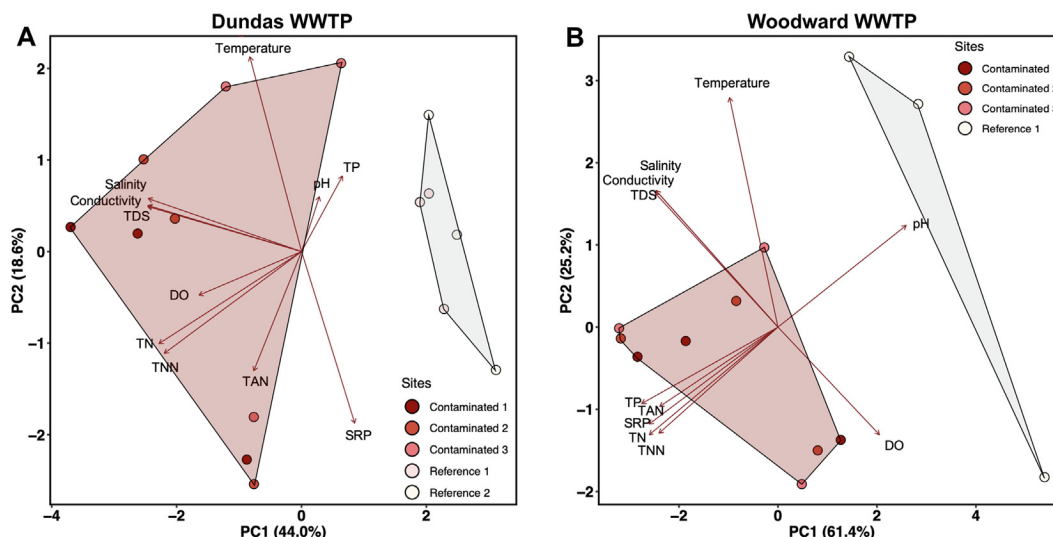


Fig. 2. Principal component analysis (PCA) biplot of water quality parameters measured in the contaminated and reference sites, site groupings (i.e., “contaminated” or “reference”) delineated in red and grey polygons, respectively, at the A) Dundas Wastewater Treatment Plant (WWTP) and B) Woodward WWTP. Data points represent each site-specific sampling events ($n = 3$ per site). Arrows represent the weight and direction of the loadings in two-dimensional space. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Average (\pm standard deviation) time-weighted concentrations of anthropogenic contaminants measured to characterize wastewater pollution at each site via polar organic chemical integrative samplers (POCIS) deployed for two weeks at each site in August 2017 (all ng/L). See Methods, Section 2.5 for time-weighting procedure. ND = not detected. P = present but below quantification limit.

	Dundas WWTP					Woodward WWTP			
	Contaminated 1	Contaminated 2	Contaminated 3	Reference 1	Reference 2	Contaminated 1	Contaminated 2	Contaminated 3	Reference 1
Antibiotics									
Trimethoprim	99.5 \pm 6.7	121.8 \pm 25.5	88.1 \pm 9.2	15.8 \pm 2.2	30.7 \pm 3.2	86.9 \pm 24.1	101.2 \pm 19.1	119.1 \pm 6.0	8.2 \pm 1.0
Sulfamethoxazole	1.6 \pm 0.1	1.5 \pm 0.4	0.6 \pm 0.2	0.4 \pm 0.0	0.8 \pm 0.2	46.5 \pm 18.6	56.1 \pm 12.0	59.2 \pm 4.1	1.4 \pm 0.1
Pharmaceuticals									
Gemfibrozil	ND	ND	ND	ND	ND	P	P	P	ND
Naproxen	ND	ND	ND	ND	ND	0.5 \pm 0.1	0.5 \pm 0.1	0.5 \pm 0.1	0.2 \pm 0.0
Carbamazepine	5.7 \pm 0.3	1.1 \pm 0.1	3.7 \pm 1.2	0.6 \pm 0.1	1.2 \pm 0.1	3.0 \pm 2.2	5.0 \pm 1.9	5.4 \pm 0.2	1.9 \pm 0.4
Ibuprofen	ND	ND	ND	ND	ND	P	P	1.6 \pm 0.3	ND
Acetaminophen	ND	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine									
Caffeine	890.8 \pm 107.1	201.9 \pm 35.2	167.8 \pm 16.4	319.7 \pm 35.9	315.3 \pm 21.2	859.0 \pm 246.1	1162.8 \pm 165.2	1202.3 \pm 48.1	583.1 \pm 44.4
Artificial Sweeteners									
Sucralose	119.3 \pm 9.5	115.4 \pm 18.6	71.6 \pm 4.7	91.6 \pm 14.4	68.2 \pm 5.5	138.6 \pm 6.5	146.0 \pm 6.5	323.4 \pm 7.4	62.3 \pm 10.8
Acesulfame	0.9 \pm 0.1	P	P	P	P	1.8 \pm 0.3	1.4 \pm 0.2	1.9 \pm 0.2	P
Steroids									
Androstenedione	P	P	P	P	P	0.2 \pm 0.0	0.4 \pm 0.1	0.3 \pm 0.0	P
Estrone	ND	ND	ND	ND	ND	ND	P	P	ND
Antibacterials									
Triclosan	0.0 \pm 0.0*	ND	ND	ND	ND	0.6 \pm 0.1	0.4 \pm 0.5	0.4 \pm 0.1	0.1 \pm 0.0
Triclocarban	ND	ND	ND	ND	ND	ND	ND	ND	ND

* Triclosan was detected above the limit of quantification on the POCIS disks; however, after the time-weighting correction, the value was 0.

ESM Appendix S2 for further results, discussion, and recommendations.

Discussion

We evaluated how three common fish species in Laurentian Great Lakes watersheds are impacted by living in environments contaminated by wastewater effluent, focusing on somatic investment, haematocrit and critical thermal tolerance. Our water quality findings show clear water quality differences between contaminated and reference sites with nitrogenous compounds being consistently higher at the contaminated sites at both the Dundas and Woodward WWTPs. Moreover, the POCIS samplers revealed higher concentrations of numerous anthropogenic contaminants commonly discharged in wastewater effluent (e.g., pharmaceuticals, food-additives, personal care products; see Table 3) downstream from both WWTPs compared to the reference sites. Our results confirm previous studies that have quantified similar patterns of nutrients and other anthropogenic contaminants in the discharges from these two WWTPs (Csiszar et al., 2011; McCallum et al., 2017a; Mehdi et al., 2021; Metcalfe et al., 2003; Muir et al., 2017). Such contaminants, especially nitrogenous compounds, have long been linked to adverse effects in aquatic organisms, suggesting that wastewater inputs can significantly impair the health of organisms living nearby (Holeton et al., 2011; Ip and Chew, 2010; Randall and Tsui, 2002).

Considering first how wastewater affected somatic investment, we found that all three fish species (bluegill sunfish, green sunfish, and round goby) were significantly larger by mass when collected from wastewater-contaminated sites than from reference sites. Green sunfish and round goby also had higher body condition at contaminated sites, and round goby and bluegill sunfish had greater liver investment at contaminated sites. These findings agree with our previous findings in bluegill sunfish (Du et al., 2019) and support the general trend that exposure to wastewater

effluent can often increase somatic investment (Melvin, 2016; Porter and Janz, 2003; Pottinger et al., 2013; Reinling et al., 2017; Tetreault et al., 2011). There are several potential reasons for these findings. First, fish living near the WWTPs were exposed to higher nutrient inputs (nitrogen, total phosphorus) which may also increase food availability, hence the higher body mass and body condition observed in these fish. Furthermore, sites closer to the effluent are composed of cobble, a substrate that may retain higher food availability (increased heterogeneity and surface area) compared to the softer sediment observed in the reference sites (e.g., Duan et al., 2008). The higher liver investment observed may also be caused in part by nutrient availability because the liver acts as a storage organ for glycogen and other metabolic fuels (Du et al., 2019; Polakof et al., 2012; Tetreault et al., 2011). Second, larger (possibly older) fish might be more competitive in obtaining resources and territories, outcompeting and displacing smaller individuals at these nutrient-rich sites (Ward et al., 2006). Alternatively, the population size at wastewater-contaminated sites may be small, and therefore reduced resource competition could result in more resources per individual fish and therefore to larger fish. However, we think this last suggestion is unlikely as recent fish community monitoring at these two WWTPs by our team revealed that fish abundance was higher at sites closer to the outfall (McCallum et al., 2019; Mehdi et al., 2021). Finally, another explanation for the increase in liver size is that fish may selectively invest in livers around WWTPs, due to this organ's important role in detoxification (Chambers and Yarbrough, 1976). Regardless of the mechanism causing increased body size and tissue investment, the effects of WWTP effluent on body mass and investment is at odds with expectations from the potential metabolic costs of exposure (Du et al., 2018; Mehdi et al., 2018). Increases in metabolic demands would be expected to lead to allocation trade-offs that might reduce investment in organ and tissue growth. This clearly was not the case for fish in this study, suggesting that increases in food and nutrient availability prevent growth constraints resulting from increased metabolic rate.

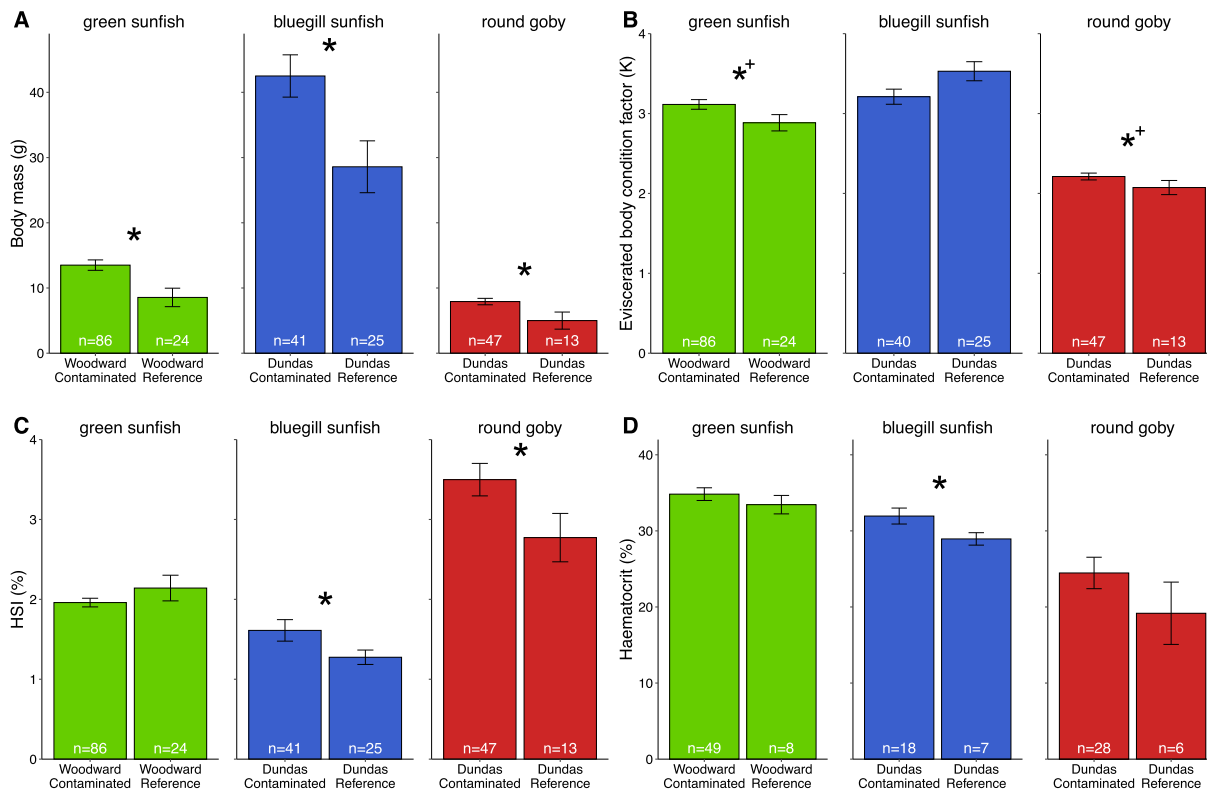


Fig. 3. Somatic measures on fish exposed to wastewater effluent. A) Body mass (g). B) Eviscerated Fulton's K body condition factor (body mass (g) – gonad mass (g)/standard length (mm)³ × 10⁵). *symbol indicates that the statistical analysis was conducted using an ANCOVA of eviscerated body mass against standard length (see *Statistical analyses* section). C) Hepatosomatic index (HSI), liver mass relative to body mass. D) Haematocrit (% of blood volume composed of red blood cells) for the fish that were dissected immediately after field collection. Error bars represent ± 1 SEM. Asterisks indicate statistical differences ($p < 0.05$) between contaminated and reference sites within each species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

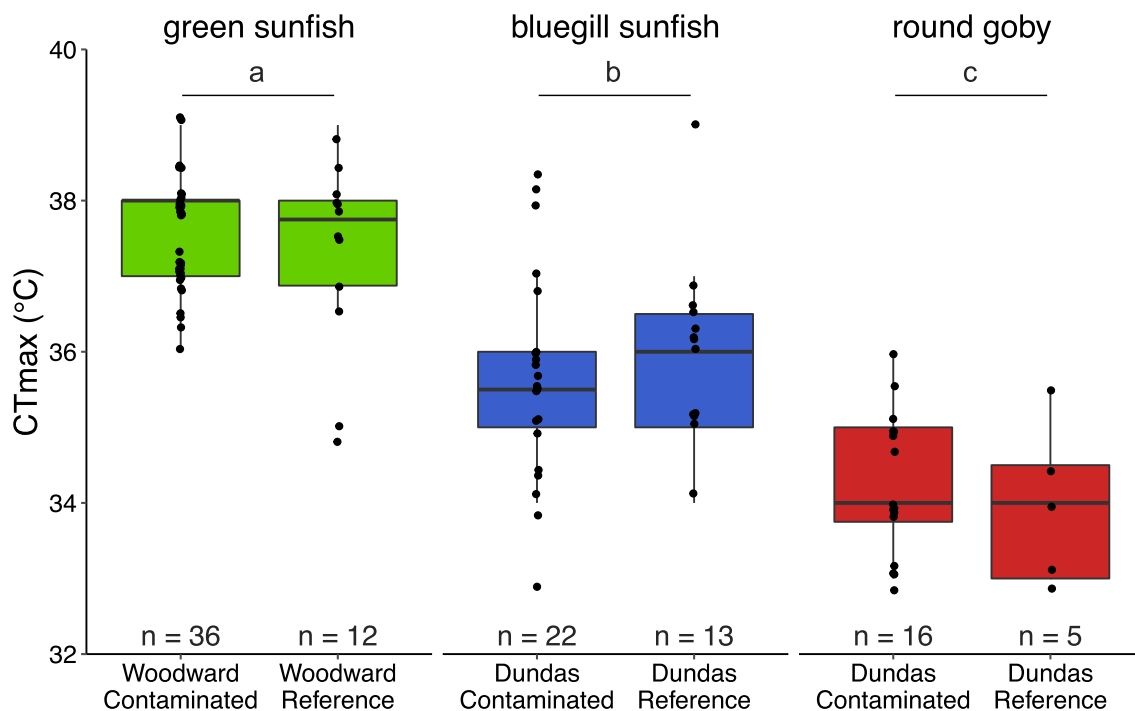


Fig. 4. Critical thermal tolerance (CT_{max}) of green sunfish, bluegill sunfish, and round goby collected from sites contaminated with wastewater effluent or from reference sites. Boxplots show the median and interquartile range. Whiskers extend to the farthest data point within 1.5 × interquartile range. Dissimilar letters indicate statistical differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

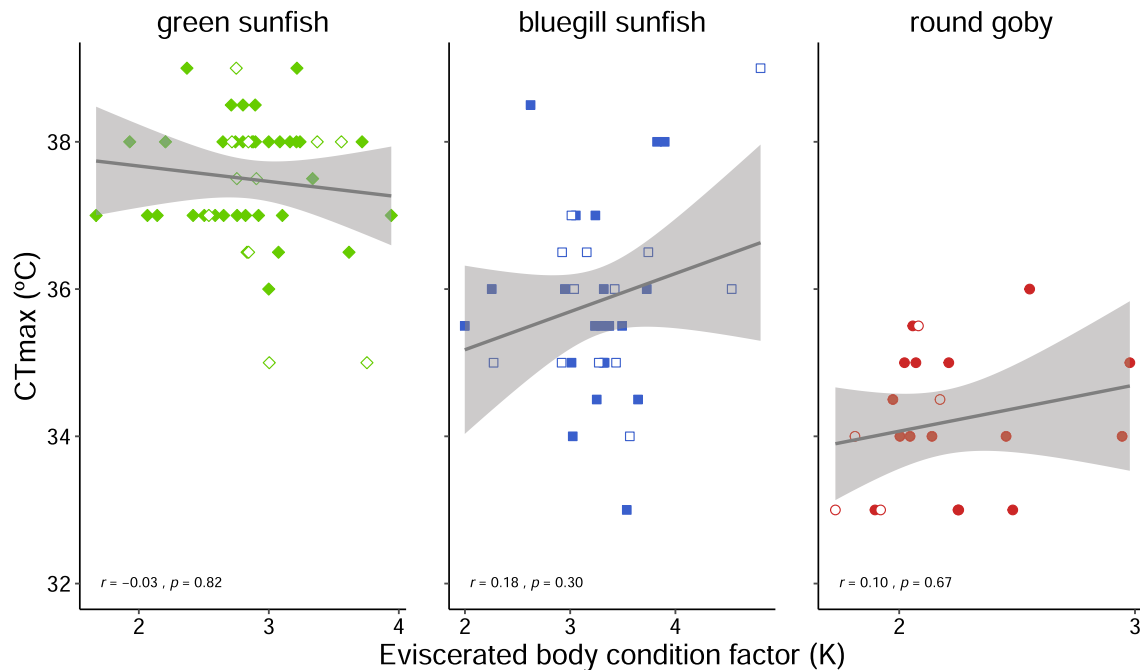


Fig. 5. Correlations between critical thermal tolerance (CT_{max}) and eviscerated Fulton's body condition factor for all fish studied (r - and p -values for body condition were conducted using the residuals of eviscerated body mass against standard length, see *Statistical analyses* section). Open shapes = fish from reference sites, filled shapes = fish from contaminated sites.

Haematocrit was higher in green sunfish downstream of the Woodward WWTP and in bluegill sunfish downstream of the Dundas WWTP. Haematocrit, which reflects the percentage of red blood cells in a volume of blood, was also elevated after several weeks of exposure to effluent from the Dundas WWTP in a recent *in situ* caging study using bluegill sunfish in 2015 (Du et al., 2018), but not in bluegill sunfish in 2016 (Du et al., 2019) or in round goby in 2015 (McCallum et al., 2017a). Furthermore, in the former study, the observed increase in haematocrit was not associated with an increase in blood haemoglobin content, suggesting that erythrocyte swelling was the likely cause of the elevated haematocrit and that the changes in haematocrit did not serve to increase the oxygen carrying capacity of the blood. The variation in haematocrit noted above is mirrored by findings from other studies that also suggest that changes in haematocrit in response to wastewater effluent exposure can be variable, showing no change (Kakuta and Murachi, 1997; Landman et al., 2006; Vajda et al., 2008), decreases (Cazenave et al., 2014; Hemming et al., 2002, 2001), or an inconsistent response over time (Grizzle et al., 1988). More research is clearly needed to fully understand the reasons and mechanisms for changes in haematocrit in certain fish species living near WWTPs.

Fish living downstream of WWTPs in our study had a similar critical thermal tolerance (CT_{max}) to fish living at reference sites, and there was no clear correlative relationship between CT_{max} and body condition. Initially, we proposed two contrasting predictions for how living in wastewater-contaminated environments might affect the ability of fish to withstand acute heat stress. The general stress and health impacts of exposure could result in a reduction in CT_{max} , or increases in body condition and nutrient/food access for effluent exposed fish might contribute to greater tolerance of higher temperatures and higher CT_{max} values (which should also result in a positive correlation between CT_{max} and body condition). However, our findings did not provide support for either prediction. Although we did find evidence of higher body

mass, condition, and/or liver investment at wastewater-exposed sites, these measures were not clearly related to CT_{max} values. Jayasundara et al. (2017) similarly found that fish collected at sites contaminated with polyaromatic hydrocarbons (PAHs) showed no obvious changes in their CT_{max} values when compared to fish from the reference site. Despite no change in CT_{max} , these researchers also found that fish in contaminated sites were less able to tolerate acute exposure to a static, high thermal stressor (i.e., fish showed a shorter time until loss of equilibrium at 36 °C). Therefore, it is possible that other aspects of thermal tolerance beyond the scope of our study may be more sensitive to pollutant or wastewater stressors (e.g., thermal safety margins, thermal performance curves, heat shock proteins) would be valuable to investigate in the future. Alternatively, it is possible that fish living in wastewater-polluted sites may have acclimatised or adapted to local conditions, counteracting the potential effects of wastewater exposure such that there were no measured differences. Numerous prior studies have documented the effects of single or even mixtures of contaminants on CT_{max} (Carrier and Beiting, 1988; LeBlanc et al., 2011; Op de Beeck et al., 2017; Patra et al., 2007, reviewed in Beiting, 1990), but to the best of our knowledge, this is the first study to determine if wastewater effluent affects CT_{max} . Studying how thermal tolerance is impacted by external stressors, such as contaminant exposure, still warrants further investigation, because thermal tolerance has long been thought of as a proxy of physiological capacity and whole-organism performance (Nguyen et al., 2017; Speers-Roesch and Norrin, 2016). More broadly, water temperatures well below an organism's CT_{max} are still important for fitness in ectotherms such as fish, whose body temperature, metabolic rate, and growth rate reflects that of the surrounding environment.

While our work did not uncover site differences in CT_{max} associated with wastewater exposure, we did observe significant differences between species, and previous studies with these species reported similar values of CT_{max} from uncontaminated areas [33.4 °C for round goby (Cross and Rawding, 2009); ~36–38 °C in

bluegill and green sunfish, depending on acclimation temperature (Beitinger et al., 2000)]. Bluegill sunfish and green sunfish are commonly found in shallow environments (e.g., wetlands) that are typically warmer than the sites inhabited by round goby (Scott and Crossman, 1998). This may explain why round goby had the lowest CT_{max} of the three species we tested, despite the round goby's reported ability to tolerate diverse environmental conditions and their invasion success through a wide range of habitats (Kornis et al., 2012).

Our study was conducted with wild-collected fish. It is always possible that the fish caught near WWTPs moved among sites and altered their level of exposure. While the fish may be sedentary during specific seasons (e.g., breeding; Ray and Corkum, 2001; Gatz, 2007; Midwood and Chow-Fraser, 2015), they may also exhibit seasonal movements between offshore and inshore regions (Blair et al., 2019; Suski and Ridgway, 2009). Future fish tracking studies (e.g., telemetry) would be beneficial to ascertain the extent of seasonal fish movement across gradients of effluent exposure (e.g., Hellström et al., 2016). Although studies of wild organisms are useful to understand the real-world implications of wastewater exposure to animals living nearby, controlled exposures (e.g., caging studies) are required to disentangle the influence of wastewater from other factors such as adaptations to local conditions or dispersal (Du et al., 2018; McCallum et al., 2017a; Oikari, 2006; Palace et al., 2005).

We selected endpoints for this study that would be general indicators of body condition and thermal tolerance but that could also be easily measured in multiple species in largescale sampling or environmental monitoring program (Dale and Beyeler, 2001; Kilgour et al., 2005). These endpoints can be measured non-lethally on-site (body mass, body condition) or quickly collected and measured in the laboratory with minimal specialized equipment (liver size, haematocrit, thermal tolerance). The results of our pilot high-throughput behavioural assays were inconclusive (see [ESM Appendix S2](#)). While these on-site behavioural endpoints would have been advantageous as measures of whole-organism performance, behaviour is likely too sensitive and/or variable for quick, on-site measurements without longer habituation or acclimation periods.

Overall, we found that fish species collected from wastewater-contaminated environments had higher somatic investment as reflected by higher body mass, body condition, liver investment, and/or haematocrit, but we found no evidence that wastewater exposure affected thermal tolerance. In this study, we tested three commonly encountered species. If there were indeed disruptive effects of WWTP effluent exposure on the underlying physiological determinants of critical thermal maxima, then our results suggest that these three fish species may have some capacity to acclimatise and/or locally adapt and thus overcome these effects in the wild. Indeed, round goby and green sunfish were often abundant in fish community samplings at effluent-affected sites (McCallum et al., 2019; Mehdi et al., 2021). We must also consider that less common, rare, and/or intolerant species may be more sensitive to such exposures. Our current study focused on one year of sampling, but multi-year studies are always preferable for understanding the responses of wildlife to dynamic wastewater effluents and how exposure interacts with seasonal life-history events (e.g., reproduction, parental care; Fuzzen et al., 2016; Muir et al., 2017). Nonetheless, when the present results are also combined with our previous studies at these sites (Du et al., 2019; Du et al., 2018; McCallum et al., 2019, 2017a; McLean et al., 2019; Mehdi et al., 2021), we begin to establish a clearer picture of how wastewater effluent affects some of the most common fish species across scales of biological organization in the Hamilton Harbour Area of Concern (Lake Ontario). Namely, that bluegill sunfish appear to be more sensitive to wastewater exposure when compared to

round goby, and that wild-caught fish are more tolerant to exposure than naïve, caged fish. Wastewater discharge remains one of the largest point sources of pollution to aquatic ecosystems worldwide and will continue to impact Laurentian Great Lakes watersheds (Holeton et al., 2011). Knowledge of the many ways in which anthropogenic wastewater inputs can alter receiving environments and fish habitats will help to inform wastewater treatment upgrades, local remediation plans, and to more broadly better manage and protect these ecosystems.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jglr.2021.01.017>.

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