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1 **Evaluating bivalve cytoprotective responses and their regulatory pathways in a**
2 **Climate Change Scenario**

3

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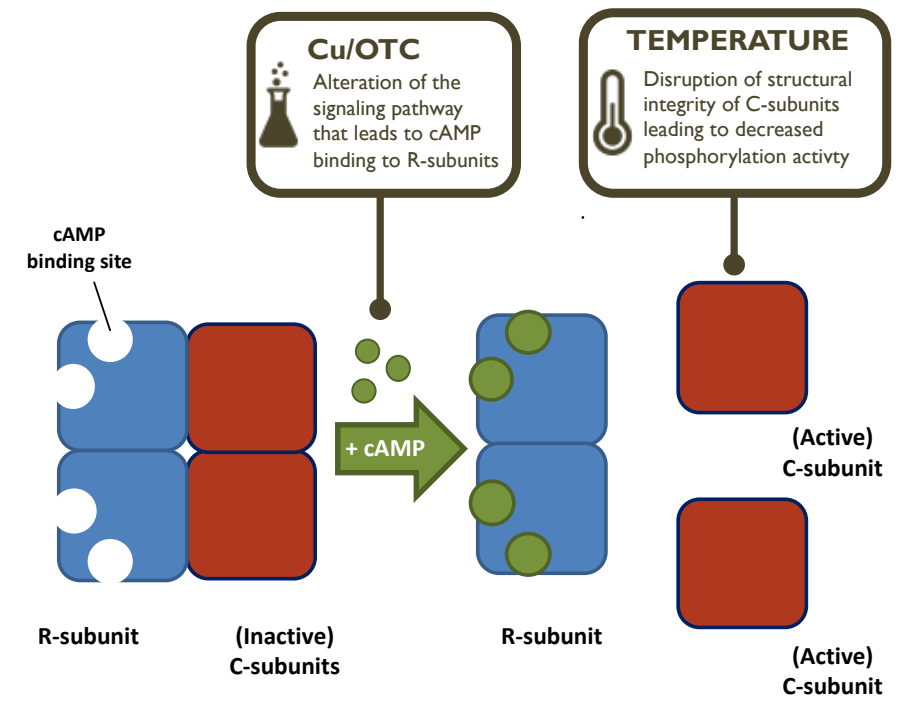
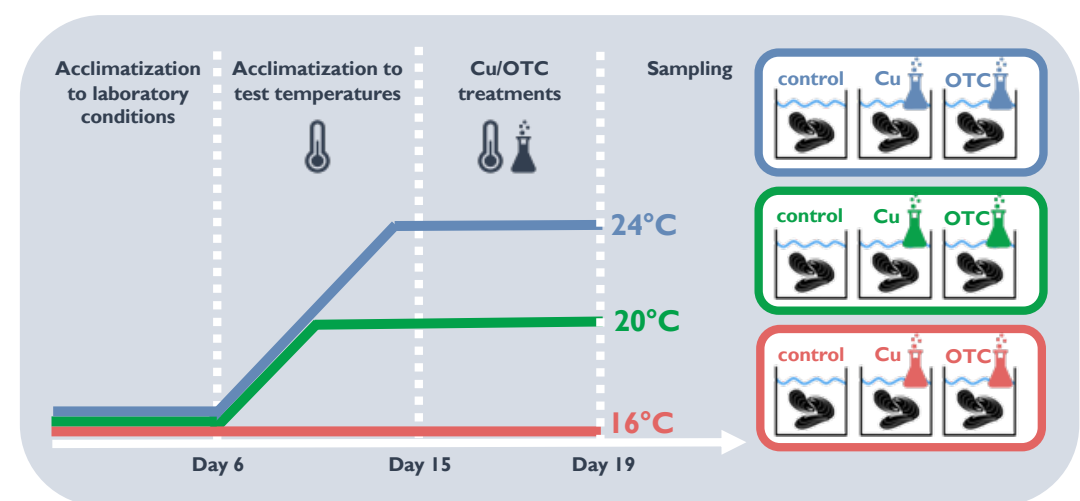
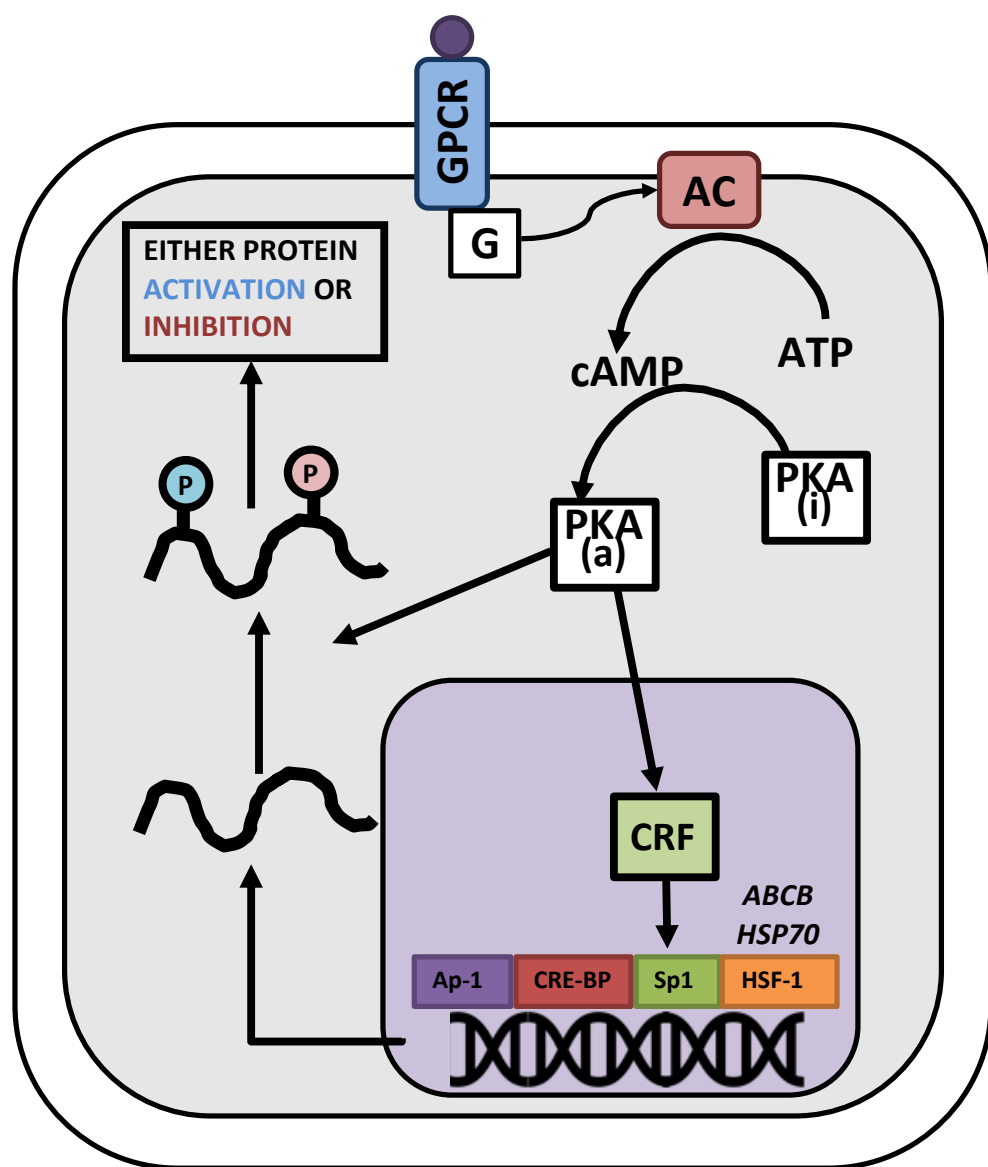
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21 **Abstract**

22 Temperature is a relevant abiotic factor affecting physiological performance and
23 distribution of marine animals in natural environments. The changes in global seawater
24 temperatures make it necessary to understand how molecular mechanisms operate under
25 the cumulative effects of global climate change and chemical pollution to promote/hamper
26 environmental acclimatization. Marine mussels are excellent model organisms to infer the
27 impacts of those anthropogenic threats on coastal ecosystems. In this study,
28 Mediterranean mussels (*Mytilus galloprovincialis*) were exposed to different concentrations
29 of the metal copper (Cu as CuCl₂: 2.5, 5, 10, 20, 40 µg/L) or the antibiotic oxytetracycline
30 (OTC: 0.1, 1, 10, 100, 1000 µg/L) at increasing seawater temperatures (16°C, 20°C,
31 24°C). Transcriptional modulation of a 70-kDa heat shock protein (HSP70) and of the ABC
32 transporter P-glycoprotein (P-gp, encoded by the ABCB gene) was assessed along with
33 the cAMP/PKA signaling pathway regulating both gene expressions. At the physiological
34 temperature of mussels (16°C), Cu and OTC induced bimodal changes of cAMP levels
35 and PKA activities in gills of exposed animals. A correlation between OTC- or Cu- induced
36 changes of PKA activity and expression of *hsp70* and *ABCB* was observed. Temperature
37 increases (up to 24°C) altered *ABCB* and *hsp70* responses to the pollutants and disrupted
38 their relationship with cAMP/PKA modulation, leading to loss of correlation between the
39 biological endpoints. On the whole, the results indicate that temperature may impair the
40 effects of inorganic and organic chemicals on the cAMP/PKA signaling pathway of
41 mussels, in turn altering key molecular mediators of physiological plasticity and
42 cytoprotection.

43

44 **Keywords:** temperature; antibiotic; metal; stress response; transcriptional control; marine
45 mussel.

46

47 **1. INTRODUCTION**

48 Owing to its impact on biochemical and cellular machinery, temperature is a key
49 abiotic factor affecting organism physiological performances and environmental distribution
50 (Judge et al., 2018; Pörtner and Gutt, 2016). The changes in seawater temperatures
51 associated with global climate change are fostering the research to understand the
52 potential interactive effects of global warming with other sources of physiological stress in
53 marine animals (Freitas et al., 2019; Sokolova and Lannig, 2008). To predict future
54 scenarios, physiological studies attempt to determine the key physiological processes that
55 set the limits of stress tolerance, how these operate in natural conditions where complex
56 exposure scenarios occur, and whether species differ in acclimatization capacities for
57 modifying their stress tolerances (Somero, 2012; Sulmon et al., 2015). In this regard,
58 investigations of the regulatory mechanisms governing acclimatory and stress responses
59 may provide early-warning molecular markers of animal-environment interaction and
60 elucidate how animal acclimatization is hampered under the cumulative effects of
61 global warming and chemical pollution.

62 Contamination by metals is a typical anthropogenic footprint in coastal areas (Hatje
63 et al., 2018). Increasing temperatures can influence distribution and fate of metals in
64 sediments and seawater, as well as their bioaccumulation in marine organisms. For
65 example, temperature affects metal bioaccumulation by enhancing bioavailability
66 (Sokolova and Lannig, 2008) or by increasing or decreasing animal uptake through altered
67 ventilation and feeding activity that support the enhanced energy demand (Coppola et al.,
68 2018; Nardi et al., 2018; Negri et al., 2013).

69 Amongst the emerging pollutants, antibiotics are attracting particular attention since
70 relatively high concentrations are detected in various aquatic ecosystems as a
71 consequence of their worldwide use to treat microbial infections and enhance the growth
72 and feeding efficiency of livestock in aquaculture (Flandroy et al., 2018; Scott et al., 2016),

73 resulting in the induction and spread of antibiotic resistance genes in natural microbial
74 communities (Dantas et al., 2008; Zhang and Zhang, 2011). However, potential risks to
75 non-target aquatic organisms via mechanisms that are apparently not related to the
76 therapeutic actions of antibiotics are emerging (Stengel et al., 2016; Van Trump et al.,
77 2010). Chemical stability of these compounds is thought to decrease with increasing
78 temperatures, thus modifying their environmental concentrations, bioavailability, and
79 animal accumulation (Chang et al., 2012, 2019).

80 Marine mussels (*Mytilus* spp.) are sessile organisms and often dominate coastal
81 environments. They live in environments characterized by a wide array of salinities and
82 temperatures, and are extremely tolerant to sudden changes of abiotic and biotic
83 parameters, which makes them ideal model organisms for studying physiological
84 alterations driven by environmental changes (Franzellitti et al., 2010; Viarengo et al.,
85 2007).

86 This study aims to investigate whether temperature may influence the effects of the
87 metal copper (Cu) or the antibiotic oxytetracycline (OTC), both commonly detected in
88 coastal marine environments (Farajnejad et al., 2017; Scott et al., 2016), on the regulatory
89 pathways that control cytoprotective responses contributing to physiological plasticity of
90 the Mediterranean mussel (*Mytilus galloprovincialis*). Cu is an essential element released
91 in the marine environment through a variety of anthropogenic sources (Wang et al., 2018).
92 At elevated concentrations, Cu can induce redox reactions that generate reactive oxygen
93 species (ROS) capable of direct damage towards cellular proteins, lipids, and DNA (Wang
94 et al., 2018). OTC is extensively used in aquaculture because of its broad-spectrum
95 efficacy in the treatment of infections caused by microorganisms (Limbu et al., 2018).
96 Aquatic toxicity of OTC has been observed on several marine organisms, from algae to
97 crustaceans and fish (Kołodziejaska et al., 2013; Limbu et al., 2018; Wu and He, 2019). We
98 assessed mRNA expression changes of a stress-inducible 70 kDa heat shock protein

99 (*hsp70*) and an *ABCB* transcript encoding the ABC (ATP-binding cassette) transporter P-
100 glycoprotein (P-gp) along with temperature and/or pollutant induced modulations on the
101 cAMP/PKA signaling pathway that may exert both transcriptional and post-transcriptional
102 control on these proteins (Fig 1). Specifically, the cAMP/PKA pathway is involved in the
103 activation of the heat shock transcription factor 1 (HSF1) (Murshid et al., 2010), one of the
104 main mediators inducing *hsp70* gene transcription. It is also involved in *ABCB*
105 transcriptional activation through several transcription factors (Franzellitti and Fabbri,
106 2013; Yao et al., 2009). P-gp and HSP70 are important players in the core stress response
107 machinery that operates as a broad-spectrum cell protective mechanism allowing marine
108 mussels to tolerate thermal stress (Luedeking and Koehler, 2004), exposure to pollutants
109 (Franzellitti and Fabbri, 2006) or physiologically-demanding environments (Franzellitti et
110 al., 2010). Induction of *hsp70* expression is a common response to temperature developed
111 by mussels in variable thermal regimes (Lockwood et al., 2015; Morris et al., 2013).
112 However, HSP70s are also induced by mussel exposure to metals and organics (Fabbri et
113 al., 2008). The function of HSP70s under stress conditions is to assist in repairing,
114 refolding, and protecting cellular proteins from damages, to minimize protein aggregation,
115 or to facilitate degradation of irreparably damaged proteins, thus contributing to cell
116 homeostasis (Fernández-Fernández et al., 2017). P-gp is the best characterized amongst
117 the bivalve ABC transporters (Franzellitti and Fabbri, 2006). It is a phase 0 membrane
118 transporter mediating the ATP-dependent extrusion of unmetabolized organic compounds,
119 although it may be also involved in the response to further biotic and abiotic stressors
120 (Buratti et al., 2013; Fu et al., 2019; Minier et al., 2000).

121 Mussels were acclimated to 16°C, 20°C, and 24°C under laboratory conditions and
122 subsequently exposed for 4 days to a wide range of copper (Cu as CuCl₂) or
123 oxytetracycline (OTC) nominal concentrations. Transcriptional levels of *ABCB* and *hsp70*,
124 as well as cAMP tissue levels and activity of the cAMP dependent protein kinase A (PKA)

125 were assessed in gills of exposed mussels. For the purpose of this study, this
126 experimental setup attempts to discriminate the contribution of the chemical and the
127 physical stressors on the observed molecular outcomes and the underlying regulatory
128 impairments, and to drive hypotheses on critical mechanisms that challenge
129 acclimatization of marine organisms to anthropogenically modified environments.

130

131 **2. METHODS**

132

133 *2.1. Mussel handling and experimental setup*

134 Specimens of *M. galloprovincialis* (5-7 cm in length) were collected from the northwestern
135 Adriatic Sea by professional fishermen of the “Cooperativa Copr.al.mo” (Cesenatico, Italy).
136 They were transferred to the laboratory in seawater tanks with continuous aeration and
137 kept for 6 days in aquaria containing 60 L of aerated 35 psu seawater at 16 °C, under a
138 natural photoperiod (30 animals per aquarium). Mussels were fed once a day with an algal
139 slurry (Koral filtrator, Xaqua, Italy). The duration of the acclimation proved suitable to
140 stabilize the mussel physiological responses at the reference temperature of 16 °C (Banni
141 et al., 2015; Viarengo et al., 2007). Fifteen mussels were sampled at zero time to assess
142 parameters at the onset of each experiment. A scheme of the experimental setup is
143 reported in Fig 2. Following the acclimation period, mussels were randomly selected and
144 divided into groups of 20 animals each and transferred to aquaria containing 20 L of
145 seawater. One liter of seawater per mussel is the suitable volume to avoid overloading and
146 the onset of stress conditions. Four aquaria for each experimental condition were the 4
147 replicates (N = 4). One group of 44 aquaria was maintained at the reference temperature
148 (16°C) throughout the experimental treatment, while the other 2 groups (each of 44
149 aquaria) were subjected to a gradual seawater temperature increase up to 20°C or 24°C
150 (1°C per day) and maintained for 24 h at the settled temperature before exposure to the

151 chemical treatment. The reference temperature (16°C) and the highest exposure
152 temperature (24°C) were derived from the time-series of monthly average SSTs recorded
153 at the sampling area in the North-West Adriatic Sea (retrieved at the Copernicus Marine
154 Service web portal, <http://marine.copernicus.eu/>), where 16°C matches the average
155 annual temperature, and 24°C approaches the maximum annual recorded values. The
156 20°C represents a projection of the average annual temperature estimated for the end of
157 the century in the North-West Adriatic Sea (Shaltout and Omstedt, 2014). In each
158 aquarium, water temperature was monitored throughout the acclimation and the
159 experimental periods using FT-800 thermometers (Econorma, Treviso, Italy). Once the
160 selected temperatures in the aquaria were established, mussels were treated for 4 days
161 with nominal 2.5, 5, 10, 20, 40 µg/L Cu (as CuCl₂) or 0.1, 1, 10, 100, 1000 µg/L OTC. OTC
162 is found in seawater at the ng/L to µg/L concentrations (max concentration about 15 µg/L)
163 (Chen et al., 2015). Cu concentrations tested in this study encompassed the range of
164 values detected in the Adriatic Sea (from 0.5 µg/L to about 7 µg/L) (Munari and Mistri,
165 2007; Zago et al., 2000). The selected Cu concentrations were previously shown to
166 decrease lysosomal membrane stability (LMS) in exposed mussels, and to exert further
167 sub-lethal health effects (Negri et al., 2013). OTC effects on LMS were assessed in
168 preliminary experiments in haemocytes of mussels exposed *in vivo* to the antibiotic at
169 16°C (Supplemental material, Fig S1). LMS was selected as the reference parameter in
170 these preliminary evaluations on chemical concentration ranges to be tested as it is a
171 sensitive and reliable biomarker of general health status in bivalves (Viarengo et al., 2007).
172 All selected OTC concentrations significantly reduced LMS, a sign that mussels were
173 subjected to a physiological stress. Acclimation periods at the selected temperatures and
174 duration of chemical exposures were selected considering the dynamic ranges of the
175 investigated biological endpoints, that constrained our experimental setup. Indeed, cell
176 signaling pathways and transcriptional regulation of stress related genes, such as *ABCB*

177 and *hsp70*, are early and fast responses to environmental stimuli. Furthermore, according
178 to our previous studies, a 4- to 7-day exposure proved suitable to develop measurable
179 changes in the selected endpoints (Franzellitti et al., 2019, 2014, 2013). A group of
180 unexposed (0 µg/L OTC or 0 µg/L Cu) mussels was maintained in parallel to the treatment
181 groups within each temperature. Mussels exposed to 0 µg/L OTC or Cu at 16°C served as
182 the reference condition for data comparisons and statistics. Seawater was renewed each
183 day and the chemicals added from stock solutions along with mussel feeding. Exposures
184 were conducted under dimmed light to minimize possible photodegradation, in particular of
185 OTC (Jiao et al., 2008).

186 For all experimental treatments, the gills were dissected from individuals, snap-
187 frozen in liquid nitrogen, and stored at -80°C. Gills were selected as they are the mussel
188 filter-feeding organs and the major barriers between the external environment and internal
189 organs, where physiological conditions are mostly imposed by the external environment
190 (Musella et al., 2020). Therefore, gills are supplied with effective protective mechanisms
191 (Franzellitti et al., 2016; Luckenbach and Epel, 2008). There was no mortality during the
192 exposure period. Mussels at zero time were immediately analyzed for the biological
193 endpoints to assess their initial health status; no significant differences compared to
194 mussels maintained for ~~4 days~~ under the reference treatment was observed (data not
195 shown).

196

197 *2.2. Measurements of cyclic AMP (cAMP) levels and PKA activity in mussel gills*

198 For the evaluations of cAMP tissue content, samples (about 200 mg of pooled gills)
199 were homogenized with 6% trichloroacetic acid and further processed as reported by
200 Franzellitti et al. (2014). cAMP contents were assessed in the aqueous extracts through
201 the DetectX™ direct cyclic AMP enzyme immunoassay kit (Arbor Assay, USA) according

202 to the manufacturer's protocol. Results were finally expressed as pmol cAMP/g fresh
203 tissue.

204 For the evaluations of PKA activity, samples (about 200 mg of pooled gills) were
205 homogenized in cold extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM
206 EGTA, 10 mM β -mercaptoethanol and proteinase inhibitor cocktail P8340 from Sigma
207 Aldrich), and further processed according to Franzellitti et al. (2014). Supernatants were
208 assayed for PKA activity using the non-radioactive PepTag PKA assay kit (Promega,
209 Milan, Italy) according to manufacturer's protocol. Results are expressed as nmol/min/mg
210 total protein, with total protein content being estimated with Lowry's method (Lowry et al.,
211 1951).

212

213 2.3. Mussel *ABCB* and *hsp70* mRNA expressions

214 Gills (200 mg tissue) were homogenized in a suitable volume of the TRI Reagent
215 (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo
216 Research, Freiburg, Germany) following the manufacturer's instructions. RNA
217 concentration and quality were confirmed using the Qubit system with the Qubit RNA
218 assay kit (Thermo Scientific, Milan, Italy), electrophoresis using a 1.2% agarose gel under
219 denaturing conditions, and analysis of UV absorbance spectra of the samples ($\lambda = 200 -$
220 340 nm) for the calculation of Absorbance (A) ratio A_{260}/A_{280} (cut-off values > 1.8 and $<$
221 2.0). First strand cDNA for each sample was synthesized from $1 \mu\text{g}$ total RNA using the
222 iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's
223 instructions.

224 *ABCB* and *hsp70* mRNA expressions were assessed by quantitative real-time PCR
225 (qPCR) as reported in previous studies (Balbi et al., 2016; Franzellitti and Fabbri, 2013).
226 Primer sequences and PCR conditions are reported in Supplemental material, Table S1.
227 18S rRNA and elongation factor 1α were selected as reference gene products for qPCR

228 data normalization by a preliminary stability analysis of 6 established candidate transcripts
229 (Balbi et al., 2016). Relative expression values of target mRNAs were inferred by a
230 comparative C_T method (Schmittgen and Livak, 2008) using the StepOne and DataAssist
231 softwares (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold
232 change) with respect to the reference treatment (0 $\mu\text{g/L}$ Cu and 0 $\mu\text{g/L}$ OTC at 16°C).

233

234 *2.4. Statistical analysis*

235 Statistical analysis of cAMP level and PKA activity data was performed using
236 GraphPad Prism 8 (GraphPad Inc.). Significant differences between treatment groups
237 were determined through the non-parametric one-way ANOVA (Kruskal-Wallis test)
238 followed by the Mann-Whitney U-test, after deviations from parametric ANOVA
239 assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: Bartlett's test).
240 qPCR data were analyzed using the REST software (Pfaffl et al., 2002) to test for
241 statistical differences in mRNA levels of the treatment groups vs the reference condition.
242 Further pairwise comparisons were performed with the Mann-Whitney U-test. Correlation
243 analyses (Spearman's test), data visualization and graphics were obtained with the
244 ggplot2 R package in R (R Development Core Team, 2018). In any case, statistical
245 differences were accepted when $p < 0.05$.

246 The complete datasets from Cu or OTC treatments were further analyzed by a 2-
247 way permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6
248 (Anderson et al., 2008) to test for the interactive effects of temperature and Cu or OTC
249 treatments. Log-transformed variations of the target transcripts and log-transformed cAMP
250 levels and PKA activities were used to calculate similarity matrices based on the Euclidean
251 distance (999 permutations; $P_{\text{perm}} < 0.05$).

252 The Cu/OTC concentration-dependent trends of the biological endpoints were
253 employed to calculate the Area Under the Curve (AUC) that gives a metric describing the

254 overall magnitude of cAMP, PKA, *ABCB* and *hsp70* variations at the different
255 temperatures. Values of AUC were computed by the trapezoidal formula (Fekedulegn et
256 al., 2007) and using GraphPad Prism 8. Details for AUC calculation are reported by
257 Franzellitti et al. (2018).

258

259 **3. RESULTS**

260

261 *3.1. Variations of cAMP-related signaling parameters in gills of mussels exposed to Cu or* 262 *OTC at increased seawater temperatures*

263 Results from PERMANOVA analyses demonstrated that temperature and OTC had
264 an overall significant effect on both cAMP levels and PKA activity, while the effects of Cu
265 on cAMP were statistically significant ($P < 0.05$; Table 1). PERMANOVA analysis also
266 showed a significant interaction between each chemical and temperature ($P < 0.05$; Table
267 1).

268 Cu treatments at 16°C showed significant increases of cAMP levels and PKA
269 activities up to 5 µg/L Cu, with values decreasing to control levels thereafter (Fig 3A,B).
270 cAMP gill content increased both at 20°C and 24°C in 0 µg/L Cu samples (Fig 3A), while
271 increasing (20°C) and decreasing (24°C) PKA activities compared to the reference
272 condition (0 µg/L Cu at 16°C) were found (Fig 3B). Significant differences of tissue cAMP
273 content between Cu-treated samples and the 0 µg/L Cu samples at the respective
274 temperatures were significant at 2.5, 5.0 and 40 µg/L Cu (20°C), and at 40 µg/L Cu (24°C)
275 ($p < 0.05$; Fig 3A). Significantly different PKA activity values compared to the 0 µg/L Cu
276 samples at the respective temperatures were observed at 2.5 and 5 µg/L Cu ($p < 0.05$; Fig
277 3B).

278 OTC treatment at 16°C resulted in a bell-shape trend for both parameters, with
279 values increasing up to 10 µg/L OTC and decreasing thereafter (Fig 4A,B). Mussels

280 acclimated at 20°C and 24°C showed no significant increase of cAMP levels compared to
281 the 0 µg/L OTC samples at the respective temperatures (Fig 4A). For PKA, significant
282 differences were observed at 1 to 1000 µg/L OTC (20°C) ($p < 0.05$; Fig 4B).

283 Correlation plots reported in Fig 2C and Fig 3C show that values of PKA activities
284 were significantly correlated with variation of cAMP tissue content across Cu or OTC
285 treatments only at 16°C ($p < 0.05$).

286

287 3.2. Variations of *ABCB* and *hsp70* mRNA expressions in gills of mussels exposed to Cu 288 or OTC at increased seawater temperatures

289 Results from PERMANOVA analyses demonstrated that Cu and OTC had an
290 overall significant effect on both *ABCB* and *hsp70* expression, whereas temperature was
291 effective on *ABCB* in the Cu treatment, while not in the OTC treatment ($P < 0.05$; Table 1).
292 PERMANOVA analysis also showed a significant interaction between both chemicals and
293 temperature ($P < 0.05$; Table 1).

294 Levels of *ABCB* expression were significantly higher in gills of Cu-exposed mussels
295 at 16°C (5 – 40 µg/L) ($p < 0.05$; Fig 5A and Fig 6A). Expression levels were significantly
296 higher in 0 µg/L Cu samples at 20°C and 24°C (Fig 5A). Significant differences between
297 Cu-treated samples and controls at the respective temperatures were observed at 2.5 and
298 5 µg/L Cu (24°C) (Fig 4A). At 16°C, the *hsp70* gene product was significantly up-regulated
299 in samples exposed to 2.5, 5, and 40 µg/L Cu, while down-regulated at 20 µg/L Cu ($p <$
300 0.05 ; Fig 5B). Expression levels were significantly increased in 0 µg/L Cu samples at 20°C
301 and 24°C (Fig 5B), while significant differences between Cu-exposed samples and
302 controls at the respective temperatures were observed at 40 µg/L Cu (24°C) (Fig 5B).

303 At 16°C, *ABCB* up-regulation at 1 µg/L OTC and down-regulation at 100 and 1000
304 µg/L OTC was observed ($p < 0.05$; Fig 6A). Expression levels were significantly increased
305 in 0 µg/L OTC at 20°C and 24°C (Fig 6A), while significant differences between OTC-

306 treated samples and controls at the respective temperatures were observed at 0.1, 1, 10,
307 1000 µg/L OTC (20°C), and 1000 µg/L OTC (24°C) (Fig 6A). At 16°C, the *hsp70* gene
308 product was significantly regulated in gills of OTC-exposed mussels, with down-regulation
309 at 0.1 and 1 µg/L OTC, and up-regulation at 10 – 1000 µg/L OTC ($p < 0.05$; Fig 6B).
310 Significantly different *hsp70* expression levels (down-regulation) between OTC-treated
311 samples and the 0 µg/L OTC samples at the respective temperatures were observed at
312 0.1 – 100 µg/L OTC (20°C), and 1.0 -1000 µg/L OTC (24°C) (Fig 6B). Correlation plots
313 reported in Fig 5C and Fig 6C show that both *ABCB* and *hsp70* expressions were
314 significantly correlated with variation of PKA activity across Cu treatments only at 16°C (p
315 < 0.05).

316

317 3.3. Analysis of temperature related trends of the biological responses to Cu and OTC

318 Calculation of AUC was employed to address changes of cAMP, PKA, *ABCB* or
319 *hsp70* response to Cu or OTC at the different temperatures (Fig 7). cAMP and PKA
320 showed a decreased response to both pollutants at increasing temperatures, whereas
321 *ABCB* and *hsp70* showed a decreasing trend towards Cu response and an increasing
322 response to OTC (Fig 7).

323

324 4. DISCUSSION

325 Both Cu and OTC affected the mussel cAMP/PKA pathway with significant changes
326 of cAMP levels and PKA activities in gills of *in vivo* exposed animals. These results are in
327 line with previous reports showing that Cu is a modulator of the cAMP signaling in bivalves
328 (Fabbri and Capuzzo, 2010). Specifically, *in vitro* treatment of mussel gill membranes with
329 Cu^{2+} (10^{-10} – 10^{-5} M) induced a bell-shape modulation of adenylyl cyclase (AC) activity,
330 suggesting a putative direct effect of the metal on the cAMP forming mechanism (Fabbri
331 and Capuzzo, 2006). Although the mechanism of action of OTC is not directly related to

332 the cAMP pathway, tetracyclines are considered pluripotent drugs in mammals with proved
333 non-antibacterial related effects on inflammation, cell proliferation, cell migration, and
334 apoptosis (Bendeck et al., 2002; Ci et al., 2011). Together with previous data (Banni et al.,
335 2015), results of this study show that OTC in mussels may impair the cAMP signaling, an
336 effect that is likely to broaden the spectrum of the physiological functions potentially
337 impacted by the antibiotic in non-target marine species. Indeed, regulatory pathways
338 mediated by cAMP underpin a variety of vital physiological processes in bivalves as well
339 as in other aquatic species (Fabbri and Capuzzo, 2010; Fabbri and Moon, 2016). A
340 correlation between Cu- or OTC- induced changes of PKA activity and expression of
341 stress-related transcripts *ABCB* and *hsp70* was observed, in agreement with the
342 occurrence of a common cAMP/PKA regulatory pathway (Fig 1) and the finding that *ABCB*
343 (P-gp) and *hsp70* transcripts may be co-regulated as a generalized response to stress
344 (Franzellitti et al., 2010; Luedeking and Koehler, 2004; Minier et al., 2000).

345 Acclimation to increased seawater temperatures affected the response to Cu and
346 OTC. AUC calculations showed that temperature reduced the magnitude of the cAMP and
347 PKA responses to both pollutants. The response of cAMP is likely linked to the relatively
348 higher tissue levels observed in Cu- or OTC- unexposed samples at the increased
349 temperatures, whereas reduction of PKA activity at 24°C is accompanied by the abolished
350 response to the chemicals; furthermore, a loss of PKA vs cAMP correlation is observed at
351 increasing temperatures. cAMP is the direct activator of PKA, and the mechanism by
352 which cAMP regulates PKA activity is conserved from bacteria to humans (Kim et al.,
353 2007). Inactive PKA is a tetrameric holoenzyme composed of two functionally distinct
354 subunits: a dimeric regulatory subunit (R-subunit) and two monomeric catalytic subunits
355 (C-subunits). The main function of the R-subunit is to lock the C-subunits in the inactive
356 state through formation of the holoenzyme inhibitory complex. Binding of cAMP to the R-
357 subunit unleashes the C-subunits, thereby allowing phosphorylation of PKA substrates

358 (Kim et al., 2007). Several C-subunit isoforms and two distinct isoforms of the R-subunit
359 have been identified in mussels (Bardales et al., 2008, 2007; Díaz-Enrich et al., 2003),
360 showing biochemical properties typical of mammalian type I and type II R-subunits,
361 respectively. Furthermore, the known pharmacological modulators of PKA activity through
362 cAMP in mammals are also effective on the cAMP/PKA system of mussel haemocytes
363 (Franzellitti and Fabbri, 2013), suggesting the conservation of the activation mechanism.
364 An *in vitro* study showed no effects of temperature increases (up to 40°C) on the cAMP-
365 binding ability of PKA purified from the posterior adductor muscle and the mantle of *M.*
366 *galloprovincialis*. This suggests that temperature does not affect conformation of R-
367 subunits of mussel PKAs, at least not at the cAMP-binding regions (Bardales et al., 2004).
368 Nevertheless, the same temperature increase significantly modified the protein kinase
369 activity at saturating concentrations of cAMP, when the holoenzyme was completely
370 dissociated, indicating an effect on the conformation of the C-subunits (Bardales et al.,
371 2004). We may hypothesize that while OTC and Cu affected the pathway leading to
372 modulation of PKA activation (i.e. rate of cAMP binding to the R-subunit), temperature may
373 impair the catalytic activity (i.e. altered conformational stability of C-subunits), causing the
374 observed reduced activities at 24°C, the loss of correlation with changes of cAMP levels,
375 and the consequent reduced responsiveness to the pollutants. This apparent uncoupling of
376 the PKA activation mechanism from the enzyme catalytic activity may also explain the
377 observed loss of correlation with *ABCB/hsp70* expressions in response to the pollutants
378 observed at 20°C and 24°C. AUC calculations also show that temperature reduced (Cu) or
379 increased (OTC) the response of *ABCB* and *hsp70*. On the whole, these results indicate
380 that when an increase of temperature disrupts the cAMP/PKA mediated pathway that
381 normally contributes to *ABCB* and *hsp70* transcription, Cu and OTC may act through
382 alternative pathways on the onset of P-gp and HSP70 responses. Interestingly, an
383 opposite temperature-related response between Cu and OTC was also observed on

384 survival, replication rate, and lysosomal membrane stability of the ciliated protozoa
385 *Euplotes crassus* exposed to the chemicals under thermal stress (25-33 °C) (Gomiero and
386 Viarengo, 2014). Those biological endpoints pointed to a reduced toxicity of OTC but an
387 increased toxicity of Cu with temperature increases, likely stemming from decreased
388 stability of the molecule or the production of less toxic metabolites (OTC), or increased
389 accumulation due to temperature-enhanced feeding activity (Cu) (Gomiero and Viarengo,
390 2014).

391 A further event that may operate in limiting the induction of *ABCB* and *hsp70*
392 transcription is the so-called constitutive gene frontloading (Barshis et al., 2013). *ABCB*
393 and *hsp70* up-regulation by Cu has been observed under *in vivo* exposure of oysters and
394 mussels (Shi et al., 2015; Xu et al., 2018). Specifically, in gills of surviving oysters
395 (*Crassostrea angulata*) exposed to high concentrations of Cu (30, 100, and 300 µg/L),
396 *abcb1* was continuously over-expressed likely to aid the transport of Cu out of the cell (Shi
397 et al., 2015). Mussels (*M. galloprovincialis*) exposure to low and environmentally relevant
398 Cu concentrations (2 and 8 µg/L) resulted in *hsp70* over-expression which precedes Cu-
399 induced oxidative damage, as evidenced by the induction of antioxidant enzymes activities
400 and increased DNA damage (Xu et al., 2018). These data demonstrate that those proteins
401 are components of the molecular machinery that maintain cellular Cu homeostasis (*ABCB*)
402 and prevent its proteotoxic effects (*hsp70*). Nevertheless, since mussels are used to thrive
403 in extremely variable environments, they are well known to retain a minimal constitutive
404 *hsp70* expression that confers them enhanced physiological resilience by means of faster
405 reaction at the protein level during transient stress events (Franzellitti and Fabbri, 2005).
406 Although *hsp70* gene frontloading is largely acknowledged in marine intertidal
407 invertebrates (Barshis et al., 2013; Fabbri et al., 2008; Morris et al., 2013), our data
408 suggest ~~also~~ that the mussel *ABCB* may also display a frontloading behavior. For both
409 transcripts, mussel acclimation to increased temperatures promoted increased basal

410 expression that limited the need for further induction due to Cu treatment. Apparently,
411 constitutive gene frontloading was not effective enough to limit the need for further
412 *hsp70/ABCB* mRNAs in OTC-exposed mussels, suggesting that OTC may affect gene
413 transcription through mechanisms that are not biased by or not related to the stress
414 response, which lead to overall independent effects between thermal stress and exposure
415 to the antibiotic.

416

417 **5. CONCLUSION**

418 Results of this study show that temperature may change the outcome of the mussel
419 cAMP/PKA signaling response to inorganic and organic chemicals, in turn altering the
420 molecular mediators of physiological plasticity and environmental acclimatization, such as
421 HSP70s and P-gp. However, the general consideration that temperature prevails over
422 chemical stressors in eliciting physiological responses in marine organisms (Sokolova and
423 Lannig, 2008) is not fully supported by our results, since a signature for the chemical
424 effects can be observed at hyperthermic conditions. We further hypothesized that the
425 temperature-related loss of correlation between changes in cAMP/PKA signaling and
426 *ABCB/hsp70* transcriptional profiles may be due to the uncoupling of the PKA activation
427 mechanism and the enzyme catalytic activity. Considering the key role of the cAMP/PKA
428 pathway in mussel physiology (Fabbri and Capuzzo, 2010), this finding highlights the
429 importance of considering the regulatory pathways upstream stress response processes
430 when addressing the complex patterns of interactions in multiple stressor scenarios.

431

432 **Conflict of interest**

433 None

434

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669 **Table 1. Two-way PERMANOVA results on the effects of oxytetracycline (OTC) or**
 670 **copper (Cu) *in vivo* exposure on cAMP levels, PKA activities, ABCB and HSP70**
 671 **expressions in gills of mussels at different temperatures (T) (998 permutations).**

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	df	cAMP		PKA		ABCB		HSP70	
		Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
<i>Cu treatment</i>									
Cu	5	13.85	0.001	1.27	0.288	19.12	0.001	25.30	0.001
T	2	8.02	0.002	58.66	0.001	23.16	0.001	65.17	0.001
Cu x T	10	6.50	0.001	4.53	0.001	7.35	0.001	4.31	0.002
<i>OTC treatment</i>									
OTC	5	4.02	0.002	16.08	0.001	24.99	0.001	8.46	0.001
T	2	17.19	0.001	95.54	0.001	0.60	0.513	12.02	0.001
OTC x T	10	2.18	0.029	2.68	0.006	17.03	0.001	4.40	0.003

673 df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P(perm):
 674 probability of pseudo-F.

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682 **Figure legends**

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684 **Fig. 1. Schematic representation of the known cAMP/PKA signaling pathway**

685 **upstream HSP70 and P-gp expression.** (1) *Transcriptional control:* PKA-mediated

686 regulation of several transcription factors (CRF) that initiate HSP70/ABCB (P-gp)

687 transcription (Franzellitti and Fabbri, 2013; Murshid et al., 2010). (2) *Post-transcriptional*

688 *control:* PKA-mediated phosphorylation of target HSP70/P-gp protein residues resulting in

689 either protein activation (HSP70, P-gp) or inhibition (HSP70) (Arana and Altenberg, 2019;

690 Nitika and Truman, 2017). GCPR, G-protein coupled receptor; G, G-protein; AC, adenylyl

691 cyclase; cAMP, cyclic-AMP; PKA(i), inactive cAMP-dependent protein kinase (PKA;

692 holoenzyme); PKA(a), active PKA (catalytic subunit); CRF, cAMP-responsive factors

693 (amongst others: AP-1, CRE-BP Sp1, HSF1). **Colored figure is intended only for the**

694 **online and PDF version.**

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696 **Fig. 2. Schematic flowchart of the experimental setup for the impact of thermal**

697 **stress on the mussel responses to copper (Cu) or oxytetracycline (OTC).** The aquaria

698 represent the replicates for each condition (N = 4). **Colored figure is intended only for**

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701 **Fig. 3. Changes of cAMP/PKA signaling in gills of Cu-exposed mussels at different**

702 **temperatures.** Bar plots report mean \pm SEM values for (A) cAMP tissue levels and (B)

703 PKA activities (N = 4). * $p < 0.05$ vs samples at 0 Cu and at 16°C; ^a $p < 0.05$ vs sample group

704 at 0 Cu within the 16°C group; ^b $p < 0.05$ vs sample group at 0 Cu within the 20°C treatment

705 groups; ^c $p < 0.05$ vs sample group at 0 Cu within the 24°C treatment groups. (C) Correlation

706 plots show the relationships between cAMP levels and PKA activities at the different

707 temperatures. Correlation analyses are based on data from individual mussels (N = 24

708 within each temperature). Average values for each data point have been used only for the
709 graphic representation. Only significant Spearman correlations ($*p<0.05$) are reported in
710 the graphs. Shaded areas show the 95% confidence intervals. **Colored figure is intended**
711 **only for the online and PDF version.**

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713 **Fig. 4. Changes of cAMP/PKA signaling in gills of OTC- exposed mussels at**
714 **different temperatures.** Bar plots report mean \pm SEM values for (A) cAMP tissue levels
715 and (B) PKA activities (N = 4). $*p<0.05$ vs samples at 0 OTC and at 16°C; $^ap<0.05$ vs
716 sample group at 0 OTC within the 16°C group; $^bp<0.05$ vs sample group at 0 OTC within
717 the 20°C treatment groups; $^cp<0.05$ vs sample group at 0 OTC within the 24°C treatment
718 groups. (C) Correlation plots show the relationships between cAMP levels and PKA
719 activities at the different temperatures. Correlation analyses are based on data from
720 individual mussels (N = 24 within each temperature). Average values for each data point
721 have been used only for the graphic representation. Only significant Spearman
722 correlations ($*p<0.05$) are reported in the graphs. Shaded areas show the 95% confidence
723 intervals. **Colored figure is intended only for the online and PDF version.**

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725 **Fig. 5. ABCB (A) and hsp70 (B) expressions in gills of Cu-exposed mussels**
726 **acclimatized at different temperatures.** Bar plots report mean \pm SEM values of fold
727 change variations. $**p<0.05$ vs samples at 0 Cu and at 16°C; $^ap<0.05$ vs sample group at
728 0 Cu within the 16°C group; $^bp<0.05$ vs sample group at 0 Cu within the 20°C treatment
729 groups; $^cp<0.05$ vs sample group at 0 Cu within the 24°C treatment groups. (C,D)
730 Correlation plots show the relationships between PKA activity and ABCB/hsp70
731 expression at the different temperatures. Correlation analyses are based on data from
732 individual mussels (N = 24 within each temperature). Average values for each data point
733 have been used only for the graphic representation. Only significant Spearman

734 correlations ($*p<0.05$) are reported in the graphs. Shaded areas show the 95% confidence
735 intervals. **Colored figure is intended only for the online and PDF version.**

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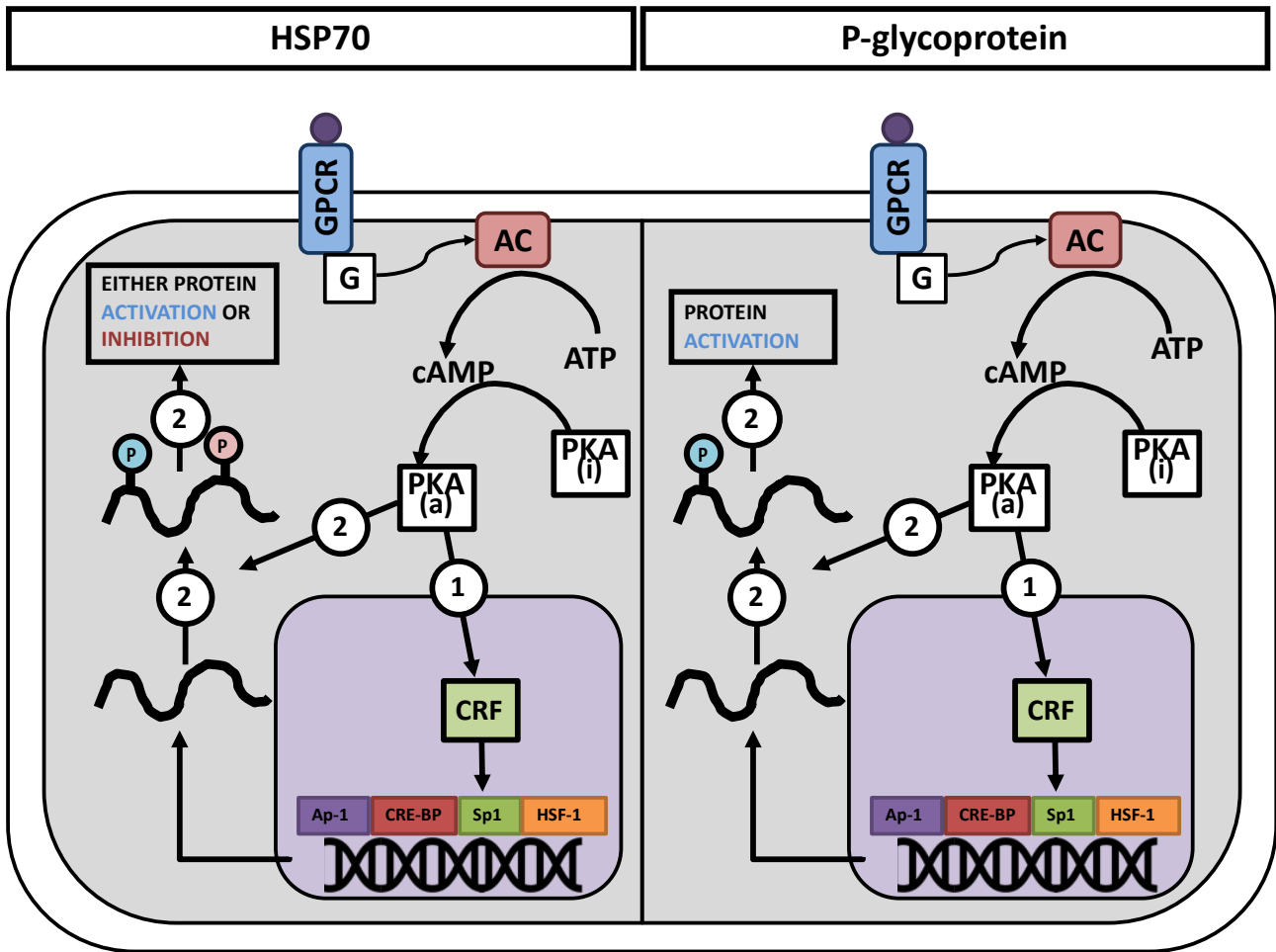
737 **Fig. 6. ABCB (A) and hsp70 (B) expressions in gills of OTC-exposed mussels**
738 **acclimatized at different temperatures.** Bar plots report mean \pm SEM values of fold
739 change variations. $**p<0.05$ vs samples at 0 OTC and at 16°C; $^ap<0.05$ vs sample group
740 at 0 OTC within the 16°C group; $^bp<0.05$ vs sample group at 0 OTC within the 20°C
741 treatment groups; $^cp<0.05$ vs sample group at 0 OTC within the 24°C treatment groups.
742 (C,D) Correlation plots show the relationships between PKA activity and ABCB/hsp70
743 expression at the different temperatures. Correlation analyses are based on data from
744 individual mussels (N = 24 within each temperature). Average values for each data point
745 have been used only for the graphic representation. Only significant Spearman
746 correlations ($*p<0.05$) are reported in the graphs. Shaded areas show the 95% confidence
747 intervals. **Colored figure is intended only for the online and PDF version.**

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749 **Fig. 7. Temperature related trends of AUC (Area Under the Curve) values.** For each
750 biological endpoint, Cu or OTC concentration-related variation at each temperature is
751 expressed by the Area Under the Curve (AUC) according to Franzellitti et al. (2018).
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754 Fig 1



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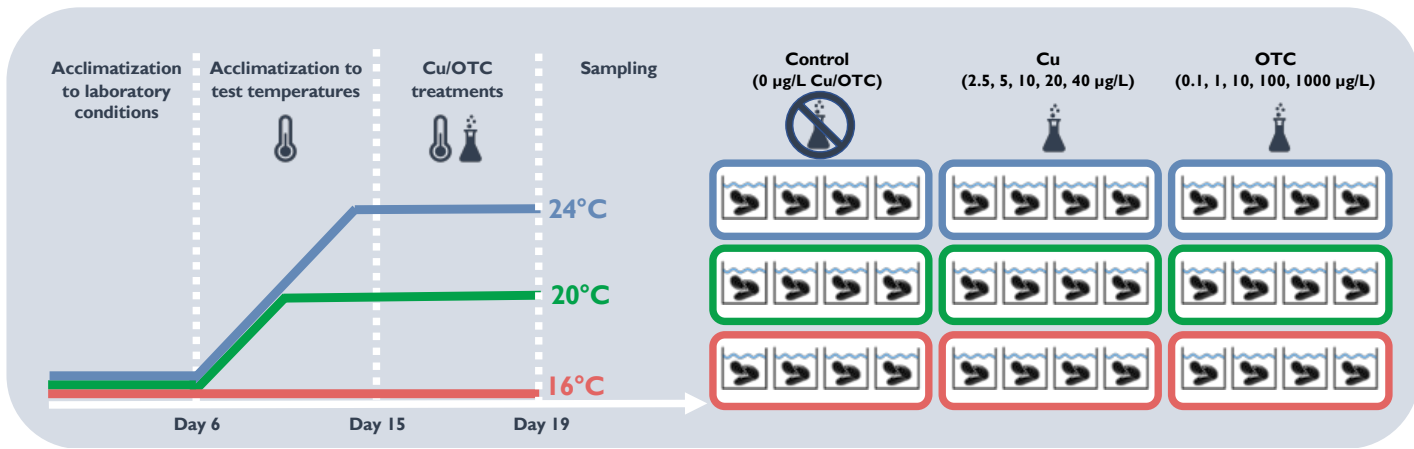
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766 **Fig 2**

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777 **Fig 3**

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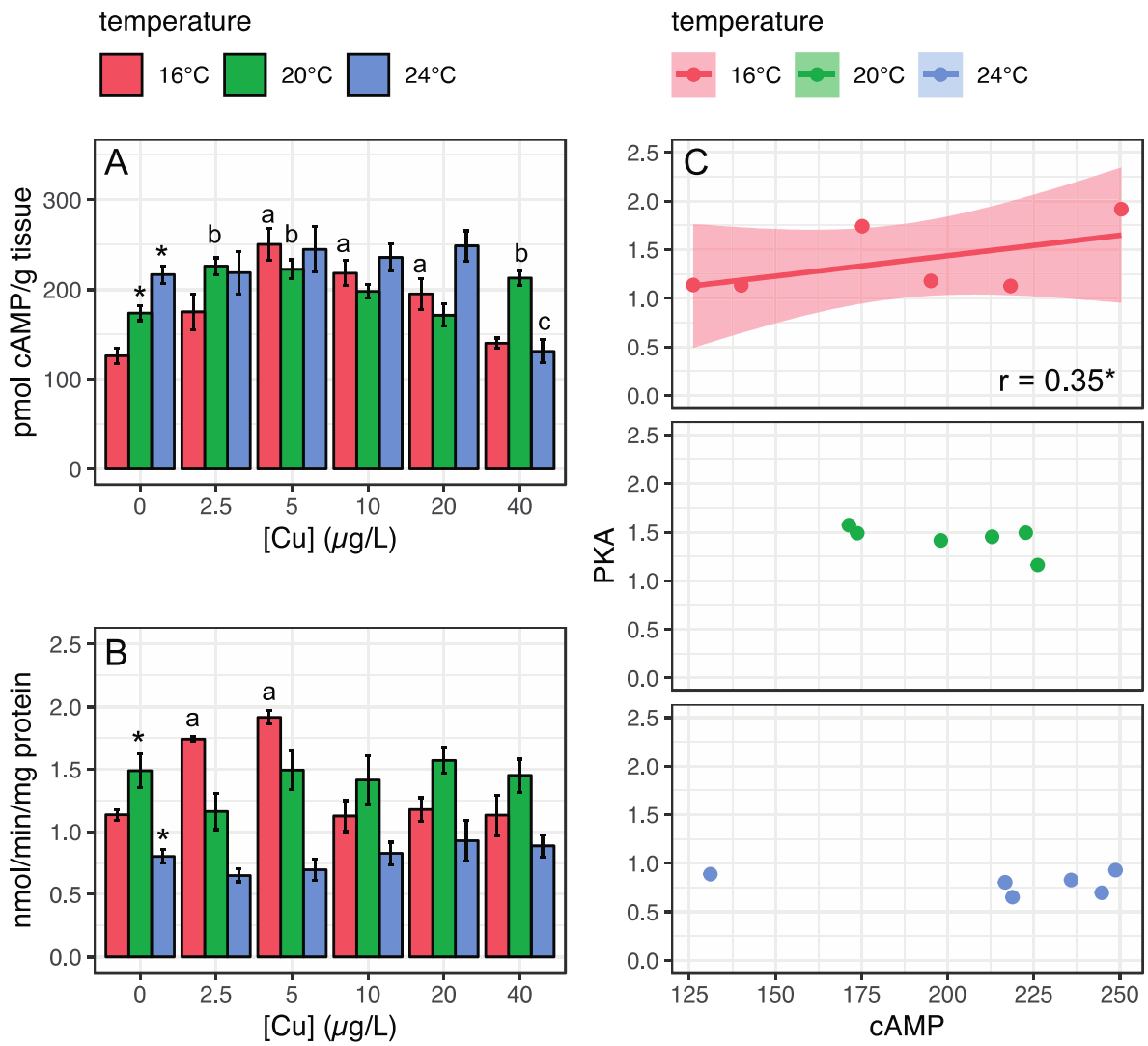
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797 **Fig 4**

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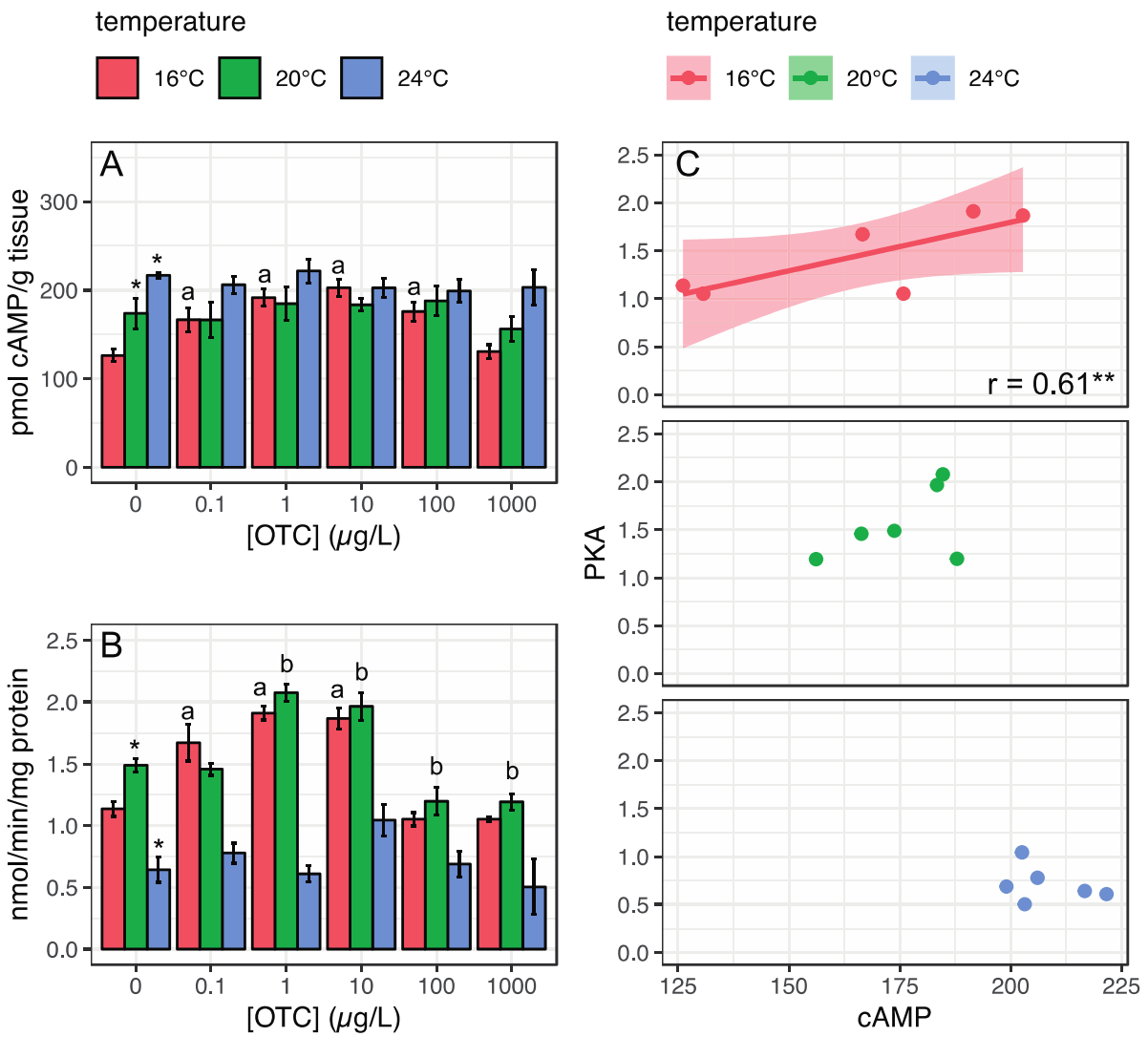
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815 **Fig 5**

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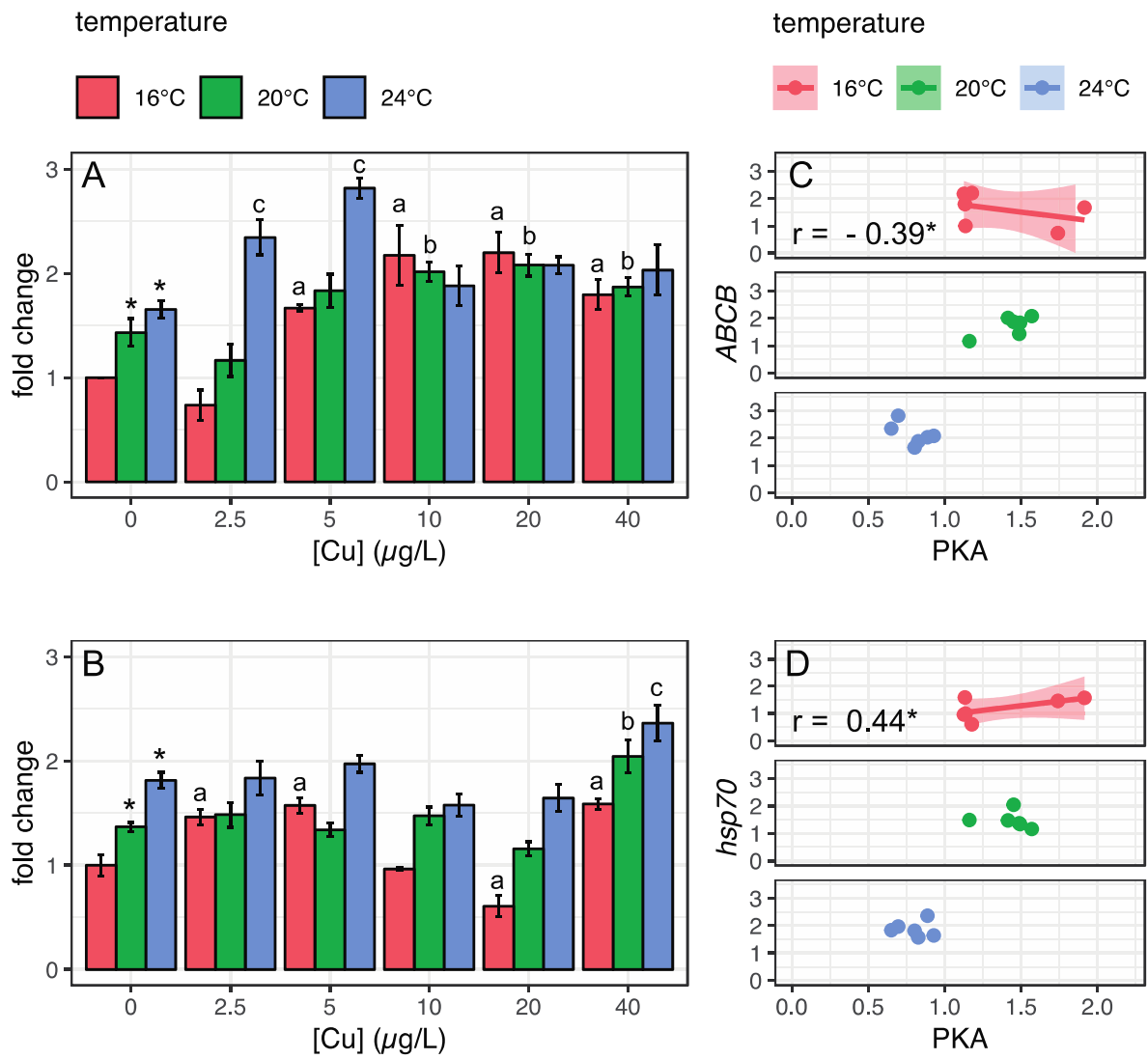
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835 **Fig 6**

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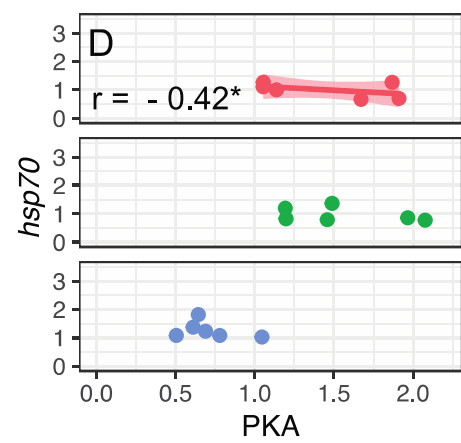
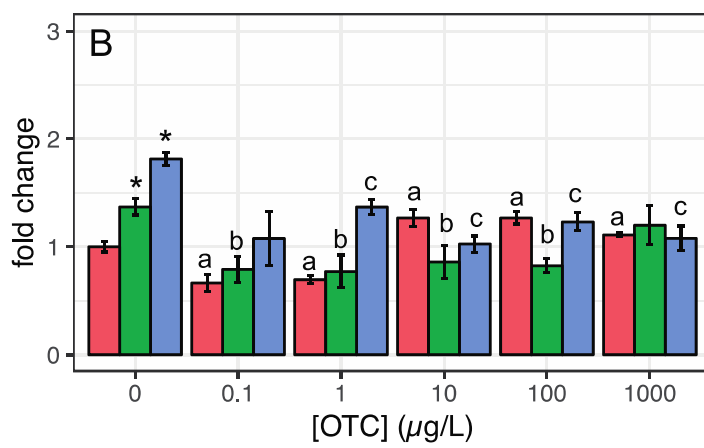
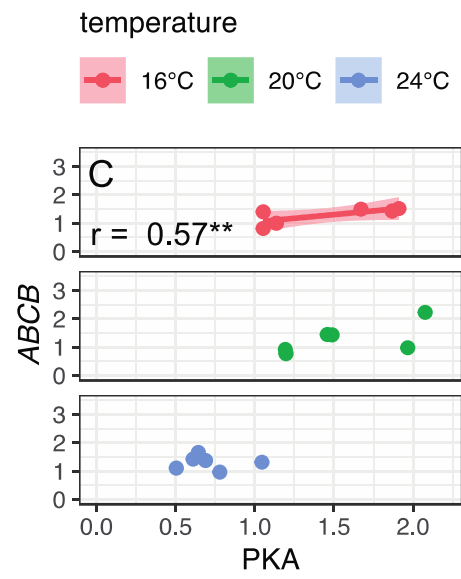
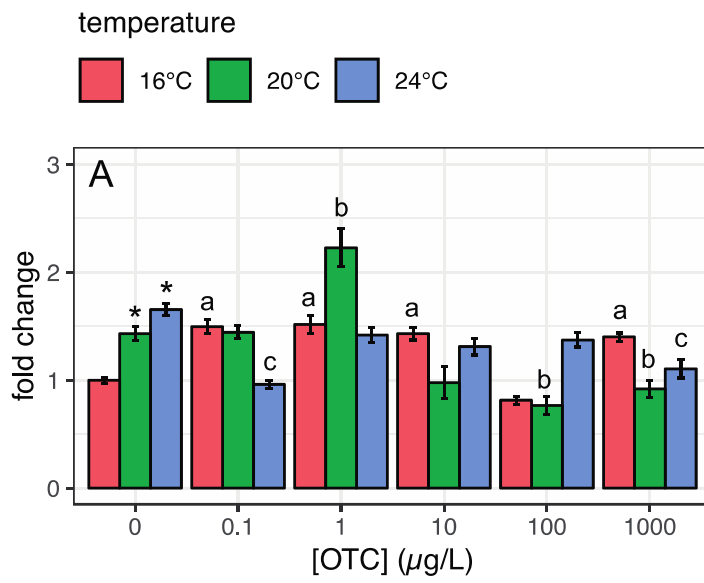
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855 **Fig 7**

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