Does exposure to testosterone significantly alter endogenous metabolism in the marine mussel *Mytilus galloprovincialis?*

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Abstract

Mussels (*Mytilus galloprovincialis*) were exposed to different concentrations of testosterone (T: 20, 200 and 2000 ng/L) in a semi-static water regime (1-day dosing intervals) for up to 5 days in an attempt to see whether endogenous steroid levels and steroid metabolism were altered by exogenous exposure to testosterone. Whole tissue levels of total-testosterone (free + esterified) sharply increased in a concentration-dependent manner, from 2 ng/g in controls to 290 ng/g in organisms exposed to the highest concentration. In contrast, levels of free-testosterone were only significantly elevated at the high-exposure group (5-fold increase respect to controls). Increased activity of palmitoyl-CoA:testosterone acyltransferase (ATAT) was detected in organisms exposed to the highest concentration of testosterone, while those exposed to low and medium concentrations showed significant alterations in their polyunsaturated fatty acids profiles. The obtained results suggest that esterification of the excess of T with fatty acids might act as a homeostatic mechanism to maintain endogenous levels of free-T stable. Interestingly, a dose-dependent decrease in CYP3A-like activity was detected in T-exposed mussels together with a significant decrease in the metabolism of the androgen precursor androstenedione to dihydrotestosterone (5α-DHT). Overall, the work contributes to the better knowledge of androgen metabolism in mussels.

Keywords: Testosterone, *Mytilus galloprovincialis,* testosterone acyltransferase, CYP3A, androstenedione, 5α-reductase, 17β-hydroxysteroid dehydrogenase, esterification

1. Introduction

The presence of vertebrate-type sex steroids in molluscs (e.g. progesterone, androstenedione (AD), testosterone (T), and estradiol (E2)) together with some enzymatic steps involved in sex steroid biosynthesis (e.g. 3β/17β-hydroxysteroid dehydrogenase –3β/17β-HSD–, 5α-reductase and P450-aromatase) has been demonstrated in several mollusc species (Reis-Henriques et al., 1990; Janer and Porte, 2007; Lafont and Mathieu, 2007). Nonetheless, the role of steroids in mollusc reproductive endocrinology has yet to be established. Annual variations in steroid content, which are often considered as an argument for their role in the control of reproductive activity, have often been reported. Such variations were detected for progesterone, AD and T in the mussel *Mytilus edulis*, but not for E2 (De Longcamp et al., 1974; Reis-Henriques et al., 1990). Mud snails *Ilyanassa obsoleta* showed an increase in free T levels at the onset and end of their reproductive phase concomitant with a decrease in the levels of esterified T (Gooding and LeBlanc, 2004). Injection of sex steroids (E2, T, progesterone, and dehydroepiandrosterone) into the sea scallop *Placopecten magellanicus* accelerated gonad differentiation and increased male/female ratio, with all four steroids acting similarly by inducing masculinization (Wang and Croll, 2004).

Despite a number of studies being performed to better understand the endocrine functions of steroids in molluscs, the knowledge is still fragmentary, and more information is needed regarding steroid biosynthesis, hormonal action and further catabolism. Thus, the androgen precursor androstenedione (AD) is readily converted to testosterone in vertebrates by the action of 17β-HSDs (Mindich et al., 2004). However, AD was mainly metabolized to 5α-reduced metabolites (5α-dihydroandrostenedione

(5α-DHA) and 5α-dihydrotestosterone (5α-DHT)) and minor amounts of T in mussels (Janer et al., 2005; Lavado et al., 2006). Significant differences both in terms of activity and metabolic profile have been reported for gastropods: AD was mainly converted to 5α-DHA by microsomal fractions isolated from *Bolinus brandaris* and *Marisa cornuarietis* whereas it was primarily metabolized to T by *Hexaplex trunculus* (Janer et al., 2006; Lyssimachou et al., 2009).

Moreover, based on analogy to vertebrates and insects, it is likely that P450s play a pivotal role in the physiology of molluscs by catalyzing the biosynthesis and oxidative metabolism of signal molecules, including steroids. However, as above, the information available so far is fragmentary. Digestive gland microsomes of the clam *Ruditapes decussata* catalyzed the oxidative metabolism of testosterone; the metabolites detected were AD, 5α-DHT, 5α-DHA, 6β-, 6α-, 2α- and 2β-hydroxytestosterone, estrone and 17β-estradiol (Morcillo et al., 1988). Among the hydroxylated metabolites, 6β-hydroxytestosterone was the predominant one. CYP3A has been reported as the major catalyst of 6β-hydroxylation of steroid hormones (progesterone and testosterone) in rat and fish (Miranda et al., 1989; 1991). Although information for invertebrates is still scarce, the presence of CYP3A-like genes and proteins in the mussel *Mytilus edulis* has been indicated (Wooton et al., 1995, Shaw et al., 2004) and the associated activity determined in freshwater mussels by using dibenzylfluorescein as a probe (Gagné et al., 2007).

Together with biosynthetic and oxidative pathways, conjugation reactions can play a key role in endocrine homeostasis and may regulate levels of active steroids in target tissues (Hochberg, 1998). Among those reactions, fatty acid esterification mediated by microsomal acyl-coenzyme A acyltransferases have been shown to regulate levels of free hormones in molluscs. Exposure to exogenous testosterone increased the

retention of testosterone as fatty acid esters in the mud snail *Ilyanassa obsoleta*, while unconjugated testosterone levels did not appreciably change (Gooding and LeBlanc, 2001). Similarly, exogenously administrated estradiol was extensively esterified by the mussel *Mytilus galloprovincialis*, whereas levels of free estradiol remained almost unaltered (Janer et al., 2005). Despite the key role of steroid acyltransferases in molluscs, little is known about the regulation of these enzymes, substrate specificity and/or the selective use of some fatty acyl-CoAs as substrates (Janer et al., 2004).

Within this context, the present study was designed to better characterize the response of the mussel *Mytilus galloprovincialis* to exposure to exogenous testosterone (20, 200 & 2000 ng/L for 5-days) by investigating the effect on those enzymatic pathways potentially involved in the synthesis, oxidative metabolism and conjugation of testosterone. Endogenous testosterone and estradiol were determined in the whole tissue of control and exposed mussels; a mild saponification step was included in the extraction protocol in order to distinguish between free and esterified steroids (released after saponification) (Gooding et al., 2003). CYP3A-like activity was determined in digestive gland microsomal fractions by using 7-benzyloxy-4-trifluoromethyl-coumarin as a fluorescent probe. 17β-Hydroxysteroid dehydrogenase (17β-HSD) and 5α-reductase were determined in digestive gland microsomal fractions, since previous studies have indicated their key role in the metabolism of the androgen precursor AD in mussels (Janer et al., 2005). Activity of palmitoyl-CoA:testosterone acyltransferase (ATAT) was determined together with fatty acid fingerprints in an attempt to better understand the regulation and physiological role of this enzymatic activity. Overall, this work aimed at investigating mussel endogenous metabolism and its modulation by exposure to different concentrations of testosterone.

2. Material and Methods

2.1. Chemicals

Unlabelled steroids were obtained from Sigma (Steinheim, Germany) and Steraloids (Wilton, NH, USA). Palmitoyl-CoA was obtained from Sigma (Steinheim, Germany). [1β-^{3}H]Androstenedione (15-30 Ci/mmol) was purchased from Perkin-Elmer Life Science Inc (Boston, MA, USA) and [4-^{14}C]testosterone (50-60 mCi/mmol) was obtained from Amersham (Buckinghamshire,UK). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany). Radioimmunoassay (RIA) kits for testosterone and 17β-estradiol were obtained from Radim, Inc (Pomezia, Italy).

2.2. Experimental design

Mussels (*Mytilus galloprovincialis*) (3 to 5 cm) were collected in December 2007 from the bivalve farms located in the Ebro Delta (NE Spain), carried to the laboratory and randomly placed into 50 L glass aquaria (30 mussels/tank) filled with filtered sea-water, and fitted with constant air bubbling. The mussels were acclimated in the laboratory for 2 days. Environmental conditions, i.e. temperature (18ºC), salinity (35‰) and photo-period simulated the original conditions of mussels.

After acclimation, mussels were exposed to different concentrations of testosterone: 20 ng/L (L; low), 200 ng/L (M; medium) and 2000 ng/L (H; high concentration). There were two set of controls: non-exposed mussels (C) and mussels exposed to 0.002% (v/v) triethyleneglycol which was used as a carrier (solvent control, SC). Water was changed every day and fresh testosterone added. Mussels were fed

every 48 h with a commercially available plankton preparation (Advanced Invertebrate 1, Marine Enterprises, INC, Baltimore, MD, USA). After a 5-day exposure, mussels were dissected; the digestive glands and the rest of the tissue stored at -80 ºC for determination of enzyme activities and steroid levels, respectively.

Water samples were collected immediately after dosing and 24 h later (on days 3rd and 5th). Approximately 100 ml of water was extracted with dichloromethane (3 x 20 ml), evaporated to dryness under nitrogen and stored at -20ºC until RIA analyses.

2.3. Sex hormone analysis

Tissue levels of free testosterone and estradiol were analysed as described in Janer et al. (2005). Briefly, tissue samples (1 g wet weight; n = 8) were homogenized in ethanol, and frozen overnight at –80 ºC. Homogenates were then extracted with 2 ml of ethyl acetate (x 3), the organic extracts recombined and reduced under a nitrogen stream. Dry residues were redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in 4 ml milli-Q water and passed through a C18 cartridge (Isolute, International Sorbent Technology, Mid Glamorgan, UK; 1 g, 6 ml), that had been sequentially pre-conditioned with methanol (4 ml) and milli-Q water (8 ml). After finishing the concentration step, cartridges were washed with milli-Q water (8 ml), dried and connected to a NH2 cartridge (Sep-Pack® Plus; Waters, Milfold, MA, USA). The C18-NH2 system was then washed with 8 ml *n*-hexane and the steroids eluted with 9 ml dichloromethane:methanol (7:3). This fraction was collected and evaporated to dryness.

Total testosterone and estradiol (free + esterified) were measured as described by Gooding et al. (2003), with some modifications. Tissue, homogenized as for free steroid determination (see above), was extracted with ethyl acetate (3 x 2 ml). The organic extract was evaporated under nitrogen, resuspended in 1.0 ml methanol containing 1% KOH, and incubated at 45 ºC for 3h. After the saponification step, milli-Q water (4.0 ml) was added, and the sample extracted with dichloromethane (3 x 3 ml).

The efficiency of the extraction and delipidation procedure was 74 ± 3% for testosterone and 80 ± 3% for estradiol (Morcillo et al., 1999). The recovery for the purification step (SPE cartridges), evaluated with radiolabelled steroids was in the range 95-97% for both testosterone and estradiol (Janer et al., 2005).

Dry extracts (tissue and water samples) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and assayed for testosterone and estradiol concentration using commercial RIA kits. Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were 30 pg/g for testosterone and 80 pg/g for estradiol in mussel tissue, and 0.1 ng/L testosterone in water. Intra-assay coefficients of variation were of 6.1% (T) and 3.3% (E_2). Inter-assay coefficients of variation were of 9.3% (T) and 3.5% (E_2).

2.4. Subcellular fractionation

Digestive glands (each sample a pool of 4 to 6 digestive glands) were homogenized in 4 ml of ice-cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 x *g* for 15 min, the fatty layer removed and the obtained supernatant centrifuged at 12,000 x *g* for

45 min. After centrifugation at 100,000 x *g* for 60 min, the resulting pellet was resuspended with the same buffer and centrifuged again at 100,000 x *g* for 30 min. Microsomal pellets were resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 20% (w/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. Protein concentrations were determined by the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

2.5. BFC-O-debenzyloxylase (BFCOD) activity

BFCOD (CYP3A-like) activity was determined according to the procedure described by Thibaut et al. (2006) and optimized for mussel digestive gland microsomes. The assay consisted in incubating 200 µg of digestive gland microsomal protein with 25 µM of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) –a characteristic CYP3A substrate– and 200 µM of NADPH in 100 mM potassium phosphate buffer pH 7.6 (final volume 250 µl) at 30ºC for 1h. The reaction was stopped by addition of 75 µl of 0.5 M Tris-base/acetonitrile (20:80, v/v) and after centrifugation (10,000 *g*/5 min), a 200 µl aliquot of the supernatant was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelength pairs of 409 and 530 nm using a microplate reader (Varioskan, Thermo Electron Corporation). Quantification was made using 7-hydroxy-4-(trifluoromethyl)-coumarin (HFC) as standard and the activity calculated as the amount of HFC (pmol) generated per milligram of protein per minute of reaction time.

2.6. Androstenedione metabolism

Assays were carried out by incubating 0.4 mg of digestive gland microsomal protein (n = 4) in 50 mM Tris-HCl buffer pH 7.4, 10 mM $MgCl_2$, and 0.1 µM ^{3}H-androstenedione (0.1 µCi). Assays were started by addition of NADPH (0.32 mM), and samples incubated for 60 min in a shaking water bath maintained at a 30ºC (final volume 250 µl). Incubations were stopped by adding 250 µl of acetonitrile, and after centrifugation (1500 x *g*, 10 min), 200 µl of supernatant were injected onto the RP-HPLC column.

2.7. Palmitoyl-CoA: testosterone acyltransferase activity

Palmitoyl-CoA:testosterone acyltranferase activity was determined by a modification of the method described by Janer et al. (2005). Digestive tube microsomal proteins (0.25 mg; n = 5) were incubated in 0.1 M sodium acetate buffer pH 6.0 with 5 µM [^{14}C]testosterone, 100 µM palmitoyl-CoA and 5 mM $MgCl_2$ in a final volume of 250 µl. The reaction was initiated by the addition of palmitoyl-CoA, and samples were incubated for 90 minutes at 30ºC. Reaction was stopped by adding 1 ml of ethyl acetate, and extracted 3 times. The ethyl acetate fraction was evaporated to dryness, the dry residue dissolved in 500 µl methanol, and 200 µl injected into the RP-HPLC system.

2.8. HPLC system

HPLC analyses were performed on a PerkinElmer Binary 250 LC pump system equipped with a 250 mm x 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 µm). Separation of [^{14}C]testosterone and its palmitoyl-ester was performed at

1.2 ml/min with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3), 13% acetonitrile and 31% methanol, and (B) 100% methanol. The run consisted of 9 min isocratic 100% A, 6 min of linear gradient from 100% A to 100% B, and 25 min isocratic 100% B. Separation of [^{3}H]androstenedione metabolites was performed at 1 ml/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 25% water and 75% acetonitrile. The run consisted on a linear gradient from 100% A to 100% B (0-30 min), isocratic mode at 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode at 100% A (5 min). Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks.

2.9. Fatty acid analysis

Total lipids from freeze dried mussels were extracted by the method of Folch et al. (1957), using chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. Lipid content was determined gravimetrically (Mettler Toledo, 0.1 mg). Total lipids (2 mg) were fractionated by thin layer chromatography (TLC) (Silica gel G 60, 20 x 20 cm glass plates; Merck, Darmstadt, Germany) using hexane:diethyl-ether:acetic acid (85:15:1.5) as a solvent system. Phospholipid bands (PL) were scraped and extracted with chloroform:methanol (2:1) containing 0.01% BHT. After the addition of nonadecanoic FA (Sigma, Poole, Dorset, UK) as internal standard, PL were subjected to acid-catalysed transmethylation for 16 h at 50ºC using toluene (1 mL) and 1% (vol/vol) sulphuric acid in methanol (2 mL) (Christie, 1982).

The FA methyl esters (FAME) were extracted with hexane:diethyl ether (1:1) and further purified by TLC using hexane:diethyl-ether:acetic acid (85:15:1.5) as a solvent system. The FAME were then analyzed with a gas chromatograph (Trace GC Ultra, Thermo Scientific, Madrid, Spain) equipped with a fused silica 30 m x 0.25 mm open tubular column (Tracer, TR-WAX; film thickness: 0.25 μm; Teknokroma, Barcelona, Spain) and a cold on-column injection system. Helium was used as a carrier gas and temperature programming was from 50 to 180ºC at 40ºC/min and then to 220ºC at 3ºC/min. Peaks were recorded in a personal computer using a software package (version 4.6.0.0. Azur, Datalys, St Martin d'Heres, France). Individual FAME were identified by reference to a well characterized fish oil standard and FAME 37 mix (Supelco, Sigma-Aldrich, Madrid, Spain), and the relative amount of each FA was expressed as a percentage of the total amount of FA in the analyzed sample.

2.10. Statistical procedures

Results are mean values ± SEM of n = 8 for whole tissue samples and n = 4-5 for pooled samples of 4 to 6 digestive glands. Statistical significance was assessed by using one-way ANOVA, followed by Dunnett's test for differences from control. Level of significance was $P \leq 0.05$. No statistically significant differences were detected among the two control sets (C and SC); therefore, only data from SC is presented in the paper.

3. Results

3.1. Testosterone concentration in water

Water testosterone levels determined in the experimental tanks right after dosing were close to nominal concentrations, *viz*. 17 ± 1, 157 ± 2 and 1294 ± 5 ng/ L (values are mean ± range of two different sampling days). However, 24 h after dosing, testosterone levels in water decreased to 14 ± 3, 74 ± 5 and 777 ± 23 ng/L. Detectable levels of testosterone, in the range of 0.3 – 1 ng/L, were recorded in the control tank.

3.2. Tissue steroid levels

Steroid levels were determined in the whole tissue of mussels, with or without a mild saponification step, in order to differentiate between free and esterified forms. No differences in free-testosterone levels were observed between SC, and L & M exposure groups (0.14 – 0.18 ng/g, w/w), whereas the H-group exhibited a significant 5-fold increase in free-testosterone levels (0.73 ng/g, w/w) (Fig. 1A). Nonetheless, total testosterone (free + esterified) increased in a dose-dependent manner, from 2.3 ng/g in SC to 290 ng/g in the H-group (Fig. 1B). Thus, individuals from tanks L, M & H had significantly high amounts of esterified testosterone that was released after saponification. In contrast, free (0.5 – 1.1 ng/g w/w) and total estradiol (8 – 12 ng/g w/w) levels did not differ between control and exposed groups (Fig. 1C).

3.3. Palmitoyl-CoA: testosterone acyltransferase (ATAT) activity

ATAT activity increased in a concentration-dependent manner in exposed mussels (Fig. 2). However, the increase was only statistically significant in organisms exposed to the highest concentration of testosterone (H: 392 ± 17 *vs*. SC: 287 ± 11

pmol/h/mg protein), which is in agreement with the high levels of esterified testosterone detected in mussels from tank H (Fig. 1B).

3.4. Fatty acid profile and lipid content

Total percentages of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids from the polar lipid fraction of control and T-exposed mussels together with fatty acid profiles are shown in Table 1. Palmitic (16:0) and stearic acid (18:0) were the predominant saturated fatty acids. Among the monounsaturaded, 20:1n-5 and 20:1n-9 were the most abundant. While eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, 22:2 NMID and 22:3 NMIT were prevalent among polyunsaturated fatty acids. Most of the polyunsaturated fatty acids underwent statistically significant changes after 5-days exposure to T. Thus, 20:3n-6; 20:3n-3; 22:2 NMID and 22:3 NMIT decreased in organisms exposed to L & M concentrations; while 20:4n-6 (arachidonic acid); 20:5n-3 and 22:6n-3 significantly increased in those organisms. However, no significant changes were detected in mussels exposed to the highest concentration of T, with the exception of 20:1-n3/20:2n-3 and 20:2n-6 that were minor components of the fatty acid mixture. Overall, polyunsaturated fatty acids, amounting to about 41–46% of total fatty acids, were more abundant than saturated (26–32%) and monounsaturated ones (13-14%). Percentage levels of total SFA, MUFA and lipid content were not altered by T-exposure ($P>0.05$). Percentage levels of total PUFA were not altered by T-exposure either, despite of the observed changes in individual fatty acids.

3.5. Androstenedione metabolism

Mussel digestive gland microsomes metabolized androstenedione into testosterone (T), 5α-dihydrotestosterone (5α-DHT) and 5α-dihydroandrostenedione (5α-DHA) (Fig 3). The synthesis of endogenous testosterone from androstenedione was not affected by T exposure (SC: 1.32 ± 0.29 pmol/h/mg protein; exposed organisms: 1.18 to 1.24 pmol/h/mg protein). Similarly, the formation of 5α-DHA –a 5α-reductase catalyzed pathway- was not significantly altered by exposure despite a tendency towards higher 5α-DHA formation rates in T-exposed mussels (1.35 to 1.73 pmol/h/mg protein) if compared to controls (0.74 ± 0.37 pmol/h/mg protein). However, a statistically significant decrease in the formation of 5α-DHT was detected in organisms from tank H (Fig. 3).

3.6. BFCOD activity

T-exposed mussels showed a decrease in BFCOD activity with increasing concentration of exposure; the decrease was statistically significant in organisms from tanks M & H (0.20 & 0.11 pmol/min/mg protein) when compared to controls (0.31 ± 0.05 pmol/min/mg protein) (Fig. 4).

4. Discussion

The analysis of water revealed daily oscillations in testosterone levels, which right after dosing were close to nominal concentrations (15-21% loss) in tanks L & M, and a higher loss (35%) in tank H, which could be attributed to the relatively low water

solubility of testosterone. Interestingly, testosterone concentrations in water decreased after 24 h of exposure (30, 63 and 61% loss in tanks L, M & H, respectively), an indication that the compound was taken up by the organisms along the exposure experiment. A low concentration of testosterone (0.3–1 ng/L) was detected in water from control tanks (C & SC); the source of the compound is uncertain as the experiment was carefully designed to avoid cross contamination between tanks. The excretion of minor amounts of testosterone into the water by mussels cannot be discarded as sulphate conjugates of testosterone have been previously described in molluscs (Ronis and Mason, 1996).

Regarding uptake of testosterone by exposed mussels, it is important to state that levels of free-testosterone in whole tissue of mussels did not increase with testosterone exposure, except for the highest exposure group that exhibited a significant 5-fold increase. Testosterone levels detected in control and exposed mussels were in the range of those naturally occurring along their reproductive cycle (0.1–1.4 ng/g) (C. Porte, unpublished data). However, when tissue extracts were saponified in order to release esterified steroids, mussels from tanks L, M & H showed a 3.4-, 16- and 140-fold increase in total testosterone (Fig. 1B). These results suggest that esterification of the excess of T with fatty acids might help to maintain endogenous levels of free-T stable. The increased esterification of T in exposed organisms was partially supported by the significantly high activity of palmitoyl-CoA:testosterone acyltransferase detected in organisms from tank H (Fig. 2). However, it is worth mentioning that estradiol levels (free and total) were similar in control and exposed organisms, suggesting that neither the synthesis nor the esterification of estradiol were altered by T-exposure (Fig. 1C). Similarly, in a previous experiment where mussels were exposed to estradiol -same range of concentrations- for 7-days, endogenous testosterone levels (free or esterified)

were not altered by exposure, while total-estradiol (free + esterified) sharply increased in a concentration dependent manner, from 2 ng/g in controls to 258 ng/g at the high exposure group (Janer et al., 2005). Results from both experiments suggest the existence of mechanisms that allow mussels to maintain their hormonal levels stable, and the important role that fatty acid esterification may play within those mechanisms.

Apolar conjugation was shown to be a major pathway of testosterone metabolism (more than 70%) in the snail *Ilyanassa obsoleta* exposed to 1μM [^{14}C]testosterone in water (Gooding and LeBlanc, 2001). Since then, several studies have indicated that esterification of testosterone with fatty acids might be a mechanism by which free steroid levels are regulated, and that hormone esterification could be a target for endocrine disrupters action (Gooding et al., 2003; Janer et al., 2006; Lyssimachou et al., 2008). Nonetheless, the physiological role of those steroid esters in molluscs and the mechanisms by which steroid esterification is regulated remain uncertain. In a previous work, we observed that oysters esterified E2 and DHEA with different fatty acyl-CoA moieties, including totally saturated (C18:0 and C16:0), monounsaturated (C18:1 and C16:1) and polyunsaturated fatty acids (C18:2 and C20:4) (Janer et al., 2004). Besides, several steroid fatty acids were tentatively identified to participate in endogenous esterification in oyster namely, palmitoleoyl- (C16:1), stearoyl- (C18:0), oleoyl/palmitoyl- (C18:1, C16:0) and linoeloyl- (C18:2), apart from some other esters that were not identified although their retention times suggested they might be polyunsaturated esters. Interestingly, palmitic acid (16:0) represented 19% of the total fatty acids detected in *M. galloprovincialis* and an increase –although not statistically significant–was observed in organisms exposed to the highest concentration of T (Table 1). Palmitic acid is one of the first fatty acids produced during lipogenesis and the main fatty acid in molluscs (Fuentes et al., 2009). Interestingly, testosterone was

reported to regulate lipid metabolism in the liver of *Oreochromis mosambicus* by stimulating the activity of lipogenic enzymes after 30 min exposure to 0.1 μg/g body wt. in vivo (Sunny et al., 2002). This is a rapid non-genomic response, independent of gene transcription that has not been reported in mussels so far, but it will be worth investigating.

Although the hypothesis that increases in the availability of certain fatty acids might modulate levels of esterified steroids is a challenging one, no support was obtained from the obtained data. In fact, almost no alteration in the fatty acid profile was observed in mussels from tank H when compared to controls, despite of the fact that those organisms showed the highest levels of esterified testosterone and the highest ATAT activity. In contrast, fatty acid profiles were mainly altered in mussels exposed to L & M testosterone concentrations (Table 1). Thus, a decrease in the synthesis of Non Methylene Interrupted (NMI) fatty acids was observed in organism from tanks L & M. These are the only long-chain PUFAs that mollusc can biosynthesize and may act as inhibitors of lipid peroxidation processes (Pirini et al., 2007). On the other hand, arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) significantly increased after T-exposure. C20 fatty acids, AA and EPA are substrates in the synthesis of biologically active eicosanoids (i.e. prostagladins, thromboxanes and leukotrienes), which exert different physiological actions in molluscs, including reproduction, e.g. stimulation of egg production and induction of spawning (Stanley-Samuelson, 1994). However, the question remains of why mussels exposed to the highest concentration of T showed almost not alteration in their fatty acid profile. One may hypothesize that at L & M concentrations, testosterone might behave as an endogenous steroid regulating key physiological functions in mussels, while at high concentration, T acts as a 'xenobiotic' (a) by significantly increasing ATAT as a

mechanism to 'inactivate' the excess of T that is taken up by mussels, and (b) by significantly altering androgen metabolism as well as the BFCOD activity.

Regarding androgen metabolism, mussels metabolized AD to similar amounts of T, 5α-DHT and 5α-DHA (0.7 to 1.5 pmol/h/mg protein). The synthesis of T and 5α-DHA remained unchanged in exposed mussels, while the formation 5α-DHT was significantly reduced in organisms exposed to the highest concentration of T. Conversely, Janer et al. (2005) reported a significant increase in the formation of 5α-reduced metabolites (5α-DHT and 5α-DHA) in mussels exposed to 200 & 2000 ng/L estradiol for 7-days. In mussels, 5α-DHT is mainly formed from 5α-DHA and not from testosterone (Janer et al., 2005). Thus, exposure to both testosterone and estradiol modulates 5α-reductase activity, with no significant effect on 17β-HSD. The reason why exposure to testosterone decreased and exposure to estradiol increased the synthesis of 5α-reduced androgens in mussels is intriguing. Whether this is a compensatory mechanism to respond to an androgenic/estrogenic exposure, the regulatory mechanisms involved as well as the potential physiological role of 5α–reduced androgens in mussels are some of the open questions. It is interesting to note that mussels from the present experiment had ripe gonads (Galimany et al., 2005) and the synthesis of 5α-DHA (0.7 to 1.7 pmol/h/mg protein) from AD was up to 50-fold lower than previously reported for mussels having mainly reabsorbing and resting gonads (20-35 pmol/h/mg protein) (Janer et al., 2005), which suggest the involvement of androgens, and possibly 5α-DHA, in gametogenesis.

Additionally, CYP3A activity, which is responsible for the 6β-hydroxylation of testosterone and thus participates in the turnover of androgen steroids in fish (James et al., 2005), was modulated by T exposure, leading to a concentration-dependent decrease in CYP3A-like activity in exposed mussels (Fig. 4). Interestingly, our results are in

agreement with fish experiments that showed that immature rainbow trout implanted with pellets containing T and then examined after 21 days responded with an increase in the hepatic P450 content but with decreases in steroid 6β-hydroxylation (Hansson, 1982; Hansson *et al.*, 1982). Although CYP3A-like activity has been previously described in mussels, this is the first report of decreased CYP3A-like activity as a consequence of testosterone exposure. However, BFC is just a probe substrate for CYP3A and so far, there is no information available on the metabolic pathways (hydroxylation of progesterone, testosterone, etc.) that could be inhibited in mussels as a consequence of T-exposure. In fact, CYP3A enzymes are among the most versatile forms of cytochrome P450 and target a variety of chemically diverse endogenous and exogenous lipophilic organic compounds, including steroid hormones, eicosanoids, retinoids and xenobiotics.

Overall, this works contributes to the better knowledge of testosterone metabolism in mussels and its modulation by exogenous exposure. The study provides the first evidence of CYP3A modulation in mussels following testosterone exposure and highlights fatty acid esterification as a key mechanism that allows mussels to maintain their hormonal levels stable following T exposure; this mechanism was only overloaded in mussels exposed to the highest concentration of T (2000 ng/L). On the other hand, fatty acid profiles from polar lipids were altered in mussels exposed to L & M concentrations of T, but not in mussels from tank H. It is therefore unlikely that changes in fatty acid availability modulate T-esterification. Further efforts are then needed to elucidate the mechanism by which testosterone esterification is regulated and to better understand the physiological role of steroid esters in molluscs.

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Figue 1. Whole tissue levels of (A) free testosterone, (B) total testosterone and (C) free and total estradiol in the whole tissue of control and testosterone-exposed mussels. Low: 20 ng/L; Medium: 200 ng/L; High: 2000 ng/L. Data expressed as mean ± SEM (n= 8). Significant differences with respect to controls indicated by *p<0.05 (one-way ANOVA followed by Dunnett's test).

Figure 2. Palmitoyl-CoA:testosterone acyltransferase activity in control and testosterone-exposed mussels. Low: 20 ng/L; Medium: 200 ng/L; High: 2000 ng/L. Data expressed as mean ± SEM (n= 5). Significant differences with respect to controls indicated by *p<0.05 (one-way ANOVA followed by Dunnett's test).

Figure 3. Metabolism of androstenedione by digestive gland microsomal fractions of control and testosterone-exposed mussels. Low: 20 ng/L; Medium: 200 ng/L; High: 2000 ng/L. Formation of testosterone (T), 5α-dihydrotestosterone (5α–DHT) and 5α-dihydroandrostenedione (5α–DHA). Data expressed as mean ± SEM (n= 4).

Figure 4. (A) BFC-*O*-debenzyloxylase (BFCOD) activity in control and testosterone exposed mussels. Low: 20 ng/L; Medium: 200 ng/L; High: 2000 ng/L. Data expressed as mean ± SEM (n= 4). (B) In-vitro effect of testosterone on BFCOD activity following incubation of digestive gland microsomal fractions with different concentrations of testosterone Values expressed as percentage of control activity as mean ± SEM (n= 5). Significant differences with respect to controls indicated by *p<0.05 (one-way ANOVA followed by Dunnett's test).

Table 1. Fatty acid profile in the digestive gland of mussels *Mytilus galloprovincialis* exposed to different concentrations of testosterone for 5-days. Data expressed as percentage of polar fatty acids (mean ± SEM; n=6). *Significant differences respect to control. [a]These fatty acids could not be definitely identified and information on the number and position of double bounds is not provided.

Fatty acid	Control	20 ng/L	200 ng/L	2000 ng/L
14:0	0.40 ± 0.11	0.28 ± 0.05	0.26 ± 0.06	0.36 ± 0.09
14:1	0.14 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.02
15:0	0.70 ± 0.05	0.67 ± 0.07	0.82 ± 0.05	0.80 ± 0.09
16:0	19.85 ± 0.84	18.94 ± 1.38	18.69 ± 0.61	22.53 ± 0.89
16:1n-9	0.22 ± 0.05	0.42 ± 0.07	0.38 ± 0.12	0.32 ± 0.05
16:1n-7	0.31 ± 0.15	0.77 ± 0.33	0.40 ± 0.16	0.67 ± 0.34
16:2	0.40 ± 0.29	0.11 ± 0.03	0.11 ± 0.01	0.10 ± 0.01
17:0	1.87 ± 0.10	1.50 ± 0.14	1.33 ± 0.42	1.97 ± 0.10
16:3	0.24 ± 0.05	0.40 ± 0.04	0.31 ± 0.05	0.23 ± 0.06
16:?[a]	4.20 ± 0.73	6.62 ± 0.59	8.04 ± 1.04*	4.86 ± 1.17
18:0	6.06 ± 0.29	6.11 ± 0.32	5.60 ± 0.12	6.62 ± 0.33
18:1n-9	0.74 ± 0.07	0.80 ± 0.08	0.89 ± 0.10	0.67 ± 0.07
18:1n-7	1.54 ± 0.14	1.77 ± 0.12	1.38 ± 0.10	1.69 ± 0.18
18:2n-6	0.43 ± 0.04	0.58 ± 0.01	0.55 ± 0.03	0.44 ± 0.07
18:3n-3	0.34 ± 0.05	0.56 ± 0.04*	0.44 ± 0.04	0.39 ± 0.07
18:4n-3	0.28 ± 0.07	0.39 ± 0.04	0.41 ± 0.06	0.28 ± 0.03
20:0	0.09 ± 0.01	0.09 ± 0.02	0.15 ± 0.07	0.08 ± 0.005
20:1n-11	1.58 ± 0.15	1.25 ± 0.18	1.61 ± 0.14	1.67 ± 0.19
20:1n-9	3.27 ± 0.15	2.89 ± 0.37	3.05 ± 0.10	3.55 ± 0.12
20:1n-7	0.85 ± 0.03	0.97 ± 0.06	0.83 ± 0.03	0.90 ± 0.06
20:1n-5	4.21 ± 0.18	3.20 ± 0.19*	4.36 ± 0.27	3.75 ± 0.27
20:1n-3/20:2n-3	1.49 ± 0.03	1.04 ± 0.05*	1.32 ± 0.12	1.07 ± 0.13*
20:2n-6	0.23 ± 0.01	0.26 ± 0.02	0.25 ± 0.02	0.36 ± 0.04*
20:3n-6	0.77 ± 0.15	0.58 ± 0.07*	0.50 ± 0.08*	0.85 ± 0.06
20:3n-3	1.30 ± 0.12	0.81 ± 0.16*	0.81 ± 0.12*	1.18 ± 0.14
20:4n-6	2.62 ± 0.30	3.22 ± 0.28	3.79 ± 0.21*	2.07 ± 0.25
20:4n-3	0.41 ± 0.03	0.29 ± 0.03	0.26 ± 0.04*	0.38 ± 0.04
20:5n-3	4.26 ± 1.04	7.10 ± 1.01*	6.05 ± 0.67*	3.75 ± 0.98
22:0	0.35 ± 0.07	0.24 ± 0.03	0.32 ± 0.03	0.35 ± 0.11
22:2 NMID	7.70 ± 0.41	5.71 ± 0.58*	6.98 ± 0.43	6.96 ± 0.44
22:2n-6/22:4n-6	0.18 ± 0.03	0.10 ± 0.01*	0.11 ± 0.01	0.13 ± 0.02
22:?[a]	0.60 ± 0.05	0.52 ± 0.06	0.82 ± 0.08	0.53 ± 0.05
22:3 NMIT	14.90 ± 1.50	9.42 ± 0.96*	9.07 ± 1.61*	14.02 ± 1.79
22:5n-3	0.46 ± 0.22	0.57 ± 0.08	0.43 ± 0.20	0.29 ± 0.15
22:6n-3	4.45 ± 1.08	8.35 ± 1.65*	6.62 ± 0.94*	4.19 ± 1.21
ΣSFA	28.33 ± 1.30	27.29 ± 1.60	26.43 ± 1.03	32.27 ± 1.32
ΣMUFA	14.29 ± 0.41	13.26 ± 0.81	14.40 ± 0.51	14.44 ± 0.71
ΣPUFA	44.98 ± 1.53	44.38 ± 0.78	46.51 ± 1.19	41.78 ± 1.24
Σn-3	12.76 ± 2.15	18.74 ± 2.39	16.05 ± 1.51	11.37± 2.18
Σn-6	4.23 ± 0.30	4.53 ± 0.15	5.16 ± 0.19*	3.85 ± 0.23
Total lipid (% of dry weight)	10.81 ± 0.81	10.55 ± 0.57	10.05 ± 0.38	9.93 ± 0.50

Figure 1.

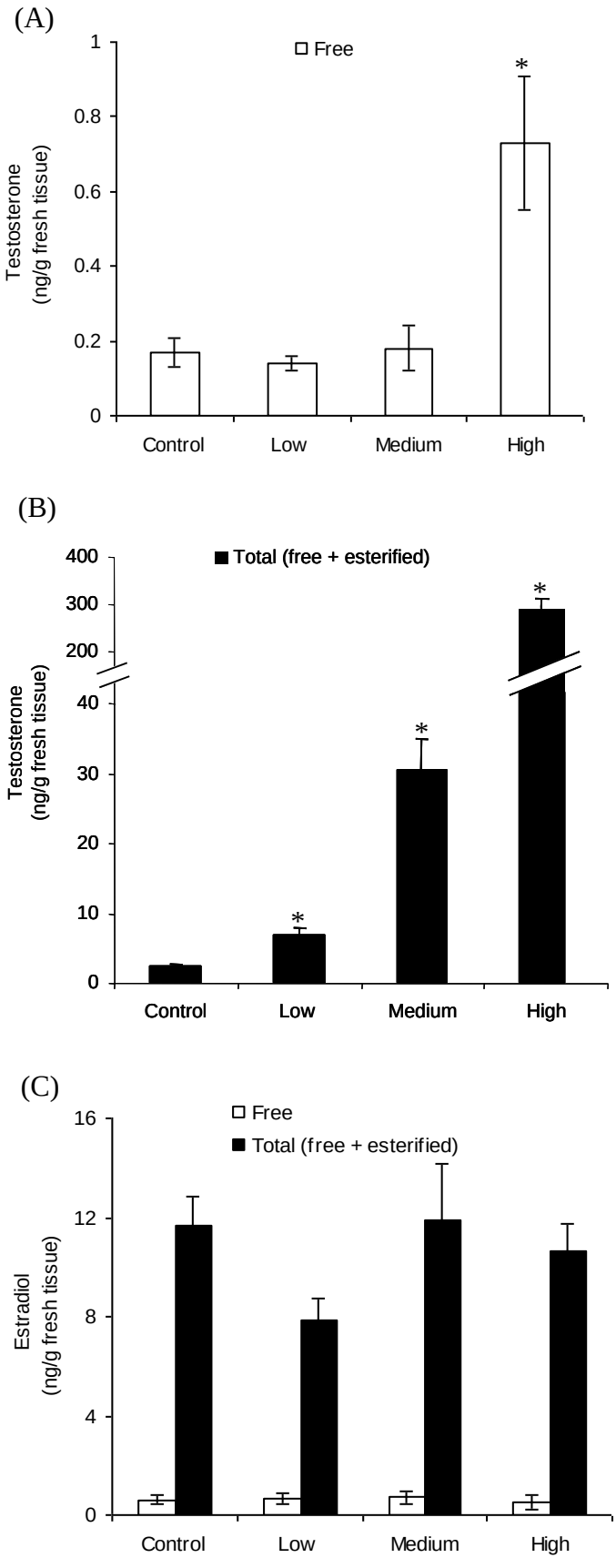
(A)
Free
Testosterone (ng/g fresh tissue)
1
0.8
0.6
0.4
0.2
0
*
Control
Low
Medium
High
(B)
Total (free + esterified)
Testosterone (ng/g fresh tissue)
400
300
200
40
30
20
10
0
*
*
*
Control
Low
Medium
High
(C)
Free
Total (free + esterified)
Estradiol (ng/g fresh tissue)
16
12
8
4
0
Control
Low
Medium
High

Figure 2.

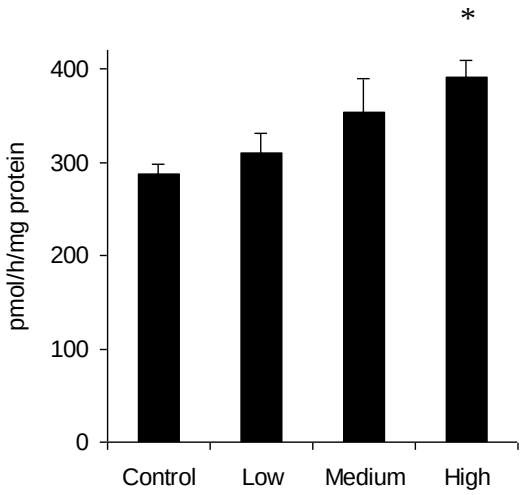

Figure 3.

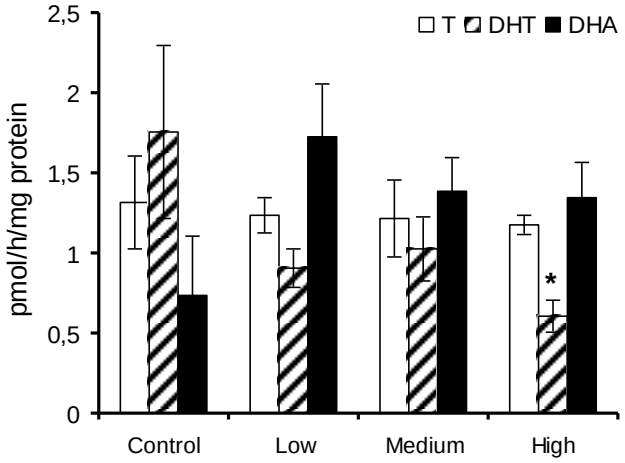
T
DHT
DHA
pmol/h/mg protein
2,5
2
1,5
1
0,5
0
*
Control
Low
Medium
High

Figure 4.