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Interactive effects of elevated temperature and venlafaxine on mitochondrial respiration and enzymatic capacity in Nile tilapia (*Oreochromis niloticus*)

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Abstract

Warming events are becoming more frequent and extreme in aquatic environments worldwide. Concurrently, many environments are polluted with biologically active compounds such as pharmaceuticals. Understanding how these challenges interact is critical for understanding the climate crisis, as contaminants may modulate how ectotherms respond to heat stress or vice versa. One potential site for these heat × contaminant interactions is the mitochondrion, which is central to metabolism, implicated in thermal tolerance, and evolutionarily conserved. Using high-resolution respirometry, we investigated how acute warming (to 35 °C, 40 °C, or 45 °C from 25 °C) impacted the respiration, coupling, and metabolic capacity of liver mitochondria isolated from Nile tilapia, and how exposure to environmentally relevant levels of the ubiquitous antidepressant venlafaxine modulated those effects. Mitochondria exposed to hotter temperatures had higher respiration rates and decreased respiratory control ratio compared to mitochondria exposed to cooler temperatures. The depressive effects of venlafaxine on respiration rates through complex I and II or complex II only (State 3 and State 4), as well as complex IV-linked respiration, were mild except in mitochondria exposed to high temperatures, suggesting an interactive effect of warming and contaminant exposure. Finally, we found that the maximal enzyme activity of intact mitochondria (represented by mitochondrial respiration) showed a different pattern of response to warming and venlafaxine compared to its underlying components (as reflected by the activity of succinate dehydrogenase [complex II] and cytochrome c oxidase [complex IV]), demonstrating the value of incorporating both interactive and reductive approaches in understanding how mitochondria cope with anthropogenic changes in the environment.

Keywords: high-resolution respirometry, metabolism, thermal tolerance

Introduction

Aquatic organisms must contend with several challenges to cope with ongoing anthropogenic changes in the environment, including more intense and frequent heatwaves (Laufkötter et al., 2020; Oliver et al., 2018) and increasing exposure to pharmaceuticals and personal care products associated with human communities (Madikizela et al., 2017; Metcalfe et al., 2010). Simultaneous exposure to elevated temperatures and contaminants is not uncommon in the wild (Kinouchi et al., 2007; Mehdi et al., 2022), and it is foreseeable that warming temperatures could modulate how animals respond to pollution or vice versa (Noyes et al., 2009) by impacting the function of various biochemical and physiological processes. Despite their clear relevance to the contemporary realities of the global ecosystem, investigations specifically focused on the interactive effects of multiple stressors such as contaminants and warming are relatively uncommon, leaving a considerable knowledge gap.

One area of interest for understanding these interactions is the mitochondrion, as given the highly conserved nature of drug targets and physiological pathways across vertebrates (Mehdi et al., 2019) as well the potential role of the mitochondria in thermal limits of ectotherms (Chung & Schulte, 2020), it is likely that alterations in mitochondrial function may occur in fish that encounter pharmaceuticals or their metabolites from treated wastewater, agricultural runoff, or other sources. Oxidative phosphorylation, the central reactions of aerobic metabolism, is catalyzed by multi-subunit enzymes ("complexes") situated along the mitochondrial respiratory chain. Briefly, mitochondrial electron transport complexes I, III, and IV use electrons harvested from reducing equivalents such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) to generate a proton gradient across the inner mitochondrial membrane, which powers adenosine triphosphate (ATP) synthesis by complex V (Blier et al., 2014; Brand & Nicholls, 2011; Dreier et al., 2019; Nolfi-Donegan et al., 2020; Zhao et al., 2019). More specifically, complex I (NADH: ubiquinone oxidoreductase) takes electrons directly from the NADH generated by the tricarboxylic acid (TCA) cycle to pump protons into the inner membrane space. Complex II (succinate dehydrogenase) is a member of both the TCA cycle and the electron transport system, but fulfills a similar function as

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complex I by oxidizing succinate and ultimately accepting electrons from $FADH_2$. Electrons originating from these reactions converge at the Q-pool before passing to complex III, which also contributes to the proton gradient, before transferring electrons to cytochrome c. Electrons are passed from cytochrome c to the final proton pump, complex IV (cytochrome c oxidase [COX]), before reacting with O_2, the terminal electron acceptor of the system (Blier et al., 2014; Chung & Schulte, 2020; Nolfi-Donegan et al., 2020; Zhao et al., 2019).

The role of the mitochondria in setting the thermal limits of ectotherms is unresolved. In ectotherms, upper thermal tolerance (as reflected by the critical thermal maximum [CT_{max}]) has clear positive relationships with both State 3 (oxidative phosphorylation [OXPHOS]) respiration and mitochondrial coupling (respiratory control ratio [RCR]), two key indicators of electron transport system functionality (Chung & Schulte, 2020). However, mitochondria in vitro often outperform whole-animal estimates of thermal tolerance by maintaining normal function near and beyond CT_{max} (Pörtner, 2002; Somero, 2002), suggesting that the respiratory capacity of the electron transfer system may not be a rate-limiting step in whole-animal thermal tolerance. Several other mitochondrial processes are implicated as possible contributors to organismal thermal limits, including altered reactive oxygen species (ROS) homeostasis (Banh et al., 2016; Christen et al., 2018), loss of membrane integrity and/or coupling (Iftikar & Hickey, 2013), increased membrane fluidity (Dahlhoff & Somero, 1993; O'Brien et al., 1991), loss of ATP synthetic capacity (Harada et al., 2019; Healy & Burton, 2023; Iftikar & Hickey, 2013; Melzner et al., 2006), and declines in the enzymatic capacity of mitochondrial complexes or TCA cycle enzymes (Blier et al., 2014; Ekström et al., 2017; Jørgensen & Mustafa, 1980; Léger et al., 2024; Lemieux et al., 2010). However, the overall evidence for each of these processes is fragmentary (Chung & Schulte, 2020) and, in some cases, counterbalanced by other work suggesting little to no link between them and thermal tolerance (Chung & Schulte, 2015).

As complex organelles integrated into various metabolic pathways (e.g., the urea cycle, heme and fatty acid synthesis, aerobic metabolism), mitochondria have multiple sites of potential vulnerability to environmental toxicants (Meyer et al., 2013) and have been shown to be sensitive to a wide range of compounds (Burbenskaya et al., 1998; Dreier et al., 2019; Meyer et al., 2013; Reddam et al., 2022; Roubicek & Souza-Pinto, 2017; Souza et al., 1994; Wallace & Starkov, 2000). The potential for xenobiotics to negatively impact mitochondrial function has been most extensively investigated in the context of the biomedical field, as several mitochondrial toxicants (e.g., adriamycin, nucleoside reverse transcriptase inhibitors, the antidepressants fluoxetine and imipramine; Burbenskaya et al., 1998; Meyer et al., 2013; Souza et al., 1994; Wallace & Starkov, 2000) have important therapeutic effects. Due to inefficiencies in drug metabolism, many of these therapeutic compounds with known mitochondrial effects are introduced into aquatic ecosystems, where they have the potential to exert their effects on non-target organisms such as fish (Meyer et al., 2013).

One common contaminant in aquatic ecosystems is venlafaxine, a selective serotonin-norepinephrine reuptake inhibitor (SNRI). Concentrations of venlafaxine in the environment can meet or even exceed concentrations of 1 μg/L in certain locations, especially downstream of wastewater treatment plants (Chen et al., 2022; Khan et al., 2020; Mehdi et al., 2021; Metcalfe et al., 2010; Mheidli et al., 2022; Weng et al., 2023) as it is typically not entirely removed by contemporary treatment procedures. Venlafaxine has been demonstrated to have a variety of developmental, physiological, and behavioral effects on fishes, including modulating mitochondrial respiration and the abundance of mitochondrial microRNAs in crude brain homogenates (Robichaud et al., 2024), the expression of handling stress-induced genes and gill enzymatic activities (Best et al., 2014), development of the nervous and endocrine systems (Thompson & Vijayan, 2022; Thompson et al., 2017), and possibly alterations in the heat shock (Weber et al., 2023) and oxidative stress responses (Mehdi et al., 2019), among other things (Gould et al., 2021; Salahinejad et al., 2022). The specific effects of venlafaxine on mitochondrial function are less understood than the effects of older antidepressants such as fluoxetine (de Oliveira, 2016; Emmerzaal et al., 2021; Souza et al., 1994), but there is some evidence that venlafaxine negatively impacts the electron transport system (Gardea-Resendez et al., 2023). As little as a 30-min incubation with 500 μmol/L venlafaxine leads to decreases in the enzymatic activities of complex II and IV in mitochondria isolated from pig brains (Hroudova & Fisar, 2010). Contrastingly, chronic venlafaxine administration (10 mg/kg for 15 days), which is more representative of clinical usage, leads to increased complex II and IV enzymatic activity in the prefrontal cortex (Scaini et al., 2010, 2011), as well as increased complex II enzymatic activity in hippocampus and striatum of male rats (Scaini et al., 2010). All of the above effects occurred without a significant loss of in the activity of citrate synthase (CS), a classic marker of mitochondrial content (Hroudova & Fisar, 2010; Scaini et al., 2010, 2011), implying that venlafaxine affects complex activity directly as opposed to mitochondrial abundance. It is therefore unclear if venlafaxine administration enhances or inhibits the enzymatic activity of the electron transport system, and therefore how it may interact with other stressors known to alter metabolism in ectotherms, such as warming.

In ectotherms, the general expectation is that contaminant handling and detoxification processes will be impacted by thermal environment, such that higher temperatures will enhance the toxicity of most contaminants (Noyes et al., 2009) due to increased metabolic rate and/or altered metabolite profiles. For example, juvenile meagre (*Argyrosomus regius*) exposed to venlafaxine and warming show enhanced venlafaxine uptake by the brain as well as both decreased uptake and impaired elimination in the liver, emphasizing not only that warming alters the compound's pharmacokinetics, but that those alterations are tissue-specific (Maulvault et al., 2018). In addition, exposure to contaminants may limit capacity of species or populations to acclimate to increased temperatures (Noyes et al., 2009), including through the modulation of metabolic processes, which may be especially damaging for animals living near their thermal limits. For example, venlafaxine exposure alters the cortisol response (Best et al., 2014) and epinephrine-stimulated glucose production in rainbow trout (Ings et al., 2012), both of which are representative of alterations in metabolic capacity that may impair the ability to cope with acute stressors such as predation or warming.

Tilapia are the second-most important farmed finfish species group: world production was in excess of 6 million tons in 2018 (Miao & Wang, 2020) and has increased year-over-year since the 1990s. Understanding how tilapia cope with high temperatures and pollution, both emerging issues of concern for global food production, is therefore of great interest to the global aquaculture community. As an abundant tropical fish generally regarded to be tolerant of pollution (Linde et al., 2008; Linde-Arias et al., 2008) and warm temperatures (Leonard & Skov, 2022; Panda et al., 2022), tilapia are an accessible model for studies on the

interactive effects of warming and contaminants. We hypothesize that mitochondria are an important site for heat × contaminant interactions in ectotherms due to their dual roles in metabolism and thermal tolerance as well as their close similarities across taxa (i.e., as mitochondria are evolutionarily conserved, compounds "designed" to affect human physiology are likely to affect other animals). In this context, our objectives took a mechanistic approach to (1) investigate the effect of acute temperature increases on the respiration, coupling, and maximal enzymatic capacity of mitochondria isolated from the livers of adult Nile tilapia (*Oreochromis niloticus*) and (2) characterize how exposure to a ubiquitous contaminant, the antidepressant venlafaxine (brand name Effexor), modulates these effects. We predicted that acute venlafaxine exposure would impair mitochondrial function (e.g., reduced respiration rates, RCR, and/or enzymatic activities), and that these effects would be most apparent at higher temperatures, as mitochondrial metabolic reactions at these temperatures would be approaching their maximal capacity and therefore be more sensitive to any perturbations in the underlying metabolic processes that contribute to that capacity.

Methods

Study animals

Adult male Nile tilapia (*Oreochromis niloticus*; wet mass: 1130.8 ± 68.8 g, total length 36.9 ± 0.1 cm, standard length of 30.8 ± 0.7 cm, $n = 20$ total in this study) were obtained from a commercial supplier (Sand Plains Aquaculture, ON) and maintained at the University of Waterloo's WATER Facility. The animals were divided across two 680 L fiberglass tanks that were continuously aerated and supplied with dechlorinated City of Waterloo water in a dedicated Recirculation Aquatic System that had daily 10% water exchanges. Water temperature (25.86 ± 0.13 °C), conductivity (407.84 ± 3.64 μS), and pH (7.78 ± 0.03 units) were continuously monitored by dedicated systems associated with each tank (ProMinent; Heidelberg, Germany). Tilapia were maintained on a 12:12-hr light: dark cycle and fed to satiation daily with commercially available fish food (Bluewater 42-14 (P) 5 mm floating fish food). Large black plastic tubes at the bottom of the tanks provided additional shelter and enrichment to the animals. Adult tilapia were not exposed to venlafaxine (nor any other contaminants) while being maintained in the WATER Facility. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the University of Waterloo Animal Care Committee (AUP #42167, 42944).

Mitochondrial isolation

Mitochondria were isolated from the liver, a mitochondria-rich tissue that is important in the uptake and detoxification of xenobiotic compounds including venlafaxine (Maulvault et al., 2018), using protocols similar to those described previously (Borowiec et al., 2022). A tilapia was removed from the holding tank using a large net and directly placed in a bucket filled with an overdose of tricaine mesylate buffered with $NaHCO_3$. Once the animal was unresponsive to gentle prodding and gill ventilation had ceased, the whole liver (8.51 g ± 1.33) was removed and finely diced in approximately 15 ml of ice-cold isolation buffer (250 mM sucrose, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid [HEPES], 1 mM ethylene glycol tetraacetic acid [EGTA], 1% mass: volume fatty-acid free bovine serum albumin; pH 7.4 when measured at 20 °C). To avoid interference from normal biological rhythms, the tilapia were always sampled in the morning (~9 a.m. to 10 a.m. local time); mitochondrial isolation and experiments were performed immediately thereafter and completed over the next several hours of the same day.

The tissue was homogenized by hand using a glass Dounce homogenizer and then centrifuged at 1,000 g for 10 min at 4 °C. The resulting supernatant was filtered through two layers of cotton cheesecloth and then centrifugated and filtered again under the same conditions. The twice-filtered supernatant was centrifuged at 9,700 g for 10 min at 4 °C. The resulting pellet was washed with isolation buffer and gently resuspended in approximately 15 ml of fresh isolation buffer using a pipette. The suspension was centrifuged again, and the resulting pellet was resuspended in 1 ml of isolation buffer (final suspension concentrations ranged from 15 to 30 mg protein/ml). The suspension was held on ice and immediately used for high-resolution respirometry experiments. Mitochondrial respiration data (as well as enzyme activity data, see below) are expressed per milligram mitochondrial protein, and protein content was determined in thawed mitochondrial suspensions using a bicinchoninic acid assay following instructions from the supplier (Pierce, Thermofisher Scientific, Waltham, MA, USA).

High-resolution respirometry of coupled mitochondria

The respiration of isolated mitochondria was assessed using high-resolution respirometry (Oxygraph-2k using DatLab software, Ver. 7.4.0.4, Oroboros Instruments, Innsbruck, Austria) at 25 °C, 35 °C, 40 °C, or 45 °C, based on previously described methods (Borowiec et al., 2022). This temperature range is larger than is typical for work on Nile tilapia (Burggren et al., 2019) but is ecologically relevant for this species with the exception of 45 °C (Nivelle et al., 2019). Respiration measurements were conducted in 2 ml of "MiR05" respiration buffer (110 mM sucrose, 20 mM HEPES, 10 mM KH_2PO_4, 20 mM taurine, 60 mM lactobionic acid, 3 mM $MgCl_2$, 0.5 mM EGTA, 0.1% mass: volume fatty-acid free bovine serum albumin; pH 7.4 at 20 °C). We modulated the amount of mitochondrial suspension added to the chamber to avoid the need to frequently reoxygenate the chamber at higher temperatures, such that 20 μl of suspension was added for respiration measured at 25 °C, 10 μl for 35 °C, and 5 μl for 40 °C and 45 °C (differences in the amount of mitochondrial suspension were accounted for when calculating respiration rates). Preliminary assays confirmed that substrate (pyruvate, malate, glutamate, and succinate), uncoupler (carbonyl cyanide m-chlorophenyl hydrazone [CCCP]), and inhibitor (oligomycin, rotenone, sodium azide, antimycin A) concentrations used in the present study were sufficient to stimulate the expected oxidative phosphorylation, uncoupling, or inhibitory responses from the mitochondria, that the substrate concentrations used achieved maximum respiration rates, and that mitochondria remained viable during the length of time of a typical experiment. Respiration data were corrected for background respiration (antimycin-resistant respiration) as well as autoxidation (sodium azide-resistant respiration, for complex IV-linked respiration only).

After the addition of the mitochondrial suspension to the chamber and stabilization of respiration rate at the selected temperature (~20 min), the complex I-linked substrates pyruvate (2 M stock, final concentration in the 2 ml respirometry chamber of 5 mM), malate (400 mM stock, 2 mM final concentration), and glutamate (2 M stock, 20 mM final concentration) were added to induce State 2 respiration (LEAK respiration without adenylates in the presence of pyruvate, malate, and glutamate, equivalent to $L_{[n]}$; Gnaiger & Group, 2020). We then measured respiration through complex I and II (State 3 or State 4), complex II alone (State 3 or State 4), or complex IV-linked respiration (see below

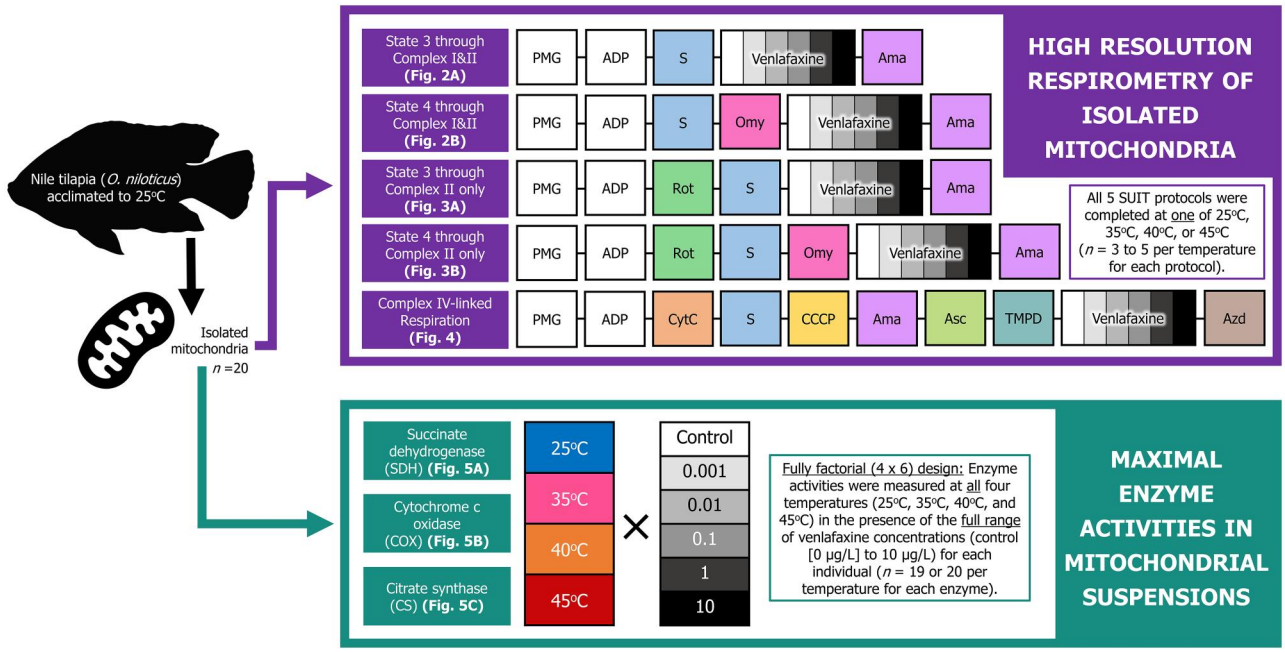

Figure 1. Schematic of the experimental design of this study, including the Substrate-Uncoupler-Inhibitor Titration (SUIT) protocols used for mitochondrial respiration. PMG, pyruvate, malate, glutamate; ADP, adenosine diphosphate; Ama, antimycin A; Asc, ascorbic acid; Azd, sodium azide; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CytC, cytochrome c; Omy, oligomycin; Rot, rotenone; S, succinate; TMPD, N, N, N',N'-Tetramethyl-p-phenylenediamine dihydrochloride. See text for further details. Graphic created by B.G. Borowiec.

for conditions for each assay) in the presence of increasing concentrations of venlafaxine (Figure 1) at the test temperatures. Because isolated mitochondria are only viable for a few hours after isolation, each temperature group for the mitochondria respiration experiments is composed of separate individuals (e.g., each temperature group in Figures 2–4 is composed of five unique individuals each). Excess mitochondrial suspensions were frozen and stored at −80°C for use in maximal enzyme activity assays.

Respiration through complexes I and II or complex II only

Because the Oxygraph-2k instrument can measure respiration in two replicates of the same mitochondria preparation simultaneously, we used a paired design to capture the effects of venlafaxine on State 3 (OXPHOS, oxidative phosphorylation; Gnaiger & Group, 2020) and State 4 (LEAK respiration induced by oligomycin in the presence of ATP, $L_{[Omy]}$; Gnaiger & Group, 2020) respiration. State 3 respiration is induced by the addition of adenosine diphosphate (ADP; 500 mM stock, 5 mM final concentration) followed by the addition of complex II-linked substrate succinate (1 M stock, 10 mM final concentration), enabling measurement for State 3 respiration through both complex I and II. In one of the paired chambers, State 4 respiration was then induced by the addition of oligomycin (5 mM stock, 2.5 µM final concentration), a complex V (ATP synthase) inhibitor.

We then added successive 2 µl additions of serial dilutions of 1 to 10,000 µg/L stock solutions of venlafaxine, such that the final concentrations of venlafaxine within the respiration chambers were 0.001 µg/L, 0.011 µg/L, 0.111 µg/L, 1.111 µg/L, and 10.111 µg/L. We also conducted preliminary control using nanopore water to account for possible effects of volume (or time) on respiration, and confirmed that these effects were negligible. Immediately after measurement of State 3 or State 4 mitochondrial respiration in the presence of the maximum dose of venlafaxine (10 µg/L), we added antimycin A (5 mM stock, 2.5 µM final concentration), a complex III inhibitor, to quantify antimycin-resistant (background) rates of respiration ("State 5," corresponding to residual oxygen consumption; Gnaiger & Group, 2020). To measure State 3 and State 4 respiration through complex II alone, we followed the same Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol outlined above, except that the complex I inhibitor rotenone (1 mM stock, 0.25 µM final concentration) was added to both chambers immediately after ADP and before succinate.

Complex IV-linked respiration (COX assay)

In replicates planned to be used to measure complex-IV linked respiration, we added cytochrome c (4 mM stock, 10 µM final concentration) between the addition of ADP and succinate (Figure 1) to avoid potential activity limitation by cytochrome c release. Then, we added the mitochondrial uncoupler CCCP (1 mM stock solution in ethanol, 0.5 µM final concentration) followed by the complex III inhibitor antimycin A (as above) and the reducing agent ascorbate (800 mM stock, 2 mM final concentration). After a brief (~2 min) pause, we added tetramethyl-p-phenylenediamine (TMPD; 200 mM stock, 0.5 mM final concentration), which acts as an electron donor and artificial substrate for reducing cytochrome c. We were then able to measure maximally stimulated complex IV-linked respiration in response to increasing doses of venlafaxine (or water control) that was added to the chamber as described for other respiration states. Because O_2 is a substrate for complex IV, we reoxygenated the chamber before each addition of venlafaxine so that complex IV-linked respiration was measured at a similar oxygen concentration for each dose. After measuring respiration in the presence of 10 µg/L of venlafaxine, we added complex IV inhibitor sodium azide (4 M stock solution, 100 mM final concentration) to quantify background rates of autooxidation of TMPD.

All reagents and chemicals for the mitochondrial isolation and respirometry experiments were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Bioshop Canada(Burlington, ON, Canada) and prepared as recommended by Bioblast Wiki (https://www.bioblast.at/index.php/MitoPedia).

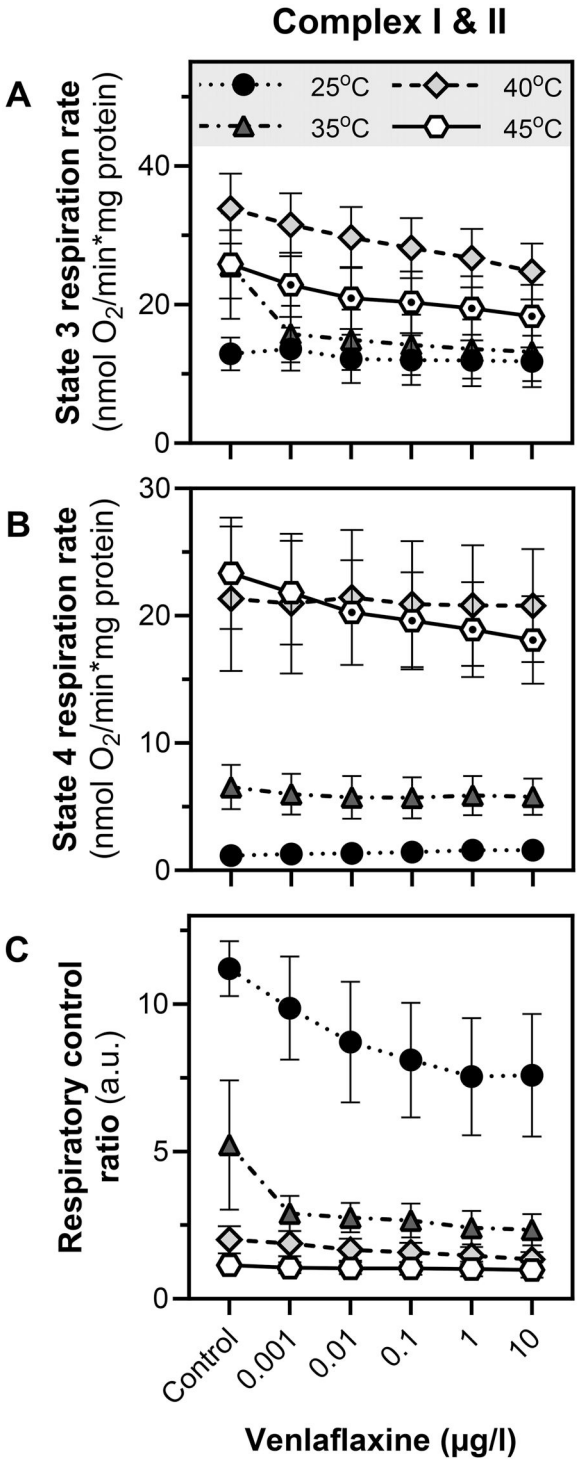

Figure 2. Effect of increasing temperature and venlafaxine concentration on respiration rate through complex I and II in mitochondria isolated from the liver of Nile tilapia. (A) State 3 (OXPHOS) respiration, (B) State 4 (LEAK) respiration, and (C) respiratory control ratio (RCR). Symbols with a black circle in their center (•) represent a significant pairwise difference in the respiration rate compared to venlafaxine-free conditions (control) within the same temperature treatment. Sample sizes of $n = 5$ per group. See the text for further statistical details.

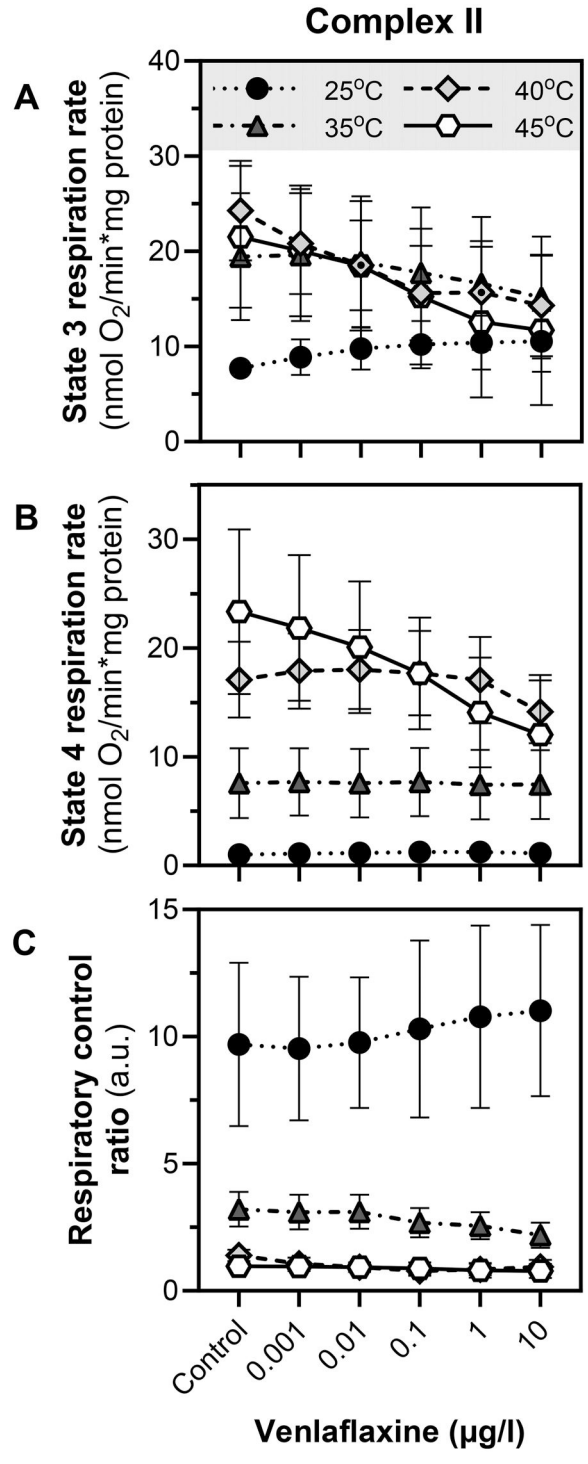

Figure 3. Effect of increasing temperature and venlafaxine concentration on respiration rate through complex II only in mitochondria isolated from the liver of Nile tilapia. (A) State 3 (OXPHOS) respiration, (B) State 4 (LEAK) respiration, and (C) respiratory control ratio (RCR). Symbols with a black circle in their center (•) represent a significant pairwise difference in the respiration rate compared to venlafaxine-free conditions (control) within the same temperature treatment. Sample sizes of $n = 5$ (25 °C, 40 °C [B]), $n = 4$ (35 °C and 40 °C [A, C]), or $n = 3$ (45 °C). See the text for further statistical details.

Venlafaxine treatments

Working venlafaxine stocks (1, 10, 100, 1,000, and 10,000 µg/L) were made by diluting a 200 mg/L stock solution of venlafaxine hydrochloride (MilliporeSigma, ≥98% CAS 99300-78-4) dissolved in nanopore water (stored at −20°C) and validated in a previous experiment (Robichaud et al., 2024). For each concentration, 2 µl of stock venlafaxine was added to the 2 ml chamber, resulting in nominal exposure concentrations for the mitochondria between

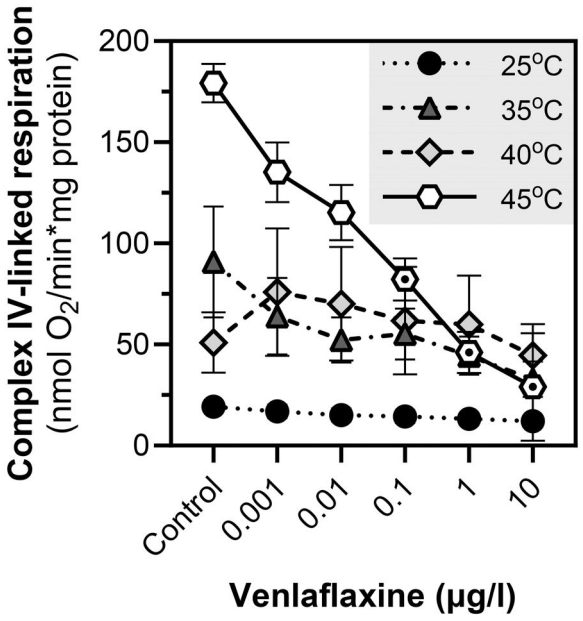

Figure 4. Effect of increasing temperature and venlafaxine concentration on complex IV-linked respiration (cytochrome c oxidase assay) in mitochondria isolated from the liver of Nile tilapia. Symbols with a black circle in their center (•) represent a significant pairwise difference in the respiration rate compared to venlafaxine-free conditions (control) within the same temperature treatment. Sample sizes of $n = 5$. See the text for further statistical details.

0.001 and 10 ug/L (excluding the venlafaxine-free control), an approach that has been previously validated (Robichaud et al., 2024). Venlafaxine levels can meet or exceed >1 ug/L in the aquatic environment (Chen et al., 2022; Khan et al., 2020; Mehdi et al., 2021; Metcalfe et al., 2010; Mheidli et al., 2022; Weng et al., 2023), and the compound has been demonstrated to accumulate in fish tissues, including the liver, after chronic exposure (Grabicova et al., 2014; Lin et al., 2022). Because mitochondria are potential sites of xenobiotic accumulation (Meyer et al., 2013), the range of exposure concentrations used in this study encompasses concentrations that may be present in fish liver mitochondria.

Calculation of respiratory control ratio RCR and temperature coefficient (Q_{10})

We calculated the RCR as an index of mitochondria coupling and integrity (Brand & Nicholls, 2011), where:

$$RCR = \frac{State\ 3\ Respiration}{State\ 4\ Respiration}$$

We calculated the temperature coefficient (Q_{10}) as an index of how sensitive mitochondrial respiration or coupling was to changes in temperature. We calculated Q_{10} as follows:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{t_2 - t_1}}$$

where R_2 is the rate of a biological process (e.g., State 4 respiration) at temperature t_2 (e.g., 25 °C), and R_1 is the rate of the same process at a different temperature, designated t_1 (e.g., 40 °C). The Q_{10} values can vary considerably for biological reactions, but they typically fall between 2 and 3, indicating that the reaction rate doubles or triples with a 10 °C change in temperature.

Enzyme activity assays

We assayed the maximal activities (metabolic capacities) of three enzymes important to aerobic respiration of the mitochondria: succinate dehydrogenase (SDH; complex II of the electron transport system and part of the TCA cycle), COX (complex IV of the electron transport system), and citrate synthase (CS, a TCA cycle enzyme) based on published methods (Borowiec et al., 2015; Bundgaard et al., 2019; Michaelsen et al., 2021; Spinazzi et al., 2012) under the same range of temperatures (25 °C, 35 °C, 40 °C, and 45 °C) and venlafaxine concentrations (0.001 to 10 µg/L) used during high-resolution respirometry (Figure 1). Frozen mitochondrial suspensions were thawed on ice and diluted in 10 to 75 volumes of homogenization buffer (100 mM phosphate buffer [21 parts 100 mM K_2HPO_4: 4 parts 100 mM KH_2PO_4], 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM EGTA, and 0.1% Triton X-100) at pH 7.2. The number of freeze–thaw cycles was held consistent for each enzyme, and all temperatures and venlafaxine concentrations for a given enzyme were assayed within a few hours of each other for each individual sample. In contrast to the respiration data, where the short-lived nature of the mitochondrial suspensions precluded us from taking measurements at more than one temperature for each individual, we were able to measure enzyme activity at all four temperatures in all individuals used in the study. Note that the frozen mitochondrial suspensions used for enzyme activity assays were not exposed to warming or venlafaxine prior to their use in the enzyme assays (i.e., they were isolated and held on ice for a few hours before being flash frozen).

Assays were performed to determine the maximal activity of each enzyme in the diluted mitochondrial suspension by measuring the rate of change in absorbance at 600 nm (SDH), 550 nm (COX), or 412 nm (CS) for at least 5 min. Assay conditions were as follows: SDH, 0.1 mM decylubiquinone in 100 mM phosphate buffer containing 0.08 mM 2,6-dichlorophenolindophenol, 20 mM succinate, 1 mM EDTA, 1 mM EGTA, and 0.3 mM KCN at pH 7.2; COX, 50 µM of fully reduced cytochrome c in 50 mM Tris containing 0.5% Tween-20 at pH 8.0; CS, 0.5 mM oxaloacetate in 50 mM Tris containing 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.15 mM acetyl-coenzyme A at pH 8.0. Preliminary assays determined the minimum substrate concentrations that would stimulate maximal activity and confirmed that the observed activities were repeatable with a linear relationship between enzyme concentration (as represented by the amount of mitochondrial suspension in the well) and enzyme activity.

Enzyme activity assays were run in triplicate (except SDH, which was run in duplicate due to high amounts of sample required per well) in a 96-well microplate spectrophotometer (SpectraMax 190, Molecular Devices, San Jose, CA, USA) with temperature control. Activities were determined by subtracting the background reaction rate, determined by replacing the diluted mitochondrial suspension with homogenization buffer, from the rates measured in the presence of the mitochondria suspension (i.e., enzyme). We used the following standard extinction coefficients (ε, OD/mM/cm): 19.1 for SDH assays, 28.5 for COX assays, and 13.6 for CS assays.

Statistical analysis

Data are reported as means ± standard error. For most analyses, we used a repeated measures two-way analysis of variance (ANOVA) to test for main effects of temperature, venlafaxine, the interaction between temperature and venlafaxine, and individual on mitochondrial function. When missing values precluded the use of a two-way ANOVA (State 3 respiration through complex II

only [Figure 3A] and RCR through complex II only [Figure 3C]), we used a mixed-effects model with fixed effects of temperature, venlafaxine, and their interaction and random effects of individual and residual. We detected a significant main effect of individual as a source of variation for all comparisons where it was tested.

We did not assume sphericity (equal variability of differences) for our analyses, so degrees of freedom were adjusted using the Geisser-Greenhouse correction. We used a Dunnett's multiple comparisons post-test to conduct pairwise comparisons between different doses of venlafaxine to their venlafaxine-free control within the same temperature. A significance level of $p < 0.05$ was used throughout the analysis. All data presentation and statistical analyses were conducted in GraphPad Prism 10.0 for Windows 8.

Results

Respiration through complexes I and II

State 3 (OXPHOS) respiration (Figure 2A) tended to increase between 25 °C and 40 °C, although this effect was marginally nonsignificant (main effect of temperature on respiration: $F_{[3,16]} = 3.179$, $p = 0.0527$). The average Q_{10} for State 3 respiration though complex I and II was 1.77 under venlafaxine-free conditions (see online supplementary material, Table S1), indicating that it was weakly sensitive to temperature. There was a significant main effect of venlafaxine concentration ($F_{[1.16,18.56]} = 8.609$, $p = 0.0068$), and this was driven largely by the 45 °C group, which showed small but consistent decrease in State 3 respiration rate with increasing doses, such that respiration was approximately 30% less when exposed to the highest dose of venlafaxine compared to controls. However, we did not detect a statistically significant main effect of interaction between temperature and venlafaxine ($F_{[15,80]} = 1.125$, $p = 0.3486$).

Temperature had a significant effect on State 4 (LEAK) respiration in the presence of substrates for both complex I and II (main effect of temperature on respiration: $F_{[3,16]} = 9.195$, $p = 0.0009$), and this was driven by very high respiration rates in the 40 °C and 45 °C groups compared to the other groups (Figure 2B). In line with this, the Q_{10} of State 4 respiration (average Q10 = 5.72) was much higher than for State 3 respiration under control conditions (see online supplementary material, Table S1), indicating that State 4 respiration is far more vulnerable to acute temperature changes than the average biological reaction. Similar to State 3 respiration, there was a significant main effect of venlafaxine concentration ($F_{[1.59,25.48]} = 5.600$, $p = 0.0141$), but unlike State 3 respiration, we also detected a significant main effect of interaction between temperature and venlafaxine concentration ($F_{[15,80]} = 4.161$, $p < 0.0001$). We suspect both of these effects were driven by the considerable (up to 22%) decline in respiration observed in the 45 °C group at high doses.

The quotient of State 3 respiration and State 4 respiration, RCR, declined sharply with increasing temperatures (main effect of temperature: $F_{[3,16]} = 15.51$, $p < 0.0001$; Figure 2C), with the vast majority of the decrease occurring between 25 °C and 35 °C. We also detected a significant main effect of venlafaxine concentration on RCR ($F_{[1.31,20.99]} = 4.741$, $p = 0.0323$). Although the decrease in RCR in response to contaminant exposure seemed most apparent in the 25 °C group, we did not detect a significant main effect of interaction between temperature and venlafaxine ($F_{[15,80]} = 1.201$, $p = 0.2887$).

Respiration through complex II only

We did not detect a significant main effect of temperature on State 3 respiration involving substrates for complex II only ($F_{[3,13]} = 0.7606$, $p = 0.5360$; Figure 3A), although the 25 °C group tended to have lower respiration rates compared to the other groups, at least initially. Unlike respiration through complex I and II, which peaked at 40 °C, respiration rates through complex II alone were very similar across the 35 °C, 40 °C, and 45 °C groups, perhaps suggestive of a lower plateau or breakpoint temperature. Regardless, the low Q_{10} value of 2.1 for State 3 respiration through complex II only (see online supplementary material, Table S1) indicated relatively little scope for this respiration rate to respond to warming. There was a significant main effect of venlafaxine concentration on State 3 respiration through complex II only ($F_{[1.66,\ 20.90]} = 8.362$, $p = 0.0033$), and this was driven by significantly lower respiration rates for the 40 °C at 0.1 μg/L and 1 μg/L of venlafaxine. We also detected a significant main effect of the temperature × concentration interaction ($F_{[15,63]} = 3.206$, $p = 0.0006$) for State 3 respiration through complex II only, which may be underlaid by respiration in the 25 °C group increasing slightly at higher doses in contrast to the other temperatures groups.

State 4 respiration through complex II increased with temperature ($F_{[3,13]} = 7.306$, $p = 0.0041$) and did not appear to approach a plateau even at the highest test temperature. Similar to what was observed for respiration through complex I and II, State 4 respiration through complex II had a Q_{10} of 6.3, approximately triple that of the corresponding value for State 3 respiration through complex II (see online supplementary material, Table S1), indicating an above-average sensitivity to warming than a typical biological reaction. The effect of venlafaxine concentration alone was marginally nonsignificant ($F_{[1.31,\ 17.01]} = 3.975$, $p = 0.0531$; Figure 3B), although there was a significant main effect of the interaction between temperature and venlafaxine ($F_{[15,65]} = 1.980$, $p = 0.0305$). Although there were no significant pairwise differences detected between groups, the 45 °C group seemed to be more sensitive to increasing concentrations of venlafaxine than other groups, as reflected by a relatively large 48% decline in respiration rate with 10 μg/L venlafaxine compared to the control (vs. 17% for the 40 °C group and 2% for the 35 °C group).

Respiratory control ratio for respiration through complex II only was significantly affected by temperature ($F_{[3,13]} = 5.823$, $p = 0.0095$). As before, the RCR for the 25 °C group was considerably higher than all other groups, suggesting a breakpoint below 35 °C. Neither venlafaxine concentration ($F_{[1.14,14.36]} = 0.077$, $p = 0.8168$) or the interaction between temperature and venlafaxine ($F_{[15,63]} = 1.020$, $p = 0.4473$) had significant main effects on RCR for complex II alone (Figure 3C).

Complex IV-linked respiration

Cytochrome c oxidase activity in venlafaxine-free conditions was relatively low in the 20 °C group, increased to a relatively stable plateau at the intermediate temperature groups, and was very high in the 45 °C group (main effect of temperature: $F_{[3,16]} = 7.202$, $p = 0.0028$; Figure 4, see online supplementary material, Table S1). We also detected significant main effects of venlafaxine ($F_{[2.25,\ 35.96]} = 12.17$, $p < 0.0001$), which was apparent in the 45 °C group and to a lesser extent in the 35 °C, as well as a significant main effect of interaction between temperature and venlafaxine ($F_{[15,80]} = 4.940$, $p < 0.0001$), likely driven by the 45 °C group.

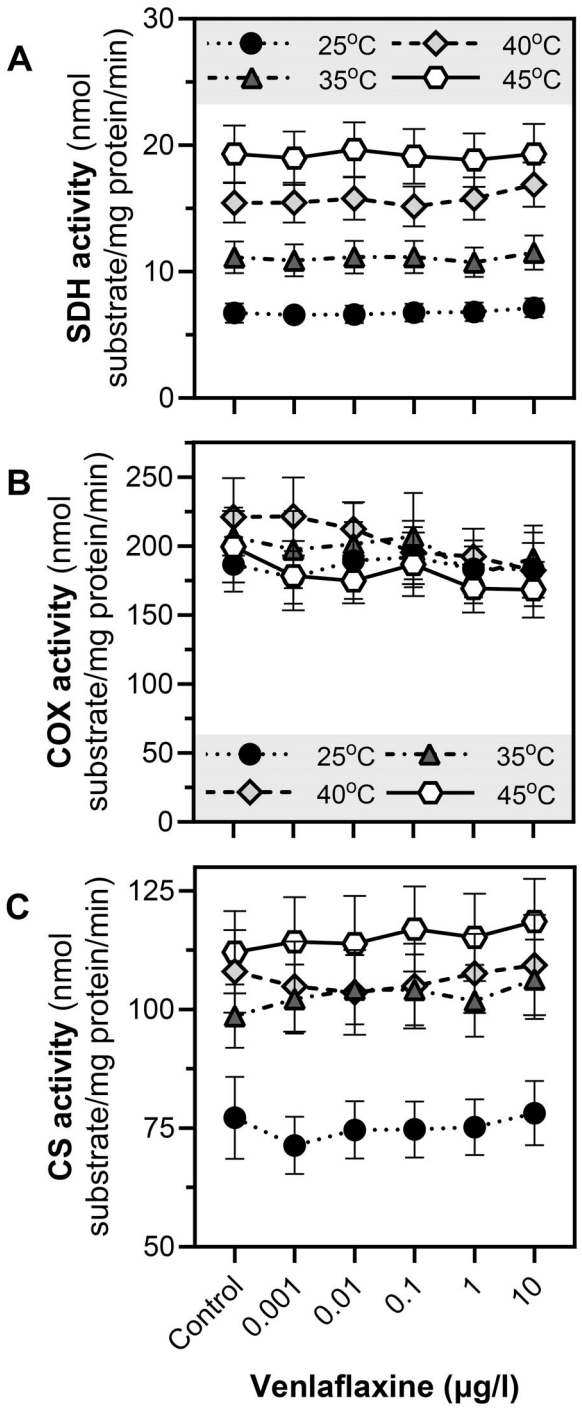

Figure 5. Effect of increasing temperature and venlafaxine concentration on the maximal activity of enzymes of the tricarboxylic acid cycle and/or electron transport system in mitochondrial suspensions obtained from the liver of Nile tilapia. (A) Succinate dehydrogenase (SDH), (B) cytochrome c oxidase (COX), and (C) citrate synthase (CS). Sample sizes of $n = 20$, except for B ($n = 19$). See the text for further statistical details.

Enzymes associated with complexes of the electron transport system

Unlike its closest comparable, State 3 respiration through complex II only (Figure 3A), there was significant main effect of temperature on the activity of SDH, a member of the TCA cycle and electron transport system (complex II; $F_{[3,\ 72]} = 12.53$, $p < 0.0001$). Of the enzymes examined, SDH was the most sensitive to increasing temperature ($Q_{10} = 1.66$), although as a general rule maximal enzyme activities were generally insensitive to warming (see online supplementary material, Table S2), especially compared to respiration measurements. Like State 3 respiration through complex II only, there was also a significant main effect of venlafaxine concentration on SDH ($F_{[3.315,238.7]} = 3.827$, $p = 0.082$; Figure 5A). Although neither the temperature nor venlafaxine main effect was associated with significant pairwise differences in SDH activity, the effects of venlafaxine were much less readily apparent than the effects of temperature with only a subtle trend for SDH activity to increase in the presence of high doses of venlafaxine, especially in the intermediate temperature ranges. We detected no significant main effect of interaction for SDH activity ($F_{[15,\ 360]} = 1.150$, $p = 0.3096$), which is also reflected in there being very little change in Q_{10} across concentrations of venlafaxine (see online supplementary material, Table S2).

In contrast to complex-IV linked respiration, which showed significant main effects of temperature, venlafaxine, and their interaction, the activity of COX quantified using standard spectrophotometric techniques was unaffected by assay temperature ($F_{[3,\ 76]} = 0.3562$, $p = 0.7848$), venlafaxine concentration ($F_{[3.979,\ 302.4]} = 1.258$, $p = 0.2868$), or their interaction ($F_{[15,\ 380]} = 0.3291$, $p = 0.9922$; Figure 5B). As expected, given the lack of significant main effect of temperature, all Q_{10} values for COX activity were approximately 1, indicating very little change in the rate of reaction with warming (see online supplementary material, Table S2).

The activity of the TCA cycle enzyme CS moderately increased with temperature ($F_{[3,76]} = 4.794$, $p = 0.0041$; Figure 5C, see online supplementary material, Table S2) but was unaffected by increasing concentrations of venlafaxine ($F_{[4.195,\ 318.8]} = 1.986$, $p = 0.0931$) or the interaction between venlafaxine and temperature ($F_{[15,\ 380]} = 0.6146$, $p = 0.8633$). Because CS is often used as a quantitative marker of protein content, we also compared the linear relationship between suspension protein content and uncorrected CS activity in venlafaxine-free controls (see online supplementary material, Figure S1), finding that the regression lines for each temperature had statistically indistinguishable slopes ($F_{[3,72]} = 0.2345$, $p = 0.8720$) but different y-intercepts ($F_{[3,75]} = 3.563$, $p = 0.0181$). Therefore, the temperature-associated increase in CS activity remained predictable and consistent within the range tested here (i.e., within the thermal optima of CS), confirming that CS is a reasonable indicator of mitochondrial protein content in the context of this investigation. Contrastingly, we found that the activity of COX did not correspond to homogenate protein content (see online supplementary material, Figure S2).

Discussion

In this study, we focused on the mitochondria as a potential mediator for interactions between warm temperatures and a common model contaminant (venlafaxine). Although the overall effects of venlafaxine were mild, they also were clearly enhanced by warming, emphasizing the value of testing the effects of contaminants under a variety of environmental conditions. More broadly, our work also stands as an example of how direct measures of "living" (respiring) mitochondria, an evolutionarily conserved organelle implicated in tolerance of temperature alterations as well as other metabolic stressors, can add novel dimension to studies of the modes of action and mechanisms of toxicity of contaminants of concern. We encourage future investigations to consider the influence of increasing temperature (and other environmental fluctuations that may alter toxicity) on

the effects of contaminants and the mitochondria as a potential site where contaminant × environment interactions could be mediated in animals.

Effects of venlafaxine on mitochondrial respiration and coupling

As far as we are aware, this is the first investigation to document the physiological effects of venlafaxine through the respiration of isolated ("living") mitochondria. The closest comparable study examined the effects of the compound on crude brain homogenates in zebrafish and demonstrated that venlafaxine exposure lead to decreased State 3 (OXPHOS) respiration through complex I and II, although this effect did not appear to be driven by effects specific to either complex I or complex II alone (Robichaud et al., 2024). In general agreement with this work, we also report a depressive effect of venlafaxine on the respiration (Figures 2A and B, 3A, and 4) as well as coupling (Figure 2C) of isolated liver mitochondria, although these effects were milder than observed in zebrafish brain homogenates. This was not unexpected, as Nile tilapia are generally regarded as a relatively tolerant species that can persist in highly polluted habitats (Linde et al., 2008; Linde-Arias et al., 2008), and therefore are presumably resistant the disruptive effects of common contaminants. Therefore, we suspect that the major mode of toxic action of venlafaxine in fishes such as tilapia is unlikely to involve the mitochondria, especially at lower concentrations.

Similar to previous studies in mammals (Hroudova & Fisar, 2010; Scaini et al., 2010, 2011), we also examined the effects of venlafaxine on the maximal enzymatic activities of succinate dehydrogenase (SDH, complex II),COX (complex IV), and CS, (Figure 5). We found COX and CS activities to be insensitive to the effects of venlafaxine (Figure 5B and C). Alhough SDH activity did have a statistically significant effect of venlafaxine concentration, those effects were also very mild and unlikely to be physiologically impactful (Figure 5A). Therefore, the depressive effects of venlafaxine on mitochondrial respiration are unlikely to be caused by limitations to or decline in the maximal capacity of these enzymes or their associated complexes. This does not agree with previous work, which interestingly has shown that venlafaxine exposure can either decrease (Hroudova & Fisar, 2010) or increase (Scaini et al., 2010, 2011) complex II and/or complex IV enzymatic activity in the brain. There are several potential explanations for these disparate results such as choice of model organism (ectothermic tilapia vs. endothermic rats or pigs), tissue examined (liver vs. brain), dosage levels used (environmental vs. therapeutic), and other aspects of experimental design. Future studies aimed at addressing these limitations would be valuable for assessing the biochemical and physiological effects of chronic environmental exposure to SNRIs in ectotherms.

Contribution of mitochondria to the thermal limits of organisms

As expected for an ectotherm, mitochondrial respiration rates increased with temperature (Figures 2 and 3), albeit with some interesting subtleties. State 4 respiration, which largely represents the routine costs of compensating for proton leak, was more sensitive to warming than State 3 respiration, as reflected by the scope of respiration rate associated with warming (Figures 2 and 3) as well as Q_{10} values (see online supplementary material, Table S1). This broadly agrees with theoretical expectations (Chung & Schulte, 2020) and experimental results (Abele et al., 2002; Martinez et al., 2016; Michaelsen et al., 2021; Pörtner et al., 1999) for State 4 respiration to increase exponentially with temperature as the costs of maintaining the proton gradient compounds with higher biochemical reaction rates. Another interesting subtlety in the data is the disparate response of RCR to warming compared to its underlying components. Whereas temperature-induced changes in State 3 and State 4 respiration were more or less evenly distributed across the thermal range, the vast majority of the decrease in RCR occurred between 25 °C and 35 °C (Figures 2C and 3C). This fits with the predicted effects of temperature on RCR as, unlike State 4 respiration, RCR should exhibit a clear breakpoint and a sudden decrease once mitochondrial coupling fails at high temperatures (Chung & Schulte, 2020). Based on the pattern observed here, the RCR breakpoint is conservatively above 25 °C and below 35 °C for respiration through complexes I and II (which best reflects the situation in a living animal). Because this is well within the ecological range of Nile tilapia, it raises interesting questions about the significance of RCR as an indicator of mitochondria function and its contribution to the thermal limits in tropical ectotherms.

Although mitochondrial efficiency was lost at relatively mild temperatures, the same cannot be said for the capacity of the mitochondria to maintain high respiration rates, because they typically plateaued (Figures 2 and 3) or even increased (Figure 4) in the 40 °C and 45 °C groups. Maintenance of mitochondrial function at temperatures near and even beyond whole-animal thermal limits (typically reflected by the critical thermal maximum, CT_{max}) is known to occur (Pörtner, 2002; Somero, 2002), although this is not universal, especially when specific complexes or enzymes are examined (Ekström et al., 2017; Léger et al., 2024; Lemieux et al., 2010). We suspect that processes other than the respiratory capacity of the electron transport system are involved in setting the upper thermal limits of tilapia, and that these may include mitochondrial (e.g., ROS homeostasis, membrane fluidity, ATP synthetic capacity; Banh et al., 2016; Christen et al., 2018; Dahlhoff & Somero, 1993; Harada et al., 2019; Healy & Burton, 2023; Iftikar & Hickey, 2013; Melzner et al., 2006; O'Brien et al., 1991) and extra-mitochondrial processes (e.g., neural activity; Andreassen et al., 2022). Further research incorporating acclimation of a subset of individuals to warm temperatures and/or investigation of ATP synthesis or other critical mitochondrial processes that could be impacted by warming would be valuable in identifying potential points of vulnerability to further stress (e.g., contaminant exposure) or even failure in thermally challenged mitochondria in ectotherms.

Interactive effects of temperature and venlafaxine

Aquatic organisms routinely encounter simultaneous exposure to elevated temperatures and contaminants in the wild (Kinouchi et al., 2007; Mehdi et al., 2022). Assessing the interactive effects of multiple stressors is likely to be a more ecologically relevant representation of life in environments experiencing anthropogenic changes. Our data set demonstrates the value of this approach as the depressive effects of venlafaxine on respiration were most significant in mitochondria also exposed to warming (Figures 2A, B and 3A, B), suggesting that the compound is more toxic and/or mitochondria are more sensitive to it when concurrently exposed to thermal stress. These effects were especially noticeable at 45 °C, a temperature that exceeds what would be considered a viable temperature for this species (Burggren et al., 2019), but the effects were also not entirely dissipated at more reasonable temperatures. This fits with concerns that exposure to contaminants may limit capacity of species and populations to acclimate to altered temperatures (Noyes et al., 2009). It is also possible that there are additional, as of yet uncharacterized mechanisms by which overall toxicity of venlafaxine is enhanced

at higher temperatures, as is expected to occur for most contaminants (Noyes et al., 2009). For example, warming decreases venlafaxine elimination in the liver of meagre fish (*A. regius*; Maulvault et al., 2018), and this could foreseeably modulate the effective dose of venlafaxine across temperature groups (Santos et al., 2020). At the whole-animal level, venlafaxine exposure is associated with alterations in carbohydrate metabolism and the capacity for steroidogenesis (Best et al., 2014; Ings et al., 2012), both of which could interact with its potential direct effects on the mitochondria by interfering with the coordination of metabolic response to either warming or pollution.

Lack of concordance between mitochondrial respiration and enzyme activity data

A key innovation of our study is directly comparing mitochondrial respiration data and enzymatic capacity data to evaluate the effects of venlafaxine on fish exposed to warming. To our knowledge, all previous work on the effects of venlafaxine on the mitochondria relies on only one or the other approach (Hroudova & Fisar, 2010; Robichaud et al., 2024; Scaini et al., 2010, 2011). This is unfortunate, because alterations in the metabolic capacity (e.g., enzyme activity) do not always translate to alterations in routine function (e.g., respiration), especially if capacity is sufficiently in excess to meet demands across a variety of conditions. The relationship, if any, between mitochondrial respiration through specific complexes ex vivo and the metabolic capacity of those complexes in vitro has only been sporadically investigated, but available data generally suggest a lack of concordance between respiration and activity data (Jørgensen & Mustafa, 1980; Lemieux et al., 2010), although exceptions do occur (Dawson et al., 2016). We found that neither SDH (Figure 5A) nor COX activity (Figure 5B) agreed with their most comparable mitochondrial respiration measurement (State 3 respiration thorough complex II only [Figure 3A] and complex-IV linked respiration [Figure 4], respectively). Arguably, the disparity between the two measures is to be expected considering the greater complexity (and perhaps sensitivity to environmental perturbations) of the full electron transport system compared to its individual components, as well as the potential for other mitigating factors to further influence observed reaction rates (e.g., the presence of biotransformation enzymes in crude homogenates but not mitochondrial isolates). Moreover, thermal sensitivity appears to be influenced by exposure time (Rezende et al., 2014, 2020; Sokolova, 2023), which naturally varied between enzymatic and mitochondrial assay protocols. Despite these caveats, we argue that reductive approaches still add value in uncovering the specific components involved in organismal responses to environmental challenges, especially in the cases where the literature is scant (e.g., effects of contaminants on mitochondria; Dreier et al., 2019; Reddam et al., 2022) or lacking in clear consensus (e.g., the role of mitochondria in thermal limits of ectotherms). Therefore, we encourage investigators to combine enzymatic capacity measurements with respiration data whenever possible.

Limitations and opportunities of in vitro experiments

A thematically similar issue to resolving differences between enzymatic activity and mitochondrial respiration is the applicability of in vitro experiments performed here to animals in the wild (e.g., in vivo). In vivo, animals can bioaccumulate, metabolize, and excrete xenobiotics, all of which can change the contaminant in question and potentially its biological effects (Matthee et al., 2023). For example, both the uptake and elimination of venlafaxine are altered by warming, but these effects vary across tissues (e.g., warming impairs uptake and elimination by the liver but enhances brain uptake; Maulvault et al., 2018). Additionally, due to a combination of its wide use (Singh et al., 2022) and its insufficient removal by many wastewater treatment plants (Rúa-Gómez & Püttmann, 2012a,b; Singh et al., 2022), aquatic animals in the wild tend to be exposed to venlafaxine chronically as opposed to the acute exposure used in this investigation. Chronic exposure allows more time for animals to physiologically respond to the stressors in their environment by inducing compensatory responses (some of which can take days or weeks to be fully established) to lessen the effect of the environmental challenge (e.g., phenotypic plasticity), and this capacity is not accounted for when examining the acute and in vitro effects of contaminants and elevated temperatures.

Although in vivo studies are perhaps best for determining ecologically relevant effects, in vitro studies can provide a more ethical and economically feasible alternative because fewer fish are required to satisfy experimental sample sizes (Grunow et al., 2021). Most notably, in vitro studies are unparalleled in their capacity to elucidate precise mechanisms using a reductionist approach (Rehberger et al., 2018), which can help in explaining why specific effects are seen in vivo by unraveling the exact cellular pathways involved in a given response. At the same time, reductionist approaches can lean toward "oversimplifying" a biological system, and caution should be taken when applying their findings to whole animals or populations. For example, chronic in vivo exposure to venlafaxine and elevated temperatures has demonstrable interactive and cumulative effects to zebrafish metabolic physiology (Mehdi et al., 2019), and these effects were different from those observed in this study at the level of the mitochondria. We encourage toxicologists and physiologists to consider employing both in vivo and in vitro approaches in their work, especially in the context of providing a more comprehensive view of the role of mitochondria in mediating temperature and contaminant interactions in ectotherms.

Conclusion

In this study, we focused on mitochondria as a potential mediator for interactions between warm temperatures and a common model contaminant. We found that venlafaxine alone has generally mild effects on the mitochondria, but also that those effects are enhanced by simultaneous exposure to high temperatures, highlighting the value of multiple stressor experiments in moving toward a more holistic approach in predicting the effects of climate change. Because mitochondria are evolutionarily conserved, the effects measured in this study may be useful in predicting the mitochondrial mechanisms associated with the response to xenobiotics and/or thermal challenges occurring in other fish exposed to these stressors. Additionally, if the reported effects on mitochondrial respiration also occur during in vivo exposure, fish may experience changes to overall metabolic rate and have insufficient energy production to succeed in the environmental conditions. Thus, the findings from this study may drive further exploration into the potential implications mutistressor exposure may have on energy metabolism in fish exposed in vivo.

Characterizing the interactive effects of simultaneous stressors on animal physiology is important for understanding how they will cope with the many challenges caused by anthropogenic changes in the environment. As far as we know, we are the first to report the effects of venlafaxine, a common and relatively abundant contaminant in aquatic ecosystems, on the respiration

of "living" mitochondria and to compare those effects on the enzymatic capacities of complexes of the mitochondrial electron transport system. The methods used in this study may serve as a useful tool to assess the effects of contaminants on mitochondrial function in fish and other organisms, by adding a novel dimension to studies of the mode of action and mechanism of toxicity of contaminants of concern. We suggest that future efforts to understand the physiological and biochemical effects of contaminants on ectotherms, including venlafaxine, incorporate the influence of increasing temperature, and that more toxicologists specifically focus on the mitochondria as a potential site whereby these interactions could be mediated in animals.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

Raw data are included in the online supplementary material.

Author contributions

Brittney Borowiec (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing—original draft, Writing—review & editing), Karyn Robichaud (Conceptualization, Investigation, Methodology, Writing—review & editing), and Paul Craig (Funding acquisition, Resources, Supervision, Writing—review & editing)

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Conflicts of interest

The authors declare no conflicts of interest.

Ethics statement

All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the University of Waterloo Animal Care Committee (AUP #42167 and #42944).

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