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Seasonal and diurnal variability in carbon respiration, calcification and excretion rates of the abalone *Haliotis tuberculata* L.

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Abstract :

Abalone (*Haliotis* spp.) are commercially important marine shellfish species worldwide. Knowledge about the physiology of abalone that impacts life-history traits is important for a better understanding of the biology of the species and the impact of stressful husbandry procedures at different seasons. The present study quantified the seasonal and diurnal variations in four physiological parameters of the European species *Haliotis tuberculata*, i.e. carbon aerial and aquatic respiration, calcification and excretion rates, and the effect of prolonged aerial exposure upon abalone aerial respiration. We also investigated the effect of individual size upon these physiological parameters. Aquatic respiration and calcification rates showed an allometric relationship with biomass. All parameters showed lower rates in cool season and higher rates in warmer season. Temperature was assumed to be the primary driver of the reported seasonal variability in physiological parameters, although reproductive needs and nutrition may also contribute to the observed patterns. Importantly, abalone did not stop calcifying in winter, and calcified more at night than during the day. Abalone did not respire more underwater at night-time than at daytime, however they excreted more overnight. The low air:aquatic ratio (0.2) is likely to be an energy-saving strategy for emerged *H. tuberculata* individuals. This study highlights the temporal heterogeneity in physiological rates of *H. tuberculata*, which constitutes a species

Keywords : ormer, metabolism, carbon fluxes, physiology

36 INTRODUCTION

37 Abalone (*Haliotis* species) are commercially important marine gastropod species worldwide.
38 Whilst abalone fisheries declined from 19 720 to 7 486 metric tonnes between 1970 and 2013,
39 farming production exploded from 50 to 103 464 metric tonnes over the same period (Cook,
40 2014). The decrease in abalone fisheries has been triggered by overexploitation, illegal
41 catches and disease that lessened wild abalone stocks (Cook, 2014). *Haliotis tuberculata* L. is
42 the only species present in Europe (van Wormhoudt *et al.*, 2011). This species is relatively
43 abundant on the Channel Islands and the Atlantic coast of France (Clavier & Richard, 1982;
44 Gaty & Wilson, 1986). In the wild, *H. tuberculata* is sedentary (Forster, 1967) and found at
45 the subtidal and low intertidal levels in shallow rocky habitats (Hayashi, 1983). Their biology
46 and ecology such as their reproductive cycle (Hayashi, 1980a), population structure and
47 growth (Hayashi, 1980b; Clavier & Richard, 1986; Roussel *et al.*, 2011) have been well
48 studied. Respiration rates of *H. tuberculata* from hatcheries (oxygen consumption rates in mg
49 or ml O₂ h⁻¹) have previously been investigated in regards to factors such as *e.g.* body size
50 (10-50mm, Gaty & Wilson, 1986; 10-90g, Basuyaux *et al.*, 2001) and simulated temperature
51 (8, 16 and 24°C in Gaty & Wilson, 1986, and 12, 15, 18, 21, 28°C in Basuyaux *et al.*, 2001).
52 Nonetheless, the natural variations (day-night and season cycles) in the metabolism of abalone
53 from the natural habitat and the effect of body size upon respiratory rates in mature
54 individuals (*i.e.* size over 50mm, Hayashi, 1980a), are still not well understood and represent
55 important biological information for the recently developed industry in Europe (Roussel *et al.*,
56 2013; Cook, 2014; Lachambre *et al.*, 2017). Indeed, many abalone farms rely on coastal
57 seawater and hence factors such as seawater temperature or dissolved oxygen which vary
58 according to season, day-night and tidal cycles are likely to impact abalone physiology
59 (Morash & Alter, 2015). A better understanding of the natural temporal variations in
60 physiological and metabolic rates which affect life-history traits like growth and reproduction

61 can thus contribute to improving fisheries management and aquaculture development (Young
62 *et al.*, 2006; Cooke *et al.*, 2014; Ragg & Watts, 2015; Gao *et al.*, 2016).

63 Several factors can modify abalone metabolism such as light quality (*H. discus discus*, Gao
64 *et al.*, 2016), infection (*H. rufescens* and *H. discus hannai*, González *et al.*, 2012; *H.*
65 *diversicolor*, Lu *et al.*, 2017), farm stressors such as density, high temperature and ammonia
66 concentration (see review from Morash & Alter, 2015). To increase our understanding of the
67 effects of environmental and farm stressors upon abalone physiology, it is first necessary to
68 better comprehend the natural temporal variability in abalone metabolism and biology. In
69 particular, seasons have a strong effect on chemical constituents in the muscle and viscera (*H.*
70 *diversicolor*, Chiou *et al.*, 2001; *H. laevigata* and *Haliotis rubra*, Su *et al.*, 2006; farmed Jade
71 Tiger hybrid abalone, Mateos *et al.*, 2010), metabolic activity of digestive-gland cells (*H.*
72 *kamtschatkana*, Carefoot *et al.*, 1998), textural proprieties (*Haliotis discus*, Hatae *et al.*, 1995)
73 and even immunity parameters of abalone (*H. tuberculata*, Travers *et al.*, 2008). These
74 variations are partly related to reproductive cycle and energy transfer. However, to our
75 knowledge, the season effect upon abalone (including *H. tuberculata*) metabolism has been
76 poorly investigated to date.

77 Abalone metabolism might also vary according to diurnal rhythm. Indeed, abalone are
78 nocturnal gastropods. They are active, move and feed mainly over-night in laboratory
79 conditions, and in their natural habitat (*H. discus hannai*, Momma & Sato, 1970; *H. laevigata*,
80 *H. roei*, *H. ruber*, *H. cyclobates*, *H. scalaris*, Shepherd, 1973). One can thus expect that
81 abalone (and thus *H. tuberculata*) metabolism is greater at nighttime in order to fulfill the
82 metabolic needs related to individuals' nocturnal behavior.

83 Finally, others stressors related to commercial practices such as high water ammonia
84 concentration, high density, and air exposure (see review from Morash & Alter, 2015) are
85 likely to impact the physiological rates of abalone. Nitrogenous waste can be toxic and limit

86 production in aquaculture (Ahmed *et al.*, 2008) and thus likely impact the physiology of
87 abalone. Both fished abalone (*H. iris*, Wells & Baldwin, 1995; Ragg & Watts, 2015, *H.*
88 *australis*, Wells & Baldwin, 1995) and farmed abalone (*H. tuberculata*, Lachambre *et al.*,
89 2017) can be exposed to air during handling procedures. Abalone have been demonstrated to
90 remain metabolically active during air exposure at a much lower rate than during immersion
91 (*H. iris*, Baldwin *et al.*, 1992). *H. tuberculata* can be found at low shore levels on rocky
92 shores during extreme spring tides or even at higher levels in large rockpools (Crofts, 1929).
93 It is thus expected that *H. tuberculata* is physiologically adapted to aerial exposure.
94 Nevertheless, periods of exposure to air longer than those experienced in natural conditions,
95 and at different temperature, can occur especially during live shipments. A greater
96 understanding of abalone (and hence *H. tuberculata*) metabolic capacities under aerial
97 conditions is therefore important to improve the transport and handling of live animals.

98

99 This study proposed to study the natural temporal variations in physiological parameters of *H.*
100 *tuberculata*. We quantified the seasonal, diurnal variations as well as the effect of individual
101 size on four physiological parameters *i.e.* carbon aerial and aquatic respiration, calcification
102 and excretion rates which are key parameters to understand abalone physiology (Morash &
103 Alter, 2015). In addition, aerial respiration was studied during a 6-h exposure and in different
104 temperature conditions to study the effect of temperature on the aerial metabolic rate.

105 MATERIALS AND METHODS

106 **Sampling**

107 Abalone were collected by divers equipped with diving tanks in the Bay of Brest, in Brittany,
108 NW of France. The sampling site was situated on the east part of the “Ile des Morts” in the
109 Bay of Roscanvel located on the SW part of the Bay of Brest (48° 18,225’ N, 4° 32,134’ W,
110 France). Abalone were found under rocks at a depth between 3m and 10m depending on the
111 tide. They were carefully removed by hand, placed into a net until the end of the diving
112 session and subsequently placed within a closed transportable tank containing seawater.

113

114 **Laboratory conditions**

115 In the laboratory, abalone were placed into a flow through system and kept unhandled for at
116 least 24 hours before starting any measurements to minimize handling stress. Filtered
117 seawater was provided from the Bay of Brest by the nearby pumping station of the Institut
118 français pour l’exploitation de la mer (Ifremer) at Sainte-Anne du Portzic. Seawater
119 temperature was thus at the same temperature as *in situ*. A mix of algae from their natural
120 habitat was added for food.

121

122 **Methodology**

123 SEASON COMPARISON IN ALL PHYSIOLOGICAL PARAMETERS

124 Aquatic and aerial respiration rate, calcification rate and ammonium excretion rate were
125 calculated in adult abalone collected in the four seasons (Figure 1, n = 16 in summer, n = 15
126 in autumn, n = 17 in winter and in spring). These sampling periods were especially chosen to
127 test the effect of seawater temperature upon the measured parameters. For instance, seawater
128 temperature recorded in 2014-2015 was on average 17.1 ± 0.4 °C ($\bar{x} \pm SD$, mean \pm standard
129 deviation) in summer (18.3°C in September experimental day), 15.1 ± 2.2 °C in autumn

130 (14.6°C in November experimental day), 10.5 ± 0.9 °C in winter (11°C in January
131 experimental day) and 13.2 ± 1.8 °C in spring (14.1°C in May experimental day) (SOMLIT:
132 Service d'Observation en Milieu Littoral, INSU-CNRS, St Anne du Portzic; Figure 1).

133 Abalone ranging from 70 mm to 80 mm in length were sexually mature *i.e.* size over 50
134 mm (Hayashi, 1980a). This size range, corresponding to adult individuals, is the most
135 abundant in the sampling area and was selected to facilitate sampling. Aquatic incubations
136 were performed around midday in each season. Aerial respiration rates were measured upon
137 emersion as soon as individuals were removed from their aquatic incubation bottle. As
138 animals were free to feed prior to experiments, measured metabolic rate may include
139 postprandial metabolic demand.

140

141 DIURNAL COMPARISON IN AQUATIC PHYSIOLOGICAL PARAMETERS

142 In order to compare metabolic rates which can vary through time at a diurnal scale (Lorrain *et*
143 *al.*, 2015), aquatic incubations were repeated overnight in autumn (November 2014) on the
144 same individuals to compare aquatic respiration, calcification and excretion rates during day
145 and night periods.

146

147 INDIVIDUAL SIZE EFFECT UPON AQUATIC PHYSIOLOGICAL PARAMETERS

148 Calcification, aquatic respiration and ammonium excretion rates were calculated in abalone (n
149 = 45) from a wide range of size (length: 35 to 106 mm) in September 2014. Smaller
150 individuals (< 50mm, $n=15$ abalone for a total of 107 abalone tested) were provided by the
151 France Haliotis abalone farm (48°36'46N; 4°33'30W, Plouguerneau, France). These abalone
152 were the third generation bred in the farm and resulted from systematic mating between wild
153 and farmed broodstock (either males or females were wild broodstock) for each generation.

154

155 AERIAL RESPIRATION DURING 6H AIR EXPOSURE AND TEMPERATURE EFFECT

156 Abalone ($n = 8$) were gently detached from the laboratory tank and emersed for 6 hours.
157 Individual aerial respiration rates were measured each hour in spring (May 2015) at 14°C.
158 Incubations were also conducted at 18°C and 10°C on successive days. These air temperatures
159 were chosen as they are representative of the temperature that can be experienced by abalone
160 during either ambient temperature or cooled temperature transport. The spring season was
161 chosen because it corresponds to usual fishing period for *H. tuberculata* (fishing is forbidden
162 in summer in Europe and practically difficult for the fisherman in winter due to poor weather
163 conditions).

164

165 **Physiological measurements**

166 Aquatic incubations of 1.5 to 2 hours (dark conditions) were conducted to calculate abalone
167 aquatic respiration (dissolved inorganic carbon, DIC), calcification/decalcification (CaCO_3)
168 and ammonium excretion (NH_4^+ fluxes). Individuals were gently detached and placed into 2-L
169 watertight plastic bottles filled with natural filtered seawater (1 individual/bottle) immersed
170 into a 580 L tank filled with running seawater to maintain a constant temperature during
171 incubations. Bottles were gently rotated once in the middle of incubations to ensure
172 homogenization.

173

174 AQUATIC RESPIRATION RATE

175 Aquatic respiration within each bottle (R , $\mu\text{mol DIC h}^{-1}$) was calculated as the variation in the
176 dissolved inorganic carbon (DIC) concentration between the start and the end of the
177 incubation using the following equation: $R = (\Delta DIC \times v) / (\Delta t \times 10^3) - G$, where ΔDIC is
178 the variation in DIC during incubation ($\mu\text{mol DIC l}^{-1}$), Δt is the incubation time (h), v is the
179 bottle volume (l) and G is the calcification rate ($\mu\text{mol CaCO}_3 \text{ h}^{-1}$). To determine the dissolved

180 inorganic carbon (DIC) concentration ($\mu\text{mol L}^{-1}$), seawater samples were taken from
181 incubation bottles at the start and at the end of each incubation in order to measure total
182 alkalinity (TA), pH and seawater temperature.

183 TA samples were obtained by filtering water through $0.7\mu\text{m}$ Whatman GF/F filters.
184 Samples were then stored with 250ml plastic bottles in the dark. Laboratory analysis consisted
185 in estimating TA ($\mu\text{mol L}^{-1}$) within 20ml subsamples (average of 6 subsamples per bottle) by
186 automatic potentiometric titration (Radiometer, Titrilab TIM 865). Subsamples were titrated
187 by adding small increments of 0.01 mol L^{-1} HCL with 0.7 mol kg^{-1} NaCl to approximate the
188 ionic strength of sea water (Dickson & Goyet, 1994) until about pH 3. TA was determined
189 using the modified Gran method by determining the second endpoint of the titration curve.

190 The pH (total scale) was measured using a pH probe (Radiometer pHC2401) which was
191 standardized with buffer solutions in synthetic seawater of 35‰ (Tris-HCL: 2-amino-2
192 hydroxymethyl-1,3-propanediol hydrochloride; 2-amminopyridine/HCL). pH values were
193 measured immediately after opening bottles in order to prevent effects of CO_2 exchange with
194 the air.

195 DIC concentration was calculated from pH, TA, temperature, salinity, phosphate and silicate
196 concentrations using the CO2SYS program (Pierrot *et al.*, 2006). The natural salinity,
197 phosphate and silicate concentrations were obtained using data collected in the Bay of Brest
198 by the French coastal observation service (SOMLIT: Service d'Observation en Milieu
199 Littoral, INSU-CNRS, St Anne du Portzic). Dissociation constants for carbonic acid K1 and
200 K2 were taken from Roy *et al.* (1993).

201

202 CALCIFICATION RATE

203 Calcification was determined using the alkalinity anomaly technique (Smith & Key, 1975) in
204 each incubated bottle. Calcification rate (G in $\mu\text{mol CaCO}_3\text{ h}^{-1}$) was calculated as follows:

205 $G = (\Delta TA \times v) / (2 \times \Delta t)$. ΔTA , Δt and v corresponds to the variation in TA during
206 incubation ($\mu\text{mol l}^{-1}$), incubation time (h) and bottle volume (L), respectively. This equation is
207 based on the evidence that the precipitation of one mole of CaCO_3 implies the consumption of
208 2 moles of HCO_3^- which decreases TA by 2 equivalents (Frankignoulle *et al.*, 1994). $G > 0$ is
209 indicative of calcification, whilst $G < 0$ indicates carbonate dissolution.

210

211 AMMONIUM EXCRETION RATE

212 Ammonia is assumed to be the primary end product of catabolism of amino acids in molluscs
213 (Bayne and Newell, 1983). Besides providing us information regarding *H. tuberculata*
214 ammonium excretion, NH_4^+ concentrations were measured to correct TA since ammonium
215 excretion is one of the processes that may affect TA in our experiments (Gazeau *et al.*, 2014,
216 2015). Ammonium excretion was obtained by collecting seawater samples at the beginning
217 and end of each incubation in 10 ml vials and kept at -20°C until analysis. The phenol-
218 hypochlorite method (Koroleff, 1969; Sororzano, 1969) was used to determine NH_4^+
219 concentration ($\mu\text{mol L}^{-1}$). After adding reagents, the coloration of each sample was measured
220 at 630 nm using a spectrophotometer. Nitrification process was not assessed here since it can
221 be considered to be negligible under short incubations (Tagliarolo *et al.*, 2013b; Lorrain *et al.*,
222 2015).

223

224 AERIAL RESPIRATION MEASUREMENTS

225 Each abalone were detached and placed into a 0.1-L airtight dark chamber connected to a
226 closed circulation system with an integrated infrared CO_2 analyser (Li-Cor, Li-820) and a
227 desiccation column filled with anhydrous calcium sulphate (Drierite, Xenia, USA) just after
228 emersion. An adjustable pump maintained air flow at $0.8\text{-}0.9 \text{ L min}^{-1}$. Aerial respiration
229 within each chamber was estimated by the linear slope of CO_2 concentration increase

230 measured every 5 seconds over 3 minutes using the Li-820 software (Clavier *et al.*, 2009).
231 Fluxes of CO₂ (μmol CO₂ h⁻¹) were corrected for the net volume of the system and incubation
232 time. The aerial temperature was maintained at that of the seawater during aquatic
233 incubations.

234

235 **Data analysis**

236 SHELL COMMUNITY CONTRIBUTION AND INDIVIDUAL BIOMASS

237 Calcification, aquatic and aerial respiration measurements were repeated on empty shells of
238 the same individuals. Shells were washed, cleared of any flesh and carefully dabbed inside
239 with a paper towel damped with 70% ethanol. The measured parameters for the shell
240 community were then subtracted to the total calcification and respiration rates. The parameter
241 values presented in this study are the shell community corrected values. The contribution of
242 the dissolution of the inner shell part was considered as negligible (maximum empty shell
243 calcification rate of 0.17 μmol CaCO₃.h⁻¹.g⁻¹ in September, and a minimal flux of -0.03 μmol
244 CaCO₃.h⁻¹.g⁻¹ in January).

245 All the studied parameters were further normalized to biomass as described below.
246 Individual biomass was estimated as ash-free dry weight (AFDW) through loss on ignition (4
247 h combustion at 450°C of 60°C dried individuals until constant weight) and expressed in g
248 AFDW⁻¹.

249

250 STATISTICAL ANALYSIS

251 The relationship between the biomass (AFDW) and the physiological aquatic parameters were
252 assessed using the following allometric equation $Y = a \times W^b$ (Marsden *et al.*, 2012) where a
253 corresponds to the physiological rate per gramme AFDW, and b shows the rate at which the
254 physiological parameter evolves with the biomass.

255 Normality and homogeneity of variances of the data distributions of calcification, aquatic
256 and aerial respiration, and ammonium excretion were investigated using the Shapiro-Wilk and
257 Bartlett tests, respectively. An ANOVA was performed to study the seasonal variability.
258 When normal distribution of the data distributions were not verified, the Kruskal Wallis test
259 was used. When the assumption of homogeneity of variance was not verified, a Welch's
260 ANOVA test was performed as recommended by Day and Quinn (1989). Subsequent non
261 parametric post hoc analyses were conducted with the Tukey and Kramer (Nemenyi) test
262 (Pohlert, 2014) to distinguish differences between seasons. The Wilcoxon Signed-Ranks test
263 was run to compare aquatic and aerial respiration at each season.

264 The relationships between the aquatic respiration, calcification and excretion rates, and the
265 seawater temperature experienced at each season were investigated using the Arrhenius
266 equation after logarithmic transformation as a function of T^{-1} as follows:

$$\ln Flux = \ln a - \frac{E_A}{K} \times \frac{1}{T}$$

267 where *Flux* is respiration (Δ DIC aquatic or Δ CO₂ in the air, μ mol g⁻¹ h⁻¹), *a* is a normalization
268 constant, *E_a* is the activation energy (J mole⁻¹), *K* is Boltzmann's constant (8.31 J K⁻¹ mol⁻¹),
269 and *T* is the absolute temperature (°K).

270 Comparison of calcification, aquatic respiration and ammonium excretion between day-
271 and night-time were conducted with the Wilcoxon Signed-Rank test.

272 As the data were normally distributed ($p > 0.05$), hourly variations in aerial respiration
273 over 6 hours emersion at different ambient temperatures (10°C, 14°C and 18°C) were
274 assessed using repeated measures ANOVA with a Greenhouse-Geisser Correction when the
275 data violated the assumption of sphericity (Mauchly's test of sphericity). Post hoc tests
276 incorporating the Bonferroni correction were done to distinguish differences in aerial
277 respiration at different times of emersion. Also, ANOVA tests were conducted to compare
278 abalone respiration rates between different temperatures (10°C, 14°C and 18°C) at each hour

279 of emersion. When significant, a subsequent posthoc test (Tukey's HSD test) was run to
280 distinguish which group(s) significantly differed.

281

282

283 RESULTS

284 **Seasonal variations**

285 A seasonal effect was observed on aquatic respiration (Welch test, $F_{3,32} = 19.25$, $P < 0.001$)
286 and aerial respiration (Anova test, $F_{3,60} = 3.63$, $P < 0.05$). Aquatic respiration was significantly
287 lower in autumn ($8.41 \pm 1.34 \mu\text{mol DIC g AFDW}^{-1} \text{ h}^{-1}$) and winter ($6.34 \pm 1.93 \mu\text{mol DIC g}$
288 $\text{AFDW}^{-1} \text{ h}^{-1}$) than in summer ($13.17 \pm 3.66 \mu\text{mol DIC g AFDW}^{-1} \text{ h}^{-1}$, Figure 2). Aerial
289 respiration was significantly lower in winter ($1.49 \pm 0.66 \mu\text{mol CO}_2 \text{ g AFDW}^{-1} \text{ h}^{-1}$) than in
290 summer ($2.21 \pm 0.83 \mu\text{mol CO}_2 \text{ g AFDW}^{-1} \text{ h}^{-1}$, Figure 2).

291 Aerial respiration was significantly lower than aquatic respiration over the 4 seasons with
292 abalone respiring between 5 and 6 times more underwater than during emersion (Table 1;
293 Figure 2).

294 Calcification rates of abalone (net CaCO_3 fluxes) had a mean annual value of 0.55 ± 0.53
295 $\mu\text{mol CaCO}_3 \text{ g AFDW}^{-1} \text{ h}^{-1}$. A seasonal effect was observed (Welch test, $F_{3,27.5} = 26.78$, $P <$
296 0.001) with CaCO_3 fluxes significantly lower in winter (Figure 2) but with still 53% of
297 individuals exhibiting shell calcification ($G > 0$). CaCO_3 fluxes were positive in 88% of
298 individuals in spring, and 100% of individuals in summer and autumn.

299 Ammonium fluxes were on average $0.29 \pm 0.26 \mu\text{mol NH}^{4+} \text{ AFDW}^{-1} \text{ h}^{-1}$ over the year. A
300 seasonal effect was found (Welch test, $F_{3,27.4} = 5.88$, $P < 0.01$) with lower values measured in
301 spring ($0.17 \pm 0.20 \mu\text{mol NH}^{4+} \text{ AFDW}^{-1} \text{ h}^{-1}$) and winter ($0.19 \pm 0.07 \mu\text{mol NH}^{4+} \text{ AFDW}^{-1} \text{ h}^{-1}$)
302 than in summer ($0.36 \pm 0.22 \mu\text{mol NH}^{4+} \text{ AFDW}^{-1} \text{ h}^{-1}$) and/or autumn ($0.47 \pm 0.36 \mu\text{mol NH}^{4+}$
303 $\text{AFDW}^{-1} \text{ h}^{-1}$, Figure 2).

304

305 A good relationship was observed between aquatic respiration and calcification, and
306 temperature (respectively, $R^2 = 0.88$ and $R^2 = 0.70$, table 2). The relationship between aerial
307 respiration and temperature was rather high but lower than that of aquatic respiration ($R^2 =$
308 0.68 , table 2). A poor relationship was observed between ammonium excretion and
309 temperature ($R^2 = 0.35$, table 2).

310

311 **Day-night variations (in autumn only)**

312 Day- and night-time aquatic respiration rates were 8.41 ± 1.34 and 9.10 ± 3.74 $\mu\text{mol DIC g}$
313 $\text{AFDW}^{-1} \text{h}^{-1}$, respectively, and did not differ significantly (Wilcoxon signed rank test, $P =$
314 0.64).

315 CaCO_3 fluxes were significantly higher (Wilcoxon signed rank test, $P < 0.01$) during the
316 night than during the day (1.19 ± 0.72 , and 0.71 ± 0.47 $\mu\text{mol CaCO}_3 \text{ g AFDW}^{-1} \text{h}^{-1}$,
317 respectively).

318 Likewise, NH_4^+ fluxes were significantly greater (Wilcoxon signed rank test, $P < 0.05$)
319 during the night than during the day (0.50 ± 0.17 , and 0.47 ± 0.36 $\mu\text{mol NH}_4^+ \text{ AFDW}^{-1} \text{h}^{-1}$,
320 respectively).

321

322 **Individual size effect upon aquatic physiological parameters**

323 Both aquatic respiration and calcification rates per unit biomass decreased as the individual
324 biomass increased (aquatic respiration: $y = 28.066 \times x^{-0.484}$, $R^2 = 0.71$; calcification:
325 $y = 3.831 \times x^{-0.885}$, $R^2 = 0.76$; Figure 3). No distinguishable pattern was found between
326 the individual biomass and ammonium excretion rate ($R^2 = 0.004$).

327

328 **Hourly variations in aerial respiration rates**

329 At 10°C, aerial respiration rates were lower at one hour of emersion than at 3, 5 and 6 hours
330 of aerial exposure (Repeated measure Anova test, $F_{5,35} = 13.98$, $P < 0.001$, Figure 4). At 14°C,
331 abalone significantly respired less after one hour of emersion than at 4 and 6 hours of
332 emersion (repeated measures Anova test, $F_{3,1,21.6} = 5.43$, $P < 0.01$, Figure 4). No difference in
333 respiration rate was observed over the 6 hour emersion at 18°C ($F_{2,1,6.7} = 1.13$, Figure 4).

334 Significant differences in aerial respiration rates between temperatures were obtained at 1
335 hour (Anova test, $F_{2,21} = 5.46$, $P < 0.05$), 2 hours (Anova test, $F_{2,21} = 5.25$, $P < 0.05$), 4 hours
336 (Anova test, $F_{2,20} = 3.94$, $P < 0.05$) and 6 hours (Anova test, $F_{2,20} = 5.54$, $P < 0.05$) of emersion
337 : abalone significantly displayed lower respiration rates at 10°C than at 18°C after 1 hour of
338 emersion (Test HSD de Tukey, $P < 0.01$, Figure 4). At 2 and 4 hours of emersion, abalone
339 displayed lower rates at 10° than 14°C (Test HSD de Tukey, $P < 0.05$, Figure 4). At 6 hours
340 of emersion, abalone displayed lower rates at 18° than 14°C (Test HSD de Tukey, $P < 0.05$,
341 Figure 4).

342

343 DISCUSSION

344 **Effect of *Haliotis tuberculata* biomass upon its physiological rate**

345 Body size has been identified as a good proxy for metabolic rates of animals (Newell, 1973).
346 Abalone respiration rates increase as weight increases (Gaty & Wilson, 1986; Basuyaux *et al.*,
347 2001; Cunningham *et al.*, 2016). An allometric relationship between aquatic respiration and
348 calcification rates per unit biomass was observed with negative b values much less than unity
349 which indicates that respiration and calcification do not decrease in direct proportion to
350 biomass (Marsden *et al.*, 2012). Our results *i.e.* higher aquatic respiration and calcification
351 rates per unit biomass in smaller *H. tuberculata* individuals, corroborate the evidence that
352 metabolic demand and growth decrease with age (at least during the first years of growth) like
353 in others molluscs such as *Tectus niloticus* (Lorrain *et al.*, 2015), *Crepidula fornicata* (Martin

354 *et al.*, 2006), *Mytilus edulis* and *M. galloprovincialis* (Tagliarolo *et al.*, 2012). Previous
355 findings in *H. tuberculata* have also found that growth rate, underpinned by metabolism and
356 energy budgets, decreases progressively as its size increases (Hayashi, 1980b; Clavier &
357 Richard, 1986; Roussel *et al.*, 2011). However, because one part of the small abalone were
358 from a farmed origin, resulting from systematic mating between wild and farmed broodstock,
359 it cannot be excluded that acclimation to the farm may have slightly modified physiological
360 rates compared to the wild abalone. In addition, because respiration was tested in still water
361 conditions, both respiration and calcification values may be greater in animals in moving
362 water on farm or in the wild (Taylor and Ragg, 2005).

363

364 No relationship was observed between *H. tuberculata* ammonium excretion rate and
365 biomass as in the trochus *T. niloticus* (Lorrain *et al.*, 2015). This may indicate a similar
366 excretion rate per unit biomass for adults and juveniles. The rate of excretion per unit of
367 biomass diminishes in mussels as individuals grow larger (Vaughn & Hakenkamp, 2001;
368 Tagliarolo *et al.*, 2012). A positive correlation however has been reported between body size
369 and ammonium excretion rates per individual in *Haliotis discus discus*, *H. gigantea* and *H.*
370 *madaka* (Ahmed *et al.*, 2008).

371

372 **Seasonal variations in *Haliotis tuberculata* physiological parameters**

373 *H. tuberculata* aquatic respiration showed a 2-fold seasonal difference with minimal rates in
374 winter and maximal rates in summer. Seasonal variations in aquatic respiration have been
375 observed in many others benthic molluscs. From 1.6 up to five-fold differences in aquatic
376 respiration rates between winter and summer have been observed in *Patella vulgata*
377 (Tagliarolo *et al.*, 2013b), *C. fornicata* community (Martin *et al.*, 2007), *Crassostrea gigas*

378 (Lejart *et al.*, 2012), the trochus *T. niloticus* (Lorrain *et al.* 2015) and the abalone *H.*
379 *tuberculata* (Gaty & Wilson, 1986).

380 In our experiment, aerial respiration was minimal in winter ($1.49 \pm 0.66 \mu\text{mol CO}_2 \text{ g}$
381 $\text{AFDW}^{-1} \text{ h}^{-1}$) and maximal in summer ($2.21 \pm 0.83 \mu\text{mol CO}_2 \text{ g AFDW}^{-1} \text{ h}^{-1}$) with an average
382 5-fold higher for aquatic respiration compared to aerial respiration. Seasonal aerial respiration
383 has not been extensively studied in molluscs. However, this experiment showed that season
384 has similar effects on underwater and aerial respiration. This can have direct consequences on
385 live transport procedure. As respiration rates and energy demand are higher in summer, it
386 might be more appropriate to transport live abalone during winter when energy needs are
387 lower and when the recovery time post-air exposure may be less.

388 CaCO_3 accretion showed that 53% of *H. tuberculata* individuals did not stop their growth
389 over winter in Brittany temperature conditions. The seasonal growth pattern reported in this
390 study is consistent with previous observations in *H. tuberculata* : abalone growth rate was
391 found to drop in winter whilst 70% of annual growth occurred between May and November
392 (Clavier & Richard, 1986). Similarly, more recent studies using stable oxygen isotopes
393 techniques have found that *H. tuberculata* does not stop its growth in winter (Roussel *et al.*,
394 2011; Jolivet *et al.*, 2015). CaCO_3 accretion in *H. tuberculata* was greater in warmer months
395 when the temperature and the development of the gonad maturation are near its maximum
396 (Hayashi, 1980a) and decreased over winter leading to a net calcification rate over the year
397 ($0.55 \pm 0.53 \mu\text{mol CaCO}_3 \text{ g AFDW}^{-1} \text{ h}^{-1}$). In contrast, the abalone *Haliotis discus hannai*
398 showed retardation in growth during gonad maturation and spawning (Sakai, 1960). Seasonal
399 variation in CaCO_3 accretion is common in molluscs; higher CaCO_3 fluxes have been found in
400 warmer months (Martin *et al.*, 2006; Lejart *et al.*, 2012) whilst CaCO_3 accretion decreases or
401 stops in winter (Lejart *et al.*, 2012; Tagliarolo *et al.*, 2013b).

402 Finally, ammonium excretion rates in *H. tuberculata* also varied seasonally; lower average
403 values were measured in spring and winter ($0.17\text{-}0.19 \mu\text{mol NH}_4^+ \text{AFDW}^{-1}\text{h}^{-1}$, respectively)
404 whilst maximal average values were found in summer and autumn ($0.37\text{-}0.47 \mu\text{mol NH}_4^+$
405 $\text{AFDW}^{-1}\text{h}^{-1}$, respectively). These results are consistent with the variations in excretion rates
406 reported for the limpet *P. vulgata*; low shore individuals had fluxes ranging from 0.5 to 0.7
407 $\mu\text{mol NH}_4^+ \text{AFDW}^{-1}\text{h}^{-1}$ in winter and in summer, respectively (Tagliarolo *et al.*, 2013b). In
408 contrast, other mollusc species showed maximum excretion rates in spring (Martin *et al.*,
409 2006) and minimum rates in winter (Bayne & Scullard, 1977; Martin *et al.*, 2006).
410 This seasonal pattern in *H. tuberculata* physiological rates can be explained by both
411 environmental stressors (*e.g.* temperature), and biogenic factors (*e.g.* reproductive needs,
412 resource abundance, distribution and availability).

413 Seasonal variation in temperature was most likely the primary driver of the observed
414 seasonal variations in carbon fluxes in this study. This hypothesis is consistent with the strong
415 relationships observed between temperature and the studied physiological rates (except
416 ammonium rates); lower rates occurred in winter when the temperature was 11°C and
417 maximal rates were recorded in summer when the temperature reached 18.3°C . Temperature
418 is known to impact all physiological rates in ectotherms (Somero, 2002) and it has been
419 showed to influence the aquatic respiration of many mollusc species (Lejart *et al.*, 2012;
420 Tagliarolo *et al.*, 2013a, 2013b). As temperature increases, the oxygen demand increases as
421 more energy is required to fulfill physiological requirements. This has already been reported
422 in *H. tuberculata*, which exhibited lower oxygen consumption rates at 8°C compared to 16°C
423 or 18°C (Gaty & Wilson, 1986).

424 Biotic factors such as food abundance can contribute to respiration rates. Abalone are
425 herbivorous (Stephenson, 1924) and mostly sedentary (Clavier & Richard, 1982). Abalone
426 increase their feeding activity in summer (Allen *et al.*, 2006) when their resources *i.e.* drifting

427 seaweed (Clavier & Chardy, 1989) are more abundant following summer macroalgal blooms
428 on the Atlantic coast (Dion & Le Bozec, 1996). Fresh algae were provided *ad libitum* before
429 the experiment in order to reduce variability due to starvation since at the same temperature,
430 oxygen consumption rate is 30% lower in 2-weeks starved abalone compared to fed *H.*
431 *tuberculata* (Gaty & Wilson, 1986). Because *H. tuberculata* are more inclined to forage in
432 summer (Roussel, pers. comm.), higher aquatic and aerial respiration rates in summer may be
433 related to higher metabolic demands as more energy is required for digestion, absorption and
434 assimilation processes (Widdows & Shick, 1985). Higher respiration rates may also be related
435 to the production of large gonad. Indeed, the spawning period has been associated with
436 increased metabolic demand as energy is required to produce gametes (Hayashi, 1983).
437 Finally, the low growth and the decrease in excretion rates may be associated with less
438 abundant resources over winter (Allen *et al.*, 2006).

439

440 **Day-night variations in *H. tuberculata* physiological parameters**

441 *H. tuberculata* is characterised like other abalone species (Momma & Sato, 1970; Barkai &
442 Griffiths, 1987) by nocturnal behaviour with movements that are initiated an hour after sunset
443 (Werner *et al.*, 1995). However, unlike other gastropods such as the abalone *H. discus hannai*
444 (Uki & Kikuchi, 1975), and the trochus *T. niloticus* (Lorrain *et al.*, 2015) which display
445 higher metabolic rates whilst being active overnight, no circadian rhythm in *H. tuberculata*
446 aquatic respiration was observed. Movement of the animal could not be controlled in most of
447 the experiment design. The apparatus used in this experiment allowed movement of the
448 abalone inside the bottle, and no movement restraint was used. However, no abalone
449 movements were observed during visual observation of the bottle, even if punctual movement
450 cannot be completely excluded. This result probably indicates that aquatic respiration is

451 similar during night and day periods. However, we cannot exclude that aquatic respiration
452 would be in average higher at night when abalone is crawling to get food.

453 Intrinsic higher metabolic rates overnight may also explain the counterintuitive greater
454 ammonium fluxes at night than during the day, even though the difference was small (0.03
455 $\mu\text{mol NH}_4^+$ AFDW $^{-1}\text{h}^{-1}$). Indeed since *H. tuberculata* has a nocturnal feeding habit like the
456 abalone *H. midae*, ammonium excretion was expected to be higher during the diurnal
457 elimination phase (Barkai & Griffiths, 1987). Further research is required to determine the
458 factors driving day-night patterns of ammonium fluxes in *H. tuberculata*.

459 *H. tuberculata* calcifies 1.7 fold more at night than during the day. Diurnal growth ridge
460 formation in other gastropods like the limpet *Acmaea antillarum* have been related to the
461 light-dark cycle (Kenny, 1977). Since incubations at both day- and night-times were
462 conducted in dark conditions, the calcification process in *H. tuberculata* may be intrinsic and
463 be related to its nocturnal behaviour and related higher metabolic activity in the same way as
464 the trochus *T. niloticus* (Lorrain *et al.*, 2015). More research is required to examine the
465 day/night pattern in calcification rates in shelled gastropods, which has to date been poorly
466 documented.

467

468 ***H. tuberculata* aerial respiration during 6-h exposure**

469 *H. tuberculata* aerial carbon respiration represents ca. 20% of aquatic carbon respiration,
470 which is in the range of what has been observed in intertidal mussels (19%-23%, Tagliarolo *et*
471 *al.*, 2012) but lower than others intertidal gastropod species (40%-230%) (Tagliarolo *et al.*,
472 2013a). The low emersion/immersion ratio *i.e.* 0.2, may be an energy saving strategy for
473 emerged *H. tuberculata* individuals which potentially implies a switch to anaerobic processes
474 (Widdows & Shick, 1985). Another explanation of this lower aerial respiration would be the
475 collapse of the gill in the air, so that the respiration would be limited. This indicates that *H.*

476 *tuberculata* can physiologically adapt to emersion like others intertidal gastropods. However,
477 regardless carefully handling of abalone, it cannot be excluded that acute stress response due
478 to detachment modified, at least partly, aerial respiration at the beginning of the measurement
479 period. Further research is however required to better understand aerobic and anaerobic
480 pathways during emersion in *H. tuberculata*.

481 Some intertidal gastropods have been shown to either decrease aerial respiration over
482 emersion or to maintain stable rates (Tagliarolo *et al.*, 2013a, 2013b). Here, abalone increased
483 their aerial respiration over time at 10°C and 14°C. At 18°C, aerial respiration was already
484 high in the first hour of aerial exposition and no further increase was observed over time.
485 Similarly, mussels (*M. edulis*) showed an increase in aerial energy expenditure when exposed
486 to the air for longer than under natural conditions (Widdows & Shick, 1985). Abalone are
487 mostly found in subtidal areas or at the low shore level of intertidal rocky shores, therefore
488 intertidal abalone are emerged for short periods of time in nature *i.e.* less than 3 hours, rather
489 than the imposed 6 hours of emersion of this study. Respiration rates in *H. tuberculata* started
490 to increase after 3 hours of emersion at the anticipated time of re-immersion. This result
491 showed that *H. tuberculata* can handle emersion during 3 hours with probably very limited
492 energetic cost. However, after 3 hours of emersion, abalone increased their respiration rate (at
493 10°C and 14°C) which suggests higher metabolic cost of longer emersion period at these
494 temperatures (up to 6 hours in this study) and potential physiological impacts on the recovery
495 time (and higher post-emersion mortality). Respiration rates at 10°C were lower than at
496 greater temperatures after 1, 2 and 4 hours of emersion. This suggests that transporting live
497 ormers at low temperature (10 °C) using ice packs for short time period may minimize the
498 metabolic cost of emersion and hence may improve survival rates during the post-emersion
499 recovery period (Buen-Ursua & Ludevese, 2011).

500

501 CONCLUSION

502 This study has underscored that (i) aquatic carbon respiration and calcification rates per unit
503 biomass are higher in smaller ormers, (ii) rates of physiological parameters are lower in
504 winter than in summer, in particular, ormers do not stop calcifying in winter, (iii) calcification
505 and excretion rates are higher at night than during the day, (iv) aerial carbon respiration
506 corresponds to 20% of aquatic respiration which indicates that *H. tuberculata* is adapted to
507 periods of emersion. The clear patterns of variations in *H. tuberculata* physiological rates
508 reported in the present study may constitute important information to understand the
509 relationship between growth, survival and metabolism during farming procedures.

510

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522

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- 709

710 TABLE CAPTIONS

711 **Table 1.** Comparison of aquatic (UW) and aerial (A) respiration in *Haliotis tuberculata* over
712 4 seasons (Wilcoxon Signed Ranks Test).

713

714 **Table 2.** Relationships between metabolic parameters and temperature described by
715 Arrhenius equation $\ln Flux = \ln a - \frac{E_A}{K} \times \frac{1}{T}$. Standards errors are in brackets.

716

717 FIGURE CAPTIONS

718 **Fig. 1.** Sea water temperature variations recorded at the SOMLIT station (St Anne du Portzic
719 site) over 12 months in 2014-2015. Grey squares indicate the field seawater temperature at
720 each sampling period (Sum: summer; Aut: autumn; Win: winter; Spr: spring).

721

722 **Fig. 2.** *Haliotis tuberculata* aquatic and aerial respiration, calcification and ammonium
723 excretion over 4 seasons in regards to seawater temperature (white dots: summer; dark grey
724 dots: autumn; black dots: winter; light grey dots: spring.) in 2014-2015. Values are means
725 and error bars are standard deviations. n = 16 in summer, n = 15 in autumn, n = 17 in winter
726 and in spring. Significant differences (p < 0.05) between seasons from Tukey and Kramer
727 post hoc tests are indicated by letters.

728

729 **Fig. 3.** Allometric relation ($aAFDW^b$) between individual abalone biomass (AFDW) and
730 aquatic respiration (top graph), and calcification (bottom graph) in summer 2014.

731

732 **Fig. 4.** *Haliotis tuberculata* aerial respiration during emersion at 10°C, 14°C and 18°C.
733 Values are means and error bars are standard deviations (n = 8 at 10°C and 14°C; 6 < n < 8 at
734 18°C).

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Season	<i>Z</i>	<i>P</i>	Results
Summer	-3.516	< 0.001	UW > A
Autumn	-3.408	0.001	UW > A
Winter	-3.574	< 0.001	UW > A
Spring	-3.516	< 0.001	UW > A

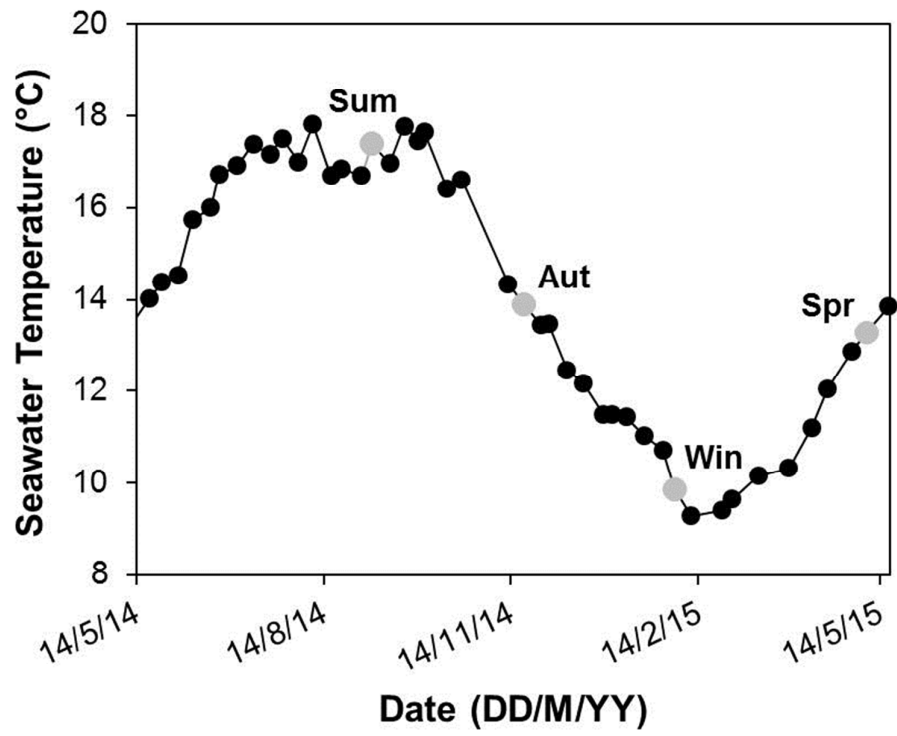
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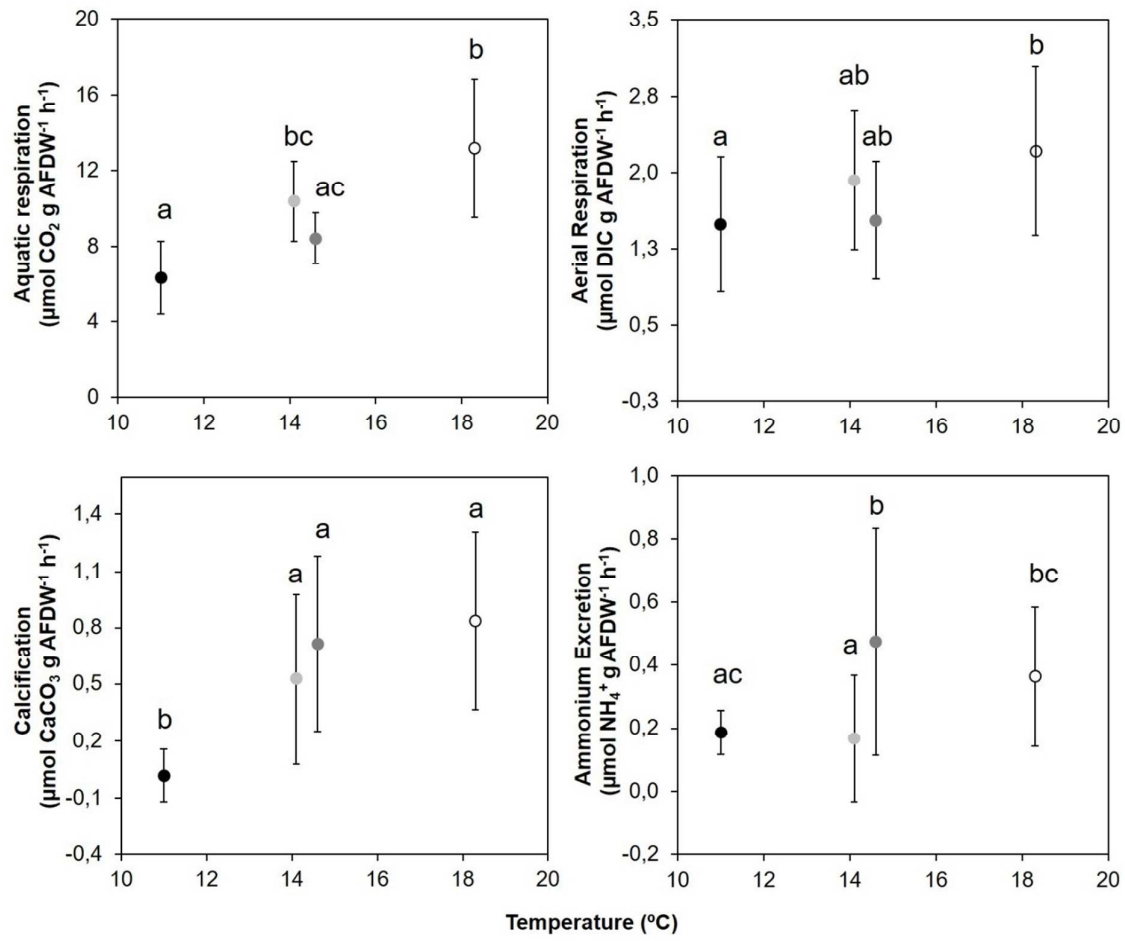
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Parameter	<i>ln a</i>	<i>Ea/K</i>	R²
Aquatic respiration	30.23 (7.46)	8.06 (2.15)	0.88 (0.13)
Calcification	143.42 (67.31)	41.62 (19.36)	0.70 (1.22)
Ammonium excretion	27.47 (27.96)	8.28 (8.04)	0.35 (0.50)
Aerial respiration	15.65 (7.27)	4.34 (2.10)	0.68 (0.13)

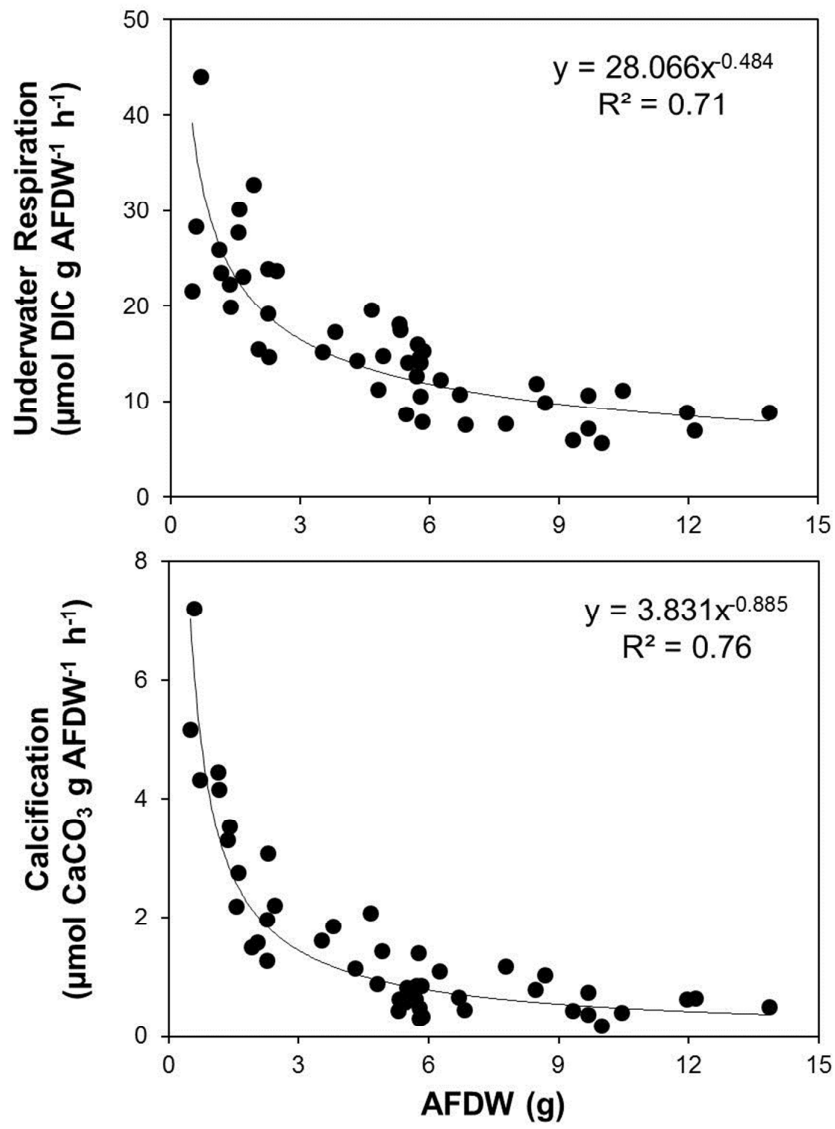
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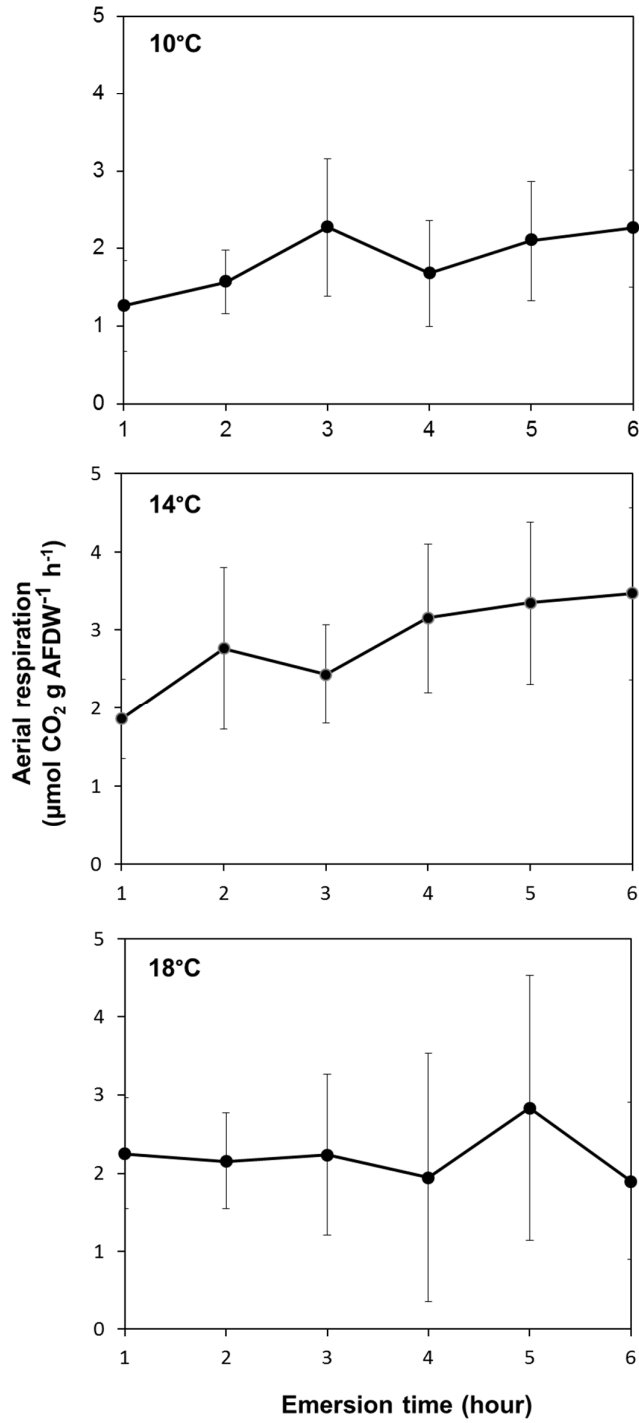
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