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Laboratory conditioning modifies properties of gills mitochondria from the Pacific oyster *Crassostrea gigas*

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Abstract :

Although laboratory experiments allow greater control of environmental conditions than field studies, they have several drawbacks. To analyze physiological responses to forcing environmental variables, experimental conditions should mimic natural conditions as closely as possible. For filter-feeding organisms in particular, diet quality and quantity is one of the environmental parameters that can differ markedly between experimental and field conditions. In the hatchery, Pacific oysters, *Crassostrea gigas*, commonly show good physiological performance and growth on a mixed algal diet of *Tisochrysis lutea*, formerly Isochrysis aff. galbana clone Tahiti (*T-Iso*), and *Chaetoceros calcitrans*, presumably as it provides a good supply of essential polyunsaturated fatty acids (PUFA) as 20:4n-6, 20:5n-3 and 22:6n-3. The present study tests whether the fluctuating biotic and abiotic conditions in the field modify the structure and function of oyster mitochondria. One group of oysters was maintained in the intertidal zone, and the other group was fed the mixed diet in a nearby experimental hatchery under salinity and temperature conditions equivalent to those in the field. After 4 weeks of conditioning, the functional capacities and membrane lipid composition of gill mitochondria were measured. For essential polyunsaturated fatty acids, only the proportion of 20:5n-3 differed between field and laboratory oysters, and confirmed the capacity of the mixed diet *T-Iso* + *C. gracilis*, to provide the essential PUFA. Nevertheless, proportions of other FA (e.g., 22:5n-6 and non-methylene-interrupted FA) differed markedly between laboratory and field-conditioned oysters. Mitochondrial oxygen uptake, cytochrome c oxidase activity, content of cardiolipin and concentration of cytochrome b were significantly lower in laboratory-conditioned than in field-conditioned oysters. These results indicate that laboratory conditioning, although allowing similar growth and gonad maturation, only partially mimics conditions that allow *C. gigas* to maintain mitochondrial function. Although our experimental design cannot ascertain what difference between experimental laboratory and field conditions led to changes in membrane composition and mitochondrial function, differences in nutritional quality (other than known essential PUFA) and abiotic factors (e.g., oxygen availability, emersion or daily temperature fluctuations) had a major impact on mitochondrial properties in oysters.

56 **Introduction**

57

58 Although laboratory experiments permit much more control over environmental conditions
59 than field studies, they have drawbacks, including the adequacy and realism of laboratory
60 conditions. Experiments in artificial settings attempt to simplify natural complexity by
61 limiting variation to a small number of factors. However, the constant environmental
62 conditions used in laboratory experiments may be inappropriate for animals that have adapted
63 to fluctuating conditions and may impair physiological and cellular processes (Guerra et al.
64 2012). Oysters generally inhabit the intertidal zone where they are exposed to large
65 fluctuations in oxygen concentration, temperature, food availability and quality, on hourly,
66 daily and seasonal basis. For marine bivalves, changes in abiotic conditions, such as
67 temperature, oxygenation, salinity and desiccation lead to regulated physiological adjustments
68 that can affect growth and reproduction. At the cellular level, changes in mitochondrial
69 function and membrane composition in response to abiotic factors have been demonstrated in
70 bivalves (Glémet and Ballantyne 1995; Gillis and Ballantyne 1999; Guderley 2004;
71 Sussarellu et al. 2013; Dudognon et al. 2013). This ability to adjust mitochondrial structure
72 and function is thought to be critical in controlling energy production under changing
73 environmental conditions (Guderley 2004; Bremer and Moyes 2011). However, whether
74 fluctuating abiotic and biotic conditions affect mitochondrial structure and function in marine
75 bivalves is unknown.

76

77 The use of an appropriate diet in the laboratory is primordial, given the influence of diet on
78 energy acquisition for physiological acclimation and growth. Formulating an adequate diet for
79 filter feeding organisms in hatcheries represents a particular challenge, as filter feeders
80 typically ingest living (mainly phytoplankton) and inert particles suspended in the water
81 column. Cultured phytoplankton is generally used during laboratory conditioning of bivalves
82 (Martínez et al. 2000; Fariás and Uriate 2006). Algal shape, size, toxicity, digestibility and
83 biochemical composition affect the nutritional value of microalgal species (Brown et al.
84 1997). Trial and error has shown that microalgal species used to rear bivalves in hatcheries do
85 not have the same nutritional value and lead to different rates of growth, survival,
86 gametogenesis, embryogenesis, hatching and metamorphosis (Epifanio 1979; Enright et al.
87 1986; Delaunay et al. 1993; Soudant et al. 1996a, 1996b; Utting and Millican 1997; Rico-
88 Villa et al. 2006; Pronker et al. 2008; Marshall et al. 2010).

89 A major biochemical difference among algae lies in their lipid and, particularly, fatty acid
90 (FA) composition that in turn, affects the composition of the bivalves feeding upon them.

91 Various studies have established that the polyunsaturated fatty acid (PUFA) composition of
92 bivalve cell membranes changes with microalgal FA composition (Delaunay et al. 1993;
93 Soudant et al. 1999; Pennarun et al. 2003; Delaporte et al. 2003, 2005, 2006; Marshall et al.
94 2010; Dudognon et al. 2014). Because of their structural role in membranes, modifications of
95 membrane FA composition can modulate membrane protein activities and physiological
96 parameters in mammals (Yamaoka et al. 1988; Senault et al. 1990; Hulbert and Else 1999;
97 Leonard et al. 2001; Hirunpanich et al. 2007), fish (Moya-Falcón et al. 2004; Hamza et al.
98 2008), and bivalves (Soudant et al. 1996a, 1996b; Delaporte et al. 2003; Delaporte et al.
99 2006). Given the compositional variety of microalgal FA, a mixture of the microalgae
100 *Tisochrysis lutea*, formerly *Isochrysis* aff. *galbana* clone Tahiti (*T-Iso*) and *Chaetoceros*
101 *calcitrans* or *Chaetoceros gracilis* is often used as an optimal supply of essential FA that
102 allows adequate performance of oysters under laboratory conditions (Rico-Villa et al. 2006;
103 Dudognon et al., 2014). Nonetheless, comparison of adult oysters fed two algae as mono-
104 specific diets (*i.e.* *T-Iso* and *C. gracilis*), shows that although the FA composition of
105 mitochondrial membranes is strongly modified by the FA composition of monoalgal diet,
106 very few functional parameters are modified (Dudognon et al. 2014). Such insensitivity of the
107 oxidative capacities of oyster gill mitochondria to major diet-induced changes of membrane
108 composition suggests that regulated changes and homeostatic mechanisms maintain critical
109 mitochondrial membrane characteristics despite major differences in nutritional quality.

110

111 In the present study, we tested how laboratory conditions perform to mimic environmental
112 conditions regarding oyster mitochondrial function. For this purpose, we compared
113 physiological performance and mitochondrial properties of oysters kept under controlled
114 laboratory conditions, with salinity and temperature in the experimental tanks similar to field
115 conditions), and a same lot of oysters reared during the same period in the field where they
116 were exposed to natural fluctuations of biotic and abiotic conditions. Physiological
117 performance was assessed through growth and reproductive investment after 4 weeks of
118 conditioning. Gill mitochondria were isolated from oysters before and after conditioning. We
119 characterized mitochondrial oxidative capacities, cytochrome c oxidase (CCO) and citrate
120 synthase (CS) activity, as well as contents of cytochrome *a*, *b*, *c*₁ and *c*, and membrane lipid
121 composition.

122

123 **Materials and methods**

124

125 **Chemicals**

126 All chemicals were purchased from Sigma (Saint Quentin Fallavier, France), unless
127 mentioned otherwise.

128

129 **Sampling procedures**

130 In May 2010, one hundred Pacific oysters *C. gigas*, shell length ranging from 6 to 8.5 cm
131 (7.3 ± 0.2 , mean \pm standard error), originating from the same batch of spat, were obtained from
132 a hatchery in Arcachon, France. After arriving in Brittany, oysters were placed in a mesh bag
133 on a tray in the intertidal zone at Aber Benoît (North Brittany, 48° 32'N, 4° 30'W) near the
134 Argenton experimental station. One day after arriving, 50 were placed in a 800 L flow-
135 through seawater tank at the Argenton experimental station (IFREMER, Argenton, France),
136 with a water flow of 120 L.h⁻¹. In parallel, the other 50 oysters were kept in the mesh bag in
137 the intertidal zone. The experimental tank was supplied with seawater pumped at mid-depth in
138 a tidal dock and partly filtered (10 μ m) through a bag filter. Thus, salinity and temperature in
139 the experimental tank were similar to field conditions (from 12°C on May 20th, to 16°C on
140 June 22nd). The tank was drained and cleaned three times a week.

141 Samples were taken at the start of the experiment (T0, 20th of May 2010) and after 4 weeks of
142 conditioning (Tf, 22nd of June 2010). Total weight was measured on whole animals (flesh +
143 shells) before dissection and mitochondrial preparation. Laboratory oysters were fed *ad*
144 *libitum* with a mix of two microalgae: *Tisochrysis lutea* formerly *Isochrysis* aff. *galbana*
145 clone Tahiti (T-Iso) and *Chaetoceros gracilis*, supplied in equal cell volumes. Cultures were
146 produced in 300 L cylinders containing 1 μ m filtered seawater enriched with Conway
147 medium at 24 \pm 1°C, air-CO₂ (3%) and mix aerated, under continuous light. Microalgae were
148 harvested at exponential growth phase (6–8 days).

149

150 **Reproductive activity**

151 *Qualitative analysis of gametogenic stage*

152 A visceral mass slice (2-3 mm thick) was cut upstream of the adductor muscle, put in a
153 histology cassette and transferred into a modified Davidson's fixative (Latendresse et al.
154 2002) for 48 h. Fixed tissues were dehydrated in ascending ethanol solutions, cleared with
155 Claral[®] and embedded in paraffin wax. Five micrometer thick sections were cut, mounted on
156 glass slides, and stained with Harry's hematoxylin–eosin Y. Slides were examined under a
157 light microscope to determine gametogenic stage according to the reproductive scale reported

158 by Mann (1979) : stage 0 (inactive), stage 1 (early gametogenesis), stage 2 (late
159 gametogenesis) and stage 3 (ripe).

160

161 *Quantitative analysis of gonad occupation area*

162 Percentage of gonad occupation area was determined on each histological section as described
163 by Enríquez-Díaz et al. (2009). Briefly, slides were scanned with a digital scanner. Gonad
164 area was measured by using image analysis software (Imaq Vision Builder, National
165 Instruments Corp., Austin, Texas, USA).

166

167 *Mitochondrial analysis*

168

169 Mitochondrial analyses were performed on mitochondria isolated from gills. For each group
170 (field and laboratory), six pools containing gills of five individuals were used (each pool
171 contained 2.5 ± 0.1 g of gill tissue).

172

173 *Isolation of mitochondria*

174 Procedures for mitochondrial isolation are detailed in Dudognon et al. (2013). Oxygen
175 consumption was measured immediately on the fresh mitochondrial preparations.

176 A subsample of mitochondrial pellets was stored at -80°C for subsequent assays of enzymatic
177 activities, cytochrome concentrations and lipid extraction.

178

179 *Measurement of oxygen consumption*

180 Mitochondrial oxygen consumption was measured polarographically using a water-jacketed
181 O_2 monitoring system (Qubit System, Kingston, Ontario, Canada). Temperature was
182 controlled at 10°C by a circulating refrigerated water bath. For each assay, around 0.8 mg of
183 mitochondrial protein (40 μl mitochondrial preparation) was added to 0.4 ml reaction buffer
184 containing 400 mM sucrose, 30 mM HEPES, 90 mM KCl, 10 mM KH_2PO_4 , 50 mM taurine
185 and 50 mM β -alanine, pH 7.5. On the day of the experiment, 0.5% BSA was added to the
186 assay medium. For measurement of maximal oxidative capacities, glutamate (40 mM) or
187 succinate (20 mM) was added to fuel oxygen consumption through complex I or II,
188 respectively. The maximal respiration rate (state 3) was obtained after addition of ADP to a
189 final concentration of 600 μM . Preliminary experiments showed that glutamate alone
190 stimulates respiration through complex I after addition of ADP (Dudognon et al. 2013). Non-
191 phosphorylating oxygen consumption (state 4) rate was measured after ADP depletion. Each
192 measurement was performed in simultaneous triplicates using three polarographic chambers.

193 RCR (respiratory control ratio) was defined as the ratio between state 3 and state 4. Oxidative
194 phosphorylation efficiency (relation between ADP added and oxygen consumption) was
195 calculated as ADP/O ratio according to Estabrook (1967).

196

197 *Enzymatic activities*

198 Cytochrome *c* oxidase was measured in mitochondrial preparations and citrate synthase (CS)
199 activity was measured in gill extracts according to Dudognon et al. (2013) and Dudognon et
200 al. (2014), respectively.

201

202 *Cytochrome concentrations*

203 Cytochromes *a*, *b*, *c*₁, and *c* concentrations were quantified by difference spectra according to
204 Leary et al. (2003) except that mitochondrial suspensions (around 0.8 mg of mitochondrial
205 protein) were diluted in phosphate buffer (NaH₂PO₄ and Na₂HPO₄ 50 mmol pH 7.8 at 25°C)
206 and Triton-X was not used. Accordingly, the electron transport chain complex was reduced by
207 adding a few grains of sodium dithionite and incubated for 15 min at room temperature. The
208 reduced samples were read against air-oxidized samples between 400 and 630 nm. We used
209 the solution to the simultaneous equations required to assess individual cytochrome
210 concentrations (Williams Jr. 1964).

211

212 *Protein concentration*

213 Aliquots of 20 µl of mitochondria preparations were suspended in reaction buffer without
214 BSA and centrifuged for 10 min at 12,000 g at 4°C. The supernatant was discarded and the
215 pellet re-suspended, washed and centrifuged twice again to remove the BSA. Pellets were
216 maintained in 0.5 ml of ultra-pure water and frozen at -80°C until protein analysis. The
217 protein concentration was determined with the RC DC Protein Assay Kit (BIORAD) using
218 BSA as standard.

219

220 *Membrane lipid analysis*

221 The membrane lipids of mitochondrial suspensions were extracted according to the method of
222 Folch et al. (1957). Before lipid extraction, the aliquot of mitochondrial preparation was
223 resuspended in reaction buffer minus BSA and centrifuged at 9000 g at room temperature for
224 10 min. The supernatant was discarded and the pellet resuspended, washed in reaction buffer
225 and centrifuged again two times. The final extract was stored at -80°C under nitrogen after
226 adding 0.01% w/v butylated hydroxytoluene (BHT, antioxidant).

227

228 *Isolation of membrane lipids on silica gel microcolumn*

229 Lipid extract was evaporated to dryness under nitrogen, recovered with three washings of
230 500 µl of chloroform/methanol (98:2, v/v) and deposited at the top of a silica gel micro-
231 column (30 mm x 5 mm i.d.), packed with Kieselgel 60 (70–230 mesh, Merck, Darmstadt,
232 Germany) previously heated at 450°C and deactivated with 6 % H₂O (Marty et al. 1992).
233 Neutral lipids were eluted with 10 ml of chloroform:methanol (98:2, v/v). The polar lipid
234 fraction (membrane lipids) was recovered with 15 ml methanol, and C23:0 solution was
235 added as internal standard for further fatty acid analysis.

236

237 *Fatty acid analysis of membrane lipids*

238 The polar lipid fraction was evaporated to dryness under nitrogen and transesterified for 10
239 min at 100°C after adding 1 ml of methanol/BF₃ (10%). After cooling, 1 ml of ultra-pure
240 water and 1 ml of hexane were added. Tubes were mixed thoroughly for 2 min and
241 centrifuged at 1,000 g for 10 min. The organic phase containing fatty acid methyl esters
242 (FAME) was washed three times with 1 mL of water. Fatty acid methyl esters were quantified
243 by gas chromatography and identified by comparing their retention times with those of a
244 standard mixture containing 37 FAME (SUPELCO/Sigma–Aldrich, St-Quentin Fallavier,
245 France), and other lab-made standard mixtures of marine bivalve. A total of 40 FA were
246 quantified and expressed as the molar percentage of the total FA content.

247

248 *Separation of membrane lipid classes*

249 Phospholipid classes were analyzed by high-performance thin-layer chromatography
250 (HPTLC) as described in details in Dudognon et al. (2014). Mass of each identified
251 phospholipid class was determined by comparison with standards of known amounts. The
252 technique used did not separate phosphatidylinositol (PI) and
253 ceramideaminoethylphosphonate (CAEP) (unpublished preliminary results), and proportions
254 of these phospholipids were presented as the sum PI + CAEP (CAEP being predominant over
255 PI in oyster gill mitochondria (data not shown)). The sum of PI+CAEP was quantified using
256 sphingomyelin as standard because sphingomyelin has a similar structure to CAEP although
257 is absent in bivalves (Le Grand et al. 2011). Results were expressed as mass% of total
258 phospholipids.

259

260 *Statistical analysis*

261 ANOVA was performed if normality and homogeneity of variances were respected. ANOVA
262 was followed by post hoc Fisher's least significant difference (LSD) test. For data that did not

263 follow normality or meet homoscedasticity, non-parametric Kruskal-Wallis analysis was
264 performed. For each test, the significance threshold was $P<0.05$. Percentage data were
265 transformed (arcsin of the square root) before ANOVA but are presented in figures and tables
266 as untransformed percentage values. All analyses were performed with the Statgraphics
267 software, version Plus 5.1. (Manugistics, Inc, Dallas, USA). Results are expressed as mean \pm
268 standard error (SE).

269
270

271 **Results**

272

273 *Oyster weight*

274 Initial oyster weight in trays was 41.7 ± 2.6 g (T0) and increased significantly after 4 weeks
275 under both field and laboratory conditions (Figure 1). The two groups of oysters did not differ
276 at the end of conditioning in June (mean values of 56.2 ± 1.7 g and 61.0 ± 2.3 g for field and
277 laboratory oysters, respectively). No mortality occurred during the experiment.

278

279 *Gonad maturation stages and gonad coverage area*

280 Oysters sampled in May were in late gametogenesis (stage 2), whereas those sampled in June
281 were mostly (about 80%) reproductively ripe (stage 3) (data not shown). Similarly, percentage
282 of gonad coverage area increased significantly ($P<0.01$) from initial values in May (43.4 ± 3.5)
283 to final values of $65.1\pm 3.1\%$ and $68.8\pm 2.1\%$ in oysters from laboratory and field conditions,
284 respectively. There was no difference in gonad occupation area between laboratory and field
285 oysters (Figure 1).

286

287 *Mitochondrial oxidative capacities*

288 Oxidative capacities of mitochondria isolated from oyster gills were assessed using glutamate
289 or succinate as substrate. Overall, the capacities of mitochondria isolated from field oysters at
290 Tf were similar to those of oysters at T0; both were systematically higher than those of
291 laboratory conditioned oysters at Tf (Fig. 2). Maximal state 3 rates of glutamate and
292 succinate oxidation were significantly lower in laboratory oysters than in field oysters for
293 both substrates (around 40% lower) (Figure 2A). The same pattern was observed with the
294 non-phosphorylating rate (state 4) (Figure 2B). State 4 was significantly higher with succinate
295 than with glutamate (Figure 2B, $P<0.05$).

296 As a consequence of these modifications of state 3 and state 4 rates, RCR with glutamate was
297 lower in laboratory than in field oysters at the end of the experiment (- 25%) (Table 3). On the
298 other hand, RCR with succinate did not differ at Tf between field and laboratory oysters.
299 ADP/O did not differ between field and laboratory oysters for glutamate and succinate, but
300 values were significantly higher for field oysters at T0 compared to Tf for both substrates.
301 RCR and ADP/O were always significantly higher with glutamate than with succinate as
302 substrate ($P<0.05$).

303

304 *Mitochondrial enzyme activities and protein composition*

305 *Cytochrome c oxidase and citrate synthase activity*

306 CCO activity, expressed in $U \cdot mg^{-1}$ mitochondrial protein, was significantly lower in
307 laboratory conditioned oysters (-33%), than in field oysters sampled at T0 and Tf (Table 4).
308 As cyt *a* is located in complex IV (CCO) of the respiratory chain, activity of CCO was also
309 expressed per nmole of cytochrome *a* to reflect its catalytic activity. In that case, CCO
310 activity did not differ between groups.

311 CS activity, measured on isolated gill tissue and expressed in $U \cdot mg^{-1}$ gill tissue, did not differ
312 between the three groups of oysters.

313

314 *Mitochondrial cytochrome concentrations*

315 Cytochrome *b* concentration did not differ between field oysters at T0 and Tf; both groups
316 had significantly higher values than laboratory conditioned oysters (-24%) (Table 5).
317 Cytochrome *a*, *c*₁ and *c* concentrations did not differ between the 3 groups. There was no
318 difference between oyster groups when cytochrome quantities were expressed relative to
319 cytochrome *c*₁. Similarly, there was no difference in the ratio $(b + c_1)/a$ between the three
320 groups of oysters.

321

322 *Fatty acid composition of phospholipids from gill mitochondria*

323 Four weeks under field and laboratory conditions significantly changed the FA composition
324 of membrane phospholipids of oyster gill mitochondria compared to their initial status (T0)
325 (Table 1). More importantly, FA composition differed between rearing conditions at Tf.

326 The proportions of 20:5n-3 decreased throughout the experiment but was higher in
327 mitochondria of field-conditioned oysters than in laboratory-conditioned oysters at Tf. The
328 proportions of 22:6n-3 and 20:4n-6 were also higher at T0, but did not differ between the two
329 groups of oysters at Tf. The proportion of 22:5n-6 was 0.4% at the start of the experiment and

330 did not change in oysters after 4 weeks in the field. However, it rose by more than 5 fold
331 (2.1%) in oysters after laboratory conditioning.

332 After 4 weeks conditioning, total NMI (non-methylene interrupted) FA were more abundant
333 in laboratory-conditioned oysters than in field oysters. This difference mainly reflected higher
334 levels of 22:2NMI(7,15) in laboratory-conditioned oysters.

335 The proportion of 18:1n-7 (NMI FA precursor) reached 5.6% in oysters after 4 weeks in the
336 laboratory while this FA was lower (3.4%) in oysters from the field at the same time. There
337 was no difference in the proportion of the monounsaturated FA (MUFA) 16:1n-7 between the
338 three groups of oysters. The level of 18:1n-9, 20:1n-9 and 20:1n-7 reached similar levels
339 between laboratory and field oysters after 4 weeks conditioning.

340 The proportion of total saturated FA (SFA), monounsaturated FA (MUFA) and
341 polyunsaturated FA (PUFA) changed markedly throughout the experiment. SFA were higher
342 in mitochondria of oysters from the field than from the laboratory at Tf. At Tf, MUFA were
343 higher in mitochondria of laboratory oysters as compared to field oysters. On the other hand,
344 total PUFA did not differ between the two groups sampled at Tf. Within PUFA, mitochondria
345 of oysters from the laboratory showed the highest proportion of n-6 PUFA but the lowest
346 proportion of n-3 PUFA at the end of the experiment.

347

348 *Phospholipid classes of mitochondrial membranes*

349 Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the main phospholipid
350 classes in mitochondrial membranes of oysters accounting for 64-66.8% of total
351 phospholipids (Table 2). Phosphatidylinositol (PI) and ceramideaminoethylphosphonate
352 (CAEP) together represented around 20% and phosphatidylserine (PS) accounted for about
353 8% of the total phospholipids. The phospholipid-to-protein ratio (i.e. total phospholipid
354 content expressed per mg of mitochondrial proteins), as well as proportions of PE, PC and PS
355 did not differ among treatments at Tf. The proportions of PI + CAEP increased slightly but
356 significantly after 4 weeks of experimentation and oysters from the laboratory showed a
357 higher proportion of PI + CAEP than oysters from the field. The proportions of CL changed
358 significantly between rearing conditions and laboratory oysters showed lower levels in
359 mitochondria than field oysters at Tf (by about -25%).

360 PC/PE ratio decreased after 4 weeks conditioning and reached significantly lower values in
361 laboratory as compared to field oysters, whereas the ratio PE/CL was higher in mitochondria
362 of oysters from the laboratory than field oysters.

363

364

365 **Discussion**

366

367 The similar increase in weight and gonadal occupation between oysters grown in the
368 laboratory and those left in a mesh bag in their natural environment confirmed the suitability
369 of laboratory conditioning for oyster growth and gonad development. Most feeding studies on
370 bivalves use such overall criteria, while few investigate cellular or sub-cellular responses. The
371 present results showed that rearing oysters in laboratory-controlled conditions rather than
372 under fluctuating field conditions decreased the capacities of gill mitochondria, while overall
373 growth and gonad development did not differ.

374 Major differences of mitochondrial capacities were observed between the groups.
375 Phosphorylating rates of oxygen consumption (state 3) were almost two fold lower in gill
376 mitochondria from laboratory-conditioned oysters. This was true both when isolated
377 mitochondria were fueled with glutamate through complex I or with succinate through
378 complex II. Non-phosphorylating oxygen consumption (state 4) which partly reflects proton
379 leak (St. Pierre et al. 2000) was also consistently lower in laboratory-conditioned oysters.

380 Differences in mitochondrial oxidative capacities can reflect changes in concentrations
381 or relative levels of electron transport chain complexes (Guderley et al. 2005). Cytochrome *b*
382 was 24% less abundant in gill mitochondria from oysters reared in laboratory tanks than in the
383 field. A decrease in cytochrome *b*, a component of complexes II and III of the respiratory
384 chain, may be implicated in the diminished mitochondrial oxidative capacities in laboratory
385 oysters. A concomitant decrease in mitochondrial respiration and in CCO activity is observed
386 in response to diet in rats (Yamaoka et al. 1988), temperature in fish (Blier and Lemieux
387 2001); (Kraffe et al. 2007), and hypoxic hibernation in frogs (Boutilier and St-Pierre 2002).
388 CCO is the last complex of the respiratory chain and can control, at least in part, rates of
389 electron transport through the electron transport chain (Groen et al. 1982). Therefore,
390 diminished CCO activity could be one cause of reduced mitochondrial oxidative capacity in
391 gills of laboratory conditioned oysters.

392 Phospholipid classes and their FA composition influence the activity of membrane-
393 bound proteins, such as respiratory chain complexes in mitochondria (Robinson 1993; Stuart
394 et al. 1998; Frick et al. 2010). Phospholipid proportions in mitochondrial membranes differed
395 between laboratory and field oysters. Among these differences, the lower levels of CL in gill
396 mitochondria of laboratory oysters could explain the lower activity of CCO. Indeed, CL is
397 specifically located in the inner membrane of mitochondria and its association with
398 respiratory chain enzymes is of major importance for regulation of their activity and the
399 generation of the inner membrane potential (Schlame et al. 2000; Haines and Dencher 2002;

400 Sedlák et al. 2006; Zhou et al. 2011; Böttinger et al. 2012; Kagan and Epanand, 2014). A
401 reduced level of CL is associated with decreased mitochondrial activity in mammals
402 (Paradies et al. 1997), snails (Stuart et al. 1998) and fishes (Frick et al. 2010). CL deficiency
403 also induces destabilization, decreased activity and loss of complex III (Schlame et al. 2000;
404 Lange et al. 2001; Petrosillo et al. 2003; Böttinger et al. 2012), potentially explaining the
405 lower cytochrome *b* content in laboratory conditioned oysters

406 The FA composition of phospholipids influence the activity of membrane-bound
407 proteins, either through overall or micro-environment localized effects (Robinson 1993;
408 Phillips et al. 2009; Hulbert and Else 1999). Proportions of SFA were higher and those of
409 MUFA lower in gill mitochondria from field oysters than laboratory conditioned oysters.
410 These overall differences may well have changed mitochondrial oxygen uptake and CCO
411 activity. The results of a partner study indicate that maintenance of overall aspects of
412 membrane FA composition (total SFA, MUFA and PUFA) despite marked changes in the
413 levels of individual FA may be key for the maintenance of mitochondrial function in oyster
414 gills (Dudognon et al., 2014) as reported in mammals (Astorg and Chevalier 1991; Lemieux
415 et al. 2008; Abbott et al. 2012) and fish (Guderley et al. 2008; Martin et al. 2013).

416
417 In the experimental tank, feeding oysters *ad libitum* with a mixture of the two microalgae
418 *T.Isochrysis lutea*, and *Chaetoceros gracilis* permitted a balanced supply of essential FA as
419 20:4n-6, 20:5n-3 and 22:6n-3 for oysters (Delaunay et al., 1993; Soudant et al. 1996a;
420 Delaporte et al., 2005; Rico-Villa et al. 2006; Marshall et al., 2010; Dudognon et al. 2014).
421 Laboratory and field conditioned oysters had indeed similar levels of the essential FA in gill
422 mitochondria, with the exception of 20:5n-3, confirming that the mixed diet *T-Iso* + *C.*
423 *gracilis* provided sufficient essential FA. Nevertheless, differences of some particular FA (*i.e.*
424 22:5n-6 and NMI FA) between laboratory and field oysters may be important for the
425 regulation of mitochondrial membrane protein activities (Zhou et al. 2011; Khairallah et al.
426 2012). Whereas 22:5n-6 appears to be preferentially incorporated from the microalgae *T. Iso*
427 (Soudant et al, 1996a; Soudant et al. 1996c; Dudognon et al. 2014), NMI FA are the only long
428 chain PUFA that bivalves synthesize *de novo* (Zhukova 1991). Both 22:5n-6 and NMI FA
429 have been identified as potentially significant for the physiology of bivalve larvae and the
430 structure and fluidity of membranes in female gametes (Soudant et al. 1996a; Soudant et al.,
431 1996c; Pernet et al. 2005; Mike et al. 2008), for membrane functions of bivalve hemocytes
432 (Le Grand 2011, 2013; Dudognon et al. 2014) as well as for peroxidation resistance and
433 activities of gills and gill mitochondria of bivalves (Dudognon et al. 2014; Munro and Blier
434 2012; Barnathan 2009; Delaporte et al. 2005; Kraffe et al. 2004).

435 Changes in several characteristics, ranging from cytochrome contents to phospholipid
436 proportions and FA characteristics, could explain the loss of capacity of gill mitochondria
437 from oysters conditioned in the laboratory. A loss of capacity after laboratory conditioning
438 also occurred in mitochondria from various tissues of the scallop, *Argopecten purpuratus*
439 (Guderley et al. 2011). One hypothesis questions whether the bi-specific algal diet contains
440 what oysters need to maintain mitochondrial activity. Beside their content in FA,
441 carbohydrate or amino acids, microalgal species also vary significantly in contents of
442 vitamins, minerals and trace elements (Brown 2002). In mammals, these compounds are
443 critical cofactors that support mitochondrial functions. Deficiencies in some minerals and
444 vitamins can reduce activities of electron transport complexes and cardiolipin content in
445 mammals (Ames et al. 2005; Oliveros et al. 2007). As growth rates and gametogenesis did not
446 differ between laboratory and field oysters, the bispecific algal diet should have provided
447 enough energy for somatic growth and gametes maturation, but may have been lacking
448 materials for mitochondrial activity. Finally, the continuous *ad libitum* feeding we used in the
449 laboratory can be unfavourable for intertidal bivalves such as oysters (Utting et Spencer 1991;
450 Racotta et al. 1998; Hurtado et al. 2009), and may have decreased mitochondrial performance.

451 Nutrient supply may not be the only environmental factor that could have impacted
452 mitochondrial functions and compositions in oysters. Laboratory conditioned oysters were
453 constantly immersed in well-aerated filtered seawater whereas field oysters had to cope with
454 marked variations in physical, chemical and biological parameters (e.g. temperature,
455 oxygenation and feeding activity during emersion and immersion cycles), linked to tidal
456 cycles. Modifications of environmental conditions, including salinity, hypoxia and
457 temperature, change the structure and function of bivalve mitochondria (Glémet and
458 Ballantyne 1995; Gillis and Ballantyne 1999; Guderley 2004; Sussarellu et al. 2013;
459 Dudognon et al. 2013). Our observations suggest that variable environmental conditions in the
460 field may have stressed changes in specific lipid constituents of mitochondrial membranes
461 and modified mitochondrial capacities.

462

463

464 **Conclusion**

465

466 Standardized conditions and *ad libitum* feeding of oysters with a mixed plankton diet do not
467 fully mimic field conditions. Changes of specific FA and phospholipid classes in
468 mitochondrial membranes may explain the lower mitochondrial capacities in laboratory-
469 conditioned oysters. Field conditions impose fluctuations and differences in abiotic and biotic

470 factors that may have led structural and functional properties of gill mitochondria to differ
471 from those of laboratory conditioned oysters. Nonetheless, overall “fitness” was not affected
472 as oysters showed similar growth and gonadal development in the field and laboratory.
473 Nevertheless, the changed mitochondrial capacities suggest that cellular processes in oysters
474 held in constant laboratory conditions are reduced compared to those of oysters living in the
475 intertidal zone. This could impair other physiological and cellular processes. It may be for
476 example questionable why oysters fed *ad libitum* and probably having access to more food
477 and for a longer period of time did not grow better in the laboratory.

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483

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492

493

494 **References**

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Figure 1

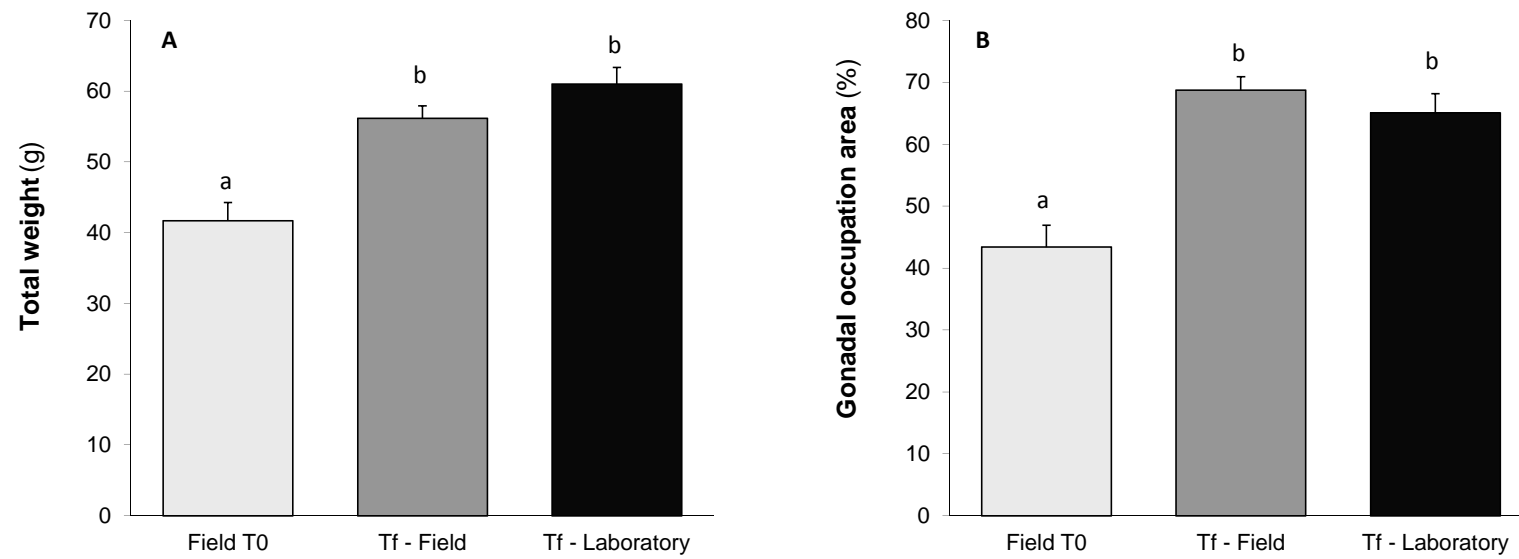


Figure 1: Total weight (expressed as g) (A) and gonad occupation area (expressed as %) (B) of oysters (n = 30) grown on their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf - Field, Tf - Laboratory). Values are mean \pm SE. Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups.

Figure 2

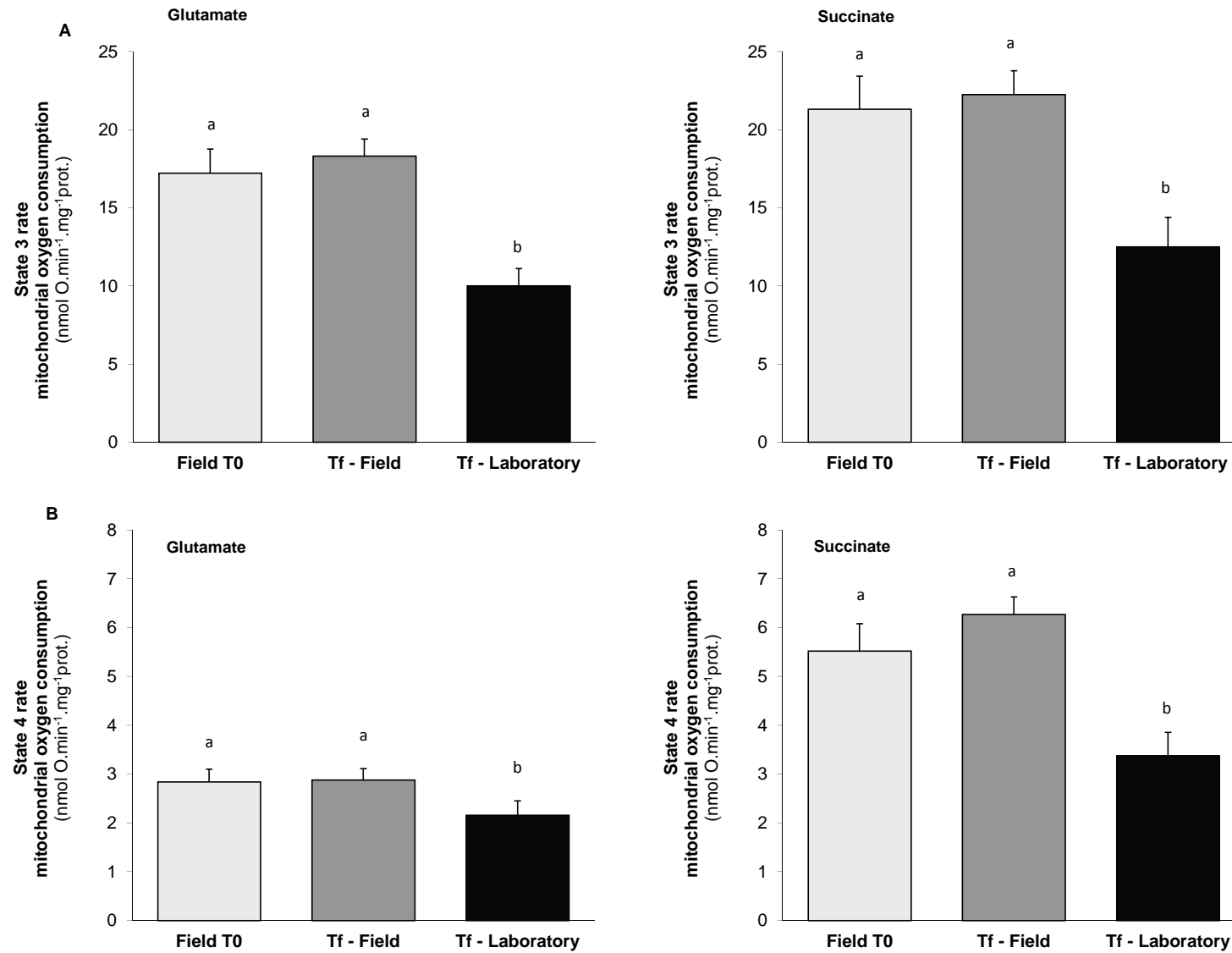


Figure 2 : Rates of oxidative phosphorylation (state 3) (A) and non-phosphorylating oxygen consumption (state 4) (B) with glutamate 40 mM or succinate 20 mM on mitochondria isolated from oysters grown in their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf . Field, Tf . Laboratory). Assay temperature was 10°C. Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups (mean \pm SE, $n = 6$ pools of 5 oysters).

Table 1: Fatty acid composition of gill mitochondria phospholipids of oysters grown in their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf – Field, Tf – Laboratory). The results are expressed as mol percentages of total fatty acids. Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups (mean \pm SE, $n = 6$ pools of 5 oysters).

	Field T0		Tf - Field		Tf - Laboratory	
	Mean	SE	Mean	SE	Mean	SE
14:0	1.4 ^a	0.0	1.5 ^a	0.1	1.8 ^b	0.1
16:0	10.3 ^a	0.1	12.2 ^b	0.4	11.1 ^c	0.1
18:0	5.7 ^a	0.1	9.5 ^b	0.6	5.5 ^c	0.0
16:1n-7	2.2	0.1	1.9	0.1	2.1	0.1
18:1n-9	1.3 ^a	0.0	1.6 ^b	0.1	2.0 ^b	0.1
18:1n-7	4.2 ^a	0.1	3.4 ^b	0.1	5.6 ^c	0.1
20:1n-11	1.5 ^a	0.0	2.1 ^b	0.0	2.6 ^c	0.1
20:1n-9	1.7 ^a	0.0	1.4 ^b	0.0	1.4 ^b	0.1
20:1n-7	5.4 ^a	0.0	4.7 ^b	0.1	5.0 ^b	0.0
18:2n-6	0.6 ^a	0.0	0.8 ^b	0.0	1.2 ^c	0.1
18:4n-3	1.3 ^a	0.1	2.1 ^b	0.3	1.0 ^c	0.0
20:4n-6	5.6 ^a	0.1	4.6 ^b	0.1	4.4 ^b	0.1
20:5n-3	19.4 ^a	0.2	16.4 ^b	0.3	14.9 ^c	0.5
22:2NMI(7,13)	1.9	0.1	1.9	0.1	2.2	0.1
22:2NMI(7,15)	5.9 ^a	0.2	5.5 ^b	0.1	7.4 ^c	0.1
22:3NMI(7,13,16)	1.2 ^a	0.0	1.0 ^b	0.1	0.9 ^b	0.0
22:4n-6	0.6 ^a	0.0	0.5 ^b	0.0	0.5 ^b	0.0
22:5n-6	0.4 ^a	0.0	0.4 ^a	0.0	2.1 ^b	0.2
22:5n-3	1.7 ^a	0.0	1.7 ^a	0.0	1.0 ^b	0.0
22:6n-3	20.9 ^a	0.3	18.7 ^b	0.6	20.1 ^{ab}	0.3
Others*	3.8 ^a	0.1	4.9 ^b	0.2	3.7 ^a	0.1
Total BR	0.5 ^a	0.0	0.5 ^a	0.0	0.3 ^b	0.0
Total SFA	17.6 ^a	0.2	23.4 ^b	1.0	18.5 ^c	0.2
Total MUFA	16.5 ^a	0.1	15.4 ^b	0.3	19.1 ^c	0.1
Total n-9	3.2 ^a	0.1	3.1 ^a	0.2	3.7 ^b	0.2
Total n-7	11.7 ^a	0.1	10.0 ^b	0.2	12.7 ^c	0.1
Total PUFA	65.4 ^a	0.3	60.6 ^b	0.8	62.1 ^b	0.3
Total n-4	0.3 ^a	0.0	0.3 ^a	0.0	0.2 ^b	0.0
Total n-6	8.4 ^a	0.1	7.7 ^b	0.2	9.5 ^c	0.3
Total n-3	47.3 ^a	0.3	43.7 ^b	0.7	41.1 ^c	0.5
Total NMI	9.4 ^a	0.2	9.0 ^a	0.1	11.2 ^b	0.2

*Others : total of 21 fatty acids detectable (iso17:0, ant17:0, 20:0, 22:0, 24:0, 18:1n-11, 22:1n-9, 16:2n-7, 16:2n-4, 16:3n-6, 16:4n-3, 18:2n-4, 18:3n-6, 18:3n-3, 20:2NMI(5,11), 20:2NMI(5,13), 18:5n-3, 20:2n-6, 20:3n-6, 20:4n-3, 21:5n-3), none of which were more than 1%.

BR: branched FA; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; NMI: non-methylene-interrupted FA

Table 2: Content of total phospholipids and proportion of phospholipid classes in gill mitochondria of oysters grown in their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf – Field, Tf – Laboratory). The results are expressed as mol percentages of total fatty acids. Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups (mean \pm SE, $n = 6$ pools of 5 oysters).

	Field T0		Tf - Field		Tf - Laboratory	
	Mean	SE	Mean	SE	Mean	SE
PE	29.8	0.3	30.3	0.2	30.6	0.3
PI + CAEP	19.3 ^a	0.2	20.1 ^b	0.2	21.9 ^c	0.4
CL	6.8 ^a	0.3	6.4 ^a	0.2	5.0 ^b	0.2
PC	37.0 ^a	0.5	35.2 ^{ab}	0.6	33.4 ^b	0.9
PS	7.1	0.3	8.0	0.7	9.0	1.0
PC/PE	1.24 ^a	0.02	1.16 ^b	0.03	1.09 ^c	0.02
PE/CL	4.4 ^a	0.2	4.8 ^a	0.2	6.1 ^b	0.2
Total phospholipid content ($\mu\text{g} \cdot \text{mg}^{-1} \text{prot.}$)	471.3	39.2	450.4	7.9	394.7	32.5

PE : phosphatidylethanolamine; PI : phosphatidylinositol; CL : cardiolipin; CAEP : ceramide aminoethylphosphonate; PC : phosphatidylcholine; PS : phosphatidylserine

Table 3: Respiratory control ratio (RCR) and oxidative phosphorylation efficiency (ADP/O), with glutamate 40 mM or succinate 20 mM as substrate and ADP 0.6 mM of oysters grown in their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf – Field, Tf – Laboratory). Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups (mean \pm SE, $n = 6$ pools of 5 oysters).

	Field T0		Tf - Field		Tf - Laboratory	
	Mean	SE	Mean	SE	Mean	SE
RCR glutamate	6.2 ^a	0.5	6.4 ^a	0.2	4.8 ^b	0.4
RCR succinate	3.9 ^a	0.1	3.6 ^b	0.1	3.3 ^b	0.2
ADP/O glutamate	2.6 ^a	0.2	2.0 ^b	0.0	2.3 ^{ab}	0.3
ADP/O succinate	1.9 ^a	0.1	1.6 ^b	0.1	1.8 ^{ab}	0.2

Table 4: CCO activity, expressed per mg of mitochondrial proteins or per nmol of cytochrome *a*, and CS activity, expressed in per mg of gill tissue of oysters grown in their natural environment or in the laboratory, and sampled before (Field T0) and after 4 weeks of conditioning (Tf – Field, Tf – Laboratory). Assay temperature was 25°C. Different superscript letters indicate values that differ significantly ($p < 0.05$) between oyster groups (mean \pm SE, $n = 6$ pools of 5 oysters).

	Field T0		Tf - Field		Tf - Laboratory	
	Mean	SE	Mean	SE	Mean	SE
CCO activity (U.mg ⁻¹ prot.)	0.3 ^a	0.0	0.3 ^a	0.0	0.2 ^b	0.0
(U.nmol ⁻¹ cyt <i>a</i>)	4.4	0.9	4.5	0.7	3.2	0.4
CS activity (U.mg ⁻¹ tissue)	4.4	0.4	4.7	0.3	5.0	0.2

1 **Table 5:** Concentrations of mitochondrial cytochromes *a*, *b*, *c*₁ and *c*, expressed as nmol of cytochrome per mg of mitochondrial proteins,
 2 relative cytochrome ratios related to cytochrome *c*₁ and ratio (*b* + *c*₁)/*a* of oysters grown in their natural environment or in the laboratory, and
 3 sampled before (Field T0) and after 4 weeks of conditioning (Tf – Field, Tf – Laboratory).
 4 Different superscript letters indicate values that differ significantly (p<0.05) between oyster groups (mean ± SE, n = 6 pools of 5 oysters).
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	Field T0		Tf - Field		Tf - Laboratory	
	Mean	SE	Mean	SE	Mean	SE
Cytochrome concentrations (nmol.mg ⁻¹ prot.)						
<i>a</i>	0.07	0.01	0.06	0.01	0.05	0.01
<i>b</i>	0.21 ^a	0.02	0.21 ^a	0.02	0.16 ^b	0.01
<i>c</i> ₁	0.12	0.01	0.13	0.01	0.12	0.02
<i>c</i>	0.06	0.01	0.08	0.01	0.06	0.01
Total	0.45	0.03	0.48	0.04	0.38	0.03
Relative ratios						
<i>a/c</i> ₁	0.60	0.11	0.44	0.04	0.46	0.08
<i>b/c</i> ₁	1.85	0.22	1.62	0.11	1.48	0.15
<i>c/c</i> ₁	0.55	0.07	0.57	0.04	0.48	0.07
(<i>b</i> + <i>c</i> ₁)/ <i>a</i>	5.2	0.80	6.1	0.60	6.1	1.10

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