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1 **Metabolomics Based on UHPLC-QToF- and APGC-QToF-MS reveals metabolic pathways**
2 **reprogramming in response to tidal cycles in the sub-littoral species *Mymachlamys varia* exposed**
3 **to aerial emergence**
4

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10

11 **Abstract**

12 *Mimachlamys varia* is a sub-littoral bivalve encountered from Norway to the Mediterranean Sea,
13 which lives mostly bissally attached to rocks. During the low tide period, *M. varia* individuals, located
14 highest on the shore, may experience short time of aerial exposure and face a low availability of
15 oxygen. Here we report a comparative metabolomic profiling of gill samples of *M. varia* obtained by
16 both LC-QToF and APGC-QToF mass spectrometry, to analyze metabolic changes occurring during
17 emersion in comparison with immersion. Scallops were grown in aquaria with a simulated intertidal
18 environment mimicking short-duration air exposure that they might experience during extreme tides:
19 alternating 2h emersion and 10h immersion. Our results show a switch from aerobic to anaerobic
20 metabolism after only two hours of emersion, with the resort to different pathways: glucose-lactate,
21 glucose-succinate and aspartate-succinate pathways. Furthermore carnitine-conjugated metabolites
22 were found to accumulate during emersion, as well as urate. The level of tyrosine on the contrary was
23 found to increase. These findings indicate a complex metabolic reprogramming that occurs after a two
24 hours emersion period and upon re-immersion. Furthermore, *M. varia* is used as sentinel species in
25 pollution biomonitoring, through the assay of biomarkers to evaluate the effects of pollutants. Here we
26 show that emersion induces a significant decrease of superoxide dismutase activity, an enzyme
27 developed by bivalves to face oxidative stress and used as biomarker. These findings have to be taken
28 into account to normalize sampling during campaigns of environmental monitoring, by taking *in situ,*
29 as far as possible only immersed individuals.
30

31 **Keywords:** anaerobic, APGC-QToF mass spectrometry, hypoxia, intertidal, LC-QToF mass
32 spectrometry, metabolomics, *Mimachlamys varia*, PLS-DA.

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36

37 **Introduction**

38 *Mimachlamys varia*, commonly known as the variegated scallop, is a marine bivalve in the family
39 *Pectinidae*. It has a wide distribution from southern Norway, along the western coasts of the British
40 Isles, France, and the Iberian Peninsula and throughout most of the Mediterranean Sea (Shumway and
41 Parsons, 2016). This sub-littoral species occurs at depths ranging from very low intertidal to 100 m. It
42 is mostly byssally attached to rocks or among rough ground or more rarely free-living (Duncan et al.,
43 2016). During the low tide period, *M. varia* individuals attached in the low intertidal area, but located
44 at the highest positions on the shore, may experience short time of aerial exposure. During this period
45 of emersion, they have to face a low availability of dissolved oxygen, as they have only a small
46 reserve of seawater inside their two-part shells. Contrary to oysters and mussels, variegated scallops
47 are unable to remain tightly closed for extended periods of time. In the absence of danger, their shells
48 are therefore usually held slightly open, whether they are in or out of the water. This raises several
49 questions: is the dissolved oxygen in the seawater inside the shells sufficient to allow for breathing
50 during this short period of emersion? Is the air breathing capacity of scallops sufficient to maintain
51 aerobic metabolic pathways? Little is known about the air-breathing capacity of scallops upon
52 emersion (Shumway and Parsons, 2016), nor about their ability to shift towards an anaerobic
53 metabolism during the short emersion periods they undergo. This resort to anaerobic energy
54 production is common among intertidal bivalves upon emersion. Indeed they cannot meet their oxygen
55 requirements in air, due to inadequate gas uptake or delivery mechanisms and the danger of
56 desiccation when exposing moist surface. The result is that many inhabitants of the marine intertidal
57 area, such as mussels and oysters, undergo a number of physiological and metabolic changes
58 associated with alternating periods of immersion and emersion. These changes include a shift from
59 aerobic to anaerobic metabolism, as scallops close shell valves to prevent desiccation and undergo a
60 sharp decrease of dissolved oxygen availability (Akberali and Trueman, 1985; Connor and Gracey,
61 2012; De Zwaan and Wijsman, 1976; Dudognon et al., 2013; Isani et al., 1995; Shick et al., 1986).
62 The aim of this paper is thus to provide a global insight into the metabolic changes occurring during
63 short aerial emergence in comparison with periods of immersion, for the particular species
64 *Mimachlamys varia*. This will help in a better understanding of physiological processes in scallops
65 upon emersion.

66 In addition to providing insight into this physiological aspect for *Mimachlamys varia*, this study may
67 contribute meaningfully to improve ecotoxicological studies using this same marine organism. Indeed,

68 due its relative abundance along the coasts and also because this species has been shown to have a
69 very elevated pollutants incorporation and retention capacity, it has been proposed as a potential
70 ecotoxicological biomonitoring species for the marine environmental watch (Metian et al., 2009a,
71 2009b; Milinkovitch et al., 2015). However, during campaigns of environmental monitoring using *M.*
72 *varia* as sentinel species, samples of these scallops are taken *in situ* on the shore at low tide at high
73 tidal coefficient, indifferently emerged or still immerged and biomarkers are assayed to evaluate the
74 sub-lethal effects of pollutants (Breitwieser et al., 2018, 2016). In order to provide normalized
75 sampling methods, during environmental biomonitoring using biomarkers in *M. varia*, it is important
76 to exactly know how metabolism of *M. varia* individuals living in the low intertidal is modified during
77 emersion period. Indeed these metabolic changes are likely to lead to confounding factors and to
78 increase the variability of biomarker responses. For example, it was shown that hypoxia induced
79 tissue-specific responses in both antioxidant and immune systems in mussels (Nogueira et al., 2017).
80 In order to reveal new insight into the metabolic adaptations to emersion of *M. varia*, we grew
81 scallops in aquaria with a simulated intertidal environment and a metabolomics study was performed
82 during six complete tidal cycles. These experimental conditions were chosen to mimic short-duration
83 air exposure that might be experienced by variegated scallops, located highest on the foreshore, during
84 extreme spring and autumn tides: alternating 2h air exposure and 10h immersion. A comparative
85 metabolomics study was performed in gills samples, using both LC-QToF and APGC-QToF mass
86 spectrometry, in order to provide a temporal overview of the relationship between tidal cycles and
87 metabolome changes. Besides the effect of these tidal cycles on metabolome, the variation of SOD
88 (superoxide dismutase) activity was measured all along the experiment. This enzyme is a part of the
89 enzymatic antioxidant system developed by bivalves to face oxidative stress. It is considered as a
90 precocious biomarker linked to this particular stress (Valavanidis et al., 2006) and it is used to monitor
91 and evaluate the possible health impairment in marine organisms, following for example acute or
92 chronic pollution (Breitwieser et al., 2018, 2016).
93
94
95 **Materials and Methods**
96
97 *Animals and experimental design*
98 *M. varia* of 4 to 5 cm length were collected in the Pertuis Charentais (France) in December 2016. They
99 were then maintained in aquaria in the laboratory at a constant temperature of 15°C. Food as liquid
100 algal culture (Shellfish Diet 1800®, Reed Mariculture, Campbell, CA, USA) was added to the water.
101 Intertidal conditions were simulated using water pumps that filled and emptied the aquaria with natural
102 seawater. Once a day, at the end of low tide period, the seawater was totally replaced by filling the
103 aquaria with fresh seawater. A tidal regime of alternating periods of 10 h in and 2 h out of seawater

104 was established. This last duration corresponds to a maximal emersion time endured by *M. varia*
105 individuals living in the low intertidal area. Low tides occurred from 8:30 to 10:30 A.M. and 8:30 to
106 10:30 PM. A light-dark cycle was also programmed with periods of darkness occurring from 8:00 PM
107 to 8:00 AM (Figure 1). Scallops were acclimated during four weeks to alternating high and low tides,
108 before sampling. Samples were collected 0.25 h before the change in tidal episode (at 8:15 AM, 10:15
109 AM, 8:15 PM and 10:15 PM) during 3 days corresponding to 6 total tidal cycles. The first sampling
110 occurred at 8:15 AM. Nine individual scallops were collected at each time point. Immediately after
111 sampling, gill tissue was dissected from each individual, drained on absorbent paper and put in
112 cryovial on ice. Gill tissues from three individuals were pooled, snap-frozen in liquid nitrogen and
113 stored in liquid nitrogen.
114
115 *Total protein concentration and SOD activity: sample processing and assays*
116 For each pool of three individuals, 100 mg of gills were collected after dissection and mixing. They
117 were then homogenized in ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm). The
118 homogenates were centrifuged at 12,500g at 4 °C for 15 min and the supernatants were used for
119 protein and SOD activity assays.
120 Total protein concentrations were determined using an adaptation of the BCA Kit method
121 (Bicinchononique Acid Kit, Sigma Aldrich). The kit contained bovine serum albumin (BSA) as a
122 standard and involved the reduction of alkaline Cu^{2+} by proteins (Smith et al., 1985) at absorbance 562
123 nm using a spectrofluorometer (SAFAS Flx-Xenius).
124 SOD activity was assessed in the homogenate fractions using the method developed by Paoletti et al.
125 (1986). The assay, involving EDTA, $MnCl_2$ and mercaptoethanol, measures the decrease of
126 nicotinamide adenine dinucleotide (NADH) oxidation. This inhibition of oxidation was monitored at
127 340 nm (using SAFAS Flx-Xenius spectrofluorimeter) and is a function of SOD activity. Fifty percent
128 inhibition of oxidation corresponds to one unit of SOD. The results are presented in UI of SOD/mg of
129 proteins. Statistical analyses were carried out using R language (v. 3.1.2, R Core Team, 2016).
130 Normality was first tested on residuals using Kolmogorov-Smirnov tests and homogeneity of
131 variances was assessed using Bartlett tests. For SOD values, tides (low and high) were compared at
132 two times per day for each day, using One-Sample t-tests.
133
134 *Sample preparation for metabolomics study*
135 The efficiency of the following technique, inspired by a metabolomics study in Phycotoxines
136 laboratory (Ifremer, Nantes, France), has previously been established (Mondeguer et al., 2015).
137 Samples were thawed on ice, homogenized by manual grinding with a mini pestle and then crushed
138 with a homogenizer T 10 basic Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Germany) at low
139 speed. Each sample was precisely adjusted to 1g and 100mg of homogenized sample were also put

140 aside in a separate tube in order to perform SOD activity measurement. Tubes were then stored at -
141 80°C for one night before extraction procedure.
142 Samples were then subjected to a triple solvent extraction protocol by successively using acetone
143 twice and methanol. For each extraction, the following protocol was used: addition of 2.5 mL of
144 solvent to the sample, resuspension and dispersion with homogenizer T 10 basic Ultra-Turrax,
145 agitation 140 rpm 10 minutes, centrifugation at 3000 g during 5 minutes, removal of supernatant.
146 Ultra-Turrax was rinsed in the solvent of the next step. The three supernatants were then pooled, put at
147 4°C and subjected to centrifugation at 3000 g during 5 minutes. The supernatant was recovered and
148 dried with a stream of nitrogen at 30°C until reduction of the volume to 50% and at 50°C until
149 complete drying. The dry extract was finally re-suspended with 2 mL methanol/water 20/80 and stored
150 at -80°C prior filtration with 0.2 µm filters and LCMS analysis.
151
152 *LC-QToF MS analysis of metabolites*
153 The analysis of metabolite compounds in gills was performed by ultra-high performance liquid
154 chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). Analyses were
155 carried out using an UHPLC system "Acquity UPLC H-class" (Waters, Milford, USA) coupled to a
156 high resolution mass spectrometer "XEVO-G2-S Q-TOF" equipped with an electrospray ionization
157 source (Waters, Manchester, England). The UHPLC system was formed by a quaternary pump
158 (Quaternary Solvent Manager, Waters) and an automatic injector (Sample Manager-FTN, Waters)
159 equipped with a 10 µL injection loop. 5 µL of the samples were injected in a column "Acquity UPLC
160 HSST3" (Waters) (2.1 × 150 mm, 1.7 µm), and the products were eluted at a flow rate of 200 $\mu L.min^{-1}$
161 using a gradient composed of solvents A (water/formic acid 100/0.001 (v:v)) and B (acetonitrile/
162 formic acid 100/0.001 (v:v)), according to the following procedure: 0–3 min, 100% A; 3–8 min 0%–
163 50% B ; 8–13 min 50% B; 13–20 min 50–95% B; 20–30 min, 95% B, 30-31 min 95-0% B, 31-36 min
164 100% B . During the analysis, the column and the injector were maintained at 25°C and 7°C,
165 respectively. The analyses were performed in positive and negative ionization mode with MS^E
166 function in a centroid mode. The MS parameters was applied in the ESI source for the two ionization
167 mode were: source temperature 120°C, desolvation temperature 500°C, gas flow-rate of the cone 50
168 $L.h^{-1}$, desolvation gas flow-rate 800 $L.h^{-1}$, capillary voltage 2.5 kV, sampling cone 130 V and source
169 compensation 80 V. The instrument was adjusted for the acquisition on a 50–2100 m/z interval, with a
170 scan time of 0.15 s.
171 The mass spectrometer was calibrated before analysis using 0.5mM sodium formiate solution and the
172 Leucine Enkephalin (M = 555.62 Da,1 ng. μL^{-1}) was used as a lock-mass.
173 Compound identification was performed by matching to a database of authentic compound standards.
174 Metabolites were finally expressed in relative abundance, which was calculated for each metabolite by
175 dividing the abundance in each sample by the median abundance across all the samples.
176

177 *Quality Control*
178 A pool sample was prepared by combining 100 µL of each gills tissue extract. The pool sample was
179 divided into several vials that were used as quality-control samples (QCs) and regularly injected
180 within samples (one injection every five samples) to ensure analytical repeatability. Samples of gills
181 tissues extracts were measured in randomized order to avoid any possible time-dependent changes in
182 LC/MS analysis. Blanks were prepared with the last extraction solvent and injected at the beginning
183 and at the end of the samples sequence. These blanks allowed the subsequent subtraction of
184 contaminants or components coming from the extraction solvent.
185
186 *Chemicals*
187 Acetonitrile, Methanol, Acetone with HPLC grade purity were acquired from Carlo Erba. Water was
188 prepared using a Milli-Q reagent water system. Succinate, malate, uric acid, aspartate, alanine, citrate,
189 glutamate, propionylcarnitine , isovalerylcarnitine, isobutyrylcarnitine were purchased from Sigma-
190 Aldrich.
191
192 *Sample and standards preparation for APGC-QToF MS*
193 20 µL of samples prepared for metabolomics studies as described above and QC or of 10 ng μL^{-1}
194 standard solutions were dried under nitrogen at 40°C in vials without inserts. For methoximation–
195 trimethylsilylation (MeOx–TMS), 20 µL of a 20 mg mL^{-1} methoxylamine hydrochloride in pyridine
196 solution were added to dissolve the dried extract. The vials were then vortex mixed for 15 s and heated
197 at 80°C for 15 min in derivatization block. Vials were the removed from the block and 20 µL of
198 MSTFA (N-methyl-N-(trimethylsilyl trifluoroacetamide)) was added to the samples and QC, vortex
199 mixed for 15 s again and heated at 80°C for 15 min in the block. Vials were removed from the block
200 and allowed to cool for 5 min. They were then centrifuged at 2000 rpm for 5 min. Supernatants were
201 transferred to autosampler vials using pipet with filter tips.
202
203
204 *APGC-QToF MS analysis of metabolites*
205 Analyses were carried out using an Agilent GC 7890A (Agilent Technologies, Santa Clara, CA)
206 coupled to a Waters Synapt G2-S ToF (Waters Corporation, Manchester, UK), equipped with APGC
207 (atmospheric pressure gas chromatography) ionization. A DB5-MS (Agilent Technologies) analytical
208 column (30 m × 0.25 mm i.d. x 0.25 µm film thickness consisting of 5% phényl-diméthylpolysiloxane)
209 was used. The carrier gas was helium; it was used at constant flow equal to 1 mL min^{-1}. The
210 temperature program for the gas chromatography was as follows: initial temperature 120°C for 0.5
211 min, increase of temperature by two different rates: 8°C.min^{-1} up to 280°C at 22 min and 20°C.min^{-1}
212 up to 300°C, then temperature was held at 300 °C for 5 min. Total time for analysis was 28 min. The
213 injector temperature was 250°C. Injection was performed in the split mode (1/50) and the injection

214 volume was 2µL. The parameters for APGC were: Corona current 1.3µA, probe temperature 20°C,
215 source temperature 150°C, sampling cone 30V (different sampling cone voltages (from 10 to 40 V)
216 were tested and 30 V was retained), source offset 80V, cone gas flow 170 L/Hr, auxillary gas flow 220
217 L/Hr. The instrument was adjusted for the acquisition on a 50–700 m/z interval, with a scan time of
218 0.5 s.
219
220 *Statistical analysis*
221 Data were obtained and treated as ion peak intensity after conversion of raw spectra (.RAW) to
222 .NetCDF format, compatible with Galaxy software, using "Databridge" software (Waters).
223 Metabolites data were processed on the online and freely available Workflow4Metabolomics (W4M)
224 platform (http://workflow4metabolomics.org) for data pretreatments and analyzed with the web server
225 MetaboAnalyst 4.0 (www.metaboanalyst.ca) for multivariate analyses processing: the data were
226 normalized and log-transformed, and Pareto scaling was applied prior to multivariate analyses. The
227 data were analyzed using both unsupervised (Principal Component Analysis, PCA) and supervised
228 (Projections to Latent Structures Discriminant Analysis, PLS-DA) methods. Unsupervised method
229 allowed to detect general spectral trends and natural clustering between samples justifying the PLS-
230 DA model reliability. It also highlighted the potential sample outliers. The supervised method is a
231 multivariate regression and prediction based on the separation between two classes: high and low tide
232 samples. It was used to select the metabolites involved in the class separation in order to identify
233 metabolites with a tidal rhythmic pattern. Selection of metabolites was based on the importance of
234 their contribution as variable in the component prediction in PLS-DA model, described as Variable
235 Importance in Projection parameter (VIP). A variable with a VIP-value > 1 can be considered as a
236 metabolite providing a significant contribution to the PLS-DA model.
237 Predictability performance was evaluated for each built PLS-DA model. To assess the statistical
238 significance, t-tests were performed on each metabolite intensities difference between low tide
239 samples and high tide samples. Normality and homoscedasticity were previously checked.
240
241 *Identification and relative level of metabolites*
242 The identification of metabolites, in case of LC-MS analyses, was performed by using the human
243 metabolome database (http://www.hmdb.ca). A research was performed with the mass value of
244 metabolites and a limit with a mass error lower 5ppm. Moreover, corresponding analytical standards
245 were used with the LC-MS method to verify this identification.
246 The characterization of compound present in case of APGC analyses depends on the chemical reaction
247 used for derivatization. Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de) and the
248 supplementary data of the paper of Jaeger et al. (2016) were used to identify metabolites of interest in
249 APGC. In APGC mode, ion fragmentation frequently occurs, making it possible to identify
250 compounds through their fragments.

251 Relative metabolite levels were expressed in relative abundance obtained by dividing each metabolite
252 intensity by the median intensity of the same metabolite across all the samples.
253
254
255 **Results**
256
257 *Data processing*
258 Mass spectrometry untreated analyses detected 9105 *m/z* features for negative ionization mode results
259 in LCMS, 3087 for positive ionization mode and 5426 for APGC results. These large sets of data
260 obtained for metabolomics profiling were preprocessed with a batch correction to eliminate instrument
261 signal drift and offset differences between batches. Sample intensities were adjusted using a Lowess
262 regression model fitting with the pool values (Van Der Kloet et al., 2009).
263 A set of generic filters allowed to remove qualitatively blanks and pool samples and/or quantitatively
264 variables corresponding to specific values regarding designated factors or numerical variables (as in
265 PSL-DA).
266 Quality metrics tool (supplied by W4M Core Development Team) provided visualization of the data
267 matrices and supplied a rapid highlight of potential sample outliers.
268 After preprocessing, the database of metabolites counted for 2171, 700 and 1461 compounds detected
269 with negative and positive ionization modes in LCMS and APGC respectively. They were analyzed to
270 highlight a potential rhythmic pattern of their intensities.
271
272 *PCA Analysis*
273 PCA was performed as a first-pass method to confirm metabolite composition differences between
274 tide levels and detect eventual outlier samples. The score plots of PCA analysis are shown in Figure 2.
275 Each point represented an individual sample, and the scatter of points indicated the similarities or
276 differences of metabolic compositions. Samples having similar metabolite contents are clustered
277 together, whereas those having different metabolites are dispersed. The PCA performed with datasets
278 from both low and high tides showed that two groups were well separated according to tidal level.
279 Thus, compound intensities, considered as variables, contributed to the separated structure observed
280 between high and low tide samples. The two first axes accounted for 25.4%-30.3%-31% and 16.8%-
281 12.1%-5.3% of the total variability among samples for negative, positive and APGC datasets
282 respectively. High and low tide samples were clustered and distinguished mainly along the first axis
283 for positive ionization condition and APGC and along the second axis for negative mode. The model
284 fitted the data well. However, too many variables were considered to highlight a specific contribution
285 to the total variability within both datasets.

286 Hotelling's T^2 statistic tested the presence of outliers measuring the variation within the PCA model.
287 Samples with large Hotelling T^2 had an unusual variation inside the model. In this case, they were not
288 representative of the modeled data and can be considered as outliers if P-value < 0.05. The PCA
289 results showed three outlier samples in the negative (3B1, 6B1 and 3H2) and four outliers in the
290 positive mode (6B1, 1H1, 2H3 and 3H2) of datasets. No outlier samples were found in APGC dataset.
291 All outlier samples were removed from each sample matrix datasets to perform PLS-DA.
292
293 *PLS-DA analyses*
294 PLS-DA analyses were then performed to identify metabolites whose abundance was linked to the
295 tidal cycle. First PLS-DA models were built to force the separation between the two tidal patterns in
296 the three datasets (negative and positive LC-MS and APGC).
297 The performances of the model was proven, as the results of both analyses for negative, positive
298 modes and APGC datasets showed a relative data consistency with R^2 (cumulative) = 0.99, 0.99 and
299 0.99 respectively. Moreover, the prediction performance of the models reached Q^2 (cumulative) =
300 0.84, 0.85 and 0.67 for negative and positive modes and APGC datasets respectively, estimating a
301 good predictive capacity. However, the permutation test was not significant (P-value > 0.05) for these
302 first models due to the very large number of variables.
303 To improve the prediction performance of the model and to select the variables presenting the most
304 important contribution in the classification model, successive PLS-DA models were built selecting
305 each time only variables with VIP > 1. Model succession was stopped when their predictive
306 performances were the highest. In the negative mode dataset, a total of 3 successive models selecting
307 687, 262 and then 87 metabolites with VIP > 1 were performed reaching Q^2 = 0.95. In the positive
308 mode dataset, a total of two successive models selecting 231 and 76 metabolites with VIP > 1 were
309 performed reaching Q^2 = 0.90. Finally in the APGC dataset, a total of three successive models
310 selecting 396, 103 and 27 metabolites with VIP > 1 were performed reaching Q^2 = 0.87.
311
312 All these selected metabolites showed a clear tidal rhythmic pattern, with a significant difference
313 between low tide and high tide samples for each metabolite (t-test, P-value < 0.05). Among them, 15
314 metabolites were identified by using one of the identification methods described in the "material and
315 methods" section. Interestingly, the relative abundance of 14 metabolites peaked in low tide samples
316 while only tyrosine peaked in high tide samples.
317
318 *Carbohydrate/energy metabolism*
319 Within the gill tissue of *M. varia*, succinate was found to be the most oscillating metabolite and malate
320 to a lesser extent. Both compounds exhibited a strong rhythmic fluctuation, with high levels at the end
321 of the two hours emersion period and lower levels at the end of the ten hours immersion periods
322 (Figure 3A). For succinate a difference of about five in relative abundance was found between end of

323 emersion and immersion (P-value < 0.0001). Levels of malate, which is an intermediate metabolite
324 formed during the reduction of oxaloacetate to succinate, also increased at low tide (P-value = 0.0016),
325 with a difference of 2.64. Citrate presented the same oscillation pattern, with a difference of two in
326 relative abundance (P-value = 0.01) (Figure 3A).
327 Similarly levels of lactate oscillated and increased at the end of low tide, with a difference of about
328 three between low and high tides (P-value < 0.0001) (Figure 3B).
329 Glutamate and alanine were also found to clearly oscillate with high levels at the end of low tide and
330 low level at the end of high tide (amplitude respectively equal to 8.4 and 1.3 and P-values respectively
331 less than 0.0001 and 0.015) (Figure 3B).
332 Propionate, which is produced by a further reduction of succinate, could not be detected in any of the
333 samples.
334
335 *Carnitine-conjugated metabolites*
336 Another striking fact is the increase of carnitine-conjugated metabolites at the end of the two hours
337 emersion periods, with a clear oscillating pattern of relative abundance, with high level at low tides
338 and low level at high tides. A carnitine derivative of a short fatty acid, propionylcarnitine, was first
339 identified as an oscillating metabolite (Figure 4A). It is a carnitine derivative of an end product of β-
340 oxidation of fatty acids. Four other derivatives of long fatty acids, stearoylcarnitine,
341 palmitoylcarnitine, hexadecenoylcarnitine and elaidoylcarnitine were also identified and followed the
342 same trend (Figure 4B). Long chain acyl-CoenzymeA, like stearoyl-,palmitoyl-, hexadecenoyl- and
343 elaidoyl-CoenzymeA cannot penetrate the mitochondrial inner membrane and have to be conjugated to
344 carnitine, which acts as a carrier from the cytoplasm to the mitochondrion. Finally isovalerylcarnitine
345 and isobutyrylcarnitine were also identified as metabolites with a rhythmic pattern linked to tide cycles
346 (Figure 4A). These compounds are intermediates of leucine and valine catabolism respectively, that
347 are conjugated to carnitine.
348
349 *Other metabolites*
350 Another metabolite whose abundance peaked during low tide was urate. The level of this metabolite,
351 which is an intermediate metabolite formed during the degradation of purines in presence of the
352 enzyme xanthine oxidase, increased at low tide (P-value < 0.0001), with a difference of about 3.5
353 (Figure 5).
354 Conversely, tyrosine was more abundant at high tide compared to low tide, with a ratio of relative
355 abundance low/high equal to 0.74 with a P-value < 0.0001 (Figure 5).

356

357 *Antioxidant enzyme activities*

358 The comparison of SOD specific activities measured in the gills of *Mimachlamys varia* between low
359 and high tide can be made for both tidal cycle during the three days of experiment and is presented in
360 the Figure 6. Regarding the three days, each tidal cycle presents a similar pattern between the low and
361 the high tides. Indeed, specific activities of SOD level increases significantly (approximatively 25%)
362 at high tide compared to low tide, even if there is a contrary result for the first tidal cycle of day 1. It
363 was interesting to compare the first low tide of this study which has a regular signal (32.43 ± 0.89
364 UI/mg of proteins) in comparison with the second (33.29 ± 0.33 UI/mg of proteins) and the third day
365 (35.17 ± 0.86 UI/mg of proteins). Moreover, a down modulation in variegated scallop gills was
366 observed at the first high tide (first day) which showed average values of 27.80 ± 1.3 UI/mg of
367 proteins. Finally, a high modulation was noted at the second high tide of the first day (39.49 ± 1.9
368 UI/mg of proteins), then the SOD specific activity remained relatively constant afterwards during
369 experimentation.

370

371 **Discussion**

372 The gills in bivalves are the first interface between external and internal environments and thus, firstly
373 exposed to changes in oxygen concentrations during the different phases of immersion and emersion.
374 In the present study gill samples collected in scallops at the end of six consecutive immersion and
375 emersion periods, simulating a tidal regime, presented significant oscillating variations in the levels of
376 different metabolites. These variations reflected a switch from aerobic to anaerobic energy metabolism
377 at low tide, an accumulation of many different carnitine-conjugated components and the appearance or
378 disappearance of some other metabolites at low tide. The specific activity of SOD presented also an
379 oscillating pattern between low and high tide. These different effects are successively discussed below.

380

381 *Carbohydrate/energy metabolism*

382 At low tide, the increase in succinate and malate to a lesser extent on the one hand and of lactate,
383 glutamate and alanine on the other hand suggests that scallops resort to anaerobic energy production
384 after a two hours' time period of emersion. This strategy has been identified since the 1970s in
385 bivalves that undergo periods of hypoxia (De Zwaan and Van Marrewijk, 1973; De Zwaan and
386 Zandee, 1972; Kluytmans et al., 1977). The most used anaerobic pathways for marine bivalves over an
387 emersion period of hours are (1) glucose-lactate pathway with lactate as end-product, (2) glucose-
388 opines pathways, with opines, such as alanopine, strombine and octopine, as end-products, (3)
389 glucose-succinate pathway, with succinate as end-product and (4) aspartate-succinate pathway with
390 succinate and alanine as end products (Hochachka, Peter W. and Somero, G. N., 1984).

391 The present study indicates that emerged scallops use alternative methods (1), (3) and (4) that do not
392 require oxygen for producing ATP. Indeed, lactate accumulates after 2 hours of emersion, coming
393 from the reduction of pyruvate by the enzyme lactate dehydrogenase with simultaneous oxidation of
394 NADH (pathway 1); succinate and malate, respectively 5 and 2 times more abundant after 2 hours of
395 emersion, are produced via the part of the Krebs cycle between oxaloacetate and succinate running in
396 reverse (Muller et al., 2012). The transformation of oxaloacetate into malate is possible as the enzyme
397 malate dehydrogenase that would oxidize malate into oxaloacetate during "normal" Krebs cycle,
398 catalyze the inverse reaction in anoxic conditions (De Zwaan, 1983) (pathway 3). Finally, the pyruvate
399 is transformed into alanine utilizing an amino group donated via transaminase reactions from
400 aspartate; the resulting oxaloacetate is further metabolized into succinate. This leads to an increase of
401 both alanine and succinate at the end of the emersion period (pathway 4).
402 Energy is produced faster in pathways (1) but in a less efficient way (two moles of ATP per glucose
403 unit) than pathways (3) and (4), which can yield two times more ATP. Pathway (2) with opines as end
404 products has been identified in scallops in case of temporary muscle anoxia, associated for example
405 with high energy production following burst activity, such an escape swimming (Muller et al., 2012).
406 This pathway has not been observed in case of long term anaerobiosis (livre scallops) and opines were
407 not identified in the present study as oscillating metabolites between low and high tides.
408
409 Citrate is another metabolite that was found to accumulate at low tide in the present study. This may
410 be because a minor proportion of malate is following the tricarboxylic acid cycle in the forward
411 direction, while the major portion of malate is metabolized via the reverse of that portion of the Krebs
412 cycle : malate-fumarate-succinate (De Zwaan et al., 1981).
413
414 Finally, glutamate was also found to accumulate at the end of the emersion period. This metabolite is
415 used with aspartate to maintain the glycolytic flux, through the transformation of pyruvate to alanine
416 by transamination reactions of these free amino acids. In this process, glutamate is transformed into
417 alanine by the action of glutamate pyruvate transaminase and regenerated from ketoglutarate in the
418 glutamate dehydrogenase reaction (Grieshaber et al., 1994). As the relative abundance of alanine was
419 shown to increase at low tide, it is very likely that this metabolic cycle occurs in *Mimachlamys varia.*
420 This pathway is a characteristic feature of many anoxia-tolerant marine invertebrates (Muller et al.,
421 2012). Nevertheless it cannot explain the observed accumulation of glutamate at low tide, as this
422 process involves simultaneous formation and consumption of glutamate.
423
424 Furthermore, glutamate plays a key role in the catabolism of amino acids, during removal of α-amino
425 nitrogen from almost all of the amino acids via transdeamination: amino groups from most amino
426 acids are transferred to α-ketoglutarate to form glutamate and an α-keto-acid. Glutamate is then
427 transported into the mitochondria, where the amino group may be removed to form ammonia. This last

428 compound is the major nitrogenous excretory product in bivalves, unlike in vertebrates where excess
429 ammonia is excreted as urea or uric acid. Ammonia excreted by bivalves while immersed diffuses into
430 the surrounding water, but bivalves in general exhibit a reduction in rate of ammonia excretion during
431 air exposure and ammonia accumulates in the hemolymph and in the fluid trapped in the mantel cavity
432 (De Vooys and De Zwaan, 1978; Mingoa-licuanan, 1993; Sadok et al., 1999; Thomsen et al., 2016).
433 The increase in glutamate concentration instead of ammonia could then be a way to avoid an excessive
434 accumulation of ammonia, which can strongly affect the animal. However this explanation remains
435 hypothetical and requires confirmation in further studies.
436
437 Propionate is a frequently observed metabolite during anaerobiosis in bivalves, in particular under
438 conditions of prolonged anaerobiosis (Muller et al., 2012). In the present study, the absence of
439 propionate at the end of the 2 hours' time period of emersion is consistent with studies performed with
440 other bivalves, showing that propionate production is initiated only after longer periods of anoxia. In
441 *Mytilus edulis*, during the early phase of hypoxia, succinate is produced via the part of the Krebs cycle
442 between oxaloacetate and succinate running in reverse and under conditions of prolonged
443 anaerobiosis, propionate is preferentially formed instead of succinate (Muller et al., 2012). The same
444 delay between succinate and propionate production during hypoxia was observed in *Mytillus*
445 *galloprovincialis*. After exposure of this mussel to anaerobiosis in laboratory, propionate was not
446 detected after 6 h in anoxic seawater, whereas a sevenfold increase in succinate was observed, but
447 accumulated after 24 h of incubation. After 48 h, propionate concentrations increased by a factor of
448 two with regard to 24 h for individuals incubated in anoxic seawater and appeared, for the first time, in
449 emersed individuals (Babarro et al., 2007).
450
451 In summary, our results show a clear signal of increased anaerobic capacity after only 2h of aerial
452 exposure. Low oxygen concentrations during emersion will decrease the oxidative phosphorylation of
453 ATP, which will induce the activation of alternative metabolic pathways of ATP production. This
454 strategy does not discard other ones like, for example, lowering metabolism and the recourse to
455 gaseous air breathing, but it means that scallops cannot meet their total energy requirements without
456 using anaerobic metabolism.
457
458 *Carnitine-conjugated metabolites*
459 As shown in the results section, many carnitine-conjugated metabolites were shown to accumulate at
460 the end of the 2 hours emersion periods.
461 The accumulation of carnitine conjugates during low tides has already been observed in the case of
462 *Mytilus californianus* (Connor and Gracey, 2012; Gracey and Connor, 2016). This was considered as a
463 way to regulate different metabolic pathways for the organism. In particular, it prevents acyl-
464 CoenzymeA accumulation in the cytoplasm and mitochondria that could lead to serious disorders.

465 Concerning fatty acid metabolism, acyl-CoenzymeA molecules remain blocked in the form of acyl-
466 carnitine at low tide and therefore fatty acid metabolism seems to be paused at low tide. In the present
467 study, propionyl-, stearoyl-, elaidoyl-, hexadecenoyl- and palmitoyl-carnitine accumulated after 2
468 hours of emersion. Concerning branched amino acids catabolism, intermediate acyl-CoenzymeA are
469 also stored in the form of carnitine conjugates (isovalerylcarnitine, isobutyrylcarntine in the present
470 study). It was reported that the ratio of acetyl-CoenzymeA to CoenzymeA has important effects on
471 overall mitochondrial metabolism and therefore the accumulation of carnitine-conjugated metabolites
472 at low tides would be a way to regulate this ratio (Connor and Gracey, 2012). Indeed conjugation to
473 carnitine is not a necessary step in branched amino acid catabolism, nor in the metabolism in
474 propionyl-CoenzymeA. Nevertheless the increase of these metabolites at low tide remains cryptic
475 (Connor and Gracey, 2012).
476
477 *Other metabolites*
478 Finally two supplementary metabolites were found to oscillate with tidal cycles: urate was higher at
479 the end of the 2 hours emersion period and tyrosine higher at the end of the immersion period.
480 Concerning the accumulation of urate during emersion, this metabolite was shown to accumulate in
481 crustaceans, scallops and cockles during hypoxia and would arise from adenylate degradation in
482 presence of the enzyme xanthine oxidase (XOD) (Dykens and Shick, 1988). In the catabolism of
483 purine nucleotides, AMP is degraded to inosine and then by phosphorolysis to hypoxanthine. In
484 presence of XOD, xanthine is formed from oxidation of hypoxanthine. Xanthine is again oxidized by
485 XOD to form the final product: urate and in presence of oxygen superoxide radicals (O_2^-). XOD
486 activity has been assayed in different bivalves after periods of anoxia. It was not detected in members
487 of the orders *Mytiloida* and *Myoida*, but it was found in scallops (*Pecten maximus and Placopecten*
488 *magellanicus*) (Dykens and Shick, 1988). So it appears that anoxia-tolerant bivalves, like *Mytilus*
489 *edulis*, avoid accumulating hypoxanthine, because they present low activity of XOD. Conversely
490 anoxia-sensitive (or more intolerant to anoxic-normoxic transitions) bivalves, like *Pecten maximus*,
491 undergo hypoxanthine accumulation and are subjected to superoxide radicals when oxygen is available
492 again, as they present XOD activity.
493 So, as urate accumulates during emersion in gills of *Mimachlamys varia*, this species likely displays
494 XOD activity during emersion, as urate is the end product of this enzyme. According to the study of
495 Dykens, this is linked to a low tolerance to anoxic environment or to anoxic-normoxic transitions. This
496 is consistent with the fact that *pectinidae* are not well-adapted to emersion, compared to *mytilidae*.
497 They are adapted to brief functional hypoxia, for example during swimming, but do not survive
498 prolonged environmental hypoxia (Riedel 2012).
499 As far as the accumulation of tyrosine after 2 hours of emersion is concerned, this may be linked to the
500 role of this amino acid in the formation of catecholamines, neurotransmitters that are present in both
501 central nervous system and hemolymph of bivalves. The enzyme tyrosine hydroxylase enables the

502 conversion of tyrosine to the precursor of catecholamines L-dihydroxyphenylalanine (L-DOPA).It was
503 shown that under the effect of unfavorable environmental factors, the quantitative catecholamine
504 contents vary (Kotsyuba, 2011). Moreover these substances exert marked cilio-excitatory or cilio-
505 inhibitory actions when applied to the bivalve gill (Malanga et al., 1972). Lateral cilia of bivalve gills
506 serve a vital function by generating the water currents that allow gas exchange as well as regulate food
507 intake and waste removal. Numerous studies indicate that the beating of the lateral cilia of bivalve
508 gills is under nervous or neurohormonal control in various bivalves. For example, lateral cilia of the
509 gill of *Mytilus edulis* are controlled by a reciprocal serotonergic-dopaminergic innervation from their
510 ganglia (Carroll and Catapane, 2007). Taking these facts into account, the accumulation of tyrosine
511 may be linked to this regulation system, in order to enhance the beating of cilia in case of hypoxia,
512 through a reduced transformation of tyrosine to dopamine, that would have indirectly a cilio-inhibitory
513 effect (Carroll and Catapane, 2007). This hypothesis is however only one assumption at this stage of
514 the study.
515

516 *SOD activity*

517 In the present study, SOD activity measurements shown lower levels of antioxidant enzyme specific
518 activity during low tides, after two hours of emersion, compared to the high tides. The alternation of
519 immersion–submersion for intertidal organisms implies fluctuations in temperature, radiation exposure
520 and oxygen supply as well, which have an impact on free radical and reactive oxygen species (ROS)
521 production. For example, the elevation of O_2 respiration in reoxygenation during re-immersion has
522 been suggested to result in the enhanced ROS production in intertidal organisms (Yin et al., 2017). To
523 protect themselves against ROS, intertidal organisms have developed a system with enzymatic
524 antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx),
525 and ascorbate peroxidase (APX). The oxidative stress occurring when higher level of oxygen is
526 introduced during re-immersion is intense for these organisms. Thus, it is therefore suggested that the
527 higher level in SOD activity in gills of scallops observed in the present study could be a response of
528 acclimation to a higher level of encountered oxidative stress upon re-immersion. In previous studies,
529 opposite results were obtained : increased activities of SOD and CAT were shown in clams under air
530 exposure compared to clams without air exposure (Yin et al., 2017) and an increase in antioxidant
531 defenses was shown to be induced by short-term hypoxia in marine invertebrates including bivalves
532 (Nogueira et al., 2017). So it appears that hypoxia associated with air exposure, is not systematically
533 accompanied by low SOD activity level compared to normoxia obtained upon re-immersion.
534 Nevertheless in any case, alternating periods of immersion and aerial emergence seems to be
535 accompanied by variations of SOD activity in intertidal inhabitants.
536
537

538 **Conclusion**

539 The purpose of this study was to investigate the metabolic strategy of *Mimachlamys varia* under
540 alternating short period of emersion and longer period of immersion, mimicking conditions that might
541 be experienced by scallops, located highest on the shore, during extreme spring and autumn tides. Our
542 results show that there is a switch from aerobic to anaerobic metabolism after only two hours of
543 emersion, with the resort to different pathways used by other bivalves to face hypoxia: glucose-lactate
544 pathway with lactate as end-product, glucose-succinate pathway, with succinate as end-product and
545 aspartate-succinate pathway. Furthermore carnitine-conjugated metabolites were found to accumulate
546 after two hours of emersion, as well as urate. The level of tyrosine on the contrary was found to
547 increase upon emersion. Moreover SOD activity was found to increase upon re-immersion. These
548 different findings concerning oscillating metabolites and enzymatic response indicate a complex
549 metabolic reprogramming that occurs after an emersion period of two hours only and upon re-
550 immersion. To our knowledge, it is the first time that this physiological adaptation to emersion is
551 studied in *Mimachlamys varia*. It appears that, like many bivalve organisms undergoing hypoxia–
552 normoxia transitions, variegated scallops have developed a high metabolic flexibility and may have
553 evolved tissue-specific abilities to deal with the abrupt exposure of gills to molecular oxygen upon re-
554 immersion. In accordance with previous studies in mussels in the same organ (Letendre et al., 2008),
555 the gills of the variegated scallop seem to be an adapted organ to assess hypoxia response after a short
556 emersion (two hours) for future experiments.
557 Further studies will be required to completely elucidate this metabolic and physiologic reorganization.
558 In particular, absolute quantification of metabolite levels and flux measurements are needed, and also
559 the evaluation of a possible global lowering metabolism upon emersion and the measure of the exact
560 recourse to gaseous air breathing.
561 These findings have to be taken into account to homogenize sampling during campaigns of
562 environmental monitoring, by taking *in situ,* as far as possible only individuals in immersion. The
563 biomarker response depends as well as on the moment of the tidal cycle, and the ambient environment
564 in which are living the bivalves. This could be related to different abiotic factors such as oxygenation
565 and temperature conditions which change at the upper side of the shore compared to the lower part. It
566 results in different impacts on the metabolism and per consequence on the physiology of the
567 variegated scallop.

568

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586
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