Steroids 78 (2013) 268–281

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Review

Do mollusks use vertebrate sex steroids as reproductive hormones? II. Critical review of the evidence that steroids have biological effects

Alexander P. Scott *

Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, Weymouth, Dorset DT4 8UB, UK

ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form 22 October 2012
Accepted 2 November 2012
Available online 3 December 2012

Keywords:
Vertebrate steroid
Invertebrate steroid
Mollusc
Steroid bioassay
Physiological role

ABSTRACT

In assessing the evidence as to whether vertebrate sex steroids (e.g. testosterone, estradiol, progesterone) have hormonal actions in mollusks, ca. 85% of research papers report at least one biological effect; and 18 out of 21 review papers (published between 1970 and 2012) express a positive view. However, just under half of the research studies can be rejected on the grounds that they did not actually test steroids, but compounds or mixtures that were only presumed to behave as steroids (or modulators of steroids) on the basis of their effects in vertebrates (e.g. Bisphenol-A, nonylphenol and sewage treatment effluents). Of the remaining 55 papers, some can be criticized for having no statistical analysis; some for using only a single dose of steroid; others for having irregular dose–response curves; 40 out of the 55 for not replicating the treatments; and 50 out of 55 for having no within-study repetition. Furthermore, most studies had very low effect sizes in comparison to fish-based bioassays for steroids (i.e. they had a very weak 'signal-to-noise' ratio). When these facts are combined with the fact that none of the studies were conducted with rigorous randomization or 'blinding' procedures (implying the possibility of 'operator bias') one must conclude that there is no indisputable bioassay evidence that vertebrate sex steroids have endocrinological or reproductive roles in mollusks. The only observation that has been independently validated is the ability of estradiol to trigger rapid (1–5 min) lysosomal membrane breakdown in hemocytes of *Mytilus* spp. This is a typical 'inflammatory' response, however, and is not proof that estradiol is a hormone – especially when taken in conjunction with the evidence (discussed in a previous review) that mollusks have neither the enzymes necessary to synthesize vertebrate steroids nor nuclear receptors with which to respond to them.

Contents

* Tel.: +44 1305 206600; fax: +44 1305 206601.
E-mail address: alexander.scott@cefas.co.uk

0039-128X
http://dx.doi.org/10.1016/j.steroids.2012.11.006

1. Introduction

There have been three main discoveries that have driven research on vertebrate steroids in mollusks. The first was the discovery of the presence of vertebrate steroids in molluscan tissues [1–3]. It was not unreasonable at the time that people should have assumed that these steroids were of endogenous origin and were possibly used as hormones in the same way as they are in vertebrates. The second big discovery was that the anti-fouling compound tributyltin (TBT) was the main causative agent of penis growth in female snails living in harbors and estuaries (reviewed by [4,5]). It was also not unreasonable for people to link this overtly 'androgenic' effect with the fact that testosterone (T) could be extracted from the flesh of the same animals (reviewed by [6]) and then work on the hypothesis that penis growth was mediated by alteration of T production or metabolism by TBT (reviewed by [4]). The third big discovery was that sewage treatment works (STWs) in the UK at the end of the 1980s were emitting large amounts of estrogenically active compounds that were inducing massive production of egg yolk protein (vitellogenin; VTG) by immature fish [7]. It was not long after this paper was published that studies started appearing in which researchers attempted to show similar changes in egg yolk protein production in mollusks that had been exposed to effluents and/or to synthetic estrogens. The results of such studies were felt by some people to be so encouraging [8–10], that a new driver for research on steroids in mollusks appeared. This was to develop bioassays for estrogenic (and androgenic) endocrine disrupters using mollusks, rather than fishes or mammals, as test animals.

The foundations of the first two research drivers have been crumbling for some time now. It has been known for over twenty years that vertebrate-type steroids can be extracted from all living organisms [11,12] including plants [13]. In other words, there is nothing necessarily special or unusual about the presence of steroids in mollusks. Also, ever since it was discovered that mollusks are rather good at absorbing steroids from the environment and storing them for many days in the form of fatty acid esters [14,15], the long-standing assumption that any of the steroids that are found in molluscan tissues are actually made by the animals themselves, or that they have functional receptors, is increasingly being challenged [6,16,17]. It is now also known that T has little or no role to play in penis development in female mollusks [18,19]. As will be seen, this negative evidence contrasts with the relatively large amount of positive evidence that vertebrate steroids are hormonally active when administered to mollusks. This present review was undertaken to critically assess the strength of this evidence.

Unfortunately, there is much confusion and obfuscation in this area of research, because many people working on mollusks make frequent use of terms that are strictly relevant to vertebrate endocrinology such as 'endocrine disruption', 'androgens', 'estrogens' and 'xenoestrogens'. However, these terms are misleading, as they are based purely on assumptions – one being that because STW effluents can affect reproductive traits in mollusks and because STW effluents contain compounds that behave as estrogens to vertebrates, then it must be estrogens that are affecting the mollusks – another being that just because certain compounds in the environment such as Bisphenol-A (BPA) and nonylphenol (NP) elicit effects in vertebrates via binding to the estrogen receptor, then any effects they might have in mollusks must be via the same mechanisms.

2. What is the current view on the involvement of steroids in mollusk reproduction?

There have been over twenty reviews that have dealt in one way or another with the putative role of vertebrate steroids in mollusks. These reviews can be split into those that contain:

a strong positive conclusion – i.e. they include firm statements such as 'the evidence that steroids play a functional role is strong' [20] 'sex steroids play important roles in molluscan reproductive control' [21]; modulation of vertebrate-type steroid levels in prosobranchs plays a key role in imposex development' [5]; 'essentially, molluscs use 'true' hormones for chemical signaling within their body tissues, including vertebrate-type sex steroids, which they are able to produce *de novo* in the gonad' [22]; 'steroid production in molluscs is undisputedly similar to that in vertebrates' [8]; 'steroids play important roles in the regulation of reproduction in both vertebrates and invertebrates' [23]; 'vertebrate-type steroids and steroid receptors are present, performing roles in molluscs which are similar to those which they play in vertebrates' [24];

a cautious positive conclusion – i.e. the authors make similar positive statements, but qualify them with words such as 'hope', 'indicate', 'suggest', 'potentially', 'possible', 'might' and 'may'; and also tend to suggest that gaps in the data are likely to be resolved (in a positive direction) by further research [9,10,12,25–31].

a cautious negative conclusion – i.e. the authors imply that an endocrine role is 'unlikely' or that there is insufficient evidence to draw a conclusion one way or the other [11,32]

a strong negative conclusion – i.e. the authors imply that an endocrine role is 'improbable' [6,16,17].

From the above, it is hard to argue against the contention that, among most people working in this field, there is a positive expectation (i.e. a preconception) that estrogens and androgens will cause reproductive effects when administered to mollusks. Carrying out an experiment with any preconception at all means that it is potentially 'biased', and this is something that has implications for the handling, statistical analysis and presentation of experimental data. It is emphasized that the word 'bias' in relation to experimental science does not imply 'bigotry' on the part of scientists, nor should it be interpreted in any way whatsoever that any person referred to directly or indirectly in this review has ever consciously or deliberately manipulated data. Bias is basically the potential for an experimenter to influence the outcome of an experiment in any way at all (whether consciously or subconsciously) and is a problem that applies to all fields of experimental science.

3. Evaluation of bioassay data

In order to evaluate the 'quality' of the bioassay data that underpin the belief that steroids have hormonal actions in mollusks, the following information was obtained from each paper: species, effect measured, compound and route of exposure, time of exposure, dose, whether there was an effect and if so, the size of the effect, the shape (if any) of the dose–response curve, whether or nor the data were statistically analyzed (though a positive answer does not necessarily mean that the analysis was correct or appropriate), whether the finding was repeated within-study, the number of replicates, the number of animals and whether actual, as opposed to nominal concentrations of the compound were used. Complete information is provided in a set of tables in Supplementary Information. Only a brief summary, with key points of interest, is included in Tables 1–3 in the main body of the review. These are split into studies that have directly tested the effects of steroids (Table 1); those that have tested compounds that are not themselves steroids (Table 2), but are known to: either act as weak estrogens (e.g. nonylphenol [NP] and Bisphenol A [BPA]) or anti-androgens in vertebrates, are drugs that modify the actions of steroids in vertebrates (e.g. the androgen receptor antagonist, cyproterone acetate or aromatase inhibitors such as Fadrozole), or are compounds that have been previously hypothesized to exert their actions via affecting endogenous steroid

Table 1
Responses of mollusks to vertebrate-type steroids.

Species	Steroids	Effect claimed?	Maximum effect size	Number of doses (shape of response curve)	Within study repetition?	Statistics?	Effect(s) measured
A. Gonad growth, fecundity, hatchability, sex ratio and secondary sexual characteristics							
Dogwhelk, *Nucella lapillus* & netted dogwhelk, *Nassarius reticulata* [53]	T	Y	4	1	N	N	Imposex/penis growth
Mediterranean land snail, *Theba pisana* [76]	T	Y	?	1	N	N	Gonad histology
Pacific oyster, *Crassostrea gigas* [64]	EB	Y	2	1	Y[a]	N	Sex ratio
Sea snail, *Murex trunculus* [77]	EB, TP	N	–	1	N	N	Gonad histology
Slug, *Ariolimax californicus* [78]	DHEA, KT	Y	2	1	N	N	Gonad weight
Slug, *Arion rufus* [79]	P, T, E2	N	–	1	N	N	Gonad histology
Slug, *Milax gagates* [80]	EB, TP	N	–	1	N	N	Gonad histology
Slugs, *Derocers reticulates* & *Limax flavus* [81]	E1, E2, T, DHEA, Preg	Y	2	1	N	N	Induction of egg-laying
Terrestrial snail, *Euhadra peliomphala* [82]	T, E2	Y	?	1	N	N	Induction of egg-laying
Coot clam, *Mulinia lateralis* [83]	MT	Y	?	3 (top dose only)	N	N	Sex ratio
Giant ramshorn snail, *Marisa cornuarietis* [84]	EE2	Y	1.5	1	N	Y	Fecundity
Giant ramshorn snail, *Marisa cornuarietis* [85]	MT, EE2	Y	5	1	N	Y	Imposex/penis growth
Giant ramshorn snail, *Marisa cornuarietis* [86]	EE2	N	–	1	N	Y	Hatching success
Bloodfluke planorb, *Biomphalaria glabrata* [87]	T, EV	Y	4	1	N	Y	Fecundity
Peppery furrow shell, *Scrobicularia plana* [88]	E2, EE2	Y	1.5	1	N	Y	Oocyte diameter
Dogwhelk, *Nucella lapillus* [55]	T	Y	?	1	N	Y	Imposex/penis growth
Edible snail, *Helix pomatia* [89]	T, E1, P	Y	2	1	N	Y	Gonad histology
Edible snail, *Helix pomatia* [90]	Cortisol, DOC, NorT	Y	2	1	N	Y	Gonad histology
NZ Mudsnail, *Potamopyrgus antipodarum* [49,56]	EE2	Y	3	1	N	Y	Embryo production
Scallop, *Mizuopecten yessoensis* [91]	T, E2, P	Y	?	1	N	Y	Gonad histology
Scallop, *Placopecten magellanicus* [92]	T, E2, P, DHEA	Y	2	1	N	Y	Gonad differentiation
Giant ramshorn snail *Marisa cornuarietis* [93]	MT	Y	2	2	N	Y	Penis growth
Eastern mud snail, *Ilyanassa obsoleta* [94]	T	Y	3	2	N	Y	Imposex/penis growth
Great ramshorn snail *Planorbarius corneus* [63]	E2	N	–	2	N	Y	Fecundity
	E2	N[b]	–	3	N	Y	Fecundity
	E2	Y[c]	2	3 (high dose only)	N	Y	Fecundity
River snail, *Viviparus viviparus* [63]	E2	Y	2	2 (low dose only)	N	Y	Unhatched embryos[d]
Great pond snail, *Lymnaea stagnalis* [95]	MT	N	–	3	N	Y	Fecundity
Dogwhelk, *Nucella lapillus* [54]	T	Y	2	3 (top two doses)	N	Y	Penis growth
NZ Mudsnail, *Potamopyrgus antipodarum* [10]	EE2	Y	10	4 (inverted-U)	N	Y	Embryo production
B. Induction of egg yolk protein production							
Freshwater mussel, *Elliptio complanata* [96]	E2, T	Y	5	1	N	Y	ALP in hemolymph
Pacific oyster, *Crassostrea gigas* [97]	E2	Y	1.5	1	N	Y	VT
Scallop, *Patinopectin yessoensis* [98]	E2	Y	2	1	N	Y	VT using EIA
Sydney rock oyster, *Saccostrea glomerata* [45]	EE2	Y	7	2	N	Y	VT using HPLC & UV
Freshwater mussel, *Elliptio complanata* [99]	E2, T, P, Ad	N	–	3	Y	Y	VT using PAGE
Freshwater mussel, *Elliptio complanata* [100]	E2	Y	2	3 (monotonic)	N	Y	VT mRNA & ALP
Freshwater mussel, *Elliptio complanata* [101]	E2	Y	1.5	3 (monotonic)	N	Y	ALP in hemolymph
NZ Mudsnail, *Potamopyrgus antipodarum* and *Valvata piscinalis* [102]	MT	Y	2	3 (flat)	N	Y	ALP
Mussel, *Mytilus edulis* Swan mussel, *Anodonta cygnea* [103]	E2	N	–	4	N	Y	VT on PAGE & proteomics
	E2	N	–	4	N	Y	
Soft-shell clam, *Mya arenaria* [104]	E2	Y	1.5	5 (inverted-U)	N	Y	ALP in hemolymph
C. Miscellaneous physiological and molecular biological effects							
Venus mercenaria and *Mactra solidissima* [1]	E2	N	–	1	N	N	Respiratory measures
Giant ramshorn snail, *Marisa cornuarietis* [86]	EE2	N	–	1	N	Y	Heart rate
Mussel, *Mytilus edulis* [57,58]	E2	N	–	1	N	Y	VT & nER mRNAs

Table 1 (*continued*)

Species	Steroids	Effect claimed?	Maximum effect size	Number of doses (shape of response curve)	Within study repetition?	Statistics?	Effect(s) measured
Mussel, *Mytilus edulis* [52]	E2	Y[e]	100	2	N	Y	Serotonin receptor & COX mRNA
	E2	Y[f]	100	2	N	Y	
	EE2	Y[e]	100	2	N	Y	
	EB	Y[e]	100	1	N	Y	
Mussel, *Mytilus edulis* [51]	E2	Y[e]	1000	3 (inverted-U)	N	Y	VT & nER mRNA
	E2	N[f]	–	2	Y	Y	
	EE2	Y[e]	100	2 (both doses)	N	Y	
	EB	Y[e]	1000	1	N	Y	
Mussel, *Mytilus galloprovincialis* [105,106]	E2	Y	3	3 (monotonic, U-shaped)	N	Y	Esterifying enzymes & aromatase
Mussel, *Mytilus galloprovincialis* [31]	T	Y	3	3 (monotonic)	N	Y	Esterifying enzymes
D. Short-term effects (minutes to hours; mainly in vitro)							
Octopus, *Octopus vulgaris* [107]	P	Y	2	1	N	Y	VT & oocyte proliferation marker
Octopus, *Octopus vulgaris* [108]	P	Y	?	1	N	N	Sperm activation
Mussel, *Mytilus edulis* [43]	E2	Y	15	3 (monotonic)	N	N	NO production
Mussel, *Mytilus galloprovincialis* [109]	EE2	Y	3	1 & 3 (monotonic)	N	Y	Lysosomal membrane stability & kinases
Mussel, *Mytilus edulis* [66]	E2, P, Cortisol	Y	3	1	N	Y	Lysosomal membrane stability
Mussel, *Mytilus galloprovincialis* [67]	E2	Y	10	1 & 4 (monotonic)	N	Y	Lysosomal membrane stability & cytosolic Ca^{++}
Slug, *Laevicaulis alte* [110]	E2, T	Y	?	1	N	Y	Various enzymes
Mussel, *Mytilus galloprovincialis* [44]	E2	Y	9	3 (monotonic, inverted-U)	N	Y	Lysosomal membrane stability
Scallop, *Placopecten magellanicus* [111]	E2, T, P	Y	3	5 (inverted-U)	N	Y	*In vitro* gamete release

Note: This is an abbreviated table. The full table on which it is based and which contains extra information (such as dosage, routes of exposure, experimental duration and whether doses were nominal or actual) is available in Supplementary Information.

Abbreviation used: Y, Yes; N, No; Ad, Androstenedione; ALP, Alkaline-labile phosphate (i.e. phosphate assumed to be part of the egg yolk protein); Aromatase, CYP19, the enzyme that converts T → E2; COX, Cyclooygenase; DHEA, Dehydroepiandrosterone (part of the steroid biosynthetic pathway in vertebrates; precursor to androstenedione; DOC, 11-deoxycorticosterone; E1, Estrone (main metabolite of E2 and common in the environment); E2, 17β-Estradiol (the main vertebrate estrogen); EB, 17β-Estradiol benzoate (a more water-soluble version of E2); EE2, Ethinyl estradiol (a potent synthetic estrogen used in 'the Pill'); EIA, Enzyme Immunoassay; EV, 17β-Estradiol valerate (a more water-soluble version of E2); HPLC, High Performance Liquid Chromatography; Imposex, the existence of male characteristics (e.g. a penis) in female mollusks; KT, 11-ketotestosterone (the main androgen in teleost fishes); mRNA, messenger RNA; MT, Methyltestosterone (a potent synthetic androgen); nER, nuclear estrogen receptor, NO, Nitric oxide; norT, 19-nortestosterone (intermediate in the aromatization of T → E2); P, Progesterone (the main progestin in mammals); PAGE, Polyacrylamide Gel Electrophoresis; Preg, Pregnenolone; T, Testosterone (the main androgen in mammals); TP, Testosterone propionate (a more water soluble version of T); UV, Ultraviolet; VT, Vitellin (egg yolk).

[a] Effect noted in 2 out of 3 experiments (animals in negative experiment were immature).
[b] Simulated summer conditions.
[c] Simulated autumn conditions.
[d] No effect noted for neonates or hatched eggs.
[e] Immature animals.
[f] Mature animals.

production in mollusks (e.g. TBT); or are studies that have tested the effects of effluents (Table 3), on the basis that, in the majority of these papers, it was suggested that effects were due to the presence of 'estrogens' or 'anti-androgens' in the effluent (or of compounds that were presumed to affect endogenous steroid production).

Within Tables 1 and 2, the studies are divided into those that show: (A) the effects of steroids on gonad development and secondary sexual characteristics in mollusks; (B) the effects of steroids on vitellin (egg yolk protein) production in mollusks; (C) the effects of steroids on various other physiological and molecular endpoints in mollusks; and (D) very short-term (minutes to hours) effects of steroids in mollusks.

From the outset, it must be made absolutely clear that none of the studies that are included in Tables 2 and 3 can be accepted as evidence for the involvement of vertebrate steroids in mollusk reproduction. Although compounds like NP and BPA are weak estrogen agonists in vertebrates, it has only ever been an assumption that any effect that they might have in mollusks is via similar mechanisms. It is also only an assumption that drugs, such as cyproterone acetate and aromatase inhibitors, which affect the binding or synthesis of steroids in vertebrates, necessarily exert their actions in the same way in mollusks. Similarly, it has never been more than a hypothesis (Section 12) that TBT exerts its undeniable effects on mollusc reproduction via interference with T production in mollusks. Up until about 2007, these assumptions could be described as plausible – as they were based mainly on the widespread presence of vertebrate steroids in molluscan tissues. However, in the past five years it has become increasingly clear that the presence of steroids in mollusks can be readily explained by their penchant for taking up (and then storing) vertebrate steroids from the environment (see review by [6]); and that the 'mollusk genome' does not contain the genes for critical steroid synthetic enzymes (such as aromatase) or for functional steroid nuclear receptors [17,33]. In the face of this negative evidence, it is apparent that if (as they appear to do) compounds such as aromatase inhibitors and NP are able to elicit effects in mollusks, then they must do so by mechanisms which are unrelated to their demonstrated mechanism of action in vertebrates (i.e. interfering with steroid synthesis or receptor binding).

The total number of publications in Table 1 (i.e. those that have specifically tested natural or synthetic vertebrate steroids) is 55 (of which two are abstracts). Since quite a few of these papers have investigated more than one steroid in more than one species, there have actually been ca. 100 exposure experiments on steroids. Several of these papers have measured more than one end-point, which means that there are data on ca. 150 steroid-effect relationship experiments in mollusks.

Table 2
Responses of mollusks to compounds that are not themselves vertebrate steroids (but are assumed, whether rightly or wrongly, to modify their function).

Species	Compounds	Effect claimed?	Maximum effect	Number of doses (shape of response curve)	Within study repetition?	Statistics?	Effect(s) measured
A. Gonad growth, fecundity, hatchability, sex ratio and secondary sexual characteristics							
Dogwhelk, *Nucella lapillus* & netted dogwhelk, *Nassarius reticulata* [53]	TBT	Y	2	3 (monotonic)	N	N	Imposex/penis growth
	CPA		6	1			
Giant ramshorn snail, *Marisa cornuarietis* [85]	TBT	Y	3	1	N	Y	Imposex
	CPA	Y	2	1			
Netted dogwhelk, *Hinia reticulata* [85]	CPA	Y	1.5	1	N	Y	Penis growth
Peppery furrow shell, *Scrobicularia plana* [88]	NP, OP (+ E2, EE2)	Y	1.3	1	Y[a]	Y	Intersex & oocyte diameter
Dogwhelk, *Nucella lapillus* [112]	TBT	Y	10	1	N	Y	Imposex/penis growth
	CPA	Y	2–8	1			
Dogwhelk, *Nucella lapillus* [55]	TBT	Y	10	1	N	Y	Imposex/penis growth
	APGW	N	–	1	N	Y	
Dogwhelk, *Nucella lapillus* [75]	TBT	Y	5	1	N	Y	Imposex
	CPA	Y	2	1			
Dogwhelk, *Nucella lapillus* [85]	TBT	Y	1.2	3 (flat)	N	Y	Imposex/penis growth
	CPA			1			
Giant ramshorn snail, *Marisa cornuarietis* [86]	BPA	Y	1.1	1	N	Y	Hatching
Eastern mud snail, *Ilyanassa obsoleta* [94]	TBT	Y	4	3 (inverted-U)	N	Y	Imposex/penis growth
	APGW	Y	3	4 (flat)			
Great pond snail, *Lymnaea stagnalis* [95]	NP	N	–	3	N	Y	Fecundity & hatching
Dogwhelk, *Nucella lapillus* [85]	VZ	Y	1.5	3 (flat)	N	Y	Penis & prostate growth
Dogwhelk, *Nucella lapillus* [39]	BPA, OP	Y	1.3	3 (flat)	N	Y	Imposex
NZ mudsnail, *Potamopyrgus antipodarum* [113]	FAD	Y	2–5	4 (flat)	Y	Y	Embryo production
NZ Mudsnail, *Potamopyrgus antipodarum* [10]	BPA, OP	Y	10	4 (inverted-U)	N	Y	Embryo production
NZ Mudsnail, *Potamopyrgus antipodarum* [49,56]	BPA	Y	2	5 (monotonic)	N	Y	Embryo production
	OP	Y	1.3	5 (monotonic)			
	NP	Y	1.3	6 (inverted-U)			
Giant ramshorn snail, *Marisa cornuarietis* [93]	TBT	Y	3.5	5 (flat)	N	Y	Imposex
	FEN	Y	2.5	2	N	Y	
Giant ramshorn snail, *Marisa cornuarietis* [35,36]	BPA	N	–	5	Y	Y	Fecundity & hatchability
Giant ramshorn snail, *Marisa cornuarietis* [39,84]	BPA	Y	1.8	4 & 6 (partly monotonic, flat)	Y	Y	Fecundity
Great pond snail, *Lymnaea stagnalis* [114]	VZ	Y	3	6 (top two doses only)	N	Y	Fecundity & fertility
NZ Mudsnail, *Potamopyrgus antipodarum* [56,115]	TPT	Y	4	7 (monotonic)	N	Y	Embryo production
	TBT	Y	8	7 (monotonic)			
B. Induction of egg yolk protein production							
Mussel, *Mytilus edulis* [116]	BPA	N	–	1	N	N	ALP & histology
NZ Mudsnail, *Potamopyrgus antipodarum* and *Valvata piscinalis* [102]	BPA	Y	2	3 (flat)	N	Y	ALP & VT on PAGE
	OP	Y	2–8	3 (lowest dose)	N	Y	
Freshwater mussel, *Elliptio complanata* [96]	Cop	Y	2.5	3 (inverted-U)	N	Y	ALP in hemolymph
Mussel, *Mytilus galloprovincialis* [117]	NP	Y	30	4 (one dose)	N	Y	ALP
Sydney rock oyster, *Saccostrea glomerata* [45]	NP	Y	1.5	2 (top dose only)	N	Y	VT using HPLC & UV
Cockle, *Cerastoderma glaucum* [50]	NP	Y	2–10	4 (all shapes)	Y	Y	ALP
Manila clam, *Tapes philippinarum* [118]	NP	Y	3	4 (monotonic)	N	Y	ALP
Soft-shell clam, *Mya arenaria* [104]	NP	Y	4	3 (monotonic)	N	Y	ALP
Freshwater mussel, *Elliptio complanata* [101]	NP	Y	1.6	3 (monotonic)	N	Y	ALP
Valvata piscinalis [102]	BPA	N	–	3	Y	Y	ALP
	OP	N	–	3			
C. Miscellaneous physiological and molecular biological effects							
Giant ramshorn snail, *Marisa cornuarietis* [86]	BPA	Y	1.2	2	N	Y	Heart rate
Freshwater mussel, *Dreissena polymorpha* [119]	NP	Y	2	3 (flat)	N	Y	Oxidative stress enzymes, T & E2
Dogwhelk, *Nucella lapillus* & netted dogwhelk, *Nassarius reticulata* [53]	TBT	Y	2	3 (monotonic)	N	N	T & E2
Giant ramshorn snail, *Marisa cornuarietis* [93]	TBT	Y	5	5 (flat)	N	Y	Esterification of T & E2
	FEN	N	–	2			

Table 2 (*continued*)

Species	Compounds	Effect claimed?	Maximum effect	Number of doses (shape of response curve)	Within study repetition?	Statistics?	Effect(s) measured
D. Short-term effects (minutes to hours; mainly in vitro)							
Mussel, *Mytilus galloprovincialis* [109]	DES, BPA, Gen, NP	Y	2.5	3 (monotonic)	N	Y	Lysosomal membrane breakdown & kinases
Mussel, *Mytilus edulis* [43]	TAM	Y	15	1	N	N	NO release from pedal ganglion
Mussel, *Mytilus galloprovincialis* [67]	TAM	Y	10	1	N	Y	Lysosomal membrane breakdown

The full table on which it is based and which contains extra information (such as dosage, routes of exposure, experimental duration and whether doses were nominal or actual) is available in Supplementary Information. [a]10-fold difference in doses between experiments.
Abbreviations used: Y, Yes; N, No; ALP, Alkaline-labile phosphate (i.e. phosphate assumed to be part of the egg yolk protein); APGW, Alanine-proline-glycine-tryptophan, a neuropeptide found in mollusks; BPA, Bisphenol-A (an exceedingly weak estrogen in vertebrates); Cop, Coprostanol, a metabolite of cholesterol formed in the gut (common constituent of sewage effluent); CPA, Cyproterone acetate (an antagonist of the vertebrate nuclear androgen receptor; DES, Diethylstilbestrol (a potent non-steroidal estrogenic drug no longer in use); FAD, Fadrozole (an aromatase inhibitor); FEN, Fenitrothione (an insecticide with anti-androgenic properties in vertebrates); Gen, Genistein, a weak estrogen present in some plants; HPLC, High Performance Liquid Chromatography; Imposex, the existence of male characteristics (e.g. a penis) in female mollusks; mRNA, messenger RNA; nER, nuclear estrogen receptor; NP, Nonylphenol (a synthetic surfactant – once widely used, but now banned in Europe); OP, Octylphenol (a synthetic surfactant – once widely used, but now banned in Europe); PAGE, Polyacrylamide Gel Electrophoresis; TAM, Tamoxifen (an estrogen receptor antagonist used as a medicine); TBT, Tributyl Tin (an anti-foulant); TPT, Triphenyl Tin (another anti-foulant); UV, Ultraviolet; VT, Vitellin (egg yolk); VZ, Vinclozolin (a fungicide with anti-androgenic activity in vertebrates).

Table 3
Field exposure studies in mollusks in which the effects have been ascribed to the presence of steroid-mimicking or steroid-modifying endocrine disrupters.

Species	Exposure to:	Effect	Effect size	Within study repetition?	Effect(s) measured
Calico scallop, *Argopecten gibbus* [120]	Municipal dump	Y	2	N	ALP
Dogwhelk, *Nucella lapillus* [121]	Raw sewage	Y (incremental, inverted-U)	3	N	% Mature females & nER mRNA
Dogwhelk, *Nucella lapillus* [122]	Sewage effluent + TBT	N	3	N	Imposex/penis growth
Freshwater mussel, *Elliptio complanata* [123]	Sewage effluent	Y	1.5	N	ALP & sex ratio
Great ramshorn snail *Planorbarius corneus* [38]	Sewage effluent	Y (U-shaped)	3	Y	Fecundity
Mussel, *Mytilus edulis* [124]	North sea oil + AP + PAH	Y	3	N	Steroids & aromatase
Mussel, *Mytilus edulis* [125]	North sea oil + AP + PAH	Y (oil alone)	7	N	ALP
NZ mudsnail, *Potamopyrgus antipodarum* [126]	River sediments	Y	2	N	Embryo production
NZ mudsnail, *Potamopyrgus antipodarum* [127]	Sediment fractions	Y	3	N	Embryo production
NZ mudsnail, *Potamopyrgus antipodarum* [10]	Sewage effluent	Y (city skyline)	2	N	Embryo production
NZ mudsnail, *Potamopyrgus antipodarum* [128]	Sewage effluent	Y	4	N	Embryo production & steroids
NZ mudsnail, *Potamopyrgus antipodarum* [129]	Zinc ore treatment plant	Y	3	N	Embryo production & steroids
NZ mudsnail, *Potamopyrgus antipodarum* [130]	Coal waste sediments	Y (U-shaped)	2	N	Embryo production
Peppery furrow shell, *Scrobicularia plana* [131]	Pollution	Y	2	N	Gonad weight & steroids
Soft-shell clam, *Mya arenaria* [132]	Inshore seawater	Y	1.5	N	ALP
Zebra mussel, *Dreissena polymorpha* [133]	Sewage effluent	Y	3	N	ALP

Abbreviations used: Y, Yes; N, No; ALP, Alkaline-labile phosphate (i.e. phosphate assumed to be part of the egg yolk protein); AP, Alkylphenols; mRNA, messenger RNA; nER, nuclear estrogen receptor; PAH, Polyaromatic Hydrocarbons; TBT, Tributyltin.

4. Why should one doubt results even if they are statistically significant at the 5% level?

Twelve out of the 55 papers listed in Table 1 lack any form of statistical analysis. These studies must be considered as providing the lowest grade of proof of the actions of steroids on mollusks (and have been sorted so that they are listed at the top of each section in Table 1).

If one dismisses publications that do not include statistical analysis, one must consider whether the studies that do include statistical analysis are necessarily any more reliable. In a paper entitled 'Why most published research findings are false', Ioannidis [34] states that 'the high rate of non-replication (lack of confirmation) of research discoveries is a consequence of the convenient yet ill-founded strategy of claiming conclusive research findings solely on the basis of a single study assessed by formal statistical significance, typically for a p value less than 0.05. The author demonstrates this mathematically and proposes an equation to calculate a much more conservative value (which he terms the 'Positive Predictive Value') that goes beyond the p value by adding another factor, which, in effect, is whether the experiment could have been open to bias. Ioannidis lists six 'corollaries' that arise from the new equation (all of which are as appropriate to studies on mollusks as they are to all other areas of experimental science). These are, quoted directly from his paper:

(1) 'The smaller the studies conducted in a scientific field, the less likely the research findings are to be true'.
In the case of mollusk studies, the majority have involved fairly reasonable animal numbers (15+). However, there is

a problem in that at least 40 out of 55 of the papers involving steroids (full details in Table 1 in Supplementary Information) appear not to have used any replication (i.e. all the test animals within each treatment were held together in one tank or enclosure). When all animals in any one treatment are held together in the same container, it is impossible to determine whether any results were due to the treatment or to 'tank effects' (which could be any number of things, including the position of the tanks in a room, or the presence of a single individual carrying an infectious disease in one of the tanks). In such a situation (referred to by many people as 'pseudoreplication'), it does not matter how many animals are in each container, the results of statistical analysis are effectively meaningless.

(2) 'The smaller the effect sizes in a scientific field, the less likely the research findings are to be true'.
As will be discussed in more detail below, the effect size in most mollusk steroid bioassays is very small in comparison to the estrogen and androgen bioassays conducted with fish. The combination of sample size, effect size and expected standard deviation of the data effectively determines the 'statistical power' of the study. The larger the effect size, the smaller the number of animals needed and *vice versa*. Only one research group in the mollusk field [35–37], has taken into account statistical power at the stage of designing experiments. Their sobering conclusion (from the point of view of effort and cost) was that to show that a 26% increase in fecundity of the Giant Ramshorn (or Apple) snail, *Marisa cornuarietis* was significant at the 5% level, one needed to use at least ten animals per tank, four treatment doses and one control, with six replicates per treatment and twelve replicates for the control.

(3) 'The greater the number and the lesser the selection of tested relationships in a scientific field, the less likely the research findings are to be true'.
Just one example in the mollusk research field is that on the study of treated sewage effluent on reproduction in the Great Ramshorn snail, *Planorbarius corneus* [38]. In that study, the animals were measured every two weeks over a three-month period. This effectively gave the authors seven separate opportunities to demonstrate significant differences between the treatments (with an eighth opportunity provided by combining all the data). Basically, the more opportunities there are to make measurements and the more things that can be measured, the more likely one is to find significant differences at the $p = 0.05$ level.

(4) 'The greater the flexibility in designs, definitions, outcomes, and analytical modes in a scientific field, the less likely the research findings are to be true'.
An examination of Table 1 exposes the fact that very little, if any, of the methodology is standardized. There are only a handful of papers that are based on studies carried out on any one species. There is no consistency in how the experiments are carried out. The animals are more likely than not to have been derived from wild stocks (with potential disease problems that might affect the outcome of the experiment). To be fair, this deficiency – the lack of a standardized operating procedure for testing chemicals (i.e. not necessarily 'vertebrate-type endocrine disrupters') in mollusks is well recognized [8] and steps are being taken to resolve the problem.

(5) 'The greater the financial and other interests and prejudices in a scientific field, the less likely the research finding are to be true'.
In most countries nowadays, experiments on vertebrates are covered by complex rules and hugely expensive legislation (and continuous pressure from animal welfare groups). The prospect of finding an invertebrate that is responsive to vertebrate-type estrogens and androgens is very attractive to scientists and regulators alike (not least because the financial rewards would be considerable). In fact, the existence of an invertebrate bioassay for a vertebrate-type steroid could be described as the 'holy grail' of endocrine disruption research.

(6) 'The hotter a scientific field (with more scientific teams involved), the less likely the research findings are to be true'. Work on mollusks can probably not be called 'hot'. The closest the field has come to be being 'hot' is when it was reported [39] that relatively low doses of BPA were able to induce 'superfeminization' (basically an increase in female fecundity) in *M. cornuarietis*. This sparked a great deal of interest among environmentalists (as it appeared to add considerable weight to their arguments that this chemical was bad not just for human health and vertebrate wildlife [40], but also for invertebrate wildlife). When the observation was not independently verified [35] it sparked controversy and ongoing arguments over how the experiments should be carried out (including the correct temperature, the source of animals and the use or lack of use of positive controls) [41,42].

5. The lack of within-study repetition

Astonishingly, only 5 out of the 55 papers listed in Table 1 appear to have attempted to repeat any findings 'within-study' or 'between-study'. The majority of papers have published the results of single experiments only. Although, several of these [43–45] repeated procedures several times, the results were combined for statistical analysis (i.e. they were effectively part of a single experiment). It is difficult to understand (a 'lack of funding' notwithstanding) why anyone should want to carry out and then publish the results of just one experiment. It shows an extraordinary trust in one's own or ones collaborators' abilities not to have made any mistakes or miscalculations; and also a faith that there were no confounding factors that might have produced the final results (e.g. uncontrolled variables such as whether a tank was near a door or not; or, more prosaically, whether a passing workman might have dropped a cigarette in one of the tanks and fished it out before anyone noticed!).

6. The importance of a monotonic dose–response relationship

If one wants to test the effect of potential endocrine disrupters in any species, it is very important that the effect is related in some way to the dose. Ideally, one would like to find a 'dose–response' relationship that is regularly incremental (or decremental) – i.e. for each increase in dose, there should be a graded increase (or decrease) in response, except of course when doses are too low to cause a response or are so high that the factor being measured can respond no further. Essentially, when such response data is plotted against dose on a logarithmic scale, it should ideally produce a 'sigmoid' (i.e. S-shaped) curve. Good examples of bioassays that produce sigmoid curves are those involving estrogen stimulation of VTG production in teleosts [46] and androgen stimulation of spiggin production in the stickleback, *Gasterosteus aculeatus* [47]. For the purposes of this review, such curves will be referred to hereafter as 'monotonic'. A key outcome of bioassays with monotonic dose–response curves (providing they can be repeated consistently) is that it is possible to calculate accurately the 'Lowest effect' and 'No effect' concentrations of compounds. These are considered important for accurate risk assessment (in, for example, current European Chemical Legislation).

One of the most perplexing questions in the endocrine disruption field is how one copes with data in which the dose–response

is non-monotonic. Although there are numerous examples of non-monotonic curves in research papers in the field of ecotoxicology [48], there are no known examples of statutory test procedures using vertebrates that operate with non-monotonic response curves. Of the 150 individual steroid-effect relationships tested in mollusks, 99 elicited an effect. Of these, 57 involved a single dose only, 12 involved two doses and 30 involved three or more doses. In this final group, only eight had curves that could be described as monotonic (almost exclusively in the short-term effect category), four had 'inverted-U' curves and the remainder consisted of flat line responses, U-shaped curves or irregular responses (e.g. responses were elicited by one or two doses only – not necessarily the highest, and not necessarily adjacent to each other).

In a field in which there is no clear understanding of the mechanisms that link cause and effect (and in which effect sizes are so low), studies using a single dose (and this could arguably be extended to two doses) are open to more than one interpretation, as it is impossible to determine whether any difference between control and treatment represents a true effect of the treatment or of uncontrolled variables.

Yet another problem with interpretation of dose–response curves has been noted in studies in which the same set of animals is sampled at more than one time interval. It is not at all unusual for the curves to be different shapes at each sampling period. For example, when octylphenol (OP) was tested on fecundity of *Potamopyrgus antipodarum*, it was noted that there was no relationship at two weeks, a monotonic/incremental response at four weeks and a more or less decremental relationship at 8 weeks [49]. In another study [50], when NP was tested on egg yolk production (by measuring levels of alkaline-labile phosphate; ALP) in the cockle, *Cerastoderma glaucum*, the dose–response relationships all differed from each other depending on the sampling time and maturity stage of the animals (but were never monotonic).

If a non-monotonic response curves is genuine (and not, for example, due to chance or error), it implies that more than one mechanism is at work. In this situation, the burden of proof is on any researcher who publishes such a relationship, firstly, to show that the dose–response relationship really is genuine (i.e. it can be consistently repeated) and secondly, at some stage in the research process, to reveal the underlying mechanisms that causes the mixed effect. It adds nothing useful to the debate to state that the presence of an inverted-U dose–response curve is 'probably due to a toxic effect of the highest dose(s)', or the presence of a U-shape is 'possibly due to the existence of a "low dose effect" [48]'. Although these might be highly plausible explanations (and turn out to be true), 'plausibility' is no substitute for proof.

7. Endpoints and 'effect size'

As can be seen from Table 1, there is a limited range of measurable and meaningful possible endpoints for vertebrate steroids in mollusks. By far the most sensitive estrogen endpoint in teleost fish, with a massive effect size of >1 million, is VTG induction in males. It is not surprising therefore to see why this has become a popular target (24 studies at least) as a reproductive endpoint in mollusks, in both laboratory and field studies. Whereas in fish, VTG is mainly measured by immunoassay, in mollusks, it is determined mainly (but not exclusively) by measuring ALP (because phosphate is a normal component of yolk proteins). It is evident that the effect size for egg yolk induction by putative 'estrogens' in mollusks is extremely low (the average of those shown in Table 1 is ca. 2.5; compared to >1,000,000 in fish).

As shown in Tables 1–3, other possible endpoints for vertebrate steroids that have been explored in mollusks include those related directly to the reproductive capability of the animals, including gonad size, oocyte diameter, fecundity, embryo production, hatchability, secondary sexual characteristics (such as occurrence of imposex and penis growth in the female dogwhelk, *Nucella lapillus*; or 'head wart' growth in the terrestrial snail, *Euhadra peliomorpha*) and shifts in sex ratio. The majority of these have also reported very low effect sizes (with an average of ca. 2.7).

There have also been a few studies that have targeted an eclectic range of physiological, biochemical and molecular endpoints. These also have mostly yielded relatively low effect sizes (average 2.2) apart from two studies, based on the same set of samples, that reported very large changes (25–1000 fold) in mRNA expression levels coding for yolk protein, nuclear estrogen receptor and serotonin receptor mRNAs in the mussel, *Mytilus edulis* [51,52].

There is also a small body of literature on 'rapid response' endpoints such as lysosomal membrane stability, cytosolic Ca^{2+} concentrations and nitric oxide (NO) production by mollusk tissues (Section 4 in Tables 1 and 2). The average of the effect sizes reported for these effects is a more encouraging 4.3.

Why is a large effect size important? As already discussed, it is an important part of the 'statistical power' equation (i.e. the larger the effect, the fewer the number of animals that are required in the experiment and the more innately variable the animals can be). Unfortunately, in many of the listed studies, not only were there low effect sizes, low numbers of animals and a lack of replication, but also high variability. Such assays can be described as having a low 'signal-to-noise' ratio and, as such are more likely to generate spurious results than assays with a high signal-to-noise ratio. This is one reason why 'within-study' repetition is so important.

A study using female *N. lapillus* [53] showed a 5-fold effect for penis growth in response to T. However only a single dose of T was used, there was no statistical analysis, there was no within-study repetition, and actual concentrations of the compounds were not measured. Although this appeared to verify the finding in the same species several years earlier (that used three doses and recorded a twofold increase in penis length at the top two doses) [54], a more recent study (albeit using a single dose) found that T had no effect at all on penis growth [55]. In other words, in regard to the ability of T to stimulate penis growth in *N. lapillus*, the response is inconsistent.

A study using *P. antipodarum* [10] showed a maximum effect size of 10 for EE2 on embryo production. However, the dose–response curves were of the inverted-U shape, the actual concentrations of the compound were not measured and there was no within-study repetition. An independent experiment using the same species [56] found an effect size of only 2, the EE2 was only tested at one dose, and actual concentration measurements were also not measured.

A study on the Sydney rock oyster, *Saccostrea glomerata* [45] found a 7-fold increase in vitellin (VT) production following administration of EE2. However, a major problem with this study is that the 'effect' was the amount of ultraviolet absorption found in a peak eluting from a High Performance Liquid Chromatography column. Any organic compound with at least one double (i.e. unsaturated) bond will absorb UV, thus there is no guarantee that the authors were actually measuring a protein, let alone VT, as they claimed.

Finally, there is the above-mentioned study on *M. edulis* [51] that found very large changes in VT and nER mRNA expression after treatments with E2 or EE2. In fact, this study was a follow-on from a previous study on the same species [57,58] that reported no effect at all of estrogen treatment on mRNA expression. The authors ascribed the difference in findings to the different state of maturity of the animals. However, this was only speculation (i.e. it has not yet been proved to be the case). The experiment was only carried out once, and the dose–response curve was of the inverted-U type because,

and rather oddly, the highest dose of E2 was totally ineffective (in contrast to the same dose of E2-benzoate).

Most of the above-mentioned studies also failed to replicate with the exception of [55] and [45], that used 2 and 18 tanks, respectively for every treatment.

8. The importance of negative and positive controls

It goes without saying that all experiments need negative controls. If the test substance has to be dissolved in an organic solvent, then the appropriate negative control would be the same volume of solvent that was used for the test substances – and, preferably a further negative control involving no solvent at all (in case the solvent might affect the animals). There are not many studies that have not included a negative control. It is essential however, that controls as well as treatments should be replicated. The replicates should give the same results and, if they do not, it means that there are confounding factors within the experimental set up.

What about positive controls? These are just as important as negative controls. If a test substance has no effect in a bioassay, it could be either that the substance was genuinely inactive or that there was something wrong with the bioassay (e.g. that batch of animals were unresponsive or already naturally at the top range of their response). In this case, as elegantly argued by vom Saal & Welshons [59] a positive control (using a substance at a dose that is known to have an effect) is a very valuable, if not essential, inclusion in any study. However, when studying effects like egg yolk production, fecundity and penis growth in mollusks, what is an appropriate positive control? If one decides that E2 or EE2 should be used as positive controls for the first two effects and T or methyltestosterone (MT) for the third effect, one is operating under the expectation (i.e. preconception) that the endocrine system of mollusks is the same as that of vertebrates; whereas in the case of mollusks, this is actually the question! The recent research that failed to confirm that BPA would induce superfeminization in *M. cornuarietis* [35] was criticized by the people who originally reported the effect [39] for not including EE2 as a positive control [42]. However, as pointed out [41] by the scientists who conducted the latter studies, it would only be an appropriate control if the fecundity in *M. cornuarietis* was already known beyond doubt to be controlled by vertebrate estrogens.

9. Evidence of bias?

As mentioned before, it has been clear for about the past five years that the mollusk genome does not contain the genes for any of the key enzymes that are involved in the biosynthesis of vertebrate steroids, nor for functional steroid nuclear receptors (see reviews by [6] and [17]). Also, it has been pointed out in a personal communication from Dr. Toshihiro Horiguchi that noone has ever demonstrated cells in mollusc tissues that are structurally equivalent to the steroid-producing cells of vertebrates. All this negative evidence contrasts with the fact that 46 of the 55 papers cited in Table 1 report at least one statistically significant effect of steroids in molluscs. This makes it legitimate to query whether the preponderance of papers with an apparently positive outcome might be due to 'publication bias'. In his book called 'Bad Science' [60], Ben Goldacre contends that there is a tendency among researchers to preferentially publish research with a positive outcome. Lest the reader might dismiss this as 'just personal opinion', Goldacre cites a well-conducted study [61] that examined the outcome of seventy officially pre-registered clinical trials on a class of antidepressant drugs. Thirty-seven studies reported a positive effect of which all were published (thirty-six of them in the peer-reviewed literature). However, there were a nearly equal number of studies (thirty-three) that gave negative results. Revealingly, twenty-two were not published at all and another eleven were written up and published in a way that implied that they had a positive outcome! In other words, not a single paper was published in the peer-reviewed literature that concluded that the drug had no effect. Anyone not knowing about the pre-registration process, would, if reviewing the peer-reviewed literature, conclude that the drug was 100% effective. Since molluscan studies do not use any form of pre-registration, it is of course impossible to tell whether they have been subject to the same phenomenon. However, there are at least two unpublished studies that, though small in number, were (from what little is written about them) possibly the two best-funded and best-replicated studies that have ever been carried out on the effect of any steroid on mollusks. Both were carried out on *Limnaea stagnalis*, and both concluded that EE2 at concentrations ranging from 1 to 10,000 ng L^{-1} had no effects on reproductive endpoints. The only clue to the existence of these two studies is that they are mentioned in two review papers [32,62]. Although the first of these reviews included a small summary of one of the studies, the detail was insufficient for it to be included in Table 1.

While it is not possible to accurately quantify the extent of publication bias in the mollusc field, it is possible to argue that there is clear evidence in those studies where findings have been contradictory, of a tendency for authors to rationalize (i.e. explain away) or downgrade the importance of the negative (i.e. no effect) data and enhance the importance of the positive data. Experiments that have yielded negative data have, for example, been explained as being due to 'the experiment not being carried out at the right time of year' [63,64], 'the animals not being at the right stage of maturation' [51], 'the experiment not being done at the right temperature' or 'the animals not being of the correct origin' [42]. There is one paper that even justified showing the results of only one of two (repeat) experiments on the basis that this was the experiment in which 'effects were most pronounced' [38]. The same group [63] based the title of another on their papers ('17β-oestradiol may prolong reproduction in seasonally breeding freshwater gastropod molluscs') on a minor observation (that occurred at the lower of two doses only and was not statistically significant) rather than on the major observation of their main experiment, which was that E2 actually had no effect on the fecundity of *N. lapillus*.

It must be stressed that most if not all of the explanations given above (e.g. seasonal differences) are highly plausible (and might well, after further research, turn out to be the correct answer). However, as already mentioned elsewhere, 'plausibility' is not a substitute for proof. The fact stands that in virtually no cases involving experiments with contradictory results (the one exception being the group that failed to confirm any effect of BPA on fecundity in *M. cornuarietis* [35]), have authors attempted to rationalize those experiments that have had a positive outcome (by, for example, ascribing them to chance, poor experimental design, problems with statistical analysis etc.).

10. Avoiding the possibility of bias

In the field of medicine, published clinical trials are nowadays assigned what is known as a 'Jadad Score' [65]. This is a five-point scale that involves giving one point if the study was described as randomized, another if the study was described as double blind (i.e. neither doctors nor patients know which is the drug and which the placebo) and yet another if there was a description of withdrawals and dropouts. Additional points are given if the method of randomization was actually described in the paper (and was appropriate); and, if the method of 'blinding' was described (and was appropriate). Points, however, are deducted if: the method

of randomization was described, but was inappropriate; and the method of 'blinding' was described, but was inappropriate. Only those that have a score of five can be considered to have totally eliminated bias. Clinical trials that have a Jadad score of zero are considered as good as useless. The reason this particular evaluation system is mentioned now and not at the beginning of the paper is that none of the studies listed in Tables 1 or 2 mentioned anything about having carried out any of the procedures 'blind' and only a handful of papers mentioned randomization, but only in relation to allocation of animals to tanks. However, in regard to future studies, the principles of randomization and 'blinding' are well-worth taking on board for all animal research (not just mollusks). It is only by carrying out procedures in this way (and, most importantly, describing how they were done in 'Materials and Methods'), can scientists avoid the charge of consciously or subconsciously biasing their data towards a 'preferred' conclusion.

11. Importance of independent verification

This is the final criterion to be considered in this review, but possibly the most important. An absolutely essential element for acceptance of any scientific finding is that it should be independently verifiable (i.e. repeatable by another scientist or group of scientists). Apart from the possibility of bias, as discussed above, mistakes are very easily made in science and exciting and convincing findings can be the result of factors as diverse as chance, experimental artefact, contamination, calculation error, bias and even fraud. In this respect, factors such as whether a paper is published in a 'high' or a 'low' impact journal, whether it comes from a well established research group with a 'world class reputation' or whether or not the paper appears to have been properly peer-reviewed are irrelevant. None of these is a reliable guarantee that what is reported in a paper is necessarily correct or truthful. The only way that one can be totally convinced that a finding is robust is to be presented with evidence that the results can be consistently repeated within and between research groups.

The only finding concerning the effect of steroids on mollusks that can be considered to have met the criterion of independent verification is the ability of E2 to cause rapid changes (with a relatively large effect size) in lysosomal membrane stability [66,67] in *Mytilus* spp. Does this then mean that E2 really is a hormone in mollusks? The answer is not necessarily. Firstly, as pointed out by Canesi et al. [44], a decrease in lysosomal membrane stability is considered to be part of an inflammatory reaction to an 'unwelcome' (therefore probably exogenous) compound rather than to an endogenous hormone. There is certainly no evidence to suggest that the effect is endocrine in nature (nor that it has anything to do with reproduction). Furthermore, very little is as yet known about the specificity of the response other than that an increase in nitric oxide (NO) production (which occurs in conjunction with lysosomal membrane breakdown) can be triggered in *M. edulis* tissues not just by E2 [68] but also by a completely unrelated cannabinoid compound [69]. Determining whether E2 is the 'preferred' ligand for a putative receptor that triggers this type of response or whether the E2 cross-talks with a receptor for another compound altogether is something that will be very difficult to establish. If one tests only one compound (and this applies to vertebrate as well as invertebrate studies) and that compound binds to the receptor (or elicits an effect via that receptor), it is impossible (even if one generates monotonic dose–response curves) to conclude that that compound is the natural ligand for that receptor. In this respect, one has to bear in mind that it took at least ten years of research (with massive backing from the pharmaceutical companies) to establish that it was cortisol, and not cortisone, that was the natural stress hormone in humans [70].

12. If testosterone does not mediate the effects of TBT in mollusks, then what does?

As mentioned in the Section 1, a major driving force for research on vertebrate steroids in mollusks was the discovery about thirty years ago that organotin compounds (such as TBT) were causing the females of marine snails (neogastropods) to develop male characteristics (of which the most obvious symptom was the appearance of a penis). Bolstered by the reports of vertebrate steroids being found in mollusk tissues, it was entirely reasonable that researchers at that time came up with the hypothesis that the effects were mediated by T (which is the main androgen in mammals). Aided by a trickle of mainly circumstantial evidence, and in the absence of any alternative hypothesis, the androgen hypothesis has held sway for many years [4]. In truth, the hypothesis that penis growth was caused by T transformed very early into a 'fact'. What people were mainly interested in was how TBT was able to elevate T levels in females so that the hormone would exert its proposed androgenic effect. Several sub-hypotheses were developed [4]), including: TBT inhibits aromatase activity (the consequence of which is a buildup in T, the immediate precursor of E2, in the flesh); TBT inhibits the conjugation of T by sulfotransferases and/or fatty acids; and TBT prevents the excretion of T. All these papers have been mentioned already in either this or the previous review [6]; and as one might expect in a field based on a premise that was never properly substantiated, there has been evidence for and against all these hypotheses; with most, probably all, of the positive evidence being based on low effect sizes.

A big breakthrough in the organotin story came only a few years ago when a group in Japan [71], studying the binding properties of the human Retinoid X Receptor (RXR); similar to steroid nuclear receptors, but derived from a different ancestral gene [72] discovered that TBT enhanced the protein–protein interaction between RXR and its coactivator to a greater extent than its natural ligand 9-cis retinoic acid (RA). This led the group to investigate whether the rock shell, *Thais clavigera* also possessed an RXR and to also investigate what happened when RA was injected into live animals [71]. The outcome of these experiments was that the mollusks did indeed express an RXR gene, and that 1 μg injections of RA were almost as effective at inducing penis growth as 1 μg injections of triphenyl tin (TPT). Although the proportion of affected animals induced by RA was only 50%, the data were sufficiently convincing for the authors to propose a new hypothesis that TBT and TPT exerted their actions via their interaction with the RXR (and not via androgens). The new hypothesis appeared to receive a setback when a group in Germany injected RA into *N. lapillus* and found it did not have any effect on penis development in this species at all [73]. However, a group in Portugal, working with the Japanese and also using *N. lapillus*, came to the exact opposite conclusion that RA was just as effective as TBT in inducing penis development in *N. lapillus*; and unlike the original study on *T. clavigera*, the proportion of affected animals was >90% [55]. Since then, the Japanese group have carried out more investigations on the RXR in *T. clavigera*, including all-important dose–response studies, that strengthen the hypothesis that it is the RXR that mediates the effects of organotins [74]. Very recently, the German research group produced a paper in which they reported that they were still unable to induce imposex with RA in *N. lapillus* [75]. However, they obtained a strong effect with a stable analogue of RA (implying that the negative results were probably due to the instability of RA during its handling). Interestingly, while now agreeing that retinoid signaling is involved in inducing imposex in mollusks, the German group are reluctant to abandon the hypothesis that steroids are also involved in imposex development. The title of their paper is 'Imposex development in *N. lapillus* – Evidence for the involvement

of retinoid X receptor and androgen signaling pathways *in vivo*'. However, their evidence in that paper for the involvement of the androgen signaling pathway is based purely on the ability of the drug cyproterone acetate to diminish the effects of simultaneously added TBT. Although cyproterone acetate is an androgen receptor inhibitor in vertebrates, the lack of evidence for the presence of an androgen receptor in mollusks means that some other explanation for its actions must be sought.

A far more detailed discussion of the role of the RXR in mediating the effects of organotins in molluscs can be found in two recent reviews by scientists with direct experience in the field [18,19]. Both also conclude that there is no functional role for T in the induction of imposex in snails.

13. Conclusions

Any studies that do not actually involve the administration of steroids to mollusks cannot be accepted as evidence for their involvement as hormones in mollusks. Of the 55 studies that have information on the bioassay of vertebrate steroids (Table 1), 14 cannot be relied upon because there was no statistical analysis and 21 cannot be relied upon because they used only single doses of the test compound (i.e. no dose–response data). However, this does not mean that remaining papers necessarily provide reliable evidence. Only a handful of papers in Table 1 had within-study repetition, and the majority also appear not to have used any replication. There has also, except in regard to the ability of E2 to trigger lysosomal membrane breakdown *in vitro* in *Mytilus* spp., been no firm independent verification of the positive effects of steroids. Most of the mollusk bioassays (excluding some of the very short-term ones in section D of Table 1) have a very low signal-to-noise ratio (i.e. low effect size and high variability). When this is taken in combination with the fact that none of the studies (to date) have used rigorous randomization and 'blinding' procedures, the possibility of 'operator bias' has to be treated as another potential source of error.

It must be stressed that none of the above criticisms necessarily mean that any findings that have been published in any paper are wrong. The important point is that if a paper contains any weaknesses in experimental design (however minor) or the findings have not been repeated or there was any opportunity (if not any intent) for operator bias, then the findings cannot necessarily be accepted as right. The solution to this problem is for scientists to use more robust experimental designs, eliminate any possibility of bias, demonstrate that the findings can be repeated consistently and, finally, insist on independent verification.

13.1. A statement from the author

Although this review makes mention of the word 'bias', this is not a criticism of any individual or organization; and I do not for one moment accuse anyone of 'intentional' bias or of 'manipulating' their data. Also, none of my criticisms should be interpreted as implying that I personally believe that any findings in any paper are necessarily wrong.

The author's expertise is in the field of fish steroids. He has been directly involved in several bioassay-type studies designed to test the effects of steroids in fish.

Acknowledgements

The author especially thanks Dr. Alan Pickering and Dr. Christina Lye for twice refereeing the manuscript (and providing many useful suggestions) prior to its submission. The author also expresses gratitude to Dr. Mike Roberts of the Chemicals and Nanotechnology Division, Defra, UK for encouraging the project and providing the necessary financial assistance; and to Paul Haywood of Cefas, Weymouth for providing efficient administration.

The views expressed in this review are the author's own and not necessarily that of any organization or institution.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2012.11.006.

References

[1] Hagerman DD, Wellington FM, Villee C. Estrogens in marine invertebrates. Biol Bull 1957;112:180–3.
[2] Rohlack S. Über das vorkommen von sexualhormonen bei der Meeresshnecke *Littorina littorea* L. Z Vergl Physiol 1959;42:161–80.
[3] Gottfried H, Lusis O. Steroids of invertebrates: the *in vitro* production of 11-ketotestosterone and other steroids by the eggs of the slug, *Arion ater rufus* (Linn.). Nature 1966;212:1488–9.
[4] Matthiessen P, Gibbs PE. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ Toxicol Chem 1998;17:37–43.
[5] Oehlmann J, Di Benedetto P, Tillmann M, Duft M, Oetken M, Schulte-Oehlmann U. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 2007;16:29–43.
[6] Scott AP. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I. Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. Steroids 2012;77:1450–68.
[7] Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. Chem Ecol 1994;8:275–85.
[8] Matthiessen P. An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. Integr Environ Assess Manag 2008;4:274–84.
[9] Hutchinson TH. Small is useful in endocrine disrupter assessment – four key recommendations for aquatic invertebrate research. Ecotoxicology 2007;16:231–8.
[10] Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, et al. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat Toxicol 2004;66:207–22.
[11] Lafont R. Reverse endocrinology, or "hormones seeking functions". Insect Biochem 1991;21:697–721.
[12] Janer G, Porte C. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology 2007;16:145–60.
[13] Janeczko A, Skoczowski A. Mammalian sex hormones in plants. Folia Histochem Cytobiol 2005;43:71–9.
[14] Gooding MP, LeBlanc GA. Biotransformation and disposition of testosterone in the Eastern Mud Snail *Ilyanassa obsoleta*. Gen Comp Endocrinol 2001;122:172–80.
[15] Peck MR, Labadie P, Minier C, Hill EM. Profiles of environmental and endogenous estrogens in the zebra mussel *Dreissena polymorpha*. Chemosphere 2007;69:1–8.
[16] Ketata I, Denier X, Hamza-Chaffai A, Minier C. Endocrine-related reproductive effects in molluscs. Comp Biochem Physiol C 2008;147:261–70.
[17] Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. Independent elaboration of steroid hormone signaling pathways in metazoans. PNAS 2009;106:11913–8.
[18] Sternberg RM. Organotins as endocrine disruptors: an examination of tributyltin-induced imposex in neogastropods. In: Pagliarani A, Trombetti F, Ventrella V, editors. Biochemical and biological effects of organotins. Bentham Science Publishers; 2012. p. 75–82.
[19] Horiguchi T. Ecotoxicological impacts of organotins: an overview. In: Pagliarani A, Trombetti F, Ventrella V, editors. Biochemical and biological effects of organotins. Bentham Science Publishers; 2012. p. 3–24.
[20] Pinder LCV, Pottinger TG, Billinghurst Z, Depledge MH. Endocrine function in aquatic invertebrates and evidence for disruption by environmental pollutants. R&D Technical Report E67; Institute for Freshwater Ecology, Report number: WI/T11068I7/1, 1999. 147pp. Institute for Freshwater Ecology, UK. http://nora.nerc.ac.uk/7206/.
[21] Croll RP, Wang C. Possible roles of sex steroids in the control of reproduction in bivalve molluscs. Aquaculture 2007;272:76–86.
[22] Roast SD, Benstead R. Endocrine disruption horizon scanning: aquatic invertebrates review (report number SC030276/SR1), 2007. 56pp. Environment Agency, UK. http://publications.environment-agency.gov.uk/PDF/SCHO0507BMQA-E-E.pdf.
[23] Morishita F, Furukawa Y, Matsushima O, Minakata H. Regulatory actions of neuropeptides and peptide hormones on the reproduction of molluscs. Can J Zool 2010;88:825–45.

[24] Kortenkamp A, Evans R, Martin O, McKinlay R, Orton F, Rosivatz E. State of the art assessment of endocrine disrupters Final Report Project Contract Number 070307/2009/550687/SER/D3 Annex 1 Summary of the state of the science, 2012. 486pp. European Commission http://ec.europa.eu/environment/endocrine/documents/4_Annex%201%20Summary%20State%20of%20Science%20ED%20V6.pdf.

[25] Lehoux JG, Sandor T. The occurrence of steroids and steroid metabolizing enzyme systems in invertebrates. A review. Steroids 1970;16:141–71.

[26] Sandor T. Steroids in invertebrates. In: Clark Jr WH, Adams TS, editors. Advances in invertebrate reproduction. Amsterdam: Elsevier North Holland Inc.; 1981. p. 81–96.

[27] Marin MG, Matozzo V. Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. Mar Pollut Bull 2004;48:835–9.

[28] Lafont R, Mathieu M. Steroids in aquatic invertebrates. Ecotoxicology 2007;16:109–30.

[29] Köhler H-R, Kloas W, Schirling M, Lutz I, Reye AL, Langen J-S, et al. Sex steroid receptor evolution and signalling in aquatic invertebrates. Ecotoxicology 2007;16:131–43.

[30] Matozzo V, Gagné F, Marin MG, Ricciardi F, Blaise C. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ Int 2008;34:531–45.

[31] Fernandes D, Navarro JC, Riva C, Bordonali S, Porte C. Does exposure to testosterone significantly alter endogenous metabolism in the marine mussel *Mytilus galloprovincialis*. Aquat Toxicol 2010;100:313–20.

[32] Segner H, Caroll K, Fenske M, Janssen CR, Maack G, Pascoe D, et al. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicol Environ Saf 2003;54:302–14.

[33] Callard GV, Tarrant AM, Novillo A, Yacci P, Ciaccia L, Vajda S, et al. Evolutionary origins of the estrogen signaling system: insights from amphioxus. J Steroid Biochem 2011;127:176–88.

[34] Ioannidis JPA. Why most published research findings are false. PLoS Med 2005;2:e124.

[35] Forbes VE, Warbritton R, Aufderheide J, van der Hoeven N, Caspers N. Effects of bisphenol A on fecundity, egg hatchability, and juvenile growth of *Marisa cornuarietis*. Environ Toxicol Chem 2008;27:2332–40.

[36] Forbes VE, Aufderheide J, Warbritton R, van der Hoeven N, Caspers N. Does Bisphenol A induce superfeminization in *Marisa cornuarietis*? Part II: Toxicity test results and requirements for statistical power analyses. Ecotoxicol Environ Saf 2007;66:319–25.

[37] Forbes VE, Selck H, Palmqvist A, Aufderheide J, Warbritton R, Pounds N, et al. Does Bisphenol A induce superfeminization in *Marisa cornuarietis*? Part I. Intra- and inter-laboratory variability in test endpoints. Ecotoxicol Environ Saf 2007;66:309–18.

[38] Clarke N, Routledge EJ, Garner A, Casey D, Benstead R, Walker D, et al. Exposure to treated sewage effluent disrupts reproduction and development in the seasonally breeding ramshorn snail (Subclass: Pulmonata, *Planorbius corneus*). Environ Sci Technol 2009;43:2092–8.

[39] Oehlmann J, Schulte-Oehlmann U, Tillmann M, Markert B. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 2000;9:383–97.

[40] vom Saal FS, Hughes C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ Health Perspect 2005;113:926–33.

[41] Forbes VE, Aufderheide J, Warbritton R, van der Hoeven N, Caspers N. Response from Forbes et al. to Oehlmann et al. Ecotoxicol Environ Saf 2008;69:580–1.

[42] Oehlmann J, Schulte-Oehlmann U, Oetken M. Superfeminization as an effect of bisphenol A in *Marisa cornuarietis*. Ecotoxicol Environ Saf 2008;69:577–9.

[43] Stefano GB, Cadet P, Mantione K, Cho JJ, Jones D, Zhu W. Estrogen signaling at the cell surface coupled to nitric oxide release in *Mytilus edulis* nervous system. Endocrinology 2003;144:1234–40.

[44] Canesi L, Borghi C, Fabbri R, Ciacci C, Lorusso LC, Gallo G, et al. Effects of 17β-estradiol on mussel digestive gland. Gen Comp Endocrinol 2007;153:40–6.

[45] Andrew MN, Dunstan RH, O'Connor WA, Van Zwieten L, Nixon B, MacFarlane GR. Effects of 4-nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock oyster, *Saccostrea glomerata*: vitellogenin induction and gonadal development. Aquat Toxicol 2008;88:39–47.

[46] Sumpter JP, Jobling S. Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. Environ Health Perspect 1995;103(Suppl. 7):173–8.

[47] Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, Scott AP. A sensitive, *in vivo* test for the detection of environmental antiandrogens, using the three-spined stickleback (*Gasterosteus aculeatus*). Environ Health Perspect 2006;114(Suppl. 1):115–21.

[48] Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs Jr DR, Lee D-H, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrinol Rev 2012;33. http://dx.doi.org/10.1210/er.2011-1050.

[49] Duft M, Schulte-Oehlmann U, Weltje L, Tillmann M, Oehlmann J. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. Aquat Toxicol 2003;64:437–49.

[50] Marin MG, Rigato S, Ricciardi F, Matozzo V. Lethal and estrogenic effects of 4-nonylphenol in the cockle *Cerastoderma glaucum*. Mar Pollut Bull 2008;57:552–8.

[51] Ciocan CM, Cuberro-Leon E, Puinean A-M, Hill EM, Minier C, Osada M, et al. Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. Environ Pollut 2010;158:2977–84.

[52] Cuberro-Leon E, Ciocan CM, Hill EM, Osada M, Kishida M, Itoh N, et al. Estrogens disrupt serotonin receptor and cyclooxygenase mRNA expression in the gonads of mussels (*Mytilus edulis*). Aquat Toxicol 2010;98:178–87.

[53] Bettin C, Oehlmann J, Stroben E. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgolander Meeresun 1996;50:299–317.

[54] Spooner N, Gibbs PE, Bryan GW, Goad LJ. The effect of tributyltin upon steroid titres in female dogwhelk, *Nucella lapillus*, and the development of imposex. Mar Environ Res 1991;32:37–49.

[55] Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J-I, et al. Imposex induction is mediated through the retinoid X receptor signalling pathway in the neogastropod *Nucella lapillus*. Aquat Toxicol 2007;85:57–66.

[56] Duft M, Schmidt C, Bachmann J, Brandelik C, Schulte-Oehlmann U, Oehlmann J. Prosobranch snails as test organisms for the assessment of endocrine active chemicals – an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology 2007;16:169–82.

[57] Puinean A-M, Rotchell JM. Vitellogenin gene expression as a biomarker of endocrine disruption in the invertebrate, *Mytilus edulis*. Mar Environ Res 2006;62:S211–4.

[58] Puinean A-M, Labadie P, Hill EM, Osada M, Kishida M, Nakao R, et al. Laboratory exposure to 17β-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. Aquat Toxicol 2006;79:376–83.

[59] vom Saal FS, Welshons WV. Large effects from small exposures II. The importance of positive controls in low-dose research on bisphenol A. Environ Res 2006;100:50–76.

[60] Goldacre B. Bad science. London: Fourth Estate; 2008. 370pp.

[61] Turner EH, Matthews AM, Linardatos E, Tell RA, Rosenthal R. Selective publication of antidepressant trials and its influence on apparent efficacy. N Engl J Med 2008;358:252–60.

[62] Lagadic L, Coutellec M-A, Caquet T. Endocrine disruption in aquatic pulmonate molluscs: few evidences, many challenges. Ecotoxicology 2007;16:45–59.

[63] Benstead RS, Baynes A, Casey D, Routledge EJ, Jobling S. 17β-Oestradiol may prolong reproduction in seasonally breeding freshwater gastropod molluscs. Aquat Toxicol 2011;101:326–34.

[64] Mori K, Muramatsu T, Nakamura Y. Effect of steroid on oyster – III. Sex reversal from male to female in Crassostrea gigas by estradiol-17β. Bull Jpn Soc Sci Fish 1969;35:1072–6.

[65] Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJM, Gavaghan DJ, et al. Assessing the quality of reports of randomized clinical trials: is blinding necessary? Contr Clin Trials 1996;17:1–12.

[66] Moore MN, Lowe DM, Fieth EM. Responses of lysosomes in the digestive cells of the common mussel, *Mytilus edulis*, to sex steroids and cortisol. Cell Tissue Res 1978;188:1–9.

[67] Canesi L, Ciacci C, Betti M, Lorusso LC, Marchi B, Burattini S, et al. Rapid effects of 17β-estradiol cell signaling and function of *Mytilus* hemocytes. Gen Comp Endocrinol 2004;136:58–71.

[68] Stefano GB, Zhu W, Mantione K, Jones D, Salamon E, Cho JJ, et al. 17-β estradiol down regulates ganglionic microglial cells via nitric oxide release: presence of an estrogen receptor β transcript. Neuroendocrinol Lett 2003;24:130–6.

[69] Stefano GB, Liu Y, Goligorsky S. Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia and human monocytes. J Biol Chem 1996;271:19238–42.

[70] Quirke V. Making British cortisone: Glaxo and the development of corticosteroids in Britain in the 1950s–1960s. Stud Hist Philos Biol Biomed Sci 2005;36:645–74.

[71] Nishikawa J-I, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ Sci Technol 2004;38:6271–6.

[72] Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M. Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Evol Biol 2004;21:1923–37.

[73] Oehlmann J, Oetken M, Schulte-Oehlmann U. A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. Environ Res 2008;108:140–9.

[74] Horiguchi T, Ohta Y, Nishikawa T, Shiraishi F, Shiraishi H, Morita M. Exposure to 9-cis retinoic acid induces penis and vas deferens development in the female rock shell, *Thais clavigera*. Cell Biol Toxicol 2008;24:553–62.

[75] Stange D, Sieratowicz A, Oehlmann J. Imposex development in *Nucella lapillus* – evidence for the involvement of retinoid X receptor and androgen signalling pathways *in vivo*. Aquat Toxicol 2012;106–107:20–4.

[76] Sakr AA, Osman GY, Abo-Shafey AE. Effect of testosterone on the ovotestis of the land snail *Theba pisana*. Funct Dev Morphol 1992;2:99–101.

[77] Rose MM, Hamon M. Sur l'action des hormones sexuelles de synthèse chez le Mollusque Gastéropode *Murex trunculus* Linne. C R Biol 1939;131:106–8.

[78] Gottfried H, Dorfman IV RI. On the optic tentacle-gonadal axis in the control of the male-phase ovotestis in the slug (Ariolimax californicus). Gen Comp Endocrinol 1970;15:101–19.
[79] Laviolette P. Rôle de la gonade dans le déterminisme humoral de la maturité glandulaire du tractus génital chez quelques gastéropodes *Arionidae* et *Limacidae*. Bull Biol 1954;88:310–31.
[80] Rose MM, Hamon M. Sur l'influence des hormones sexuelles de synthèse chez le Mollusque Gastéropode pulmoné, *Milax (Lallemantia) gagates* Draparnaud. C R Biol 1939;131:937–9.
[81] Takeda N. Induction of egg-laying by steroid hormones in slugs. Comp Biochem Physiol A 1979;62:273–8.
[82] Takeda N. Hormonal control of head-wart development in the snail, *Euhadra peliomphala*. J Embryol Exp Morphol 1980;60:57–69.
[83] Moss SM. Effects of exogenous androgens on growth, biochemical composition, and reproduction of the coot clam, *Mulinia lateralis*. Pac Sci 1989;43:200.
[84] Oehlmann J, Schulte-Oehlmann U, Bachmann J, Oetken M, Lutz I, Kloas W, et al. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. Environ Health Perspect 2006;114:127–33.
[85] Tillmann M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III. Cyproterone acetate and vinclozolin as antiandrogens. Ecotoxicology 2001;10:373–88.
[86] Schirling M, Bohlen A, Triebskorn R, Köhler H-R. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential development and endocrine disruptors. Chemosphere 2006;64:1730–8.
[87] De Souza CP, Martins EV, Dias EP, Katz N. Influencia de hormônios de vertebrados sobre reprodução de *Biomphalaria glabrata* e na infecção pelo *Schistosoma mansoni*. Rev Bras Pesqui Med 1978;11:135–40.
[88] Langston WJ, Burt GR, Chesman BS. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Mar Ecol Prog Ser 2007;333:173–84.
[89] Csaba G, Bierbauer J. Effect of oestrogenic, androgenic and gestagenic hormones on the gametogenesis (oogenesis and spermatogenesis) in the snail *Helix pomatia*. Acta Biol Med Ger 1979;38:1145–8.
[90] Csaba G, Bierbauer J. Effect of cortisol, deoxycorticosterone and 19-nortestosterone on the gametogenesis of the snail *Helix pomatia*. Acta Biol Acad Sci Hung 1981;32:15–8.
[91] Varaksina GS, Varaksin AA, Maslennikova LA. Role of sex steroid hormones in spermatogenesis of the scallop *Mizuhopecten yessoensis*. Biol Morya-Vlad 1992;1–2:77–83.
[92] Wang C, Croll RP. Effects of sex steroids on gonadal development and gender determination in the sea scallop, *Placopecten magellanicus*. Aquaculture 2004;238:483–98.
[93] Janer G, Lyssimachou A, Bachmann J, Oehlmann J, Schulte-Oehlmann U, Porte C. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. Steroids 2006;71:435–44.
[94] Oberdörster E, McClellan-Green P. The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. Peptides 2000;21:1323–30.
[95] Czech P, Weber K, Dietrich DR. Effects of endocrine modulating substances on reproduction in the hermaphroditic snail *Limnaea stagnalis* L. Aquat Toxicol 2001;53:103–14.
[96] Gagné F, Blaise C, Lachance B, Sunahara GI, Sabik H. Evidence of coprostanol estrogenicity to the freshwater mussel *Elliptio complanata*. Environ Pollut 2001;115:97–106.
[97] Li Q, Osada M, Suzuki T, Mori K. Changes in vitellin during oogenesis and effect of estradiol-17β on vitellogenesis in the Pacific oyster *Crassostrea gigas*. Invert Reprod Dev 1998;33:87–93.
[98] Osada M, Takamura T, Sato H, Mori K. Vitellogenin synthesis in the ovary of scallop, *Patinopecten yessoensis*: control by estradiol-17β and the central nervous system. J Exp Zool 2003;299A:172–9.
[99] Won S-J, Novillo A, Custodia M, Rie MT, Fitzgerald K, Osada M, et al. The freshwater mussel (*Elliptio complanata*) as a sentinel species: vitellogenin and steroid receptors. Integr Comp Biol 2005;45:72–80.
[100] Gagné F, André C, Blaise C. Increased vitellogenin gene expression in the mussel *Elliptio complanata* exposed to estradiol-17β. Fresenius Environ Bull 2005;14:861–6.
[101] Gagné F, Blaise C, Salazar M, Salazar S, Hansen PD. Evaluation of estrogenic effects of municipal effluents to the freshwater mussel *Elliptio complanata*. Comp Biochem Physiol C 2001;128:213–25.
[102] Gagnaire B, Gagné F, André C, Blaise C, Abbaci K, Budzinski H, et al. Development of biomarkers of stress related to endocrine disruption in gastropods: alkali-labile phosphates, protein-bound lipids and vitellogenin-like proteins. Aquat Toxicol 2009;92:155–67.
[103] Riffeser M, Hock B. Vitellogenin levels in mussel hemolymph – a suitable biomarker for the exposure to estrogens? Comp Biochem Physiol C 2002;132:75–84.
[104] Blaise C, Gagné F, Pellerin J, Hansen PD. Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Fjord, Canada): a potential biomarker for endocrine disruption. Environ Toxicol 1999;14:455–65.
[105] Janer G, Lavado R, Thibaut R, Porte C. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: a possible regulating role for steroid acyltransferases. Aquat Toxicol 2005;75:32–42.
[106] Janer G, Lavado R, Thibaut R, Porte C. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*. Mar Environ Res 2004;58:443–6.
[107] Di Cristo C, Paolucci M, Di Cosmo A. Progesterone affects vitellogenesis in *Octopus vulgaris*. Open Zool L 2008;1:29–36.
[108] Tosti E, Di Cosmo A, Cuomo A, Di Cristo C, Gragnaniello G. Progesterone induces activation in *Octopus vulgaris* spermatozoa. Mol Reprod Dev 2001;59:97–105.
[109] Canesi L, Lorusso LC, Ciacci C, Betti M, Zampini M, Gallo G. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. Gen Comp Endocrinol 2004;138:58–69.
[110] Chinoy NJ, Seethalakshmi L. Biochemical evidence for the occurrence of a possible steroidal mechanism in the reproductive organs of slugs (*Laevicaulis alte* Ferussac). In: Adiyodi KG, Adiyodi RG, editors. Advances in invertebrate reproduction, vol. 133. India: Peralam-Kenoth; 1977. p. 356–66.
[111] Wang C, Croll RP. Effects of sex steroids on *in vitro* gamete release in the sea scallop, *Placoplecten magellanicus*. Invert Reprod Dev 2003;44:89–100.
[112] Santos MM, Castro LFC, Vieira MN, Micael J, Morabito R, Massanisso P, et al. New insights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*. Comp Biochem Physiol C 2005;141:101–9.
[113] Gust M, Garric J, Giamberini L, Mons R, Abbaci K, Garnier F, et al. Sensitivity of New Zealand mudsnail *Potamopyrgus antipodarum* (Gray) to a specific aromatase inhibitor. Chemosphere 2010;79:47–53.
[114] Ducrot V, Teixeira-Alves M, Lopes C, Delignette-Muller M-L, Charles S, Lagadic L. Development of partial life-cycle experiments to assess the effects of endocrine disruptors on the freshwater gastropod *Lymnaea stagnalis*: a case study with vinclozilin. Ecotoxicology 2010;19:1312–21.
[115] Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J. Toxicity of triphenyltin and tributyltin to the freshwater mudsnail *Potamopyrgus antipodarum* in a new sediment biotest. Environ Toxicol Chem 2003;22:145–52.
[116] Aarab N, Lemaire-Gony S, Unruh E, Hansen PD, Larsen BK, Andersen OK, et al. Preliminary study of responses in mussel (*Mytilus edulis)* exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether. Aquat Toxicol 2006;78S:S86–92.
[117] Ricciardi F, Matozzo V, Marin MG. Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. Mar Pollut Bull 2008;57:365–72.
[118] Matozzo V, Marin MG. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? Environ Pollut 2005;97:43–9.
[119] Riva C, Porte C, Binelli A, Provini A. Evaluation of 4-nonylphenol *in vivo* exposure in *Dreissena polymorpha*: bioaccumulation, steroid levels and oxidative stress. Comp Biochem Physiol C 2010;152:175–81.
[120] Quinn B, Gagné F, Weber J-P, Blaise C. Ecotoxicological effects of a semi-submerged municipal dump (Castle harbour, Bermuda) on the Calico scallop *Argopecten gibbus*. Mar Pollut Bull 2005;51:534–44.
[121] Castro LFC, Melo C, Guillot R, Mendes I, Queirós S, Lima D, et al. The estrogen receptor of the gastropod *Nucella lapillus*: modulation following exposure to an estrogenic effluent? Aquat Toxicol 2007;84:465–8.
[122] Santos MM, Reis-Henriques MA, Guillot R, Lima D, Franco-Duarte R, Mendes I, et al. Anti-androgenic effects of sewage treatment plant effluents in the prosobranch gastropod *Nucella lapillus*. Comp Biochem Physiol C 2008;148:87–93.
[123] Gagné F, Bouchard B, André C, Farcy E, Fournier M. Evidence of feminization in wild *Elliptio complanata* mussels in the receiving waters downstream of a municipal effluent outfall. Comp Biochem Physiol C 2011;153:99–106.
[124] Lavado R, Janer G, Porte C. Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: the modulating effect of dispersed crude oil and alkylphenols. Aquat Toxicol 2006;78S:565–72.
[125] Aarab N, Minier C, Lemaire S, Unruh E, Hansen P-D, Larsen BK, et al. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. Mar Environ Res 2004;58:437–41.
[126] Schmitt C, Balaam J, Leonards P, Brix R, Streck G, Tuikka A, et al. Characterizing field sediments from three European river basins with special emphasis on endocrine effects – a recommendation for *Potamopyrgus antipodarum* as test organism. Chemosphere 2010;80:13–9.
[127] Schmitt C, Streck G, Lamoree M, Leonards P, Brack W, de Deckere E. Effect directed analysis of riverine sediments – the usefulness of *Potamopyrgus antipodarum* for *in vivo* effect confirmation of endocrine disruption. Aquat Toxicol 2011;101:237–43.
[128] Gust M, Buronfosse T, Geffard O, Mons R, Queau H, Mouthon J, et al. *In situ* biomonitoring of freshwater quality using the New Zealand mudsnail *Potamopyrgus antipodarum* (Gray) exposed to waste water treatment plant (WWTP effluent discharges). Water Res 2010;44:4517–28.
[129] Gust M, Buronfosse T, Geffard O, Coquerry M, Mons R, Abbaci K, et al. Comprehensive biological effects of a complex field poly-metallic pollution gradient on the New Zealand mudsnail *Potampyrgus antipodarum* (Gray). Aquat Toxicol 2011;101:100–8.
[130] Mazurová E, Hilscherová K, Jálová V, Köhler H-R, Triebskorn R, Giesy JP, et al. Endocrine effects of contaminated sediments on the freshwater snail

Potamopyrgus antipodarum in vivo and in the cells bioassays *in vitro*. Aquat Toxicol 2008;89:172–9.
[131] Mouneyrac C, Linot S, Amiard J-C, Amiard-Triquet C, Métais I, Durou C, et al. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. Gen Comp Endocrinol 2008;157:133–41.
[132] Gagné F, Blaise C, Pellerin J, Fournier M, Gagnon C, Sherry J, et al. Impacts of pollution in feral *Mya arenaria* populations: the effects of clam bed distance from the shore. Sci Total Environ 2009;407:5844–54.
[133] Quinn B, Gagné F, Costello M, McKenzie C, Wilson J, Mothersill C. The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). Aquat Toxicol 2004;66:279–92.