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Review

Biosynthesis and metabolism of steroids in molluscs☆

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ABSTRACT

Molluscs are the second most diverse animal group, they are ecologically important and they are considered excellent indicators of ecosystem health. Some species have been widely used in pollution biomonitoring programs; however, their endocrinology is still poorly known. Despite some studies reporting the presence of (vertebrate-type) steroids in molluscs, information regarding enzymatic pathways involved in steroid synthesis and further catabolism of those steroids is still fragmentary. Regarding steroidogenesis, a number of excellent studies were performed in the 70s using different radio-labelled steroid precursors and detecting the formation of different metabolites. But, since then a long gap of research exist until the late 90s when the 'endocrine disruption' issue raised the need of a better knowledge of mollusc (and invertebrate) endocrinology in order to assess alterations caused by pollutants. Here we summarize past and recent studies dealing with steroid biosynthesis and metabolism in different mollusc species. Most of these studies suggest the involvement of steroids in mollusc reproduction. However, the knowledge is still fragmentary and many questions remain to be answered.

Contents

1. Introduction

An increasing number of studies show that sex steroids are widespread in molluscs [1]. It was initially thought that they were taken up through the diet as many plant species contain vertebrate-like sex steroids [2]. However, several studies have demonstrated that the main classes of molluscs, i.e. cephalopods, gastropods and bivalves, are able to synthesize sex steroids from precursors such as cholesterol or pregnenolone [3–5]. Actually, most of the steroidogenic pathways described for vertebrates have been demonstrated to occur in molluscs either by directly exposing the organisms to steroid precursors or by incubating homogenates with those steroid precursors. Together with steroidogenesis, steroid metabolism plays an important role in the regulation of endogenous steroid levels. Most of the enzymes involved in steroid metabolism can metabolize a variety of steroids and, some of them (e.g., hydroxylases, phase II enzymes), can also metabolize a wide range of structurally unrelated molecules. An overview of the major pathways of steroidogenesis and steroid metabolism reported in different mollusc species is given in Fig. 1, and these pathways are described in detail below. Usually, incubation of tissue extracts or subcellular fractions with labelled vertebrate-type precursors have been undertaken, and although information is fragmentary and comes from different species, most crucial enzymatic activities have been detected.

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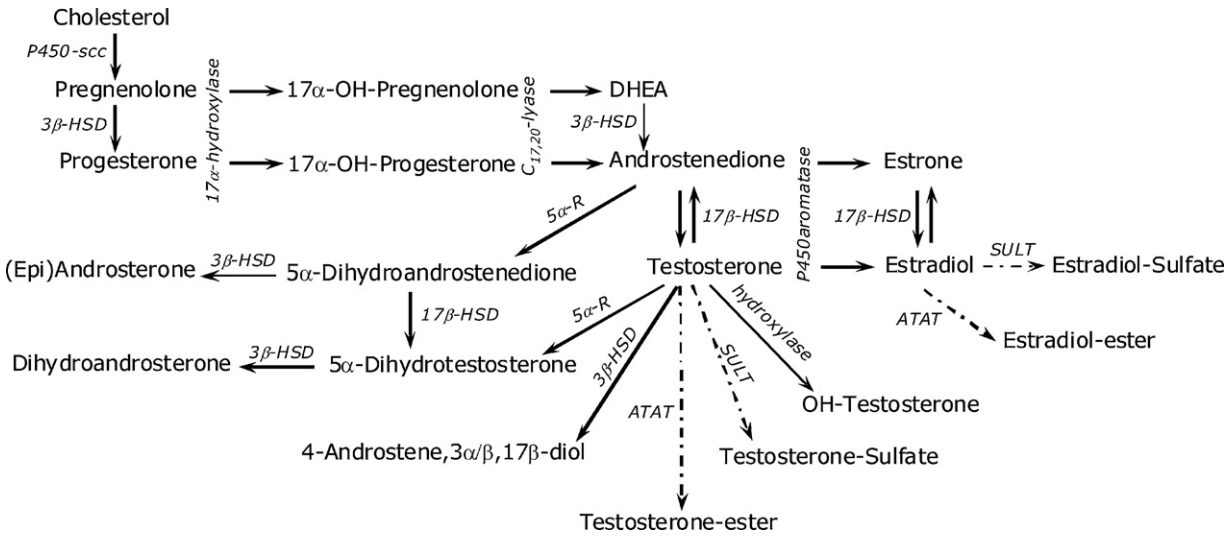

Fig. 1. Steroidogenic and metabolic pathways described in molluscs through the measurement of the metabolism of labelled steroids. P450scc: P450 side chain cleavage; 3β- or 17β-HSD: 3β- or 17β-hydroxysteroid dehydrogenases; 5α-R: 5α-reductase; ATAT: fatty acid acyl-CoA acyltransferase; SULT: sulfotransferase; DHEA: dihydroepiandrosterone; dihydroandrosterone: 5α-androstane-3α,17β-diol. (➖ · ➖ · ➖ ➤) phase II metabolism of steroids.

2. P450 side chain cleavage (P450scc)

In vertebrates, P450scc converts cholesterol to pregnenolone by catalyzing three distinct sequential reactions on a single active site. First, cholesterol undergoes 20-hydroxylation, then 22-hydroxylation followed by scission of the 20,22 C–C bond to yield both pregnenolone and isocaproaldehyde [6]. While a P450scc homolog has not yet been identified in molluscs, side chain cleavage of labelled precursors has been detected. The conversion of cholesterol to pregnenolone was first described by Gottfried and Dorfman [7], although with a very low efficiency. They incubated cholesterol in the presence of 700 × *g* supernatant fractions obtained from the male phase ovotestis of *Ariolimax californicus* and detected the formation of pregnenolone (Fig. 1). More recently, Alonso Martínez et al. [8] examined the localization of P450scc in different tissues of the mussel *Mytilus galloprovincialis* along a gonadal cycle by using a polyclonal antibody against rat P450scc. Interestingly, immunoreactivity specific for P450scc was found only in the cytoplasm of basophilic cells from the digestive gland; SDS-gel electrophoresis and western blot analysis revealed that this protein was mainly located in the microsomal fraction. In bivalves, as well as in other molluscs, the ability to synthesize cholesterol does not exist or is very low [9]; therefore, the authors assumed that the cholesterol content was diet dependent, and that an exogenous origin of cholesterol could explain the presence of P450scc in the digestive system and the lack of immunoreactivity in gonadal tissues of *M. galloprovincialis*. However, the unusual subcellular location of the enzyme in microsomes instead of in the inner mitochondrial membrane as in mammals and the use of heterologous antisera for immunohistochemistry, makes necessary to ascertain the identity of this P450scc recognized protein and its role in pregnenolone biosynthesis.

3. 3α/β-Hydroxysteroid dehydrogenases (3α/β-HSDs)

3β-Hydroxysteroid dehydrogenases (3β-HSDs) are enzyme complexes that catalyze both the reduction/oxidation of the 3-keto/hydroxyl and the Δ5–Δ4-isomerization of steroids, such as the conversion of pregnenolone to progesterone. They catalyze the transformations of 5-ene-3β-hydroxy-steroids (Δ5 steroids) into 4-ene-3-oxosteroids (Δ4 steroids) and are involved in the synthesis of all classes of active steroids [10]. The Δ5–Δ4-isomerase activity associated with 3β-HSD was also demonstrated by Gottfried and Dorfman [7]; the authors detected the formation of progesterone in 700 × *g* supernatant fractions of male phase ovotestis from the land slug *A. californicus* using pregnenolone as substrate (Fig. 1). De Longcamp et al. [11] demonstrated the presence of this enzymatic complex in gonad homogenates of the marine mussel *Mytilus edulis* by using pregnenolone, 17α-hydroxypregnenolone, and dehydroepiandrosterone (DHEA) as precursors, but the conversion to the 4-en,3-keto products was rather low. This enzyme complex was also found in the gonad and hepatopancreas of the marine gastropod *Aplysia depilans* [12], in the testis of the cephalopod *Octopus vulgaris* [13] and in the dorsal body complex, ovotestis and buccal ganglia of the land snail *Helix pomatia* [14] among other molluscs. These findings comprise a series of preliminary indications of steroidogenesis in different mollusc species and tissues. Interestingly, Krusch et al. [14] detected higher 3β-HSD activity in the ovotestis of *H. pomatia* before oviposition than after using dehydroepiandrosterone as a substrate. However, 30 years later, the physiological significance of these results remains to be investigated.

In contrast, the 3α-HSD family of enzymes catalyzes the reduction/oxidation of the keto/hydroxyl group at the 3α-position. They generally act upon 5α-reduced steroids and play a central role in regulation of steroid levels by inactivating 5α-dihydrotestosterone (DHT) to 5α-androstane-3α,17β-diol, a weak androgen in vertebrates [15]. Although 5α-reduced steroids have been described in molluscs, the presence of 3α-HSD has not been definitely depicted. Several studies have identified 3α/β-hydroxylated metabolites in molluscs, but, they did not distinguish between 3α- and 3β-isoforms [16–18]; e.g. Morcillo et al. [16] described the formation of 5-androstene-3α(β),17βdiol from testosterone by microsomal fractions isolated from the digestive gland of the clam *Ruditapes decussata*.

4. 5α-Reductases

These enzymes act upon steroids containing a 4-en,3-keto configuration by reducing the double bond in the A ring. Thus, androstenedione (AD) and testosterone (T) can be converted to 5α-dihydroandrostenedione (DHA) and 5α-dihydrotestosterone (DHT), respectively. 5α-Reduction precludes the aromatization of androgens to estrogens and promotes intracellular accumulation

Table 1
Evidences of 5α-reductase activity in different molluscs species. AD: androstenedione; T: testosterone; DHA: 5α-dihydroandrostenedione; DHT: 5α-dihydrotestosterone.

Species	Tissue (conc. protein)	Substrate	Cofactor	Metabolites formed	Methods[a]	References
Gastropods						
Marisa cornuarietis	Gonad digestive gland complex (microsomes, 150–400 μg)	[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	DHA: 0.6–14.4 (males) pmol/h/mg prot. DHA: 1.1–48.6 (females) DHT: 0.7–46.4 (males) DHT: 2.7–27.1 (females)	HPLC/TLC/GC–MS (EI)	[19–21]
		[^{14}C]-T (10 μM; 150,000 dpm)		DHT: n.d.		[22–24]
Littorina littorea	Digestive gland (microsomes)	[^{14}C]-T: 7 mM	NADPH (1 mM)	DHT: 401 ± 174 pmol/h/mg protein	TLC	[17]
			Absence of NADPH	DHT: n.d.		
Helix aspersa	Gonad homogenate	[^{3}H]-AD (10 μCi)	NAD^+, NADPH mix (2 mM)	DHT: 6.8% (juveniles) DHT: 4.4 ± 0.4% (adults)	HPLC-RD/TLC/GC–MS (EI)	[25]
Hexaplex trunculus	Gonad-digestive gland complex (microsomes, 200 μg)	[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	DHT: n.d. (males and females) DHA: n.d. (males and females)	HPLC-RD	[26]
Bolinus brandaris	Gonad-digestive gland complex (microsomes, 200 μg)	[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	DHT: 6.5–56 pmol/h/mg protein (males) DHT: 8.7–127 pmol/h/mg protein (females) DHA: 428–1693 pmol/h/mg protein (males) DHA: 192–949 pmol/h/mg protein (females)	HPLC-RD	[26]
Bivalves						
Ruditapes decussata	Digestive gland (microsomes, 1.5–2 mg)	[^{14}C]-T (18 nmol; 1 μCi)	NADPH (0.5 mM)	DHT: 9.5 ± 0.7 pmol/h/mg protein DHA: 83.5 ± 55.0 pmol/h/mg protein	TLC	[16]
			Absence of NADPH	DHT: 0 DHA: 8.0 ± 3.1 pmol/h/mg protein		
Mytilus galloprovincialis	Digestive gland (microsomes, 0.4 mg)	[^{3}H]-AD (0.1 μM; 0.1 μCi)	NADPH (320 μM)	DHA: 25 pmol/h/mg protein (stage 0 – not developed gonads) DHT: 1.8 pmol/h/mg protein (stage 0 – not developed gonads)	HPLC-RD	[21]

n.d. = not detected.
[a] HPLC-RD or -ECD: high performance liquid chromatography-radiometric detector or -electrochemical detector; TLC: thin layer chromatography; GC–MS (EI): gas chromatography–mass spectrometry (electron impact mode).

of androgens [19]. In molluscs, 5α-reductase activity has been demonstrated when exposing the gastropods *Clione antarctica* and *Ilyanassa obsoleta* to progesterone, AD or T *in vivo* [18,20]. In addition, a 5α-reductase activity was reported by incubating T or AD with digestive gland microsomes from the bivalves *R. decussata* [16] and *Mytilus* sp. [21], the gastropods *Littorina littorea* [17] and *Marisa cornuarietis* [22–24], and with gonad whole homogenates of the gastropod *Helix aspersa* [25] (see Table 1 for details on the substrates and methods used). Although 5α-reductase plays a key role in the metabolism of AD in molluscs, significant differences, both in terms of activity and metabolic profile, have been observed, confirming the wide diversity of this invertebrate phylum. Thus, Lyssimachou et al. [26] reported that AD was mainly metabolized to DHT by microsomal fractions isolated from the gastropod *Bolinus brandaris*, whereas no evidence of 5α-reductase activity was detected in *Hexaplex trunculus*. Additionally, sex differences in the metabolism of AD were detected in *B. brandaris* where the formation of DHA occurred at a higher rate in males than in females, while the opposite trend was reported for *M. cornuarietis* [22–24,26].

5. 17β-Hydroxysteroid dehydrogenases (17β-HSDs)

The last steps of steroid synthesis and its primary metabolism are catalyzed by 17β-HSDs. These enzymes catalyze the reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids using NAD(P)H or NAD(P)+ as cofactor: e.g. they catalyze the intercoversion of androstenedione and testosterone, estrone and 17β-estradiol, or androstanedione and dihydrotestosterone. In vertebrates, 17β-hydroxy forms of androgens and estrogens are active steroids, while those with a 17-keto group have substantially less activity [27]. Most 17β-HSDs belong to the short chain dehydrogenase/reductase family of enzymes, which are known to be

Table 2
Detection of 17β-HSD activity in different mollusc species. AD: androstenedione; T: testosterone; E1: estrone; E2: estradiol; DHEA:dihydro-epiandrosterone.

Specie	Tissue (conc. protein)	Substrate	Cofactor	Metabolites formed	Methods[a]	References
Gastropods						
Marisa cornuarietis	Gonad-digestive gland complex (microsomes 150–400 μg)	[^{14}C]-T (10 μM; 150,000 dpm)	NADPH (1 mM)	AD: 0.8 ± 0.1 pmol/h/mg protein	HPLC-RD/TLC/GC–MS (EI)	[22–24]
		[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	T: 4.9–7.4 pmol/h/mg protein (males) T: 0.6–0.8 pmol/h/mg protein (females)		
Ilyanassa obsoleta	Gonad-digestive gland complex and viscera	[^{14}C]-T (40 μM; 80,000 cpm)	Not specified	AD: 40 pmol/h/g	TLC/scintillation counting	[18]
Hexaplex trunculus	Gonad-digestive gland complex (microsomes 200 μg)	[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	T: 12–27 pmol/h/mg protein (males) T: 12–40 pmol/h/mg protein (females)	HPLC-RD	[26]
Bolinus brandaris	Gonad-digestive gland complex (microsomes 200 μg)	[^{3}H]-AD (0.2 μM; 150,000 dpm)	NADPH (1 mM)	T: n.d. (males) T: 4–76 pmol/h/mg protein (females)	HPLC-RD	[26]
Helix aspersa	Gonad whole homogenates	[7n-^{3}H]-AD (10 μCi)	NAD^+, NADPH mix (2 mM)	T: 60% (juveniles) T: 7% (adults)	HPLC-RD/ TLC/GC–MS (EI)	[25]
Bivalves						
Crassostrea gigas	Ovarian tissue homogenate	E1: 0.5 μg E2: 0.5 μg	NADPH, $NADP^+$, NADH, NAD^+ mix (5 μM)	E2: 15–80 ng/g E1: 20 ng/g	HPLC-ECD	[29]
	Gills, digestive gland, gonad-mantle (tissue homogenates)	[4-^{14}C]-AD, [4-^{14}C]-T, [4-^{14}C]-E2, [4-^{14}C]-E1 (0.1–1 μM; 0.5–5 × 10^6 dpm)	NADPH (100 μM)	E2 ⇄ E1[b] T ⇄ AD	HPLC-RD/TLC	[30]
Crassostrea virginica	Sperm preparations	[4-^{14}C]-E2: 0.01–0.1 μCi [4-^{14}C]-T: 0.01–0.1 μCi	Diphospho-pyridine solution (20 mM)	E1: 122 μM/10^{10} cells AD: 18.3 μM/10^{10} cells	PC	[31]
Mytilus edulis	Gonad whole homogenates (500 mg)	[7α-^{3}H]-DHEA: 16 Ci [7α-^{3}H]-AD: 65 Ci [4-^{14}C]-T: 2 Ci [^{14}C]-E_1: 1 Ci [6,7-^{3}H]-E_2: 5 Ci	NADPH-generating system	Androstenediol: 6.6–7.3% T: 1.5–2.2% AD: 0.37–0.44% E2: 45–50% E1: 11.7–15.3%	TLC/PC	[11]
Mytilus galloprovincialis	Digestive gland (microsomes 400 μg)	[^{3}H]-AD (0.1 μM; 0.1 μCi)	NADPH (320 μM)	T: 0.9 pmol/h/mg protein	HPLC-RD	[21]
Ruditapes decussata	Digestive gland (microsomes 1.5–2 mg)	[^{14}C]-T (18 nmol; 1 μCi)	NADPH (0.5 mM) Absence of NADPH	AD: 749.2 ± 67.7 pmol/h/mg protein AD: 14.5 ± 9.2 pmol/h/mg protein	TLC	[16]
Patinopecten yessoensis	Ovarian tissue homogenate (2.0 ml)	E1: 0.5 μg E2: 0.5 μg	NADPH, $NADP^+$, NADH, NAD^+ mix (5 μM)	E2: 10–60 ng/g E1: 55–120 ng/g	HPLC-ECD	[29]
Cephalopods						
Sepia officinalis	Gonad (300 mg)	[7α-^{3}H]-DHEA: 4 × 10^{-2} μCi/mg [7α-^{3}H]-AD: 0.6 × 10^{-2} μCi/mg [4-^{14}C]-T: 2 × 10^{-2} μCi/mg	NADPH-generating system	Androstenediol: 0.5% T: 1.7–1.0% AD: 0.5%	TLC/PC/scintillation counting	[32]

n.d. = not detected.
[a] HPLC-RD or -ECD: high performance liquid chromatography-radiometric detector or -electrochemical detector; TLC: thin layer chromatography; GC–MS (EI): gas chromatography–mass spectrometry (electron impact mode); PC: paper chromatography.
[b] Quantification not available.

present in bacteria, fungus, plants and animals [28]. Several studies have reported the presence of 17β-HSD activity in molluscs; an overview of those studies including the different methods, organisms and tissues investigated, substrates used, and metabolites detected is given in Table 2. Incubations of digestive gland microsomes or gonad whole homogenates with labelled vertebrate-type precursors have evidenced 17β-HSD activity in different bivalves, e.g. mussels (*M. edulis* and *M. galloprovincialis*), oysters (*Crassostrea gigas* and *Crassostrea virginica*) and clams (*R. decussata*) [11,16,21,29–31]. Thus, gonad homogenates of *M. edulis* metabolized 17β-estradiol into estrone (12–15% conversion), and estrone to 17β-estradiol (45–50% conversion), also dehydroepiandrosterone was metabolized to androstenediol (7%); in contrast, the conversion of androstenedione into testosterone and vice versa was comparatively low (0.37–2.2%) [11]. *In vitro* incubations of ovarian homogenates with estrogens at a concentration of ~1 μM demonstrated the conversion of 17β-estradiol into estrone and vice versa, indicating the presence of 17β-HSD in the ovaries of the scallop *Patinopecten yessoensis*. Interestingly, changes in 17β-HSD activity were associated to the reproductive cycle of the scallop: the activity was 2-fold higher in individuals at the early differentiation stage than in those at post-spawning [29]. Also in oysters (*C. virginica* and *C. gigas*), the conversion of 17β-estradiol into estrone and vice versa by 17β-HSD has been well demonstrated [29–31]. Le Curieux-Belfond et al. [30] reported the conversion of testosterone into androstenedione and vice versa in several tissues of *C. gigas*,

i.e., gills, digestive gland and gonad-mantle, using labelled precursors and analyzing the metabolites by TLC and HPLC coupled to radiometric detector (HPLC-RD). The activity of 17β-HSD increased with sexual maturation and declined after spawning, which reinforces the role of 17β-HSD as a hormonal biosynthesis pathway in bivalves.

17β-HSD activity has been also reported in other classes of molluscs. In cephalopods, gonads of the cuttlefish (*Sepia officinalis*) metabolized androstenedione to testosterone and vice versa as well as DHEA to androstenediol although at relatively low rates [32]. In gastropods, the activity was detected in microsomal fractions isolated from the gonad-digestive gland complex of the freshwater gastropod *M. cornuarietis* that metabolised androstenedione to testosterone, but no evidence of 17β-HSD activity was detected in cytosolic fractions; the metabolism of androstenedione was NADPH-dependent and linear up to 60 min of incubation [22]. In addition, males of *M. cornuarietis* had higher 17β-HSD activity than females; this observation is consistent with sexual dimorphism in the metabolism of androgens in this species [23,24]. However, no significant differences between males and females were observed for *H. trunculus* regarding 17β-HSD activity, and the opposite trend – high activity in females and no activity in males – was reported for *B. brandaris* [26].

Overall, 17β-HSDs are widespread enzymes in molluscs. Phylogenetic studies suggest that some of the vertebrate forms descended from an ancestral retinoid dehydrogenase in invertebrates, but those proteins implicated in estrogen synthesis arose from vertebrate-specific duplications and have no orthologues in protostomes [28]. Thus, further work regarding the endogenous role of these enzymes in molluscs is needed, mainly considering the wide substrate spectrum of HSDs, ranging from steroids to retinoids, but also alcohols, sugars, aromatic compounds and xenobiotics.

6. Cytochrome P450-dependent biotransformations: aromatization and hydroxylations

Cytochrome P450 monooxygenase enzymes (CYP) comprise an ancient and widely distributed protein superfamily. P450-type enzymatic activities have been reported in the digestive gland of molluscs [33]. Typically, total P450 protein and associated enzymatic activities in invertebrates are found to be 10-fold lower than in mammals [34]. In vertebrates, testosterone is hydroxylated in a regiospecific and stereospecific manner by many different CYP isozymes [35]. Testosterone hydroxylation has been demonstrated in digestive gland/digestive tube microsomes of molluscs [16,17], but the metabolic rates were much lower than those usually found in vertebrates.

In vertebrates, conversion of androgens (C19 steroids) to estrogens (C18 steroids) is catalyzed by CYP19; this enzyme requires NADPH as a cofactor and involves hydroxylations and dehydrations that culminate in aromatization of the A ring of the androgens. Aromatase activity has been reported in different mollusc species [29,30,36,37] by using the tritiated water release assay, which is based on the quantification of the tritiated water released during [^{3}H]-androstenedione aromatization. Aromatization rates described so far in molluscs are very close to the detection limit of the technique (0.3–3.5 pmol/h/mg protein); however, significant inhibition of aromatase activity by specific CYP inhibitors (miconazole, MR20494) as well as 4-hydroxyandrostenedione suggests the presence in molluscs of a P450 aromatase enzyme similar to that in vertebrates [30]. It would have been interesting to check whether inhibition would have been overcome with an excess of estrogen. Regarding tissue distribution, P450 aromatase specific activity was 3-fold higher in digestive gland than in gonads of *M. edulis*, indicating a more active role of the digestive gland in the aromatization of androgens [34]. Interestingly, Matsumoto et al. [29] described the immunohistochemical localization of 3β-HSD, P450 aromatase and 17β-estradiol in extra-gonadic cells adhering to the wall of the acini in the gonad of the Japanese scallop *P. yessoensis*, whereas neither hemocytes nor germ cells were labelled. The authors concluded that estrogens can be synthesized in the gonad, and that their levels vary with the reproductive cycle, and therefore they might have a role in the development of gametes. More recently Osada et al. [38] reported immunoreactivity against P450 aromatase and estradiol-17β in the cells along the inside of the acinar wall of the testis of the same species, and suggested that testicular estrogen may play a physiological role in spermatogenesis. The antibodies used in both studies were rabbit anti-3β-hydroxysteroid dehydrogenase, rabbit anti-17β-estradiol and rabbit anti-human P450 aromatase, and their specificity for Japanese scallop might be questioned.

Actually, despite intensive research on *CYP19* genes, no orthologue has been described from fully sequenced invertebrate genomes, like *Drosophila melanogaster*, *Ciona intestinalis* or *Caenorhabditis elegans* [39]. Thus, it has been suggested that the *CYP19* gene arose at the origin of vertebrates [39,40]. Nevertheless, an aromatase homolog has recently been identified in the invertebrate amphioxus [41].

7. Sulfotransferases

Sulfate conjugation modulates the metabolism and biological activity of endogenous substances, including steroids [42]. Sulfation of low-molecular weight compounds such as hydroxysteroids, estrogens, and catecholamines is catalyzed by cytosolic sulfotransferases belonging to a gene superfamily designated as SULT [43]. These cytosolic enzymes utilize 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfate donor [42]. The sulfation of steroids is considered to have an important role in inhibiting their biological activity and increasing their excretion. In mammalian studies, the sulfated form of the steroids may also serve as soluble, inactive transporters, from which the active steroid may be regenerated by sulfatase activity [42]. Sulfate conjugates of steroid hormones have been observed in molluscs [17,20]. Identification of these compounds was based on their susceptibility to hydrolysis by sulfatases. *In vitro* sulfation of steroid hormones occurs at rather high metabolic rates in echinoderms, but not in molluscs or crustaceans [44]. Lavado et al. [37] determined the sulfation of estradiol (E2: 110 nM) in cytosolic fractions isolated from both gonads and digestive gland of *M. edulis*; the enzymatic activity showed a maximum at pH 9.0 in both tissues, and the specific activity was always higher in digestive gland than in gonads (up to 2-fold). Interestingly, at pH 9.0, sulfotransferase activity was linear for at least 1 h at concentration of proteins in the assay ranging from 0.05 to 0.4 mg. Under the selected conditions (0.1 mg of proteins, pH 9.0, 110 nM E2, 1 h incubation), the sulfation of E2 by digestive gland cytosolic fractions of control organisms was in the range of 0.5–5.1 pmol/h/mg protein. In another study, Janer et al. [21] showed that digestive gland cytosolic fractions of *M. galloprovincialis* can form estradiol sulfates at a rate of 6–12 pmol/h/mg protein; however, estradiol sulfation was not significantly altered by estradiol exposure.

8. Fatty acid acyl-CoA acyltransferases

Fatty acid conjugation (or esterification) renders steroids to an apolar form, which is retained within the lipoidal matrices of the body, and reduces their bioactivity, bioavailability, and susceptibility to elimination [45]. Esterification might have a regulatory function by inactivating steroids. Steroid esters do not bind steroid

receptors, but they can be hydrolyzed by esterases liberating the active steroid; they are considered to be long-acting steroids [46] and esterification is known to occur in both vertebrate and invertebrate species. Sex steroid esters have been reported in molluscs [44,47,48]. It has been suggested that esterification is the major biotransformation pathway for testosterone in snails, based on the reports that exogenously provided testosterone or estradiol are converted to fatty acid esters and retained in the tissues of the organism by the mud snail *I. obsoleta* [47] or the mussel *M. galloprovincialis* [21]. In addition, steroid esterification has been implicated in the regulation of free steroid levels. Gooding and LeBlanc [49] observed that, irrespective of the amount of testosterone administered to the snails, the amount of free testosterone measured in the tissues of the organism remains relatively constant and all excess of testosterone is converted to the fatty acid ester. Similar results were obtained when *M. galloprovincialis* were exposed to 20–200 ng/L estradiol [21]. However, future research is required to study the mechanism of esterification of steroids as well as the esterases responsible for releasing steroids from the fatty acid moiety, and the process that affect/regulate the equilibrium between synthesis and hydrolysis of this family of steroids.

9. Final remarks

In the last decade, the presence of pollutants in the aquatic environment has lead to an increasing number of endocrine disruption studies concerning both vertebrates and invertebrates and involving physiological processes controlled by steroid hormones. Thus, a better understanding of the role of steroids in invertebrates became a strong need for ecotoxicologists. Nonetheless, in spite of a number of studies being performed to better understand the endocrine functions of steroids in molluscs, the knowledge is still fragmentary. As shown in the present review, although the information obtained from different species strongly suggests the involvement of (vertebrate related) steroids in the control of mollusc reproduction, a complete scheme of vertebrate-related steroid biosynthesis (enzymatic pathways and steroidogenic cells and tissues), transport, target tissues, and further catabolism is lacking. In spite of recent advances, most questions on the action and function of sex steroids in molluscs remain to be answered: (1) most of the enzymes mentioned in this review (3α/β-HSDs, 5α-reductases, 17β-HSDs) have not been functionally characterized and their genes have not been cloned; (2) efforts to identify an androgen receptor from molluscs have been so far unsuccessful [50,51]. Although estrogen receptor orthologs have been found in representatives of the major groups of molluscs (*M. edulis*, *C. gigas*, *Aplysia californica*, *O. vulgaris*), ligand studies have shown that these receptors do not respond to estrogens [52–55]. Altogether, this opens the question of whether alternative mechanisms of action for androgens and estrogens may exist in molluscs [56,57], and points out the need to deeply investigate those mechanisms.

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