Liquid chromatography-mass spectrometry based metabolomics investigation of different tissues of Mytilus galloprovincialis

Lea James, Elena Gomez, Gaëlle Ramirez, Thibaut Dumas, Frédérique Courant

Liquid chromatography-mass spectrometry based metabolomics investigation of different tissues of *Mytilus galloprovincialis*

Lea James, Elena Gomez, Gaelle Ramirez, Thibaut Dumas and Frédérique Courant *

HydroSciences Montpellier, University of Montpellier, IRD, CNRS, Montpellier, France

HydroSciences Montpellier, 15 avenue Charles Flahault, 34090 Montpellier

* Corresponding author : email: frederique.courant@umontpellier.fr; tel: +0033411759414

Acknowledgements & funding

This research was funded by SANOFI France and the French Agence Nationale de la Recherche (IMAP ANR-16-CE34-0006-01). The authors thank the Platform Of Non-Target Environmental Metabolomics (PONTEM) of the Montpellier Alliance for Metabolomics and Metabolism Analysis (MAMMA) consortium facilities.

Abstract

The Mediterranean mussel (*Mytilus galloprovincialis*) is widely used in monitoring programs and in ecotoxicological studies to examine the biological effects of physicochemical parameter changes and the impact of chemical pollutants. Metabolomics has recently demonstrated high potential to gain further insight into the molecular effects of chemical exposure and the success of its application is dependent on the extent of prior metabolomics knowledge available on the target organism. Therefore, the purpose of this study was the investigation of the metabolites of five different functional tissues of male and female Mediterranean mussels (digestive gland, foot, gill and gonad tissues and in the remaining soft tissues) accessible to the analysis using the most common sample preparation recommended for tissue analysis (i.e. Bligh & Dyer). Metabolic fingerprints were acquired via liquid chromatography high-resolution mass spectrometry and the identification was based on an internal database developed in the laboratory. It led to the identification of 110 metabolites, among which amino acids, carboxylic acids, purine and pyrimidine metabolites were often the most abundant. The metabolic contents of the five tissues quantitatively and qualitatively differed, with a clear distinction between male and female contents observed in the gonads and digestive glands. These results underline the importance of selecting the most suitable tissue and sex to study the impact of contamination on metabolism and the need for further research to deeper characterize the metabolome of this organism.

Keywords: metabolomics, bivalve, gonad, foot, digestive gland, gill, soft tissue

1. Introduction

Bivalve molluscs—a major taxon in marine waters—include the Mediterranean mussel (*Mytilus galloprovincialis*). The distribution range of this species extends from southwestern Britain to the Mediterranean Sea. This mollusc is considered to be a crucial ecosystem engineer since it is responsible for enhancing the presence of small invertebrates by increasing the habitat complexity and improving the environmental conditions for coastal communities (Arribas et al., 2014). Mussels filter large volumes of water and can bioaccumulate pollutants, thereby facilitating monitoring of their exposure to a variety of chemical contaminants (Moreno-González et al., 2016; Faggio et al., 2018; López-García et al., 2019). Moreover, these organisms are particularly interesting for monitoring programs and laboratory ecotoxicological studies due to their wide geographical distribution, ability to tolerate different environmental conditions and suitability for *in situ* cage experiments. Mussels actively respond to environmental changes via dynamic modifications in their physiology, gene expression and metabolism (Connor & Gracey, 2011, 2012; Gracey & Connor, 2016; Yang et al., 2019; Keskinbalta & Çelik, 2020). The ability of these organisms to rapidly react to changes in their surrounding environment provides a basis for molecular profiling geared towards identifying specific transcriptomic, proteomic or metabolomic profiles in response to different stresses.

Although the application of omics techniques in environmental science studies is recent, information can be provided by these approaches to allow the assessment of molecular effects of environmental stressors on aquatic organisms (Campillo et al., 2019; Lockwood et al., 2015; Tomanek & Zuzow, 2010). One of the most recent omics approaches, i.e. metabolomics, concerns the metabolic profile of a cell or tissue or entire organism. Investigated metabolites are generally of low molecular weight (50-1,500 Da) and occur in many essential biological pathways. Their levels vary according to the physiological, developmental or pathological status of cells, tissues, organs or whole organisms (Courant et al., 2014; Lin et al., 2006; Zhang et al., 2021). Two analytical methods for metabolic fingerprinting are commonly used: nuclear magnetic resonance (NMR) and mass spectrometry (MS) combined with component separation methods such as liquid chromatography (LC-MS) and gas chromatography (GC-MS). Many ecotoxicology studies involving metabolomics have been successfully carried out to unravel the effects of environmental stressors on mussels (Bonnefille et al., 2018; Campillo et al., 2019; Cappello et al., 2015, 2017, 2020; Dumas et al., 2022; Ramirez et al., 2022).

However, although mussels are a key species for ecotoxicological studies, information on their metabolome is still fragmentary. The choice of the tissue to be studied is important in ecotoxicological studies. Indeed, different stressor exposure signatures may be observed depending on the investigated tissue (Jordan et al., 2012). Insight into the metabolome of the different *M. galloprovincialis* tissues is therefore essential. Two studies already reported knowledge on mussel metabolome for the purpose of species discrimination (Rochfort et al., 2013; Utermann et al., 2018). The latter identified 76 major compounds dominated by pigments, alkaloids and polyketides in whole tissue extracts. As the digestive gland and gills are the tissues focused on most commonly in ecotoxicological studies, some metabolites have already been reported in these tissues following environmental contaminant exposure (Song et al., 2016; Dumas et al., 2020a; Dumas et al., 2022). Gonads have also been investigated to determine sex-specific differences in the mussel metabolome for the purpose of reproductive physiology studies (Cubero-Leon et al., 2012) or to highlight hormonal differences (Martínez-Pita et al., 2012). One NMR-based study provided an overview of concentrations of the main metabolites of three functional tissues (digestive gland, gills and posterior adductor muscle) (Cappello et al., 2018). A total of 44 metabolites were thereby reported, with 27 being common to all three tissues, at 0.1–100 mM concentration.

The purpose of this study was to give information on male and female mussel metabolome and particularly on mussel metabolites that can be accessible to the analysis using the most common sample preparation recommended for tissue analysis (i.e. Bligh & Dyer) (Vuckovic, 2020) and Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS). Mass spectrometry (MS) is more sensitive than NMR and may therefore offer new opportunities for the detection of minor yet important compounds. This study was also designed to estimate concentrations of the main metabolites of four different functional tissues of the Mediterranean mussel (*M. galloprovincialis*), namely digestive gland (DG), foot (F), gill (G) and gonad (Go) tissues and in the remaining soft tissues (S) which include mantle, muscles and palpes.

2. Material and methods

2.1 Chemicals

Ultrapure water was generated by a Simplicity UV system from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ.cm at 25°C. Pesticide analytical-grade solvents (methanol, dichloromethane and ethanol) and LC/MS grade solvents (water, acetonitrile, formic acid 99%) were obtained from Carlo Erba (Val de Reuil, France). Analytical pure standards were purchased from the four following suppliers: Sigma-Aldrich (now part of Merck), Santa Cruz Biotechnology, Toronto Research Chemicals and LGC Standards. All chemicals used in this study were analytical grade (> 95% purity).

2.2 Animals

Mytilus galloprovincialis mussels were obtained from a Mediterranean shellfish farm (Bouzigues, France) in February 2017. Twenty mussels were collected and uniformly selected according to their shell size (6-8 cm). They were depurated for 7 seven days in filtered seawater (provided by IFREMER, Palavas, France; filter GF/F Ø 100 µm) and dissected. Sex determination was done by microscopy and 3 mature females and 3 males were selected. Mussels were dissected on ice and five tissues were collected (digestive gland, foot, gill, gonad and soft tissues). The samples were directly frozen and stored at -80°C prior to analysis.

2.3 Tissue sample preparation

This study was performed on 5 different tissues with 3 replicates for male and 3 for female mussels (n = 30). Mussel tissues were freeze-dried and ground into a fine powder. The tissue sample preparation protocol was previously described by Bonnefille et al. (2018). A biphasic methanol/dichloromethane/water (16/16/13; *v/v/v*) solvent system was used. Briefly, 30 mg of each tissue sample (± 0.40 mg) were first homogenised and extracted in a glass tube with 240 µL of methanol and 75 µL of water and vortexed for 1 min. In a second step, 240 µL of dichloromethane and 120 µL of water were added and again vortexed. The samples were left at 4°C for 15 min and then vortexed and centrifuged at 2,000× *g* for 15 min at 4°C. 50 µL volumes of the supernatant (polar phase) were collected and evaporated to dryness under a nitrogen stream and finally re-suspended in 200 µL of water/acetonitrile (ACN) (95/5; *v/v*). The extracts were subsequently filtered directly into an analysis vial using a 0.20 µm PTFE syringe filter (Minisart SRP 4, Sartorius). Five quality control (QC) samples corresponding to each tissue were prepared by pooling 40 µL of each sample extract included in the experiment.

2.4 LC-HRMS analysis

The injections were randomly performed on a Vanquish HPLC (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (HESI). A reversed phase pentafluorophenylpropyl (PFPP) analytical column (100 × 2.1 mm; 3 µm particle size; Sigma Aldrich, PA, Bellefonte, USA) was used for LC separation. 10 µL volumes of each sample were loaded onto the column with full loop injection. The mobile phases were two mixtures of water/ACN (99/1; *v/v*) (phase A) or ACN/water (99/1; *v/v*) (phase B), both modified with 0.1% formic acid. The flow rate was 250 µL/min and the following gradient was applied (A/B): 95/5 from 0 to 3 min, 60/40 at 8 min, 50/50 at 9 min, 30/70 at 13 min and 5/95 from 15 to 18 min. From 18 to 21 min, the system was left to return to the starting conditions followed by a re-equilibration period (95/5) of 7 min (total run time, 28 min).

The Q Exactive HRMS was tuned to a mass resolution of 35,000 (FWHM, m/z 200), with a mass spectrum range of 50–750 m/z. Data were acquired simultaneously in both positive (ESI^+) and negative (ESI^-) ionization modes with the following settings: 3.35 |kV| spray voltage, 55 sheath gas flow rate, 10 aux gas flow rate, 50 S-Lens RF level, 300°C capillary temperature and 250°C heater temperature. Data independent acquisition (DIA) was also applied on the QC_{tissue} samples with a high energy collision dissociation (HCD) cell set at 20 eV, with a mass resolution of 17,500.

2.5 Quality control and semi-quantification

The QC samples were injected at the beginning, end and during the sequence to assess the analytical repeatability of the acquisitions. In order to estimate the concentrations of the identified metabolites in mussel tissues (ND: not detected; +/-: <0.1 mg/kg; +:0.1-1 mg/kg; ++:1-10 mg/kg; +++:10-100 mg/kg; ++++:>100 mg/kg), calibration curves were plotted by injecting increasing quantities of the pure target analytes from 0.015 to 15 ng injected on-column (corresponding to 0.01 to 10 mg/kg dw when considering a 30 mg sample size).

2.6 Data processing and statistical analysis

2.6.1 Data processing

Raw data were converted to .mzXML files with MSConvert freeware (ProteoWizard 3.0, Chambers et al., 2012). Data were then processed using the XCMS package (Smith et al., 2006) in the R environment. Two treatments were performed: all tissues together (n=30) and each tissue separately (n=6 for each tissue, n=3 per sex). Optimized XCMS parameters were used: 0.002 m/z interval for peak picking, signal-to-noise ratio threshold of 10, the group bandwidth of 15, and 0.5 minimum fraction. XCMS returned results as a peak table containing variable identity (i.e. m/z and retention time) and feature abundances (i.e. peak area). One file per tissue was generated and one file containing the peak integrations in all tissues. Each extracted ion chromatogram (EIC) was visually assessed to verify that each integrated signal corresponded to a Gaussian chromatographic peak. All features corresponding to baseline drift or background noise were discarded from the dataset.

2.6.2 Multivariate statistical analysis

Principal component analysis (PCA) was performed on the table containing all the tissues results (SIMCA 13.0.3., Umetrics, Sweden) in order to reduce the dataset complexity and highlight mussel tissue- and sex-specific metabolic differences. Datasets were log transformed and Pareto scaled prior to analysis.

2.7 Metabolite identification

Annotation was performed using an in-house database. This database consisted of 272 metabolites and their characteristics (chemical formula, log P, retention time Rt with the same chromatographic column, positive and negative adducts). Therefore, all annotations performed were of level 1 according to Sumner et al., (2007). Level 1 corresponds to annotation confirmed by injection of the analytical standard on the same analytical platform under the same conditions (validation based on both accurate mass and retention time). Moreover, standard addition was made in the matrix to confirm the retention time. Identified metabolites were assigned to subclasses and direct parents according to HMDB. For identified metabolites, integrations were performed again manually with Thermo FreeStyle software (Thermo Fisher Scientific, USA).

2.8 Molecular network

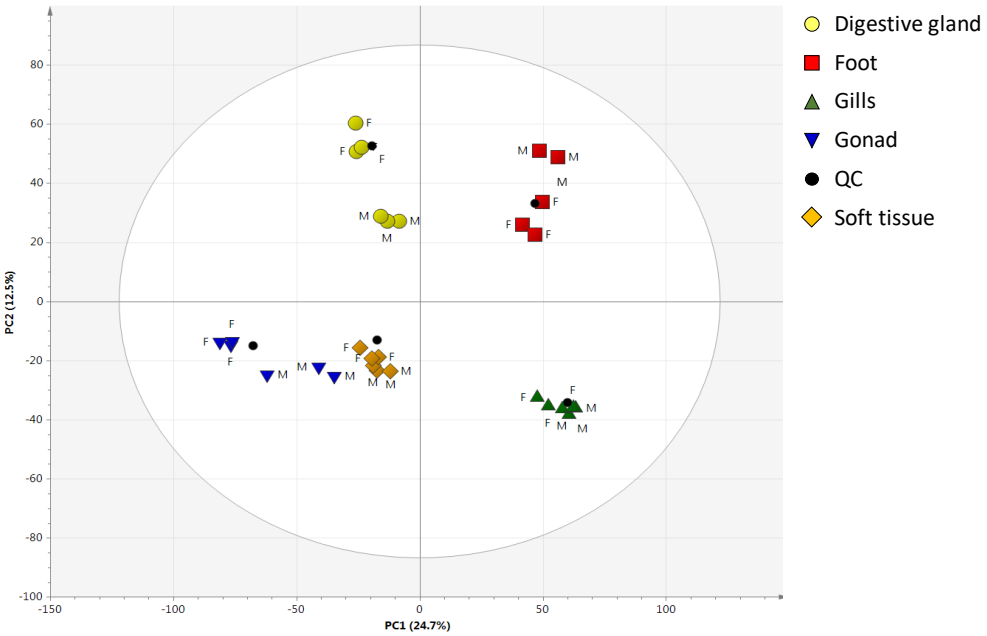
Using all MS/MS data (converted in mgf format with MZmine software) obtained for the five tissues, a molecular network was created using the Global Natural Products Social Molecular Networking (GNPS) online

workflow (http://gnps.ucsd.edu). With MS-Cluster, the data were clustered with a parent mass tolerance of 0.002 Da and a MS/MS fragment ion tolerance of 0.002 Da. A network was then created where the edges were filtered to have a cosine score > 0.6 with more than four matched fragment ions. Molecular networks were visualized using Cytoscape 3.6.0. (NRNB & NIGMS, USA).

3. Results

LC-HRMS analyses of the 5 male and female mussel tissues were carried out in both negative and positive electrospray ionization modes (ESI- & ESI+). The metabolic fingerprints provided 10,625 signals detected in ESI- and 8,878 signals in ESI+. The analytical repeatability was acceptable, as demonstrated on the PCA score plots in both ESI+ (Fig. 1) and ESI- (Supplementary Fig. 1), where the $QC_{tissues}$ samples (n=3 per tissue) were plotted together and along with their respective tissue samples.

Distinct clusters were observed between the 5 tissues (Fig. 1). According to the first principal component (PC1), and the second component (PC2), there was a clear separation between the metabolome of the 5 tissues. Moreover, a clear separation between male and female samples was noted in some tissues. Sexual metabolic differentiation in gonad tissues was visible according to PC1, whereas this differentiation was observable according to PC2 for digestive gland and foot tissues. Similar patterns were noted in ESI- (Supplementary Fig. 1).



194

195 ***Fig. 1*** *PCA score plot of ESI + metabolic fingerprints from digestive gland (yellow circle), foot (red square),*
196 *gill (green triangle), gonad (inverted blue triangle) and soft (orange diamond) mussel tissues showing a clear*
197 *separation between these tissues. In black (circle), the* QC_{tissue} *are shown. (Note that interpretation of the*
198 *references to colour in this figure legend are provided in the web version of this article)*

199

200 A molecular network was obtained, showing clusters of compounds with spectral similarities (Supplementary
201 Fig. 2). Interestingly, there were clusters shared between all tissues, thus demonstrating the absence of tissue
202 specificity for these metabolites, while other clusters appeared to be specific to one tissue (Fig. 2).

203

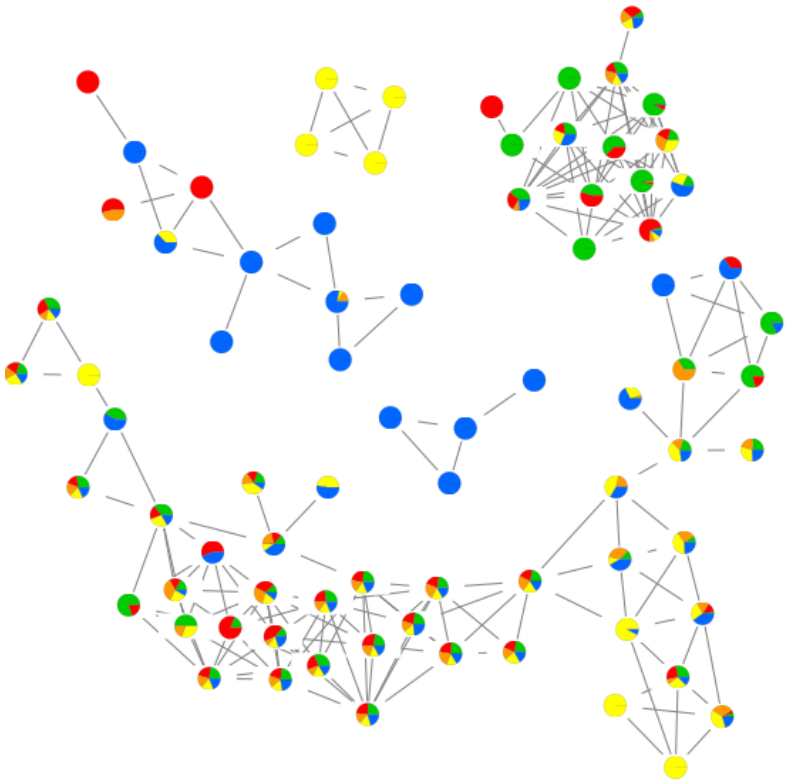


Fig. 2 *Small selection of clusters highlighted by GNPS analysis based on MS/MS fragmentation spectra obtained from the different Mediterranean mussel tissues. The color code is as follows: digestive gland (yellow), foot (red), gill (green), gonad (blue) and soft (orange) tissues. The relative intensity of the metabolite in each tissue is represented as a pie chart on each node*

Based on our in-house database, 110 metabolites were identified in the 5 tissues (87 in gill, 102 in gonad, 102 in digestive gland, 90 in foot and 105 in soft tissues). A list of metabolites identified in the different tissues with a distinction between male and female samples is given in Table 1, along with their characteristics in positive and negative ESI and their abundances. Some metabolites were found to be present in large quantities in all tissues compared to others, such as betaine, proline betaine and succinic acid (> 100 mg/kg dw). These metabolites were present at 100- and 50-fold higher concentrations than most of the other identified compounds. Other compounds were also noted at high concentrations specifically in one or more tissues, such as glycerophosphorylcholine, adenosine related compounds or deoxyribose. Three amino acids (glycine, arginine and L-aspartic acid) were also abundant, with concentrations of >100 mg/kg dw. A large proportion of the identified metabolites belonged to the amino acid subclass, such as serine, threonine and tyrosine metabolites. Purine and pyrimidine subclasses were also detected with many intermediates, including their respective

221 nucleotides and nucleosides, which were identified. Differences in abundance of specific metabolites were noted
222 when comparing male or female samples (Table 1, e.g. 2-methylglutaric acid in gonad tissues or adenosine in
223 foot, digestive gland and soft tissues). Indeed, as depicted in Fig. 1, gonad and digestive gland tissues showed
224 differentiation between male and female tissues.
225
226

227 *Table 1: Estimation of the concentrations of metabolites (mg/kg dw, ppm) observed in gill, gonad, digestive gland, foot and soft tissues in female and male mussels (mean*

228 *abundances for each sex; ND: not detected; +/-: <0.1 ppm; +:0.1-1 ppm; ++:1-10 ppm; +++:10-100 ppm; ++++:>100 ppm)*

Sub-class (HMDB)	Direct parent (HMDB)	Metabolite	Rt (min)	ESI +		ESI-		Abundances in tissues									
				Adduct	m/z	Adduct	m/z	Male gills	Female gills	Male Gonads	Female Gonads	Male digestive glands	Female digestive glands	Male feet	Female feet	Soft Male tissues	Soft Female tissues
Alcohols and polyols	Secondary alcohols	Pantothenic acid	2,94	$[M+H]^+$	220,1179	$[M-H]^-$	218,1034	+	+	++	++	+	+	+	+	++	++
Alkaloids and derivatives	Alkaloïds and derivatives	Trigonelline	1,55	$[M+H]^+$	138,0550	$[M+FA-H]^-$	182,0459	ND	+	++	++	++	++	+/-	+/-	++	++
Alkyl-phenylketones	Alkyl-phenyl ketones	DL-kynurenine	9,17	$[M+H]^+$	209,0921			+	+	+	+	+	+	+/-	+/-	+	+
		N-Acetyl-5-methoxykynuramine (AMK)	11,33	$[M+H]^+$	237,1234			ND	ND	+	+	+	+	+	+	ND	ND
Amines	2-arylethylamines	Histamine	5,34	$[M+H]^+$	112,0869			+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Amino acids, peptides and analogues	Alanine and derivatives / Alpha amino	L-Alanine/Sarcosine	1,49	$[M+H]^+$	90,0550	$[M-H]^-$	88,0404	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Alpha amino acids	Betaine	1,41	$[M+H]^+$	118,0863	$[M+FA-H]^-$	162,0772	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
		Glycine	1,37	$[M+H]^+$	76,0393	$[M-H]^-$	74,0248	+++	+++	++++	+++	++++	+++	++++	+++	++++	++++
		Pipecolic acid / pipecolinic acid	2,52	$[M+H]^+$	130,0863			+/-	+/-	+	+	+	+	+	+	+	+
	Alpha amino acids and derivatives	Pyroglutamic acid = 5-oxoproline	1,91	$[M+H]^+$	130,0499	$[M-H]^-$	128,0353	+	+	++	++	++	++	+/-	+/-	++	++
	Aspartic acid and derivatives	L-Aspartic acid	1,33	$[M+H]^+$	134,0448	$[M-H]^-$	132,0302	+++	+++	++++	++++	+++	++++	++++	++++	+++	++++
		N-Acetyl-L-aspartic acid	1,65	$[M+H]^+$	176,0553	$[M-H]^-$	174,0408	+	+	+	+	+	+	+	ND	+	+
	Beta amino acids and derivatives	(s)-b-aminoisobutyric acid	2,83	$[M+H]^+$	104,0706			+	+	++	+	+	+	+	+	+	+
		beta-Alanine	2,28	$[M+H]^+$	90,0550			+	+	++	++	++	++	++	++	++	++
		Gamma-l-glutamyl-l-leucine	9,91	$[M+H]^+$	261,1445	$[M-H]^-$	259,1299	+	+	+	+	+	+	+	+	+	+
	Gamma amino acids and derivatives	gamma-Aminobutyric acid (GABA)	2,58	$[M+H]^+$	104,0706			+	+	+	+	+	+	++	++	+	+
	Glutamic acid and derivatives	L-Glutamic acid	1,47	$[M+H]^+$	148,0604	$[M-H]^-$	146,0459	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		N-Acetyl-L-glutamic acid	1,92			$[M-H]^-$	188,0564	ND	ND	ND	+/-	+/-	+	ND	ND	+/-	ND
	Histidine and derivatives	L-Histidine	2,33	$[M+H]^+$	156,0768	$[M-H]^-$	154,0622	+	+	++	++	++	++	++	++	++	++
	Indolyl carboxylic acids and	L-Tryptophan	11,77	$[M+H]^+$	205,0972	$[M-H]^-$	203,0826	++	++	+++	+++	+++	+++	++	++	+++	+++
	Isoleucine and derivatives	L-Isoleucine	4,60	$[M+H]^+$	132,1019	$[M-H]^-$	130,0874	+	+	++	++	++	++	++	++	++	++
		N-Acetylisoleucine	9,72	$[M+H]^+$	174,1125	$[M-H]^-$	172,0979	ND	ND	+/-	+/-	+/-	+/-	ND	ND	+/-	+/-
	L-alpha amino acids	L-Allothreonine	1,35	$[M+H]^+$	120,0655	$[M-H]^-$	118,0510	++	++	+++	+++	+++	+++	++	+++	+++	+++
		L-alpha-Aminobutyric acid/2-aminoisobutyric acid	2,30	$[M+H]^+$	104,0706			++	++	++	+	++	+	++	++	++	+
		L-Arginine	2,62	$[M+H]^+$	175,1190	$[M-H]^-$	173,1044	+++	+++	++++	+++	++++	+++	++++	++++	++++	+++
		L-Glutamine	1,35	$[M+H]^+$	147,0764	$[M-H]^-$	145,0619	+	++	+++	+++	+++	++	++	++	+++	+++
		L-Homocysteic acid	1,13	$[M+H]^+$	184,0274			+	+	++	++	+	++	+	+	+	+
		L-Lysine	2,54	$[M+H]^+$	147,1128	$[M-H]^-$	145,0983	++	++	+++	+++	+++	++	++	++	+++	+++
		L-Ornithine	2,38	$[M+H]^+$	133,0972			+	+	++	++	++	+	++	++	++	++
		L-Threonine/L-Homoserine	1,43	$[M+H]^+$	120,0655	$[M-H]^-$	118,0510	ND	ND	++	+++	+++	++	++	++	++	++
		N6-Acetyl-L-lysine	2,01	$[M+H]^+$	189,1234	$[M-H]^-$	187,1088	+/-	+/-	+	+/-	+	+/-	+/-	ND	+/-	+/-
	Leucine and derivatives	L-Leucine	5,07	$[M+H]^+$	132,1019			++	++	++	++	+++	++	++	++	++	++
	Methionine and derivatives	L-Methionine	2,70	$[M+H]^+$	150,0583			+	+	+	ND	++	++	++	++	++	++
	N-acyl-L-alpha amino acids	N-Acetyl-L-alanine	1,98	$[M+H]^+$	132,0655	$[M-H]^-$	130,0510	+	+	+	+	+	+	+	+/-	+	+
		N-Acetyl-L-threonine	1,67	$[M+H]^+$	162,0761	$[M-H]^-$	160,0615	++	++	+	+	++	++	++	++	++	++
	Oligopeptides	Ophthalmic acid	2,02	$[M+H]^+$	290,1347	$[M-H]^-$	288,1201	+/-	+/-	+	+/-	ND	ND	ND	ND	+	+
	Peptides	Oxidized glutathione	2,27	$[M+2H]^+$	307,0833	$[M-H]^-$	611,1447	++	++	++	++	++	++	++	++	++	++
	Phenylalanine and derivatives	L-Phenylalanine	8,25	$[M+H]^+$	166,0863	$[M-H]^-$	164,0717	++	++	+++	+++	+++	+++	+++	+++	+++	+++
		N-Acetyl-L-phenylalanine	11,06	$[M+H]^+$	208,0968	$[M-H]^-$	206,0823	ND	ND	+/-	+/-	+/-	+/-	ND	ND	+/-	+/-
	Proline and derivatives	4-Hydroxyproline	1,33	$[M+H]^+$	132,0655	$[M-H]^-$	130,0510	+	+	++	++	++	++	+	+	++	++
		L-Proline	1,51	$[M+H]^+$	116,0706			++	++	+++	+++	+++	+++	++	++	+++	+++
		Proline betaine	1,59	$[M+H]^+$	144,1019	$[M+FA-H]^-$	188,0928	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Sub-class (HMDB)	Direct parent (HMDB)	Metabolite	Rt (min)	ESI +		ESI-		Abundances in tissues									
				Adduct	m/z	Adduct	m/z	Male gills	Female gills	Male Gonads	Female Gonads	Male digestive glands	Female digestive glands	Male feet	Female feet	Soft Male tissues	Soft Female tissues
	Serine and derivatives	L-Serine	1,35	$[M+H]^+$	106,0499	$[M-H]^-$	104,0353	++	++	+++	+++	+++	++	++	++	+++	+++
	Tyrosine and derivatives	3,4-Dihydroxy-L-phenylalanine (L-Dopa)	2,81	$[M+H]^+$	198,0761	$[M-H]^-$	196,0615	ND	ND	ND	ND	ND	ND	++	++	ND	++
		Gamma-glutamyltyrosine	7,65	$[M+H]^+$	311,1238			ND	ND	ND	+	+	+	ND	ND	ND	ND
		L-Tyrosine	3,65	$[M+H]^+$	182,0812	$[M-H]^-$	180,0666	++	+++	++++	+++	++++	+++	+++	+++	+++	+++
		N-Acetyl-L-tyrosine	8,44	$[M+H]^+$	224,0917	$[M-H]^-$	222,0772	ND	ND	+/-	+/-	+/-	+/-	ND	ND	+/-	+/-
	Valine and derivatives	L-Valine	2,43	$[M+H]^+$	118,0863	$[M-H]^-$	116,0717	++	++	++	++	++	++	++	++	++	++
		N-Acetylvaline	5,45	$[M+H]^+$	160,0968	$[M-H]^-$	158,0823	ND	ND	+	+	+/-	+	ND	ND	+/-	+/-
Benzene and substituted derivatives	2(hydroxyphenyl)acetic acids	Homogentisic acid	4,48			$[M-H]^-$	167,0350	ND	ND	ND	ND	ND	ND	+/-	ND	ND	+/-
	Benzoic acids	Phthalic acid	9,88	$[M-H_2O+H]^+$	149,0228	$[M-H]^-$	165,0193	+/-	+/-	+/-	+/-	+/-	+	+/-	+/-	+/-	+/-
	dimethoxybenzenes	Homoveratric acid	11,58	$[M+H]^+$	197,0808	$[M-H]^-$	195,0663	ND	ND	+/-	+/-	ND	ND	ND	ND	+/-	+/-
	Phenylacetamides	2-Phenylacetamide	9,30	$[M+H]^+$	136,0757			+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Benzoic acids and derivatives	Benzoic acids	Formylanthranilic acid	11,83	$[M+H]^+$	166,0499	$[M-H]^-$	164,0353	+/-	+/-	+	+/-	+/-	+	+/-	+/-	+	+
Carbohydrates and carbohydrate conjugates	Acylaminosugars	N-Acetyl-D-glucosamine	1,30	$[M+H]^+$	222,0972	$[M+Cl]^-$	256,0593	+++	+++	++	++	+++	+++	++	++	++	++
		N-Acetylmannosamine	1,32	$[M-H_2O+H]^+$	204,0861	$[M+FA-H]^-$	266,0881	+	+	+	+	++	++	+/-	ND	+	+
	Sugar acids and derivatives	Gluconic acid	1,21			$[M-H]^-$	195,0510	ND	ND	+	+	++	++	+	+	+	+
		Glyceric acid	1,32			$[M-H]^-$	105,0193	+	+	++	++	++	++	++	+	++	++
Carboxylic acids	Carboxylic acids	Glyoxylic acid	1,44			$[M-H]^-$	72,9931	++	++	++	++	++	++	+++	++	++	++
	Dicarboxylic acids and derivatives	Fumaric acid	2,17			$[M-H]^-$	115,0037	+	+	+	+	+	+	+	+	+	+
		Glutaric acid	2,77			$[M-H]^-$	131,0350	+	+	+++	+++	++	++	+	+	++	++
		Maleic acid	2,85			$[M-H]^-$	115,0037	+/-	+/-	+	+	+	+	+/-	+/-	+	+
		Malonic acid	1,65			$[M-H]^-$	103,0037	+	+	++	++	+	++	+	+	++	++
		Succinic acid	2,07	$[M+2Na-H]^+$	162,9978	$[M-H]^-$	117,0193	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	N-acetyl-2-arylethylamines	N-Acetylhistamine	3,26	$[M+H]^+$	154,0975			+/-	+/-	ND	ND	ND	ND	ND	ND	ND	ND
	Tricarboxylic acids and derivatives	Citric acid	1,55			$[M-H]^-$	191,0197	+	+	+	+	+	+	++	++	++	++
Fatty acid and conjugates	Long chain fatty acids	Tetradecanedioic acid	14,43			$[M-H]^-$	257,1758	+	+	+	+	+	+	+	+	+	+
	Medium chain fatty acids	Pimelic acid	9,18	$[M-H_2O+H]^+$	143,0697	$[M-H]^-$	159,0663	+	+/-	++	++	+	++	+/-	+/-	+	+
	Methyl-branched fatty acids	2-Methylglutaric acid	4,80			$[M-H]^-$	145,0506	+	+	++	+++	++	++	+/-	+/-	++	++
	Straight chain fatty acids	Succinic acid semialdehyde	9,64			$[3M-H]^-$	305,0878	ND	ND	+/-	+/-	ND	ND	ND	ND	+/-	+/-
Fatty acids esters	Acyl carnitines	L-Acetylcarnitine	6,32	$[M+H]^+$	204,1230	$[M+FA-H]^-$	248,1140	+	+	+	+	+	++	++	++	++	++
Glycerophospholipids	Glycerophosphocholines	Glycerylphosphorylcholine	1,20	$[M+H]^+$	258,1101	$[M+Cl]^-$	292,0722	+++	+++	+++	++++	++++	++++	+++	+++	+++	++++
Beta hydroxy acids and	Beta hydroxy acids and derivatives	L-Malic acid	1,44			$[M-H]^-$	133,0142	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Keto acids and derivatives	Alpha-keto acids and derivatives	Pyruvic acid	1,58			$[M-H]^-$	87,0088	+	+	+	++	++	+	+	+	++	++
	Medium chain keto acids and	Oxoadipic acid	1,92			$[M-H]^-$	159,0299	ND	ND	+	+	+/-	+/-	ND	ND	+/-	+
	Short-chain keto acids and derivatives	3-Methyl-2-oxovaleric acid	8,35			$[M-H]^-$	129,0557	ND	ND	ND	ND	+	+	+	+	+	+
Methoxyphenols	Methoxyphenols	Normetanephrine	5,38	$[M-H_2O+H]^+$	166,0857			ND	ND	+	+	ND	ND	ND	ND	+	+
Organic phosphoric acids and	Monoalkylphosphates	Phosphoglycolic acid	1,00	$[M+H]^+$	156,9897			+	ND	++	++	++	++	+	ND	++	++
Pentoses	Pentoses	Deoxyribose	1,29			$[M+FA-H]^-$	179,0561	+++	++	+++	+++	+++	++++	+++	+++	+++	+++
Phenethylamines	Phenetylamines	Phenylethylamine	14,10	$[M+H]^+$	122,0964			+/-	+/-	+	+	+/-	+/-	+/-	+/-	+	+/-
		Tyramine	8,50	$[M+H]^+$	138,0913			+/-	+/-	+/-	+	+/-	+/-	ND	ND	+/-	+/-
Phenols	1-hydroxy-2-unsubstituted	p-octopamine	3,67	$[M-H_2O+H]^+$	136,0751			+	+	++	++	++	++	+	++	++	++
Phenylacetaldehydes	Phenylacetaldehydes	3,4-Dihydroxymandelaldehyde	5,10			$[M-H]^-$	167,0350	ND	ND	+	+	++	++	ND	ND	++	++
		L-3-phenyllactic acid	10,88			$[M-H]^-$	165,0557	ND	ND	ND	+/-	+/-	+/-	+/-	ND	+/-	+/-
Phenylsulfates	Phenylsulfates	Dopamine 4-sulfate	2,39			$[M-H]^-$	232,0285	+	+	+	ND	+	+	+	+	+	+
Purine nucleosides	Purine 2'-deoxyribonucleosides	2'-Deoxyguanosine	2,48	$[M+H]^+$	268,1040	$[M-H]^-$	266,0895	+/-	ND	+	ND	+	+	ND	ND	+	ND
	Purine nucleosides	Adenosine	3,32	$[M+H]^+$	268,1040	$[M+FA-H]^-$	312,0950	++	++	++++	++++	++++	+++	+++	++	+++	++
		Guanosine	2,12	$[M+H]^+$	284,0989	$[M-H]^-$	282,0844	++	+	++	++	++	++	+	+	++	++
		Inosine	1,92	$[M+H]^+$	269,0880	$[M-H]^-$	267,0735	++	++	++	++	+++	+++	++	++	++	++
	Purine ribonucleoside diphosphates	Adenosine diphosphate (=ADP)	1,12	$[M+H]^+$	428,0367	$[M-H]^-$	426,0221	+++	+++	++++	++++	++++	++++	+++	++++	++++	++++

Sub-class (HMDB)	Direct parent (HMDB)	Metabolite	Rt (min)	ESI +		ESI-		Abundances in tissues									
				Adduct	m/z	Adduct	m/z	Male gills	Female gills	Male Gonads	Female Gonads	Male digestive glands	Female digestive glands	Male feet	Female feet	Soft Male tissues	Soft Female tissues
Purines and purines derivatives	Purine ribonucleoside	Adenosine monophosphate (=5'AMP)/3'AMP	1,31	$[M+H]^+$	348,0704	$[M-H]^-$	346,0558	++	++	++++	++++	+++	+++	+++	+++	+++	+++
	6-aminopurines	Adenine	3,03	$[M+H]^+$	136,0618	$[M-H]^-$	134,0472	+	+	++	++	+	+	+	+	++	+
	Hypoxanthines	Hypoxanthine	1,91	$[M+H]^+$	137,0458	$[M-H]^-$	135,0312	+	+	+	+	+	++	+	+/-	+	+
	Purines and purine derivatives	Guanine	2,46	$[M+H]^+$	152,0567	$[M-H]^-$	150,0421	+	+	++	+	+	+	+	+	++	++
	Xanthines	Xanthine	1,90	$[M+H]^+$	153,0407	$[M-H]^-$	151,0261	+/-	ND	+/-	+/-	+/-	+/-	+	+/-	+	+
Pyridinecarboxylic acids and derivatives	Nicotinamides	Niacinamide (=nicotinamide)	2,62	$[M+H]^+$	123,0553	$[M+FA-H]^-$	167,0462	+	+	++	+	+	+	+	+	++	++
	Pyridine carboxylic acids	Nicotinic acid (vitamine B3)	2,02	$[M+H]^+$	124,0393	$[M-H]^-$	122,0248	++	++	++	++	++	++	+	+	++	++
Pyrimidine nucleosides	Pyrimidine nucleosides	Cytidine	2,78	$[M+H]^+$	244,0928	$[M+FA-H]^-$	288,0837	+	+	++	+++	++	++	+	+	++	++
		Uridine	1,91	$[M+H]^+$	245,0768	$[M+FA-H]^-$	289,0677	++	++	++	++	++	+++	++	+	++	++
	Pyrimidine-2'-deoxyribonucleosides	Deoxycytidine	3,23	$[M+H]^+$	228,0979	$[M+FA-H]^-$	272,0888	ND	ND	+	+	+	+	ND	ND	+	+
		Deoxyuridine	1,96			$[M+FA-H]^-$	273,0728	ND	ND	+/-	ND	ND	+/-	ND	ND	+/-	ND
		Thymidine	2,71	$[M+H]^+$	243,0975	$[M+FA-H]^-$	287,0885	ND	ND	+	ND	+	+	ND	ND	+	ND
Pyrimidines and pyrimidine derivatives	Hydroxypyrimidines	Thymine	1,60	$[M+H]^+$	127,0502	$[M-H]^-$	125,0357	++	++	+++	+++	++	++	+	+/-	+++	++
	Pyrimidones	Cytosine	2,55	$[M+H]^+$	112,0505			+/-	+/-	+	+	+	+	ND	ND	+	+
		Uracil	1,64			$[M-H]^-$	111,0200	+	+	+	+	+	++	++	+	++	++
Pyrrolidones	Pyrrolidine-2-ones	2-Pyrrolidinone	2,03	$[M+H]^+$	86,0600			++	++	++	++	+	+	++	++	++	+
Quaternary ammonium salts	Carnitines	L-Carnitine	2,80	$[M+H]^+$	162,1125	$[M+FA-H]^-$	206,1034	++	++	++	++	++	++	++	++	++	++
Quinolines and derivatives	Quinoline carboxylic acids	Kynurenic acid	10,88	$[M+H]^+$	190,0499			ND	ND	+/-	+/-	+/-	+/-	ND	ND	+/-	+/-
Sulfinic acids	Sulfinic acids	Hypotaurine	1,22	$[M+H]^+$	110,0270	$[M-H]^-$	108,0125	++	++	ND	ND	+++	+++	+++	+++	++	++
Tryptamines and derivatives	Serotonins	Serotonin	10,78	$[M+H]^+$	177,1022			+	++	+/-	+/-	+	+/-	++	++	+	+

229

4. Discussion

The purpose of this study was to give information on mussel metabolome and particularly on mussel metabolites that can be accessible to the analysis using the most common sample preparation recommended for tissue analysis (i.e. Bligh & Dyer). Acquisitions were performed with LC-HRMS and identifications were conducted with an internal database (suspect screening approach).

Main metabolites belong to amino acids, carboxylic acids and purine nucleosides sub-classes

One hundred and ten metabolites were identified. The most detected subclasses were amino acids, carboxylic acids and purine and pyrimidine metabolites. Three amino acids were present in large quantities in tissues (glycine, L-aspartic acid and arginine) which had already been found in significant quantities in digestive glands according to Cappello et al. (2018). Betaine and proline betaine markedly prevailed over the other metabolites in all 5 tissues. Indeed, osmolytes such as betaine as well as some free amino acids, are the most abundant metabolites in *Mytilus galloprovincialis* according to Rochfort et al. (2013) and Cappello et al. (2018). These osmolytes are natural compounds that actively accumulate to allow the cells to adapt quickly to variations, such as osmotic stress (Pulliainen et al., 2010). Glycerophosphorylcholine has also been detected in large quantities, and is noteworthy for its osmolytic nature but also for its involvement, as a choline derivative, in cellular health (Kidd, 2005). Cappello et al. (2018) reported significant concentrations of glutamic acid, as also confirmed by our results. Indeed, glutamic acid is a precursor of glutamine, which is itself a constituent of oligopeptides such as glutathione. This amino acid has a major role in the protection mechanism against oxidative stress. Intermediates involved in energy metabolism (ADP, succinic acid, aspartic acid) are also widespread in the different tissues along with purine nucleosides.

Sensitivity of mass spectrometry is an asset

Metabolomics data on *M. galloprovincialis* are fragmentary, although some studies have already been conducted to gain knowledge on circulating metabolites in mussels (Belivermiş et al., 2020; Cappello et al., 2018; Rochfort et al., 2013; Utermann et al., 2018). Most of the metabolites detected by Cappello

et al. (2018) were identified in this study, along with those identified by Rochfort et al. (2013) and Belivermiş et al. (2020). Some other metabolites were specifically detected in this study, e.g. cytosine and DL-kynurenine. Cytosine is a pyrimidine base, found in DNA as a nucleotide. The kynurenine pathway is in Human connected to the immune system and the *de novo* synthesis of nicotinamide adenine dinucleotide (NAD+), which plays a critical role in many enzymatic redox reactions and in mitochondrial energy production (Castro-Portuguez & Sutphin, 2020). Immune system and energy metabolism disruption along with a down modulation of DL-kynurenine were observed in mussels exposed to a WWTP extract (Dumas et al., 2020b). The detection of such compounds was probably due to the higher detection sensitivity of mass spectrometry over NMR. Conversely, several compounds, such as glucose, glycogen, taurine and homarine which were found in high concentrations in the studies of Belivermiş et al. (2020), Cappello et al. (2018) and Rochfort et al. (2013), were not screened and reported in Table 1 since data on these particular compounds were not available in our internal database.

Specificity between tissues was observed

A certain specificity between tissues was observed as already described by Cappello et al. (2018). However, we cannot ensure that concentrations in tissues are not impaired by lower recoveries or matrix effects for certain specific metabolites. Some identified metabolites (Table 1) were specifically detected in one or two tissues. For example, L-dopa was detected in feet and soft tissues. This result was not surprising given the fact that L-dopa is a component of adhesive proteins that occur in high proportions in byssus tissues (Bandara et al., 2013) formed in a gland located in the foot. Serotonin, which was detected only in gills by Cappello et al. (2018) was detected predominantly in gills (and foot) in this study. Serotonin having several physiological roles, e.g. contraction or relaxation of the adductor muscles responsible for valves opening or closing, involvement in reproductive functions and ciliary activity, its detection in all the investigated tissues of this study was then not surprising. Many other detected metabolites seemed to be tissue-specific according to the molecular network results, where clusters specific to one tissue were observed (Fig. 2, Supplementary Fig. 2). Metabolic similarities between gonad and soft tissues were observed that may be due to the fact that mussels were collected in their reproductive period, during which the mantles of mussels (i.e. the main soft tissue component)

285 fill with gamete-containing follicles (Anantharaman & Craft. 2012). Since the metabolic content is to
286 some extent different between tissues, these metabolic differences should be taken into consideration
287 when studying stressor effects in this species. For a large number of metabolites, significantly higher
288 concentrations were observed in three tissues (digestive gland, gonad and soft tissues). In order to be as
289 representative as possible of the effects of stress in ecotoxicological studies (when no prior hypotheses
290 enable target tissue selection), a maximum number of metabolites should be detected, which is easier
291 when there are high tissue concentrations. Studying these three tissues will preferentially facilitate the
292 detection of many metabolites. In particular, soft tissue is the tissue with the greatest mass available for
293 analysis. However, it is less specific to a biological function due to its complex composition involving
294 many organs (mantle, various muscles, heart and mouth) and the results may hence be hard to interpret.
295 The digestive gland—where high concentrations are also observed and from which it is possible to
296 obtain a reasonable sample size for analysis (unlike gonad tissues) is thus a very interesting target tissue
297 in ecotoxicological studies. In particular, the digestive gland is a tissue commonly used to measure
298 biological parameters (Bonnefille et al., 2018; Campillo et al., 2019; Serra-Compte et al., 2019) because
299 it has a higher absorption and bioaccumulation capacity than other tissues (Faggio et al., 2018).
300
301 ***Sexual dimorphism was confirmed***
302 The sexual metabolic differentiation observed in gonads (Fig. 1) has already been described by Cubero-
303 Leon et al. (2012) and Martínez-Pita et al. (2012). Both highlighted quantitative differences in fatty acid
304 (Martínez-Pita et al., 2012) and eicosanoid (Cubero-Leon et al., 2012) compositions. These differences
305 were explained in terms of the specific needs of males and females during gametogenesis and/or of the
306 differences in sperm and oocyte composition. The sexual metabolic differentiation observed in gonad
307 and digestive gland tissue samples may explain the different responses between males and females
308 observed following contaminant exposure (Dumas et al.,2020b; Liu et al., 2014; Zhang et al., 2017;
309 Ramirez et al., 2022). Notably, a different response of male and female mussels to venlafaxine exposure
310 was demonstrated by our group (Ramirez et al., 2022) in digestive gland tissues. Male and female
311 mussels showed several sex-specific impacted metabolites as well as differences in metabolite
312 modulation, such as an increase (glycine) or decrease (L-tyrosine) in only one of the two sexes, thereby

implying that they have different protective mechanisms in response to stress, especially with regard to energy metabolism. These metabolites were quantified at different concentrations for the two sexes in our study. The sexual metabolic differentiation in non-exposed organisms—although we cannot confirm that the mussels were not exposed to a variety of contaminants in the Thau lagoon where they had been reared and that the 1-week depuration period in the lab was sufficient to recover a baseline metabolome—due to differences in their physiological needs may explain the sex-specific responses observed after stress exposure. Further studies on the metabolome of digestive glands and gonads in a large number of samples along with ecotoxicology studies on both sexes would be interesting to precisely explain these metabolic differences and thus enhance the overall understanding of sex-specific responses to pollutants.

5. Conclusion

One hundred and ten metabolites belonging to different metabolic pathways were identified via liquid chromatography high resolution mass spectrometry in five tissues collected from *Mytilus galloprovincialis*. All tissues were dominated by the osmolytes betaine and proline betaine, as well as intermediates that have a role in energy metabolism (ADP, succinic acid and aspartic acid). This study revealed marked differences between the 5 studied tissues. The choice of appropriate tissue according to the target objectives of ecotoxicological studies is thus important. Digestive gland, gonad and soft tissues showed higher levels of all metabolites. Sex-specific differences between the digestive gland and gonad tissues in relation to the specific needs and metabolisms of males and females were also observed. Overall, the results of this study will help enhance mussel tissue characterization, while also being useful for future metabolomics studies focused on the effects of environmental contaminants on *Mytilus* spp.

References

Anantharaman, S., & Craft, J. A. (2012). Annual Variation in the Levels of Transcripts of Sex-Specific Genes in the Mantle of the Common Mussel. Mytilus edulis. PLoS ONE, 7(11), e50861. https://doi.org/10.1371/journal.pone.0050861

Arribas, L. P., Donnarumma, L., Palomo, M. G., & Scrosati. R. A. (2014). Intertidal mussels as ecosystem engineers : Their associated invertebrate biodiversity under contrasting wave exposures. Marine Biodiversity, 44(2), 203-211. https://doi.org/10.1007/s12526-014-0201-z

Bandara, N., Zeng, H. & Wu, J. (2013). Marine mussel adhesion: biochemistry, mechanisms, and biomimetics. Journal of Adhesion Science and Technology 27, 2139–2162. https://doi.org/10.1080/01694243.2012.697703

Belivermiş, M., Swarzenski, P. W., Oberhänsli, F., Melvin, S. D., & Metian, M. (2020). Effects of variable deoxygenation on trace element bioaccumulation and resulting metabolome profiles in the blue mussel (Mytilus edulis). Chemosphere, 250, 126314. https://doi.org/10.1016/j.chemosphere.2020.126314

Bonnefille, B., Gomez, E., Alali, M., Rosain, D., Fenet, H., & Courant, F. (2018). Metabolomics assessment of the effects of diclofenac exposure on Mytilus galloprovincialis : Potential effects on osmoregulation and reproduction. Science of The Total Environment, 613-614, 611-618. https://doi.org/10.1016/j.scitotenv.2017.09.146

Campillo, J. A., Sevilla, A., González-Fernández, C., Bellas, J., Bernal, C., Cánovas, M., & Albentosa, M. (2019). Metabolomic responses of mussel Mytilus galloprovincialis to fluoranthene exposure under different nutritive conditions. Marine Environmental Research, 144, 194-202. https://doi.org/10.1016/j.marenvres.2019.01.012

Cappello, T., Giannetto, A., Parrino, V., Maisano, M., Oliva, S., De Marco, G., Guerriero. G., Mauceri, A., & Fasulo, S. (2018). Baseline levels of metabolites in different tissues of mussel Mytilus galloprovincialis (Bivalvia : Mytilidae). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 26, 32-39. https://doi.org/10.1016/j.cbd.2018.03.005

Cappello, T., (2020). NMR-Based Metabolomics of Aquatic Organisms. In: eMagRes. John Wiley & Sons, Ltd. 81–100. https://onlinelibrary.wiley.com/doi/abs/10.1002/9780470034590.emrstm1604.

Castro-Portuguez, R., Sutphin, G.L., (2020) Kynurenine pathway, NAD(+) synthesis, and mitochondrial function: Targeting tryptophan metabolism to promote longevity and healthspan. Experimental Gerontology, 132,110841. https://doi.org/10.1016/j.exger.2020.110841

Connor, K. M., & Gracey, A. Y. (2011). Circadian cycles are the dominant transcriptional rhythm in the intertidal mussel Mytilus californianus. Proceedings of the National Academy of Sciences, 108(38),16110-16115. https://doi.org/10.1073/pnas.1111076108

Connor, K. M., & Gracey, A. Y. (2012). High-resolution analysis of metabolic cycles in the intertidal mussel Mytilus californianus. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 302(1), R103-R111. https://doi.org/10.1152/ajpregu.00453.2011

Courant. F., Antignac. J.-P., Dervilly-Pinel, G., & Le Bizec, B. (2014). Basics of mass spectrometry based metabolomics. PROTEOMICS. 14(21-22), 2369-2388. https://doi.org/10.1002/pmic.201400255

Cubero-Leon. E., Minier. C., Rotchell. J. M., & Hill, E. M. (2012). Metabolomic analysis of sex specific metabolites in gonads of the mussel, Mytilus edulis. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 7(2), 212-219. https://doi.org/10.1016/j.cbd.2012.03.002

Dumas, T., Boccard, J., Gomez, E., Fenet, H. & Courant, F. (2020a). Multifactorial Analysis of Environmental Metabolomic Data in Ecotoxicology: Wild Marine Mussel Exposed to WWTP Effluent as a Case Study. Metabolites, 10, https://doi.org/10.3390/metabo10070269

Dumas, T., Bonnefille, B., Gomez, E., Boccard, J., Castro, N.A., Fenet, H. & Courant, F. (2020b). Metabolomics approach reveals disruption of metabolic pathways in the marine bivalve Mytilus galloprovincialis exposed to a WWTP effluent extract. Science of The Total Environment, 712, 136551. https://doi.org/10.1016/j.scitotenv.2020.136551

Dumas, T., Courant, F., Almunia, C., Boccard, J., Rosain, D., Duporté, G., Armengaud, J., Fenet, H., & Gomez, E. (2022). An integrated metabolomics and proteogenomics approach reveals molecular alterations following carbamazepine exposure in the male mussel Mytilus galloprovincialis. Chemosphere, 286, 131793. https://doi.org/10.1016/j.chemosphere.2021.131793

Dumas, T., Courant, F., Fenet, H., Gomez, E. (2022). Environmental Metabolomics Promises and Achievements in the Field of Aquatic Ecotoxicology: Viewed through the Pharmaceutical Lens. Metabolites 12, https://doi.org/10.3390/metabo12020186

Faggio, C., Tsarpali, V., & Dailianis, S. (2018). Mussel digestive gland as a model tissue for assessing xenobiotics : An overview. Science of The Total Environment, 636, 220-229. https://doi.org/10.1016/j.scitotenv.2018.04.264

Gracey, A. Y., & Connor, K. (2016). Transcriptional and metabolomic characterization of spontaneous metabolic cycles in Mytilus californianus under subtidal conditions. Marine Genomics, 30, 35-41. https://doi.org/10.1016/j.margen.2016.07.004

Jordan, J., Zare, A., Jackson. L.J., Habibi, H.R. & Weljie, A.M. (2012). Environmental Contaminant Mixtures at Ambient Concentrations Invoke a Metabolic Stress Response in Goldfish Not Predicted from Exposure to Individual Compounds Alone. Journal of Proteome Research 11, 1133–1143. https://doi.org/10.1021/pr200840b

Keskinbalta, M. A., & Çelik, M. Y. (2020). Proximate Composition of Freshwater Mussels (Unio Pictorum, Linnaeus 1758) in Karasustream, Sinop. Turkish Journal of Agriculture - Food Science and Technology, 8(9), 1948-1951. https://doi.org/10.24925/turjaf.v8i9.1948-1951.3584

Kidd, P. M. (2005). GlyceroPhosphoCholine (GPC), Mind-Body Nutrient for Active Living And Healthy Aging. 21.

Lin, C. Y., Viant, M. R., & Tjeerdema, R. S. (2006). Metabolomics : Methodologies and applications in the environmental sciences. Journal of Pesticide Science, 31(3), 245-251. https://doi.org/10.1584/jpestics.31.245

Liu, X., Sun, H., Wang, Y., Ma, M., & Zhang, Y. (2014). Gender-specific metabolic responses in hepatopancreas of mussel Mytilus galloprovincialis challenged by Vibrio harveyi. Fish & Shellfish Immunology, 40(2), 407-413. https://doi.org/10.1016/j.fsi.2014.08.002

Lockwood, B. L., Connor, K. M., & Gracey, A. Y. (2015). The environmentally tuned transcriptomes of Mytilus mussels. Journal of Experimental Biology, 218(12). 1822-1833. https://doi.org/10.1242/jeb.118190

López-García, E., Postigo, C., & López de Alda, M. (2019). Psychoactive substances in mussels : Analysis and occurrence assessment. Marine Pollution Bulletin, 146, 985-992. https://doi.org/10.1016/j.marpolbul.2019.07.042

Martínez-Pita, I., Sánchez-Lazo, C., Ruíz-Jarabo, I., Herrera, M., & Mancera, J. M. (2012). Biochemical composition, lipid classes, fatty acids and sexual hormones in the mussel Mytilus

galloprovincialis from cultivated populations in south Spain. Aquaculture, 358-359, 274-283. https://doi.org/10.1016/j.aquaculture.2012.06.003

Moreno-González, R., Rodríguez-Mozaz, S., Huerta, B., Barceló, D., & León, V. M. (2016). Do pharmaceuticals bioaccumulate in marine molluscs and fish from a coastal lagoon? Environmental Research, 146, 282-298. https://doi.org/10.1016/j.envres.2016.01.001

Pulliainen, K., Nevalainen, H., Väkeväinen, H., Jutila, K., & Gummer, C. L. (2010). An analytical method for the determination of betaine (trimethylglycine) from hair. International Journal of Cosmetic Science, 32(2), 135-138. https://doi.org/10.1111/j.1468-2494.2009.00554.x

Ramirez, G., Gomez, E., Dumas, T., Rosain, D., Mathieu, O., Fenet, H., & Courant, F. (2022). Early Biological Modulations Resulting from 1-Week Venlafaxine Exposure of Marine Mussels Mytilus galloprovincialis Determined by a Metabolomic Approach. Metabolites 12. https://doi.org/10.3390/metabo12030197

Rochfort, S. J., Ezernieks, V., Maher, A. D., Ingram, B. A., & Olsen, L. (2013). Mussel metabolomics—Species discrimination and provenance determination. Food Research International, 54(1), 1302-1312. https://doi.org/10.1016/j.foodres.2013.03.004

Serra-Compte, A., Álvarez-Muñoz, D., Solé, M., Cáceres, N., Barceló, D., & Rodríguez-Mozaz, S. (2019). Comprehensive study of sulfamethoxazole effects in marine mussels : Bioconcentration. enzymatic activities and metabolomics. Environmental Research, 173, 12-22. https://doi.org/10.1016/j.envres.2019.03.021

Song, Q., Chen, H., Li, Y., Zhou, H., Han, Q. & Diao, X. (2016). Toxicological effects of benzo(a)pyrene, {DDT} and their mixture on the green mussel Perna viridis revealed by proteomic and metabolomic approaches. Chemosphere, 144, 214–224. http://dx.doi.org/10.1016/j.chemosphere.2015.08.029

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W.-M., Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., … Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis : Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics, 3(3), 211-221. https://doi.org/10.1007/s11306-007-0082-2

Tomanek, L., & Zuzow, M. J. (2010). The proteomic response of the mussel congeners Mytilus galloprovincialis and M. trossulus to acute heat stress : Implications for thermal tolerance limits and metabolic costs of thermal stress. Journal of Experimental Biology, 213(20), 3559-3574. https://doi.org/10.1242/jeb.041228

Utermann, C., Parrot, D., Breusing, C., Stuckas, H., Staufenberger, T., Blümel, M., Labes, A., & Tasdemir, D. (2018). Combined genotyping, microbial diversity and metabolite profiling studies on farmed Mytilus spp. From Kiel Fjord. Scientific Reports, 8(1), 7983. https://doi.org/10.1038/s41598-018-26177-y

Vuckovic, D. (2020). Chapter 4 - Sample preparation in global metabolomics of biological fluids and tissues. In: Issaq HJ, Veenstra TD, editors. Proteomic and Metabolomic Approaches to Biomarker Discovery (Second Edition). Second Edition. Boston: Academic Press. 53–83. https://www.sciencedirect.com/science/article/pii/B9780128186077000049.

Yang C, Du X, Hao R, et al (2019) Effect of vitamin D3 on immunity and antioxidant capacity of pearl oyster Pinctada fucata martensii after transplantation: Insights from LC-MS-

based metabolomics analysis. Fish Shellfish Immunol 94:271–279. https://doi.org/10.1016/j.fsi.2019.09.017

Zhang J, Xiong X, Deng Y, et al (2021) Integrated application of transcriptomics and metabolomics provides insights into the larval metamorphosis of pearl oyster (Pinctada fucata martensii). Aquaculture 532:736067. https://doi.org/10.1016/j.aquaculture.2020.736067

Zhang, L., Sun, W., Zhang, Z., Chen, H., Jia, X., & Cai, W. (2017). Gender-specific metabolic responses in gonad of mussel Perna viridis to triazophos. Marine Pollution Bulletin, 123(1-2), 39-46. https://doi.org/10.1016/j.marpolbul.2017.09.032

Statements & declarations

Acknowledgements & funding: This research was funded by SANOFI France and the French Agence Nationale de la Recherche (IMAP ANR-16-CE34-0006-01). The authors thank the Platform Of Non-Target Environmental Metabolomics (PONTEM) of the Montpellier Alliance for Metabolomics and Metabolism Analysis (MAMMA) consortium facilities.
.

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Author contributions: Lea James: investigation, formal analysis and writing. Elena Gomez: supervision and review. Gaëlle Ramirez : investigation and review. Thibaut Dumas : investigation and review. Frédérique Courant: conceptualization, methodology, supervision and review.

Ethics declarations

Ethical approval: Ethical approval is exempted in accordance with the European Union directive on the protection of animals used for scientific purposes.

Consent to participate: Not applicable.

Consent to publish: All authors agree to publish.

Availability of data and materials: Not applicable.