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1 # Physiological Performance of Warm-Adapted Marine Ectotherms:

2 # Thermal Limits of Mitochondrial Energy Transduction Efficiency

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12 **Running Title**: Mitochondrial energetics of warm-adapted teleosts

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22 **Abstract**

23 Thermal regimes in aquatic systems have profound implications for the physiology of
24 ectotherms. In particular, the effect of elevated temperatures on mitochondrial energy
25 transduction (i.e. energy from carbon substrates to ATP) in tropical and subtropical teleosts may
26 have profound consequences on organismal performance and population viability. Upper and
27 lower whole-organism critical temperatures for teleosts suggest that subtropical and tropical
28 species are not susceptible to the warming trends associated with climate change, but sub-lethal
29 effects on energy transduction efficiency and population dynamics remain unclear. The goal of
30 the present study was to compare the thermal sensitivity of processes associated with
31 mitochondrial energy transduction in liver mitochondria from the striped mojarra (*Eugerres*
32 *plumieri*), the whitemouth croaker (*Micropogonias furnieri*) and the palometa (*Trachinotus*
33 *goodei*), to those of the subtropical pinfish (*Lagodon rhomboides*) and the blue runner (*Caranx*
34 *crysos*). Mitochondrial function was assayed at temperatures ranging from 10° to 40 °C and
35 results obtained for both tropical and subtropical species showed a reduction in the energy
36 transduction efficiency of the Oxidative Phosphorylation (OXPHOS) system in most species
37 studied at temperatures below whole-organism critical temperature thresholds. Our results show
38 a loss of coupling between $O_2$ consumption and ATP production before the onset of the critical
39 thermal maxima, indicating that elevated temperature may severely impact the yield of ATP
40 production per carbon unit oxidized. As warming trends are projected for tropical regions,
41 increasing water temperatures in tropical estuaries and coral reefs could impact long-term growth
42 and reproductive performance in tropical organisms, which are already close to their upper
43 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).

67 A select number of studies have determined the critical thermal-tolerance windows in tropical
68 fishes to assess potential effects of climate change on tropical marine teleosts (Eme and Bennett,
69 2009; Eme et al., 2011; Mora and Ospina, 2001; Mora and Ospina, 2002; Ospina and Mora,
70 2004; Rajaguru and Ramachandran, 2001). Based on the wide thermal window of tolerance in
71 various estuarine species, those authors have suggested that tropical species may be better poised
72 to survive long-term warming trends associated with climate change than previously thought
73 (Eme et al., 2011). In the present study, we provide evidence that sub-lethal effects of
74 temperature at the mitochondrial level are evident, and potentially significant.

75 Our current understanding of whole-organism thermal tolerance relies heavily on critical, rather
76 than sub-lethal analyses of organismal performance as a function of temperature. The influence
77 of environmental change on mitochondrial energy transduction efficiency and resulting effects
78 on whole-organism physiological performance are poorly resolved. Studies of teleost
79 mitochondria indicate that substrate flux and oxygen consumption rates poorly estimate energy
80 balance and flow in organisms whose body temperature regularly fluctuates (Weinstein and
81 Somero, 1998; Hardewig and Pörtner, 1999; Pörtner et al., 1999; Hilton et al. 2010; Mark et al.
82 2012, Martinez et al., 2013). Since energy production relies on the efficiency of mitochondrial
83 ATP production, a detailed analysis of mitochondrial performance is likely to be a more accurate
84 indicator of temperature effects on whole-organism physiological performance than the critical
85 thermal maximum (Weinstein and Somero, 1998; Pörtner at. al., 1999; Martinez et al., 2013).

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

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

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

105

106 **2. Methodology**

107 *2.1 Chemicals.* All chemicals for respiration measurements were purchased from Sigma-Aldrich
108 (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Water for solution preparation was purified
109 with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

110 *2.2 Study systems.* Subtropical specimens were collected during the fall (October) in the southern
111 portion of Tampa Bay, Florida using hook and line. Water temperature at the collection site was
112 27.9°C. After collection, all specimens were transported in aerated 19 L containers to the
113 aquarium facility of the University of South Florida, College of Marine Science. Specimens were
114 transferred to holding tanks equipped with a flow-through water system for at least two weeks
115 prior to analysis, and fed pathogen-free frozen mysid shrimps every 48 hours. Holding tanks
116 consisted of three 570 L fiberglass rectangular tanks, and specimens were held at low densities
117 (less than 10 individuals per tank) at any given time. Temperature was controlled (28 ± 2.0°C),
118 and nutrients were monitored biweekly.

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

127 Tropical specimens were collected during the winter season (December) in neighboring waters of
128 the Punta Santiago Estuary area in Humacao, Puerto Rico. Specimens were collected using a 20-
129 meter long seine net, and later transported in aerated 19 L containers to a 190 L holding tank at
130 the University of Puerto Rico, Humacao Campus. Water temperature at the collecting site was
131 27.8°C. Specimens were held for less than 72 hours in artificial seawater at habitat salinity and
132 aquarium room temperature (25.0 ± 2.0°C) prior to experiments.

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

142 *2.3 Isolation of liver mitochondria.* Fresh livers were excised and processed according to
143 Martinez et al. (2013). Briefly, liver tissue from one or more individuals (~1.0 g of liver tissue)
144 were minced in an ice-cold petri dish, then homogenized in 8 mL of a sucrose-based isolation
145 medium (250 mM Sucrose, 1 mM EGTA, 10 mM $K_2PO_4$, 1 % BSA, pH = 7.4, 20°C) using an
146 ice-cold Dounce homogenizer (Kontes, Vineland, NJ). Five passes with a loose fitting pestle
147 were followed by two passes with a tight fitting pestle. Homogenate was transferred to 1.5 mL
148 centrifuge tubes and centrifuged at 650 *g* for 10 min at 4°C to remove cellular debris and
149 undisrupted tissue. The supernatant was collected and again centrifuged at 9,600 *g* for 15 min at

150 4°C to sediment the mitochondrial fraction. Pellets were washed with isolation medium,
151 resuspended, and twice consecutively recollected by centrifugation at 9,600 *g* for 15 min at 4°C.
152 The final pellet was suspended in 300–500 µL of isolation medium and stored on ice until
153 assayed.

154 *2.4 Mitochondrial respiration.* To assess the thermal sensitivity of mitochondrial respiration,
155 high-resolution respirometry systems were employed. Those systems comprised two 2.0 mL
156 water-jacketed respirometric chambers (DW-1, Hansatech Instruments, Norfolk, England)
157 equipped with Clark-type polarographic oxygen electrodes (C-1, Hansatech Instruments,
158 Norfolk, England). Chamber temperature was controlled using a circulating, refrigerated water
159 bath (E200, Lauda-Königshofen, Germany). Electrodes were calibrated in air- and nitrogen-
160 saturated respiration medium (500 µL – see below) at each assay temperature. Respiration
161 medium was prepared according to Martinez et al. (2013); it consisted of 100 mM KCl, 1% w/v
162 BSA, 2 mM $MgCl_2$, 1mM EGTA, 25 mM $K_2PO_4$, and 10 mM Tris-HCl, pH = 7.5 at 20°C.
163 Deviations in the pH of the assay medium (7.8 - 7.0) as a function of temperature were in the
164 lower range of pH observed for teleost blood, which ranges from 8.1 to 7.6 (Cameron, 1978;
165 Rahn and Baumgardner, 1972). Other studies evaluating mitochondrial thermal performance in
166 teleosts have performed assays at an assay pH ranging from 7.1 (Hilton et al., 2010) to 7.5
167 (Johnston et al., 1998).

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

173 monitored at assay temperatures ranging from 10-40°C. Substrate stocks were carefully prepared
174 according to Lemieux and Gnaiger (2010). Respiration associated with the activation of
175 complexes I and II of the electron transport system (ETS) was evaluated at each temperature
176 regime for *L. rhomboides* and *C. crysos* following the titration protocol and techniques described
177 by Gnaiger (2010). Briefly, non-phosphorylating respiration (LEAK) was initiated by adding 2
178 mM malate (M), 10 mM glutamate (G) and 5 mM pyruvate (P), which supplies electrons to
179 complex I via production of NADH by mitochondrial dehydrogenases. Non-phosphorylating
180 LEAK in the absence of ADP was broadly defined in this study as the respiration associated with
181 proton conductance, proton slip and cation cycling at saturating substrate concentrations. To
182 induce ATP synthesis via OXPHOS, 2 mM ADP was added, and convergent electron entry to the
183 ubiquinone pool via NADH and $FADH_2$ was initiated by addition of 10 mM succinate (S).
184 Contribution of complex II alone to OXPHOS was recorded after addition of the complex I
185 inhibitor rotenone (0.5 µM).

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

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

202 *Mitochondrial protein quantification.* Total protein in sample was quantified according to
203 (Bradford, 1976), using the commercially available Better Bradford Coomassie Stain Assay
204 (Thermo Scientific, Rockford, IL). Samples were diluted 20:1 in deionized water and absorption
205 values were determined after 10 min incubation with a Cary 1 spectrophotometer at 20°C and $\lambda$ =
206 595 nm. Protein values in the isolation buffer were measured, and samples were corrected for the
207 concentration of BSA present in the isolation buffer.

208 *2.5 Enzymatic activity.* The activity of a key enzyme associated with the citric acid cycle, Citrate
209 synthase (CS), and the enzymatic activity of an enzyme complex associated with the ETS,
210 succinate dehydrogenase (SDH), was employed as indicator of the aerobic capacity of the
211 homogenates. Enzymatic activity of CS was assayed from 10 μL of resuspended mitochondrial
212 pellet, following Childress and Somero (1979) with minor modifications (Torres et al., 2012). CS
213 activity was assayed at 20°C in a temperature-controlled Varian Cary IE UV/Vis
214 spectrophotometer, coupled with computer-based analysis software (CaryWin). CS activity was
215 assayed in a solution of 42.5 mM imidazole buffer (pH = 7.2 at 20°C), 0.2 mM 5,5'-Dithio-bis 2-
216 nitrobenzoic acid (DTNB), 1.5 mM $MgCl_2 \cdot 6H_2O$, and 124 μM acetyl-CoA. To 1 mL of the assay
217 cocktail, 10 μL of homogenate was added, and the absorbance at 412 nm was monitored until

218 reaching a plateau. The enzymatic reaction was initiated by adding 12.5 µL of 40 mM
219 oxaloacetate, and the increase in absorbance as the reduced acetyl CoA reacted with DTNB was
220 monitored for 4 min. Succinate dehydrogenase (SDH) activity in mitochondrial extracts was
221 followed using a spectrophotometric assay described by Munujos et. al (1993). Briefly, an
222 Evolution 300 UV-VIS spectrophotometer (Fisher Scientific, Pittsburgh, PA) and cuvettes with a
223 path length of 1 cm were used for the assay. The reaction mixture consisted of triethanolamine
224 (100 mM, pH = 8.3), EDTA (0.5 mM), NaCN (2 mM), iodonitrotetrazolium chloride (INT) (2
225 mM), and Kolliphor EL (12 g/L). The cuvette was charged with 10 µL of isolated mitochondria
226 dissolved in 970 µL of the reaction mixture and the assay was started through the addition of 20
227 µL of succinate (1.0 M) after the absorbance reading was set to zero. The change in absorbance
228 after the addition of substrate was recorded at room temperature every second for 6 minutes at
229 500 nm. Succinate activity was calculated from the initial linear increase in absorbance at 500
230 nm and expressed as abs $min^{-1}$ µg $protein^{-1}$.

231 *2.6 Statistical analyses*. Mitochondrial respiration and respiratory control ratios as a function of
232 temperature were tested for normality (Shapiro-Wilk test) and heteroscedasticity (equal variance
233 test) prior statistical analysis. Interactions of RCR obtained from different species and assay
234 temperature were evaluated employing a two-way analysis of variance (ANOVA). Data
235 significance was analyzed with a one-way ANOVA, followed by a pairwise comparison among
236 treatments (Holm-Sidak method). Interspecific CS and SDH enzyme activities were analyzed
237 separately using a one-way ANOVA. Interactions of enzyme activity between regions
238 (tropical/subtropical) and life habit (demersal/pelagic) were evaluated with a two-way ANOVA,
239 followed by a pairwise comparison between regions and life habits (Holm-Sidak method).
240 SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) was used for the analyses.

241

242 **3. Results**

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

248 *3.1 Thermal sensitivity of mitochondrial OXPHOS and LEAK from subtropical teleosts.* Thermal
249 sensitivity was evaluated for the demersal species *L. rhomboides* and the active pelagic *C.*
250 *crysos*. As illustrated in Figure 1a, OXPHOS and LEAK rates, fueled by NADH-generating
251 substrates, showed significant differences with temperature in *L. rhomboides*. Lowest LEAK
252 rates were found at 10°C. From 10° to 40°C both OXPHOS and LEAK rates increased with
253 increasing temperatures. Significant increases in OXPHOS rates were found between 10° and
254 40°C (one-way ANOVA, $P = 0.006$, $n = 5\text{-}8$). Similarly, LEAK rates in the absence of ADP
255 increased significantly with temperature (one-way ANOVA, $P < 0.001$, $n = 5\text{-}8$). Complex-
256 specific contributions to LEAK rates were similar at 30°C (Table 1). However, average
257 mitochondrial OXPHOS rates obtained in *L. rhomboides* by supplying complex I-activating
258 substrates were two times higher than OXPHOS rates with complex II-activating substrates
259 (Table 1; one-way ANOVA, $P = 0.032$).

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

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

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

274 *3.2 Thermal sensitivity of the mitochondrial OXPHOS system from tropical teleosts.* Significant
275 changes in LEAK rates with increasing temperature were found in the demersal species *E.*
276 *plumieri*. The average LEAK rate observed in *E. plumieri* increased with assay temperature (one-
277 way ANOVA, P < 0.001, *n* = 6-8). Similar results were observed in OXPHOS rates; OXPHOS
278 exhibited a significant increase from 40.02 nmol $O_2$ $min^{-1}$ mg $protein^{-1}$ at 10°C to 311.25 nmol
279 $O_2$ $min^{-1}$ mg $protein^{-1}$ at 40°C (Figure 3a).

280 Maximum OXPHOS and LEAK rates at 30°C in the presence of NADH and $FADH_2$-generating
281 substrates were highest in *E. plumieri* (Table 1). Respiration rates with individually activated
282 complexes I and II in *E. plumieri* were different from complex-specific OXPHOS rates obtained
283 with the pelagic species *T. goodei*. Within each species, complex I consistently elicited about
284 80% of the OXPHOS respiration rate observed with complex II activated and no significant
285 differences were found among complex I / complex II ratios (Table 1; one-way ANOVA, P =
286 0.54, *n* = 5).

287 In the demersal *M. furnieri*, LEAK rates displayed higher sensitivity to increased assay
288 temperature than those in *E. plumieri*. LEAK rates increased significantly with increasing assay
289 temperature (Fig. 3b; one-way ANOVA, P < 0.001, *n* = 6-7). OXPHOS rates increased between
290 10°C and 30°C, then decreased at 40°C (Fig. 3b; one-way ANOVA, P <0.001, *n* = 6-7).

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

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

305 *3. Citrate synthase and Succinate dehydrogenase activity in tropical and subtropical teleosts.*
306 CS activity was lower in *C. crysos* than the tropical pelagic *T. goodei* and the demersal *E.*
307 *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 ±0.13 abs $min^{-1}$ μg $protein^{-1}$ and 2.03 ± 0.20

309 abs $min^{-1}$ μg $protein^{-1}$ 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

319 **4. Discussion**

320 *4.1 Thermal sensitivity of the OXPHOS system in tropical teleosts*. Liver was the tissue of choice
321 for supplying the mitochondria assayed in this study. Protocols for extraction are well
322 established, and its multiple roles in metabolism assure its performance will mirror whole-
323 organism response to temperature. Previous studies have established its effectiveness as an
324 indicator of thermal performance in fishes (Hardewig et al., 1999; Hilton et al., 2010; Mark et al.,
325 2012; Martinez et al., 2013; Weinstein and Somero, 1998), and also provide a baseline for
326 comparing the data acquired in the present study. Likely, differences in tissue-specific thermal
327 performance will emerge as further work is performed (Kawall et al., 2002). For example, in
328 brain samples of the subtropical *L. rhomboides,* mitochondrial OXPHOS respiration rates
329 decreased sharply between 20°C and 30°C (Martinez, unpublished data).

330 Results obtained for tropical and subtropical species indicate that increasing temperatures
331 beyond 30°C reduced the efficiency of ATP production of the OXPHOS system in most species
332 studied. As warming trends are projected for tropical regions (Atwood et al., 1992; Roessig et al.,
333 2004), the lack of thermal heterogeneity in tropical estuaries and coral reefs could impact long-
334 term growth and reproductive performance of those individuals, as evidence suggests that
335 tropical marine ectotherms are already close to their upper thermal limit (Coles et al., 1976;
336 Maté, 1997; Stillman and Somero, 2000; Urban, 1994).

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

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

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

357 Mitochondrial energy transduction efficiency was sensitive to assay temperature in all species.
358 Despite some variability observed, our results indicate a breakpoint in OXPHOS respiration at
359 30°C for all species investigated; ADP-induced OXPHOS respiration rates at temperatures
360 warmer than 30°C were reduced or completely impaired. A coarse integration of the coupling
361 ratios obtained with environmental temperature data suggests that the coupling efficiency
362 maximum, close to the 30°C treatment, correlates with the average annual temperatures found in
363 both regions (Fig. 6). However, a finer-scale reassessment of mitochondrial energy transduction

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

368 In tropical Pacific reef- and estuary-associated fishes, critical thermal tolerance studies have
369 concluded that tropical fishes are well poised to overcome physiological challenges arising from
370 gradual changes in temperature, like those associated with climate change (Eme and Bennett,
371 2009; Menasveta, 1981; Mora and Ospina, 2001; Mora and Ospina, 2002; Ospina and Mora,
372 2004; Rajaguru and Ramachandran, 2001). Those conclusions are well founded for tropical
373 fishes that possess a critical thermal maximum above 40°C (Menasveta, 1981; Rajaguru and
374 Ramachandran, 2001). However, our results suggests that before the onset of the loss of whole-
375 body equilibrium, a proxy commonly employed in critical thermal maximum studies, there are
376 sub-lethal effects that could compromise mitochondrial energy transduction in tropical species.
377 Moreover, this imbalance is shown to be influenced by the loss of coupling between $O_2$
378 consumption and ATP generation, as observed in an increase in LEAK respiration that is not
379 matched by OXPHOS respiration rates. In endotherms, LEAK respiration accounts for up to
380 30% of the $O_2$ consumption, whether mitochondrial LEAK is assessed *in vivo* or *in vitro* (Brand,
381 2000; Brand et al., 1994). Moreover, our data show that LEAK in fish mitochondria *in vitro* at
382 temperatures close to the habitat's average are close to 30% of total $O_2$ consumption without
383 playing a role in body temperature regulation, and might serve to lower the mitochondrial
384 membrane potential and reactive oxygen species (ROS) formation (Buttemer et al., 2010;
385 Murphy, 2009).

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

395 The mitochondrial energy transduction efficiency of the electron transfer and phosphorylation
396 system is often employed as an indicator of mitochondrial function and dysfunction (Brand and
397 Nicholls, 2011). Studies of mitochondrial dysfunction in mammalian and insect tissues indicate
398 that membrane proton conductance constitute an important modulator of proton motive force
399 (Brand, 2000; Brand et al., 1994; Chamberlin, 2004) and, in addition to the complex dynamics
400 among substrate intermediaries, it is not well understood in teleosts. To further characterize the
401 impact of global warming trends on ectotherm fitness, studies evaluating the energy transduction
402 efficiency of isolated mitochondria and intact cells from disparate tissue types as a function of
403 temperature are needed. Both *in vivo* as well as *in vitro* approaches have benefits and
404 shortcomings when used to understand the mitochondrial proton circuit (Brand and Nicholls,
405 2011), thus further studies should address both conditions. In addition, a thorough evaluation of
406 the thermal sensitivity of mitochondrial respiratory fluxes, coupled with measurements of
407 membrane potential as indicators of proton motive force, will aid in understanding how
408 mitochondrial energy transduction in ectotherms responds to temperature.

409

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## Author Contributions

EM performed specimen collection, mitochondrial respirometry data collection and processing, citrate synthase measurements and contributed to manuscript drafting. EH preformed 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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549 Antarctic fish *Trematomus bernacchii*. Journal of Comparative Physiology B: Biochemical,
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554 **Figure Legends**

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

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

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

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

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

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

586 **Figure 6**: Thermal sensitivity of the coupling of oxidative phosphorylation system with
587 mitochondrial oxygen consumption (quantified as the RCR) in fishes from various thermal
588 regimes. Data for *Pleuragramma antarctica* was modified after Martinez et al. (2013). Water
589 temperature daily traces for the Tampa Bay are courtesy of the University of South Florida
590 Coastal Ocean Monitoring and Prediction System (USF-COMPS). Tropical water temperature
591 traces are courtesy of Dr. Ricardo Colón-Rivera. Antarctic shelf water temperature range, shown
592 by blue slotted lines, are based on the range provided by Eastman and McCune (2000).
593 Significant interactions were found between species and temperature (Two-way ANOVA, $F_{12}$ =
594 2.70, $P = 0.0036$)

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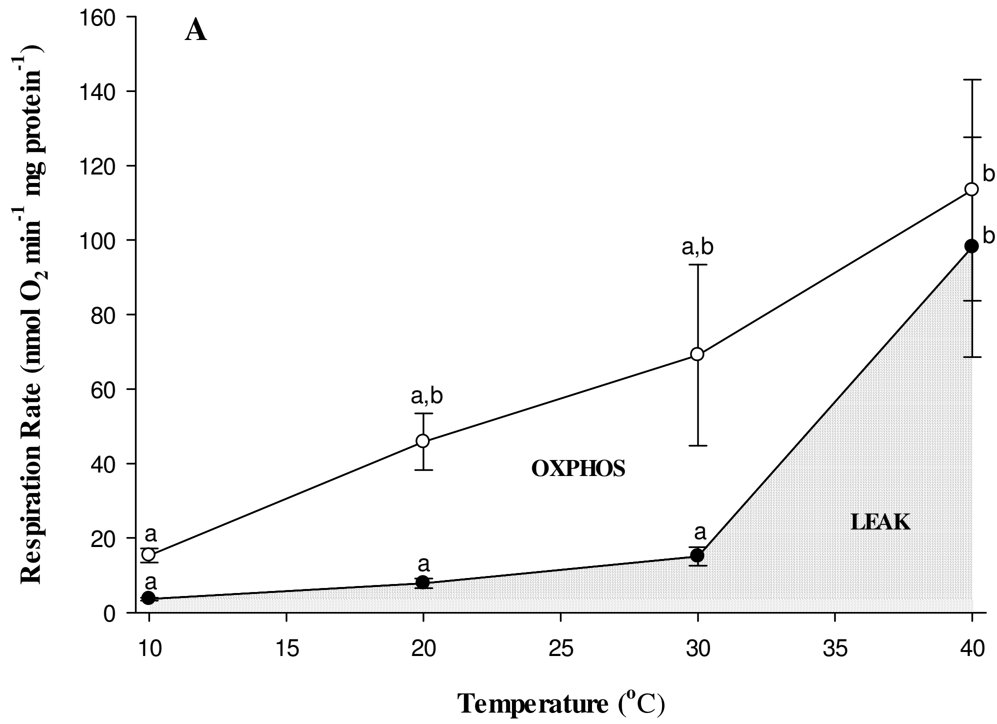
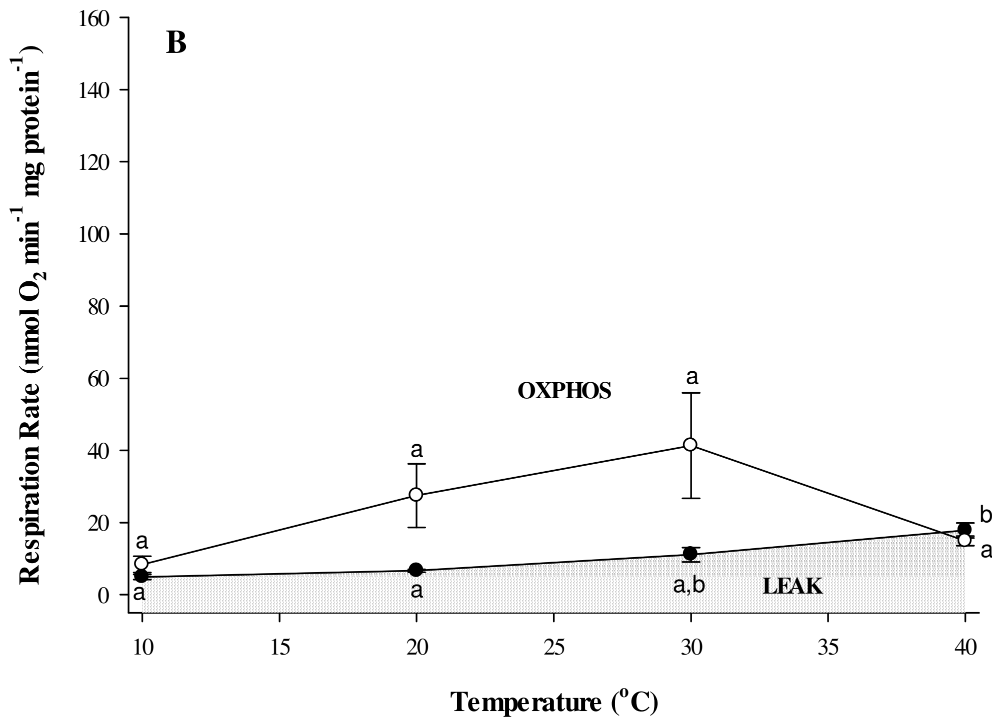
597 **Fig. 1**

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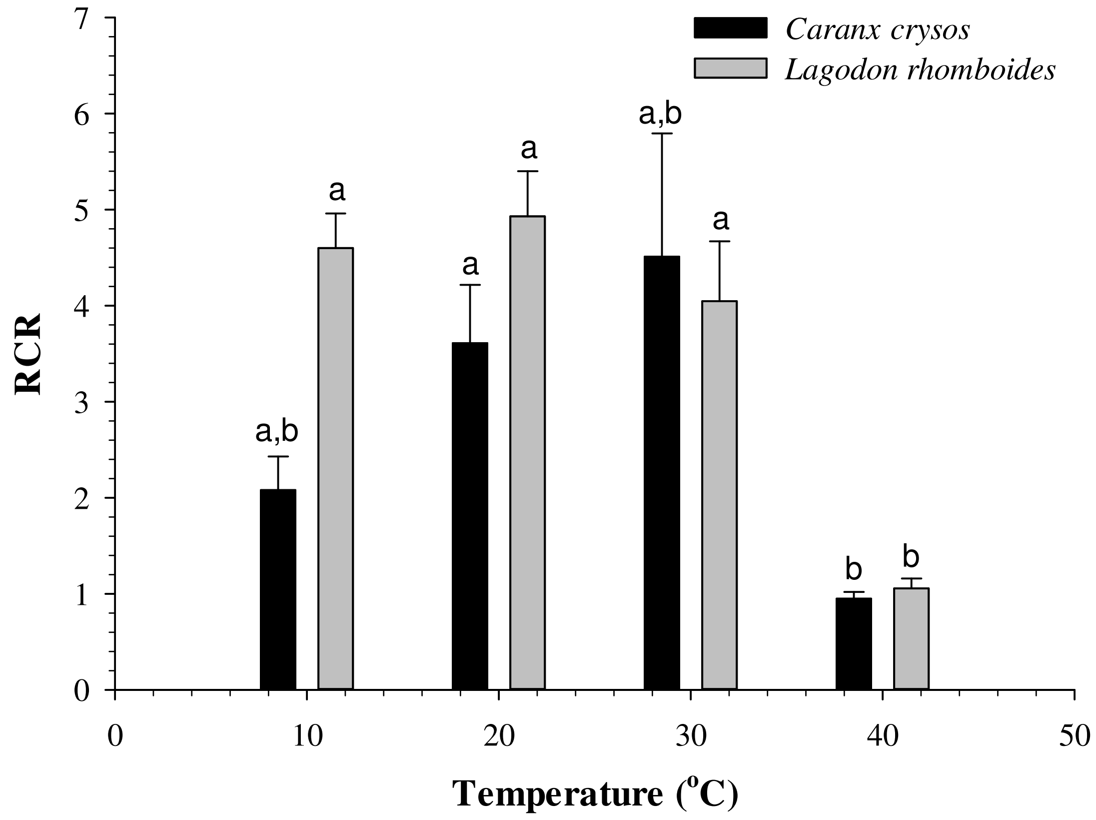
600 **Fig. 2**

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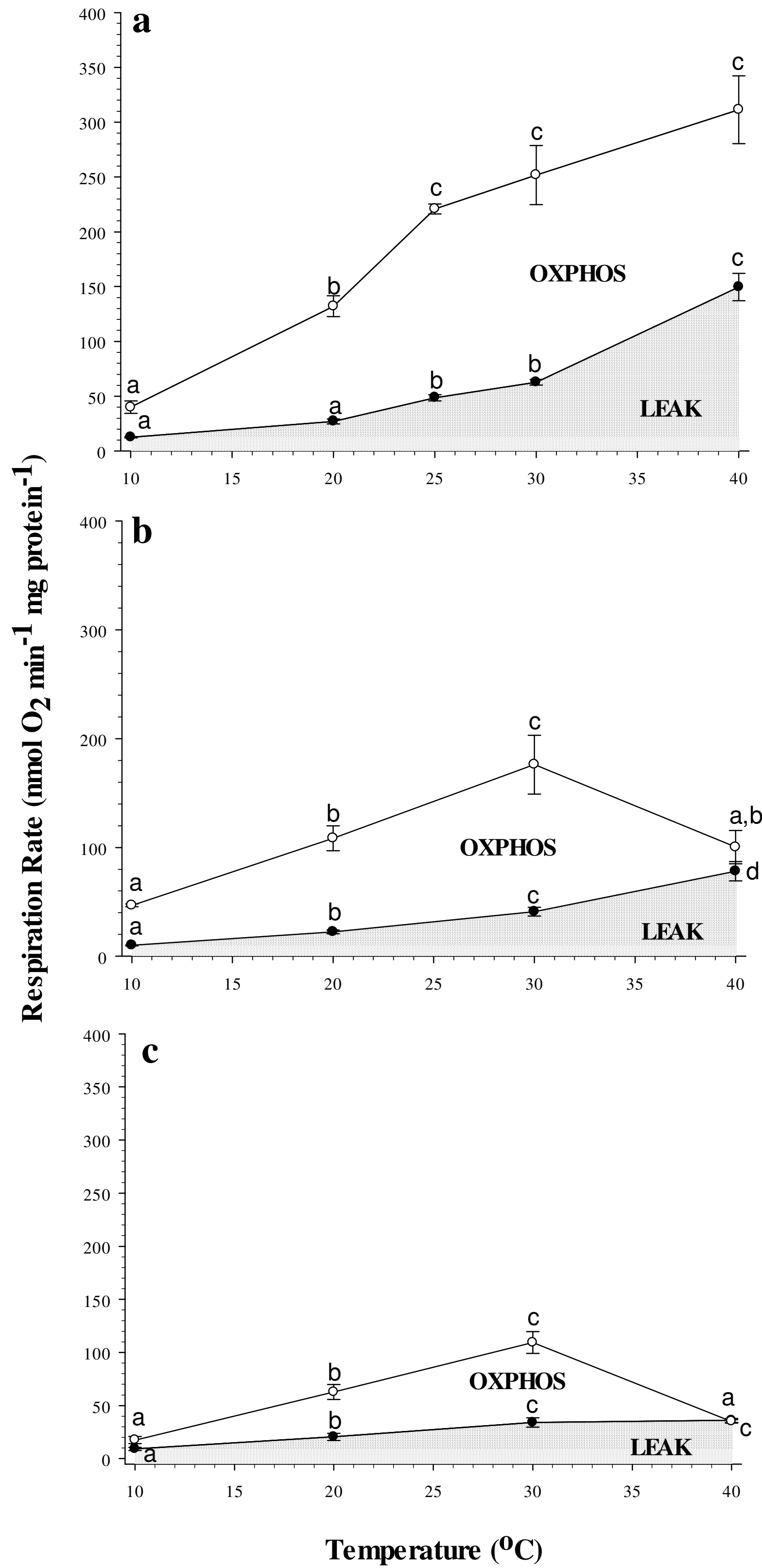
603 **Fig. 3**

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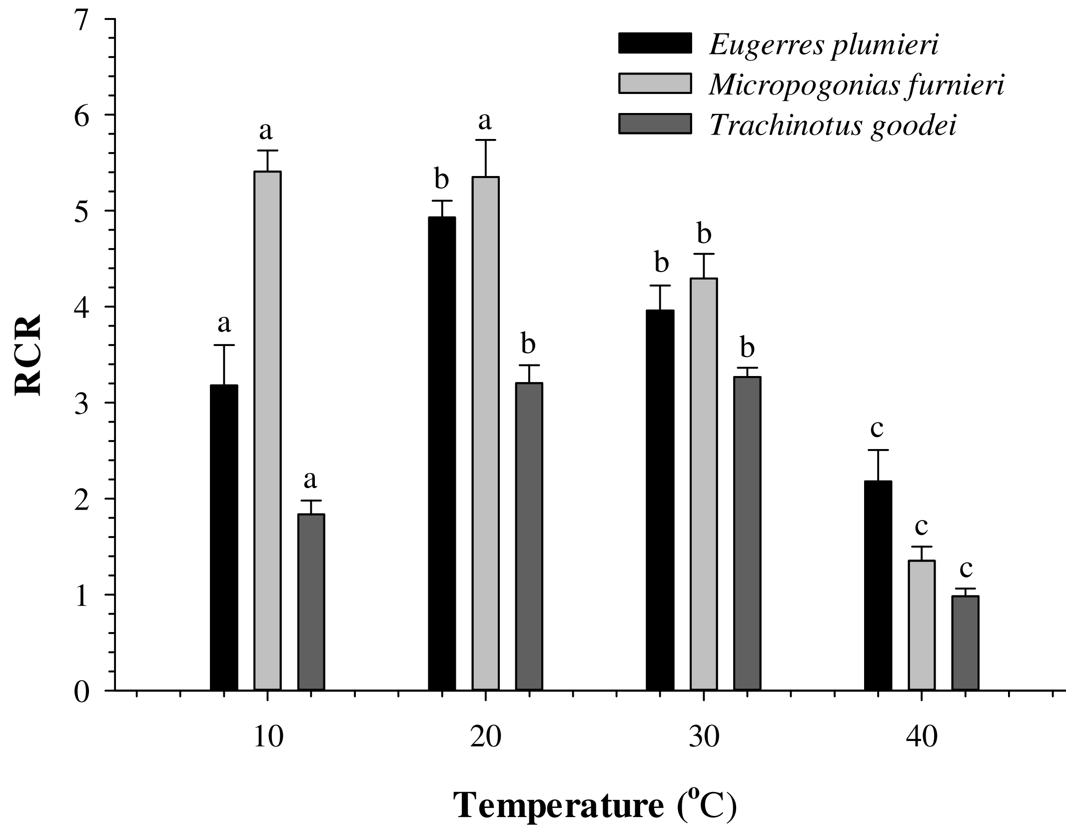
605 **Fig. 4**

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608 **Fig. 5**

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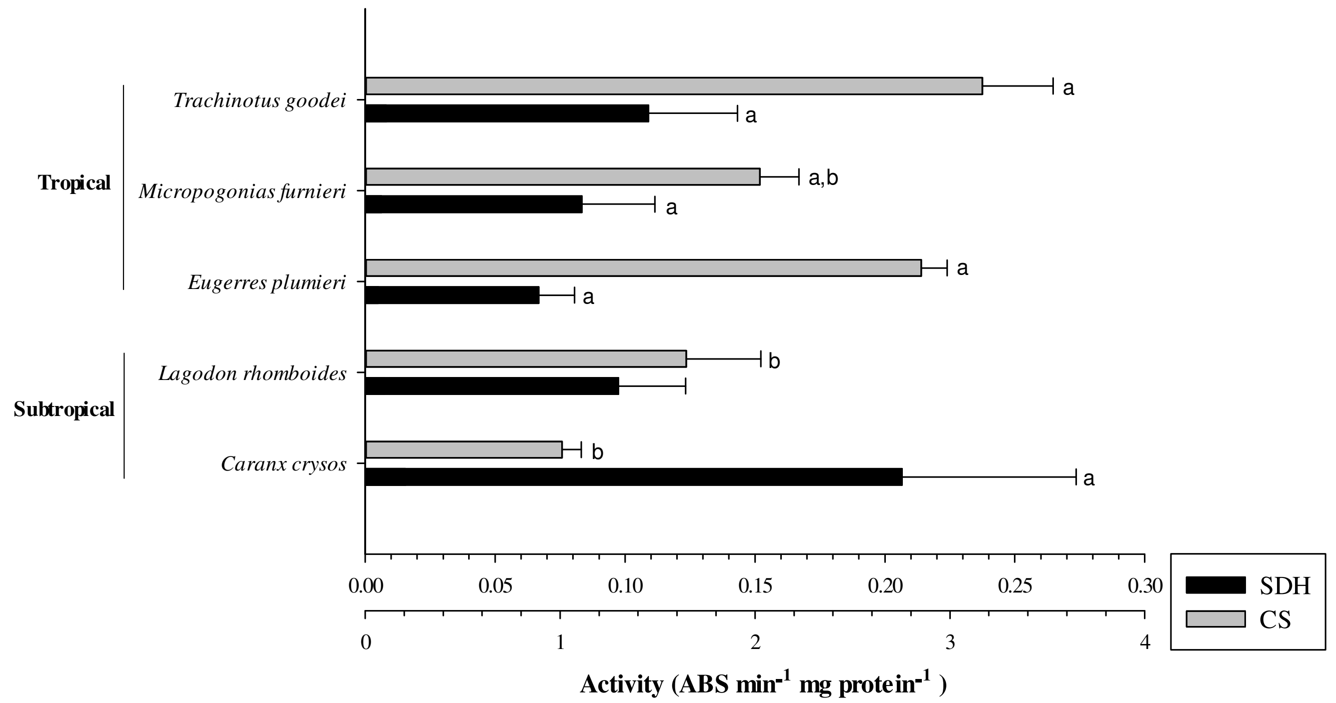


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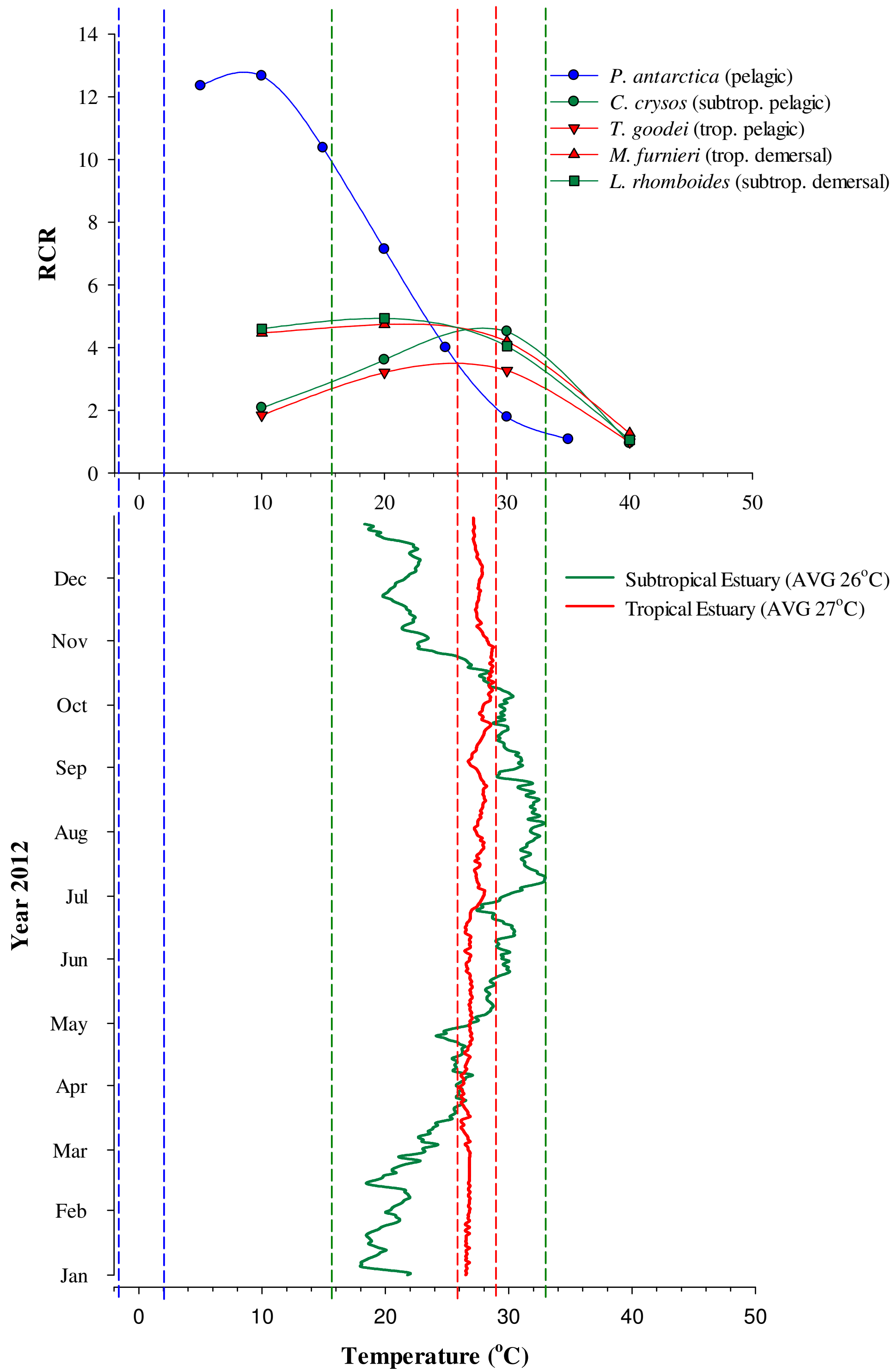
613 **Fig. 6**

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616 **Table 1:** Complex specific LEAK and OXPHOS rates, and their relative contribution to the ETS

617 in tropical and subtropical teleosts at 30°C. Oxygen consumption rates are expressed in nmol $O_2$

618 $min^{-1}$ 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 |
|---|---|---|---|---|---|---|---|
| *Eugerres plumieri* (*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 |
| *Micropogonias furnieri* (*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 |
| *Trachinotus goodei* (*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 |
| *Lagodon rhomboides* (*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 |
| *Caranx crysos* (*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 |

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