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Abstract

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

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

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Introduction

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

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

due its relative abundance along the coasts and also because this species has been shown to have a very elevated pollutants incorporation and retention capacity, it has been proposed as a potential ecotoxicological biomonitoring species for the marine environmental watch (Metian et al., 2009a, 2009b; Milinkovitch et al., 2015). However, during campaigns of environmental monitoring using *M. varia* as sentinel species, samples of these scallops are taken *in situ* on the shore at low tide at high tidal coefficient, indifferently emerged or still immerged and biomarkers are assayed to evaluate the sub-lethal effects of pollutants (Breitwieser et al., 2018, 2016). In order to provide normalized sampling methods, during environmental biomonitoring using biomarkers in *M. varia*, it is important to exactly know how metabolism of *M. varia* individuals living in the low intertidal is modified during emersion period. Indeed these metabolic changes are likely to lead to confounding factors and to increase the variability of biomarker responses. For example, it was shown that hypoxia induced tissue-specific responses in both antioxidant and immune systems in mussels (Nogueira et al., 2017).

In order to reveal new insight into the metabolic adaptations to emersion of *M. varia*, we grew scallops in aquaria with a simulated intertidal environment and a metabolomics study was performed during six complete tidal cycles. These experimental conditions were chosen to mimic short-duration air exposure that might be experienced by variegated scallops, located highest on the foreshore, during extreme spring and autumn tides: alternating 2h air exposure and 10h immersion. A comparative metabolomics study was performed in gills samples, using both LC-QToF and APGC-QToF mass spectrometry, in order to provide a temporal overview of the relationship between tidal cycles and metabolome changes. Besides the effect of these tidal cycles on metabolome, the variation of SOD (superoxide dismutase) activity was measured all along the experiment. This enzyme is a part of the enzymatic antioxidant system developed by bivalves to face oxidative stress. It is considered as a precocious biomarker linked to this particular stress (Valavanidis et al., 2006) and it is used to monitor and evaluate the possible health impairment in marine organisms, following for example acute or chronic pollution (Breitwieser et al., 2018, 2016).

Materials and Methods

Animals and experimental design

M. varia of 4 to 5 cm length were collected in the Pertuis Charentais (France) in December 2016. They were then maintained in aquaria in the laboratory at a constant temperature of 15°C. Food as liquid algal culture (Shellfish Diet 1800®, Reed Mariculture, Campbell, CA, USA) was added to the water. Intertidal conditions were simulated using water pumps that filled and emptied the aquaria with natural seawater. Once a day, at the end of low tide period, the seawater was totally replaced by filling the aquaria with fresh seawater. A tidal regime of alternating periods of 10 h in and 2 h out of seawater

was established. This last duration corresponds to a maximal emersion time endured by *M. varia* individuals living in the low intertidal area. Low tides occurred from 8:30 to 10:30 A.M. and 8:30 to 10:30 PM. A light-dark cycle was also programmed with periods of darkness occurring from 8:00 PM to 8:00 AM (Figure 1). Scallops were acclimated during four weeks to alternating high and low tides, before sampling. Samples were collected 0.25 h before the change in tidal episode (at 8:15 AM, 10:15 AM, 8:15 PM and 10:15 PM) during 3 days corresponding to 6 total tidal cycles. The first sampling occurred at 8:15 AM. Nine individual scallops were collected at each time point. Immediately after sampling, gill tissue was dissected from each individual, drained on absorbent paper and put in cryovial on ice. Gill tissues from three individuals were pooled, snap-frozen in liquid nitrogen and stored in liquid nitrogen.

Total protein concentration and SOD activity: sample processing and assays

For each pool of three individuals, 100 mg of gills were collected after dissection and mixing. They were then homogenized in ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm). The homogenates were centrifuged at 12,500g at 4 °C for 15 min and the supernatants were used for protein and SOD activity assays.

Total protein concentrations were determined using an adaptation of the BCA Kit method (Bicinchononique Acid Kit, Sigma Aldrich). The kit contained bovine serum albumin (BSA) as a standard and involved the reduction of alkaline Cu^{2+} by proteins (Smith et al., 1985) at absorbance 562 nm using a spectrofluorometer (SAFAS Flx-Xenius).

SOD activity was assessed in the homogenate fractions using the method developed by Paoletti et al. (1986). The assay, involving EDTA, $MnCl_2$ and mercaptoethanol, measures the decrease of nicotinamide adenine dinucleotide (NADH) oxidation. This inhibition of oxidation was monitored at 340 nm (using SAFAS Flx-Xenius spectrofluorimeter) and is a function of SOD activity. Fifty percent inhibition of oxidation corresponds to one unit of SOD. The results are presented in UI of SOD/mg of proteins. Statistical analyses were carried out using R language (v. 3.1.2, R Core Team, 2016). Normality was first tested on residuals using Kolmogorov-Smirnov tests and homogeneity of variances was assessed using Bartlett tests. For SOD values, tides (low and high) were compared at two times per day for each day, using One-Sample t-tests.

Sample preparation for metabolomics study

The efficiency of the following technique, inspired by a metabolomics study in Phycotoxines laboratory (Ifremer, Nantes, France), has previously been established (Mondeguer et al., 2015). Samples were thawed on ice, homogenized by manual grinding with a mini pestle and then crushed with a homogenizer T 10 basic Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Germany) at low speed. Each sample was precisely adjusted to 1g and 100mg of homogenized sample were also put

aside in a separate tube in order to perform SOD activity measurement. Tubes were then stored at -80°C for one night before extraction procedure.

Samples were then subjected to a triple solvent extraction protocol by successively using acetone twice and methanol. For each extraction, the following protocol was used: addition of 2.5 mL of solvent to the sample, resuspension and dispersion with homogenizer T 10 basic Ultra-Turrax, agitation 140 rpm 10 minutes, centrifugation at 3000 g during 5 minutes, removal of supernatant. Ultra-Turrax was rinsed in the solvent of the next step. The three supernatants were then pooled, put at 4°C and subjected to centrifugation at 3000 g during 5 minutes. The supernatant was recovered and dried with a stream of nitrogen at 30°C until reduction of the volume to 50% and at 50°C until complete drying. The dry extract was finally re-suspended with 2 mL methanol/water 20/80 and stored at -80°C prior filtration with 0.2 µm filters and LCMS analysis.

LC-QToF MS analysis of metabolites

The analysis of metabolite compounds in gills was performed by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). Analyses were carried out using an UHPLC system "Acquity UPLC H-class" (Waters, Milford, USA) coupled to a high resolution mass spectrometer "XEVO-G2-S Q-TOF" equipped with an electrospray ionization source (Waters, Manchester, England). The UHPLC system was formed by a quaternary pump (Quaternary Solvent Manager, Waters) and an automatic injector (Sample Manager-FTN, Waters) equipped with a 10 µL injection loop. 5 µL of the samples were injected in a column "Acquity UPLC HSST3" (Waters) (2.1 × 150 mm, 1.7 µm), and the products were eluted at a flow rate of 200 $\mu L.min^{-1}$ using a gradient composed of solvents A (water/formic acid 100/0.001 (v:v)) and B (acetonitrile/ formic acid 100/0.001 (v:v)), according to the following procedure: 0–3 min, 100% A; 3–8 min 0%–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 100% B . During the analysis, the column and the injector were maintained at 25°C and 7°C, respectively. The analyses were performed in positive and negative ionization mode with MS^E function in a centroid mode. The MS parameters was applied in the ESI source for the two ionization mode were: source temperature 120°C, desolvation temperature 500°C, gas flow-rate of the cone 50 $L.h^{-1}$, desolvation gas flow-rate 800 $L.h^{-1}$, capillary voltage 2.5 kV, sampling cone 130 V and source compensation 80 V. The instrument was adjusted for the acquisition on a 50–2100 m/z interval, with a scan time of 0.15 s.

The mass spectrometer was calibrated before analysis using 0.5mM sodium formiate solution and the Leucine Enkephalin (M = 555.62 Da,1 ng. μL^{-1}) was used as a lock-mass.

Compound identification was performed by matching to a database of authentic compound standards. Metabolites were finally expressed in relative abundance, which was calculated for each metabolite by dividing the abundance in each sample by the median abundance across all the samples.

Quality Control

A pool sample was prepared by combining 100 µL of each gills tissue extract. The pool sample was divided into several vials that were used as quality-control samples (QCs) and regularly injected within samples (one injection every five samples) to ensure analytical repeatability. Samples of gills tissues extracts were measured in randomized order to avoid any possible time-dependent changes in LC/MS analysis. Blanks were prepared with the last extraction solvent and injected at the beginning and at the end of the samples sequence. These blanks allowed the subsequent subtraction of contaminants or components coming from the extraction solvent.

Chemicals

Acetonitrile, Methanol, Acetone with HPLC grade purity were acquired from Carlo Erba. Water was prepared using a Milli-Q reagent water system. Succinate, malate, uric acid, aspartate, alanine, citrate, glutamate, propionylcarnitine , isovalerylcarnitine, isobutyrylcarnitine were purchased from Sigma-Aldrich.

Sample and standards preparation for APGC-QToF MS

20 µL of samples prepared for metabolomics studies as described above and QC or of 10 ng μL^{-1} standard solutions were dried under nitrogen at 40°C in vials without inserts. For methoximation–trimethylsilylation (MeOx–TMS), 20 µL of a 20 mg mL^{-1} methoxylamine hydrochloride in pyridine solution were added to dissolve the dried extract. The vials were then vortex mixed for 15 s and heated at 80°C for 15 min in derivatization block. Vials were the removed from the block and 20 µL of MSTFA (N-methyl-N-(trimethylsilyl trifluoroacetamide)) was added to the samples and QC, vortex mixed for 15 s again and heated at 80°C for 15 min in the block. Vials were removed from the block and allowed to cool for 5 min. They were then centrifuged at 2000 rpm for 5 min. Supernatants were transferred to autosampler vials using pipet with filter tips.

APGC-QToF MS analysis of metabolites

Analyses were carried out using an Agilent GC 7890A (Agilent Technologies, Santa Clara, CA) coupled to a Waters Synapt G2-S ToF (Waters Corporation, Manchester, UK), equipped with APGC (atmospheric pressure gas chromatography) ionization. A DB5-MS (Agilent Technologies) analytical column (30 m × 0.25 mm i.d. x 0.25 µm film thickness consisting of 5% phényl-diméthylpolysiloxane) was used. The carrier gas was helium; it was used at constant flow equal to 1 mL min^{-1}. The temperature program for the gas chromatography was as follows: initial temperature 120°C for 0.5 min, increase of temperature by two different rates: 8°C.min^{-1} up to 280°C at 22 min and 20°C.min^{-1} up to 300°C, then temperature was held at 300 °C for 5 min. Total time for analysis was 28 min. The injector temperature was 250°C. Injection was performed in the split mode (1/50) and the injection

volume was 2µL. The parameters for APGC were: Corona current 1.3µA, probe temperature 20°C, source temperature 150°C, sampling cone 30V (different sampling cone voltages (from 10 to 40 V) were tested and 30 V was retained), source offset 80V, cone gas flow 170 L/Hr, auxillary gas flow 220 L/Hr. The instrument was adjusted for the acquisition on a 50–700 m/z interval, with a scan time of 0.5 s.

Statistical analysis

Data were obtained and treated as ion peak intensity after conversion of raw spectra (.RAW) to .NetCDF format, compatible with Galaxy software, using "Databridge" software (Waters). Metabolites data were processed on the online and freely available Workflow4Metabolomics (W4M) platform (http://workflow4metabolomics.org) for data pretreatments and analyzed with the web server MetaboAnalyst 4.0 (www.metaboanalyst.ca) for multivariate analyses processing: the data were normalized and log-transformed, and Pareto scaling was applied prior to multivariate analyses. The data were analyzed using both unsupervised (Principal Component Analysis, PCA) and supervised (Projections to Latent Structures Discriminant Analysis, PLS-DA) methods. Unsupervised method allowed to detect general spectral trends and natural clustering between samples justifying the PLS-DA model reliability. It also highlighted the potential sample outliers. The supervised method is a multivariate regression and prediction based on the separation between two classes: high and low tide samples. It was used to select the metabolites involved in the class separation in order to identify metabolites with a tidal rhythmic pattern. Selection of metabolites was based on the importance of their contribution as variable in the component prediction in PLS-DA model, described as Variable Importance in Projection parameter (VIP). A variable with a VIP-value > 1 can be considered as a metabolite providing a significant contribution to the PLS-DA model.

Predictability performance was evaluated for each built PLS-DA model. To assess the statistical significance, t-tests were performed on each metabolite intensities difference between low tide samples and high tide samples. Normality and homoscedasticity were previously checked.

Identification and relative level of metabolites

The identification of metabolites, in case of LC-MS analyses, was performed by using the human metabolome database (http://www.hmdb.ca). A research was performed with the mass value of metabolites and a limit with a mass error lower 5ppm. Moreover, corresponding analytical standards were used with the LC-MS method to verify this identification.

The characterization of compound present in case of APGC analyses depends on the chemical reaction used for derivatization. Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de) and the supplementary data of the paper of Jaeger et al. (2016) were used to identify metabolites of interest in APGC. In APGC mode, ion fragmentation frequently occurs, making it possible to identify compounds through their fragments.

Relative metabolite levels were expressed in relative abundance obtained by dividing each metabolite intensity by the median intensity of the same metabolite across all the samples.

Results

Data processing

Mass spectrometry untreated analyses detected 9105 *m/z* features for negative ionization mode results in LCMS, 3087 for positive ionization mode and 5426 for APGC results. These large sets of data obtained for metabolomics profiling were preprocessed with a batch correction to eliminate instrument signal drift and offset differences between batches. Sample intensities were adjusted using a Lowess regression model fitting with the pool values (Van Der Kloet et al., 2009).

A set of generic filters allowed to remove qualitatively blanks and pool samples and/or quantitatively variables corresponding to specific values regarding designated factors or numerical variables (as in PSL-DA).

Quality metrics tool (supplied by W4M Core Development Team) provided visualization of the data matrices and supplied a rapid highlight of potential sample outliers.

After preprocessing, the database of metabolites counted for 2171, 700 and 1461 compounds detected with negative and positive ionization modes in LCMS and APGC respectively. They were analyzed to highlight a potential rhythmic pattern of their intensities.

PCA Analysis

PCA was performed as a first-pass method to confirm metabolite composition differences between tide levels and detect eventual outlier samples. The score plots of PCA analysis are shown in Figure 2. Each point represented an individual sample, and the scatter of points indicated the similarities or differences of metabolic compositions. Samples having similar metabolite contents are clustered together, whereas those having different metabolites are dispersed. The PCA performed with datasets from both low and high tides showed that two groups were well separated according to tidal level. Thus, compound intensities, considered as variables, contributed to the separated structure observed between high and low tide samples. The two first axes accounted for 25.4%-30.3%-31% and 16.8%-12.1%-5.3% of the total variability among samples for negative, positive and APGC datasets respectively. High and low tide samples were clustered and distinguished mainly along the first axis for positive ionization condition and APGC and along the second axis for negative mode. The model fitted the data well. However, too many variables were considered to highlight a specific contribution to the total variability within both datasets.

Hotelling's T^2 statistic tested the presence of outliers measuring the variation within the PCA model. Samples with large Hotelling T^2 had an unusual variation inside the model. In this case, they were not representative of the modeled data and can be considered as outliers if P-value < 0.05. The PCA results showed three outlier samples in the negative (3B1, 6B1 and 3H2) and four outliers in the positive mode (6B1, 1H1, 2H3 and 3H2) of datasets. No outlier samples were found in APGC dataset. All outlier samples were removed from each sample matrix datasets to perform PLS-DA.

PLS-DA analyses

PLS-DA analyses were then performed to identify metabolites whose abundance was linked to the tidal cycle. First PLS-DA models were built to force the separation between the two tidal patterns in the three datasets (negative and positive LC-MS and APGC).

The performances of the model was proven, as the results of both analyses for negative, positive modes and APGC datasets showed a relative data consistency with R^2 (cumulative) = 0.99, 0.99 and 0.99 respectively. Moreover, the prediction performance of the models reached Q^2 (cumulative) = 0.84, 0.85 and 0.67 for negative and positive modes and APGC datasets respectively, estimating a good predictive capacity. However, the permutation test was not significant (P-value > 0.05) for these first models due to the very large number of variables.

To improve the prediction performance of the model and to select the variables presenting the most important contribution in the classification model, successive PLS-DA models were built selecting each time only variables with VIP > 1. Model succession was stopped when their predictive performances were the highest. In the negative mode dataset, a total of 3 successive models selecting 687, 262 and then 87 metabolites with VIP > 1 were performed reaching $Q^2 = 0.95$. In the positive mode dataset, a total of two successive models selecting 231 and 76 metabolites with VIP > 1 were performed reaching $Q^2 = 0.90$. Finally in the APGC dataset, a total of three successive models selecting 396, 103 and 27 metabolites with VIP > 1 were performed reaching $Q^2 = 0.87$.

All these selected metabolites showed a clear tidal rhythmic pattern, with a significant difference between low tide and high tide samples for each metabolite (t-test, P-value < 0.05). Among them, 15 metabolites were identified by using one of the identification methods described in the "material and methods" section. Interestingly, the relative abundance of 14 metabolites peaked in low tide samples while only tyrosine peaked in high tide samples.

Carbohydrate/energy metabolism

Within the gill tissue of *M. varia*, succinate was found to be the most oscillating metabolite and malate to a lesser extent. Both compounds exhibited a strong rhythmic fluctuation, with high levels at the end of the two hours emersion period and lower levels at the end of the ten hours immersion periods (Figure 3A). For succinate a difference of about five in relative abundance was found between end of

emersion and immersion (P-value < 0.0001). Levels of malate, which is an intermediate metabolite formed during the reduction of oxaloacetate to succinate, also increased at low tide (P-value = 0.0016), with a difference of 2.64. Citrate presented the same oscillation pattern, with a difference of two in relative abundance (P-value = 0.01) (Figure 3A).

Similarly levels of lactate oscillated and increased at the end of low tide, with a difference of about three between low and high tides (P-value < 0.0001) (Figure 3B).

Glutamate and alanine were also found to clearly oscillate with high levels at the end of low tide and low level at the end of high tide (amplitude respectively equal to 8.4 and 1.3 and P-values respectively less than 0.0001 and 0.015) (Figure 3B).

Propionate, which is produced by a further reduction of succinate, could not be detected in any of the samples.

Carnitine-conjugated metabolites

Another striking fact is the increase of carnitine-conjugated metabolites at the end of the two hours emersion periods, with a clear oscillating pattern of relative abundance, with high level at low tides and low level at high tides. A carnitine derivative of a short fatty acid, propionylcarnitine, was first identified as an oscillating metabolite (Figure 4A). It is a carnitine derivative of an end product of β-oxidation of fatty acids. Four other derivatives of long fatty acids, stearoylcarnitine, palmitoylcarnitine, hexadecenoylcarnitine and elaidoylcarnitine were also identified and followed the same trend (Figure 4B). Long chain acyl-CoenzymeA, like stearoyl-,palmitoyl-, hexadecenoyl- and elaidoyl-CoenzymeA cannot penetrate the mitochondrial inner membrane and have to be conjugated to carnitine, which acts as a carrier from the cytoplasm to the mitochondrion. Finally isovalerylcarnitine and isobutyrylcarnitine were also identified as metabolites with a rhythmic pattern linked to tide cycles (Figure 4A). These compounds are intermediates of leucine and valine catabolism respectively, that are conjugated to carnitine.

Other metabolites

Another metabolite whose abundance peaked during low tide was urate. The level of this metabolite, which is an intermediate metabolite formed during the degradation of purines in presence of the enzyme xanthine oxidase, increased at low tide (P-value < 0.0001), with a difference of about 3.5 (Figure 5).

Conversely, tyrosine was more abundant at high tide compared to low tide, with a ratio of relative abundance low/high equal to 0.74 with a P-value < 0.0001 (Figure 5).

Antioxidant enzyme activities

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

Discussion

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

Carbohydrate/energy metabolism

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

compound is the major nitrogenous excretory product in bivalves, unlike in vertebrates where excess ammonia is excreted as urea or uric acid. Ammonia excreted by bivalves while immersed diffuses into the surrounding water, but bivalves in general exhibit a reduction in rate of ammonia excretion during air exposure and ammonia accumulates in the hemolymph and in the fluid trapped in the mantel cavity (De Vooys and De Zwaan, 1978; Mingoa-licuanan, 1993; Sadok et al., 1999; Thomsen et al., 2016). The increase in glutamate concentration instead of ammonia could then be a way to avoid an excessive accumulation of ammonia, which can strongly affect the animal. However this explanation remains hypothetical and requires confirmation in further studies.

Propionate is a frequently observed metabolite during anaerobiosis in bivalves, in particular under conditions of prolonged anaerobiosis (Muller et al., 2012). In the present study, the absence of propionate at the end of the 2 hours' time period of emersion is consistent with studies performed with other bivalves, showing that propionate production is initiated only after longer periods of anoxia. In *Mytilus edulis*, during the early phase of hypoxia, succinate is produced via the part of the Krebs cycle between oxaloacetate and succinate running in reverse and under conditions of prolonged anaerobiosis, propionate is preferentially formed instead of succinate (Muller et al., 2012). The same delay between succinate and propionate production during hypoxia was observed in *Mytillus galloprovincialis*. After exposure of this mussel to anaerobiosis in laboratory, propionate was not detected after 6 h in anoxic seawater, whereas a sevenfold increase in succinate was observed, but accumulated after 24 h of incubation. After 48 h, propionate concentrations increased by a factor of two with regard to 24 h for individuals incubated in anoxic seawater and appeared, for the first time, in emersed individuals (Babarro et al., 2007).

In summary, our results show a clear signal of increased anaerobic capacity after only 2h of aerial exposure. Low oxygen concentrations during emersion will decrease the oxidative phosphorylation of ATP, which will induce the activation of alternative metabolic pathways of ATP production. This strategy does not discard other ones like, for example, lowering metabolism and the recourse to gaseous air breathing, but it means that scallops cannot meet their total energy requirements without using anaerobic metabolism.

Carnitine-conjugated metabolites

As shown in the results section, many carnitine-conjugated metabolites were shown to accumulate at the end of the 2 hours emersion periods.

The accumulation of carnitine conjugates during low tides has already been observed in the case of *Mytilus californianus* (Connor and Gracey, 2012; Gracey and Connor, 2016). This was considered as a way to regulate different metabolic pathways for the organism. In particular, it prevents acyl-CoenzymeA accumulation in the cytoplasm and mitochondria that could lead to serious disorders.

Concerning fatty acid metabolism, acyl-CoenzymeA molecules remain blocked in the form of acyl-carnitine at low tide and therefore fatty acid metabolism seems to be paused at low tide. In the present study, propionyl-, stearoyl-, elaidoyl-, hexadecenoyl- and palmitoyl-carnitine accumulated after 2 hours of emersion. Concerning branched amino acids catabolism, intermediate acyl-CoenzymeA are also stored in the form of carnitine conjugates (isovalerylcarnitine, isobutyrylcarntine in the present study). It was reported that the ratio of acetyl-CoenzymeA to CoenzymeA has important effects on overall mitochondrial metabolism and therefore the accumulation of carnitine-conjugated metabolites at low tides would be a way to regulate this ratio (Connor and Gracey, 2012). Indeed conjugation to carnitine is not a necessary step in branched amino acid catabolism, nor in the metabolism in propionyl-CoenzymeA. Nevertheless the increase of these metabolites at low tide remains cryptic (Connor and Gracey, 2012).

Other metabolites

Finally two supplementary metabolites were found to oscillate with tidal cycles: urate was higher at the end of the 2 hours emersion period and tyrosine higher at the end of the immersion period. Concerning the accumulation of urate during emersion, this metabolite was shown to accumulate in crustaceans, scallops and cockles during hypoxia and would arise from adenylate degradation in presence of the enzyme xanthine oxidase (XOD) (Dykens and Shick, 1988). In the catabolism of purine nucleotides, AMP is degraded to inosine and then by phosphorolysis to hypoxanthine. In presence of XOD, xanthine is formed from oxidation of hypoxanthine. Xanthine is again oxidized by XOD to form the final product: urate and in presence of oxygen superoxide radicals (O_2^-). XOD activity has been assayed in different bivalves after periods of anoxia. It was not detected in members of the orders *Mytiloida* and *Myoida*, but it was found in scallops (*Pecten maximus and Placopecten magellanicus*) (Dykens and Shick, 1988). So it appears that anoxia-tolerant bivalves, like *Mytilus edulis*, avoid accumulating hypoxanthine, because they present low activity of XOD. Conversely anoxia-sensitive (or more intolerant to anoxic-normoxic transitions) bivalves, like *Pecten maximus*, undergo hypoxanthine accumulation and are subjected to superoxide radicals when oxygen is available again, as they present XOD activity.

So, as urate accumulates during emersion in gills of *Mimachlamys varia*, this species likely displays XOD activity during emersion, as urate is the end product of this enzyme. According to the study of Dykens, this is linked to a low tolerance to anoxic environment or to anoxic-normoxic transitions. This is consistent with the fact that *pectinidae* are not well-adapted to emersion, compared to *mytilidae*. They are adapted to brief functional hypoxia, for example during swimming, but do not survive prolonged environmental hypoxia (Riedel 2012).

As far as the accumulation of tyrosine after 2 hours of emersion is concerned, this may be linked to the role of this amino acid in the formation of catecholamines, neurotransmitters that are present in both central nervous system and hemolymph of bivalves. The enzyme tyrosine hydroxylase enables the

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

SOD activity

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

Conclusion

The purpose of this study was to investigate the metabolic strategy of *Mimachlamys varia* under alternating short period of emersion and longer period of immersion, mimicking conditions that might be experienced by scallops, located highest on the shore, during extreme spring and autumn tides. Our results show that there is a switch from aerobic to anaerobic metabolism after only two hours of emersion, with the resort to different pathways used by other bivalves to face hypoxia: glucose-lactate pathway with lactate as end-product, glucose-succinate pathway, with succinate as end-product and aspartate-succinate pathway. Furthermore carnitine-conjugated metabolites were found to accumulate after two hours of emersion, as well as urate. The level of tyrosine on the contrary was found to increase upon emersion. Moreover SOD activity was found to increase upon re-immersion. These different findings concerning oscillating metabolites and enzymatic response indicate a complex metabolic reprogramming that occurs after an emersion period of two hours only and upon re-immersion. To our knowledge, it is the first time that this physiological adaptation to emersion is studied in *Mimachlamys varia*. It appears that, like many bivalve organisms undergoing hypoxia–normoxia transitions, variegated scallops have developed a high metabolic flexibility and may have evolved tissue-specific abilities to deal with the abrupt exposure of gills to molecular oxygen upon re-immersion. In accordance with previous studies in mussels in the same organ (Letendre et al., 2008), the gills of the variegated scallop seem to be an adapted organ to assess hypoxia response after a short emersion (two hours) for future experiments.

Further studies will be required to completely elucidate this metabolic and physiologic reorganization. In particular, absolute quantification of metabolite levels and flux measurements are needed, and also the evaluation of a possible global lowering metabolism upon emersion and the measure of the exact recourse to gaseous air breathing.

These findings have to be taken into account to homogenize sampling during campaigns of environmental monitoring, by taking *in situ,* as far as possible only individuals in immersion. The biomarker response depends as well as on the moment of the tidal cycle, and the ambient environment in which are living the bivalves. This could be related to different abiotic factors such as oxygenation and temperature conditions which change at the upper side of the shore compared to the lower part. It results in different impacts on the metabolism and per consequence on the physiology of the variegated scallop.

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