

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

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24 25

Abstract

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 larviphagy in adult *M. edulis*. This study thus provides an insight the necessity to 40 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; Prasetiya, 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; Prasetiya 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 to Vibrio splendidus. Consequently, marennine could be potentially applied in shellfish 71 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 Prasetiya 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 physiological experiments to investigate whether marennine could influence larviphagy 83 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 autoclaved F/2 medium at 20 μ mol photons m⁻² s⁻¹. Furthermore, pilot-scale cultures 97 98 were conducted in two 100 L flat bottom circular photobioreactors (PBRs) at irradiance 99 180 µmol photons m⁻² 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 Prasetiya 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 specific extinction coefficient for extracellular marennine ($\varepsilon_{677} = 12.13 \ \text{l g}^{-1} \ \text{cm}^{-1}$), as 104 105 stated in Pouvreau et al. (2007). To achieve optimum advantage provided by marennine in aquaculture, feasibility in using this pigment needs to be taken into account. The
utilization of purified marennine obtained according to the anion-exchange
chromatography method (Pouvreau *et al.*, 2006) seem not be the best option, since it is a
costly and time-consuming process. Therefore, in this study we used the supernatant
containing the marennine (blue water, BW) concentrated 100 times (from 100 L to 1 L)
using two ultrafiltration units (30 and 3 kDa) as described in Turcotte *et al.* (2016).

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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).

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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 μ m) 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

mixed at a ratio of 10 spermatozoids $oocyte^{-1}$ 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 μ m) and UV-pretreated seawater.

141 D-larvae were collected by filtering the water on a 20 μ m 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 μ L⁻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 fed the larvae.

148

149 2.4. Greening and physiological measures

Mussel adults (20 ind) and larvae (54000 ind) were greened in BW (equivalent to 3 mg L⁻ 151 ¹ 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 μ m-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 Prasetiya *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 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 h⁻¹g⁻¹ tissue dry

chamber in order to reduce the resuspension of feces. Food particles were counted every

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 ml O_2 h⁻¹ g⁻¹ tissue dry weight on standardized mass-183 specific rate equivalent.

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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 ± 2.1 μ 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 µm 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

measures by offering high larval density (around 100 larvae mL⁻¹) of GL or NGL.
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 x = 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

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

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

217

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

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

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

Unlike the effect of natural toxic substances produced by dinoflagellates (Hégaret *et al.*,
2007; Navarro & Contreras, 2010; Jauffrais *et al.*, 2012; Basti *et al.*, 2013, 2015;
Navarro *et al.*, 2018), the studies of the effect of natural non-toxic substances such
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 Prasetiya 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 Prasetiya 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 adult oysters *Crassostrea gigas* that were exposed to the BW 5 mg L⁻¹ had approximately 262 263 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 Prasetiya et al. 268 (2017a) who observed significant effect in consumption of oxygen on juvenile of American oysters *Crassostrea virginica* following a 16 h exposure to 2 mg L⁻¹ of purified 269 270 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 $(2 \text{ mg } L^{-1})$ 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 (Prasetiya et al., 2017a).

282

283 4.2 Consequence of greening to larviphagy

Significant differences were observed in filtration activity for both treatments (control *vs* BW) when the adult mussels *M. edulis* fed on microalgae. However, no significant difference due to the presence of larvae (greened or not) was observed. Thus, this result invalidated our hypothesis that greened larvae by marennine could change the palatability of adults mussels, increasing the CR on microalage. However, no estimation of direct larviphagy (number of larvae ingested by adult mussels) has been obtained and potential 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 μ m. 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; Prasetiya 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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502									
503 504	List of Tables								
505	ned from d	ifferent							
506 treatments									
	Factors	df	SS	MS	F	р			
	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					

- 509 List of Figures
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Figure 1. Clearance rate (CR, 1 h⁻¹ g⁻¹ dry weight, mean \pm standard error, n = 10 per condition) of adult mussels *M. edulis* exposed or not to BW containing 3 mg L⁻¹ of marennine. Different lower case letters indicate significant difference.

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Figure 2. Oxygen consumption (VO₂, ml O₂ h⁻¹ g⁻¹ dry weight, mean \pm standard error, n = 10 per condition) of adult mussels *M. edulis* exposed or not to BW containing 3 mg L⁻¹ of marennine. Different lower case letters indicate significant difference.

520 Figure 3. Veliger larvae control (A) and exposed to the BW containing marennine with a521 blue light ring around the shell (B).

522

519

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









Type of diet