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# Metabolomic analysis of sex specific metabolites in gonads of the mussel, *Mytilus edulis*

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ABSTRACT

Marine mussels have been used as sentinel organisms to monitor exposure to a variety of chemical contaminants, including endocrine disrupting chemicals, in the aquatic environment. Although they are an important species for use in ecotoxicology investigations, information on their reproductive physiology and biochemistry is fragmentary. Mass spectrometry-based profiling techniques are increasingly being used to study how the metabolome of an organism changes as a result of tissue differentiation, disease or in response to environmental stressors. In this study, ultraperformance liquid chromatography–time-of-flight-mass spectrometry (UPLC–TOFMS) was used to investigate sex specific differences in the mussel metabolome in order to further investigate the reproductive physiology of this species. Using this method, a comparison of female and male mantle tissues containing mature gonad, revealed significant differences in glycerophosphatidylcholine (PC) and lysophosphatidylcholine (LPC) metabolites. A number of other unidentified metabolites, including those putatively identified as conjugated sterols, were also differentially expressed between male and female mantle/gonadal tissue.



## 1. Introduction

Metabolomics, the most recent of the "omic" approaches, refers to the metabolite profile of a cell or a tissue (Fiehn, 2002) and has been previously used to investigate pollution responses in mussels (Jones et al., 2008). Transcript levels of a gene are not always correlated to protein expression and translated proteins are not always enzymatically active. Metabolomics, however, aims to profile the end products of the regulatory processes of a cell and hence is more closely related to the biological condition of an organism (Miracle and Ankley, 2005). However, no single analytical method has been capable of extracting and detecting all the different molecules present in a tissue at once. The challenges of detecting simultaneously the whole "metabolome" of an individual arise in the variety of chemical structures, the large range of concentrations at which metabolites are present in tissues or biofluids, and the capability of the analytical platforms that have been used for this type of work. For instance, high resolution time-of-flight mass spectrometry (TOFMS) using electrospray ionization (ESI) and coupled to chromatographic techniques such as ultra performance liquid chromatography (UPLC) has been successfully used for the analysis of steroid metabolites in fish tissues (Flores-Valverde and Hill, 2008; Flores-Valverde et al., 2010).

Molluscs are key indicators of environmental pollution and there is strong evidence obtained from field studies showing that they may be exposed to biologically active concentrations of endocrine disrupting compounds that have the potential to disrupt their sexual differentiation and fertility (Goldberg, 1986; Gibbs et al., 1991; Huet et al., 1996). However, in many cases, the mechanisms of action of endocrine disrupting chemicals in aquatic invertebrates are unclear, partly because the understanding of invertebrate reproductive physiology, including the biochemistry of sexual differentiation, is often limited and fragmented (LaFont, 2000). For example, vertebrate-type sex steroids including estradiol (E2), estrone (E1), testosterone (T2) and progesterone (P) have been detected in mussels, and although their levels in the gonad seem to correlate with gonadal development and maturation (Delongcamp et al., 1974; Reishenriques and Coimbra, 1990), their physiological roles have not been fully characterised. In vertebrates, glucuronation and sulphation of steroids are considered to play an important role in inhibiting their biological activity and increasing their excretion as well as serving as an inactive transport form, from which the active steroid may be regenerated. Even though these sulfotransferase and glucuronosyl transferase activities have been described in molluscs (Lavado et al., 2006) very little is known about the nature of the products formed. Amino-acid derived hormones, such as the neuropeptide Phe-Met-Arg-Phe amide (FMRF-$NH_2$) have been found in mollusc tissues,

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mainly in pedal, visceral ganglia and gonads (Henry et al., 1995; Lopez-Vera et al., 2008). The main functions of the FMRF-$NH_2$ and related peptides in molluscs have been described as modulation of synaptic transmission, modulation of visceral and somatic muscles, stimulation of the penis and retractor muscles in gastropods and regulation of egg laying in cephalopods (Price and Greenberg, 1989; Krajniak, 2005). Lipids and eicosanoid lipid metabolites are also present in high concentrations in gonadal tissues of *Mytilus edulis* (Lubet et al., 1986; Coffa and Hill, 2000; Rowley et al., 2005). During the reproductive period of mussel, *Mytilus gallovprovinciallis*, the majority of the lipid content of the mantle is associated with the gametes and the total lipid content in the female mantle is twice as high as in the male mantle (Lubet et al., 1986). In addition, seasonal variations in the fatty acid composition of lipids and different lipid reserves of female and male *M. edulis* have been observed (Lubet et al., 1986; Hines et al., 2007) suggesting an involvement of lipids in the reproductive physiology of this species. Eicosanoids such as hydroxy fatty acids and prostaglandins, together with monoamines have been associated with gamete maturation and spawning in a number of mollusc species (Martinez et al., 2000; Rowley et al., 2005). It is not clear whether there are other, as yet undetected, metabolites present in mollusc tissues, which may play a role in their reproductive physiology and gonadal differentiation.

In this study, we used solid phase extraction (SPE) and UPLC–TOFMS methodology to extract and profile a wide range of molecules from mantle tissue (which contains the gonads) of sexually mature *M. edulis*. An SPE fractionation approach was used to separate classes of metabolites with different polarities or charge in order to reduce ion suppression effects during MS analyses and to increase analytical sensitivity (Flores-Valverde and Hill, 2008; Flores-Valverde et al., 2010). After metabolite profiling in the mantle/gonadal tissue of *M. edulis*, the MS data was analysed by multivariate statistical methods in order to compare the biochemical profiles of male and female mussels and characterise key sex-specific metabolites.

## 2. Material and methods

### 2.1. Chemicals

Internal deuterated standards [2,4,16,16-d4] 17β-estradiol (E2-$d_4$) and [1,2-$d_2$] testosterone (T2-$d_2$) (isotopic purity > 98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA), while [2,2,4,6,6,17α-21,21,21-d9] progesterone (P-$d_9$), [2,4,16,16-d4] estrone (E1-$d_4$) and [2,4,16,16-d4] estrone sodium sulphate (E1-$d_4$–S) (isotope purity > 98%) were obtained from C/D/N isotopes (Quebec, Canada). All other standards were purchased from Sigma-Aldrich (Gillingham, UK). Sodium acetate and ammonium hydroxide were supplied by Sigma Aldrich and all solvents (HPLC grade) and the formic acid were purchased from Rathburn Chemicals (Walkerburn, UK). For the method development part of this study, test compounds (standard analytes) were chosen to represent free and conjugated steroids, peptides, and lipid metabolites. Stock individual standard solutions (1000 ng/μL) were prepared in methanol and stored at −20 °C. A working standard solution containing all the test compounds was obtained by further dilution to give concentrations of 1 ng/μL of free and conjugate steroids, 10 ng/μL of eicosanoids, and 100 ng/μL of peptides. A solution of the deuterated internal standards (IS) was prepared at 1 ng/μL methanol.

### 2.2. Sample collection

#### 2.2.1. Method development

To test the method performance in biological tissues, an analysis of the recoveries of standard analytes spiked into buffer only or mussel extracts was performed. To do that, aqueous buffered samples (20 mL of 5% methanol in sodium acetate buffer solution, pH 7.0) were prepared for spiking experiments. Mantle/gonadal tissue from 4 female and 4 male mussels (*M. edulis*) were collected in February 2007 from Brighton Pier (50° 49′ longitude and 0° 8′ latitude, Brighton, UK) and extracted and spiked as described in Section 2.5.1.

#### 2.2.2. Metabolomic profiling

For the metabolomic profiling experiment, mussel samples were taken on April 2007 from Brighton Pier. After collection, mussels were kept in seawater and immediately brought to the laboratory where they were placed in a glass tank with 60 L of artificial seawater (InstantOcean, Sarrebourg, France) at a light regime of 12 h light/12 h dark. The temperature of the water was kept at 9.3 ± 0.8 °C, dissolved oxygen at 10 ± 0.5 mg/L and the conductivity at 50 ± 1.3 mS/$cm^2$. The mussels were depurated and acclimatized for a period of 2 weeks without food to allow time for any xenobiotics present in the tissues to be eliminated (Magnusson et al., 2000; Labadie et al., 2007) and the water was renewed every 24 h. One of each pair of the mantle/gonadal tissue was taken, weighed, and stored in 1.5 mL of methanol at −70 °C for metabolomic analysis. The average mass of the mantle/gonadal tissue selected for metabolomic analysis was 0.7 ± 0.3 g for the male samples and 0.6 ± 0.3 g for the female samples. The remaining half of the pair of mantle tissue was kept in RNA*later*™ (Qiagen Ltd., Crawley, UK) and stored at −20 °C for species identification.

### 2.3. Determination of gonadal stages

For both spiking and profiling experiments mussels were sized, the sex identified under a light microscope and the gonadal stage determined by histological analysis. A piece of the mantle (approximately 0.5 $cm^2$) was fixed in 4% formaldehyde prior to histology processing. The tissue was then transferred to 30% sucrose solution overnight. 10 μm cryotome sections (Bright OTF5000, UK) were stained with hematoxylin/eosin and examined under light microscopy and the samples were divided into 4 stages: resting, early gametogenesis, mature and spawning stages (Cubero-Leon et al., 2010).

### 2.4. Species identification

Species of the genus *Mytilus* are morphologically similar and shell shape can be influenced by local environment making the identification of these species by morphological characteristics difficult. Differences in the length of a sequence in the nonrepetitive domain of the foot protein 1 agree with the taxonomic rank of species and seem effective for the identification of hybrids within the *M. edulis* complex (Inoue et al., 1995; Hines et al., 2007; Ciocan et al., 2011). Therefore, to identify the species of individual mussels, an amplification of a part of the nonrepetitive region of the foot adhesive protein 1 gene (GenBank accession no. **D50553**) by RT-PCR was carried out. cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Burgess Hill, UK) from total RNA extracted from mantle/gonadal tissue using NucleoSpin RNAII (Macherey–Nagel GmbH & Co., Düren, Germany) and following manufacturer's protocol. The sequences of the primers were Me-15 5′-CCAGTATACAAACCTGTGAAGA-3′ for the sense primer and Me-16 5′-TGTTGTCTTAATAGGTTTGTAAGA-3′ for the antisense primer. PCR was performed in a 10 μL reaction volume consisting of 1.2 μM of each sense and antisense primers, 3 μL of 1:100 diluted template and 5 μL of GoTaq Colourless Master Mix (Promega Corporation, Madison, WI, USA) containing 400 μM of dNTPs, 3 mM $MgCl_2$, 2× Reaction Buffer (pH 8.5) and GoTaq DNA polymerase. Amplifications were carried out in a Piko Thermal Cycler (Finnzymes Instruments, Vantaa, Finland) equipped with a heated lid. After preheating to 95 °C for 2 min, the reaction was subjected to 35 cycles of amplification. Each cycle consisted of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C followed by a final extension step of 5 min at 72 °C. The amplified products were then electrophoresed in a 2% agarose gel and visualised with ethidium bromide (stock solution 10 mg/mL). The expected sizes of the