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Rapid uptake, biotransformation, esterification and lack of depuration of testosterone and its metabolites by the common mussel, *Mytilus* spp.

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ABSTRACT

The presence of the vertebrate steroids, testosterone (T) and 17β-estradiol in mollusks is often cited as evidence that they are involved in the control of their reproduction. In this paper, we show that a likely source of T in at least one species, the common mussel (*Mytilus* spp.), is from uptake from water. When mussels were exposed to waterborne tritiated T ([^{3}H]-T) in a closed container, the radioactivity decreased rapidly and exponentially until, by 24 h, approximately 35% remained in the water. The rate of uptake of radiolabel could not be saturated by concentrations as high as 16.5 $\mu g\,L^{-1}$ (mean measured) of non-radiolabeled T, showing that the animals have a very high capacity for uptake of T. At least 30% of the applied radioactivity could be extracted from the tissues of the animals with organic solvents and most of this (26% of the total applied radioactivity) was in the fatty acid ester fraction. Following alkaline hydrolysis, reverse phase HPLC and TLC, this fraction was shown to consist predominantly of 5α-dihydrotestosterone and 5α-androstane-3β,17β-diol, while T was a minor component. These steroids were definitively identified in the fatty acid ester fraction by mass spectrometry. Overall, less than 5% of the [^{3}H]-T applied to the system remained untransformed at the end of exposure. After ten days of depuration there was no reduction in the total amount of radioactivity in the tissues, nor any changes in the ratio of the metabolites in the ester fraction. These findings show that any association between T presence and reproductive status or sex is confounded by their significant capacity for uptake, and that T undergoes extensive metabolism in mussels *in vivo* and therefore may not be representative of the androgenic burden of the animals. Consequently, measurements of T in mussel tissue offer little utility as an indicator of reproductive status or sex.

1. Introduction

The belief that at least three human sex steroids (17β-estradiol [E_2], testosterone [T] and progesterone [P]) play an important role in the reproductive endocrinology and toxicology of mollusks has lasted over 50 years, and has been propagated in over 200 peer reviewed publications. It arose from the numerous studies confirming the presence of T and E_2 in mollusk tissues [1–4]. Further impetus was provided by the finding [5] that the anti-fouling compound tributyltin (TBT) was the causative agent of imposex, a condition in which female marine snails living in TBT-contaminated waters develop a penis and a vas deferens normally only found in males. It was assumed, though with little hard evidence (reviewed by [6,7]), that the endocrine system of mollusks was the same as that of humans and that T was responsible for mediating these effects. The interest in the potential role of vertebrate steroids in mollusks was further stimulated with the discovery that sewage treatment works in the UK, at the end of the 1980s, were emitting large amounts of estrogenically active compounds that were inducing substantial production of egg yolk protein (vitellogenin; VTG) in immature and male fish [8]. Soon after this discovery, it was thought that if the same was true in mollusks (*i.e.* E_2 was, as in vertebrates, a crucial female hormone and responsible for stimulating VTG production), it would allow the use of mollusks as both model and sentinel organisms for the evaluation of estrogenic endocrine disrupters without the ethical, monetary and legislative burden of using vertebrates [9–11]. Hence, much effort was put into measuring levels of alkali-labile phosphate (ALP), an indirect measure of VTG production in vertebrates, in a range of mollusks (reviewed by [12,13]) that had been exposed to compounds and effluents known to have estrogenic effects in vertebrates. However, a recent investigation of egg yolk production in a typical bivalve mollusk

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[14] has convincingly demonstrated that ALP is not a suitable surrogate marker for VTG in mollusks and that E_2 has no effect on VTG production. Yet another recent, well-controlled study using a gastropod snail [15] has also shown that neither 5α-dihydrotestosterone (DHT) nor 17α-methyltestosterone, which are both potent androgens in vertebrates, had any discernible effects on reproduction in this species. In fact, it is now widely accepted that the mechanism *via* which TBT induces imposex in mollusks involves binding to the retinoic acid and peroxisome proliferating receptors [16–18].

While it remains unproven that vertebrate steroids have a role in mollusks, their presence in mollusk tissue is indisputable (see Table 1 in the review by Scott [1] and, more recently [19,20]). Where do these steroids come from if, as discussed above, they are not endogenously-produced hormones? It is possible that they are acquired exogenously, and there are several lines of evidence to support this. Firstly, when laboratory-reared animals are caged in the wild, the amounts of steroid in them tend to increase, and rarely, if ever, decrease [21]. Secondly, the synthetic estrogen, ethinyl estradiol (EE_2) has been definitively identified in the tissues of mollusks caught in the wild – this is a man-made compound and it is inconceivable that it has an endogenous source [19,22–24]. Conclusively, controlled laboratory experiments have proven that a wide range of snails and bivalves can take up T and E_2 directly from the water [25–33].

It has also previously been shown, that, in addition to being able to absorb steroids from the water, snails and bivalves (including the mussel) also have the ability to covalently bind at least two of them, T [31,34–39] and E_2 [28–31,40,41], to fatty acids. This process is termed esterification and has been shown to also occur with dihydroepiandrosterone [30]. Furthermore, it has been shown in some of these studies that once these esters have been formed, there is a strong tendency for them to be retained by the animals [25,28,29]. It is important to point out that a key requirement of the esterification process is that the steroid must have a 'reactive' hydroxyl (–OH) group to conjugate to the fatty acid. In the case of T and E_2, this is the 17β-hydroxyl group.

The present study was undertaken to examine the rate and capacity of uptake, the degree of metabolism of T and the rate of depuration in the mussel, as understanding these processes was considered key in elucidating the origin of steroids in mollusk tissues.

2. Materials and methods

All scintillation counting was carried out with color quench correction on a Tricarb 2910 scintillation counter (PerkinElmer).

2.1. Chemicals

Testosterone-[1,2,6,7-^{3}H] ([^{3}H]-T) and 17β-[2,4,6,7,16,17-^{3}H]-estradiol ([^{3}H]-E_2) were purchased from American Radiolabeled Chemicals, Inc. (101 ARC Dr. St. Louis, MO 63146, USA). Cold (*i.e.* non-radiolabeled) androstenedione (Ad; CAS 63-05-8), T (CAS 58-22-0), testosterone sulfate (T-S; CAS 651-45-6), 5α-androstan-3β,17β-diol (3β,17β-A5α; CAS 571-20-0), 5α-androstan-3α,17β-diol (3α,17β-A5α; CAS 1852-53-5) and 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone; DHT; CAS 521-18-6) were purchased from Steraloids Inc. (www.Steraloids.com) and all other chemicals were purchased from Fisher-Scientific UK Ltd. (www.fishersci.co.uk). Water used for exposures was filtered (50 μm) seawater and water used for all other purposes was reverse osmosis water.

2.2. Laboratory exposures of Mytilus spp. to [^{3}H]-T and T

The animals used in the four experiments were all from Portland Harbour (Dorset, UK). To our knowledge this area is populated by *Mytilus edulis* although we cannot discount the co-existence of *Mytilus galloprovincialis* and/or their hybrids. The nearby northeast Portland Harbour breakwater is a catchment holding a long term class B shellfish harvesting classification. This means the mussels can only go for human consumption after they have been processed (with the commonest procedure being 42 h depuration in clean seawater) in order to reduce their bacterial and viral load (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). The animals were transported in a cool-box and immediately placed in a flow-through system of seawater and

Table 1
Summary of exposure conditions.

Experiment	Sampling date	Exposure conditions									
		Water vol. (L)	Number of animals	Replicates	Label conc. at first sampling point (μCi L^{-1})	Label conc. at first sampling point (ng L^{-1})	Nominal cold T conc. (ng L^{-1})	Exposure time (h)	Volume of water animal^{-1} (mL)	Mean tissue wet weight (g)	Mean shell length (mm)
Exp 1 [^{3}H]-T only	Oct 2014	2	5	6	4.22	11.08	–	24	400	4.52	56.61
Exp 1 [^{3}H]-T + low concentration cold T	Oct 2014	2	5	2	4.25	11.15	2500	24	400	5.08	56.86
Exp 1 [^{3}H]-T + high concentration cold T	Oct 2014	2	5	2	4.07	10.68	25,000	24	400	5.64	56.53
Exp 1 [^{3}H]-E_2 positive control	Oct 2014	2	5	2	5.47	12.36	–	24	400	4.44	58.48
Exp 1 [^{3}H]-T control	Oct 2014	2	0	1	4.48	11.74	–	24	-	-	-
Exp 2 [^{3}H]-T only	Nov 2015	0.2	1	8	3.20	8.40	–	6	200	4.56	60.06
Exp 2 [^{3}H]-T control	Nov 2015	0.2	0	8	2.99	7.85	–	6	200	-	-
Exp 3 [^{3}H]-T only	Nov 2015	3.2	18	1	6.86	17.98	–	24	200	4.23	60.13
Exp 4 cold T only	May 2015	2	5	1	–	–	25,000 × 2	24	400	4.24	60.70
Exp 4 solvent control	May 2015	2	5	1	–	–	–	24	400	5.28	60.70