A critical evaluation of some of the recent so-called ‘evidence’ for the involvement of vertebrate-type sex steroids in the reproduction of mollusks

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ABSTRACT

Many studies on the control of reproduction in mollusks have focused on hormones (and proteins associated with the production and signaling of those hormones) which were originally discovered in humans, in the belief that if they are also present in mollusks, they must have the same role. However, although human sex steroids can be found in mollusks, they are so readily absorbed that their presence is not necessarily evidence of endogenous synthesis. A homolog of the vertebrate nuclear estrogen receptor has been found in mollusks, but it does not bind to estrogens or indeed to any steroid at all. Antibodies against human aromatase show positive immunostaining in mollusks, yet the aromatase gene has not been found in the genome of any invertebrates (let alone mollusks). This review will deal with these and other examples of contradictory evidence for a role of human hormones in invertebrate reproduction.

1. Introduction

Ever since the first studies on molluscan reproductive processes and (neuro)endocrine systems, there have always been some conflicts largely originating from terminology. The ‘multiomics approach’ (especially genomics and transcriptomics with high-throughput next generation platforms) facilitates the identification and naming of molecules in invertebrates, the functions of which, however, are often inferred purely through homology searches in the initial studies (and in most cases without any further functional investigations). That is to say, homologous genes found in different species are presumed to perform homologous functions – those functions being those found in the organisms from which they were first characterized (usually a vertebrate). Clearly, researchers, especially newcomers, need to be fully aware of the limitations when making such assumptions when exporting ‘omics’ methodologies to emerging invertebrate model systems. Markov et al. (2008) refer to this as the “street light syndrome”. In other words, scientists sometimes find it far easier to search for clues (and, incidentally generate data for papers) in the light (where something is already known) than in the dark (where nothing, or very little, is yet known; but where they should, in all probability, be looking). The authors of that paper gave several examples of vertebrate proteins in which literal interpretation of their names had given rise to confusion in invertebrate studies. This paper gives several more examples, all related to the putative role of vertebrate-type sex steroids in mollusks, to inspire invertebrate researchers to be hypercritical and to support the contention that molluscan endocrinology differs from the well-characterized vertebrate endocrine system.

2. Gonadotropin-releasing hormone

The endocrine basis of vertebrate reproduction (and its mediating neuroendocrine system) is the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1A) (Kapprara and Huhtaniemi, 2018). A gonadotropin-releasing hormone (GnRH) liberated from the hypothalamus reaches the anterior pituitary and stimulates the release of gonadotropins. Mollusks also have a reproduction-mediating endocrine system although, morphologically, it is highly different from the vertebrate one (see next paragraph) and it is very poorly understood (i.e. knowledge about it is ‘in the dark’) (Di Cristo and Koene, 2017). However, peptides very similar to vertebrate GnRHs have been identified in all sorts of invertebrates, including mollusks. On the basis of their close similarity, these peptides have also been labelled GnRHs. The only problem with them having been given the same name is that it has led

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many researchers to assume that these invertebrate GnRHs have the same (or at least a very similar) role to the GnRHs in vertebrates (i.e. that they are reproductive hormones) (Jung et al., 2014; Minakata et al., 2009; Tsai et al., 2010). This assumption has been made in spite of the fact that when GnRH was first identified in the common octopus (Octopus vulgaris), it actually emerged from bioassay screenings of peptide fractions that had cardio-acceleratory effects (Iwakoshi et al., 2002). Recent studies have demonstrated that these peptides are multifunctional and do not necessarily (and indeed very rarely) have anything to do with reproduction in these species. This is the first of our examples where ‘searching in the light’ (i.e. working with a compound that was essentially easy to investigate) failed to answer the question about what really controls reproduction in mollusks. What is clear is that the nomenclature of this peptide in invertebrates should be changed in order to prevent continuing confusion (Fodor et al., 2020a; Hauser and Grimmelikhuijzen, 2014; Flachetzkii et al., 2016; Tsai, 2018).

As already stated, there is no homologue of the vertebrate hypothalamus in mollusks. For example, in gastropods, including the great pond snail (Lymnaea stagnalis) as the species that has been investigated in most detail in this respect (Fodor et al., 2020b), neurons and neuronal clusters responsible for regulation of reproduction are dispersed in different ganglia (Fig. 1B), instead of being organized into modules of a hierarchic system as in case of humans (Fig. 1A) (Di Cristo and Koene, 2017; Koene, 2010). Depending on the species, the exact terminology of these cells and clusters varies, but is relatively conserved across gastropods. Especially in the gastropods, the regulation is performed basically with synchronization of numerous neuropeptides through either direct innervation, neuroendocrine, or local autocrine or paracrine mechanisms (Di Cristo and Koene, 2017; Koene, 2010).

3. Gonadotropins

There are two gonadotropins in most vertebrates known as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These reach the gonads from the pituitary via the bloodstream and (directly or indirectly) stimulate gamete production as well as sex steroid biosynthesis (Fig. 1A) (Kaprara and Huhtaniemi, 2018). There is no equivalent of the vertebrate pituitary gland in mollusks and, furthermore, from a genomic viewpoint, there is zero evidence that mollusks have gonadotropins, because the genes encoding for LH and FSH-specific subunit are vertebrate-specific members of the glycoprotein hormone family (Dufour et al., 2020). Yet there are some papers claiming the presence of gonadotropins in mollusks. However, it should be pointed out that this claim has only been inferred from positive immunostaining of tissues with antibodies to human LH and/or FSH (Nuurai et al., 2020; Omran, 2012) and immunostaining has been deemed to be a highly unreliable technique for identifying proteins (Baker, 2015a,b) and over 50% of worldwide studies have in fact been described by Bradbury, Plückthun and 110 co-signatories (Bradbury and Plückthun, 2015) as ‘money down the drain’. This puts a huge question mark over the so-called evidence for the presence of gonadotropins in mollusks.

4. Presence of sex steroids in mollusks

In vertebrates, the sex steroids are made by specific cells in the gonads. Cells with the same structural characteristics have not been identified in mollusks. However, vertebrate-type steroids are present in mollusks (Scott, 2012). There is a big question mark, though, about how much if any of them are of endogenous origin (Scott, 2018). The last common precursor of animal steroids is cholesterol, which is built up from squalene in a 10-step process (Desmond and Gribaldo, 2009). The origin of the formation of cholesterol predates the protostome-deuterostome split and is broadly conserved across eukaryotes with secondary losses in at least some protostomes, like insects or nematodes (Markov et al., 2017). Cholesterol, with 27 carbon atoms, is at the base of the vertebrate sex steroid biosynthetic chain (Fig. 2). The
steroids are in fact formed by a process akin to catabolism (molecular break-down) which produces compounds (‘metabolites’) that have progressively 21 (progestins), then 19 (androgens) and then 18 (estro-gens) carbon atoms (Payne and Hales, 2004) (Fig. 2). There are numerous sex steroids in vertebrates, but the major ones in humans (which are also the only ones that receive attention in mollusks) are progesterone (P), testosterone (T), and estradiol (E2). These steroids have a multitude of powerful effects on gamete production and secondary sexual characteristics in vertebrates (Kaprara and Huhtaniemi, 2018); but, it must be pointed out, have weak, and in most case questionable, effects in mollusks (Scott, 2013). As already stated, the presence of the steroids in molluscan tissues is widely-documented (see also reviews by Fernandes et al., 2016; Giusti and Joaquim-Justo, 2013; Janer and Porte, 2007; Scott, 2018). However, it has been established numerous times, from 2000 onwards (Scott, 2018), that mollusks can readily absorb steroids from the environment. They also have the ability to store T and E2 for weeks to months in the form of fatty acid esters. Additionally, survey after survey shows that the environment (which includes laboratories where experiments are carried out (Scott, 2012)) is ‘awash’ with vertebrate steroids. These facts mean that it is not possible to conclude that just because steroids are present in molluscan tissues, that the animals have made them themselves (i.e. presence is not proof of endogenous synthesis) (Scott, 2018). Those who find it difficult to accept this inconvenient fact (having spent time and resources measuring steroid concentrations in molluscan tissues) would do well to read the story about the presence of E2 in yeast (Feldman and Krishnan, 1995).

Looking in a bit more detail at the evidence for whether mollusks do have an ability to endogenously synthesize vertebrate steroids, even though the precursor, cholesterol is undoubtedly present in mollusks (Altelaar et al., 2005; Idler and Wiseman, 1972), the evidence that it can be broken down into steroids in the same way as it is in vertebrates is very poor (Scott, 2012).

There are two generic types of reaction involved in formation of vertebrate steroids: the first in which two hydrogen atoms can be either inserted or removed (oxidoreductase reactions); and the second that involves oxygen atom insertions. The first type of reaction is relatively simple and occurs at the stage of transformation of pregnenolone (Pg) + P (catalyzed in vertebrates by the enzyme 3ß-Hydroxysteroid dehydrogenase [3ß-HSD] and involves the removal of two hydrogen atoms from the hydroxyl group at position carbon atom 3 (C-3)), androstenedione (Ad) → T or estrone (E1) → E2 (catalyzed by 17ß-HSD; involving addition of two hydrogen atoms to the oxo group at position C-3), and T → DHT (catalyzed by 5α-reductase; involving addition of two hydrogen atoms either side of the double bond between C-4 and C-5) (Fig. 2). Oxidoreductase reactions are relatively simple and easy to demonstrate in all sorts of living organisms and with all sorts of compounds (i.e. not just steroids). The evidence for their occurrence in mollusks is strong - e.g., conversion of Pg → P by Lymnaea stagnalis and Octopus vulgaris (de Jong-Brink et al., 1981; Di Cristo et al., 2010); conversion of E1 → E2 by Mytilus edulis (Labadie et al., 2007) and conversion of T → DHT by Mytilus edulis (Schwarz et al., 2017). Evidence that these reactions form part of a steroid biosynthetic pathway which is equivalent to that of vertebrates is circumstantial, however, because of the involvement of HSDs in other metabolic pathways (e.g. that of lipids [Lima et al., 2013]). This in turn means that, although enzymes have been identified in the genomes of invertebrates that are homologous to the enzymes labelled ‘hydroxysteroid dehydrogenases’ of vertebrates, it does not mean that steroids are necessarily their intended substrate in invertebrates.

The second type of reaction is relatively complex (Payne and Hales, 2004). The enzymes that carry this out belong to the Cytochrome P450

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**Fig. 2. Vertebrate-type sex steroid (progestogens, androgens, and estrogens) biosynthetic pathway with the mediating enzymes.** Five reactions, though some with low activity, appear to be present in mollusks (indicated by green check mark and question mark). The gene for cholesterol side-chain cleavage enzyme and aromatase has not been determined in any mollusk so far (marked by red X). HSD: hydroxysteroid dehydrogenase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
family (CYP). In its simplest form, the reaction involves the insertion of a single oxygen atom directly onto a carbon atom of the steroid molecule. However, in the reactions that involve the progressive formation of E2 (18 carbons) from cholesterol (27 carbons), multiple oxygen atom insertions occur. For example, to cleave the initial side-chain of cholesterol (thereby converting it into Pg), an oxygen atom must first be added to C-20, another to C-22 (forming two new hydroxyl groups) and then yet another oxygen used to effectively push these two oxygens apart by electrostatic repulsion.

In a similar way, to convert P (with 21 carbons) into Ad (with 19 carbon atoms), another side-chain cleavage must take place. A new oxygen must be inserted at C-17 (17-hydroxylase) and then yet another oxygen atom used to push this apart from the already existing oxygen atom at C-20. To convert T to E2 (a reaction catalyzed by the enzyme aromatase), two oxygens are inserted at C-19 and then a third oxygen atom used to break both these off together with the carbon atom at C-19 and at the same time as the characteristic ‘aromatic’ A ring is formed.

4.1. Evidence for the cholesterol side-change cleavage enzyme (CYP11A) in mollusks

There is no firm evidence that any metazoans other than vertebrates can carry out cholesterol side-chain cleavage (Fig. 2) (Markov et al., 2017). The gene homologue of this enzyme has not yet been identified outside of vertebrates. It is not present in the genome of the snail, Biomphalaria glabrata, for example (Adema et al., 2017) – nor in that of Lymnaea stagnalis (our own still unpublished studies). However, as hydroxyl groups occur at positions C-20 and C-22 of the insect molting hormone, 20α-hydroxyecdysone (Thummel and Chory, 2002) it is possible (though highly speculative – as plants are also able to form hydroxyl groups at these positions (Ohnishi, 2018)) that a cleavage enzyme could have been part-evolved by the point that the vertebrate and insect lines diverged, which could explain (again speculatively) the observation of a ‘partial protein equivalent’ to CYP11A in the genome of Mytilus edulis (Blalock et al., 2018).

4.2. 17-hydroxylase/17,20 lyase

Concerning 17-hydroxylase (CYP17) there is evidence for trace conversion of P to Ad in mollusks (reviewed by Scott, 2012) and (Fernandes et al., 2010); (Fig. 2). There is also evidence that, given enough time (many days as opposed to minutes) organisms in anaerobic sediments can convert P to Ad (Jenkins et al., 2004). A homologue of the vertebrate CYP17 gene appears to be present in invertebrates (Goldstone et al., 2015; Guo et al., 2010, 2013; Thongbuakaw et al., 2016; Zanette et al., 2010). This has many amino acid differences from the vertebrate enzyme though (Guo et al., 2010). This may account for its low level of activity in comparison to the vertebrate enzymes, but no one has yet checked the ability of any invertebrate CYP17 homologues to cause either 17-hydroxylolation or subsequent cleavage of the side-chain.

4.3. Aromatase

No one has yet located a gene for aromatase (CYP19A; the enzyme that converts T → E2 in vertebrates; Fig. 2) in mollusks. It is not present in the genome of the Biomphalaria glabrata (Adema et al., 2017) nor in that of Lymnaea stagnalis (our own still unpublished studies). Yet there are people who are still convinced that this enzyme exists in mollusks. There is a recent paper, for example Hallmann et al. (2019), claiming that there is substantial aromatase activity in tissues of a Baltic bivalve, Mytilus trossulus. One of the lines of evidence in their paper was the existence of enzyme activity that has an ability to remove a radioactive hydrogen (i.e. tritium) atom from the C-1β position of the vertebrate steroid androstenedione. The ability of aromatase to remove this tritium atom (which then becomes incorporated into water molecules) has been used for many years as the basis for an indirect assay for aromatase activity in vertebrate tissues. The authors claimed that this activity was stronger than in previous studies carried out on other mollusks (Le Curieux-Belfond et al., 2001; Matsumoto et al., 1997; Morcillo and Porte, 1999), especially when the experiments were conducted in the springtime and at a relatively low temperature. The other line of evidence came from another experiment which showed that live mussels could transform 13-Carbon labelled Ad and T (added to the water) into 14C-labelled E2 and E1. Dealing with this second line of evidence first, the yields of E2 and E1 (calculated on the basis that the authors exposed the animals to tens of micrograms of precursor and only recovered a few nanograms of estrogens) was <0.1%. This represents ‘trace’ production, especially when contrasted with the 21% rate of conversion of T into DHT steroids by the closely-related mussels, Mytilus edulis (Schwarz et al., 2017). In fact, trace production of estrogens has already been claimed in previous studies on mollusks (Carreau and Drosdowsky, 1977; Goto et al., 2012; Le Guellec et al., 1987; Matsumoto et al., 1997; Morcillo et al., 1998). In relation to the first line of evidence in the paper on Mytilus trossulus, there is also a problem. The authors only made an assumption that tritium release represented aromatization. At no point did they present any direct evidence that estrogens were the end-product of the reaction that released the tritium. The removal of the 1β-hydrogen from Ad or T is only one small cog in the aromatization mechanism in vertebrates and it is more than probable that there are other mechanisms (entirely unrelated to aromatization) that could cause tritium release. One possible mechanism could be the removal of two hydrogen atoms at C-1 and C-2 (to form a double bond between them). This simple dehydrogenation reaction has in fact been demonstrated under both aerobic and anaerobic conditions in certain bacteria, and shown to be the first step in the metabolic breakdown of androgens by these organisms (Chiang et al., 2019; Yang et al., 2016). Also, when Schwarz et al. (2017) exposed live Mytilus edulis to T that was labelled with tritium atoms attached to C-1, 2, 6 and 7 - none of which should theoretically have been involved in the formation of any of the major metabolites that were identified in that study, there was a considerable 22% production of tritiated water that could not be ascribed to aromatization, as there was no evidence of E2 or E1 production in either free, esterified or sulfated forms. There were, however, plenty of unidentified peaks of radioactive organic compounds in the water (supporting the existence of pathway(s) of androgen metabolism in mollusks which are still in the dark).

There have also been several papers reporting the immunostaining of cells in molluscan tissues with antibody raised against human aromatase (Matsumoto et al., 1997; Osada et al., 2004; Prisco et al., 2017) and one study that went further in reporting the immunostaining of a protein that was extractable from tissues of the aquatic snail, Lymnaea palustris. This protein formed a single band on gel electrophoresis and appeared to be modulated by treatment of the animals with the herbicide Roundup (Reddy et al., 2018). Although, in all these papers, the authors claimed that immunostaining was evidence of the presence of aromatase, one must take into account, firstly, that an aromatase gene has yet to be identified in the genome of any invertebrate (let alone any mollusks) and, secondly, that immunostaining (especially using polyclonal antibodies against mammalian proteins) is a highly unreliable procedure for identifying or localizing specific proteins in tissues (especially when applied to invertebrates).

In conclusion, there is little or no evidence that CYP19A itself, or a homologue of CYP19A, is present in any animals other than vertebrates and their immediate ancestors (Fig. 2) (Callard et al., 2011; Di Cristo and Koene, 2017; Markov et al., 2009, 2017).

5. Promiscuity of enzymes as an explanation for trace production of vertebrate steroids in mollusks

If CYP19A is not present in mollusks, then what could be the explanation for the trace production of estrogens by mollusks (as in the in vivo study carried out by Hallmann et al., 2019)? The likely explanation is that it is due to the now well-recognized phenomenon of
enzyme promiscuity (Lathe et al., 2015; Atkins, 2015). In the same way that HSDs (discussed above) can recognize more than one substrate (and, incidentally, that antibodies can cross-react with more than one ligand), there is no reason why one or other (admittedly yet to be identified) CYP enzymes in invertebrates should not have a weak affinity for T or Ad that allows them to partially convert them into estrogens. Indeed Lathe et al. (2015) and Atkins (2015) quote examples of such enzyme-substrate cross-talk taking place with both vertebrate CYP11A and CYP17. In the case of CYP19A, Markov et al. (2017) refer to the discovery of aromatized steroids (i.e. metabolites of cholesterol that have not undergone side-chain cleavage) in sponges and cnidarians. The authors termed these compounds ‘paraestrols’ and, incidentally, speculated that they might have been the original ligands for the ‘ancestral’ steroid receptor that was already present at the base of the vertebrate and invertebrate chains (see for more details at the end of section 7). In the same study, the authors further speculated that the evolution of side-chain cleavage in the vertebrates probably resulted in compounds (i.e. steroids) that were perhaps more efficient at binding to the nER than the paraestrols (and also, we would like to point out, be a lot more soluble in plasma than steroids, making them far better potential candidates as blood-borne hormones). These factors were possibly the driver for the co-evolution of a dedicated aromatizing enzyme (i.e. CYP19A) in vertebrates.

In relation to the paraestrols, it does not seem likely that they are the ‘missing estrogens’ in mollusks. However, their introduction to the discussion does highlight an underexplored (‘dark’) area in molluscan research – viz. the potential role of steroids (as opposed to steroids) as hormones. As mentioned before, there is strong 5α-reductase activity in mollusks. Also, a recent study investigating two freshwater gastropods has intriguingly demonstrated that a 5α-reductase inhibitor caused marked malformations in their shell morphology during their development (Baynes et al., 2019). Since it is known that 5α-reductase is an ancient enzyme (present in plants as well as animals) and is known to use sterols (as well as T and Ad) as substrates (Rosati et al., 2003; Ohnishi, 2018), we believe this (i.e. the study of steroid derivatives) might indeed be the way forward in throwing light on the endocrine system in mollusks (though not necessarily in relation to reproduction). Several sterols with non-hormonal/non-reproductive properties have already been identified in mollusks (Pereira et al., 2016).

In summary, three absolutely key steps (cholesterol side-chain cleavage, 17-hydroxylation and aromatization) in the vertebrate steroid biosynthetic pathway are either absent, or occur very weakly (i.e. with only trace conversion of their substrates), in mollusks (Fig. 2). Most importantly, the homologues of the enzymes that catalyze the first and third of these reactions in vertebrates are missing in molluscan genomes. One must question whether natural selection in mollusks would have favored the evolution (or the retention) of a steroid-based endocrine system that was reliant on such low rates of endogenous production of their purportedly key hormones. The implied wastage of precursors would be massive.

6. Vertebrate sex steroid receptors

Clearly, besides the synthetized steroids, the presence of their receptors is also necessary to the function of the HPG axis (Kapraa and Huhtaniemi, 2018). The main receptors in vertebrates are a) the estrogen (ER), b) the progesterone (PR), and c) the androgen (AR) receptors, both of them having membrane (small subset, approximately 5%; e.g., mPR) and nuclear (e.g., nPR) forms involved in reproductive processes (Levin and Hammes, 2016). Unlike the nuclear forms which mediate their effects via genomic mechanisms, membrane forms are cell surface receptors which rapidly alter cell signaling via modulation of intracellular signaling cascades. The nuclear forms belong to the subfamily 3 of nuclear receptors sharing a common structure of seven functional domains (termed A-F) that are functionally homologous (Levin and Hammes, 2016).

- A/B domains: beginning at the N-terminal and are the most variable domains between the different receptors. It provides a binding site for transcriptional co-regulators.
- C domain: the centrally located highly conserved DNA binding domain (DBD) consists of two non-repetitive globular motifs where zinc is coordinated with four cysteine and no histidine residues. On DNA it interacts with the hormone response element (HRE).
- D domain: Hinge region. Flexible region that connects the DBD with the LBD; along with the DBD, contains a ligand dependent nuclear localization signal controlling the movement of the receptor to the nucleus.
- E domain: ligand binding domain (LDB). Moderately conserved domain responsible for ligand/hormone binding, as well as binding sites for coactivator and corepressor proteins.
- F domain: the C-terminal connecting the molecule to its partner in the homodimer or heterodimer. It may affect the magnitude of the response.

7. Progesterone receptors in mollusks

The current theory is that the nER was the first nuclear receptor to evolve (in the ancestors of both vertebrates and mollusks) and that nPR (and nAR) did not evolve until late in vertebrate evolution (Thornton, 2001). This explains why no one has found a gene for the nPR (or nAR) receptor in molluscan species. However, there are genes that are homologous to mPRs and membrane associated progesterone receptors (MAPRs) (Ren et al., 2019). The MAPRs (mPRs, mPRβ, mPRγ, mPRδ, mPRε; they are also referred to as PAQ5S-9) are members of progestin and adipoQ receptor (PAQR) family and found only in chordates, while MAPRs occur also in invertebrates (Thomas et al., 2007). The MAPRs include progesterone receptor membrane component 1 (PGRMC1), progesterone receptor membrane component 2 (PGRMC2), neudestin, and neuferrin (Ren et al., 2019). Homologous sequences for human PGRMC1 are found in many invertebrates, including mollusks (e.g., Lymnaea stagnalis, #MT178274, supplement of this paper). Does this mean that P may likely function as a hormone in mollusks (despite the fact that specific cholesterol side-chain cleavage likely does not occur in mollusks)? The current prevailing view is that, although PGRMC1 has ‘progesterone’ in its name and has been proved to act as a chaperone protein for mPR in vertebrates, it does not actually bind progesterone itself (Cahill et al., 2016; Gonzalez et al., 2020; Pang et al., 2013; Ren et al., 2019; Thomas et al., 2007, 2014). It has also been found to chaperone other membrane receptors (e.g. nER) and potentially has been assigned many other functions (Cahill et al., 2016). Essentially, its presence in an organism does not necessarily mean that its role is to chaperone an mPR.

8. Estrogen receptors in mollusks

Homologue sequences to human nERs have been determined in many molluscan species including for example Octopus vulgaris (#ABG00286; Keay et al., 2006), Aplysia californica (#NP_001191648; Thornton et al., 2003), Lottia gigantea (#XP_009064842; Simakov et al., 2013), Parafossarulus striatulus (#MG214706; Ma et al., 2019), and Lymnaea stagnalis (#MN989918, supplement of this paper).

Ma and co-workers have performed a comprehensive sequence comparison across invertebrate and vertebrate species (Ma et al., 2019). They demonstrated that the C-domain/DBD (especially the P-box and D-box responsible for mediation of response element recognition in vertebrate receptors) shows high conservation in estrogen receptors, while the E-domain/LBD shows less conservation (except the activation function 2 (AF2) motif). For demonstration of the sequence homology that has led to the term ‘molluscan nER’, a sequence comparison between the human nERa and the ER of Aplysia californica as well as the human estrogen related receptor alpha (ERαa) and the ER of Aplysia californica is presented in Supplementary Fig. 3. At the same time, in
mollusks, it has been shown innumerable times that their nERs do not bind vertebrate-type estrogens, i.e. they are not functional estrogen receptors (Bannister et al., 2013; Kajiwara et al., 2006; Keay et al., 2006; Ma et al., 2019; Markov et al., 2008, 2009; Matsumoto et al., 2007; Pirger et al., 2018; Scott, 2018; Thornton et al., 2003).

Despite its inability to bind estrogens being known for a long time, the nER in mollusks has received an inordinate amount of attention from researchers in the mollusk field. A key driver of this interest was a study published in 2010 (Ciocan et al., 2010) that showed some very large changes (in one case up to 1 million-fold) in mRNA expression of the gene for the nER when Mytilus spp. was exposed to a variety of estrogens. These findings have led many researchers to adopt nER mRNA expression as an endpoint in studies designed to show that estrogens had a hormonal effect in mollusks. The 2010 paper is also highly cited in the Introductions and Discussions of papers in which the authors wish to justify a hormonal role for estrogens in reproduction in mollusks. This positive publication bias is a problem across the life sciences – in essence meaning that only exciting ‘positive’ studies get quoted, while ones showing ‘no effect’ or a ‘negative effect’ are ignored (Bishop, 2019). We have found sixteen other studies that have measured nER expression in mollusks and plotted their results (Fig. 3) in the same way as Ciocan et al. (i.e. relative expression in comparison to a control). It is noteworthy that the results are highly variable. Some found positive changes, some found negative change and some found no changes. Some, such as by (Tran et al., 2016a,b) found bell-shaped responses to a range of doses (not indicated in Fig. 3). None of the studies showed changes anywhere in the same order of magnitude as those reported in the Ciocan et al. study. Some of these differences between studies could obviously be due to differences in assay conditions, type and dose of estrogen and the state of the animals. However, the lack of consistency does rather call into question the usefulness of nER expression as a biomarker for estrogen exposure in mollusks.

In fact, if one examines the data in the original paper (Ciocan et al., 2010) and plots mRNA expression against E2 concentrations (Fig. 4), one finds no evidence of dose-responsiveness (and no consistency between the sexes) in their study - the regression coming out as a straight line on the zero axis. Why these results were as they were and why no one has been able to replicate the findings is still a mystery. Yet another anomalous result in the literature is that of Di Cosmo and co-workers (De Lisa et al., 2012; Di Cosmo et al., 2002), who found that tissue extracts from this species showed an ability to bind radioactive E2 with an affinity and capacity that was similar to vertebrate nERs. However, in view of the fact that the structure of octopus nER is such that it cannot bind E2 (Keay et al., 2006), it is difficult to know how to interpret the binding data. These binding results have not yet been independently replicated in octopus or any other mollusk.

Finally, Markov et al. (2017) presented two alternative versions for the steroid receptor evolution in bilaterians. The first option is that chordate ERs (also generally referred to as NR3A group) as well as other chordate receptors (e.g., PRs, ARs; also generally referred to as NR3C group) are the results of a chordate-specific ancestral duplication. In this case, the ancestral steroid receptor would be the last common ancestor predating this chordate-specific gene duplication. Consequently, vertebrate ERs (also generally referred to as NR3D group) would be orthologous to this. The second option is that the last common ancestor from the NR3A group was orthologous to the common ancestor of NR3D.

Fig. 3. A comparison of 16 studies (excluding the landmark study by Ciocan et al., 2010) that measured the relative expression of nER mRNA in mollusks. The studies are divided into those that recorded predominantly positive responses, those that recorded no response, and those that recorded predominantly negative responses. It should be noted that these experiments were all carried out on different species and under different conditions and this could well explain some of the variability. However, the data show that consistency is lacking even within some studies.

Relevant studies from where the results derive: a, Tran et al. (2016a,b); b, Tran et al. (2016b); c, Völker et al. (2014); d, Nagasawa et al. (2015); e, Stange and Oehlmann (2012); f, Stange et al. (2012a); g, Stange et al. (2012b); h, Canesi et al. (2007); i, Ciocan et al. (2015); j, Puinean et al. (2006); k, Ma et al. (2019); l, An et al. (2014); m, Bannister et al. (2013); n, Canesi et al. (2008); o, Hultin et al. (2014); p, Lei et al. (2015); q, Balbi et al. (2019); r, Hultin et al. (2016).
group implying that the gene orthologous to NR3C was secondarily lost in invertebrates. In this way, the ancestral chordate steroid receptor would also be the ancestor for the NR3D. According to the findings presented above, functional estrogen binding receptors have most likely been never present in the molluscan lineage. In this respect, delineation of a third alternative should be taken into consideration. Furthermore, just like in the case of the originally named molluscan GnRH peptides, a revision of the molluscan steroid receptor terminology needs to be performed.

9. Can steroids occurring in the environment affect mollusks?

Summarizing the above: although a homologous sequence to vertebrate nER is present in mollusks, it does not bind estrogens; nPR and nAR do not exist in mollusks; and at least two key steps in the steroid biosynthetic pathway are missing. Why is it then that papers have been, and still are being, published that claim that exposure of mollusks to vertebrate steroids affect a wide range of processes including sex ratio, fecundity, egg yolk production, egg mass production, the quality of egg masses, shell size during development, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen (Pirger et al., 2018; Scott, 2013; Zrinyi et al., 2017)?

In general, a large part of the answer is that many of the claims are probably not true, in the sense that they are based on bioassay data that are flawed in one way or another. Examples of such flaws are the use of single experiments only, lack of replication, lack of repetition, questionable endpoints, low effect sizes, lack of dose-responsiveness and failure to take steps to avoid bias (Scott, 2013). There is a salutary lesson to be learned from the numerous attempts in the 1990s and 2000s (Matozzo et al., 2008; Scott, 2012; Tran et al., 2019) to prove that egg yolk (often wrongly referred to in mollusks as ‘vitellogenin’) in mollusks was stimulated by estrogens in the same way that it is in fishes. The highly laudable aim of these studies was to replace fish by mollusks as sentinel species for detecting and measuring xenoestrogens in the environment. Despite most of these earlier studies claiming a relationship between estrogens and egg yolk production, the data was never very convincing (at least to people carrying out similar studies on fishes) – an opinion backed up by the fact no such test-procedure for xenoestrogens using mollusks has ever been developed. Furthermore, more rigorous studies carried out in the last five to six years (Fernandez-Gonzalez et al., 2020; Morthorst et al., 2014; Oliveri et al., 2014; Sanchez-Marin et al., 2017) have found zero effect of estrogens on egg yolk production in mollusks.

Another possible explanation for apparent effects of vertebrate hormones on mollusks is that the effects are real, but are due to non-specific interaction with receptors for other compounds (whether G-protein coupled receptors or ancient multifunctional nuclear receptors). This is in effect yet another example of promiscuity of another typical ‘lock and key’ mechanism, and probably explains effects induced by vertebrate GnRHs in mollusks (i.e. they are similar enough in structure to the natural molluscan peptide ligands to be able to bind to their receptors). Yet another possible reason why some steroids (T and E\textsubscript{2} being good examples) might have effects on mollusks is suggested by the fact of their being so readily absorbed and extensively metabolized and esterified. If the dose of these steroid is large enough (as they are in many experiments), it is not inconceivable that there could be a measurable change in biochemistry, physiology, and gene regulation (genetic and/or epigenetic) due to the enhanced energy usage and re-allocation of resources involved with these reactions.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110949.
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