2015

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**Physiological Performance of Warm-Adapted Marine Ectotherms:
Thermal Limits of Mitochondrial Energy Transduction Efficiency**

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Running Title: Mitochondrial energetics of warm-adapted teleosts

ms. has 34 pages, 6 figures, 1 table

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Abstract

Thermal regimes in aquatic systems have profound implications for the physiology of ectotherms. In particular, the effect of elevated temperatures on mitochondrial energy transduction (i.e. energy from carbon substrates to ATP) in tropical and subtropical teleosts may have profound consequences on organismal performance and population viability. Upper and lower whole-organism critical temperatures for teleosts suggest that subtropical and tropical species are not susceptible to the warming trends associated with climate change, but sub-lethal effects on energy transduction efficiency and population dynamics remain unclear. The goal of the present study was to compare the thermal sensitivity of processes associated with mitochondrial energy transduction in liver mitochondria from the striped mojarra (*Eugerres plumieri*), the whitemouth croaker (*Micropogonias furnieri*) and the palometa (*Trachinotus goodei*), to those of the subtropical pinfish (*Lagodon rhomboides*) and the blue runner (*Caranx crysos*). Mitochondrial function was assayed at temperatures ranging from 10° to 40 °C and results obtained for both tropical and subtropical species showed a reduction in the energy transduction efficiency of the Oxidative Phosphorylation (OXPHOS) system in most species studied at temperatures below whole-organism critical temperature thresholds. Our results show a loss of coupling between O₂ consumption and ATP production before the onset of the critical thermal maxima, indicating that elevated temperature may severely impact the yield of ATP production per carbon unit oxidized. As warming trends are projected for tropical regions, increasing water temperatures in tropical estuaries and coral reefs could impact long-term growth and reproductive performance in tropical organisms, which are already close to their upper thermal limit.

44 **Key-words:** temperature, marine, mitochondria, teleostei, *Lagodon*, *Micropogonias*, *Caranx*, *Eugerres*,
45 OXPHOS, LEAK

1. Introduction

Physiological constraints, thermal tolerance in particular, play an important role in limiting species' habitat selection and range of distribution. Most individuals inhabit environments close to their thermal optimum (Pörtner, 2001; Pörtner, 2002; Somero, 2005). Within the optimal thermal range, biochemical processes, especially enzyme-mediated processes, exhibit a higher performance than at temperatures above or below the thermal optimum. Since teleosts inhabiting tropical estuaries experience high temperatures (25-30°C) year round, it follows that their thermal optima are higher than those of ecological analogues in subtropical and temperate estuaries and are likely amongst the highest found in aquatic ectotherms.

Seasonal fluctuations in the temperature of coastal tropical regions are small in comparison to those observed in subtropical estuaries. For example, in the subtropical Tampa Bay estuary (USA), with a mean annual water temperature of 24°C, water temperatures have been observed to change by up to 15°C in a matter of weeks (Badylak et al., 2007). In contrast, the smaller tropical estuary of San Juan Bay (Puerto Rico) varies in temperature by less than 6°C throughout the year, from an annual mean of 28°C (SJBEP Program Report, 2011). Although the different thermal regimes experienced by fishes inhabiting subtropical and tropical estuaries are well documented, comparative physiological characteristics of estuarine teleosts from the two different thermal environments are not. Most of our understanding about thermal tolerance in marine tropical regions stems from invertebrate studies, where it has been established that tropical invertebrates live close to their upper thermal limit (Coles et al., 1976; Maté, 1997; Stillman and Somero, 2000; Urban, 1994).

A select number of studies have determined the critical thermal-tolerance windows in tropical fishes to assess potential effects of climate change on tropical marine teleosts (Eme and Bennett, 2009; Eme et al., 2011; Mora and Ospina, 2001; Mora and Ospina, 2002; Ospina and Mora, 2004; Rajaguru and Ramachandran, 2001). Based on the wide thermal window of tolerance in various estuarine species, those authors have suggested that tropical species may be better poised to survive long-term warming trends associated with climate change than previously thought (Eme et al., 2011). In the present study, we provide evidence that sub-lethal effects of temperature at the mitochondrial level are evident, and potentially significant.

Our current understanding of whole-organism thermal tolerance relies heavily on critical, rather than sub-lethal analyses of organismal performance as a function of temperature. The influence of environmental change on mitochondrial energy transduction efficiency and resulting effects on whole-organism physiological performance are poorly resolved. Studies of teleost mitochondria indicate that substrate flux and oxygen consumption rates poorly estimate energy balance and flow in organisms whose body temperature regularly fluctuates (Weinstein and Somero, 1998; Hardewig and Pörtner, 1999; Pörtner et al., 1999; Hilton et al. 2010; Mark et al. 2012, Martinez et al., 2013). Since energy production relies on the efficiency of mitochondrial ATP production, a detailed analysis of mitochondrial performance is likely to be a more accurate indicator of temperature effects on whole-organism physiological performance than the critical thermal maximum (Weinstein and Somero, 1998; Pörtner et al., 1999; Martinez et al., 2013).

Although the tolerance window of some estuarine fishes is beyond any temperature found in their natural habitat (Eme and Bennett, 2009; Mora and Ospina, 2001; Mora and Ospina, 2002), the long-term implications of gradual changes in temperature on physiological performance and survival are unknown. In particular, the effects of thermal heterogeneity on mitochondrial

performance are yet to be determined. Based on previous studies on terrestrial systems, thermal heterogeneity of habitats favor an organism's ability to adapt to changes in their thermal regime (Deutsch et al., 2008; Huey et al., 2009; Tewksbury et al., 2008). If we extend this to the marine milieu, it is possible that tropical organisms experiencing stable but high temperatures, such as teleosts associated with coral reefs and estuaries, could be particularly challenged by increasing habitat temperatures as they shift to a warmer sub-optimal range.

The goal of this study was to employ a series of estuarine teleosts as tropical and subtropical study systems to compare the thermal sensitivity of mitochondrial energy transduction. To achieve our goal, this study examines the oxidative phosphorylation (OXPHOS) system in liver mitochondria from the striped mojarra (*Eugerres plumieri*), the whitemouth croaker (*Micropogonias furnieri*) and the palometa (*Trachinotus goodei*), and compares them to the subtropical pinfish (*Lagodon rhomboides*) and the blue runner (*Caranx crysos*). Mitochondrial function was assayed at various temperatures, and the thermal sensitivity of mitochondrial complex I (NADH:ubiquinone reductase) and complex II (succinate dehydrogenase) activity was determined.

2. Methodology

2.1 Chemicals. All chemicals for respiration measurements were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

2.2 Study systems. Subtropical specimens were collected during the fall (October) in the southern portion of Tampa Bay, Florida using hook and line. Water temperature at the collection site was 27.9°C. After collection, all specimens were transported in aerated 19 L containers to the aquarium facility of the University of South Florida, College of Marine Science. Specimens were transferred to holding tanks equipped with a flow-through water system for at least two weeks prior to analysis, and fed pathogen-free frozen mysid shrimps every 48 hours. Holding tanks consisted of three 570 L fiberglass rectangular tanks, and specimens were held at low densities (less than 10 individuals per tank) at any given time. Temperature was controlled ($28 \pm 2.0^\circ\text{C}$), and nutrients were monitored biweekly.

The pinfish, *Lagodon rhomboides*, is a demersal estuarine species commonly associated with vegetated bottom hard structures and the brackish water surrounding mangroves (Robins and Ray, 1999). *L. rhomboides*' diet consists of vegetation as well as small mollusks, polychaetes, and juvenile fishes (Montgomery and Targett, 1992; Robins and Ray, 1999). The blue runner, *Caranx crysos*, is a schooling pelagic predator found throughout the coastal subtropical Atlantic. Despite its active pelagic habit, the species is mainly found schooling in shallow (0-100 m) water; it is most frequently observed in the estuarine pelagial where it feeds on small fishes, shrimp and other invertebrates (Cervigón et al., 1992).

Tropical specimens were collected during the winter season (December) in neighboring waters of the Punta Santiago Estuary area in Humacao, Puerto Rico. Specimens were collected using a 20-meter long seine net, and later transported in aerated 19 L containers to a 190 L holding tank at the University of Puerto Rico, Humacao Campus. Water temperature at the collecting site was 27.8°C. Specimens were held for less than 72 hours in artificial seawater at habitat salinity and aquarium room temperature ($25.0 \pm 2.0^\circ\text{C}$) prior to experiments.

Tropical species included the striped mojarra, *Eugerres plumieri*, the whitemouth croaker, *Micropogonias furnieri*, and the palometa, *Trachinotus goodei*. The striped mojarra is often found in tropical estuaries, primarily over soft bottom. It is commonplace in Caribbean estuaries with a distribution that extends to subtropical regions. The mojarra's diet comprises infaunal species of crustaceans, bivalves, and detritus (Bussing, 1998). The whitemouth croaker is , commonly found over the sandy bottom of estuaries where it feeds upon crustaceans, mollusks and fishes (Isaac, 1988). The palometa, is an active pelagic species frequently found in tropical estuaries. Analogous to the subtropical *C. crysos*, *T. goodei* is also a schooling species that feeds primarily on crustaceans and fishes (Cervigón and Los Roques, 1991).

2.3 Isolation of liver mitochondria. Fresh livers were excised and processed according to Martinez et al. (2013). Briefly, liver tissue from one or more individuals (~1.0 g of liver tissue) were minced in an ice-cold petri dish, then homogenized in 8 mL of a sucrose-based isolation medium (250 mM Sucrose, 1 mM EGTA, 10 mM K_2PO_4 , 1 % BSA, pH = 7.4, 20°C) using an ice-cold Dounce homogenizer (Kontes, Vineland, NJ). Five passes with a loose fitting pestle were followed by two passes with a tight fitting pestle. Homogenate was transferred to 1.5 mL centrifuge tubes and centrifuged at 650 g for 10 min at 4°C to remove cellular debris and undisrupted tissue. The supernatant was collected and again centrifuged at 9,600 g for 15 min at

4°C to sediment the mitochondrial fraction. Pellets were washed with isolation medium, resuspended, and twice consecutively recollected by centrifugation at 9,600 g for 15 min at 4°C. The final pellet was suspended in 300–500 µL of isolation medium and stored on ice until assayed.

2.4 Mitochondrial respiration. To assess the thermal sensitivity of mitochondrial respiration, high-resolution respirometry systems were employed. Those systems comprised two 2.0 mL water-jacketed respirometric chambers (DW-1, Hansatech Instruments, Norfolk, England) equipped with Clark-type polarographic oxygen electrodes (C-1, Hansatech Instruments, Norfolk, England). Chamber temperature was controlled using a circulating, refrigerated water bath (E200, Lauda-Königshofen, Germany). Electrodes were calibrated in air- and nitrogen-saturated respiration medium (500 µL – see below) at each assay temperature. Respiration medium was prepared according to Martinez et al. (2013); it consisted of 100 mM KCl, 1% w/v BSA, 2 mM MgCl₂, 1mM EGTA, 25 mM K₂PO₄, and 10 mM Tris-HCl, pH = 7.5 at 20°C. Deviations in the pH of the assay medium (7.8 - 7.0) as a function of temperature were in the lower range of pH observed for teleost blood, which ranges from 8.1 to 7.6 (Cameron, 1978; Rahn and Baumgardner, 1972). Other studies evaluating mitochondrial thermal performance in teleosts have performed assays at an assay pH ranging from 7.1 (Hilton et al., 2010) to 7.5 (Johnston et al., 1998).

At each measurement temperature (10°, 20°, 30° and 40 °C), the background signal was recorded prior to mitochondrial injection. For each run, 10-50 µL of purified mitochondria (0.04-0.5 mg of mitochondrial protein) were injected into the respirometer chamber containing 500 µL of respiration medium. Bennett and Judd (1992) found a critical thermal minimum (CT_{min}) for *L. rhomboides* at 11.7°C for specimens acclimated to 22°C, therefore oxygen consumption was

monitored at assay temperatures ranging from 10-40°C. Substrate stocks were carefully prepared according to Lemieux and Gnaiger (2010). Respiration associated with the activation of complexes I and II of the electron transport system (ETS) was evaluated at each temperature regime for *L. rhomboides* and *C. crysos* following the titration protocol and techniques described by Gnaiger (2010). Briefly, non-phosphorylating respiration (LEAK) was initiated by adding 2 mM malate (M), 10 mM glutamate (G) and 5 mM pyruvate (P), which supplies electrons to complex I via production of NADH by mitochondrial dehydrogenases. Non-phosphorylating LEAK in the absence of ADP was broadly defined in this study as the respiration associated with proton conductance, proton slip and cation cycling at saturating substrate concentrations. To induce ATP synthesis via OXPHOS, 2 mM ADP was added, and convergent electron entry to the ubiquinone pool via NADH and FADH₂ was initiated by addition of 10 mM succinate (S). Contribution of complex II alone to OXPHOS was recorded after addition of the complex I inhibitor rotenone (0.5 µM).

Adjustments to the mitochondrial titration protocol allowed complex-specific data collection in subtropical species. However, the thermal sensitivity of LEAK and OXPHOS respiration rates of *E. plumieri*, *M. furnieri* and *T. goodei* (tropical species) were obtained by simultaneous activation of complexes I and II according to Martinez et al. (2013). Proton conductance increases exponentially with mitochondrial membrane potential (Divakaruni and Brand, 2011). To estimate the maximal impact of temperature on LEAK respiration rates of *E. plumieri*, *M. furnieri* and *T. goodei*, measurements were obtained at saturating substrate concentrations by simultaneous activation of complexes I and II adding 2 mM M, 10 mM G, 5 mM P and 10 mM S. Phosphorylating rates were obtained by adding 2 mM ADP to the chamber.

Complex-specific LEAK and OXPHOS rates were obtained only at 30°C, the temperature closest to habitat temperature. Complex I activity was measured in the presence of P, M and G. In a separate run, succinate dehydrogenase (complex II) activity was measured after the addition of 10 mM succinate in the presence of 0.5 μ M rotenone. The relative coupling of oxygen consumption with ATP production or respiratory control ration (RCR), was calculated from average respiration rates at each temperature by dividing OXPHOS respiration rates by the LEAK rates.

Mitochondrial protein quantification. Total protein in sample was quantified according to (Bradford, 1976), using the commercially available Better Bradford Coomassie Stain Assay (Thermo Scientific, Rockford, IL). Samples were diluted 20:1 in deionized water and absorption values were determined after 10 min incubation with a Cary 1 spectrophotometer at 20°C and λ = 595 nm. Protein values in the isolation buffer were measured, and samples were corrected for the concentration of BSA present in the isolation buffer.

2.5 Enzymatic activity. The activity of a key enzyme associated with the citric acid cycle, Citrate synthase (CS), and the enzymatic activity of an enzyme complex associated with the ETS, succinate dehydrogenase (SDH), was employed as indicator of the aerobic capacity of the homogenates. Enzymatic activity of CS was assayed from 10 μ L of resuspended mitochondrial pellet, following Childress and Somero (1979) with minor modifications (Torres et al., 2012). CS activity was assayed at 20°C in a temperature-controlled Varian Cary IE UV/Vis spectrophotometer, coupled with computer-based analysis software (CaryWin). CS activity was assayed in a solution of 42.5 mM imidazole buffer (pH = 7.2 at 20°C), 0.2 mM 5,5'-Dithio-bis 2-nitrobenzoic acid (DTNB), 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 124 μ M acetyl-CoA. To 1 mL of the assay cocktail, 10 μ L of homogenate was added, and the absorbance at 412 nm was monitored until

reaching a plateau. The enzymatic reaction was initiated by adding 12.5 μL of 40 mM oxaloacetate, and the increase in absorbance as the reduced acetyl CoA reacted with DTNB was monitored for 4 min. Succinate dehydrogenase (SDH) activity in mitochondrial extracts was followed using a spectrophotometric assay described by Munujos et. al (1993). Briefly, an Evolution 300 UV-VIS spectrophotometer (Fisher Scientific, Pittsburgh, PA) and cuvettes with a path length of 1 cm were used for the assay. The reaction mixture consisted of triethanolamine (100 mM, pH = 8.3), EDTA (0.5 mM), NaCN (2 mM), iodonitrotetrazolium chloride (INT) (2 mM), and Kolliphor EL (12 g/L). The cuvette was charged with 10 μL of isolated mitochondria dissolved in 970 μL of the reaction mixture and the assay was started through the addition of 20 μL of succinate (1.0 M) after the absorbance reading was set to zero. The change in absorbance after the addition of substrate was recorded at room temperature every second for 6 minutes at 500 nm. Succinate activity was calculated from the initial linear increase in absorbance at 500 nm and expressed as $\text{abs min}^{-1} \mu\text{g protein}^{-1}$.

2.6 Statistical analyses. Mitochondrial respiration and respiratory control ratios as a function of temperature were tested for normality (Shapiro-Wilk test) and heteroscedasticity (equal variance test) prior statistical analysis. Interactions of RCR obtained from different species and assay temperature were evaluated employing a two-way analysis of variance (ANOVA). Data significance was analyzed with a one-way ANOVA, followed by a pairwise comparison among treatments (Holm-Sidak method). Interspecific CS and SDH enzyme activities were analyzed separately using a one-way ANOVA. Interactions of enzyme activity between regions (tropical/subtropical) and life habit (demersal/pelagic) were evaluated with a two-way ANOVA, followed by a pairwise comparison between regions and life habits (Holm-Sidak method). SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) was used for the analyses.

3. Results

An interesting pattern emerged in the relationship between LEAK and OXPHOS in subtropical and tropical teleosts with pelagic and demersal lifestyles. Thermal sensitivity was more dependent on the ecology of the species (pelagic vs. demersal) than region (subtropical vs. tropical). Across the species range studied, coupling efficiency of substrate oxidation with ATP synthesis was significantly compromised at 40°C.

3.1 Thermal sensitivity of mitochondrial OXPHOS and LEAK from subtropical teleosts. Thermal sensitivity was evaluated for the demersal species *L. rhomboides* and the active pelagic *C. crysos*. As illustrated in Figure 1a, OXPHOS and LEAK rates, fueled by NADH-generating substrates, showed significant differences with temperature in *L. rhomboides*. Lowest LEAK rates were found at 10°C. From 10° to 40°C both OXPHOS and LEAK rates increased with increasing temperatures. Significant increases in OXPHOS rates were found between 10° and 40°C (one-way ANOVA, $P = 0.006$, $n = 5-8$). Similarly, LEAK rates in the absence of ADP increased significantly with temperature (one-way ANOVA, $P < 0.001$, $n = 5-8$). Complex-specific contributions to LEAK rates were similar at 30°C (Table 1). However, average mitochondrial OXPHOS rates obtained in *L. rhomboides* by supplying complex I-activating substrates were two times higher than OXPHOS rates with complex II-activating substrates (Table 1; one-way ANOVA, $P = 0.032$).

In contrast to *L. rhomboides*, OXPHOS rates for the pelagic species *C. crysos* showed a significant decrease in activity at 40°C. As shown in Figure 1b, the temperature effect on respiration rates was lower in this species and no significant differences were found among OXPHOS rates (one-way ANOVA, $P = 0.10$, $n = 3$). However, there was a significant difference between LEAK rates obtained at 40°C and those values obtained at 10°C and 20°C (one-way

ANOVA, $P = 0.001$, $n = 3$). Complex-specific activation in *C. crysos* elicited variable LEAK and OXPHOS rates, with no significant differences between respiratory states (Table 1; one-way ANOVA, $P = 0.761$).

The highest respiratory coupling ratios (RCR) values above four were found for *L. rhomboides* at assay temperatures between 10°C and 30°C (Fig. 2). At an assay temperature of 40°C, both species exhibited a significant decrease in the RCR values (one-way ANOVA, $P < 0.05$, $n = 3-8$). Changes in RCR values between 30°C and 40°C were significant for *L. rhomboides* (one-way ANOVA, $P < 0.001$, $n = 5-8$). Likewise, in the pelagic *C. crysos*, a significant decrease in coupling from 20°C to 40°C was recorded (one-way ANOVA, $P = 0.05$, $n = 3$).

3.2 Thermal sensitivity of the mitochondrial OXPHOS system from tropical teleosts. Significant changes in LEAK rates with increasing temperature were found in the demersal species *E. plumieri*. The average LEAK rate observed in *E. plumieri* increased with assay temperature (one-way ANOVA, $P < 0.001$, $n = 6-8$). Similar results were observed in OXPHOS rates; OXPHOS exhibited a significant increase from 40.02 nmol O₂ min⁻¹ mg protein⁻¹ at 10°C to 311.25 nmol O₂ min⁻¹ mg protein⁻¹ at 40°C (Figure 3a).

Maximum OXPHOS and LEAK rates at 30°C in the presence of NADH and FADH₂-generating substrates were highest in *E. plumieri* (Table 1). Respiration rates with individually activated complexes I and II in *E. plumieri* were different from complex-specific OXPHOS rates obtained with the pelagic species *T. goodei*. Within each species, complex I consistently elicited about 80% of the OXPHOS respiration rate observed with complex II activated and no significant differences were found among complex I / complex II ratios (Table 1; one-way ANOVA, $P = 0.54$, $n = 5$).

In the demersal *M. furnieri*, LEAK rates displayed higher sensitivity to increased assay temperature than those in *E. plumieri*. LEAK rates increased significantly with increasing assay temperature (Fig. 3b; one-way ANOVA, $P < 0.001$, $n = 6-7$). OXPHOS rates increased between 10°C and 30°C, then decreased at 40°C (Fig. 3b; one-way ANOVA, $P < 0.001$, $n = 6-7$).

The active pelagic *T. goodei* showed lower LEAK and OXPHOS respiration rates than those found for demersal species. LEAK respiration was significantly impacted across the thermal range assayed (Fig 3c; one-way ANOVA, $P < 0.001$, $n = 5-6$). OXPHOS increased significantly from 10°C to 30°C, then a loss of coupling was observed at 40°C, where no discernible OXPHOS rates were observed (Fig. 3c; one-way ANOVA, $P < 0.001$, $n = 5-6$).

RCR values are shown for *E. plumieri*, *M. furnieri* and *T. goodei* in Figure 4. Significant differences in RCR values across the thermal range studied were found for all tropical species, indicating a reduction in coupling efficiency at temperature extremes (Fig. 4). Average RCR values for *M. furnieri* and *E. plumieri* were high between 10°C and 30°C, significantly decreasing at 40°C. RCR values for *M. furnieri* further decreased between 20°C and 30°C. In *T. goodei*, RCR values were significantly different between all temperatures but 20°C and 30°C (Fig. 4). In summary, coupling efficiency measured in all three species varied with assay temperature. *E. plumieri* exhibited the highest coupling efficiency, *M. furnieri*'s coupling efficiency extended to the lowest temperature assayed; and *T. goodei* showed the lowest coupling

3. Citrate synthase and Succinate dehydrogenase activity in tropical and subtropical teleosts.

CS activity was lower in *C. crysos* than the tropical pelagic *T. goodei* and the demersal *E. plumieri* (Fig. 5; one-way ANOVA, $P < 0.001$, $n = 3-4$). CS activity of tropical species with

308 demersal habits was similar: CS activity was $2.85 \pm 0.13 \text{ abs min}^{-1} \mu\text{g protein}^{-1}$ and 2.03 ± 0.20
309 $\text{abs min}^{-1} \mu\text{g protein}^{-1}$ in *E. plumieri* and *M. furnieri*, respectively (Fig. 5).

310 SDH activity of demersal species was significantly lower than the SDH activity measured in
311 species with pelagic habits (two-way ANOVA; $p = 0.049$, $n = 3-4$). No significant differences
312 were detected in the SDH activities of fishes from tropical and subtropical regions (Fig. 5; two-
313 way ANOVA, $P = 0.092$, $n = 3-4$). Also, no significant interactions between region and life
314 habits were found (two-way ANOVA, $P = 0.275$, $n = 3-4$). The activity of CS showed significant
315 interactions between region and life habits (two-way ANOVA, $P = 0.033$, $n = 3-4$). Tropical
316 species exhibited significantly higher CS activity than subtropical species, independent of life
317 habits (Fig. 5; two-way ANOVA, $P = <0.001$, $n = 3-4$).

318

4. Discussion

4.1 Thermal sensitivity of the OXPHOS system in tropical teleosts. Liver was the tissue of choice for supplying the mitochondria assayed in this study. Protocols for extraction are well established, and its multiple roles in metabolism assure its performance will mirror whole-organism response to temperature. Previous studies have established its effectiveness as an indicator of thermal performance in fishes (Hardewig et al., 1999; Hilton et al., 2010; Mark et al., 2012; Martinez et al., 2013; Weinstein and Somero, 1998), and also provide a baseline for comparing the data acquired in the present study. Likely, differences in tissue-specific thermal performance will emerge as further work is performed (Kawall et al., 2002). For example, in brain samples of the subtropical *L. rhomboides*, mitochondrial OXPHOS respiration rates decreased sharply between 20°C and 30°C (Martinez, unpublished data).

Results obtained for tropical and subtropical species indicate that increasing temperatures beyond 30°C reduced the efficiency of ATP production of the OXPHOS system in most species studied. As warming trends are projected for tropical regions (Atwood et al., 1992; Roessig et al., 2004), the lack of thermal heterogeneity in tropical estuaries and coral reefs could impact long-term growth and reproductive performance of those individuals, as evidence suggests that tropical marine ectotherms are already close to their upper thermal limit (Coles et al., 1976; Maté, 1997; Stillman and Somero, 2000; Urban, 1994).

In all the species investigated, the thermal tolerance of the OXPHOS system was species-specific (Figs. 1 and 3). Although species-specific variability in OXPHOS and LEAK has been established in teleosts (Hardewig et al., 1999; Hilton et al., 2010; Mark et al., 2012; Martinez et al., 2013; Weinstein and Somero, 1998), common patterns associated with the species' lifestyle

were distinguished. More specifically, species with demersal habits exhibited a more highly coupled OXPHOS system over a wider thermal range (Figs. 1a and 3a,b), and RCR values indicate highly coupled mitochondria at temperatures ranging from 10-30°C (Figs 2 and 4). Pelagic species (Fig. 1b, 3c) showed a lower response of OXPHOS to temperature than demersal species from the same region (Figs. 1a and 3a,b), also shown by a more narrow RCR profile with temperature (Figs. 2 and 4). This type of lifestyle-based coupling has not been documented in mitochondria from warm-adapted species. Although a low coupling might be a consequence of a compromised inner mitochondrial membrane due to mitochondrial isolation procedures, the low sample variance recorded and the moderate coupling of respiration at 30°C for both tropical (Fig. 4) and subtropical (Fig. 2) pelagic species are indicative of acceptable mitochondrial integrity.

Interestingly, in warm-adapted teleosts of tropical waters, CS activity was higher than in subtropical species (Fig. 5). Within regions, species-specific CS and SDH activity was highly variable, and may reflect the variability of ATP turnover rates due to locomotion and feeding activities (Killen et al., 2010). Variability in the activity level of these traits will likely affect the rates of substrate oxidation, altering the turnover rates of reducing agents (i.e. NADH) that fuel the electron transport system.

Mitochondrial energy transduction efficiency was sensitive to assay temperature in all species. Despite some variability observed, our results indicate a breakpoint in OXPHOS respiration at 30°C for all species investigated; ADP-induced OXPHOS respiration rates at temperatures warmer than 30°C were reduced or completely impaired. A coarse integration of the coupling ratios obtained with environmental temperature data suggests that the coupling efficiency maximum, close to the 30°C treatment, correlates with the average annual temperatures found in both regions (Fig. 6). However, a finer-scale reassessment of mitochondrial energy transduction

efficiency between 30°C and 40°C will be necessary to establish a more precise breakpoint in efficiency. When comparing OXPHOS coupling efficiency of warm-adapted teleosts to those from the cold-adapted stenotherm *Pleuragramma antarctica*, (Martinez et al., 2013), it suggests that OXPHOS efficiency of liver mitochondria tracks the species' thermal environment (Fig. 6).

In tropical Pacific reef- and estuary-associated fishes, critical thermal tolerance studies have concluded that tropical fishes are well poised to overcome physiological challenges arising from gradual changes in temperature, like those associated with climate change (Eme and Bennett, 2009; Menasveta, 1981; Mora and Ospina, 2001; Mora and Ospina, 2002; Ospina and Mora, 2004; Rajaguru and Ramachandran, 2001). Those conclusions are well founded for tropical fishes that possess a critical thermal maximum above 40°C (Menasveta, 1981; Rajaguru and Ramachandran, 2001). However, our results suggests that before the onset of the loss of whole-body equilibrium, a proxy commonly employed in critical thermal maximum studies, there are sub-lethal effects that could compromise mitochondrial energy transduction in tropical species. Moreover, this imbalance is shown to be influenced by the loss of coupling between O₂ consumption and ATP generation, as observed in an increase in LEAK respiration that is not matched by OXPHOS respiration rates. In endotherms, LEAK respiration accounts for up to 30% of the O₂ consumption, whether mitochondrial LEAK is assessed *in vivo* or *in vitro* (Brand, 2000; Brand et al., 1994). Moreover, our data show that LEAK in fish mitochondria *in vitro* at temperatures close to the habitat's average are close to 30% of total O₂ consumption without playing a role in body temperature regulation, and might serve to lower the mitochondrial membrane potential and reactive oxygen species (ROS) formation (Buttemer et al., 2010; Murphy, 2009).

As it is appreciable in Figure 6, the substantial difference in thermal heterogeneity between tropical and subtropical estuaries implies that tropical species are exposed to their OXPHOS optimum far more frequently than their subtropical counterparts. Within the predicted gradual warming scenario for coastal systems (Atwood et al., 1992; Roessig et al., 2004), our results suggest that tropical estuarine teleosts could be forced to accommodate increases in water temperatures which, depending upon their adaptive capacity, would impact their long-term individual performance. Additional studies evaluating the capacity for acclimation of mitochondria to water temperatures above 30°C will be instructive, to evaluate whether the observed reduction in coupling efficiency could be improved through acclimation.

The mitochondrial energy transduction efficiency of the electron transfer and phosphorylation system is often employed as an indicator of mitochondrial function and dysfunction (Brand and Nicholls, 2011). Studies of mitochondrial dysfunction in mammalian and insect tissues indicate that membrane proton conductance constitute an important modulator of proton motive force (Brand, 2000; Brand et al., 1994; Chamberlin, 2004) and, in addition to the complex dynamics among substrate intermediaries, it is not well understood in teleosts. To further characterize the impact of global warming trends on ectotherm fitness, studies evaluating the energy transduction efficiency of isolated mitochondria and intact cells from disparate tissue types as a function of temperature are needed. Both *in vivo* as well as *in vitro* approaches have benefits and shortcomings when used to understand the mitochondrial proton circuit (Brand and Nicholls, 2011), thus further studies should address both conditions. In addition, a thorough evaluation of the thermal sensitivity of mitochondrial respiratory fluxes, coupled with measurements of membrane potential as indicators of proton motive force, will aid in understanding how mitochondrial energy transduction in ectotherms responds to temperature.

Acknowledgments

The authors of the paper would like to express their gratitude to the Biology Department at the University of Puerto Rico at Humacao, in particular to Drs. Raymond L. Tremblay, Deborah Parrilla Hernández, Miguel P. Sastre Wirshing and Sylvia M. Vélez Villamil for providing laboratory space and infrastructure to carry out all mitochondrial respiration measurements of tropical specimens. We are also grateful to Bayrex M. Rosa, Marangelly Torres Mercado and Carlos M. Zayas Santiago for their valuable assistance during tropical species sampling and processing. This manuscript is dedicated to the memory of Francisco Hernández Velázquez.

Funding

This research was funded by the National Science Foundation (grant number OCE 0727883 to J.J.T.).

Author Contributions

EM performed specimen collection, mitochondrial respirometry data collection and processing, citrate synthase measurements and contributed to manuscript drafting. EH performed succinate dehydrogenase measurements and contributed to manuscript drafting. MAM provided experimental design advice, laboratory infrastructure, data analysis and manuscript preparation. JJT provided laboratory infrastructure, instrumentation, and contributed to the experimental conception, data analysis and manuscript preparation.

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Figure Legends

Figure 1: Temperature dependent contributions of complex I to the oxidative phosphorylation (OXPHOS) system and proton leakage (LEAK) of liver mitochondria from *Lagodon rhomboides* (A) and *Caranx crysos* (B). Statistically significant differences among temperature treatments are shown with letters **a** and **b**. (one-way ANOVA on temperature, $P < 0.05$, $n = 3-8$, $\pm$ SE)

Figure 2: Respiratory control ratio (RCR) as a function of temperature of liver mitochondria from the demersal *Lagodon rhomboides* (grey bars) and the active pelagic *Caranx crysos* (black bars). No significant interactions of RCR between species and temperature were found (two-way ANOVA, $F_3 = 0.97$, $P = 0.420$). Significant differences within species are highlighted with shown with letters **a** and **b** (one-way ANOVA on temperature, $P < 0.05$, $n = 3-8 \pm$ SE).

Figure 3: Thermal sensitivity of LEAK and OXPHOS respiration of liver mitochondria from *Eugerres plumieri* (A), *Micropogonias furnieri* (B) and *Trachinotus goodei* (C). Average respiration rates obtained with the addition of pyruvate, malate, glutamate and succinate (LEAK) are shown. OXPHOS respiration rates under saturating concentrations of ADP are shown for each species; *E. plumieri* exhibited the lowest thermal sensitivity, where no breakpoint in OXPHOS respiration was found throughout the thermal regime. Significant differences are highlighted with letters; **a** is statistically different from **b** and **c**, **b** is statistically different from **c** (one-way ANOVA on temperature, $P < 0.05$, $n = 5-8 \pm$ SE).

Figure 4: Respiratory control ratios (RCR) of liver mitochondria from the demersal *Eugerres plumieri* *Micropogonias furnieri* and the active pelagic *Trachinotus goodei* as a function of assay temperature. Significant interactions of RCR between species and temperature were found (two-

way ANOVA, $F_6 = 8.89$, $P < 0.001$). Different letters indicate significant differences within species (one-way ANOVA on temperature, $P < 0.05$, $n = 5-8 \pm SE$).

Figure 5: Analysis of succinate dehydrogenase (SDH) and citrate synthase (CS) activity in liver mitochondria isolated from tropical and subtropical estuarine teleosts ($n = 3-4$). SDH activity (black bars) was calculated by observing the reduction of iodonitrotetrazolium chloride by succinate dehydrogenase for each sample. CS activity (grey bars) was assayed with the addition of oxaloacetate and the subsequent increase in absorbance from the reduced acetyl CoA-DTNB reaction. SDH and CS activities were standardized based on protein content and are expressed as absorbance per minute per mg protein. No significant interspecific differences in SDH activity were found (one-way ANOVA, $P = 0.114$, $n = 3-4$, $\pm SE$). Significant differences in interspecific CS activity are identified with letters **a** and **b** (one-way ANOVA, $P < 0.001$, $n = 3-4 \pm SE$).

Figure 6: Thermal sensitivity of the coupling of oxidative phosphorylation system with mitochondrial oxygen consumption (quantified as the RCR) in fishes from various thermal regimes. Data for *Pleuragramma antarctica* was modified after Martinez et al. (2013). Water temperature daily traces for the Tampa Bay are courtesy of the University of South Florida Coastal Ocean Monitoring and Prediction System (USF-COMPS). Tropical water temperature traces are courtesy of Dr. Ricardo Colón-Rivera. Antarctic shelf water temperature range, shown by blue slotted lines, are based on the range provided by Eastman and McCune (2000). Significant interactions were found between species and temperature (Two-way ANOVA, $F_{12} = 2.70$, $P = 0.0036$)

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Table 1: Complex specific LEAK and OXPHOS rates, and their relative contribution to the ETS in tropical and subtropical teleosts at 30°C. Oxygen consumption rates are expressed in nmol O₂ min⁻¹ mg protein⁻¹; standard error is shown.

Species	Region/Lifestyle	C-I LEAK	C-II LEAK	C-I:C-II LEAK	C-I OXPHOS	C-II OXPHOS	C-I : C-II OXPHOS
<i>Eugerres plumieri</i> (n = 5)	Tropical/demersal	23.928 ± 1.00	49.420 ± 6.494	0.506 ± 0.0860	196.01 ± 11.24	243.67 ± 44.88	0.85 ± 0.09
<i>Micropogonias furnieri</i> (n = 5)	Tropical/demersal	19.169 ± 2.653	8.613 ± 4.307	0.475 ± 0.0386	126.71 ± 19.94	153.88 ± 32.79	0.80 ± 0.01
<i>Trachinotus goodei</i> (n = 5)	Tropical/pelagic	20.260 ± 4.596	41.377 ± 7.093	0.482 ± 0.0258	94.45 ± 11.58	108.34 ± 19.16	0.89 ± 0.07
<i>Lagodon rhomboides</i> (n = 7)	Subtropical/demersal	15.0630 ± 2.4630	11.8970 ± 1.7940	1.33 ± 0.17	69.1160 ± 24.2740	37.2230 ± 7.1320	1.584±0.31
<i>Caranx crysos</i> (n = 3)	Subtropical/pelagic	11.1400 ± 2.0100	6.5830 ± 0.9140	1.829 ± 0.57	41.3430 ± 14.5850	31.6300 ± 10.1200	1.293±0.19