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A trade-off between mucocytes and bacteriocytes in *Loripes orbiculatus* gills (Bivalvia, Lucinidae): a mixotrophic adaptation to seasonality and reproductive status in a symbiotic species?

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Abstract

In this study, we investigated the composition of the gill tissue relative to the reproductive status of the lucinid clam *Loripes orbiculatus* (sensus Poli, 1791) according to seasonal as well as biological parameters to provide insights into the physiological variability of this symbiotic bivalve. Temporal variation in population density was also studied. The species was investigated in *Zostera noltii* seagrass beds in the Thau lagoon (43°26'52.27" N, 3°39'6.25" E) in the south of France in a monthly sampling study from May 2013 to July 2015. A total of 257 individual adults of different sizes were analysed according to water temperature and salinity variations. The findings revealed a very stable *Loripes* density over time, with one single reproductive period during late spring/early summer. We also found that bacteriocytes and mucocytes in the gills were negatively correlated and highly variable in their respective proportions. Bacteriocytes remained dominant during cold periods, whereas mucocytes appeared mainly in the gills of large individuals when the water temperature increased in the spring. As mucocytes were also related with gonadal maturation, we hypothesize that these may allow the host to increase the proportion of heterotrophy in its nutrition during spring primary production to face the metabolic demands required for reproduction. It is possible that mucocytes may also be involved in host immunity.

31 **Introduction**

32 Lucinid clams are benthic bivalves that live in seagrass beds in coastal tropical or temperate
33 areas (van der Heide et al. 2012). These bivalves host sulphur-oxidizing chemoautotrophic
34 endobacteria in large cells called bacteriocytes located in the gills (Felbeck et al. 1981), as has
35 been described for *Loripes* (Herry et al. 1989). The gills of symbiotic species are thickened,
36 which is related to the dominance of bacteriocytes in the gill tissue and represents an
37 adaptation process to intracellular symbiosis. This symbiosis allows the bivalve's colonization
38 of seagrass sediment, conditions that are generally toxic for most invertebrates due to the high
39 level of sulphide, which originates from the anaerobic decomposition of organic matter by
40 sulphate-reducing bacteria at the bottom of seagrass bed sediments. The endosymbiotic
41 bacteria in the clam's gills use sulphide to fuel their own metabolism, i.e. to synthesize sugar
42 compounds after CO₂ fixation (van Dover 2000; Stewart et al. 2005). These compounds are
43 then transferred to the host as an important source of nutrition (Distel and Felbeck 1987; Cary
44 et al. 1989). Where lucinids are present, they mitigate sulphide stress in seagrass (van der
45 Geest et al. 2020); indeed, the sulphur-oxidizing metabolism of endosymbionts can result in
46 removing 2–16% of total sulphide in seagrass sediment (Reynolds et al. 2007), which is
47 beneficial to ecosystem function (van Gils et al. 2012). Van der Heide et al. (2012) described
48 the positive effects for all partners in the endosymbiont–host–seagrass relationship as ‘three-
49 stage symbiosis’, highlighting the ecological importance of lucinids for seagrass conservation
50 (Johnson et al. 2002).

51 Estimates of the chemoautotrophic contribution to the host's carbon requirements generally
52 reveal a high contribution – on average, 70% – from symbionts (through the translocation of
53 sugars in a process called ‘milking’), with some variation according to season, size of the
54 host, reproductive period, etc. (Cary et al. 1989; Dando and Spiro 1993; Le Pennec and
55 Beninger 2000; Rossi et al. 2013; van der Geest et al. 2014). Moreover, as has been shown in
56 starvation experiments, in addition to milking, the host's metabolic requirements can be
57 ensured by the intracellular digestion of symbionts by host cell lysosomes in a process called
58 ‘farming’ (Kádár et al. 2008; Caro et al. 2009; König et al. 2015). However, most lucinids
59 have a mixotrophic diet, allowing them to feed either on symbiotic carbon (by milking or
60 farming) or on particulate organic carbon (Le Pennec et al. 1988; Johnson and Fernandez
61 2001; Duplessis et al. 2004). Recently, it was shown that the endosymbionts of lucinids can
62 also contribute to the nitrogen metabolism of the host (*Ca. Thiodiazotropha endoloripes* and
63 *Ca. Thiodiazotropha endolucinida* were discovered as diazotrophic endosymbionts), in

64 addition to the host's ability to use ammonium, urea and nitrate as a source of nitrogen (König
65 et al. 2016; Petersen et al. 2016).

66 *Loripes orbiculatus* (previously known as *Loripes lacteus*), a bivalve abundant in the coastal
67 lagoons of the south of France (Johnson et al. 2002; Carlier et al. 2009) and also present in
68 Brittany (Herry et al. 1989; Johnson and Fernandez 2001), is one of these mixotrophic
69 species. This species lives buried in the sediment of *Zostera noltii* seagrass beds (Lebreton et
70 al. 2011), taking advantage of sulphides to fuel symbiont metabolism and dioxygen released
71 by seagrass roots. *Loripes orbiculatus* also seems to be able to meet part of its metabolic
72 requirements (an estimated 20% to 70%) by the ingestion and absorption of food particles in a
73 process of heterotrophic nutrition (Johnson et al. 1996; Rossi et al. 2013; van der Geest et al.
74 2014). Diatoms are frequently found in its digestive system (Le Pennec et al. 1988), as is the
75 case more generally in lucinids (Dando et al. 1986; Southward 1986; Herry and Le Pennec
76 1987; Duplessis et al. 2004), in spite of their reduced digestive tract (Allen 1958; Reid 1990;
77 Le Pennec et al. 1995). Recently, molecular evidence has reinforced the pathway of
78 heterotrophic nutrition, as enzymes able to digest complex polysaccharides from marine
79 phytoplankton were shown at high levels in the visceral mass of *Loripes orbiculatus* (Yuen et
80 al. 2019). All these characteristics are evidence of the ability of lucinids to feed on particles in
81 the water column or benthic zone, even if carbon and nitrogen stable isotopic ratios remain
82 low (which is typical of chemoautotrophic bivalves) and vary between individuals (Petersen
83 et al. 2016). Thus lucinids are considered to be suspension and deposit feeders, in addition to
84 their symbiotic mode of nutrition (Duplessis et al. 2004; Rossi et al. 2013; van der Geest et al.
85 2014).

86 The ability of suspension-feeding bivalves to collect and process particulate foods is generally
87 associated with the mucus secreted by mucocytes located in the gills, labial palps, digestive
88 tract, etc. In different suspension-feeding bivalves (Pectinidae, Ostreidae, Mytilidae), the
89 types, distribution and density of mucocytes in the gills give indications of the latter's
90 functional role, especially in terms of particle processing (Beninger et al. 1993; Dufour and
91 Beninger 2001). Acid-secreting mucocytes are generally involved in collecting particles on
92 the gill surface and transporting them to the ventral groove via the ciliated zone, forming a
93 'particle cord' that is conducted to the anterior labial palps (when present) for either ingestion
94 or rejection as pseudo-faeces (Beninger and Dufour 1996; Beninger et al. 1997). The
95 molecular role of mucus in the capture of food particles has been evidenced by the
96 identification of lectins, isolated from the mucus of oysters (*Crassostrea virginica*) and

97 mussels (*Mytilus edulis*); this lectin is involved in the interaction between carbohydrates
98 found at the surface of microalgae and the mucus (Pales-Espinosa et al. 2009, 2010a, 2010b).
99 However, little attention has been paid to the distribution and density of mucocytes in the gills
100 of symbiotic bivalves; mucocytes have often been reported as scarce cells, interspaced among
101 the bacteriocytes (reviewed in Duplessis et al. 2004; Pales-Espinosa et al. 2013b). Only a few
102 studies on lucinids have investigated the distribution and potential function of these
103 mucocytes regarding the filtration process (Duplessis et al. 2004).

104 Beyond the role of mucus in particle capture for host nutrition, it also represents the first line
105 of defence to fight harmful microbes in marine invertebrates. The structure and composition
106 of the mucus covering the body surface in invertebrates influence its effectiveness in
107 immunity; mucin matrices contain a large variety of bioactive molecules such as lysozymes,
108 antimicrobial peptides (Vidal-Dupiol et al. 2011, Destoumieux-Garzon et al. 2016, Rivera-
109 Ortega and Thomé 2018), and adhesion molecules such as lectins and agglutinins (Xing et al.
110 2011; Pales-Espinosa et al. 2016). The diversity of immune effectors found in mucus allows a
111 tailored immune response (Allam and Pales-Espinosa, 2016) according to the type of host-
112 microbe association. In bivalves, mucosal secretions, in addition to cellular immunity, help
113 the host to fight off diseases caused by various types of bacteria (Allam and Pales-Espinosa
114 2016, Travers et al. 2015), viruses (Arzul et al. 2017) and protozoans (Pales-Espinosa et al.
115 2013a). Moreover, mucus could favour a specific symbiont, as seen in some lucinid bivalves
116 through a C-type lectin (Gourdine and Smith-Ravin 2007) or in the association between
117 *Vibrio fischeri* and the squid *Euprymna scolopes* (Nyholm and McFall-Ngai 2003).

118 The primary objective of this study was to investigate the gill tissue composition of *Loripes*
119 *orbiculatus* (sensu Poli 1791), with a focus on the seasonal dynamics of mucocytes and
120 bacteriocytes, through a quantitative histological analysis. The second objective was to
121 decipher the environmental and biological factors responsible for gill plasticity in this species.
122 The environmental variables investigated were water temperature and salinity; the biological
123 factors considered were the different areas of the gills (on an anterior to posterior axis), and
124 the size and sex of individuals.

125

126 **Materials and methods**

127 **Sampling of the bivalves**

128 The survey was conducted in the Mediterranean Thau lagoon in the south of France near the
129 oyster-farming village of Bouzigues (43°26'52.27" N, 3°39'6.25" E). The symbiotic clam
130 *Loripes orbiculatus* (hereafter *Loripes*) was collected between May 2013 and July 2015.
131 These bivalves are located at a depth of 5 to 10 cm in the sediment surrounding the roots of
132 the seagrass *Zostera noltii*. They were collected by hand using a PVC cylinder (12 cm
133 diameter, 20 cm high). Once a month, 10 sediment cores were randomly collected in the
134 seagrass meadows and sieved over a 3 mm mesh. From one month to another, we carefully
135 avoided collecting *Loripes* in the same area, but samples were always collected in the same
136 meadow. The survey was divided into two periods for a total of 26 months of sampling: (1)
137 from May 2013 to October 2014, the total number of *Loripes* collected per core was recorded
138 and the monthly data from the 10 sediment cores was used to calculate the mean and standard
139 deviation of *Loripes* density (number of individuals m⁻²); (2) from February 2014 to July
140 2015, adult bivalves (> 7 mm) were collected for histological investigation and sorted into
141 three size classes: small adults (7–8 mm), medium adults (9–10 mm) and large adults (> 12
142 mm). For each sampling month, 5 to 10 adults were selected in each size class to perform
143 histological analyses, amounting to a total of 257 analysed individuals.

144

145 **Bouin's fixative and staining**

146 The bivalves selected for analysis for each size class were carefully dissected to keep the
147 visceral mass (gonad–digestive gland complex) and the gills associated. Entire specimens
148 extracted from the shell were fixed in Bouin's fixative for 48 h, then rinsed daily in ethanol
149 (70%) until discoloration of the tissue. After dehydration in an ascending ethanol series, the
150 whole soft tissue was then embedded in a Paraplast block and cut into serial transverse
151 sections (7-µm thick) along an anteroposterior axis, midway between the dorsal part of the
152 gills and the ventral groove. The sections were stained with Masson's trichrome (Gabe 1968):
153 bacteriocytes were stained pink or purple, depending on the individual, whereas mucocytes
154 were non-specifically stained light blue.

155 As several types of mucocytes exist, the identification of mucocytes was performed on some
156 individuals with Periodic Acid-Schiff-Alcian Blue (PAS-AB) staining. This method
157 specifically focuses on mucopolysaccharides, making acid-secreting mucocytes appear
158 intensely blue-stained, whereas bacteriocytes and ciliated zones appear weakly pink-stained
159 (Beninger et al. 2003).

160

161 **Image analysis**

162 The Masson's trichrome-stained sections of the whole tissue (i.e. visceral–gonadal mass
163 joined to both gills) were scanned with a NanoZoomer (MRI Platform, Montpellier) for
164 further image analysis of the gill tissue composition (see below) and sexual determination.
165 After microscopic examination of the visceral–gonadal mass, individuals were classified as
166 male based on the presence of spermatogonia or spermatozoid (Fig. 2d) or as female when
167 oogonia or oocytes were present (Fig. 2f). When any gametes were visible, the individuals
168 were classified as 'not determined'. In the gill analysis, three specific regions were
169 investigated: anterior ('Ant'), median ('Med') and posterior ('Post') regions (Fig. 2a).
170 For the gill tissue analysis, we used Definiens Developer 7.1 software (Definiens, Munich,
171 Germany) to quantify the proportions of the different parts of the gills. The ciliated zone was
172 included in the analysis as it is an integral part of the gill. First, 6 gill filaments were manually
173 outlined using 'Paint' free software to determine the total area of the analysed gill region in
174 order to standardize the gill surface area between individuals of varying sizes (See Electronic
175 Supplementary Material, ESM, Fig. S1). Then, Definiens was used to measure, in pixels, the
176 surface area of the two ciliated zones (CZ) and the central zone (ESM, Fig. S1a). In the
177 central zone, Definiens was used to build a set of rules to distinguish different types of cells or
178 spaces ('areas') in the stained sections: light blue for mucocytes (MC), pink/purple for
179 bacteriocytes (BC) and white (unstained) for lacunal/interfilamentar space (LIS) (ESM, Fig.
180 S1b). The different surface areas (BC, MC, CZ and LIS) were used to calculate the
181 proportions (as percentages) of bacteriocytes, mucocytes, ciliated zone and
182 lacunal/interfilamentar space in the gill tissue. These proportions were investigated in 239
183 *Loripes* individuals (out of the 257 collected), generating 647 gill images. This would have
184 yielded a total of 717 images (three gill regions – Ant, Med, Post – per individual), but some
185 images were not usable.

186

187 **Statistical analyses**

188 Descriptive statistics (mean, standard deviation, coefficients of variation) were calculated
189 using Excel (version 16.16.9). Spearman's correlations and Kruskal-Wallis mean comparison

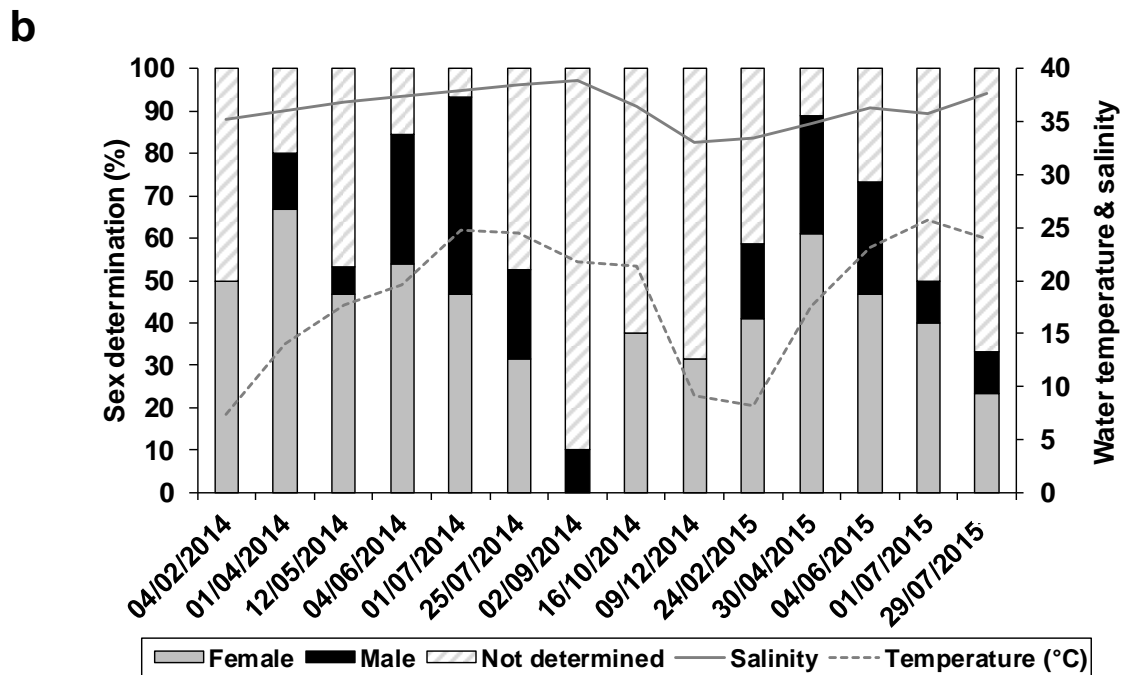
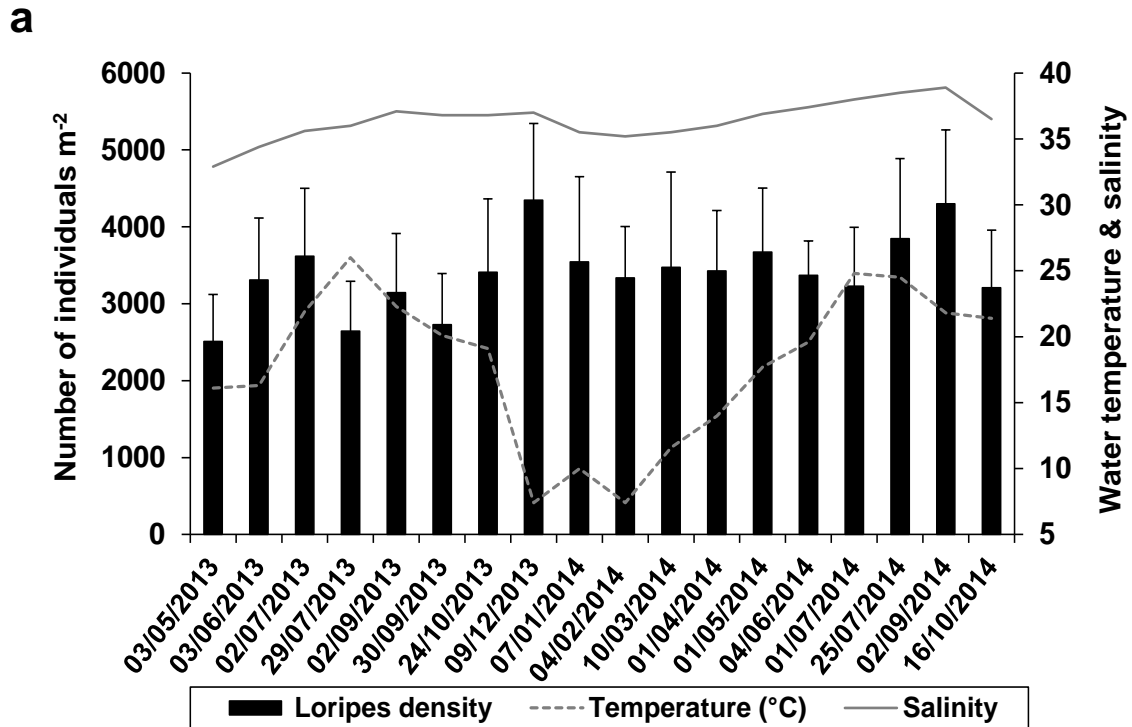
190 tests were calculated using XLSTAT software (Addinsoft 2019, Paris, France,
191 <https://www.xlstat.com>).

192

193 **Results**

194 **Population density and reproductive cycle**

195 From May 2013 to October 2014, the mean *Loripes* density in seagrass sediment was in the
196 order of 3395 ± 195 individuals m^{-2} , ranging from 2509 to 3445 (Fig. 1a).



197

198 **Fig. 1** Mean *Loripes* density in the Thau lagoon from May 2013 to October 2014 in relation to
 199 water temperature and salinity (a). Reproductive cycle based on sex determination throughout
 200 the sampling period (from February 2014 to August 2015) (b). Females were identified from
 201 oogonia or oocyte detection in the gonad, whatever the development stage; males were
 202 identified from spermatogonia or spermatozoa detection. The ‘not determined’ status
 203 corresponds to spent gonads after spawning (Seed 1969) or immature gonads.

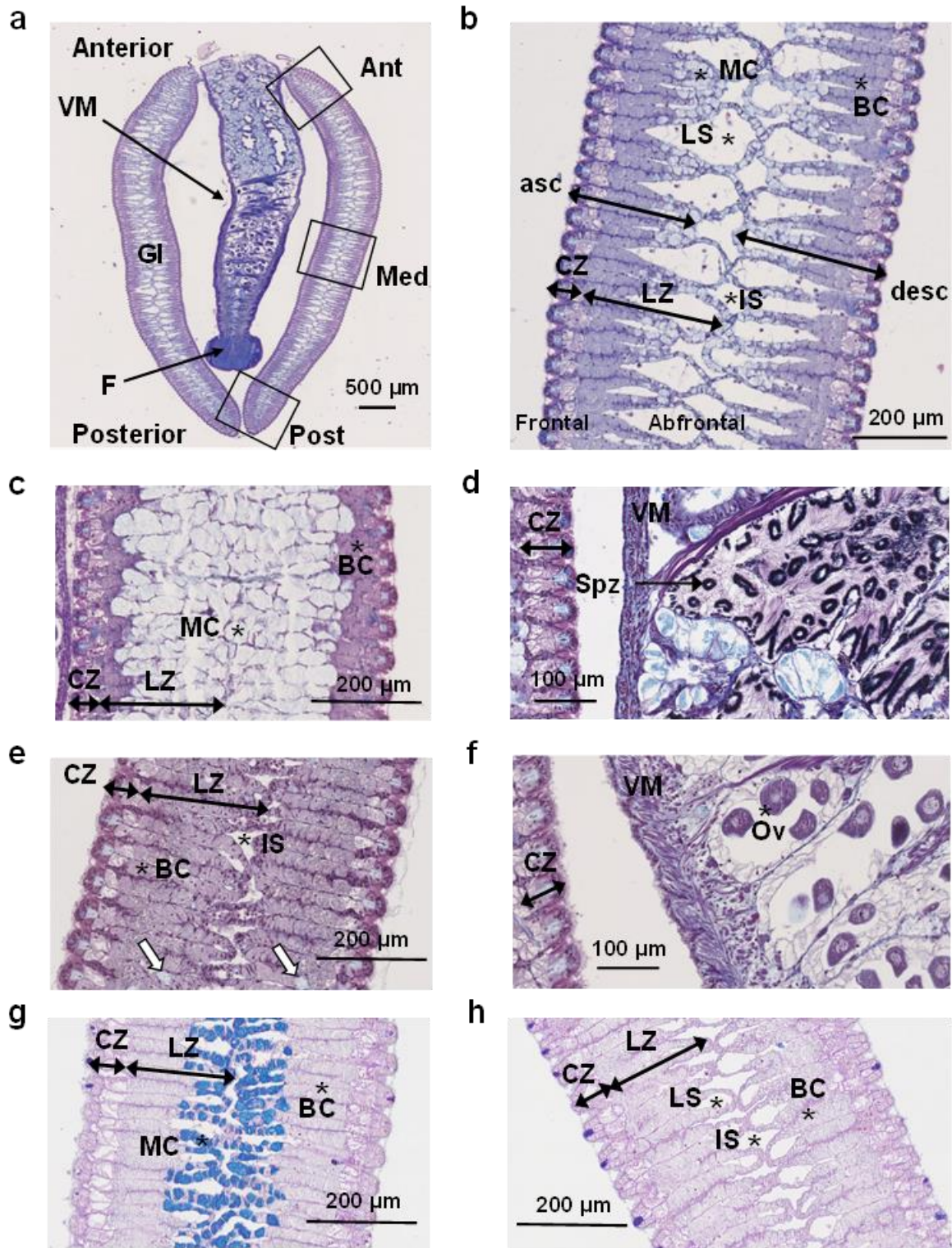
204

205 This density remained quite stable over the sampling period, whereas the water temperature
206 varied greatly, from 7.4 °C in winter to 26 °C in summer. Conversely, low variation was
207 observed for salinity in the same period (from 32.9 in May 2013 to 38.9 in September 2014).
208 To investigate the reproductive cycle of *Loripes*, sampling was performed from February
209 2014 to August 2015 on the three size classes used for histological investigations. For this
210 analysis, all size classes were pooled together, as even the smallest class included individuals
211 able to reproduce (van der Geest 2014). The distribution of sexually determined individuals
212 (see Fig. 2d for male and 2f for female) showed a clear annual gametogenesis cycle (Fig. 1b).
213 The percentage of sexually determined individuals reached a maximum in the beginning of
214 July 2014 and at the end of April 2015 (Fig. 1b). These periods were immediately followed by
215 a substantial decrease in the percentage of sexually determined individuals during summer
216 (from June-July to September), corresponding to individuals with spent gonads. This indicates
217 that spawning probably occurred in late spring/early summer. Personal observations in 2017
218 and 2018 confirmed the presence of egg masses (the consequence of spawning) in seagrass
219 beds at the beginning of July on the same site.

220

221 **Description of the gill tissue components**

222 The gills of *Loripes* consist of two demibranches, one located on each side of the visceral–
223 gonadal mass (Fig. 2a).



224

225

226 **Fig. 2** Light micrographs of *Loripes* tissue after Masson's trichrome (from **a** to **f**) and PAS-AB
 227 (**g** and **h**) staining. (**a**) Transverse section of a whole individual showing the visceral-gonadal
 228 mass (VM) and the foot (F), surrounded by both demibranch gills (GI). Three different
 229 regions of the gills were investigated for image analysis: the anterior (Ant), the median (Med)
 230 and the posterior (Post) regions. (**b**) Magnification of the median gill section showing the
 231 ascending ('asc') and descending ('desc') lamella, separated by the interfilamentar space (IS);

232 each lamella consists of a ciliated zone (CZ) (frontal position) and a lateral zone (LZ)
233 (abfrontal position). The lateral zone is composed of bacteriocytes (BC), mucocytes (MC) and
234 a lacunal space (LS). The variation in the gill histology between individuals is illustrated in
235 (b), (c) and (e). Rare mucocytes in (e) are indicated by white arrows. A close-up of the
236 visceral–gonadal mass with spermatozooids ('Spz'), typically organized in rings, is shown in
237 (d), and with oocytes in (f), which allowed sex determination. In (g) and (h), PAS-AB
238 staining shows mucocytes as blue cells (g), whereas bacteriocytes are weakly stained in pink
239 (g and h).

240

241 The digestive system and the gonads cannot be separated. Each gill is composed of an
242 ascending and a descending lamella, with a ciliated zone and a lateral zone (Fig. 2b). The
243 ciliated zone represents the gill's external surface, which is directly in contact with seawater
244 circulating in the pallial cavity. The lateral zone occupies most of the gill tissue; its
245 composition is highly variable between individuals. As shown in Fig. 2b, this lateral zone can
246 be composed of bacteriocytes and mucocytes in variable proportions. Large bacteriocytes can
247 be observed just under the ciliated zone, and thinner ones can be seen in the abfrontal part of
248 the lateral zone (Fig. 2b, 2e). As illustrated in Fig. 2b, mucocytes are generally located deep
249 inside the gill (in the abfrontal lateral zone), but in some rare cases they can totally invade the
250 gill tissue (Fig. 2c) to the detriment of bacteriocytes. More frequently, mucocytes are totally
251 absent from or rare in gill tissue (Fig. 2e). The type of mucocytes was determined with PAS-
252 AB staining of some individuals, with acid-secreting mucocytes identified in blue (Fig. 2g).
253 Deep inside the gill (the abfrontal part of the filaments), two types of spaces can be observed:
254 the interfilamentar space (IS), allowing the circulation of seawater throughout the gill from
255 the ciliated zone to the abfrontal zone, and the lacunal space (LS) between the basal
256 membrane of bacteriocytes or mucocytes. The lacunal space, which is dedicated to
257 hemolymph circulation, often has a loop-like structure and is also highly variable in volume
258 between specimens, as is the case for interfilamentar space.

259

260 **Quantification of gill tissue composition**

261 In order to quantify the different gill components described above and characterize their
262 variability, we pooled the data from individuals in all class sizes (between 15 and 30 adults
263 per month) and all gill regions (Ant, Med, Post) for the total sampling period (from February
264 2014 to July 2015). We calculated the mean value and standard deviation for each type of cell

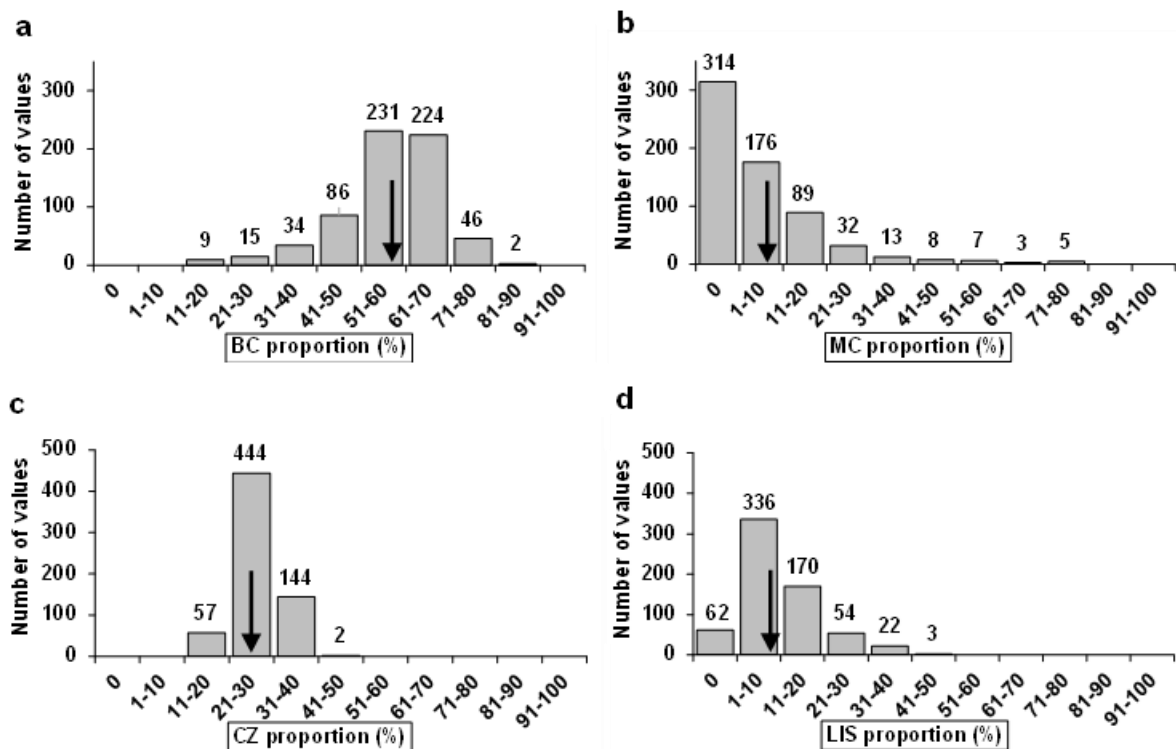
265 (bacteriocytes and mucocytes) and gill area (ciliated zone, interfilamental/lacunal space)
 266 (Table 1) and represented the data distribution for all gill components (Fig. 3).

267 **Table 1** Descriptive statistics relative to the proportions (%) of bacteriocytes, mucocytes,
 268 lacunal/interfilamentar space and the ciliated zone (N = 647) calculated over the total
 269 sampling period (from February 2014 to July 2015).

	Bacteriocytes	Mucocytes	Lacunal/inter- filamentar space	Ciliated zone
Mean value \pm standard deviation (SD)	56.4 \pm 11.5	7.6 \pm 12.6	9.8 \pm 8.5	26.2 \pm 5
Coefficient of variation (CV)	20	166	88	19

270

271 Image analysis revealed that bacteriocytes occupied on average $56.4 \pm 11.5\%$ (mean value \pm
 272 standard deviation) of gill tissue, with extreme values ranging from 11% to 83.2% (Fig. 3a,
 273 Table 1).



274

275 **Fig. 3** Data distribution of the proportion of bacteriocytes (BC) (a), mucocytes (MC) (b), the
 276 ciliated zone (CZ) (c) and lacunal/interfilamentar space (LIS) (d) in the gill tissue. The data
 277 was obtained by pooling the values from all individuals (small, medium, large) and all gill
 278 regions (Ant, Med, Post) from all sampling dates. The mean value is indicated by a black

279 arrow. The number above the bars corresponds to the exact number of values in that
280 proportional range.

281

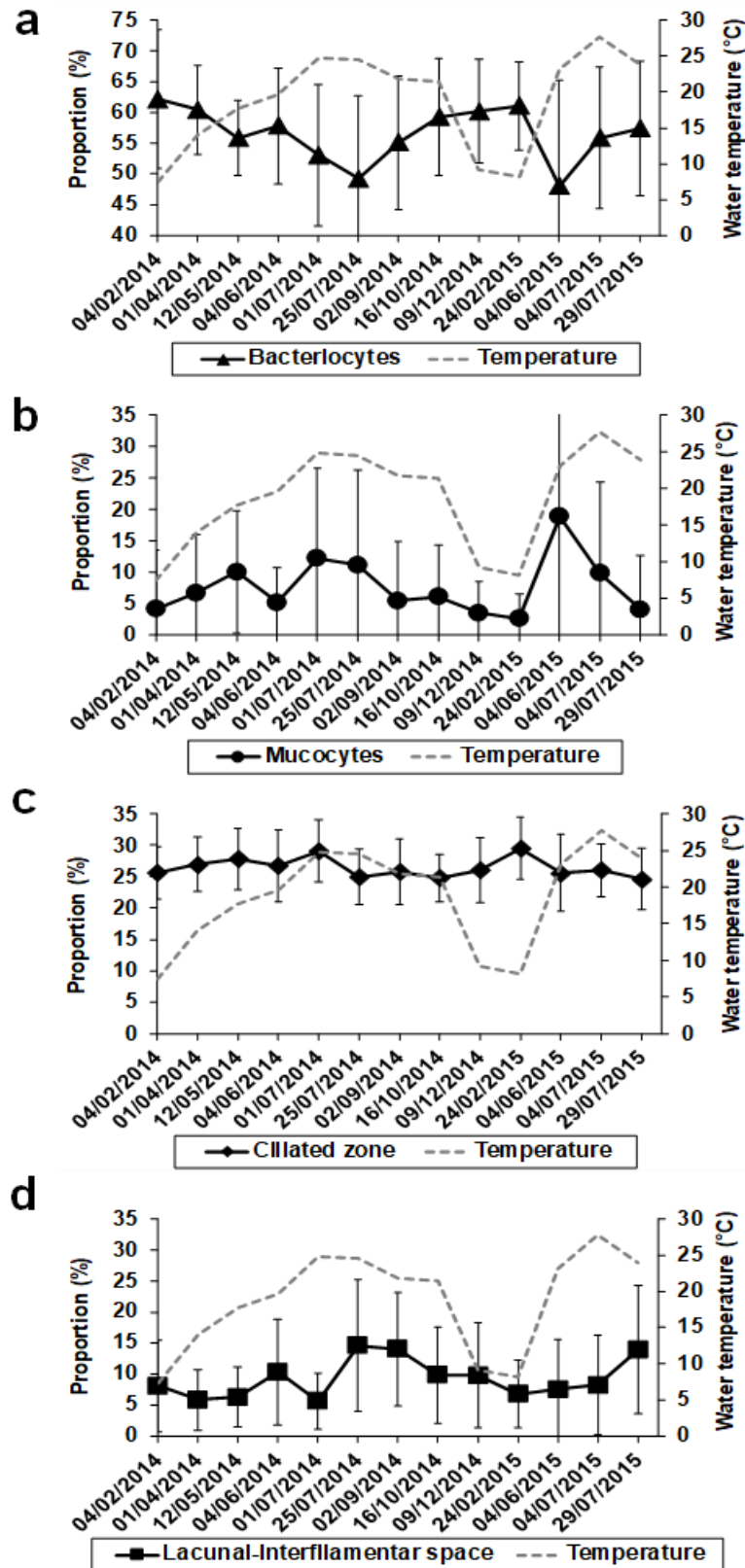
282 In approximately 85% of the gill tissue samples, the proportion of bacteriocytes fluctuated
283 between 40% and 70% (Fig. 3a). With a moderate CV value ($CV = 20\%$), bacteriocytes
284 represented a major and constant component in *Loripes* gills. The most variable gill
285 component was mucocytes. The mean proportion of mucocytes was $7.6 \pm 12.6\%$ of gill tissue,
286 but this masked significant variability ($CV = 166\%$). Fig. 3b shows that approximately half
287 the samples (314 out of 647) corresponded to individuals without mucocytes. Thus mucocytes
288 can be considered optional in the gill tissue of *Loripes*. For individuals with mucocytes, 41%
289 had a proportion lower than 20%. A few individuals showed an extremely high proportion of
290 mucocytes compared to the mean value, sometimes reaching 80% of the gill tissue. As an
291 illustration, the photo in Fig. 2c represents a gill with 70% mucocytes. The second largest
292 constant component was the ciliated zone, which accounted for $26.2 \pm 5\%$ of the gill tissue,
293 with limited variation ($CV = 19\%$) (Fig. 3c). The lacunal/interfilamentar space represented
294 $9.8 \pm 8.5\%$ (mean value) of the gill tissue, with significant variability ($CV = 88\%$). This space
295 was not a constant component of the gill, as around 10% of the values were equal to 0.
296 Around 80% of the values relative to the lacunal/interfilamentar space present in the gill were
297 in low proportions ($< 20\%$ of the gill tissue).

298

299 **Seasonal influence on gill tissue composition**

300 Over the sampling period, the data clearly showed that the average proportion of bacteriocytes
301 changed over time, ranging from $48 \pm 17.2\%$ to $62.2 \pm 11.3\%$ (Fig. 4a).

302



303

304

305 **Fig. 4** Change over time (from February 2014 to July 2015) in the proportion (%) of gill
 306 components: bacteriocytes (a), mucocytes (b), ciliated zone (c) and lacunal/interfilamentar
 307 space (d) in *Loripes* gills. Standard deviations are represented by vertical bars; for large
 308 standard deviation ranges, the lower bar could not be fully represented.

309

310 The highest values were observed in winter, whereas the lowest were observed during warm
311 periods (end of July 2014 and beginning of June 2015). The proportion of bacteriocytes in the
312 gill tissue was noticeably reduced as water temperature increased. Non-parametric tests
313 (Spearman's correlation) confirmed that the proportion of bacteriocytes was significantly and
314 negatively correlated with water temperature ($rs = -0.835$, $P = 0.001$) and with salinity (rs
315 $= -0.687$, $P = 0.012$).

316 Conversely, the proportion of mucocytes seemed to follow the trend in variation in water
317 temperature (Fig. 4b). Maximum values occurred at the beginning and end of July 2014 (11.2
318 $\pm 15.2\%$ and $12.2 \pm 14.3\%$) and at the beginning of June 2015 ($18.8 \pm 25.4\%$) when water
319 reached its maximum temperature (between $23.1\text{ }^{\circ}\text{C}$ and $24.8\text{ }^{\circ}\text{C}$). Spearman's correlation
320 tests showed that mucocytes were significantly and positively related with water temperature
321 ($rs = 0.621$, $P = 0.026$), indicating that they appeared in the gill during warm periods.
322 Moreover, the data showed that bacteriocytes and mucocytes were significantly and
323 negatively correlated ($rs = -0.808$, $P = 0.0001$), indicating that mucocytes appear in the gills
324 along with a simultaneous reduction in bacteriocytes. The proportions of the ciliated zone and
325 the lacunal/interfilamentar space did not show any correlation with temperature or indicate
326 any specific trend according to season.

327

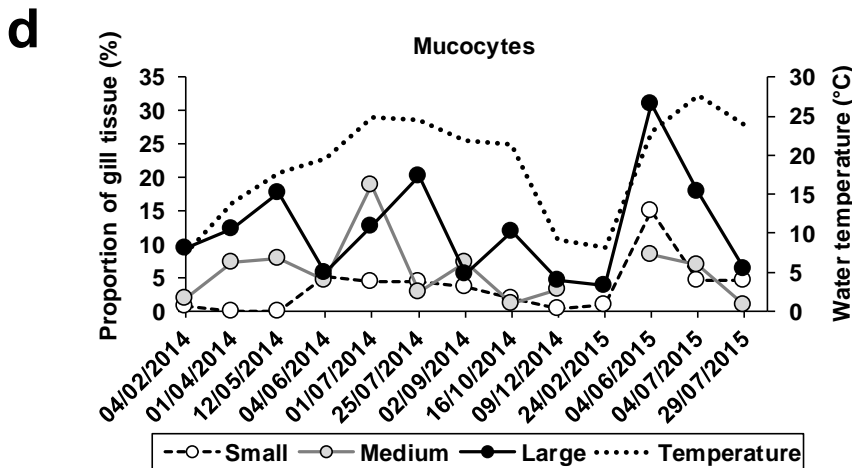
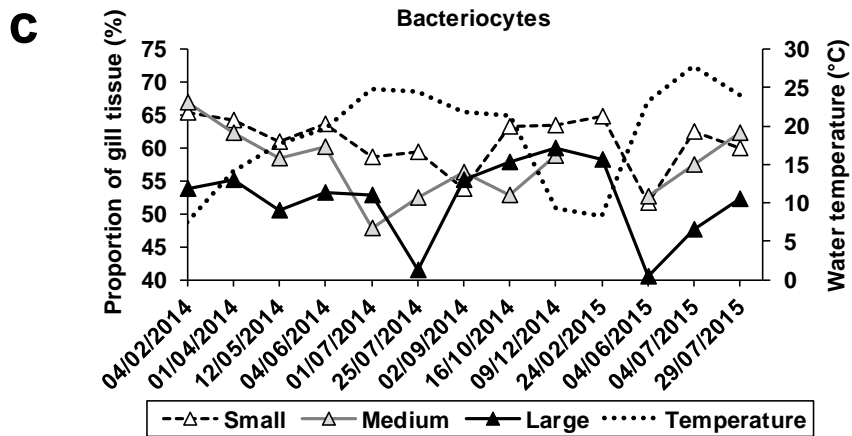
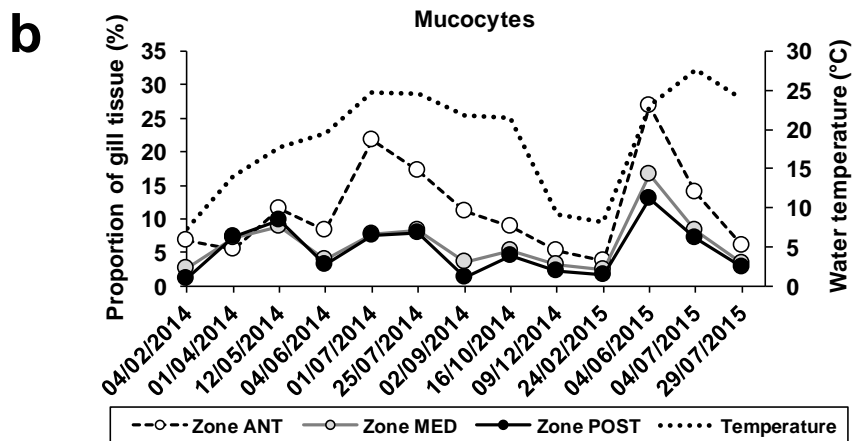
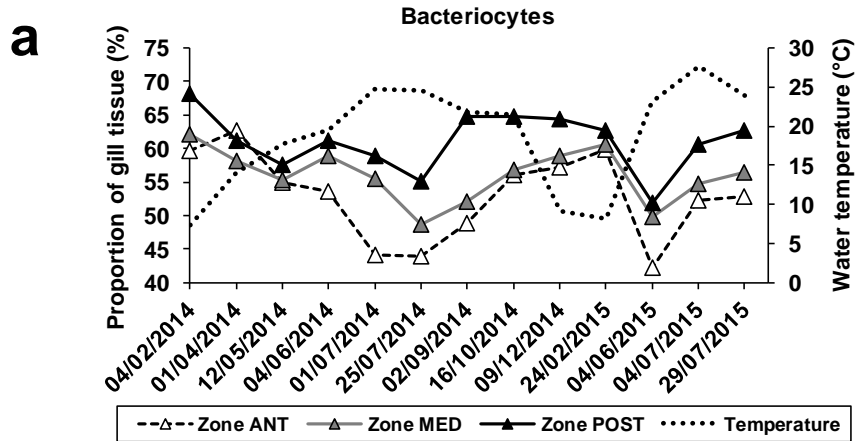
328 **Biological influence on gill tissue composition**

329 Among the biological factors that may influence the proportions of the different gill
330 components, we investigated the anterior, median and posterior regions of the gill, and the
331 size and sex of individuals.

332 **Influence of gill region**

333 Along the anteroposterior axis, all data considered and independently of season, the general
334 trend showed a significant increase in the mean proportion of bacteriocytes from the anterior
335 to the posterior region (ESM, Fig. S2a), while the proportion of mucocytes significantly
336 decreased (ESM, Fig. S2b), according to the Kruskal-Wallis test. Considering both the gill
337 region and the season, the results showed that the lowest proportion of bacteriocytes occurred
338 in the anterior part of the gills (44% in July 2014 and 42.3% in June 2015) when the water
339 temperature reached between 20°C and 25°C (Fig. 5a). In contrast, at the same periods, the

340 highest proportion of mucocytes (21.8% and 23.1%) occurred in the anterior region (during
341 early summer 2014 and late spring 2015 respectively) (Fig. 5b). Thus the influence of warm
342 temperatures on gill composition seen in Fig.4 is even more pronounced in the anterior part of
343 the gill. In contrast, the proportion of the ciliated zone remained constant throughout the
344 antero-posterior axis of the gill ($\approx 26\%$) (ESM, Fig. S2b), and the proportion of the
345 lacunal/interfilamentar space was found to be significantly higher in the median region of the
346 gill ($12.1 \pm 9.1\%$) (ESM, Fig. S2b), but was not correlated with water temperature, as stated
347 previously (Fig. 4d).



349

350 **Fig. 5** Change over time (from February 2014 to July 2015) of proportions (%) of
351 bacteriocytes (**a** and **c**) and mucocytes (**b** and **d**) in different gill regions (Zones ANT, MED,
352 POST) (**a** and **b**) and in different size classes of individual (Small, Medium, Large) (**c** and **d**).
353 Water temperature is represented by the dotted line.

354

355 **Influence of individual's size**

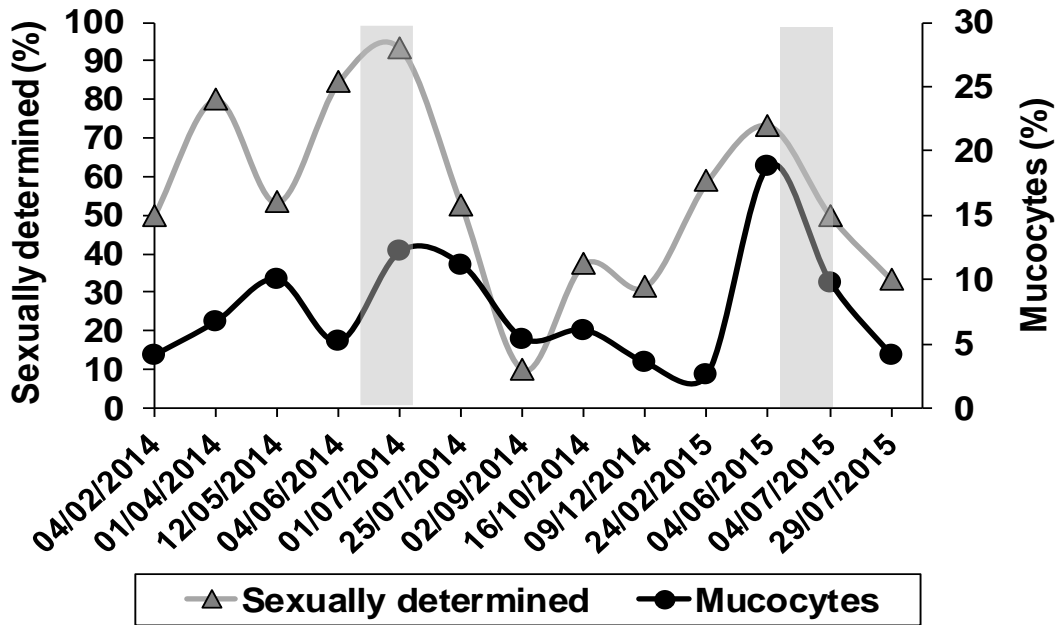
356 All the individuals examined were adults, but they ranged in size. The results showed that
357 independently of season, the proportion of bacteriocytes decreased significantly between
358 small ($60.9 \pm 9.5\%$) and large individuals ($52.6 \pm 11.9\%$) (ESM, Fig. S2c). This was also
359 confirmed through the temporal variation in bacteriocytes, with the greatest decrease
360 occurring in large individuals in late spring (41.4%) to early summer (40.6%) (Fig. 5c). An
361 opposite trend was observed for the proportion of mucocytes, which increased significantly
362 between small ($3.8 \pm 10.3\%$) and large ($11.4 \pm 14\%$) individuals (ESM, Fig. S2d). In terms of
363 temporal variation, the largest proportions of mucocytes were observed during late spring
364 (31.1%) to early summer (20.2%) in large individuals (Fig. 5d). Without considering season,
365 the highest proportion of the mean ciliated zone and the lowest proportion of
366 lacunal/interfilamentar space were found in small individuals (ESM, Fig. S2d).

367

368 **Influence of sex/reproductive status**

369 We also investigated gill tissue composition in relation to the reproductive status of
370 individuals; all data considered independently of season, no statistical differences were found
371 between females and males for any gill tissue components (ESM, Fig. S2e, S2f). Interestingly,
372 compared to sexually determined individuals, sexually undetermined individuals showed a
373 significantly higher proportion of bacteriocytes and a much lower proportion of mucocytes.
374 As this could suggest a possible relationship between the balance of mucocytes/bacteriocytes
375 and the reproductive cycle, we investigated the temporal variation, throughout the studied
376 period, in the percentage of sexually determined individuals (pooling together females and
377 males) and the percentage of mucocytes (Fig. 6). We found that variation in the proportion of
378 mucocytes followed, with a short delay, the trend of the reproductive cycle.

379



380

381 **Fig. 6** Change over time (from February 2014 to July 2015) in the percentage of sexually
 382 determined individuals and mucocytes. Grey areas correspond to estimated spawning periods
 383 according to personal observations of egg masses in the field in 2017 and 2018 at the
 384 Bouzigues site.

385

386 Spearman’s correlation tests were performed on this data, demonstrating, by shifting
 387 mucocyte data one month earlier, a positive correlation between sexually determined
 388 individuals and mucocytes ($r_s = 0.764$, $P = 0.006$). This indicates that the occurrence of
 389 mucocytes and the reproductive cycle are related.

390 **Discussion**

391 In this large field survey of a seagrass meadow in Thau lagoon near Bouzigues, we found a
 392 very stable, high density *Loripes* population throughout the year (mean of 3395 individuals m⁻²).
 393 A recent study revealed, however, that this density is spatially variable within Thau lagoon
 394 and is correlated with seagrass density and organic matter in the sediment (van der Geest et al.
 395 2020). In comparison, much lower *Loripes* density has been observed in Corsica (777 *Loripes*
 396 m⁻²) (Johnson et al. 2002) and in Mauritania (320–838 *Loripes* m⁻²); (Ahmedou Salem et al.
 397 2014). In our study, one major reproductive period was identified during late spring/early
 398 summer, with spawning estimated to occur in June/July, which is consistent with other areas
 399 (Brittany in France and Mauritania) (Johnson and Le Pennec 1994; Johnson and Fernandez
 400 2001; van der Geest et al. 2014).

401 Aside from the reproductive cycle, we also investigated seasonal variation in gill tissue
402 composition. The results showed that bacteriocytes are a key component of gill tissue (mean
403 value of 56.4%, with values ranging from 40–70%) and are always present, whatever the
404 season, the size or the sex of adult *Loripes*. This is in line with the value (55%) reported in
405 other studies (Herry et al. 1989; Johnson and Fernandez 2001; Pales-Espinosa et al. 2013b),
406 supporting the idea that symbionts are the main contributor (between 60% and 75%) to host
407 nutrition in lucinid clams via autotrophic pathways (Johnson et al. 1994; Rossi et al. 2013).

408 As our study examined a very large number of individuals, it also brings new insights about
409 the discrete occurrence of mucocytes at the population level. We found that mucocytes were
410 only present in half the individuals, in low proportions (mean value 7.6%), but with a large
411 range of variation between individuals. These results indicate that mucocytes can be
412 considered an optional and highly variable component of *Loripes* gill tissue, explaining why
413 they are mentioned as rare cells in the literature on *Loripes* and more generally in lucinids
414 (Herry et al. 1989; Liberge et al. 2001; review in Duplessis et al. 2004; Pales-Espinosa et al.
415 2013b). In comparison, the gill tissue of other lucinids (e.g. *Codakia* sp.) contains
416 bacteriocytes, but in lower proportion (30%), some rare mucocytes, and granular cells absent
417 from *Loripes* gills (Caro et al. 2009; Gros et al. 2012). Mucocytes are also mentioned in some
418 Thyasiridae bivalves, but without precise quantification (Dufour 2006). This gill tissue
419 variability in symbiotic bivalves probably corresponds to differences in host physiology and
420 modes of nutrition, potentially related to the environment. In future studies, it would be useful
421 to consider the gills of *Loripes*, and other symbiotic species more generally, not as composed
422 strictly of bacteriocytes, but as dynamic tissue with varying proportions of bacteriocytes and
423 mucocytes and potentially other cells.

424 Our findings also demonstrated a seasonal pattern in the dynamic of mucocytes and
425 bacteriocytes, with larger proportions of mucocytes in late spring/early summer (as water
426 reaches its maximum temperature). In Thau lagoon at this period, the main primary
427 production bloom occurs (Bec et al. 2005), and a possible hypothesis is that mucocytes may
428 be involved in the heterotrophic nutrition of the host as is the case for other bivalves; one of
429 the main roles attributed to the mucus secreted by mucocytes concerns particle-feeding
430 processes (Beninger and St Jean 1997). Another hypothesis concerns host immunity, as gills
431 and mucus are greatly involved in this function (Allam and Pales-Espinosa 2016; Yuen et al.
432 2019). Moreover, since 2008, massive spring mortality has been regularly recorded in oysters

433 in the Thau lagoon (Pernet et al. 2012), involving a community of pathogenic microorganisms
434 (Alfaro et al. 2019) that may trigger *Loripes*' defence systems.

435 Several arguments may support the first hypothesis that mucocytes could be involved in
436 particle processing. Indeed, *Loripes orbiculatus* is generally attributed a mixotrophic status,
437 with a significant part of chemoautotrophic nutrition (Schweimanns and Felbeck 1985; Herry
438 et al. 1989; Taylor and Glover 2006), but supplemented by heterotrophic feeding through the
439 ingestion of particulate organic matter (Le Pennec et al. 1988; Duplessis et al. 2004; Rossi et
440 al. 2013; van der Geest et al. 2014). A similar temporal fluctuation in symbiont abundance, a
441 proxy for bacteriocytes, was also observed in Thysirids according to season (Laurich et al.
442 2015). More recently, the postulate of the heterotrophic nutrition of *Loripes* has been
443 strengthened by the ability of this host's symbionts to digest complex polysaccharides (Yuen
444 et al. 2019), typically found in marine phytoplankton (Mühlenbruch et al. 2018). In
445 suspension-feeding bivalves, heterotrophic nutrition is related to the ability of the gills to
446 collect and process particulate organic matter via mucocytes' acid secretion from the anterior
447 part of the gill (Beninger and Dufour 1996; Beninger and St Jean 1997; Ward and Shumway
448 2004). Our data demonstrated a larger proportion of acid mucocytes (from PAS-AB staining)
449 in the anterior region of the gill, which Beninger et al. (2003) considers, given the type and
450 location, to be mucocytes obviously involved in particle capture. According to REPHY data
451 (2018), a spring phytoplankton bloom occurred in 2014 and 2015 at the Bouzigues site,
452 dominated both years by large diatom species (mainly *Chaetoceros*), as well as some smaller
453 benthic diatoms such as *Navicula*. As the increase in mucocytes observed in our study
454 coincided with these blooms, we cannot exclude the possibility that mucocytes could occur in
455 *Loripes* gills in order to benefit from this particulate nutrition source, after it sinks to the
456 sediment below (Souchu et al. 1998). Indeed, particulate food is known to be energetically
457 more valuable for symbiotic bivalves than chemoautotrophic nutrition (Le Pennec and
458 Beninger 2000). Small epiphytic diatoms (20µm), which cover the leaves of *Zostera noltii*, as
459 has been described in intertidal zones (Lebreton et al. 2009), could be another potential
460 particulate food source for *Loripes*, as diatoms have been found in the digestive system of
461 lucinids, among other organic particles (Le Pennec et al. 1988; Dando et al. 1986; Southward
462 1986; Herry and Le Pennec 1987; Duplessis et al. 2004). The high nutritional quality of
463 benthic diatoms, and microphytobenthos more generally, sustain the growth of non-symbiotic
464 benthic bivalves (Lebreton et al. 2011) and could also be part of the heterotrophic nutrition of
465 symbiotic species such as *Loripes*, considered a filter- and deposit-feeder. The low biomass

466 represented by microphytobenthos in *Z. noltii* seagrass beds (Lebreton et al. 2009) could still
467 be sufficient to fuel the supplemental heterotrophic nutrition of *Loripes*, as autotrophic
468 nutrition represents only 60–75% of host nutrition in lucinids (Cary et al. 1989).

469 Concerning the potential involvement of mucocytes in *Loripes* immunity, our field survey
470 was not designed to investigate this question, so we can only speculate on this point. The
471 diseases that regularly affect oyster spats in Thau lagoon when the water temperature rises to
472 16–17°C in spring include the herpes virus OsHV-1 μ var and a *Vibrio* community (see review
473 in Destoumieux-Garzon et al. 2020). Non-symbiotic benthic bivalves (e.g. the *Ruditapes*
474 clam, *Cerastoderma* cockle, *Haliotis* abalone, *Chlamys* scallop, etc.) are also affected by
475 harmful bacteria and viruses (Renault et al. 2001; Paillard et al. 2006; Arzul et al. 2017), but
476 as most studies to date have focused on farmed bivalves (Travers et al. 2015), as far as we are
477 aware, information is scarce about pathogenic agents that could infect *Loripes* (Johnson and
478 Le Pennec 1995). Nevertheless, the sediment in Thau lagoon is assumed to be a reservoir of
479 *Vibrio* species that are pathogenic for oysters during winter and, to a lesser extent, during
480 spring (Lopez-Joven et al. 2018). Even trapped in the sediment, these *Vibrio* species may also
481 trigger an immune response in *Loripes* through the appearance of mucocytes in the gills and
482 mucus secretion, yet without causing significant mortality, since our data found *Loripes*
483 density to be stable over the years. The gene expressions involved in immunity response to
484 bacteria has indeed been recently evidenced in the transcriptome of the *Loripes* gill (Yuen et
485 al. 2019). Further studies could aim to correlate transcriptomic and histological data in order
486 to clarify the role of mucocytes.

487 Our findings also highlighted that mucocytes mainly occur in large adults, and that their
488 proportion is positively correlated with water temperature and with the sexual maturation of
489 *Loripes*. This suggests that the factors underlying mucocyte emergence are related to
490 metabolic demands in line with gonadal maturation in adults able to reproduce. Johnson and
491 Fernandez (2001) noted a correlation between gametogenesis and symbiont lysis inside
492 bacteriocytes in *Loripes*; they suggested that the supplemental energy demands related to
493 gametogenesis could be sustained by ‘heterosynthetic metabolites’. Indeed, high-nutrition
494 food is required to ensure gonadal development in molluscs (Bayne and Newell 1983; Hilbish
495 and Zimmerman 1988), as well as favourable temperatures (Morgan et al. 2013). The diet of
496 other non-symbiotic benthic bivalves also varies according to the biological stage of the
497 species (from juvenile to adult) (Kang et al. 1999), possibly related to reproduction, and
498 according to the seasonal availability of microphytobenthos as a heterotrophic source of

499 nutrition (Kang et al. 2006). The beneficial effect of heterotrophic nutrition to ensure gonadal
500 maturation is also valuable for other invertebrates such as corals (Séré et al. 2010). If *Loripes*
501 mucocytes effectively contribute to particle nutrition, that would suggest that the small adults
502 in our study rely more on autotrophic nutrition and then shift to a higher amount of
503 heterotrophic nutrition, as seen in larger adults, to sustain reproduction during spring. This
504 hypothesis is in line with recent C and N stable isotope ratio findings on *Loripes*, which show
505 an increasing level of autotrophy from April to October (Cardini et al. 2019). These authors
506 suggest that host nutrition relies on heterotrophy during spring and then turns mainly to
507 chemoautotrophic nutrition during autumn. Similar variations in the C and N stable isotope
508 ratios between seasons were also reported in the same species by Carlier et al. (2007) in the
509 south of France (Lapalm lagoon) and in Mauritania (van der Geest et al. 2014). Unfortunately,
510 during our survey we did not measure phytoplankton in the water column, or particulate
511 organic carbon or sulphide levels in the sediment, so we could not correlate the proportion of
512 bacteriocytes versus mucocytes to these parameters. However, the sulphide level measured in
513 Thau lagoon during the summer of 2016 remained close to zero (van der Geest et al. 2020),
514 whereas organic matter is in theory high in late spring/early summer at the sediment surface
515 (Rossi and Gili 2007; Lebreton et al. 2009). This is in line with the generally low values of
516 sulphide found in lucinid habitats (Cary et al. 1989).

517 Based on the previous arguments, we posit that mucocytes, given their correlation with water
518 temperature and the size and reproductive status of the host, contribute to the host's
519 heterotrophic nutrition, without ruling out a potential role in host immunity. Isotopic ratio and
520 transcriptomic data (Carlier et al. 2007; van der Geest et al., 2014; Cardini et al. 2019; Yuen
521 et al. 2019) helped both to throw light on these findings and confirm them. The balance
522 between bacteriocytes and mucocytes, varying seasonally to sustain reproduction, give
523 histological support for the well-known mixotrophic status of *Loripes*, which shifts from
524 autotrophy to heterotrophy. The low proportion of mucocytes in *Loripes* gill composition
525 found in our study might be sufficient to supplement the host's autotrophic nutrition (60–
526 75%) with heterotrophic nutrition. A mixotrophic diet might represent a strategy for
527 symbiotic bivalves living in shallow water environments to benefit from variable nutrient
528 resources (sulphides, particulate organic matter, etc.). For other filter- and deposit-feeders
529 such as Cnidarians, Sponges, Ascidiarians, and some bivalve Molluscs, another strategy, called
530 the 'dormant state', is adopted to cope with the variability in composition and availability of

531 particulate organic matter at the sediment surface during spring and summer (Ribes et al.
532 1999; Coma and Ribes 2003).

533 By extrapolating our results throughout the lifetime of a *Loripes* bivalve, we suggest that
534 before being able to reproduce, this species relies on the symbionts hosted in bacteriocytes for
535 its nutrition by ‘milking’ (direct carbon transfer) (Schweimanns and Felbeck 1985; Herry et
536 al. 1989; Yuen et al. 2019) and ‘farming’ (host digestion of the symbionts), as has been
537 described for other lucinids (Caro et al. 2009; Elisabeth et al. 2014; König et al. 2015). Then,
538 when sexual maturation occurs in larger individuals, we posit that this triggers an annual
539 cycle of histological gill variation in relation to gametogenesis (summarized in ESM, Fig. S3).
540 During spring, a regression in bacteriocytes occurs along with the appearance of mucocytes,
541 to allow particle feeding during phytoplankton blooms, in order to sustain the metabolic
542 demands related with reproduction through heterotrophy. Then immediately after spawning in
543 June/July, host nutrition rapidly returns to a standard symbiotic and autotrophic metabolism
544 from July to February, with a dominance of bacteriocytes in the gill tissue (ESM, Fig. S3).
545 This flexible metabolism is of particular importance in the context of global warming, with its
546 potential increase in the frequency of extreme environmental conditions.

547 **Conclusion**

548 In this field survey, we quantified the occurrence of mucocytes and their trade-off with
549 bacteriocytes in the gill tissue of the lucinid species *Loripes orbiculatus*. Mucocytes occur in
550 low proportions in the gill tissue, except during spring in large adults. Our findings could
551 allow new insights into the biological and environmental drivers underlying this gill
552 plasticity. The reproductive cycle was identified as the main biological factor triggering
553 mucocyte appearance, coinciding with the period when primary production is high. This is the
554 first field study providing histological support to describe the biological traits and
555 environmental conditions underlying the ‘mixotrophic diet’ of this species – and potentially
556 that of other lucinids. The high variability in histological gill composition between individuals
557 and according to season could perhaps explain the variability of stable isotope measurements
558 (Cardini et al. 2019; van der Geest et al. 2014, 2020). In future studies of lucinids and other
559 symbiotic species, a large sample of individuals of the same size would be valuable. To
560 further investigate the role of mucocytes, transcriptomic investigations could be conducted on
561 individuals with and without mucocytes to compare their profiles. The microbiome of gill
562 tissue could also be investigated in small versus large individuals, as gill-secreted mucus may

563 trap and host heterotrophic bacteria from the environment that could contribute to the
564 heterotrophic nutrition of the host as part of its ‘holobiont’.

565

566

567 **Compliance with ethical standards**

568 **Conflicts of interest** All authors declare that they have no conflicts of interests.

569 **Ethical approval** All applicable international, national, and/or institutional guidelines for the
570 care and use of animals were followed.

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572

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579 **Author contributions** This study was designed by AC, CR; the field work was carried out by
580 AC and CR; CR, EG, SH, JLC and AC collected the data; CR and CS contributed to the software
581 development; MT analysed the data; AC, CR, MT and JLC wrote the manuscript. All authors
582 read and approved the final manuscript.

583 **Data availability** The datasets collected and analysed during the study are available from the
584 corresponding author.

585

586

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