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1 **Does culture supernatant of *Haslea ostrearia* containing marennine**
2 **affect short-term physiological traits in the adult blue mussel *Mytilus***
3 ***edulis*?**

4
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23

24 **Abstract**

25
26 Marennine, the blue-green pigment excreted by the pennate diatom *Haslea ostrearia* has
27 potential for applications as a natural antimicrobial agent in bivalve aquaculture.
28 However, utilization of this pigment should be assessed further prior to its application in
29 bivalve hatcheries. The present study aims to investigate the effect of the culture
30 supernatant of *H. ostrearia* containing marennine (Blue Water, BW) on physiological
31 rates of adult mussel *Mytilus edulis*. The hypothesis tested in this study was that BW
32 could alter clearance and respiration rates of adult *M. edulis* as indicative physiological
33 traits. Furthermore, the effect of BW on veligers and pediveligers larvae was assessed in
34 physiological experiments to determine whether the fixation of marennine by the larvae
35 can influence larviphagy in this species. Our results showed that BW significantly
36 lowered CR by 43% and reduced the oxygen consumption by 30% in adult mussels, as
37 compared to control. In larviphagy experiments, mussels ingested veliger and pediveliger
38 larvae without discrimination between greened or non-greened larvae. Moreover, fixation
39 of BW in bivalve gills at 3 mg L⁻¹ affected the physiological rate but not intensity of
40 larviphagy in adult *M. edulis*. This study thus provides an insight the necessity to
41 determine the potential effect of marennine on adult bivalve for the future utilization of
42 this pigment in bivalve hatcheries.

43
44 **Keywords:** Marennine, *Haslea ostrearia*, *Mytilus edulis*, Clearance rate, larviphagy

45 **1. Introduction**

46 Bivalves play a significant role in energy and nutrient flux between benthic and pelagic
47 communities in the aquatic environment (Dame, 1993, 2011). They are able to control the
48 abundance of primary producers, zooplankton and larval stages of other marine species,
49 including bivalves themselves (Fréchette & Bourget, 1985; Prins *et al.*, 1991; Coen *et al.*,
50 2007). As food and oxygen are obtained by filtering water across their gills in bivalve
51 suspension-feeders, filtration and respiration are considered useful tools to measure their
52 physiological state (Widdows & Johnson, 1988). These two physiological indicators are
53 frequently used to determine bivalve's response to the presence of dissolved chemicals
54 and particulate matters in water. To date, many studies have shown that biotoxin,
55 especially from toxic dinoflagellates, can alter bivalve behavior, physiology and cellular
56 activity (Bardouil *et al.*, 1993; Bricelj *et al.*, 1996, 2005; Hégaret *et al.*, 2007, 2011;
57 Navarro & Contreras, 2010). Nevertheless, studies on the effects of non-toxic organic
58 compounds on bivalves are less documented.

59 Marennine is a water-soluble blue pigment that is excreted by the non-toxic diatom
60 *Haslea ostrearia* (Gaillon) Simonsen. This non-toxic organic compound can be fixed
61 preferentially on the gills and labial palps of bivalves and is responsible for the greening
62 phenomenon of Pacific oysters *Crassostrea gigas* on the French Atlantic Coast
63 (Gastineau *et al.*, 2014; Prasetya, 2015). This natural greening phenomenon contributes
64 to the economic value of cultured oysters due to the organoleptic and essential fatty acids
65 modification in green oysters (Piveteau, 1999; Prasetya *et al.*, 2017a). Most importantly,
66 marennine has antibacterial properties and could act as antibacterial and natural
67 protective agent against pathogens in aquaculture (Gastineau *et al.*, 2012, 2014; Turcotte
68 *et al.*, 2016). In the mussel *Mytilus edulis* and the scallop *Placopecten magellanicus*,
69 Turcotte *et al.* (2016) revealed that culture supernatant containing marennine (hereafter
70 named as blue water, BW) can increase the survival of their larvae when they are exposed
71 to *Vibrio splendidus*. Consequently, marennine could be potentially applied in shellfish
72 aquaculture as natural antimicrobial agent and immunostimulant if the concentration is
73 maintained below the toxicity level (Falaise *et al.*, 2019). However, before the
74 development of this potential new antibacterial agent in commercial hatchery, knowledge
75 on their effect on other ontogenic stages is necessary. Recent study from Prasetya *et al.*

76 (2017a) demonstrated that marennine, affects behavior, physiology and biochemical traits
77 of juvenile blue mussel *Mytilus edulis* and the American oyster *Crassostrea virginica*.
78 However, no evidence was documented on the effect of this compound on adult bivalves.
79 The selected biological model in the present study was the blue mussel *M. edulis* that is
80 spread and cultivated worldwide. The main hypothesis of the present study was that
81 marennine could alter clearance and respiration rates of adult *M. edulis* as indicative
82 physiological traits. Additionally, a possible effect of marennine on larvae was studied in
83 physiological experiments to investigate whether marennine could influence larviphagy
84 in this species. Previous studies have revealed that most bivalve perform cannibalism by
85 ingesting veliger larvae from their species. Thus, *M. edulis*, *C. virginica* and *C. gigas*,
86 lead to larval death, as the filtered larvae are rejected in pseudofaeces or directly ingested
87 (Tamburri & Zimmer-Faust, 1996; Lehane & Davenport, 2004; Tamburri *et al.*, 2007;
88 Troost *et al.*, 2008). An increased filtration pressure could therefore reduce the number of
89 bivalve larvae. We hypothesize that the greening of larvae by marennine may perturb
90 larval recognition by the adult mussels and increase the palatability as resulting green
91 larvae could be confounded with microalgae and thus stimulate filtration in mussels.

92

93 **2. Materials and methods**

94 **2.1. *Haslea ostrearia* culture**

95 The cultures of *H. ostrearia* (NCC-136) were obtained from the Nantes Culture
96 Collection (NCC), Université de Nantes, France. The stock cultures were maintained in
97 autoclaved F/2 medium at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Furthermore, pilot-scale cultures
98 were conducted in two 100 L flat bottom circular photobioreactors (PBRs) at irradiance
99 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 14/10 h light/dark cycle, temperature of 20 °C and salinity of
100 28 ppt. Marennine concentration was estimated on cell-free culture water (syringe-
101 filtered on 0.20 μm , Sterile-R) using the Beer-Lambert law as described in Prasetya *et al.*
102 (2016). The optical density was measured at 677 nm in a 10 mm cuvette using a
103 spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies), as well as the
104 specific extinction coefficient for extracellular marennine ($\epsilon_{677} = 12.13 \text{ l g}^{-1} \text{ cm}^{-1}$), as
105 stated in Pouvreau *et al.* (2007). To achieve optimum advantage provided by marennine

106 in aquaculture, feasibility in using this pigment needs to be taken into account. The
107 utilization of purified marennine obtained according to the anion-exchange
108 chromatography method (Pouvreau *et al.*, 2006) seem not be the best option, since it is a
109 costly and time-consuming process. Therefore, in this study we used the supernatant
110 containing the marennine (blue water, BW) concentrated 100 times (from 100 L to 1 L)
111 using two ultrafiltration units (30 and 3 kDa) as described in Turcotte *et al.* (2016).

112

113

114 **2.2. Animals and diet preparation**

115 Adult *M. edulis* were obtained in June 2013 from the Havre-aux-Maisons lagoon (Iles-de-
116 la-Madeleine, QC, Canada, 47°25' N, 61°50' W), where a pure *M. edulis* population is
117 observed (Myrand *et al.*, 2009). Introduction and transfer permits from Fisheries and
118 Oceans Canada were obtained and our study did not involve endangered or protected
119 species. Animals were transported to the Station Aquicole de Pointe-aux-Pères (Institut
120 des Sciences de la Mer, Rimouski, Canada). Upon arrival, 40 individuals of adult mussels
121 (mean shell length = 63 ± 1.8 mm) were numbered with bee tags, and were acclimated to
122 laboratory conditions for 30 days prior to physiological experiment. Another 40 adults
123 were laboratory acclimated in similar condition for larval production. Animals were
124 placed in a 300 L maintenance tank with light aeration, salinity of 29 ppt, temperature of
125 16°C and natural photoperiod. Furthermore, animals were continuously fed with
126 microalgae strains from the National Center of Marine Algae and Microbiota (Bigelow
127 Laboratory for Ocean Sciences, MA, USA). The species were *Pavlova lutheri* (CCMP
128 459), *Tisochrysis lutea* (CCMP 1324) and *Chaetoceros muelleri* (CCMP 1316) at 1:1:1
129 equivalent volume and a daily ration of 7×10^8 cells animal d⁻¹ (3% dry weight algae/dry
130 weight mussel per day).

131

132

133 **2.3. Larval production**

134 Spawning was induced by thermal shock from 12 to 25°C. When gametes began to be
135 released, adult mussels were transferred to 18°C filtered (1 µm) seawater to improve
136 oocyte or sperm release. A total of 40 different individual parents were used to generate

137 gametes in a pool-cross design to produce one random larval family. Gametes were
138 mixed at a ratio of 10 spermatozooids oocyte⁻¹ and fertilized eggs were then transferred to
139 180 L tanks at densities of 30×10^6 eggs tank⁻¹ and left undisturbed at 18°C for 48 h in
140 filtered (1 µm) and UV-pretreated seawater.

141 D-larvae were collected by filtering the water on a 20 µm mesh screen and transferred at
142 densities of 10 larvae mL⁻¹ into 60 L rearing tanks. Larvae were fed every 2–3 days with
143 a mixture of the 3 microalgae species as mentioned above at 60 cells µL⁻¹ (1:1:1),
144 cultured with treated seawater, photoperiod and temperature as explained earlier. Before
145 each feeding period, water in each tank was entirely renewed. Microalgae were harvested
146 in the late exponential growth phase approximately from 6 to 10 days after flask
147 inoculation in order to feed the larvae.

148

149 ***2.4. Greening and physiological measures***

150 Mussel adults (20 ind) and larvae (54000 ind) were greened in BW (equivalent to 3 mg L⁻¹
151 of marennine) for 16-h and fed with a mixture (3% of mean mussel dry weight) of three
152 hatcheries microalgae as mentioned in the section “Animals and diet preparation”. Adult
153 mussels and larvae fed only with the microalgal mixture constituted the controls.

154 Physiological measurements were performed only on adult mussels over two days on
155 individuals randomly sampled. Twelve chambers filled with 1 µm-filtered and UV
156 treated seawater were used concurrently, which allowed us to measure 10 animals (5
157 greened and 5 non-greened) and two controls (empty shell). Mussels that remained closed
158 in the chamber during the measurement were excluded from physiological analysis.
159 Physiological rates were also standardized to mass-specific rate equivalent to individuals
160 of 1 g dry mass using weight exponents as described in (Bayne *et al.*, 1987).

161 Clearance rate (CR), which could be defined as the volume of water removed of
162 suspended particles per unit time, was measured using a static system as described in
163 Prasetya *et al.* (2017a). Briefly, adult mussels were placed in 1.3 L metabolic chambers 1
164 h prior to measurements, then fed with the mixture of the three microalgae species as
165 mentioned above at an initial concentration of 100000 cells mL⁻¹ (1:1:1 in dry weight).
166 Circulation was generated by fine bubble aeration around the wall of the metabolic

167 chamber in order to reduce the resuspension of feces. Food particles were counted every
168 10 min during 60 min of measurement using a particle counter machine (Beckman
169 Coulter Counter Z2, Mississauga, ON, Canada). The instrument was installed with a 70 μ
170 m aperture tube and was adjusted to measure particle size in the range 3.5 - 8 μ m, the size
171 of the three microalgae used in this study and known to be efficiently retained by *M.*
172 *edulis* (Barillé *et al.*, 1993; Ward and Shumway, 2004). The utmost difference between
173 two consecutive measurements was used to calculate CR as described in (Gilek *et al.*,
174 1992) and then expressed as one liter of water cleared from microalgae $\text{h}^{-1} \text{g}^{-1}$ tissue dry
175 weight after standardization to mass-specific rate equivalent (Tremblay *et al.*, 1998).
176 Respiration or oxygen consumption was measured on the same individuals in a 0.85 L
177 metabolic chambers following (Prasetiya *et al.*, 2017a). Briefly, the rate of oxygen uptake
178 was determined by sealing the metabolic chambers and measuring the decrease in
179 dissolved oxygen using an YSI (5331) polarographic electrode coupled to a YSI micro-
180 oxymeter (Yellow Springs, OH, USA). Afterwards, the output signal was monitored
181 continuously on a chart recorder until there was a 20% decrease in oxygen saturation.
182 Respiration was then expressed as $\text{ml O}_2 \text{ h}^{-1} \text{g}^{-1}$ tissue dry weight on standardized mass-
183 specific rate equivalent.

184

185 ***2.5. Larviphagy interaction experiment***

186 Experiments were also conducted within the physiological experiments on control and
187 BW exposed adult mussels to estimate if presence of greened larvae modifies their CR
188 values on microalgae. Briefly, two size ranges of larvae were used, veligers (160.1 ± 2.7
189 μ m) and pediveligers ($274.8 \pm 2.1 \mu$ m) of 8 and 18 days post-fertilization, called “small”
190 and “large” larvae, respectively. These two sizes of larvae were then subdivided into the
191 “greened larvae (GL)” and “non-greened larvae (NGL)”. After the greening larvae
192 treatment (60 000 larvae by size) in BW for 16-h as described above, larvae at the surface
193 were siphoned on 35 μ m mesh screen to obtain only live larvae, and then recovered in
194 500 mL to estimate the concentrations. Afterwards, the larviphagy experiment was
195 performed in the same 1.3 L metabolic chambers and condition described for clearance

196 measures by offering high larval density (around 100 larvae mL⁻¹) of GL or NGL.
197 Mussels exposed only to microalgae (without larvae) were used as control.

198

199 **2.6. Data analysis**

200 Univariate variables in physiological experiments were compared using a t-test to
201 compare the effect of BW on CR and respiration. The homoscedasticity was graphically
202 assessed and normality tested by a Saphiro-Wilk test. Data were transformed into square
203 root when necessary. Additionally, the two-way ANOVA was performed to determine
204 whether there were any differences in CR between the two factors (BW concentration
205 and type of larvae). Statistical analysis on CR was also performed when the diet was
206 shifted from larvae to microalgae only. All statistical analyses were conducted at a
207 significance level of $\alpha = 0.05$ and performed using Graphpad Prism 5 for Mac OS X
208 software.

209 **3. Results**

210 **3.1. Effect of BW on physiological traits of *M. edulis***

211 A significant difference of clearance rate (CR) in adult *M. edulis* was observed (t-test_{2,38}
212 = 2.649; $P = 0.008$) with CR 43% lowered for mussels exposed to BW comparatively to
213 control (Fig. 1).

214 The BW also significantly lowered the oxygen consumption in adult *M. edulis* (t-test_{2,38}
215 = 2.649; $P = 0.005$). The adult mussels exposed to 3 mg L⁻¹ of BW had 30% lower
216 oxygen consumption than that of control (Fig. 2).

217

218 **3.2. Effect of BW on CR in presence of larviphagy**

219 Larvae from both size ranges appeared to be healthy with mortality in the rearing tanks
220 less than 15% until pedivelger stage, presence of abundant food in the digestive system,
221 and larvae largely active with their velum. After the greening treatment, larvae showed a
222 blue light ring around the shell with similar behavior than non-greened larvae (Fig. 3).
223 CR estimated only on the monoalgal diet (control) showed similar values than the first
224 experiment (Fig. 1). The two-way ANOVA revealed that significant difference in CR was

225 only due to BW without interaction effect (Table 1). Regardless the size of the larvae
226 given, it was found that the CR of mussels did not change significantly when they fed
227 either on greened or on non-greened larvae (SGL and SNGL; LGL and LNGL),
228 suggesting that the greening of larvae by BW did not affect the CR in presence of
229 larviphagy (Fig. 4). Additionally, the intensity of CR in presence of larviphagy was
230 similar between veligers and pediveligers larvae (Table 1).

231

232 **4. Discussion**

233 The present work aimed to determine the consequence of greening by BW (supernatant
234 of *Haslea ostrearia* culture containing marennine) on physiological rate of adult mussels
235 *M. edulis* and to determine whether the greening of adults and larvae could affect the CR
236 in presence of larviphagy. To our knowledge, no other study has reported the short-term
237 effect of BW containing marennine on the adult mussel *M. edulis* and also the possible
238 consequence of the presence of greened larvae in the CR of adults. In this study, it was
239 found that the greening by BW marennine affects the physiological rates of adults *M.*
240 *edulis*. However, our hypothesize that the greening of larvae may perturb larval
241 recognition by the adult mussels increasing their palatability and stimulating their
242 clearance rate does not seem to be confirmed. We observe a trend of increasing CR in
243 presence of veliger or pediveliger greened larvae, but the variability between individuals
244 outweighs significant differences. As the CR in control (mussels exposed to only
245 microalgae without larvae) had similar values than other treatments for non-greened adult
246 mussels, we suggest that 1 hour of CR measurement was too short or the larviphagy rate
247 was too high to measure a potential impact of larvae on adult mussels CR.

248

249 **4.1 Physiological responses to marennine**

250 Unlike the effect of natural toxic substances produced by dinoflagellates (Hégaret *et al.*,
251 2007; Navarro & Contreras, 2010; Jauffrais *et al.*, 2012; Basti *et al.*, 2013, 2015;
252 Navarro *et al.*, 2018), the studies of the effect of natural non-toxic substances such
253 marennine are scarce. The present study provides evidence that the clearance rates of

254 adult *M. edulis* were significantly reduced when they were exposed to BW, in accordance
255 with Prasetya *et al.* (2017a), who observed that juveniles of *M. edulis* had a clearance
256 rate reduced by 56% in response to marennine. The CR reduction by BW on adults was
257 up to 43%. However, distinction should be made due to different types of marennine,
258 concentration and also the stage in the life cycle of *M. edulis* used in the experiment.
259 Indeed, the marennine solution used by Prasetya *et al.* (2017a) was the purified form and
260 the effect was observed at 2 mg L⁻¹ on the stage of juveniles, which are considered more
261 sensitive than the adults. In line with this finding, Gastineau *et al.* (2017) observed that
262 adult oysters *Crassostrea gigas* that were exposed to the BW 5 mg L⁻¹ had approximately
263 50% lower CR in comparison with control. These results suggest that marennine is not
264 only able to change coloration in bivalve oysters and mussels but also affect their
265 physiological rate.

266 Our results show, the accumulation of marennine present in the tissues of adult mussels
267 affects the consumption of oxygen. This result is in coherence with Prasetya *et al.*
268 (2017a) who observed significant effect in consumption of oxygen on juvenile of
269 American oysters *Crassostrea virginica* following a 16 h exposure to 2 mg L⁻¹ of purified
270 marennine. Nevertheless, the same authors found similar trends on reduction of oxygen
271 uptake on the juvenile of *M. edulis* at similar concentration of marennine (2 mg L⁻¹) but
272 this effect was not significant. Such inhibition of oxygen uptake and clearance rate has
273 been previously documented in studies involving toxic and non-toxic algae (Bricelj *et al.*,
274 1996; Li *et al.*, 2002; Navarro & Contreras, 2010; Navarro *et al.*, 2014, 2018). From the
275 result of our study, the blue water solution containing marennine appears to have
276 inhibitory effect on the mechanism of oxygen uptake and filtration activity on gills of
277 bivalves, presumably due to the interaction between this pigment and cilia organs. Thus
278 in reaction to an exposure to marennine, bivalves *M. edulis* and *C. virginica* adapt
279 behaviorally by closing their valves and decreasing their CR for certain period of time,
280 and biochemically, for instance, by change in the unsaturation of their cell membrane
281 phospholipids in gills (Prasetya *et al.*, 2017a).

282

283 **4.2 Consequence of greening to larviphagy**

284 Significant differences were observed in filtration activity for both treatments (control vs
285 BW) when the adult mussels *M. edulis* fed on microalgae. However, no significant
286 difference due to the presence of larvae (greened or not) was observed. Thus, this result
287 invalidated our hypothesis that greened larvae by marennine could change the palatability
288 of adults mussels, increasing the CR on microalage. However, no estimation of direct
289 larviphagy (number of larvae ingested by adult mussels) has been obtained and potential
290 selectivity of greened larvae could be possible.

291 Vulnerability of larvae to larviphagy by the adults in bivalve species could be explained
292 by the relationship between the bivalve' gills area and the size of the larvae themselves.
293 The adult mussels' gills may retain any particles above their specific size limit, which
294 ranged from 2 to 7 μm (Møhlenberg & Riisgård, 1978). In our study, the larvae of *M.*
295 *edulis* used were at the stage of D-larvae (8 and 18 days after fertilization) with size
296 ranged from 100 to 200 μm . Therefore, these larvae could mostly be retained on adult *M.*
297 *edulis* gills (Hendriks *et al.*, 2005; Sprung, 1984; Strohmeier *et al.*, 2012). However,
298 bivalve larvae could avoid being filtered by the filter feeders due to two possibilities.
299 First, by detecting the hydro-mechanical stimuli in the inhalant flow field as described in
300 Jakobsen (2001) and Kingsford *et al.* (2002). Second, by detecting the filter feeders
301 chemically (Fitt & Coon, 1992; Tamburri & Zimmer-Faust, 1996). In this study, fixation
302 of marennine by the gills of *M. edulis* could perhaps facilitate detection of this animal by
303 the mussel larvae by chemical sensors. However, further studies should be conducted to
304 address this possibility. Previous studies revealed that size and biochemical properties
305 affect the feeding behavior of bivalves, where the preference could fall to the either
306 smaller particles, higher biochemical content or a combination between them (Beninger
307 *et al.*, 2008; Bougrier *et al.*, 1997; Mafra *et al.*, 2009; Prasetya *et al.*, 2017b; Shumway *et*
308 *al.*, 1985). As Turcotte *et al.* (2016) have demonstrated that greened larvae accumulated
309 more energetic lipids, this biochemical modification could increase the palatability of
310 adult mussels on greened larvae. Thus, it could be possible that larviphagy of greened
311 larvae could be increased without increase of microalgae ingestion.

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322

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502

503 **List of Tables**

504

505 **Table 1.** Factorial ANOVA analysis for mussel’s clearance rates obtained from different
506 treatments

<i>Factors</i>	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>p</i>
Interaction	3	0.302	0.101	0.298	0.8267
[BW marennine]	1	1.892	1.892	5.595	0.0242
Type of diet	3	0.607	0.202	0.598	0.6208
Residual	32	10.82	0.338		

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509 **List of Figures**

510

511

512 **Figure 1.** Clearance rate (CR, $l\ h^{-1}\ g^{-1}$ dry weight, mean \pm standard error, $n = 10$ per
513 condition) of adult mussels *M. edulis* exposed or not to BW containing $3\ mg\ L^{-1}$ of
514 marennine. Different lower case letters indicate significant difference.

515

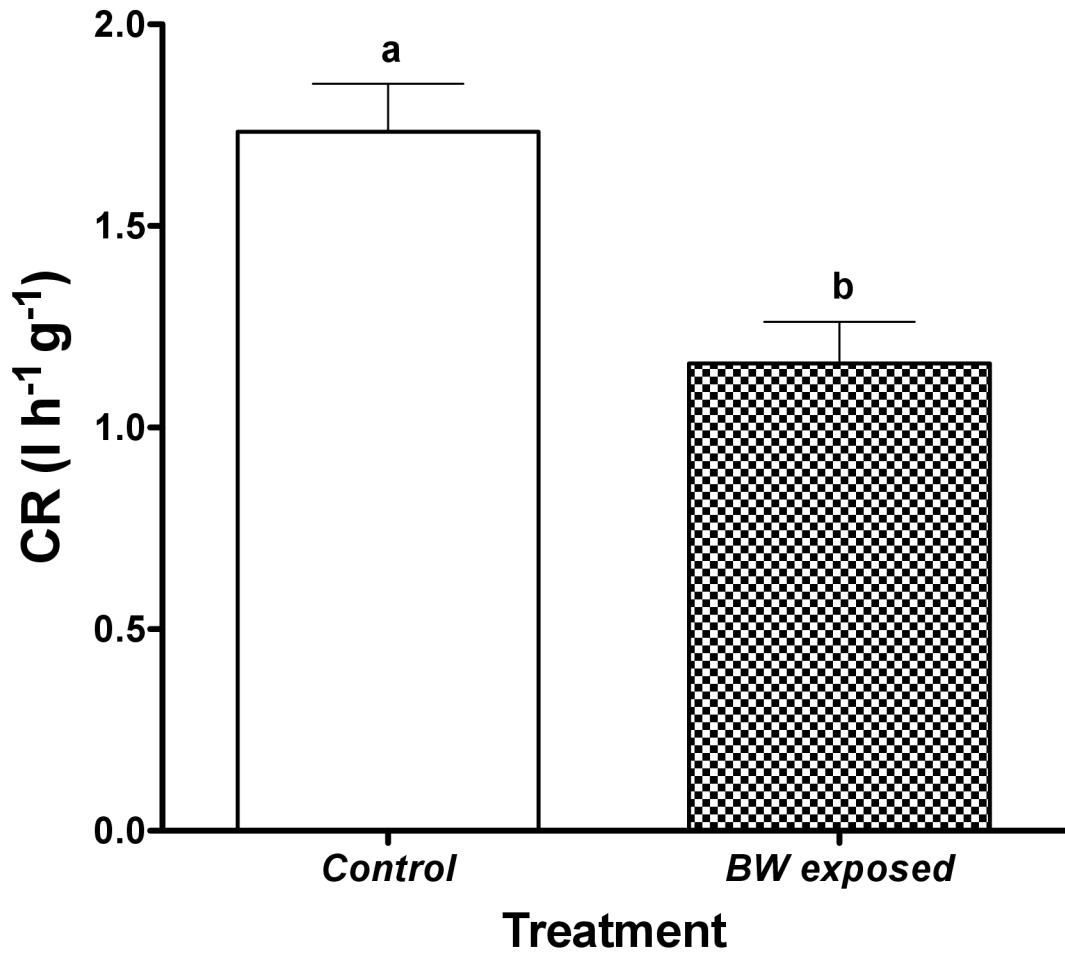
516 **Figure 2.** Oxygen consumption (VO_2 , $ml\ O_2\ h^{-1}\ g^{-1}$ dry weight, mean \pm standard error, n
517 = 10 per condition) of adult mussels *M. edulis* exposed or not to BW containing $3\ mg\ L^{-1}$
518 of marennine. Different lower case letters indicate significant difference.

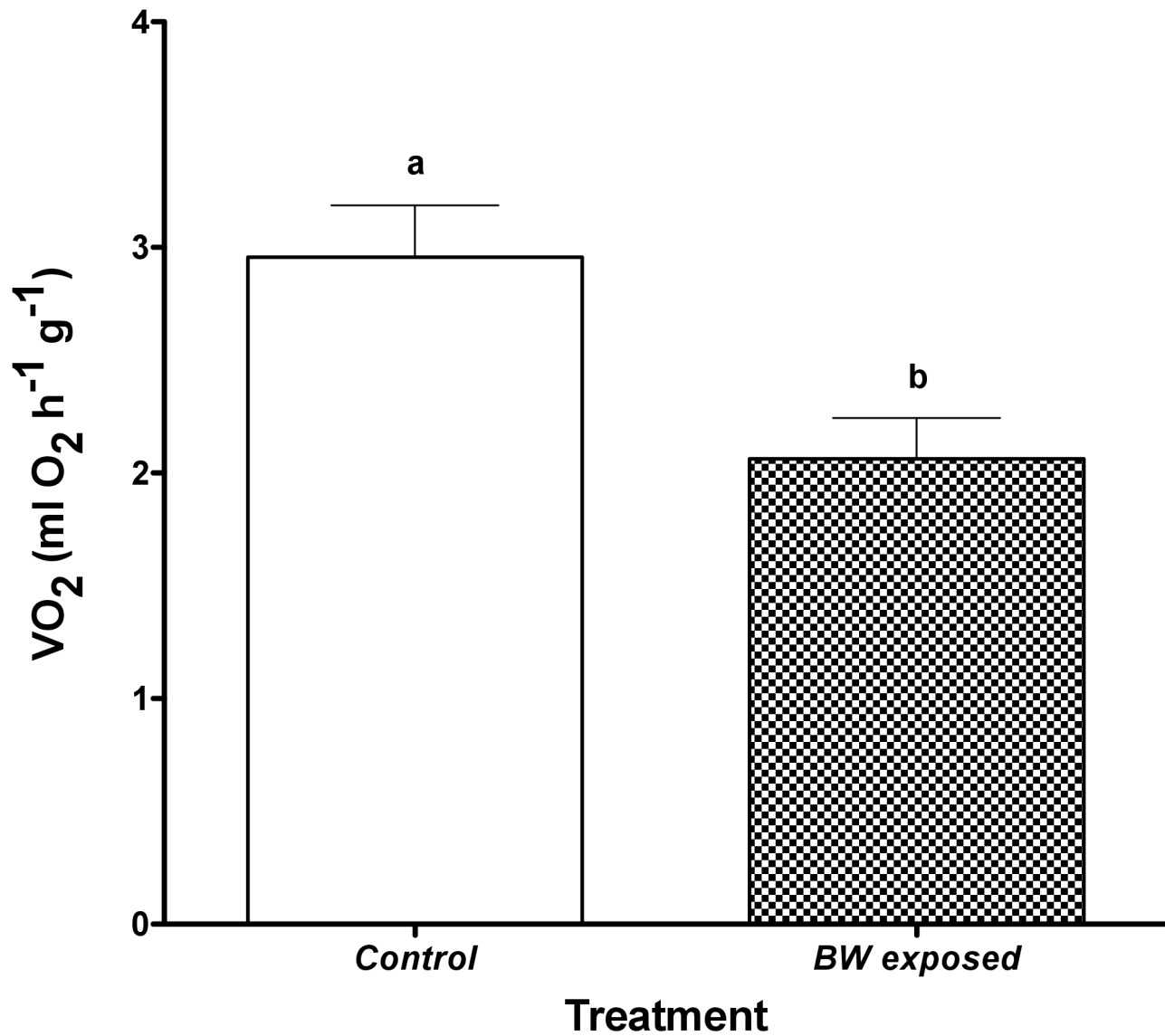
519

520 **Figure 3.** Veliger larvae control (A) and exposed to the BW containing marennine with a
521 blue light ring around the shell (B).

522

523 **Figure 4.** Mean clearance rates (CR ($l\ h^{-1}\ g^{-1}$ dry weight) of adult mussels *M. edulis*
524 exposed or not to BW in relation to different types of diet (M: only microalgae, SGL:
525 with presence of small greened larvae, SNGL: with presence of small non-greened larvae,
526 LGL: with presence of large greened larvae, LNGL: with presence of large non greened
527 larvae). Values are mean \pm standard error of mean based on 10 individuals per
528 concentration.





A



100 μm

B



100 μm

