

## Energy metabolism of juvenile scallops *Nodipecten subnodosus* under acute increased temperature and low oxygen availability

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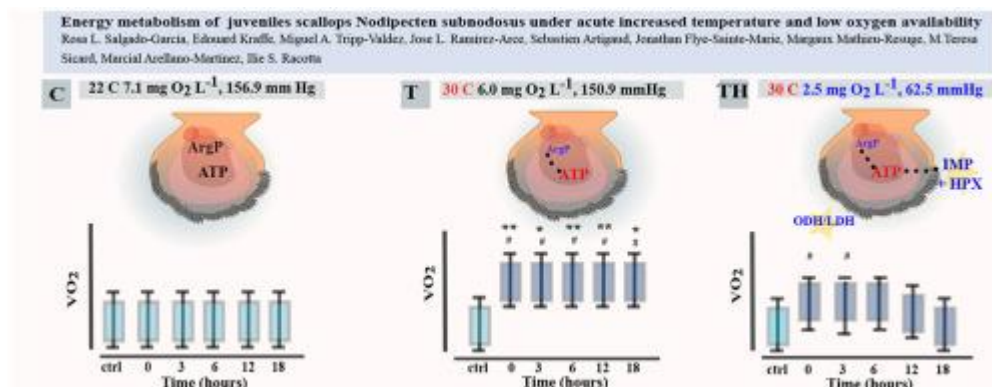
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### Abstract :

High temperature increases energy demand in ectotherms, limiting their physiological capability to cope with hypoxic events. The present study aimed to assess the metabolic tolerance of juvenile *Nodipecten subnodosus* scallops to acute hyperthermia combined with moderate hypoxia. A previous study showed that juveniles exhibited a high upper temperature limit (32 °C), but the responses of juveniles to combined hyperthermia and low dissolved oxygen are unknown. Scallops were exposed to control conditions (treatment C: 22 °C, ~7.1 mg O<sub>2</sub> L<sup>-1</sup> or PO<sub>2</sub> 156.9 mmHg), acute hyperthermia under normoxia (treatment T: 30 °C, ~6.0 mg O<sub>2</sub> L<sup>-1</sup> or PO<sub>2</sub> 150.9 mmHg) or acute hyperthermia plus hypoxia (treatment TH: 30 °C, ~2.5 mg O<sub>2</sub> L<sup>-1</sup> or PO<sub>2</sub> 62.5 mmHg) for 18 h. In T, juveniles exhibited an enhanced oxygen consumption, together with a decrease in adenylate energy charge (AEC) and arginine phosphate (ArgP), and with no changes in metabolic enzyme activity in the muscle. In TH, scallops maintained similar AEC and ArgP levels in muscle as those observed in T treatment. This response occurred along with the accumulation of inosine monophosphate and hypoxanthine. Besides, reduced citrate synthase and pyruvate kinase activities, enhanced hexokinase activity, and a higher octopine dehydrogenase/lactate dehydrogenase ratio in the mantle indicated the onset of anaerobiosis in TH. These responses indicate that juvenile scallops showed tissue-specific compensatory responses regarding their energy balance under moderate hypoxia at high temperatures. Our results give an insight into the tolerance limit of this species to combined hyperthermia and hypoxia in its northern limit of distribution.

## Graphical abstract



## Highlights

► Oxygen consumption increases at high temperatures, a response affected by hypoxia. ► Adenylate energy charge decreases, buffered by phosphagen hydrolysis and adenylate kinase. ► Tissue-specific responses of metabolic enzyme activity under hyperthermia and hypoxia.

**Keywords :** Bivalve, Growth, Hypoxia, Tolerance, Warming

48 **1. Introduction**

49 Temperature regulates virtually all physiological processes in marine ectotherms (Pörtner,  
50 2001; Abele, 2012); it can vary following diurnal, tidal, and seasonal changes. Marine  
51 ectotherms experience frequent hyperthermia events, which are expected to increase due to  
52 anthropogenic activities (IPCC, 2014). Commonly, marine organisms cope with the  
53 combined effects of rising temperatures and other environmental fluctuations. Among  
54 them, ocean warming usually occurs along with moderate ( $\leq 5 \text{ mg O}_2 \text{ L}^{-1}$ ) or severe ( $\leq 2 \text{ mg}$   
55  $\text{O}_2 \text{ L}^{-1}$ ) hypoxic events, or even almost complete oxygen depletion (anoxia), which can  
56 impair the physiological performance of organisms and affect their behavior, distribution,  
57 and abundance (Burnett and Stickle, 2001; Vaquer-Sunyer and Duarte, 2008; Basso et al.,  
58 2015).

59 Marine ectotherms may exhibit a wide range of strategies to mitigate physiological impacts  
60 and maintain energy balance when facing high temperature and hypoxic events, according  
61 to the intensity and duration of these suboptimal conditions (Vaquer-Sunyer and Duarte,  
62 2008; Sussarellu et al., 2013; Dudognon et al., 2014; Gurr et al., 2018; Bock et al., 2019).  
63 Animals increase both ventilation and circulation rates to support oxygen supply and meet  
64 the increased oxygen demand at higher temperature (Pörtner, 2001). Insufficient oxygen  
65 supply due to limited physiological capacities (functional hypoxia) or low oxygen levels in  
66 the habitat (environmental hypoxia), can compromise the energy obtained from aerobic  
67 metabolism, affecting several physiological processes (e. g., growth) (Pörtner and  
68 Langenbuch, 2005; Anestis et al., 2010; Verberk et al., 2016). Impairment of oxidative  
69 metabolism can lead to a partial switch to anaerobic pathways to maintain the adenylate  
70 energy charge (AEC) (Isani et al., 1997). In bivalve mollusks, arginine phosphate (ArgP)  
71 and carbohydrates are predominant energy reserves to cope with energy costs under these  
72 environmental constraints (Chih and Ellington, 1983; Guppy et al., 1994; Lannig et al.,  
73 2010; van Os et al., 2012; Ivanina et al., 2016; Stevens and Gobler, 2018). Metabolic  
74 responses to high temperature and hypoxia are also tissue-specific according to their role in  
75 ventilation, oxygen transport, and energy allocation for locomotion, growth, or  
76 reproduction (Chih and Ellington, 1983; Hochachka and Lutz, 2001; Brokordt et al., 2013;  
77 Gurr et al., 2018). Metabolic responses of the adductor muscle of scallops facing

78 hyperthermia alone or combined with hypoxia have been documented (for reviews, refer to  
79 Guderley, 2004; Götze et al., 2020). However, other tissues such as the mantle, which is  
80 involved in the perception of physical and environmental stimulus, have been poorly  
81 documented in these animals. In bivalves, the mantle is a sophisticated structure usually  
82 covering up to 40 % of scallop body mass, in close contact with the surrounding  
83 environment. It is mainly involved in the calcification of valves and the regulation of the  
84 water flux through the body. Also, the mantle of scallops is an aerobic tissue that has  
85 sensory structures (tentacles, eyes-stalks) that are highly responsive to surrounding  
86 environmental changes (David et al., 2005; Seibel et al., 2014; Artigaud et al., 2015;  
87 Speiser and Wilkens, 2016). However, the metabolic responses in the mantle of scallops to  
88 such changes are still poorly understood.

89 The lion's paw scallop, *Nodipecten subnodosus* (Sowerby, 1835), is distributed in coastal  
90 waters along the Pacific coast from the Ojo de Liebre lagoon, Baja California Sur, Mexico,  
91 to Peru (Keen, 1972). Wild populations from Ojo de Liebre had been harvested as a high-  
92 quality food product for several decades, until it started to decline in 2010 for reasons not  
93 yet well known (Ruíz-Verdugo et al., 2016; Velázquez-Abunader et al., 2016). In addition,  
94 reduced growth rates and mortality events in cultured populations of *N. subnodosus* from  
95 the north Pacific coast of the Baja California Peninsula have been frequently reported  
96 during summer (Osuna-García et al., 2008; Koch et al., 2015). *N. subnodosus* juveniles  
97 exhibit fast growth rates during the first two years (Villalejo-Fuerte et al., 2004), showing  
98 the highest energy balance (scope for growth) at 22 °C and an upper thermal limit close to  
99 32 °C (González-Estrada, 2003). As juveniles allocate significant energy to growth, we  
100 hypothesized that they are particularly responsive to increased temperature combined with  
101 low dissolved oxygen availability. To explore this hypothesis, the present study evaluated  
102 the metabolic capacities of juvenile *N. subnodosus* facing the combination of high  
103 temperature (30 °C) and moderate hypoxia (2.5 mg O<sub>2</sub> L<sup>-1</sup>) during acute challenges (18 h).  
104 We analyzed oxygen consumption rate, energy content, and the activities of aerobic and  
105 anaerobic enzymes, comparing the specific responses of the adductor muscle and the  
106 mantle to both environmental drivers.

## 107 **2. Materials and Methods**

### 108 *2.1 Environmental Data Collection*

109 Temperature (°C) and dissolved oxygen (DO) records were obtained from the culture site  
110 (<2 m depth) in the Ojo de Liebre lagoon, state of Baja California Sur (BCS), Mexico  
111 (27°45'00" N; 114°15'00" W) every 30 minutes using autonomous data loggers (miniDOT,  
112 Precision, Measurement Engineering Inc., US) from 16 November 2016 to 30 June 2017.  
113 One week before scallop collection in the field, the mean, minimum, and maximum  
114 temperatures were 20 °C, 13.6 °C, and 27.8 °C, respectively. The mean dissolved oxygen  
115 level was 8.8 mg O<sub>2</sub> L<sup>-1</sup>, ranging between 2.9 and 11 mg O<sub>2</sub> L<sup>-1</sup> (Fig. 1).

### 116 *2.2 Animal Collection and Maintenance*

117 Ten-month-old juveniles of *N. subnodosus* (17.3 mm ± 0.3 mm, shell height; mean ± SE),  
118 colorless and with an incipient gonad (gonadosomatic index 1.8 % ± 0.2 %) were collected  
119 in the summer of 2017 (01 July 2017) from a cultured population maintained in a  
120 suspended system (<2 m depth) located at the Ojo de Liebre lagoon, BCS. Scallops were  
121 transported to the *Centro de Investigaciones Biológicas del Noroeste* (CIBNOR) in the city  
122 of La Paz, BCS, Mexico, in polyurethane boxes with precooled aerated seawater from the  
123 culture site maintained at the mean SST in the lagoon (22 °C ± 1 °C). Seawater temperature  
124 in the container was continuously monitored during transportation (12 hours) using an  
125 autonomous data logger (HOBO, Onset UA-002-64, US). Temperature was maintained at  
126 22 °C ± 1 °C by slowly adding precooled and aerated seawater collected from the culture  
127 site from a second container using a water pump (~50 mL min<sup>-1</sup>). Once in the laboratory,  
128 animals were transferred to aquariums with filtered seawater at 22 °C ± 1 °C at a density of  
129 4 scallops L<sup>-1</sup>. During the acclimation period (eight days), scallops were kept at controlled  
130 conditions of photoperiod (12 L:12 D), temperature (HOBO, Onset UA-002-64, US),  
131 salinity (35-36 ppm; Extech Instruments, Waltham, US), and DO (7 mg O<sub>2</sub> L<sup>-1</sup>; Presens,  
132 Germany). The microalgae diet consisted of 90,000 cells mL<sup>-1</sup>, *Isochrysis galbana*, and  
133 *Chaetoceros calcitrans* (1:1) supplied and monitored daily using a particle counter  
134 (Multisizer, Beckman, US). No scallop mortality was observed during transportation and  
135 maintenance under laboratory conditions.

### 136 2.3 Experimental Design and Oxygen Consumption Measurements

137 The thermal treatments used in this study were based on seawater temperature data  
138 recorded at the culture site from 16 November 2016 to 30 June 2017. We selected 30 °C  
139 as the hyperthermia condition, which is higher than the maximum temperature recorded in  
140 the field (Fig.1), but lower than the upper thermal lethal limit reported for *N. subnodosus*  
141 juveniles (32 °C, González-Estrada, 2003). The hypoxic condition was selected according  
142 to the minimum dissolved oxygen level recorded in the culture system during the scallop  
143 culture period (Fig. 1).

144 Three experimental groups were exposed to different temperature-DO regimes. For each  
145 regime (treatment), ten scallops were randomly selected, cleaned of epibionts, and placed  
146 individually in flow-through glass respiration chambers (60 mL min<sup>-1</sup>) under controlled  
147 conditions of temperature (22 °C ± 1 °C) and DO (7.4 mg O<sub>2</sub> L<sup>-1</sup>). Scallops were fasted for  
148 12 hours before the start of the experiments. The control group (C) was kept at constant  
149 temperature (22 °C ± 1 °C) under normoxia (7.1 mg O<sub>2</sub> L<sup>-1</sup>) throughout the experiments.  
150 The second group of scallops (treatment T) was exposed to acute hyperthermia under  
151 normoxic conditions (5.4 mg O<sub>2</sub> L<sup>-1</sup>). To this end, the temperature was increased gradually  
152 (1 °C h<sup>-1</sup>) from 22 °C to the target temperature (30 °C), which was then kept constant for 18  
153 hours. In the third group (treatment TH), scallops were exposed to acute hyperthermia (30  
154 °C) in the same way as the second group (T). Then, the dissolved oxygen level of seawater  
155 was decreased gradually in steps of 1.5 mg O<sub>2</sub> L<sup>-1</sup> every two hours until reaching a final  
156 concentration of 2.5 mg O<sub>2</sub> L<sup>-1</sup>, and was subsequently kept constant for 12 hours (18 hours  
157 from the beginning until the end of the experiment (Suppl. Fig. 1). In a preliminary  
158 experiment, 50 % mortality of juvenile scallops (*n* = 10) was recorded when animals were  
159 acutely subjected to 1 mg O<sub>2</sub> L<sup>-1</sup> at 30 °C for 16 h.

160 Seawater DO level and temperature were controlled by an in-house computer system using  
161 data loggers (HOBO, Onset UA-002-64, US) and oxygen microsensors (Microx TX2,  
162 Presens, Germany), respectively (Ulaje et al., 2020).

163 The standard oxygen consumption of animals (VO<sub>2</sub>) was recorded before starting the  
164 experiment (22 °C) as a baseline condition of animals at the acclimation temperature (*ctrl*)  
165 and at 0, 3, 6, 12, and 18 hours after reaching 30 °C for conditions T and TH. During VO<sub>2</sub>

166 measurements, respiration chambers were covered with a mesh to maintain the  
167 experimental animals undisturbed. In each group, oxygen consumption was measured using  
168 an oxygen microsensor (Microx TX2, Presens, Germany) connected to the outflow of the  
169 respiration chamber for 2 minutes by switching between animal chambers at each sampling  
170 time. A chamber with an empty shell was used as a blank to correct for background  
171 respiration by microorganisms (Rosewarne et al., 2016). Standard oxygen consumption  
172 rates ( $VO_2$ ) were calculated as follows:

173

$$VO_2 = \frac{(\Delta O_2 \times V_f)}{M}$$

174

175 Where  $\Delta O_2$  is dissolved oxygen level in the respiration chamber containing one undisturbed  
176 animal ( $\mu\text{mol O}_2 \text{L}^{-1}$ ), corrected according to the DO level in the blank respiration chamber  
177 (empty shell):  $\Delta O_2 = O_{2 \text{ blank}} - O_{2 \text{ animal}}$ ;  $V_f$  is flow rate ( $\text{L h}^{-1}$ ), and  $M$  is total soft tissue wet  
178 mass (g).

#### 179 *2.4 Tissue Sampling, Metabolite Extraction, and Quantification*

180 At the end of the experiments, scallops were carefully removed from the respiration  
181 chambers, and the adductor muscle and mantle were quickly excised, weighed, and snap-  
182 frozen in liquid nitrogen. Frozen samples were ground to a fine powder with a ball mill  
183 mixer (MM400, Retsch, Germany) previously cooled with liquid nitrogen.

184 Nucleotides were extracted from grounded samples of adductor muscle (100 mg) and  
185 analyzed by reverse-phase HPLC (model 1100, Agilent Technologies, Palo Alto, CA),  
186 according to the methods described by Moal et al. (1989) modified by Robles-Romo et al.  
187 (2016). The standards used for adenosine monophosphate (AMP), adenosine diphosphate  
188 (ADP), adenosine triphosphate (ATP), guanosine triphosphate (GTP), inosine  
189 monophosphate (IMP) and hypoxanthine (HX) identification were purchased from Sigma-  
190 Aldrich-Merck, St. Louis, MO. Adenylate energy charge (AEC) was estimated according to  
191 Atkinson (1968) as follows:  $AEC = [(ATP + 0.5ADP) / (ATP + ADP + AMP)]$ . The  
192  $ATP/ADP$  ratio and the equilibrium constant of the adenylate kinase-catalyzed reaction  $K_{eq}$   
193  $= (ATP \cdot AMP) / ADP^2$  were also calculated according to Raffin and Thébault (1996).

194 Arginine phosphate (ArgP) was analyzed from the same neutralized extract of adductor  
195 muscle used for nucleotide quantification according to Viant et al. (2001) with  
196 modifications by Robles-Romo et al. (2016) by reverse phase HPLC (model 1100, Agilent  
197 Technologies, Palo Alto, CA). ArgP identification and quantification were performed at  
198 254 nm using a commercial standard with a known concentration (Santa Cruz  
199 Biotechnology, Santa Cruz, CA). All HPLC-grade reagents were purchased from Fermont.

### 200 *2.5 Enzyme Activity*

201 Activities of the metabolic enzymes hexokinase (HK), pyruvate kinase (PK), cytochrome c  
202 oxidase (CCO), citrate synthase (CS), lactate dehydrogenase (LDH), octopine  
203 dehydrogenase (ODH), and arginine kinase (AK) were quantified in fifty milligrams of  
204 frozen powder of adductor muscle and mantle samples. Tissues were homogenized with  
205 specific buffers (1:10, v/v) for each enzyme using a Polytron device (PT 6100D,  
206 Kinematica AG, Switzerland) at 180 g for 30 s at 4 °C (refer to Salgado-García et al.,  
207 2020). All homogenates were immediately sonicated (Q125-110, Qsonica Sonicators,  
208 Newtown, CT) for 30 s and a 30 % output at 4 °C. For the mantle, extracts were  
209 additionally centrifugated at 900 g and 4 °C for 5 min. Enzyme activity was expressed as U  
210 g<sup>-1</sup> tissue (wet weight), where U is  $\mu\text{mol}$  of substrate converted to product per minute.  
211 Conditions for the analysis of adductor muscle and mantle samples are described by  
212 Salgado-García et al. (2020). Only for CCO and CS activities in the mantle, phosphate and  
213 Tris-HCl buffers were changed to 200 mM Imidazole, 5mM ethylenediaminetetraacetic  
214 acid (EDTA), 5 mM (ethylene glycol tetraacetic acid (EGTA), 0.1M sodium fluoride  
215 (NaF), 0.1 % Triton X100, and 0.2 mM Phenylmethylsulfonyl fluoride (PMSF), pH 7.2. All  
216 chemicals were purchased from Sigma-Aldrich-Merck, St. Louis, MO.

### 217 *2.6 Statistics*

218 All variables were tested for normality and homoscedasticity using Shapiro and Levene  
219 tests, respectively. Logarithm transformations were performed, if necessary, before the  
220 statistical analyses. To confirm that animals from each experiment had a similar baseline  
221 VO<sub>2</sub> value, a one-way ANOVA was run with the initial values (*ctrl*) from all experiments.  
222 Then, a two-way mixed ANOVA was run to evaluate the effect of experimental treatment  
223 (C, T, and TH; between factor), experimental time (0 h, 3 h, 6 h, 12 h, and 18 h; within  
224 factor), and their interaction. Thereafter, multiple *t*-tests with Bonferroni's correction were



225 performed as post hoc analyses. A one-way ANOVA was conducted to test the effect of the  
226 experimental treatments (C, T, and TH) on nucleotide concentrations and their byproducts  
227 and metabolic enzyme activities. When a significant effect was found, only the means for  
228 each condition were compared by Tukey's HSD test. In all cases, statistical significance  
229 was accepted at  $p \leq 0.05$ . Statistics and graphs for standard respiration rate, metabolites,  
230 and enzyme activities were performed in R and with the software GraphPad Prism version  
231 8.0 for Windows (GraphPad, La Jolla, CA), respectively.

### 232 **3. Results**

#### 233 *3.1 Oxygen Consumption*

234 The baseline oxygen consumption rate of scallops ( $VO_2$ ) in all treatments (C, T, TH) at the  
235 beginning of the experiments at 22 °C (*ctrl*) showed no differences between them ( $2.8 \pm 0.3$   
236  $\mu\text{mol g}^{-1} \text{h}^{-1}$ , mean  $\pm$  SE). After correcting for sphericity, the mixed ANOVA indicated  
237 significant effects of the treatment ( $F_{(2,23)} = 19.91$ ;  $p < 0.001$ ), exposure time ( $F_{(2,76,63.53)} =$   
238  $5.58$ ;  $p < 0.01$ ), and the interaction between treatment and exposure time ( $F_{(5,52,63.53)} = 3.02$ ;  
239  $p = 0.014$ ). Animals exposed to treatment T significantly increased their  $VO_2$  from 0 h to  
240 18 h compared with their baseline  $VO_2$  level at 22 °C (*ctrl*). In contrast, animals exposed to  
241 the treatments C and TH maintained  $VO_2$  levels similar to their respective baseline values  
242 (*ctrl*). The  $VO_2$  of scallops exposed to 30 °C (T) was significantly higher than the  $VO_2$  of  
243 scallops kept at 22 °C (C) over all time points analyzed ( $p < 0.01$ ), while animals exposed  
244 to treatment TH displayed higher  $VO_2$  values than treatment C only immediately after  
245 reaching 30 °C and after 3 h, but remained at similar levels as in C after 6 hours (Fig. 2;  
246 Supplementary Figure S1).

#### 247 *3.2 Nucleotides, Adenylate Energy Charge, and ArgP Content in Adductor Muscle*

248 AMP content increased significantly in scallops exposed to T and TH compared to the  
249 control (C) ( $F_{(2,18)} = 26.42$ ,  $p < 0.01$ ; Fig. 3A). The opposite pattern was observed for  
250 adenosine triphosphate (ATP), which showed the lowest levels in treatment TH ( $F_{(2,18)} =$   
251  $3.97$ ,  $p < 0.05$ ) and intermediate levels in treatment T (Fig. 3C). While ADP showed higher  
252 values in T than in C ( $F_{(2,18)} = 4.67$ ,  $p < 0.05$ ) Fig. 3B), GTP maintained similar values in  
253 all treatments (Fig. 3D). Nucleotide byproducts, like HX and IMP, were significantly  
254 higher in T and TH than in C ( $F_{(2,18)} = 4.62$ ,  $p < 0.05$ ), with a further significant increase of

255 IMP in treatment TH compared to T ( $F_{(2,18)} = 38.06, p < 0.01$ ) (Figs. 3E- F). AEC and ArgP  
256 displayed similar levels in treatments T and TH, which were significantly lower ( $F_{(2,18)} =$   
257 13.5 and 19.8, respectively;  $p < 0.01$ ) compared to C (Figs. 4A-B). The ATP/ADP ratio  
258 also decreased ( $F_{(2,18)} = 15.5, p < 0.01$ ), while adenylate kinase  $K_{eq}$  increased ( $F_{(2,18)} =$   
259 13.9,  $p < 0.01$ ) in treatments T and TH (Figs. 4C-D). A further non-significant ( $p = 0.13$ )  
260 increase in  $K_{eq}$  was observed in TH compared to T.

### 261 3.3 Enzyme Activity in Adductor Muscle and Mantle

262 The mitochondrial enzymes CCO and CS, the glycolytic enzymes (HK, PK, and ODH) and  
263 AK in adductor muscle were not significantly affected by the experimental treatments  
264 (Figs. 5A-F). Moreover, neither CCO/CS, PK/CS, nor ODH/LDH ratios in adductor muscle  
265 were affected by T or TH compared to C (Table 1).

266 In the mantle, HK activity was significantly higher only in scallops exposed to treatment  
267 TH compared to those in T ( $F_{2,16} = 9.20, p < 0.05$ ) but similar to values observed in  
268 treatment C (Fig. 6A). Conversely, CS activity in the mantle was significantly lower only in  
269 scallops exposed to TH compared to scallops in treatment T ( $F_{2,16} = 3.46, p < 0.05$ ), but was  
270 not significantly different to the activity found in C (Fig. 6C). While a significant decrease  
271 in the activity of PK ( $F_{2,16} = 8.79, p < 0.01$ ) and ODH ( $F_{2,16} = 18.12, p < 0.01$ ) was  
272 observed in the mantle of scallops under TH compared with C and T (Figs. 6B, E), AK  
273 activity in the mantle did not show a significant difference between treatments (Fig. 6F).  
274 The CCO/CS and PK/CS ratios in the mantle were not affected by treatments T or TH  
275 compared to C. In contrast, the ODH/LDH ratio in the mantle increased in organisms  
276 exposed to TH compared to T and C ( $F_{2,16} = 4.62, p < 0.05$ ; Table 1).

## 277 4. Discussion

278 In this study, we evaluated some metabolic responses of juvenile *Nodipecten subnodosus*  
279 scallops to acute hyperthermia combined with low DO availability. Scallops were  
280 challenged by high temperature (30 °C) considering the temperature range they would be  
281 exposed in their natural environment (13.6 °C–27.8 °C) in culture systems at the Ojo de  
282 Liebre lagoon and temperatures that have been identified as deleterious for growth (26 °C)  
283 and survival (32 °C) for this species (González-Estrada, 2003). On the other hand, bivalve  
284 mollusks may exhibit metabolic adjustments to cope with moderate or severe hypoxic

285 conditions (Vaquer-Sunyer and Duarte, 2008; 2011). However, no data on tolerance  
286 regarding survival and metabolic adjustments to low DO levels are currently available for  
287 *N. subnodosus*. The hypoxic condition was selected based on the minimum DO level (2.9  
288 mg O<sub>2</sub> L<sup>-1</sup>, June 2017) recorded in summer in the culture site, indicating that *N. subnodosus*  
289 juveniles were only exposed to acutely moderate hypoxic events in the field (Fig. 1).  
290 Moreover, the low tolerance of *N. subnodosus* juveniles under found in a preliminary  
291 experiment severe hypoxic conditions (50 % mortality at 1 mg O<sub>2</sub> L<sup>-1</sup>, 30 °C) was another  
292 reason to select moderately hypoxic levels (2.5 mg O<sub>2</sub> L<sup>-1</sup>).

293 When exposed to acute hyperthermia under normoxia (T), *N. subnodosus* juveniles showed  
294 an increase in VO<sub>2</sub>, a well-known response in ectotherms reflecting a high-energy  
295 requirement (Schmidt-Nielsen, 1997). The enhanced respiratory response of juveniles  
296 contrasted with the steady VO<sub>2</sub> values in adult scallops exposed to similar acute  
297 hyperthermia challenges (Salgado-García et al., 2020). These differences in aerobic  
298 performance of organisms at different life stages may be related to shifts in energy  
299 metabolism to meet physiological requirements (i.e., locomotion, growth, reproduction) and  
300 compensatory responses to environmental conditions the local habitat (Pörtner et al., 2017;  
301 Verberk et al., 2018). Indeed, adult scallops were collected in a southern coastal lagoon  
302 with seasonal variability in environmental factors (e.g., summer temperatures) (Salgado-  
303 García et al., 2020).

304 In contrast, scallops exposed to hypoxic conditions at 30 °C (treatment TH) decreased their  
305 VO<sub>2</sub> until reaching a VO<sub>2</sub> level similar to scallops in treatment C once the hypoxic  
306 conditions were set (6 h to 12 h). *N. subnodosus* juveniles showed a constrained VO<sub>2</sub>  
307 induced by the simultaneous exposure to high temperature and moderate hypoxia (TH)  
308 compared to scallops exposed to T, as observed in other species of scallops such as *Pecten*  
309 *maximus* and *Argopecten purpuratus* (Artigaud et al., 2014; Aguirre-Velarde et al., 2016).  
310 Conversely, some bivalves like the fan mussel *Pinna nobilis* seemed to be unaffected by  
311 similar environmental conditions (~3 mg O<sub>2</sub> L<sup>-1</sup>, 30 °C) (Basso et al., 2015); while some  
312 scallops, like *A. irradians irradians*, exposed acutely to severe hypoxia (<0.1 % air  
313 saturation, 20 °C, 18 h) show damage in the oxidative capacity of mitochondria (Ivanina  
314 and Sokolova, 2016). These differential aerobic responses of organisms to both

315 environmental constraints may be associated with adaptative responses of each species to  
316 the environmental variability in their local habitat. The respiratory activity of *N.*  
317 *subnodosus* under TH suggests that this subtidal species could be vulnerable to  
318 simultaneous hyperthermia and hypoxia events.

319

320 For aquatic ectotherms, changes in temperature and DO availability beyond their critical  
321 thresholds are followed by time-dependent cellular and molecular responses to maintain  
322 homeostasis (Pörtner et al., 2017). Among the indicators of energy metabolism evaluated in  
323 *N. subnodosus* juveniles, AEC and ArgP are powerful regulatory signals that activate  
324 metabolic responses in the adductor muscle of scallops during physiological (e.g.,  
325 locomotion) or environmental (e.g., elevated temperature) challenges (Sokolova, 2003; De  
326 La Fuente et al., 2014). In adductor muscle of *N. subnodosus* juveniles, the slight decrease  
327 in AEC and ATP levels concomitant with the increase in AMP would indicate some  
328 disruption of the energy balance in cells under acute hyperthermia (T), especially  
329 concomitant with moderate hypoxia (TH). This increased use of ATP was partially buffered  
330 by a decrease of ArgP, as previously observed in *N. subnodosus* adults exposed to a similar  
331 hyperthermia challenge (Salgado-García et al., 2020). Indeed, the decrease in ATP in the  
332 adductor muscle of *N. subnodosus* between treatments C and T was around  $1.0 \mu\text{mol g}^{-1}$ ,  
333 while ArgP decreased to up to  $\geq 14 \mu\text{mol g}^{-1}$ , indicating that energy supply from  
334 phosphagen compensated by more than ten-fold the absolute decrease in ATP, as also  
335 observed in other scallop species (Bailey et al., 2003). Therefore, under acute hyperthermia  
336 (T), the energy supply was probably efficient enough for the adductor muscle of juveniles,  
337 as ArgP content was not depleted. Indeed, this is confirmed by the negligible decrease in  
338 AEC (3 %, from 0.95 to 0.92) observed for *N. subnodosus* adults (refer to Salgado-García  
339 et al., 2020). However, AEC has some limitations as an indicator of the cellular energy  
340 status; for example, due to spontaneous natural oscillations in AEC representing a  
341 homeorhetic rather than a homeostatic regulation (De la Fuente et al., 2014). Therefore,  
342 such oscillations could possibly skew the AEC levels in one or another sense, whereas the  
343 slight decrease was the result of a natural oscillation or, conversely, the natural oscillation  
344 masked a stronger decrease. In this sense, the decrease in the ATP/ADP ratio was more

345 pronounced (27%) in muscle of juveniles under T and TH, in agreement with the concept  
346 that this ratio should also be considered to estimate disruptions in cell energy status (Raffin  
347 and Thébault, 1996, Thébault et al., 2000). Moreover, the adenylate kinase equilibrium  
348 constant increased by acute hyperthermia, either alone (T) or combined with hypoxia (TH),  
349 suggesting a role of this pathway, besides the use of phosphagens, for ATP synthesis and  
350 the resulting buffering of AEC (Raffin and Thébault, 1996; De la Fuente et al., 2014).

351 Interestingly, energy content (AEC and ArgP) in the adductor muscle was not significantly  
352 different between *N. subnodosus* juveniles exposed to T and those exposed to TH. Under a  
353 lower DO availability at high temperature (TH), we could expect a further decrease in  
354 ArgP, ATP, and AEC levels in adductor muscle, as observed in previous studies on bivalve  
355 mollusks exposed to hypoxia. A decrease in AEC was observed in the mussel *Mytilus*  
356 *galloprovincialis* under anoxia (Isani et al., 1997); ATP decreased and AMP increased in  
357 the scallop *Patinopecten yessoensis* under low DO (1 mg L<sup>-1</sup>) (Enomoto et al., 2000); ArgP  
358 decreased in the scallop *A. irradians* under low DO (< 20 mmHg, 12 % Sat O<sub>2</sub>), with no  
359 difference in AEC (Chih and Ellington, 1983). Therefore, the different energy response  
360 observed in *N. subnodosus* juveniles compared to the studies just mentioned may be related  
361 to less severe hypoxic conditions or endogenous factors (e.g., age). Nevertheless, IMP and  
362 HX levels suggest that scallops are already facing an energy imbalance under hyperthermia  
363 (T). The further accumulation of IMP in the adductor muscle of individuals exposed to TH  
364 compared to T indicated a higher energetic disruption by low DO levels at high  
365 temperatures. This change in energy metabolism characterized by IMP accumulation in  
366 juveniles was also found in adductor muscle of adult scallops exposed to a similar  
367 hyperthermia challenge (Salgado-García et al., 2020). The increased IMP levels suggest the  
368 activation of the AMP deamination pathway that may occur concomitantly with adenylate  
369 kinase activation for the direct synthesis of ATP from ADP. Indeed, such reaction implies  
370 the accumulation of AMP that should be compensated by its deamination to IMP (Raffin  
371 and Thébault, 1996). In contrast, oysters *Crassostrea gigas* exposed to hypoxia by  
372 emersion at 15 °C for two days showed no change in IMP and HX levels in adductor  
373 muscle (Kawabe et al., 2010). These differences in nucleotide metabolism and degradation  
374 rate may underlie adaptive responses for maintaining the energy balance between

375 intertidal (e.g., oysters) and subtidal species (e.g., scallops) facing similar environmental  
376 constraints.

377 Juvenile scallops from the Ojo de Liebre lagoon (BCS, Mexico) can maintain energy (AEC  
378 and ArgP) in cells facing acute hyperthermia under low DO levels (TH). Therefore, we  
379 evaluated whether energy content in *N. subnodosus* juveniles exposed to both T and TH  
380 challenges could be related to the regulation of some key metabolic enzymes involved in  
381 aerobic and anaerobic metabolism in adductor muscle and mantle of juvenile scallops. To  
382 satisfy the energetic requirements of a tissue, quantitative or qualitative modifications of  
383 mitochondria could change the abundance or the oxidative capacities of mitochondria  
384 where the ATP synthase generates the vast majority of cellular ATP (St-Pierre et al., 1998,  
385 Guderley et al., 1990). The CCO/CS ratio can be a suitable indicator of size and shape of  
386 mitochondria, and thus may indicate changes in O<sub>2</sub>-dependent ATP production in tissues  
387 per unit of mitochondria (Boutilier and St-Pierre, 2002; Dudognon et al., 2015). A slight  
388 but not significant difference in the CCO/CS ratio in the adductor muscle of scallops  
389 suggests that mitochondrial aerobic metabolism was marginally affected by T or TH  
390 conditions. A similar lack of compensatory changes in mitochondrial activity was found in  
391 the adductor muscle of adult scallops exposed to hyperthermia (Salgado-García et al.,  
392 2020). Interestingly, CCO activity showed no differences in the mantle, but CS activity was  
393 significantly lower under TH compared to T. This response may indicate that mitochondrial  
394 abundance has already started to decrease in the mantle of scallops (Mantle et al., 2010) as  
395 an early sign of compensation in aerobic pathways in *N. subnodosus* only after an 18 h  
396 exposure to hyperthermia combined with low DO levels (TH). Such results may  
397 corroborate the tissue-specific shifts in mitochondrial energy metabolism found in other  
398 scallops (*P. maximus*) from temperate zones in response to acute warming combined with  
399 hypoxic events (Götze et al., 2020).

400 Additionally, we evaluated HK, PK, ODH, and AK activities as proxies of O<sub>2</sub>-independent  
401 (anaerobic) energy production (Zammit and Newsholme, 1976; de Zwaan et al., 1980;  
402 Wongso et al., 1999; Saborowski and Buchholz, 2002; Artigaud et al., 2015). Interestingly,  
403 HK activity was down-regulated in the adductor muscle and mantle of *N. subnodosus*  
404 juveniles exposed to T (although not significantly in the adductor muscle). On the other

405 hand, only ODH activity was slightly down-regulated (although not significantly) in the  
406 adductor muscle of scallops exposed to T. In contrast, glycolytic activity (i.e., HK activity)  
407 and arginine metabolism (AK) were stimulated in the adductor muscle of adult scallops  
408 exposed to a similar hyperthermia challenge (Salgado-García et al., 2020). These responses  
409 indicate that juveniles could use mostly ArgP in the adductor muscle to cope with acute  
410 hyperthermia, as observed in *P. maximus* (Artigaud et al., 2015), while adults may rely on  
411 ArgP and carbohydrates (i.e., HK activity) to cope with the combined effects of acute  
412 hyperthermia and reproductive effort (Salgado-García et al., 2020). Similarly, as for VO<sub>2</sub>,  
413 such differences in energy metabolism (ArgP, carbohydrates) may also be explained by  
414 compensatory responses to high energy demand for physiological processes in juveniles  
415 (i.e., growth) and adults (i.e., reproduction) but also as adaptative responses to  
416 environmental variability associated with the geographic zone (see above).

417 The constrained VO<sub>2</sub> of juveniles *N. subnodosus* exposed to TH occurs concurrently with  
418 decreased CS activities and pyruvate metabolism (PK and ODH) and the up-regulation of  
419 glycolytic enzymes (i.e., HK) in the mantle, suggesting an enhanced anaerobic response (Le  
420 Moullac et al., 2007) and a controlled reduction of energy-demanding processes in this  
421 tissue to maintain the energy balance (Strahl et al., 2011; Seibel et al., 2014). Alternatively,  
422 these responses may reflect the early signs of metabolic dysfunction under limited oxygen  
423 supply at high temperatures close to the upper thermal limit of the species (T<sub>c</sub>) (Gonzalez-  
424 Estrada, 2003). This response has also been observed in juveniles of the European scallop  
425 (*P. maximus*) exposed to severe hypoxia at a temperature above their upper thermal limit  
426 (25 °C) (Artigaud et al., 2015). These metabolic responses of juvenile *N. subnodosus*  
427 scallops facing acute hyperthermia under normoxia (T) or along with with low DO levels  
428 (TH) are insights into the capacity of these animals to maintain the energy supply for  
429 physiological activities (e.g., growth, locomotion) and their vulnerability to ocean warming  
430 conditions at physiological (e.g., diseases, mortality) and ecological (e.g., abundance)  
431 levels, according to the peak summer temperatures ( $\geq 30$  °C) reported over the past decade  
432 in the Ojo de Liebre lagoon, which is the northern limit of distribution of this species.

433 **5. Conclusions**

434 Our results showed that *N. subnodosus* juveniles quickly respond to T conditions with an  
435 increased respiratory response to preserve cellular energy homeostasis (AEC) by aerobic  
436 pathways and ArgP hydrolysis in the adductor muscle. Under a TH challenge, a decreased  
437 aerobic response of scallops was followed by metabolic changes in the mantle (CS, ODH,  
438 PK), highlighting the role of this tissue in animal tolerance to these environmental  
439 challenges. These results suggested that *N. subnodosus* populations from the Ojo de Liebre  
440 lagoon seem adapted to acute hyperthermia, but the simultaneous occurrence of  
441 hyperthermia and hypoxic events may have adverse impacts on energy allocation  
442 determining overall fitness (e.g., growth and reproduction), with significant consequences  
443 on the abundance of scallop populations in their northern limit of distribution.

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458 **Figure captions**

459 **Figure 1.** Thermal profile ( $^{\circ}\text{C}$ ) (upper) and seawater dissolved oxygen ( $\text{mg O}_2 \text{L}^{-1}$ ) (lower)  
460 obtained from the culture site of *Nodipecten subnodosus* juvenile scallops (Ojo de Liebre  
461 lagoon) using an autonomous data logger (miniDOT, Precision, Measurement Engineering  
462 Inc., US) during the scallop culture period (16 November 2016 to 30 June 2017).

463 **Figure 2.** Standard oxygen consumption rate ( $\text{VO}_2$ ,  $\mu\text{mol O}_2 \text{g}^{-1} \text{h}^{-1}$ ) in *Nodipecten*  
464 *subnodosus* juveniles maintained under control (C,  $22^{\circ}\text{C}$ ,  $7.1 \text{mg O}_2 \text{L}^{-1}$ ) (dark grey), acute  
465 hyperthermia (T,  $30^{\circ}\text{C}$ ,  $5.4 \text{mg O}_2 \text{L}^{-1}$ ) (light grey), or acute hyperthermia combined with  
466 moderate hypoxia (TH,  $30^{\circ}\text{C}$ ,  $2.5 \text{mg O}_2 \text{L}^{-1}$ ) (white), respectively. Symbols denote  
467 significant differences between the initial time point (*ctrl*) in each experimental treatment  
468 (\*) or significant differences for each time point between treatments T and TH compared to  
469 the control treatment (C) (#). In all cases, the significance value was  $p \leq 0.05$ . The box  
470 ranges from Q1 (first quartile, 25th) to Q3 (third quartile, 75th) of the distribution; the  
471 range represents the IQR (interquartile range). The median is indicated by a line across the  
472 box. The whiskers on box plots go from Q1 and Q3 to the most extreme data points. The  
473 number of scallops per treatment were  $N = 8$  in treatment C,  $N = 8$  in treatment T, and  $N =$   
474  $10$  in treatment TH.

475  
476 **Figure 3.** Nucleotide composition of the adductor muscle of *Nodipecten subnodosus*  
477 juveniles exposed to control conditions (C,  $22^{\circ}\text{C}$ ,  $7.1 \text{mg O}_2 \text{L}^{-1}$ ) (black), acute  
478 hyperthermia (T,  $30^{\circ}\text{C}$ ;  $5.4 \text{mg O}_2 \text{L}^{-1}$ ) (grey), or acute hyperthermia combined with  
479 moderate hypoxia (TH,  $30^{\circ}\text{C}$ ,  $2.5 \text{mg O}_2 \text{L}^{-1}$ ) (white). **A.** Adenosine monophosphate  
480 (AMP,  $\mu\text{mol g}^{-1}$ ), **B.** Adenosine diphosphate (ADP,  $\mu\text{mol g}^{-1}$ ), **C.** Adenosine triphosphate  
481 (ATP,  $\mu\text{mol g}^{-1}$ ), **D.** Guanosine triphosphate (GTP,  $\mu\text{mol g}^{-1}$ ), **E.** Inosine monophosphate  
482 (IMP,  $\mu\text{mol g}^{-1}$ ), **F.** Hypoxanthine (HX,  $\mu\text{mol g}^{-1}$ ). One-way ANOVA results are shown in  
483 the figure. Following Tukey's post hoc mean comparison, different letters indicate  
484 significant differences between treatments ( $p \leq 0.05$ ). Values are expressed as mean  $\pm$  SE.  
485 The number of scallops per treatment were  $N = 5$  (treatment C),  $N = 10$  (treatment T), and  
486  $N = 10$  (treatment TH).

487

488 **Figure 4.** Energy status of the adductor muscle of *Nodipecten subnodosus* juveniles  
489 exposed to control conditions (C, 22 °C, 7.1 mg O<sub>2</sub> L<sup>-1</sup>) (black), acute hyperthermia (T, 30  
490 °C; 5.4 mg O<sub>2</sub> L<sup>-1</sup>) (grey), or acute hyperthermia combined with moderate hypoxia (TH, 30  
491 °C, 2.1 mg O<sub>2</sub> L<sup>-1</sup>) (white). **A.** Adelynate energy charge (AEC). **B.** Arginine phosphate  
492 content (ArgP, μmol g<sup>-1</sup>). **C.** ATP/ADP ratio. **D.** adenylate kinase-catalyzed reaction ( $K_{eq}$ ).  
493  $K_{eq} = (ATP \cdot AMP) / ADP^2$ , calculated according to Raffin and Thébault (1996). Refer to  
494 Fig. 3 for statistics.

495 **Figure 5.** Activity of aerobic and anaerobic enzymes (U g<sup>-1</sup>) in the adductor muscle of  
496 *Nodipecten subnodosus* juveniles exposed to control conditions (C, 22 °C, 7.1 mg O<sub>2</sub> L<sup>-1</sup>,  
497 100% Sat O<sub>2</sub>) (black), acute hyperthermia (T, 30 °C; 5.4 mg O<sub>2</sub> L<sup>-1</sup>) (grey), or acute  
498 hyperthermia combined with moderate hypoxia (TH, 30 °C, 2.5 mg O<sub>2</sub> L<sup>-1</sup>) (white). **A.**  
499 Hexokinase activity (HK), **B.** Pyruvate kinase activity (PK), **C.** Citrate synthase activity  
500 (CS), **D.** Cytochrome c oxidase activity (CCO), **E.** Octopine dehydrogenase activity  
501 (ODH), **F.** Arginine kinase (AK). Refer to Fig. 3 for statistics.

502 **Figure 6.** Activity of aerobic and anaerobic enzymes (U g<sup>-1</sup>) in the mantle of *Nodipecten*  
503 *subnodosus* juveniles exposed to control conditions (C, 22 °C, 7.1 mg O<sub>2</sub> L<sup>-1</sup>) (black), acute  
504 hyperthermia (T, 30 °C; 5.4 mg O<sub>2</sub> L<sup>-1</sup>) (grey), or acute hyperthermia combined with  
505 moderate hypoxia (TH, 30 °C, 2.5 mg O<sub>2</sub> L<sup>-1</sup>) (white). **A.** Hexokinase activity (HK), **B.**  
506 Pyruvate kinase activity (PK), **C.** Citrate synthase activity (CS), **D.** Cytochrome c oxidase  
507 activity (CCO), **E.** Octopine dehydrogenase activity (ODH), **F.** Arginine kinase (AK).  
508 Refer to Fig. 3 for statistics.

509

510 Table 1. Relation of activity of metabolic enzymes in the adductor muscle and the mantle of  
511 juveniles *N. subnodosus* exposed to control conditions (C, 22 °C, 7.1 mg O<sub>2</sub> L<sup>-1</sup>), acute  
512 hyperthermia (T, 30 °C; 5.4 mg O<sub>2</sub> L<sup>-1</sup>) or acute hyperthermia combined with moderate  
513 hypoxia (TH, 30 °C, 2.5 mg O<sub>2</sub> L<sup>-1</sup>).

	Adductor muscle			Mantle		
	C	T	TH	C	T	TH
CCO/CS	0.24±0.07	0.43±0.08	0.46±0.12	0.40±0.17	0.29±0.04	0.7±0.20
PK/CS	15.6±1.7	30.2±6.4	24.4±5.2	12.1±2.4	18.5±3.7	14.9±3.8
ODH/LDH	47.6±15.6	49.2±8.0	49.5±9.9	18.1±4.5 <sup>a</sup>	20.4±2.3 <sup>a</sup>	46.9±9.1 <sup>b</sup>

514 One-way ANOVA. Tukey ( $p \leq 0.05$ ). Mean value ± SE. N= 5 (C), 10 (T) 10 (TH).

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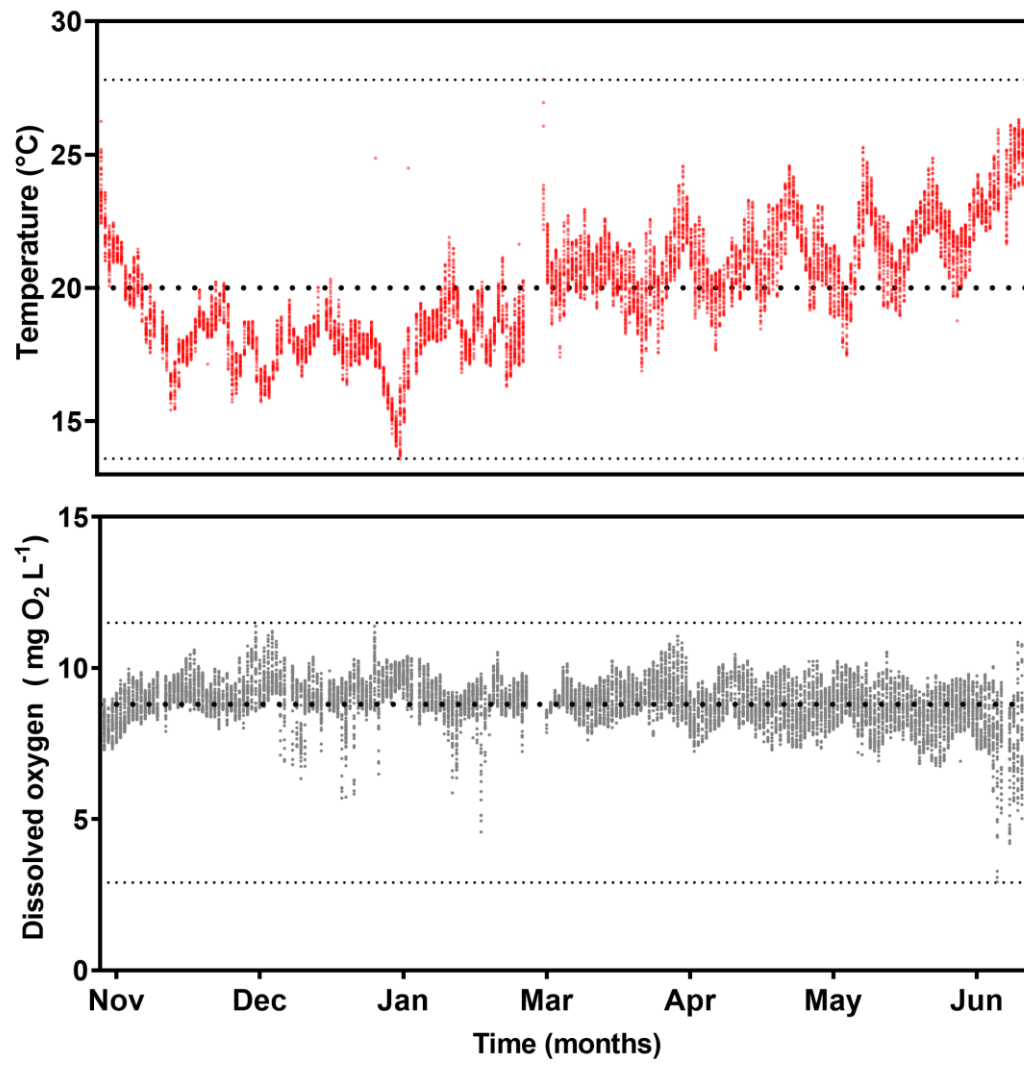
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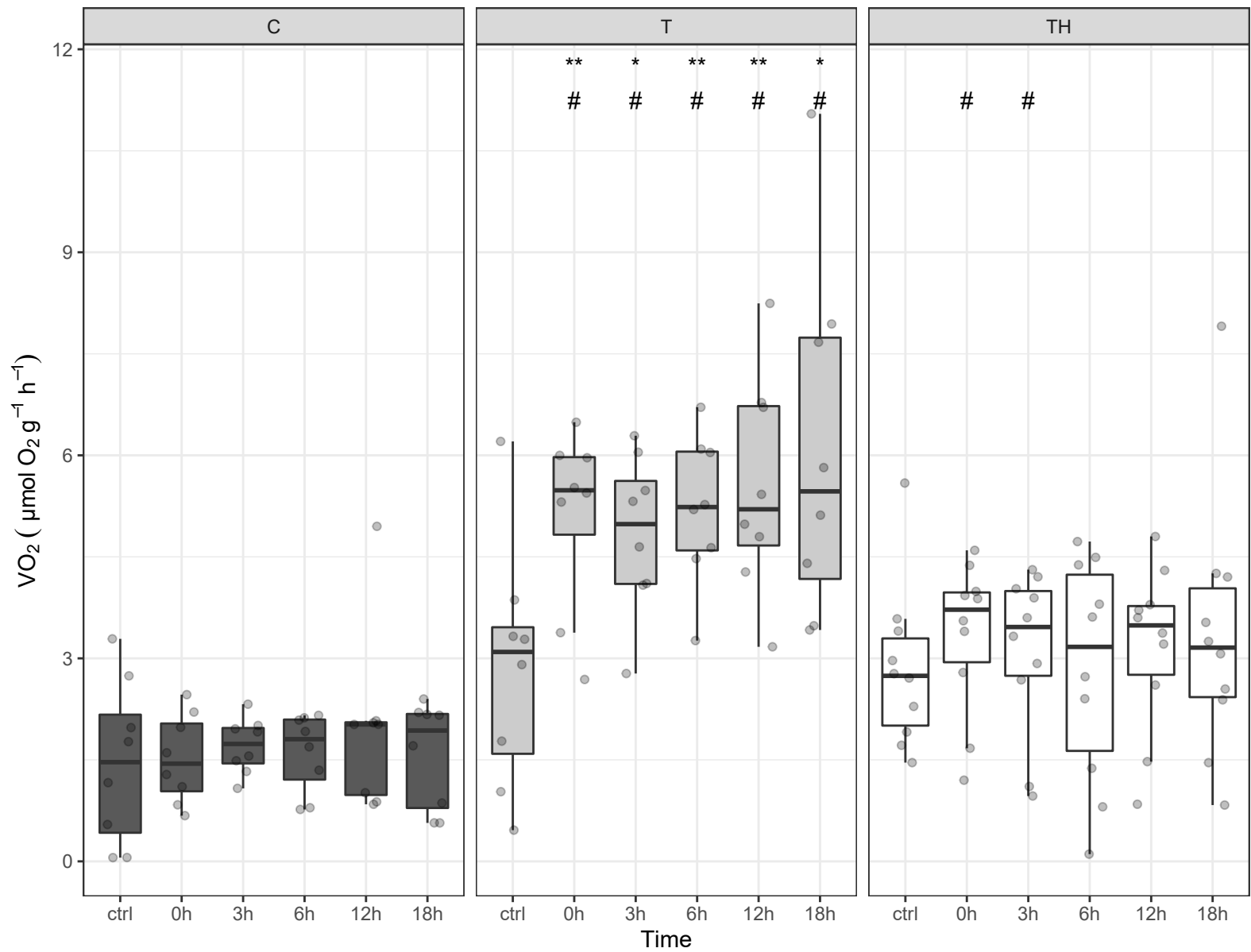
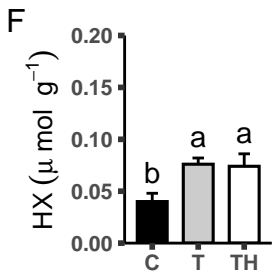
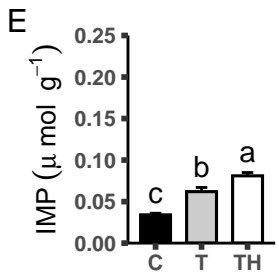
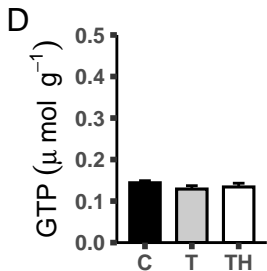
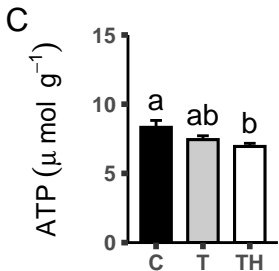
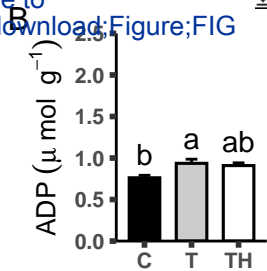
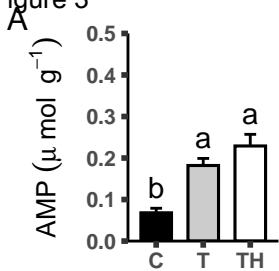


Figure 3

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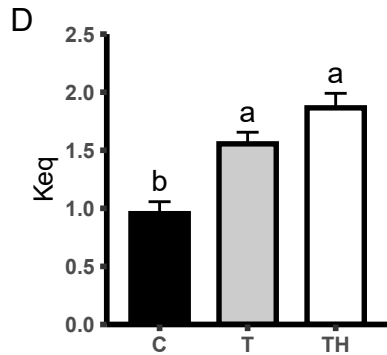
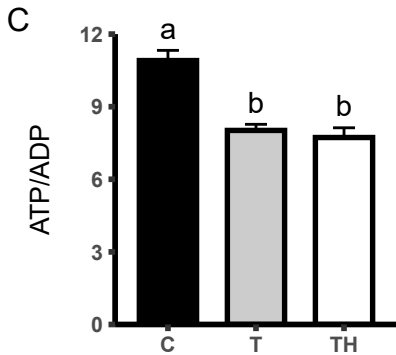
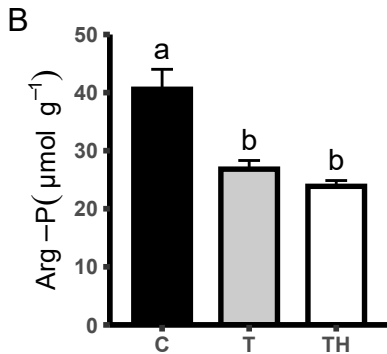
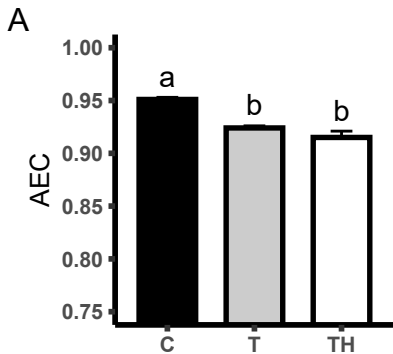




Figure 5

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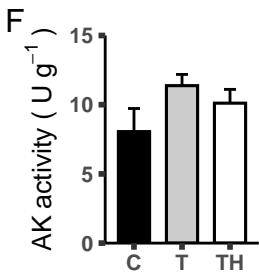
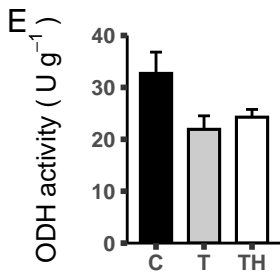
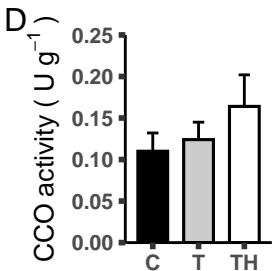
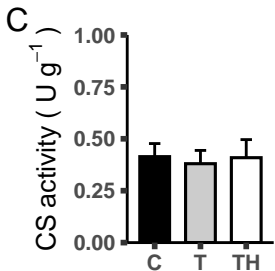
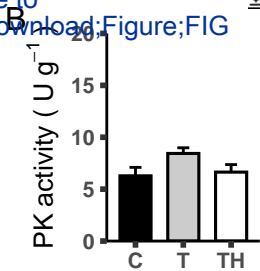
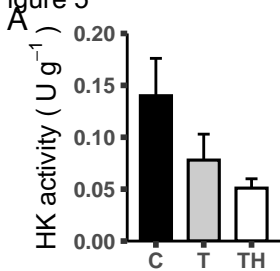
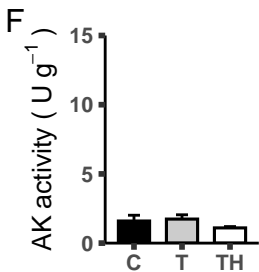
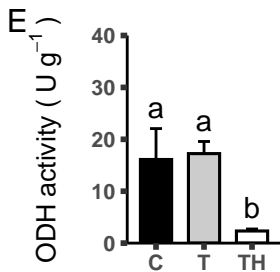
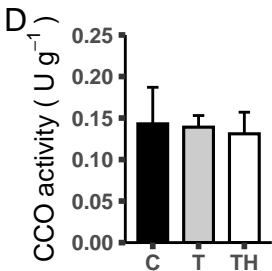
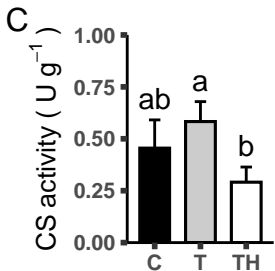
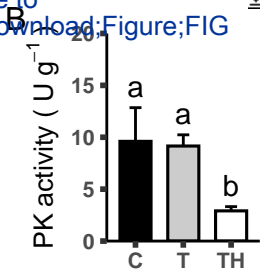
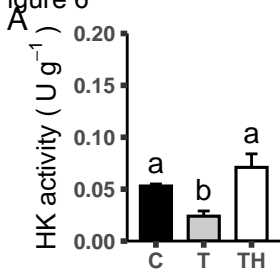
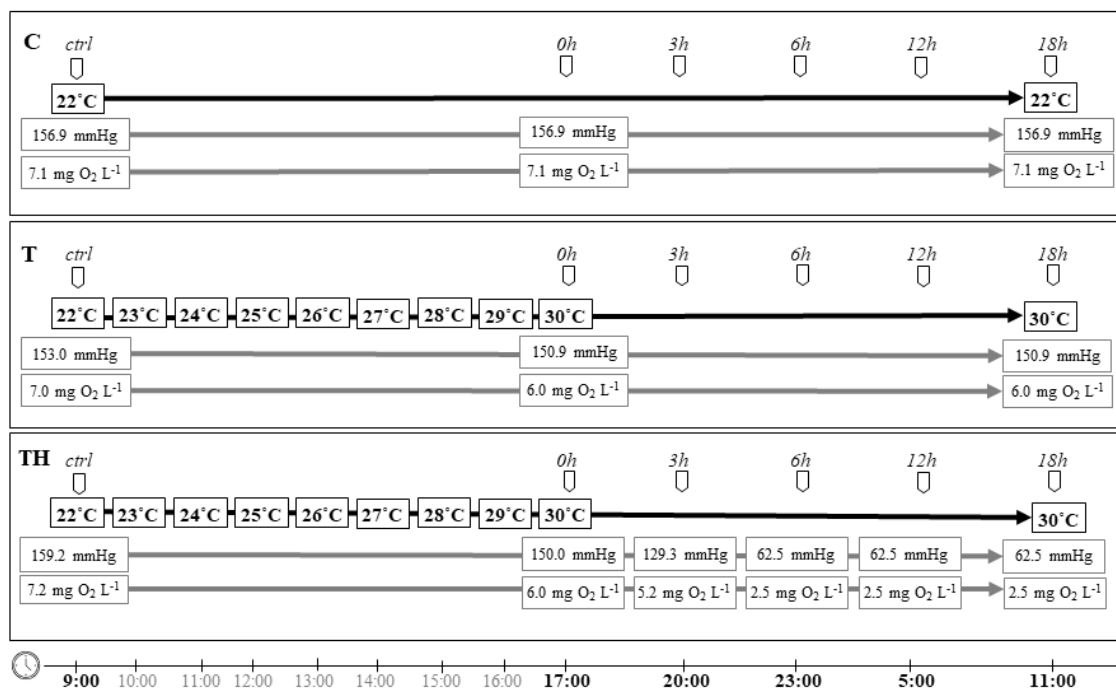


Figure 6

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Manuscript Number: CBPA-D-22-00271

**Title: Energy Metabolism of Juvenile Scallops *Nodipecten subnodosus* under Acute Increased Temperature and Low Oxygen Availability**



**FIGURE S1.** Experimental protocol for juvenile *N. subnodosus*. Symbols (□) indicate when standard metabolic rate ( $VO_2$ ) was calculated for animals exposed to the control conditions (treatment C: 22 °C;  $O_2$  ~7.1  $mg\ O_2\ L^{-1}$ , 156.9 mmHg), acute hyperthermia (treatment T: 30 °C; ~6.0  $mg\ O_2\ L^{-1}$ , 136.54 mmHg) or acute hyperthermia combined with moderate hypoxia (treatment TH, 30 °C; 2.5  $mg\ O_2\ L^{-1}$ , 62.51 mmHg).