THE ORIGIN OF VERTEBRATE STEROIDS IN MOLLUSCS: UPTAKE, METABOLISM AND DEPURATION STUDIES IN THE COMMON MUSSEL

TAMAR IMOGEN SCHWARZ

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Institute of Molecular, Cell and Systems Biology
College of Medical, Veterinary and Life Sciences
University of Glasgow
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Abstract

Many studies have found vertebrate sex steroids, such as testosterone (T), 17β-oestradiol (E_2) and progesterone (P) to be present in molluscan tissues. The underlying assumption of most, if not all, these studies has been that these steroids are formed endogenously and furthermore, act as reproductive hormones in the same way that they do in vertebrates (i.e. they bind to receptors and induce physiological processes such as egg yolk protein production in females, sex reversal in bivalves and penis formation in neogastropods). However, an in depth evaluation of the literature indicates that the evidence for endogenous formation of vertebrate steroids is rather weak (for example, mollusc genomes do not contain sequences for critical P450 enzymes such as 17-hydroxylase and aromatase nor for functional steroid nuclear receptors). The evidence for the uptake of at least two of these steroids, 17ß-oestradiol (E_2) and testosterone (T) from the environment appears, on the other hand, to be very strong and the aim of this thesis was to study this in more detail in common mussels (*Mytilus edulis* and a mixed population of *M. edulis* / *Mytilus galloprovincialis*). This involved exposing mussels to tritiated E_2, T, progesterone (P), 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P), 17α-ethinyl-oestradiol (EE_2) and cortisol (F) and then determining the rate and capacity of uptake, how much was esterified (i.e. conjugated to fatty acids), how much if any was sulphated and finally, how rapidly it was depurated. The study also required novel procedures to be developed for optimising *in vivo* radiolabel uptake and for separating free (i.e. non-conjugated), esterified and sulphated steroids. It was discovered that all steroids except, intriguingly, F, were rapidly absorbed by mussels from the water. It was discovered that the rate of uptake of radioactive steroids could not be saturated by the addition of non-radioactive steroid (even at 25µg L^{-1}), i.e. the uptake capacity was far higher than amounts found in nature. It was found that the largest proportion (> 70 %) of radioactive E_2 and T in the tissues was present as fatty acid esters. These depurated very slowly (E_2 had a half-life of ca. 12 days, while T showed no signs of depletion after 10 days). It was found that the level of esterification of EE_2, P and 17,20ß-P was noticeably lower than that of E_2 and T. Following saponification of esterified E_2, a single steroid (E_2 itself) was recovered.

However, following saponification of esterified P, two steroids, neither of them P and not yet fully identified, were recovered. Following saponification of T, four steroids were recovered. The least abundant (< 10 %) was T itself and the most abundant was 5α-dihydrotestosterone (47 %). It was discovered that sulphation played an important role in the metabolism of E_2.

While none of these results disprove endogenous synthesis of vertebrate steroids, they do call into question those studies that have claimed a cause and effect relationship between vertebrate steroid concentrations in mollusc tissues and factors such as the stage of the reproductive cycle, gender or exposure to presumed 'endocrine' disrupters (i.e. it is impossible to determine in any of these studies whether any of the steroids were of endogenous origin).

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I dedicate this thesis to my father, Hugo Schwarz and my cat, Tazzywazzy.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Tamar I. Schwarz

Definitions/Abbreviations

17,20ß-P - 17α,20ß-dihydroxypregn-4-en-3-one

3ß,17ß-A5α - 5α-androstan-3ß,17ß-diol

3ß-P5α – 3ß-hydroxy-5α-pregnan-20-one

5α-DHT, 17ß-A5α and 5α-dihydrotestosterone - 17ß-hydroxy-5α-androstan-3-one

An – androstenedione

B[a]P – benzopyrene

E_1 – oestrone

E_2 - 17ß-oestradiol

ECLIA – electro chemo-luminescent immunoassay

EE_2 - 17α-ethinyloestradiol

ELISA – enzyme-linked immunosorbent assay

ER- oestrogen receptor

F – cortisol

mER – molluscan oestrogen receptor

P – progesterone

P5α - 5α-pregnane-3,20-dione

Pn – pregnenolone

RIA – radioimmunoassay

T – testosterone

TFA – trifluoroacetic acid

YES – yeast oestrogen screen

Whenever possible, the experiments in this thesis were carried out on the native *Mytilus edulis* Linnaeus 1758 (commonly known as the blue mussel or edible mussel). However, the waters around the UK (especially in the Channel) have been invaded by *Mytilus galloprovincialis* Lamarck 1819 (commonly known as the Mediterranean mussel). The two species are so similar that they can interbreed. Together with *Mytilus trossulus* Gould 1850 (the foolish mussel), native to the Baltic Sea, they form part of the '*Mytilus edulis* complex'. Where the species is uncertain in the present study (i.e. the animals could be *M. edulis* or *M. galloprovincialis*; or hybrids), the term '*Mytilus* spp.' has been used.

Chapter 1

General introduction

Are vertebrate steroids present in molluscs and, if so, are they of endogenous or exogenous origin?

Sewage treatment effluents have long been identified as the carriers of a large number of chemicals able to disrupt the endocrine system of vertebrates (Purdom et al., 1994). These (together with other industrial and natural sources of endocrine disrupting chemicals; EDCs) can contribute to feminisation or (anti)-androgenisation of aquatic vertebrates (by binding and activating the oestrogen and androgen receptors that are in those species). On the basis of several lines of circumstantial evidence (discussed by Scott, 2012, 2013)– one of them being the fact that many people have found vertebrate steroids in the flesh of molluscs - it has been postulated many times (Jobling et al., 2004; Matthiessen, 2008; Oehlmann et al., 2007) that these steroids act as hormones in molluscs in the same way as they do in vertebrates (i.e. they are produced endogenously, they bind to receptors and they induce similar physiological and /or reproductive changes). If this were true, then the obvious corollary, as pointed out by the above-mentioned authors, is that molluscs would not only be vulnerable to the same endocrine disruptors (EDs) as vertebrates, but would also provide a superb alternative for current endocrine chemical tests. This would mean that the impact of EDCs on the aquatic environment would have to be reassessed (as there would be a much larger number of susceptible organisms) and the current vertebrate-based tests would be able to be replaced with a molluscan model (which are not regulated by the live animal testing legislation). The financial benefits of the latter (much lower costs!) would be enormous. So, what is the evidence that molluscs have the same steroid-based endocrine system as vertebrates?

1.1 Bivalve Physiology

Molluscs are soft bodied animals usually enclosed by a hard outer shell. They are an incredibly diverse phylum encompassing free moving gastropods, highly developed cephalopods to filter feeding, mostly sessile, bivalves. As the latter are the focus of this thesis, the next section will address bivalves, and more specifically *Mytilus* species, anatomy and reproductive physiology.

Mussels are comprised of two shell valves which are controlled by the posterior (the larger of the two) and anterior adductor muscles. The tissue lining the valves is called the mantle; its outer edge secretes the shell to which it is attached. The rest of the mantle contains the animal's organs, including most of the gonads.

Bivalves are dioecious and have very simple reproductive systems. The paired gonads are contained in the mantle together with their accessory sex organs. Gametes are produced in the gonads and released into the mantle cavity via ciliary movement and gonoducts. From the mantle cavity they are ejected via the exhalant opening into the water column for external fertilisation. During maturation, up to 40 % of the animal's wet weight (excluding the shell) can be made up of either oocytes or sperm. Bivalves have an annual reproductive cycle; a generalised maturation cycle consists of the following stages:

1. Maturing:
    i. Recovering
    ii. Filling
    iii. Half-full
    iv. Full
2. Spawning
3. Spent

Gonad development (maturing stages) occurs over winter (i.e. approximately November to February in the northern hemisphere). In

spring the gonads are ripe and partial spawning may occur after which gametogenesis takes place again before a second spawning at the end of the summer. The reproductive cycle described above is an approximation and varies between and within species (e.g. continuous spawning vs two spawning events) as both gametogenesis and spawning are regulated by internal and exogenous cues such as temperature, food availability and salinity.

Bivalves have gills which are used for both food intake and respiration; and which in turn are responsible for the uptake and potential bioaccumulation of pollutants.

1.2 Evidence of the presence of steroids in molluscs

Vertebrate steroids have been measured in molluscs (both tissue and haemolymph) in a large number of studies (Table 2); the focus of most of these studies has been identifying and quantifying the three classical vertebrate steroids: testosterone (T), 17β-oestradiol (E_2) and progesterone (P). Although it can now be safely said (based on some of the more rigorous publications) that vertebrate steroids most definitely can be found in mollusc tissues, the vast majority of the publications only measured free (i.e. non-conjugated) steroid concentrations and many relied purely on immunoassays (without any characterisation steps such as chromatography) to detect and quantify them.

1.2.1 Why is measurement of free steroids inadequate?

It is now known that steroids in molluscs are present not just as free steroids, but also as steroid esters and, to a lesser extent, steroid sulphates. The first study to demonstrate the production of steroid-fatty acid esters *in vivo* was (Gooding and LeBlanc, 2001). They exposed the Eastern mudsnail, *Ilyanassa obsoleta*, to radiolabelled T and found that not only did the animal take it up from the water, but also conjugated it to free fatty acids (a process known as ‘esterification’ or alternatively ‘acylation’; Figure 1).

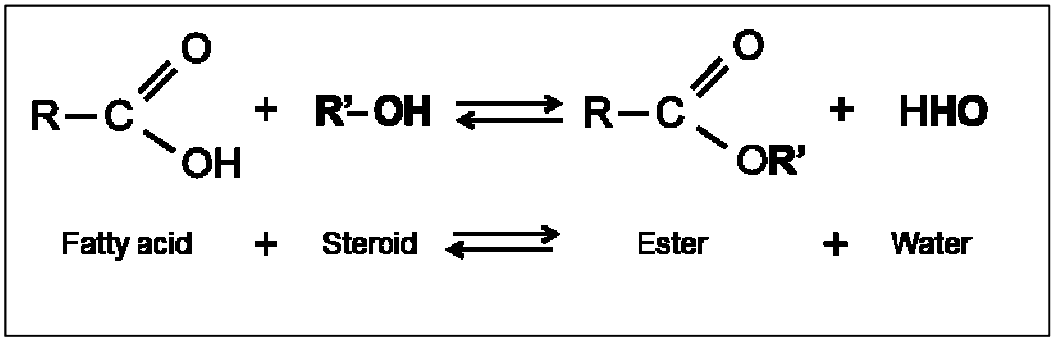

Figure 1: Illustration of steroid esterification.

The T esters then bioaccumulated in the fat stores of the snails and remained there for at least 96 h (< 4 % depuration when moved to fresh water). A number of publications since then have shown (*in vivo*) that a range of bivalves and gastropods esterify and store most of the T and E_2 that they pick up from the water (Janer et al., 2004a; Janer et al., 2004b; Labadie et al., 2007; Peck et al., 2007). It has also been shown that, after being taken up from the water, free T and E_2 account for a small proportion of the total burden in molluscan tissues and also remain at low, stable levels regardless of the concentration of steroid in the water. For example, when bivalves were exposed to a range of concentrations of T and E_2, esters were formed in a dose- and time-dependent manner while free steroid concentrations in the tissue remained the same throughout the treatments except at the very highest dose, i.e. these studies showed that free steroid values do not reflect the total steroid concentrations in the animals (Fernandes et al., 2010; Janer et al., 2004a; Puinean et al., 2006). These findings were among the first to suggest that measurement of free steroid concentrations only (which includes the majority of studies) are of little or no value. Not only can total steroid levels not be inferred from free steroid levels, but also the concentrations of the free steroids are often so low that they challenge the limits of detection (LODs) of the assays (see next section).

1.2.2 Is choice of method important?

A major issue with the studies listed on Table 2 are the methods employed to detect and quantify steroids; as seen in the 'Method' column, immunoassays appear to be the technique of choice (48 out of 62 papers).

Immunoassays, although useful (relatively cheap and not very technical), are highly susceptible to matrix interference (from the salts, fat and protein that are extracted in conjunction with the steroids) (Gust et al., 2010b; Mitchell and Lowe, 2009) and are also prone to cross-reaction with other similarly structured steroids (which could be significant when measuring free steroids which are present at pg g^{-1} wet weight (ww) concentrations) (Krasowski et al., 2014). The low levels of steroids being measured and the low specificity of the methods employed, suggest that most enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) studies that have claimed to have measured free steroid concentrations in molluscs are likely inaccurate and therefore unreliable. A partial solution to this problem would be to analyse tissue extracts with Thin Layer or High Performance Liquid Chromatography (TLC or HPLC) to check that the reactivity can be found in its expected elution position. However, only one of the immunoassay studies listed in Table 2 has carried out this simple check (Zhu et al., 2003) and that showed that only ca. 10 % of the total E_2 immunoreactivity measured in the gonads of the common mussel, *Mytilus edulis*, eluted with the E_2 standard on HPLC. However, even that study is suspect, as the total amounts of immunoreactivity that were reported were an astonishingly high 165 ng g^{-1} ww of tissue. These are more than 100-fold higher than those reported in the same species by other authors (Table 2).

So, if immunoassays yield uncertain results, what methods can one rely on? Analytical chemistry methods are the gold standard method for identification and quantification of steroids but the high costs and high levels of expertise required to operate equipment and process data make it less popular (reviewed by Stanczyk & Clarke, 2010). Other limitations are that mass spectrometry has higher detection limits than immunoassays (usually low ng vs low pg; although as technology improves, sensitivity increases). Low levels of free steroids in molluscs, coupled with sample loss during clean-up, can sometimes mean mass spectrometry is not a viable option. Gust *et al.,* (2010) compared the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to the frequently employed RIA. They found that results for both methods were in the same order of magnitude; however the RIA underestimated P and overestimated

T. The authors suggested that the lower value for P was a result of low antibody affinity but these discrepancies could also be explained by matrix interference in P measurement and cross-reactivity of T with other compounds (e.g. Ad or dihydrotestosterone (5α-DHT); Janer *et al.*, 2005). They also reported difficulties with positive ionisation of saponified (i.e. de-esterified) steroids and were therefore unable to measure steroid esters using LC-MS/MS. Despite the fact that the results obtained by the RIA differed from the more accurate mass spectrometry results, the immunoassay was employed to measure the ester fractions. So although mass spectrometry is the method of choice it is not always feasible and has to be used in conjunction with other, less precise methods.

There are other problems with the interpretation of steroid concentrations in molluscs. In the majority of vertebrate studies, steroid measurements are carried out on blood plasma samples and levels are presented as ng ml^{-1} of plasma. In most mollusc studies, steroid measurements are carried out in tissue extracts and therefore presented, usually, as ng g^{-1} wet weight of tissue. The tissue wet weight is far more likely than plasma to be affected by factors such as the amount of water, protein and fat in the tissue (something that can vary considerably in gonad tissue during reproduction). Since steroid concentration is effectively the amount of steroid divided by the wet weight of the tissue, small differences in values between males and females or between mature and immature animals might be due purely to changes in the tissue composition rather than differences in the steroid composition. Another factor is that certain proteins (examples being albumin and vitellogenin – VTG - in vertebrates) have a high capacity for low affinity binding of steroids (Baker, 2002; Scott et al., 2005; Yoshikuni et al., 1993). Thus the presence of higher amounts of steroid in gonads of mature females for example might purely be due to the egg yolk protein having a higher capacity to bind (and thereby bioconcentrate) certain steroids. However, in regard to molluscs this is as yet speculation.

1.2.3 Evidence for bioaccumulation of steroids

Uptake of steroids has been directly demonstrated *in vivo* with the use of radiolabelled steroids (Gooding and LeBlanc, 2001; Labadie et al., 2007;

Peck et al., 2007). However, there are also several studies which have indirectly shown that molluscs can accumulate steroids; moreover, that they can accumulate them from their natural environment, supporting the contention that their presence does not necessarily mean that the animals make them. For example, Peck *et al*. (2007) quantified oestrogenicity (before and after hydrolysis), using the yeast oestrogen screen (YES), in the zebra mussel, *Dreissena polymorpha* soft tissue from reference ('clean') sites and contaminated sites. Samples from the clean site had lower levels of oestrogenicity (presented as E_2 equivalents; EEQ) than those from contaminated sites. At the time of sampling, E_2 could not be measured in the water of the clean site, and on the basis of its absence, the authors attributed the presence of E_2 in those animals to biosynthesis. However, there are at least two flaws in their reasoning: firstly the fact that E_2 is below a method's detection limit in water (especially at a single time point) does not mean the chemical is not (or has not been) there (the use of long-term samplers would have been more relevant); and secondly, the same paper provided evidence at the polluted site that the animals almost certainly were able to take up compounds from the water - as 4-nonylphenol was found in their flesh (this is a synthetic chemical and it could not have been made endogenously). There are several other studies in which laboratory-reared molluscs have been placed in cages in rivers (Gust et al., 2010a; Gust et al., 2011a; Gust et al., 2014). In almost all cases (presumed 'clean' and contaminated sites), regardless of the time of year, the tissue content of T and E_2 (free + esterified) was significantly higher after *in situ* exposure for 7 to 14 days.

1.2.4 Do steroid levels change with maturation cycles?

In fish, it is usual to find that plasma concentrations of sex steroids are synchronised with the reproductive cycle (Awruch et al., 2008; Elisio et al., 2014; Espinosa et al., 2011; Scott and Sumpter, 1983; Scott and Sumpter, 1989; Vazirzadeh et al., 2014). The sex steroids in fish are after all produced by the gonads (with E_2 in particular being needed to be transported via the bloodstream to the liver in order to induce egg yolk production). One of the main arguments (albeit involving 'circumstantial evidence') that molluscs produce vertebrate steroids endogenously is that,

in some cases, an association has been found between steroid levels and a stage of the reproductive cycle. However, when the literature on seasonality is critically appraised it is fairly obvious that there is, in most cases, no clear or consistent association between steroid levels (whether free or esterified) and mollusc reproductive maturation; or of a gender-specific association of E_2 with females and T with males. There are, however, a few exceptions to this. For example, Ni *et al*. (2013), working on the oyster *Crassostrea angulata*, Zheng *et al*. (2014) and Liu *et al*. (2014a) working on the Zhikong scallop, *Chlamys farreri* and Yan *et al*. (2011), working on the razor clam, *Sinovacula constricta* all showed a rise and fall of E_2 concentrations in ovaries that followed the classic pattern found in fishes (i.e. high during the growth stage of the oocytes and low to undetectable during the rest of the year). The same authors also showed a rise and fall in T concentrations in synchrony with the development of the testis (again a situation that would be expected if T was involved in stimulation of testis development as it is in higher vertebrates). Although these studies appear to report vertebrate-like seasonality, in the majority of other studies on maturation cycles in molluscs, there is, as already mentioned, actually little or no consistency within or between species (with 'peaks' being found at the beginning, the middle, the end and sometimes totally outside the reproductive cycle). For example Gooding & LeBlanc (2004) and Sternberg *et al*. (2008) measured T by RIA in *I. obsoleta* over a one and two year period respectively. Regarding free T, the first study found two peaks (ca. 3 to 5 fold in males and females), one in April and another in November. These high levels of free T coincided with the beginning and then the end of the time that males and females had (mostly) mature gonads. The authors thus concluded that 'These periods of elevated free testosterone bracket the breeding season of the mud snail'. The second study found only one free T peak lasting from September to November and they stated that free T in males presented 'a progression from high during recrudescence to low during dormancy'. So T levels measured with the same assay, in the same species and sampled in the same country presented two different seasonal patterns, one where the reproductive season started and ended with a high concentration of free T and another where free T levels were low at the beginning of the reproductive season and only peaked at the end of it. Another example of

discrepancies between studies was the concentrations of E_2 during the annual reproductive cycle of clams. As already mentioned, Zheng *et al.* (2014) measured E_2 (and T) in the gonads of *C. farreri* by LC-MS/MS during the reproductive cycle (April to July) and Liu *et al.* (2014a) also quantified E_2 (E_1 and T too) in the gonads of the same species by ELISA (January to December). Both studies found a peak of E_2 concentration in mature / spawning females in June and May, respectively. If E_2 was indeed linked to maturation it would be expected that similar concentrations of E_2 would be found in animals of the same species, sex and maturation stage. This was not the case however, as there was approximately a 50-fold difference between the reported values of the E_2 peak. This difference could perhaps be explained by differences in the sensitivities of the two detection methods employed, although such a high difference is unlikely considering that a comparative study of immunoassay and analytical chemistry found that results, though different, were in the same order of magnitude (Gust et al., 2010b). It should be noted also that the second study on the clam reported another 'peak' in September (during restoration) but as the first study (Zheng et al., 2014) only analysed animals until June it is not possible to compare the two studies regarding this latter peak. The vast difference in concentrations of E_2 reported by both studies at any given time (not just E_2 peaks) could, on the other hand, quite fittingly be explained by different levels of uptake of E_2 from the environment. E_2 levels in surfaces waters likely vary with location (e.g. being near heavily farmed land might increase steroid availability; Matthiessen *et al.*, 2006) and seasons (e.g. increased rainfall is known to dilute the effects of sewage effluents; Johnson, 2010).

Dévier *et al.* (2010) investigated P, pregnenolone (Pn; the precursor of P in vertebrates), E_2, E_1, T, Ad, 5α-DHT and dihydroepiandrosterone (DHEA) in the digestive gland of *M. edulis* over two reproductive cycles (May '02 to May '04) using gas chromatography-mass spectrometry (GC-MS). Only P and Pn were detected - at low but similar levels in both males and females. P was always below the LOD (0.4 ng g^{-1}) except in April when the animals were in the first spawning stage (a second one occurred in August / September) and June. The peak in April was observed in both reproductive cycles but the June peak was only present in the first year. Martínez-Pita

et al. (2012) on the other hand, measured P, E_2 and T in the haemolymph of the closely related Mediterranean mussel, *Mytilus galloprovincialis* (with which *M. edulis* can interbreed) during three sampling points (October, December and March) using ELISA. In general, T and E_2 presented no particular differences between time points (which reflected maturation stages) or sexes. On the other hand males presented P peaks at sampling point one (post spawned animals) and females at sampling point one and two (post spawned and active stage). The Dévier *et al.(*2010) and Martínez-Pita *et al*. (2012) studies are another example of discrepancies in presumed correlations between the presence of vertebrate steroids and maturation stages. Firstly, study one did not detect the presence of any oestrogens or androgens and study two did. Although they used different detection methods and sampled different parts of the animal, P was measured in both haemolymph and digestive gland. So why were T and E_2 only detected in the haemolymph? Secondly, during the months that Martínez-Pita *et al*. (2012) found P peaks (October and December), Dévier *et al*. (2010) reported P to be under the LOD. And although the peaks reported by Dévier *et al*. (2010) cannot be compared - the other study did not measure P during that period of time - it must be noted that the maturation stages at which P was highest in both studies differed too (spawning stage vs post spawning stage!).

To summarise, many studies have measured vertebrate steroid concentrations over a reproductive period in the context of maturation. In some cases, the patterns are the same as one might expect to find in fish, while, in others, statistically significant increases in steroid concentrations have all the appearance of being random, although most authors attempted to link them to reproductive events. Certainly, if one were studying an annual-spawning fish, one would not expect to find (as did Knigge *et al.*, 2014 in a study on *D. polymorpha*) highly raised levels of E_2 outside the maturation period nor, once maturation had begun, to find raised levels in one month, close to zero levels in the next month and then raised levels the following month. Other factors to bear in mind when assessing the value of annual cycle data in molluscs are: a) free steroids (which most people measure) are, as explained in the previous section, independent of total steroid levels; b) whether or not a correlation has been found, it is of

course not proof of cause-and-effect; and c) many studies have very low numbers of replicates (n = 3 in some cases). By comparing data from studies investigating the same species and using similar methods (as the three examples discussed above) it has also been shown that there is very rarely a consistent association between steroid concentration and maturation stage. Once again, the theory that steroids in molluscs are of exogenous origin seems more fitting. It is known that when steroids (especially T and E_2) are picked up by molluscs they are mostly either esterified and stored, or sulphated and purged (Ronis & Mason, 1996 found a small amount of sulphated steroids in the exposure water). Therefore, any free steroid found in the animal is likely, when levels are low, to correspond to steroids that were in the water or the food of the animal close to the time of sampling (e.g. heavy agricultural input) or, more likely, bound with low affinity to one or more unknown proteins in the mollusc tissues (i.e. in the same way that steroids are loosely bound to albumin; Baker, 2002).

Finally, no section on steroid measurement would be complete without mentioning the probable contribution of calculation errors to variability in reported steroid concentrations. A recently published study (Feswick et al., 2014) involving blind steroid measurements on the same set of fish plasma samples made by seven laboratories revealed that three of the laboratories made gross (as much as 1000-fold!) calculation errors. A Defra-funded study (Defra CB0427, 2011) on blind measurement of steroid concentrations in water found that three out of the four laboratories had made one or more calculation errors (some again as much as 1000-fold). This shows not just a frighteningly high rate of human error, but also that calculation error is possibly the most likely reason for large differences in reported steroid concentrations. Certainly, there is no reason to assume that the field of steroid measurement in molluscs is free of these types of calculation errors.

1.3 Evidence for biosynthesis of vertebrate-like steroids

For the last 50 years, scientists have endeavoured to prove that molluscs synthesise vertebrate-like steroids. By so doing, they would not only be able to explain why steroids are present in molluscan tissues (i.e. they are the natural hormones of these animals) but also provide solid evidence for the long-standing hypothesis that the induction of penis growth in female dog whelks that have been exposed to Tributyl Tin (TBT) (Smith, 1981) is controlled by T (Matthiessen and Gibbs, 1998).

The vertebrate steroid biosynthetic pathway has been well characterised. Vertebrate sex steroids are derived from cholesterol. There are three groups broadly differentiated by the number of carbons that they contain: progestagens (C21), androgens (C19) and oestrogens (C18). The basic outline of the systematic structural changes and the enzymes responsible for these changes are outlined in Figure 2. There are two generic reactions that are involved in vertebrate steroid synthesis. One is **reduction / oxidation** (the insertion or removal of two hydrogen atoms; mediated by oxido-reductases [which, in relation to steroids, are termed hydroxysteroid dehydrogenases; HSD]) and the other is **oxygenation** (the insertion of an oxygen atom onto a carbon atom; mediated by P450 enzymes). The latter reaction is, in the three most important steps in vertebrate steroid biosynthesis (Figure 3), accompanied by **cleavage** (the removal of one or more carbon atoms; also mediated by P450 enzymes). The basic enzymatic reactions involved in steroid biosynthesis are 1) Cholesterol side-chain cleavage (insertion of two oxygen atoms at C20 and C22; and then cleavage between them) to form Pn; 2) Removal of two hydrogen atoms from the 3ß-hydroxyl position of Pn to form P; 3) Insertion of an oxygen atom at C17 of P and then cleavage between C17 and C20 to form Ad; 4) Addition of two hydrogen atoms at C17 of Ad to form T; 5) Addition of two oxygen atoms and cleavage of C19 to form E_2 (a process known as aromatisation); 6) There is a further important reaction in vertebrates involving the addition of two hydrogen atoms to the A ring of the T molecule to convert it into another important hormonal steroid, 5α-DHT. Attempts have been made to show all these reactions in molluscs (reviewed by Scott, 2012). It has been

pointed out by (Janer and Porte, 2007) that the complete array of transformations necessary for formation of E_2 from cholesterol has never been demonstrated unequivocally within a single molluscan species. A couple of very early studies (Lupo di Prisco and Fulgheri, 1975; Lupo di Prisco et al., 1973) purport to show the production of all intermediates between Pn and E_2, but the papers lack any information on methodology and yields (i.e. the findings are equivocal). To be more precise, the only transformations for which there is compelling evidence (high yields found in one or more species plus definitive identification of products) are those involving the addition or removal of two hydrogen atoms (i.e. Pn → P; Ad → T and T → 5α-DHT). On the other hand, the evidence for the occurrence of the three transformations (Figure 3) involving direct oxygenation and cleavage (i.e. Cholesterol → Pn; P → Ad; and T → E_2) is far less compelling. Some studies report negative findings, while others report positive findings but with extremely low yields. In other words the evidence is still questionable, especially that purporting to show the presence of aromatase activity (discussed below).

It should be noted that the oxygenation reactions (and their associated cleavage) are far more complex than the transformations involving hydrogen insertion (which in the case of Ad → T is readily reversible). They are mediated by distinct and highly specific P450 enzymes in vertebrates. No-one has yet traced the genes for these enzymes further back in evolution than the protochordates (i.e. they appear as of this moment to be specific to the vertebrate line of evolution; Callard *et al.*, 2011; Markov & Laudet, 2011).

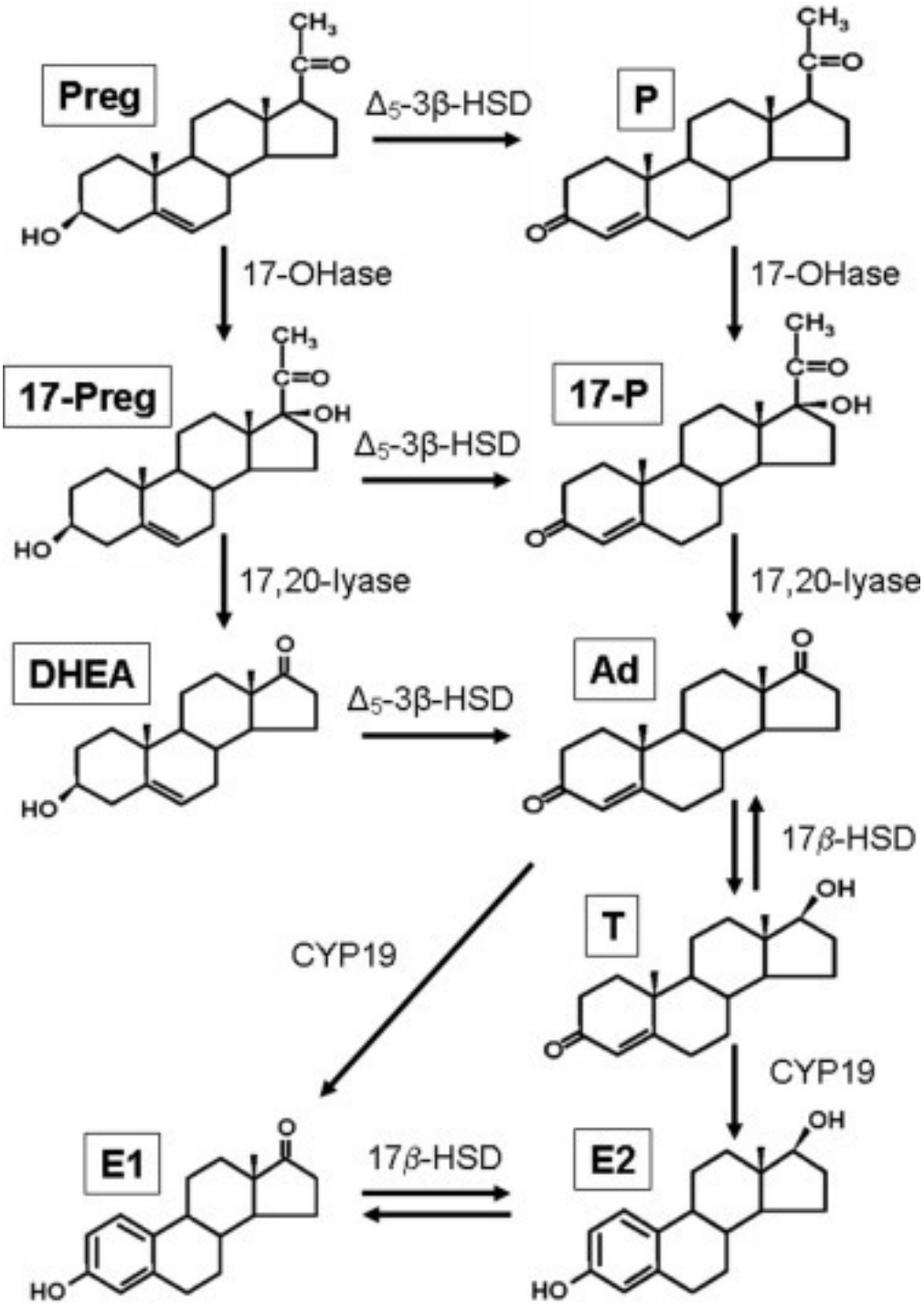

Figure 2: Diagram of steroidogenesis in vertebrates (Scott, 2012).

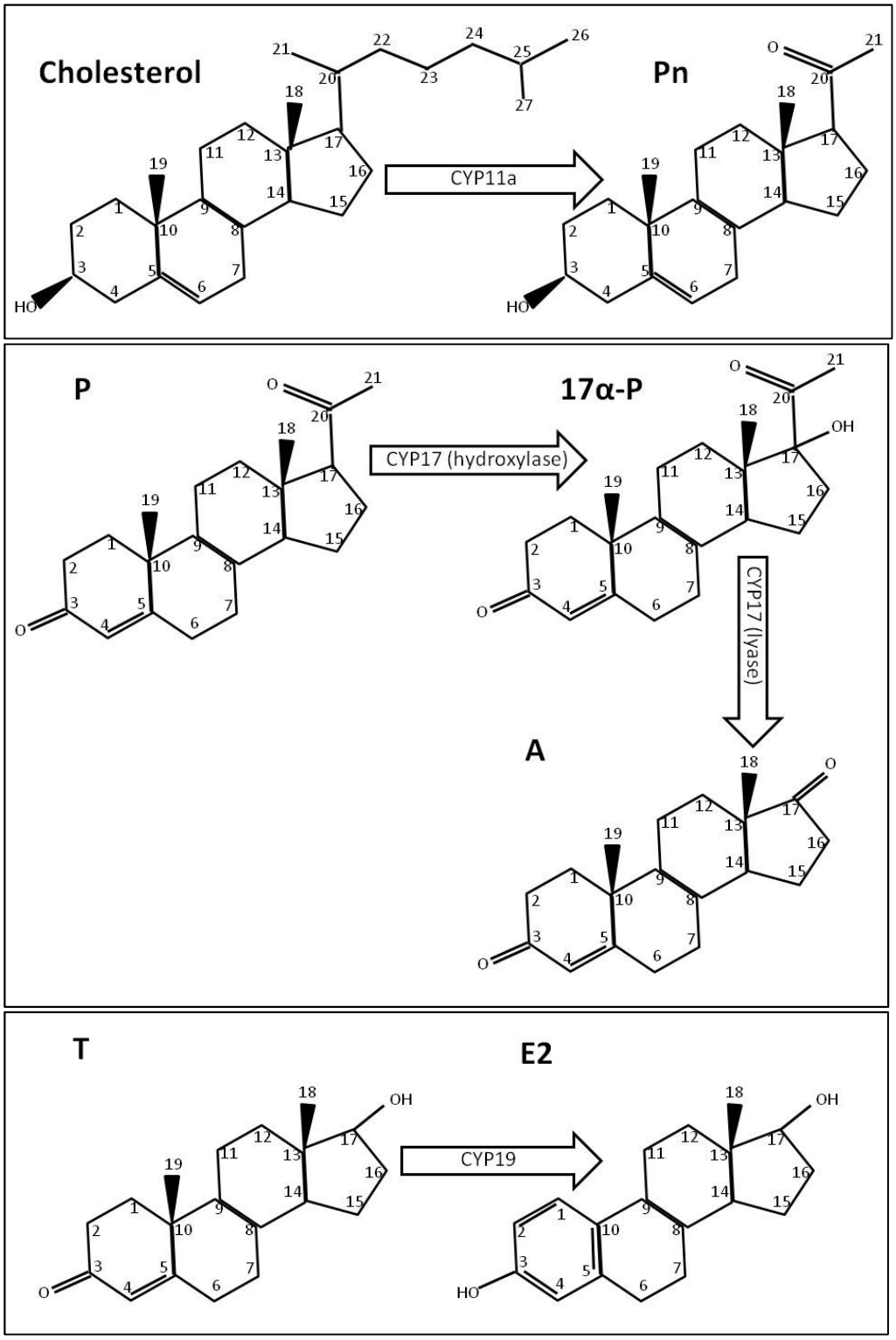

Figure 3: Diagrams of the key enzymatic reactions of steroidogenesis for which there is no solid evidence in molluscs.

1.3.1 The strength of the evidence for steroid interconversions

Steroid biosynthesis in molluscs has been mostly studied *in vitro* by incubating tissue (microsomal fractions, homogenates, etc.) with

precursors (mostly radiolabelled); metabolites are then identified with one or a combination of methods (chromatography, microchemistry, crystallisation or analytical chemistry) and the identity of the enzyme (assumed to be the same as the vertebrate enzymes describe above) is then inferred. When analysed critically, a few of these studies have flaws which render some, if not all, of the data unreliable.

As mentioned above, the literature is replete with studies showing that molluscs have the ability to carry out reactions involving addition or removal of two hydrogen atoms. However, this fact does not prove that steroids are their intended substrate (oxido-reduction reactions are involved with metabolism and synthesis of lipids, proteins and sugars and are common not just in vertebrates but also in bacteria and plants; Baker, 2001) nor that the enzymes involved are the exact genetic counterpart of the hydroxysteroid dehydrogenases found in vertebrates. For example, de Jong-Brink *et al*. (1981) reported the production of P (up to 15 %) when incubating the gonad-digestive gland complex of the great pond snail, *Lymnaea stagnalis,* with radioactive Pn. Confirmation of steroid identity was good (consisting of co-migration with P standard by TLC, resistance to formylation and re-crystallisation). However, all this study demonstrates is that *L. stagnalis* has an oxido-reductase that performs in a similar way to the 3ß-HSD of vertebrates. Most importantly, P was **not** converted into 17α-hydroxyprogesterone (17α-P) or Ad, both of which would be expected if the 3ß-HSD activity was part of a typical vertebrate steroid biosynthetic pathway.

As well as interconversion studies only showing isolated parts of the vertebrate-like steroid biosynthetic pathway, a more pressing issue with the literature is the tendency for chromatographic position to be used as the sole method of metabolite identification. For example, Janer *et al*. (2005) investigated metabolism of radioactive Ad and T by microsomal and cytosolic fractions of the gonad-digestive gland of the giant ramshorn snail, *Marisa cornuarietis* , (and other non- molluscan organisms). They reported that T was converted into Ad and an unknown polar metabolite, suggesting the presence of 17ß-HSD and possibly sulphotransferase. Ad on the other hand was converted into 5α-DHA, 5α-DHT, T and some unknown metabolites

which suggested the presence of 5α-reductase as well as 17β-HSD. The problem with these results is that TLC and HPLC were the only tools used for metabolite identification, i.e. co-migration of radioactivity with known standards was considered definitive metabolite identification. Chromatography, however, is a means of separation and can only 'presumptively' identify a compound (i.e. it can narrow down the list of possible metabolites). Some steroids will migrate together as a result of similar polarity despite being structurally different. Chromatographic methods should, therefore, be used in conjunction with other methods such as microchemistry and re-crystallisation, or analytical chemistry (e.g. LC-MS/MS). Goto *et al.* (2012) performed a study which highlights this issue perfectly. They investigated the fate of radioactive T and Ad after incubation with tissue homogenates (of gonads or digestive glands of males and females) of the marine snail *Thais clavigera*. After incubation, the homogenates were extracted and separated on a TLC plate. The radioactive spots on the plate were then extracted and analysed by GC-MS. These showed, that the major radioactive product formed from Ad contained a compound that had the same mass and disintegration pattern as E_1; while the T product had the same mass as E_2. On the surface, these data look sound, the conclusion being that *T. clavigera* appears to contain aromatase activity – the key reaction in the synthesis of oestrogens. However, the authors did not take into account the fact that E_1 and E_2 were already present in the animals at the start of their incubation experiments (as the authors themselves had shown by identifying both oestrogens in a subgroup of animals they had collected from the wild). Incidentally, the same two hormones were identified in tissues from the same species more than ten years previously (Lu *et al.*, 2001, 2002). Basically, the authors provided no proof that the E_1 and E_2 that they identified by co-migration on the TLC plates had been actually made during the incubation period (i.e. it could have been steroids that were already present in the tissues presumably absorbed from the environment prior to their capture). The fact that the E_1 and E_2 chromatographed in similar positions to the major radioactive spots formed from Ad and T is therefore irrelevant. The identification of EE_2 in *T. clavigera* and a closely related species, *Babylonia japonica* (Lu et al., 2001), further calls into question the belief that *T. clavigera* is able to make E_1 and E_2 *de novo* (strongly supporting, on the

other hand, the hypothesis that these steroids are picked up from the environment).

1.3.1.1 Could esterification explain low yields in some biosynthesis experiments?

When Scott (2012) critically reviewed the methods employed in studies regarding biosynthesis of steroids in molluscs, he highlighted the fact that many did not report yields and that, where they did, they were much lower than one might have expected. In 2001, Gooding & LeBlanc definitively discovered not only that T was readily absorbed from the water by *I. obsoleta* but that it was predominantly esterified (> 70 %). This sequestration of steroids as highly fat-soluble molecules could perhaps explain the low yields of free metabolites reported in many biosynthesis studies in the literature. *In vitro* incubations of tissue homogenates usually last 1 to 3 h, so if it is assumed that they pick up (and potentially metabolise) half of the precursor present in the water and then esterify 70 % of that, only 15 % of the original precursor would be present as potential free metabolites. Additionally, if it is considered that sulphation of steroids also occurs in molluscs (Hines et al., 1996) and that sometimes two or more metabolites are made from the same precursor (e.g. Janer *et al.* (2005) found Ad was converted to 5α-DHT and 5α-DHA) and probable extraction and clean-up losses are taken into account: the yield of any particular free steroid quickly dwindles. Of the six studies (Dimastrogiovanni et al., 2015; Goto et al., 2012; Janer et al., 2004b; Janer et al., 2005a; Janer et al., 2006a; Lyssimachou et al., 2009) that have investigated the metabolism of radioactive T (all carried out after the discovery of T esterification in *I. obsoleta* (Gooding and LeBlanc, 2001), not one investigated the nature of the metabolites in the ester fraction. They have only looked at the free fraction. One study (that also reported low yields of metabolites) did indeed mention in their Discussion that the organism in question (*M. cornuarietis*) had been found to pick up and esterify T at similar rates to *I. obsoleta* (Janer et al., 2005a). The relevant data, however, were not published and not taken into account when identifying the metabolites (mainly 5α-reduced steroids). The study by Gooding & LeBlanc, (2001) provided circumstantial evidence that esterification of T metabolites (not just T itself) is also possible. *I. obsoleta* produced five ester metabolites

(that could be separated by TLC) after T exposure *in vivo* and although the authors assumed these differences were a result of fatty acid variation, it is equally possible that they were actually different T metabolites conjugated to the same fatty acids.

In theory P cannot be esterified, as it does not have any hydroxyl groups by which the fatty acids can be attached. Thus yields of free metabolites from this steroid should in theory be high. However, there is always the possibility that P could be converted to metabolites that do have hydroxyl groups and that they then become liable to esterification. It has been shown, for example (Dimastrogiovanni et al., 2015) that the A ring of P can be reduced to the 5α configuration and that this can then be further reduced to 3ß-hydroxy-5α-pregnane-20-one (3ßP5α) (although it must be stressed that identification was based purely on chromatographic elution position). In theory, this steroid is esterifiable (via the 3ß-hydroxyl position) but the authors did not examine this possibility.

1.3.1.2 Molecular biological evidence to support steroid inter-conversions

In the last ten years, molecular methods have been used to investigate steroid biosynthesis, giving rise to conflicting evidence. Several studies have endeavoured to show transcription of 17ß-HSD-like genes that were assumed to be involved primarily in hydrogenation of steroids (e.g. A → T). The problem with these studies is that they report relative activity of transcription (in different tissues or different maturation stages) of a gene which has not yet been fully characterised and therefore cannot render information in terms of primary function. Liu *et al*. (2014b) investigated a putative 17ß-HSD (Cf-17ß-HSD8) in *C. farreri*. They measured transcription in a number of different tissues (gonads, mantle, adductor muscle, kidney and digestive gland) and reported that 'high levels of Cf-17ß-HSD8 mRNA were detected in digestive gland and kidney, while relatively low levels were detected in mantle, gill and adductor muscle'. They did not however, state whether transcription was higher or lower in gonads (compared to other tissues), instead they (only) presented transcription data highlighting the differences between ovaries and testis at different maturation stages. To add to this bias, the authors cloned, expressed and purified the enzyme

in order to investigate its activity towards Ad, T, E_1 and E_2. They reported that T and E_2 were oxidised to Ad and E_1 respectively (they found no transformation of Ad or E_1 into to T and E_2 – these reactions are readily reversible in vertebrates), however, the authors reported the data as a decrease in the substrate (measured by ELISA), not an increase in the product and in fact, nowhere in the text does it say whether or not the product was actually measured. In spite of the evidence for an association between this particular transformation and reproduction being anecdotal at best the authors concluded that 'the expression pattern of Cf-17β-HSD8 in gonads suggests that Cf-17β-HSD8 may participate in regulating the steroid-mediated gametogenesis in *C. farreri*'.

A couple of studies have cloned uncharacterised 17β-HSD homologues and expressed them in human cell lines to investigate steroid interconversions (which already makes an assumption that steroids are the natural substrates of these enzymes) (Zhai et al., 2012; Zhou et al., 2011). The first study found that Hd17β-HSD-11 from the abalone *Haliotis diversicolor supertexta* oxidised 5α-androstan-3α,17β-diol to androsterone and T to Ad. They reported no activity on E_1 and E_2 and no reduction of androsterone or Ad (Zhai et al., 2012). The other study investigated a second HSD in *H. diversicolor,* Hd17β-HSD-12. They reported reduction of E_1 to E_2 but no oxidation of E_2 to E_1 (Zhou et al., 2011). Both studies measured transcription levels in gonads and then attempted to link these results with maturation. The authors then suggested that the 17β-HSD isomers 'plays a central role' or has an 'essential function' in the abalone 'steroid-mediated reproductive process'. Not only are these conclusions drawn on circumstantial evidence (i.e. 17β-HSD transcription levels were high at the pre-reproductive stage; Zhou *et al.*, 2011) but they are assuming these enzymes were there in order to help biosynthesise vertebrate steroids. Another study investigating 17β-HSD in molluscs has presented a much more plausible role for the enzymes in question. Lima *et al.* (2012) cloned a putative 17β-HSD (Nl17β-hsd-12) from the dog whelk *Nucella lapillus* and investigated transcription of the gene in the context of TBT exposure. TBT is known to cause imposex in this species and although it was assumed for some time that this was due to endocrine disruption as known for vertebrates; it is now accepted that TBT acts via the retinoic acid pathway

(Castro et al., 2007; Horiguchi et al., 2008). Sequencing and sequence alignments of Nl17β-hsd-12 revealed that it was highly similar to the Hd-17β-hsd12 found by Zhai *et al.* (2012). They reported that mRNA of Nl17β-hsd-12 was expressed in all tissues but was higher in organs with high metabolic rates (the kidney and digestive gland). TBT exposure did not affect enzyme expression in the gonads but markedly reduced transcription levels in the digestive gland of both males and females. The authors concluded that it is likely that Nl17β-hsd-12 is involved in lipid metabolism and they argued that the differences in expression of 17β-HSD in male gonads which Zhai *et al.* (2012) linked to steroid biosynthesis, are more likely associated to known differences in distribution of lipids between male and female gonads in molluscs.

Another study investigating enzymes that in vertebrates are associated with steroidogenesis (in the context of endocrine disrupting pollutants) was by Tian *et al.*, (2013). They exposed female *C. farreri* to benzopyrene (a polyaromatic hydrocarbon; PAH) (B[a]P) for 10 days, measuring three steroids (P, T and E_2) in gonads and haemolymph as well as assessing transcription levels of 17β-HSD, 3β-HSD, CYP17 (incidentally, the first hint that this enzyme actually might exist in molluscs), oestrogen sulphotransferase (SULT), oestrogen-like receptor (ER); VTG (egg yolk precursor protein) and CYP1a1 (involved in detoxification of PAHs). The authors concluded that '... 3β-HSD, CYP17 and 17β-HSD could be potential targets of B[a]P in the *C. farreri* ovary and subsequently result in disrupted steroid levels.'. If this was in fact the case, one would expect to see a correlation between up and down regulation of genes presumably involved in steroidogenesis and relative steroid levels in the animal. Table 1 presents a simplified interpretation of the data reported by Tian *et al.* (2013) regarding gene expression and steroid levels. If for simplicity purposes, a single time point and dose for enzyme transcription (Table 1) is examined, on day 3 an increase in P (3β-HSD: Pn→P); an increase in Ad (CYP17: P→17αP→Ad) (Ad was not measured but is the precursor for T); a decrease in T (17β-HSD: Ad→T) and therefore a decrease in E_2 (the product of aromatisation of T) would be expected. Instead, there is a decrease in P and not much change in E_2 and T in gonads; and an increase in E_2 in the haemolymph. If the data are analysed for day 10 in the same way, similar

discrepancies can be found between the levels of steroids and the level of enzymes that are supposed to produce them. The author's conclusions were in fact, unfounded as there is no correlation between the changes in gene expression brought on by B[a]P and the reported levels of steroids. It is more than likely that *C. farreri* has enzymes of similar nature (i.e. oxido-reductases) to those involved in vertebrate steroidogenesis but that these play other roles (e.g. lipid metabolism). The theory that steroids are picked up from the environment is a more plausible explanation for the random levels of steroids that were found in this study. Furthermore, as only free steroids were measured, the detected variation could perhaps be the result of incidental depuration and uptake of what was already in the animal tissue, compounded by what could be added to the exposure water during handling. It would not be wise to speculate on the reason for the variation in transcription levels as not only are the genes in question not yet characterised in *C. farreri*, but there are any number of pathways B[a]P could interfere with.

Table 1: Interpretation of data output (+ increase; - decrease; 0 no change) of high dose treatment (10 µg L^{-1}) from (Tian et al., 2013) .

Target measured	Day 3	Day 10
3ß-HSD	+	-
17ß-HSD	0	-
CYP17a	+	-
P (gonad)	-	+
T (gonad)	0	-
E2 (gonad)	0	-
T (haemolymph)	0	0
E2 (haemolymph)	+	0

So, it appears to be that molluscs do indeed possess enzymes capable of hydrogenating or dehydrogenating certain steroids. This does not, however, prove that these are true orthologs of the vertebrate enzymes or that they have the same intended substrates. Both biochemical and molecular evidence have failed so far to demonstrate the presence of all the required enzymes in molluscs for full vertebrate steroidogenesis to be possible.

1.4 Evidence of steroid receptor presence and activity in molluscs

Nuclear receptors (NR) are transcription factors involved in regulating development, metabolism and homeostasis. In vertebrates they are broadly divided into six groups. Like many other NRs, steroid hormone receptors belonging to group 3 are ligand-activated. Figure 4 portrays a simple diagram of steroid receptor activation and function. Briefly, the receptors recognise and bind a specific hormone (ligand), they undergo a conformational change in order to translocate into the nucleus where they recognise and bind a DNA response element (e.g. oestrogen-response element; ERE); this in turn activates transcription of downstream genes which lead to, for example, sexual differentiation, immune responses, etc. Vertebrate steroid receptors are well characterised but their presence and role in invertebrates is still unclear. Scott (2012) reviewed the evidence for the presence of steroid receptors in molluscs and concluded that 'there is still no firm evidence for the presence of specific functional receptors for vertebrate steroids'. The next section will touch briefly on the subject, highlighting not only the lack of evidence for the presence of vertebrate-like ligand-activated steroid receptors but also the low quality of some of the data supporting it.

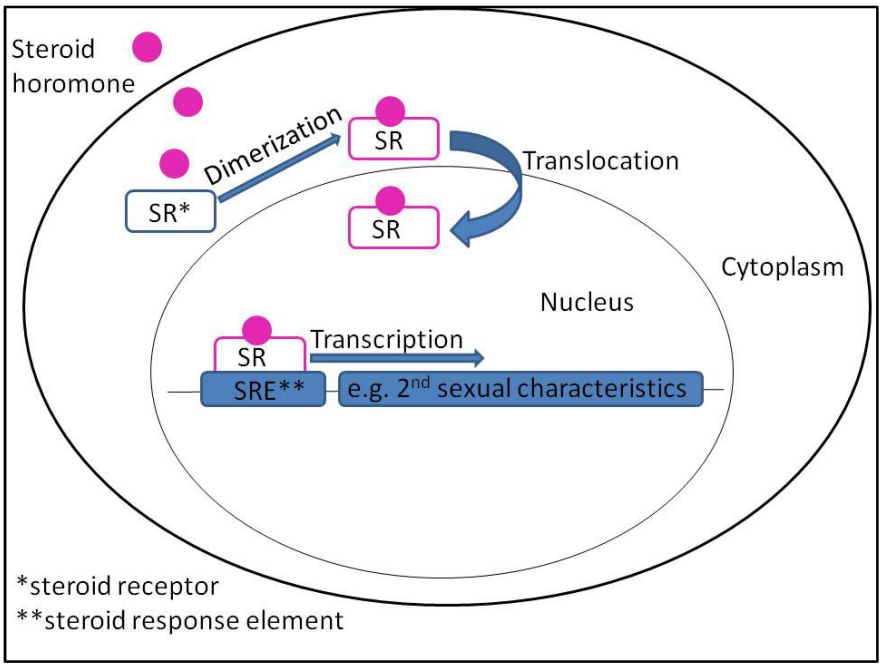

Figure 4: Diagram illustrating the mechanism of action of nuclear receptors.

1.4.1 Do molluscs have steroid receptors?

Oestrogen and oestrogen-related nuclear receptors (ER and ERR; molluscan receptors referred to as mER-like and mERR respectively from here on) have been found in several bivalves, gastropods and cephalopods (e.g. Keay *et al.*, 2006; Nagasawa *et al.*, 2015; Thornton *et al.*, 2003). Cloning and sequencing of these receptors has allowed for phylogenetic analysis to better understand evolution of ERs. It has shown that the mER-like and mERR are genuine orthologs of the vertebrate ER and ERR from nuclear receptor group 3, i.e. they evolved from a shared ancestor but in this case they do not necessarily have the same function. *In silico* analysis of the whole genome of the Pacific oyster, Crassostrea *gigas*; the freshwater snail, *Biomphalaria glabrata* and the owl limpet, *Lottia gigantea*, have demonstrated that molluscs have a high number of NRs (the two latter have representatives in all six groups), including the above mentioned mER-like and mERR. They do not, however, possess androgen receptors or any subgroup 3C receptors (the vertebrate androgen, progesterone, glucocorticoid and mineralocorticoid receptors) for that matter (Kaur et al., 2015; Vogeler et al., 2014). Attempts to detect androgen receptors (AR) have not only been done by mining genomes *in silico*; six years earlier Sternberg *et al.* (2008) designed primers based on the conserved region of the DNA binding domain (DBD; see Figure 5 for a diagram of steroid receptor gene) and sought to amplify the AR in *I. obsoleta*. Their search, however, was unsuccessful.

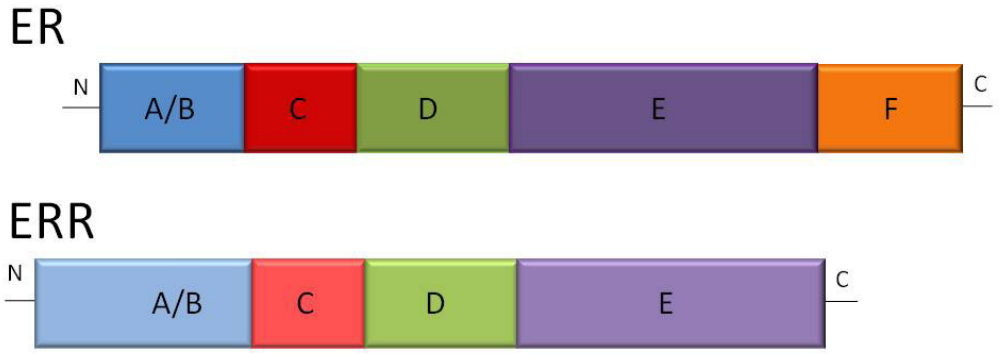

Figure 5: Diagram of the oestrogen receptor (ER) and oestrogen-related receptor (ERR) consisting of five and four domains respectively, where A/B: N-terminal domain (contains the AF-1 ligand-independent activation function); C: DNA-binding domain; D: hinge; E: Ligand-binding domain (contains the AF-2 ligand-dependent activation function; F: C-terminal domain) .

The presence of mER-like and mERR in molluscs is no longer in doubt; there are over 30 studies which have detected and cloned the genes. It is now, in fact, generally believed that the steroid nuclear receptor ancestor was a ligand-activated ER. Thornton *et al*. (2003) constructed an ancestral ER which was activated by oestrogen and Keay & Thornton (2009) reported a ligand-activated ER in an annelid; which is in a different line to both vertebrates and molluscs, suggesting ligand-activation evolved before they all split. This steroid receptor ancestor would have evolved before deuterostomes and protostomes diverged some 600 million year ago. Vertebrates then gained other steroid receptors via gene duplication and molluscs lost the ability to activate the ER (Bridgham et al., 2014).

1.4.2 Are these receptors functional?

So far, it is known that the presence of both mER-like and steroids (in particular E_2) in molluscs is irrefutable. Does this mean that the mER-like is structurally and functionally homologous to the vertebrate ER? Although the molluscan ER has the same six domains as the vertebrate ER (making it structurally homologous; for structure see Figure 5) (Bannister et al., 2007; Thornton et al., 2003); significant variation (ca. < 40 % conserved when compared to vertebrates) in the E and F domains have rendered it incapable of actually binding oestrogens (Bannister et al., 2007; Bannister et al., 2013; Bridgham et al., 2014; Kajiwara et al., 2006; Keay et al., 2006; Thornton et al., 2003). Cloned receptors (from a number of species) have been tested *in vitro* for functionality. Bannister *et al*. (2007) investigated the binding capacity of the mER-like and mERR from *M. cornuarities* to tritiated E_2 using a radiological binding assay. They reported there was no evidence of specific binding of E_2 to either receptor. Furthermore, during another study, several other substrates (of oestrogenic and anti-oestrogenic nature) were tested *in vitro* (reporter-gene assay): methyl testosterone, EE_2, hydroxytamoxifen, diethylstilbestrol and cyproterone acetate. None of the chemicals elicited an increase in transcription of the reporter gene, suggesting that the receptors were not able to bind the ligand-binding domain (LBD). The authors did, however, report transcription activation by mER-like and mERR in the presence of genistein and BPA respectively. Activity, though, was only reported at very

high concentrations, suggesting that the interaction between the LBD and the chemicals was very weak (Bannister et al., 2013). Matsumoto *et al.* (2007) also constructed a reporter-gene assay to test the binding abilities of the mER-like of *C. gigas*. Once again, the reporter gene was not activated in the presence of E_2. Furthermore, a couple of studies showed that when only the LBD was expressed in a construct (with suitable activators, etc.) it was found to activate reporter-gene transcription via the response element in the absence of a ligand; and addition of E2 (and other steroids) made no difference to the level of transcription (Keay et al., 2006; Thornton et al., 2003). This suggests that molluscan ERs-like are expressed constitutively. This is further supported by some *in vivo* data where mER-like mRNA has been found to be unresponsive (i.e. transcript levels remain the same) to E_2 exposure (Puinean et al., 2006). This lack of effect of steroids on mER-like transcription is not always the case; this however is discussed in section 1.5.1.

Despite solid evidence that the LBD is not conserved and does not bind E_2 *in vitro* (in fact it has been reported to be constitutively expressed), there is still a high output of studies that claim to show that levels of mER-like mRNA in molluscan tissue are up-regulated by exposure to oestrogens. Moreover, there are also reports of mER-like up-regulation during certain stages of reproductive maturation (e.g. egg yolk production). One can only assume that, among many scientists, there is bias towards the hypothesis that oestrogens 'should' have an effect on the mER-like because the name implies it is a 'receptor for oestrogen'.

Even though molluscs do not have functional nuclear receptors for vertebrate steroids, the possibility cannot be dismissed that signalling for vertebrate steroids is mediated via membrane-bound (i.e. non-genomic) receptors (Janer and Porte, 2007). The strongest evidence that this might in fact be so comes from a study on the rotifer, *Brachionus plicatilis* (Stout et al., 2010). Although this is not a mollusc, it is nevertheless a primitive invertebrate, and if this organism contains a membrane-bound receptor for a vertebrate steroid, then the same situation would almost certainly apply to molluscs. The paper by Stout *et al. (*2010) reports the identification of a protein that is present in vertebrates and is known as the 'membrane

progesterone receptor' or by its approved nomenclature as the 'progesterone receptor membrane component I (PGMRC1)'. However, since the publication of that paper, it has been shown that, despite its misleading name, it is not actually a P receptor; PGRMC1 is in fact an 'adaptor protein' (Thomas et al., 2014) that assists in the action of many types of membrane receptors and appears to be ubiquitous in all living organisms. The important point at this moment is that its presence in rotifers is not evidence, as claimed by the authors, that P function is conserved in invertebrates; nor, by extension, that molluscs are therefore likely to have specific membrane-bound receptors for vertebrate steroids.

1.5 Evidence for biological activity

1.5.1 Does exposure to oestrogens affect transcription of mER-like?

One of the arguments for the endogenous production of steroids in molluscs is their apparent biological response to steroids and other vertebrate endocrine disrupting compounds. Many studies have investigated this by measuring biological endpoints and molecular biomarkers in exposed and control animals (*in situ* or *in vivo*) and reported positive findings (e.g. Benstead *et al.*, 2011; Canesi *et al.*, 2008; Ciocan *et al.*, 2010). The strength of the evidence that steroids have biological effects in molluscs actually formed the basis of a review by Scott in 2013. In the conclusions to his paper, Scott (2013) stated:

'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'.

Has the situation changed in 2015? In section 1.4.2 studies were presented with strong evidence that the mER-like is not only constitutively expressed but incapable of binding E_2 (or any other steroid). Despite this evidence, studies on up-regulation or down-regulation of the mER-like in response to oestrogens form the commonest type of study. Not only are these studies based on physiologically unsound hypotheses (i.e. why investigate the effects of an oestrogen on a receptor that has been shown not to recognise it?) but the fundamental study design and data interpretation flaws mentioned by Scott (2013) are still very much present. For example, Hultin et al. (2014) exposed the fresh water snail, *Bithynia tentaculata* to 10 and 100 ng L^{-1} EE_2 and compared mER-like transcription to that of unexposed controls. Indeed, the study had good replication (three replicates with 12 animals for each dose) and controls (three water and solvent controls). However, only a 20 % decrease in mER-like transcription was found at the highest concentration compared to controls. The change was statistically significantly different, but since the experiment had not been carried out blind and there was potential for bias (a result of the receptors name), the possibility that this was a chance observation cannot be ruled out. In a very similar study on another gastropod, the New Zealand musdsnail (*Potamopyrgus antipodarum*), that was cited by Hultin *et al*. (2014) in support of their observations, mER-like was up-regulated (though again by only a very small amount) when exposed to EE_2 (Stange and Oehlmann, 2012). Hultin et al. (2014) actually reported a large amount of variability in mER-like transcription and justified the difference between studies with the fact that the latter had analysed pooled samples. Furthermore, the primers used in the Hultin study were based on the phylogenically close mER-like of the sea slug, *Aplysia californica*, which has already been shown not to be ligand-activated (Thornton et al., 2003). As previously mentioned, yet another phylogenetically related mER-like and mERR (from *M. cornuarites*) was recently shown not to be responsive (*in vitro* and *in vivo*) to E_2 or octylphenol (Bannister et al., 2013). Even then, despite such

negative results Hultin et al. (2014) concluded that 'Furthermore, the significant interaction between the dose and exposure duration of EE_2 on the er mRNA in *B. tentaculata* is worth studying further'. Another study (Nagasawa et al., 2015) cloned the mER-like and mERR of *M. galloprovincialis* and *M. edulis*, and measured their transcription in gonads after incubating them with T and E_2. They reported a two-fold up-regulation of the mER-like in the E_2 exposure treatment group (single dose) compared to the control, but also showed the same level of up-regulation following exposure to T. This suggests that either both T and E_2 regulate mER-like in blue mussels or else the differences were generated purely by chance. The latter conclusion is supported by the fact that in order for a < 2-fold change to be reliably considered up-regulation (since one cycle of PCR = 2-fold change) a large number of biological replicates are required (their study had n = 4) and at least three technical replicates (not reported in their study) (reviewed by Bustin et al., 2009). The study by Völker et al. (2014) is another good example of data misinterpretation. They found non-monotonic responses in transcription of the mER-like and VTG in *P. antipodarum* after exposure to silver nanoparticles and EE_2. They justified the non-monotonic response (e.g. transcription of both genes was higher than controls at low and high doses but down-regulated at the intermediate dose; and all changes were < 3-fold) by referring to a general review of biphasic responses to oestrogens in vertebrates (Calabrese, 2001).

1.5.1.1 Does VTG serve as an oestrogen exposure biomarker in molluscs?

VTG has been successfully used as a biomarker for oestrogenic activity in fish since the 1990s (Sumpter and Jobling, 1995). The presence of VTG-like proteins in molluscs (note that VTG-like proteins can be found in all oviparous organisms) has led to a number of studies using them as biomarkers for oestrogen exposure. The problem is that VTG-like protein in molluscs have been so far poorly characterised and they have only been assumed to be responsive to oestrogen because this is the case for vertebrates. As it is now known, there is no solid evidence that molluscs produce or use oestrogen (or any other vertebrate-like steroids) like vertebrates do, so why would it be assumed that they use and regulate VTG in the same manner? Since the 2013 review by Scott, a very well-

conducted study has been published that categorically failed to show any effect of oestrogen on egg yolk production in a fresh water bivalve (Morthorst et al., 2014). Other publications regarding VTG-like expression present similar issues as those detailed above for mER-like transcription. For example, Ni *et al.* (2014) cloned VTG-like from *C. angulata* and measured transcription after E_2 exposure. The response was not only non-monotonic for the three exposure concentrations but the changes reported were only 2 to 3-fold. The latter seems like a very minor change (physiologically speaking) when considering that during the same study VTG-like was reported to change by > 300-fold in relation to the stage of the reproductive cycle. Another study (Falfushynska et al., 2014) reported a decrease in VTG-like protein (measured indirectly by quantifying alkali-labile phosphate; ALP) when the freshwater bivalve, *Unio tumidus*, was exposed to E_1. Although the authors did acknowledge that there is no solid evidence for functional ERs or steroidogenesis in molluscs, they suggested that, like vitellogenesis (although they could not explain the reported down-regulation) synthesis of total protein may also be under the control of steroids. Note that this is the same animal for which Morthorst *et al.* (2014) reported there was no effect of E_2 on VTG-like levels and, furthermore, that ALP was not a good representation of yolk protein levels. More importantly, it should be mentioned that this study (Falfushynska et al., 2014), like many of those reviewed by Scott (2013), consisted of a single replicate for each chemical. Lack of tank replication, regardless of the number of individual replication (i.e. number of animals per tank), renders results unreliable as they are susceptible to so-called tank effects (which many would consider to be 'pseudoreplication'; Hurlbert, 1984).

The use of VTG as a biomarker in fish has been very reliable, i.e. oestrogens consistently induce up-regulation of VTG in a concentration-dependent manner in many fish species (Hahlbeck et al., 2004; Kirby et al., 2004; Panter et al., 1998; Routledge et al., 1998). In molluscs on the other hand, findings are rarely reproducible even within the same species, for example Stange & Oehlmann (2012) and Hultin *et al.* (2014) (as mentioned earlier) reported contradictory findings in two gastropod species with phylogenetically close mER-like. Gagné *et al.* (2001) reported up-regulation of VTG-like in a freshwater mussel *Elliptio complanata* after

exposure to sewage treatment effluents (containing oestrogenic chemicals), whereas Won *et al*. (2005) found no change in VTG-like levels after E_2 exposure. Moreover, Jasinska *et al*. (2015) tried to identify biomarkers for chemicals (including oestrogens – detected with passive samplers) in a freshwater mussel (*Pyganodon grandis*) and the fathead minnow (*Pimephales promelas*). They caged mussels and fish for four weeks downstream from a sewage treatment plant effluent (and two upstream reference sites) and measured, amongst other things, transcription of VTG. They reported up-regulation of VTG in the fish and down-regulation in mussels. It must be noted that the increase in fish was only 5-fold, however if vitellogenesis was indeed controlled by steroids in molluscs, as it is in vertebrates, one would expect to find the same trend in both organisms.

Another important point is that whether or not the data are conclusive, no account has been taken in any of these studies of the fact that changes in protein expression (whether it be VTG-like, mER-like or any other protein for that matter) could just be due to the fact that the steroids are, as already discussed, substrates for esterification. In other words, the authors have only assumed that any changes in transcription are due to a hormone-receptor effect (e.g. Ni *et al*., 2014) as opposed to a 'substrate availability' effect. This same criticism can be levelled at the two studies that claim to have shown changes in the metabolomic profile of molluscs that have been exposed to oestrogens (Cubero-Leon et al., 2012; Leonard et al., 2014a).

Finally, Scott (2013), pointed to the fact that the mollusc literature was biased toward positive findings, by firstly scientists writing up negative (or more strictly 'no effect') data in a way that makes it appear to be positive and secondly, by scientists not publishing negative data. On this latter point, Scott (2013) referred to the existence of two major EU-funded studies which had failed to establish any effects of EE_2 on the snail, *Helix aspersa* but that had not then been published. A recent search of Defra-funded contract projects by Dr Scott (unpublished) revealed two other studies that had found no effect of oestrogens on invertebrates (one involving E_2 and *Mytilus* spp.) and that had not been published in the peer-reviewed literature (Defra AE1146, 2002; Johnson et al., 2005). Also, Dr

Maren Ortiz-Zarragoitia of the Dept. of Zoology and Cell Biology, University of the Basque Country (UPV/EHU), Bilbao, Basque Country, Spain drew the attention of Dr Scott to a further two such unpublished studies from his Department (both showing no effect of E_2 on vitellogenesis in *M. galloprovincialis*). The absence of these studies from the peer-review literature is bound to bias people's views about whether or not steroids have hormonal actions in molluscs.

1.6 Objectives of this thesis

The major aim of this thesis is to confirm that mussels (*Mytilus* spp.) are able to take up vertebrate steroids from the environment. It must be stressed that by doing so it cannot be disproved that endogenous production of vertebrate steroids occurs in molluscs. However, by quantifying factors such as the rate and capacity of not just uptake of vertebrate steroids, but also their esterification, sulphation and depuration, it is hoped to be able to prove conclusively that steroid uptake by molluscs is a major process that has the potential to confound attempts to prove vertebrate steroids are of endogenous origin. The following aims have been set out:

Phase 1 – Answer fundamental questions such as:

- What are the ideal conditions for studying steroid uptake, esterification and depuration? The few studies that have been carried out to date have all used a variety of different procedures with little or no indication of their relative merits.

- What is the best volume of water (and ratio of animal:water) to use?

- How much radioactive steroid must one add in order to incorporate enough in the tissue so that the products can be identified?

- What is the optimum timing for exposure? Do animals need to be exposed for days as opposed to hours?

- In terms of quantifying the steroid by-products in the tissue, is there a convenient way of separating free and esterified steroids? All previous immunoassay studies have quantified esterified steroids by a process of subtracting the value for free steroid from the value for free + esterified steroid [i.e. no attempt to separate them], while previous studies with radioactive tracers have used some form of chromatography to separate free from esterified steroids; while these methods all work, they are time-consuming and / or limited in their general applicability.

- What are the optimum conditions for extraction of free and esterified steroids from molluscan tissues?

Phase 2 – A key fact in the ability of the animals to form a bond between a fatty acid and a steroid is that the steroid must have a reactive hydroxyl group on it. In the case of T and E_2, this appears to be the hydroxyl group on Carbon-17. However:

- What happens when the animals encounter other types of vertebrate steroids? (E_2 and T are not the only vertebrate steroids in the environment!). How quickly are these other steroids absorbed from the water and then how easily are they esterified?

- What about P, that has no hydroxyl group by which it can be esterified?

- What about the ubiquitous fish maturation-inducing steroid, 17α,20ß-dihydroxypregn-4-en-3-one, that has an available 20B-hydroxyl group (that could possibly also be recognised by the esterifying enzyme(s)?

- What about the stress steroid, cortisol, which has a potentially easily esterifiable hydroxyl group on C21? (NB. this steroid is particularly abundant in water)

- What about EE_2 that has an ethinyl group attached to C17, does this group hinder esterification of the 17-hydroxyl group?

Phase 3 – Even for T and E_2, there are important things that are not known:

- Are they metabolised and are the metabolites esterified as well?
- Exactly how long does it take for the animals to depurate them?
- What is the capacity of molluscs for absorbing and esterifying these steroids?
- Are there differences between males and females?*
- Are there differences due to reproductive stage, temperature, feeding etc?*
- Which particular tissues are involved with esterification?*
- Are there any implications for human health through eating shellfish contaminated with sex steroids?*

Within the time scale of this project, all these questions have been tackled experimentally except those marked with an asterisk.

Table 2: Steroids that have been found in mollusc tissue.

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
(Goto et al., 2012)	Predatory sea snail, *Thais clavigera*	Wild	Gonad	GC-MS	T A E_2 E_1	Identification only.	-	-	-	-	-
(D'Aniello et al., 1996)	Common octopus, *Octopus vulgaris*	Wild (males only)	Reprocutive tissue, highly metabolic tissue & haemolynph.	RIA, EIA & HPLC	E_2 T P	100-1000 100-6000 100-5000	-	-	-	-	-
(Liu et al., 2015)	Bivalve, *Atrina pectinata*; common orient clam, *Meretrix lusoria*; bivalve, *Trisidos kiyoni*; oyster, *Crassostrea rivularis*	Shellfishery	Soft tissue	RRLC-MS/MS	Androsta-1,4-diene-3,17-dione 4-Androstene-3,17-dione 17α-Boldenone 17β-Boldenone Epi-androsterone Methyl T 19-NorT T 17α-Trenbolone 17β-Trenbolone F Cortisone Ethynyl T MedroxyP Norgestrel P	<LOD-2800 200-2200 <LOD-2500 <LOD-1400 <LOD <LOD-900 <LOD-1500 <LOD-1100 <LOD-600 <LOD-200 <LOD <LOD <LOD-1100 <LOD-1100 <LOD-3300 <LOD-2500	-	-	-	-	-
(Zhu et al., 2003)	Blue mussel, *Mytilus edulis*	Wild (tank)	Gonad Haemolymph	HPLC RIA Q-TOF-MS	E_2 E_2	40000 50000	-	-	-	-	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
(Casatta et al., 2015)	Manila clam, *Ruditapes philippinarum*	Wild	Soft tissue	UPLC-MS/MS	E_1 E_2 E_3 EE_2	<LOD <LOD <LOD <LOD	-	-	-	-	-
(Carreau and Drosdowsky, 1977)	Common cuttle-fish, *Sepia officinalis*	Wild & lab culture	Haemolymph Soft tissue	RIA	T E_2 DHT T	150 10 0 10000-20000	-	-	-	♂=♀ ? ♂=♀ ♂>♀	-
(Avila-Poveda et al., 2013)	Mexican four-eyed octopus, *Octopus maya*	Wild	Gonad	RIA	P T	4000-10000 300-700	-	-	-	♂<♀ ♂>♀	-
(Alvarez-Muñoz et al., 2014)	Peppery furrow shell, *Scrobicularia plana*	Wild	Soft tissue	GC-MS	DHT	4.8 ng fraction^{-1}	-	-	-	♀ <LOD	-
(Zabrzańska et al., 2015)	Foolish mussel, *Mytilus edulis trossulus*	Wild	Gill Gonad	LC-MS	E_2 T E_1 E_3 E_2 T E_1 E_3	5000 7000-9000 1000 1000 5000 1000-5000 1000 1000	-	-	-	♂=♀ ♂=♀ ♂=♀ ♂=♀	-
(Reis-Henriques et al., 1990)	Blue mussel, *Mytilus edulis*	Wild	Soft tissue	TLC & GC-MS RIA	E_2 E_1 E_3 T Ad P DHT androsterone androstanediol	20-50 50-800 <LOD 200-700 800-4000 300-5000 Identified only	-	-	-	♂<LOD ♀<LOD All other s ♂=♀	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
(Bose et al., 1997)	Giant African snail, *Achatina fulica*	Wild	Haemolymph Gonad Albumin gland	RIA	E_2 T P Ad F	<LOD-180000 <LOD-60000 <LOD-50000 <LOD-4000 <LOD	-	-	-	♂<♀ ♂>♀ ♂>♀ ♀ only	-
(Cheour et al., 2014)	Sea snail, *Osilinus articulatus*	Wild	Gonad-viscera complex	RIA	E_2 T	20-80 20-90	♂Feb ♀Feb ♂Jan ♀Jan &June	Rest. & early gam. Rest. & early gam. Rest. Rest. & ripe	7 3 4.5 2	♂<♀ ♂>♀	-
(Gooding and LeBlanc, 2004)	Eastern mudsnail, *Ilyanassa obsoleta*	Wild (tank)	Soft tissue	RIA	T T T (ester)	2000-45000 1000-10000 20000-85000	♂Nov&Apr ♀Nov&Apr	Mature Mature	6-9 3-5	♂>♀	 2-3-fold decrease in T ester after exposure to nonylphenol.
(Sternberg et al., 2008)	Eastern mudsnail, *Ilyanassa obsoleta*	Wild	Gonad-viscera complex	RIA	T T (total) E_2 E_2 (total)	<LOD-50000 <LOD-300000 <LOD-1500 <LOD-8000	♂Sep-Nov ♀Sep-Nov ♂Sep ♀Aug-Sep ♂Aug-Sep ♀ Apr, Jul-Aug & Oct ♂ Aug ♀ Jul & Oct	Dev Residual Dev Residual Dev Full dev. & residual Dev Residual	4 4 10 4 2 2 3 4	♂>♀ ♂>♀	-
(Le Guellec et	Garden snail, *Helix*	Lab culture	Gonad	RIA	T	8000-25000	-	Juv>Adu	3	-	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
al., 1987)	*aspersa (Cornu aspersum)*		Haemolymph	RIA	DHT A P T A P	2000-3000 6000-20000 2500-4000 200-300 <LOD-18 1000-6000		Juv<Adu Juv<Adu Juv<Adu Juv<Adu Juv<Adu Juv<Adu	1.5 3 1.5 1.2 - 5		
				HPLC & GC	Androsterone, DHEA, 3a-androstanediol, E_1, E_2, E_3	Identified only					
(Gust et al., 2011b)	New Zealand mudsnail, *Potamopyrgus antipodarum*	Wild	Soft tissue	RIA	E_2 (total) T (total)	100-200 pg ind^{-1} 2-10 pg ind^{-1}	June-Aug lowest in Nov-Jan & Oct	Reproductive Resting	2 3	-	-
(Dévier et al., 2010)	Blue mussel, *Mytilus edulis*	Wild	Digestive gland	GC-MS	P Pn T A E_1 E_2 DHT DHA	400-9000 500-9000 <LOD <LOD <LOD <LOD <LOD <LOD	♂Jun, Apr & Apr ♀Jun, Apr & Apr ♂May-Jun & Jul-Sep ♀May-Jun & Jul-Sep	-	2-7 3-4 6-8 3-4	♂=♀ ♂=♀	-
(Martínez-Pita et al., 2012)	Mediterranean mussel, *Mytilus galloprovincialis*	Wild	Haemolymph	ELISA	T	3-5	♂no ♀no			♂>♀	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
					E_2 P	2-12 300-600	♂no ♀no ♂Oct ♀Oct&Dec	 Post spawn Post spawn & active	 2.5 20	♂=♀ ♂=♀	
(David et al., 2008)	Mediterranean mussel, *Mytilus galloprovincialis*	Wild	Soft tissue	ELISA	E_2	1000-5000 (dry weight)	♂April-May ♀April-May	Spawn & rest Spawn & rest	2.5 2.5	♂=♀	-
(David et al., 2010)	Mediterranean mussel, *Mytilus galloprovincialis*	Shellfishery	Whole animal	Reporter-gene assay (MELN cell line)	E_2 (EEQ)	200-1500 pg g^{-1} dw	♂no ♀no	-	-	♂>♀	Sediments & water showed that there was low estrogenic contamination. No reference site for comparison.
(Kaloyianni et al., 2005)	Mediterranean mussel, *Mytilus galloprovincialis*	Wild	Mantle/gonad complex	RIA	E_2	<LOD-100	Sept-Oct & Mar-May	-	4-5	-	-
(Reis-Henriques and Coimbra, 1990)	Blue mussel, *Mytilus edulis*	Wild	Gonad Soft tissue	RIA GLC	P P	1000-4000 5000-40000	Jul ♂Jun & Oct ♀Jun & Oct	Spawning Spawning	2 6 5	 ♂<♀	-
(De Longcamp, 1974)	Blue mussel, *Mytilus edulis*	Wild	Mantle/gonad	RIA	E_1 E_2	30000-45000 4000-5000	-	E1 & E_2 in spent & restoration only	-	♂=♀ ♂=♀	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
					T	1400-43000		Gametogenesis & spent/restoration		♂>♀	
(Knigge et al., 2014)	Zebra mussel, *Dreissena polymorpha*	Wild (female only)	Gonad	EIA	E_2	13000	♀Jun, Aug & Feb,	Reabsorbing, resting & early dev.	5-6	-	-
(Liu et al., 2014a)	Chinese scallop, *Chlamys farreri*	Wild	Gonads	ELISA	E_2 T	50-700 100-500	♂Mar ♀ May & Sept ♂ May & Sept ♀Jab-Feb & Nov-Dec	Grow Mat. & restoration Mat. & restoration Proliferative & resting	2 2-3 2 2	♂<♀ ♂>♀	-
(Zheng et al., 2014)	*Chinese scallop, Chlamys farreri*	Wild	Gonad	UPLC-MS/MS	E_1 E_2 T	<LOD-3000 <LOD-30000 <LOD-4000	 ♂ May ♀June ♂June ♀May &June	-	 4 30 6 4	♂=♀ ♂<♀ ♂=♀	-
(Osada et al., 2004)	Yesso scallop, *Patinopecten yessoensis*	Wild	Gonad	HPLC	E_2 E_1	1500-5000 <LOD-1000	♂Feb-Apr ♀Feb-Apr ♂no	-	2 2	♂=♀ ♂=♀	-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
							♀no				
(Matsumoto et al., 1997)	Pacific oyster, *Crassostrea gigas*; Yesso scallop, *Patinopecten yessoensis*	Wild	Gonad	HPLC	E_2 E_1 E_3	<LOD-1500 100-400 <LOD	♂no ♀Feb (P. yess.) & Jul (C. gigas) ♀no	-	 1.5 & 2	♂<♀	-
(Ni et al., 2013)	Portuguese oyster, *Crassostrea angulata*	Wild	Gonad	ELISA	E_2 T	100-300 10-30	♂no ♀Ripe ♂Ripe ♀no	 Ripe Ripe	 3 2	♂<♀ ♂>♀	-
(Siah et al., 2002)	Sand gaper, *Mya arenaria*	Wild	Gonad	ELISA LC-MS	P P	3000-5000 Identification only	♂Sep ♀Sep	Ripe Ripe	1.5 1.5	♂=♀	-
(Gauthier-Clerc et al., 2006)	Sand gaper, *Mya arenaria*	Wild	Gonad	ELISA	E_2 T	200-400 30-50	♂no ♀no ♂no ♀spawning	 spawning	 1.5	♂<♀ ♂<♀	-
(Ketata et al., 2007)	Grooved carpet shell, *Ruditapes decussatus*	Wild	Gonad	RIA	E_2 T P	10-200 40-400 500-2500	♂Feb ♀Apr ♂Nov ♀Sep ♂Feb ♀No	Spent Prev Spawning Spawning Spent	3.5 20 2 8 5	♂=♀ ♂=♀ ♂=♀	-
(Negrato et al., 2008)	Manila clam, *R. philippinarum*	Wild	Soft tissue	RIA	E_2 T P	100-350 100-200 600-1600	♂no ♀July ♂no ♀July ♂no ♀July	 Spawning Spawning Spawning	 2 2 1.5	♂= ♀ ♂= ♀ ♂= ♀	-

Study	Species	Source	Tissue	Method	Steroid	Concentration ($pg\ g^{-1}$)	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
(Yan et al., 2011)	Razor clam, *Sinonovacula constricta*	Wild	Gonad	ELISA	E_2 T	200-800 20-120	♂no ♀ Aug-Sep ♂ Oct-Nov ♀no	 Early gameto. / ripe Early spawn. / restoration	 3.5 2	♂< ♀ ♂> ♀	-
(Liu et al., 2008)	Cockle, *Fulvia mutica*	Wild	Gonad	ELISA	T E_2	-20000-80000 8000-30000	May-Jun & Nov-Dec May-Jun & Nov-Jan	Spent & undif./dev Spent & undif./dev	4 2-3	-	-
(Di Cosmo et al., 2001)	Common octopus, *Octopus vulgaris*	Wild (female only)	Gonad Haemolymph	RIA	E_2 P E_2 P	20-150 20-200 <LOD <LOD	♀May ♀May	-	9 10	-	-
(Liscio et al., 2009)	Painter's mussel, *Unio pictorum*	Wild	Gills Mantle Other tissue	LC-MS/MS	E_1 E_2 EE_2	50-1500 50-50000 <LOD	-	-	-	-	E_2 only increased in mantle at one influent site. E1 generally increased in both influent & effluent sites.
(Lazzara et al., 2012)	Zebra mussel, *Dreissena polymorpha*	Wild (tank)	Soft tissue	RIA	T T (ester) E_2 E_2 (ester)	80-140 3400-4400 40-100 5800-12800	-	-	-	-	Fluoxetine increased E_2 esters in high exposure dose.
(Riva et al., 2010)	Zebra mussel, *Dreissena polymorpha*	Wild (tank)	Soft tissue	RIA	E_2 E_2 (total) T T (total)	2000-4000 6000-10000 100-150 5000-10000	-	-	-	Prestudy showed ♂=♀ so they did	Exposure to NP lowered T (total) by 2-fold. No effect

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
										not sex during NP exposure.	on E_2.
(Peck et al., 2007)	Zebra mussel, *Dreissena polymorpha*	Wild (tank)	Soft tissue	YES (EEQ) GC-MS	E_2 E_2 (ester)	<LOD-6700 7000-300000 70-85% of EEQ was E_2	-	-	-		E_2 was higher in polluted sites than references sites (3-10-fold).
(Janer et al., 2005b)	Mediterranean mussel, Mytilus galloprovincialis	Shellfishery (tank)	Soft tissue	RIA	T T (total) E_2 (total – control)	1000-1800 1000 1000-2000	-	-	-	-	E_2 exposure caused no change in T levels.
(Fernandes et al., 2010)	Mediterranean mussel, *Mytilus galloprovincialis*	Wild (tank)	Soft tissue	RIA	E_2 E_2 (total) T (control) T (total - control)	1000 8000-12000 200 2000	-	-	-	-	No change in E_2 (free/total) after exposure to T.
(Dimastrogiovanni et al., 2015)	Mediterranean mussel, *Mytilus galloprovincialis*	Shellfishery (tank)	Digestive gland Mantle/gonad	RIA	E_2 (total) T (total) P (total – contol)	4000-6000 1000-6000 10000	-	-	-	-	After P exposure: no difference in E_2; 3-fold increase in T at high dose.
(Lavado et al., 2006b)	Blue mussel, *Mytilus edulis*	Wild (tank)	Gonad Gill/mantle	RIA	T T (ester) E_2 E_2 (ester)	150-3000 3000-12000 20-2500 3000-11000	-	-	-	-	A combination of crude oil, alkylphenols & PAHs increased T & E2 esters in

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
											the gonad; & T in the gill/mantle.
(Ronan and McHugh, 2013)	Edible mussel, *Mytilus* spp.	Wild	Soft tissue	LC-MS/MS	E_1 E_2 EE_2	<LOD <LOD <LOD	-	-	-	-	No steroids detected in any mussels from 3 locations.
(Halem et al., 2014)	Ribbed mussel, *Geukensia demissa*	Wild	Gills	EIA	E_2 T P	15-90 25-150 15-100	-	-	-	♂>♀ ♂>♀ ♂=♀	T (3-fold) & P (5-fold) were lower at the site with low levels of dissolved oxygen; whereas E_2 was 2-fold higher.
(Mouneyrac et al., 2008)	Peppery furrow shell, *Scrobicularia plana*	Wild	Gonad	ELISA	E_2 T P	100-400 100-300 1000-2000	-	-	-	♂>♀ ♂>♀ ♂<♀	E_2 & P were higher in the reference site than polluted site. T in males was lower at polluted sites.
(Tian et al., 2015)	Chinese scallop, *Chlamys farreri*	Wild (male only) (tank)	Gonad Haemolymph	ECLIA	E_2 T P E_2 T	80-100 200-450 100-600 15 190	-	-	-	-	Gonad: E_2 increased 1.2-fold, T decreased 1.5-fold & 4-fold P increase after exposure to B[a]P (non-monotonic). Haemolymph:

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
											E_2 increased on d3 & decreased d10; no change in T except on d10.
(Tian et al., 2013)	Chinese scallop, *Chlamys farreri*	Wild (mature females only; tank)	Gonad Haemolymph	ECLIA	E_2 T P E_2 T P	80-140 150-350 100-250 15-20 200 <LOD	-	-	-	-	After exposure to B[a]P: only T in gonads decreased in a dose-responsive manner (on d10 only). In haemolymph there was no change in T or E_2.
(Morcillo and Porte, 2000)	Grooved carpet shell, *Ruditapes decussatus*	Wild	Soft tissue	RIA	E_2 T	5000-20000 110000-160000	-	-	-	-	TBT pollution: E_2 decreased 4-fold in five weeks (monotonic) & T increased 1.3 –fold on week five only (non-monotonic).
(Morcillo and Porte, 1998)	Grooved carpet shell, *Ruditapes decussatus*	Wild (tank)	Soft tissue (without digestive gland)	RIA	E2 T	<LOD 100-600	-	-	-	-	T increased 6-fold at the lowest TBT; no dose-response.
(Giusti et al., 2013)	Great pond snail, *Lymnaea stagnalis*	Lab culture	Soft tissue	RIA	T T (ester)	200-500 100-400	-	-	-	-	1.5-fold increase of T in cyproterone

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
											acetate treatment.
(Gust et al., 2011a)	New Zealand mudsnail, *Potamopyrgus antipodarum*	Wild & lab culture	Soft tissue	RIA	T (total) E_2 (total)	10-70 pg ind^{-1} 1-3 pg ind^{-1}	-	-	-	-	Up to a 7-fold increase in T & 3-fold increase in E_2 over 28 days (at both reference & contaminated sites).
(Gust et al., 2010b)	New Zealand mudsnail, *Potamopyrgus antipodarum*	Wild & lab culture (tank)	Soft tissue	RIA LC-MS/MS	T (total) P (total) E_2 (total) T P E_2	500-3000 300-1000 200-600 600-1800 500-1200 <LOD	-	-	-	-	Total T & E_2 levels increased after 28 d in water (regardless of location). P remained the same.
(Gust et al., 2014)	New Zealand mudsnail, *Potamopyrgus antipodarum*; European valve snail, *Valvata piscinalis*	Wild & lab culture	Soft tissue	RIA	E_2 T E_2 T	2-20 pg ind^{-1} 10-80 pg ind^{-1} 35-45 pg ind^{-1} 100-250 pg ind^{-1}	-	-	-	-	*P. antipodarum:* T & E_2 increased after 14 days exposure at all sites; sites closest to sewage treatment plant accumulated 4-fold E_2 & T. *V. piscinalis*: 2-fold increase in

Study	Species	Source	Tissue	Method	Steroid	Concentration ($pg\ g^{-1}$)	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
											T sites close to treatment plant.
(Janer et al., 2006b)	Giant ramshorn snail, *Marisa cornuarietis*	Lab culture	Digestive gland-gonad complex	RIA	E_2 E_2 (total) T T (total)	30-200 1000-67000 130-2900 1800-42000	-	-	-	♂>♀ ♂>♀	T & E_2 esters lowered over 100 d of TBT exposure.
(Gooding et al., 2003)	Eastern mudsnail, *Ilyanassa obsoleta*	Wild (tank)	Soft tissue	RIA	T T (ester)	2000-3000 22000-27000	-	-	-	-	TBT exposure caused a 2-fold increase in % free T at high dose only.
(Morcillo and Porte, 1999)	Purple dye murex, *Bolinus brandaris*	Wild	Digestive gland/ gonad	RIA	T E_2	700-1200 3-300	-	-	-	♂=♀ ♂>♀	T was higher in males in area of high imposex incidence.
(Santos et al., 2005)	Dog whelk, *Nucella lapillus*	Wild (females only)(tank)	Soft tissue	RIA	E_2 T T (ester)	600-1500 1000-1600 10000-14000	-	-	-	-	Free T increased 1.5-fold after TBT exposure; no change in T esters. Free E_2 increased 1.5-fold in TBT, CPA & TBT/CPA exposures.
(Spooner et al., 1991)	Dog whelk, *Nucella lapillus*	Wild (females only)(tank)	Soft tissue	RIA	E_2 T P	10-500 1500-8000 1200	-	-	-	-	No change in E_2 & P after TBT exposure; T increased 1.5-

Study	Species	Source	Tissue	Method	Steroid	Concentration (pg g^{-1})	Seasonality			Differences between sexes?	Contamination effects?
							Peak	Stage	Fold increase		
											4–fold (non-monotonic)
(Bettin et al., 1996)	Dog whelk, *Nucella lapillus*; netted dog whelk, *Hinia reticulata*	Wild (females only) (tank)	Soft tissue	RIA	T (*N. lapillus*) T (*H. reticulata*)	800-1800 600-1800	-	-	-	-	TBT exposure caused monotonic response in T (max 2–fold increase) at high dose after 5 months only. And 2-fold difference in T between imposex stage 0 & 4.
(Huerta et al., 2015)	Limpet, *Ancylus fluviatili*	Wild	Soft tissue	UPLC-MS/MS	E_1	<LOD	-	-	-	-	Nonylphenol accumulated; higher downstream of waste water treatment plant.

Chapter 2

Method Development

2.1 Introduction

Many studies confirm the presence of potent vertebrate sex steroids such as testosterone (T) and 17ß-oestradiol (E_2) in molluscs (Goto et al., 2012; Liu et al., 2015; Peck et al., 2007; Reis-Henriques and Coimbra, 1990). There are also many studies purporting to show that these steroids cause biological effects when administered to molluscs. However, as has been reviewed by Scott (2013), most of these bioassay studies are flawed (e.g. single experiments only, no concentration-response curves, confounding factors not corrected for, not independently validated). Besides, there are also many studies (including some that have never been published) that report no effects. A protein that is clearly homologous to the vertebrate oestrogen nuclear receptor (ER) is present in mollusc genomes. However, the molluscan ER (mER) is not able to bind to oestrogens (Bannister et al., 2007; Thornton et al., 2003); and furthermore, there is no evidence for the presence of nuclear receptors for T and progesterone (P) (Sternberg et al., 2008).

Concerning the ability of molluscs to synthesise vertebrate steroids *de novo*, there are also publications that claim this is happening. However, as with the bioassay data, these also are subject to several flaws (e.g. non-definitive identification of steroid metabolites) and cover only limited parts of the biosynthetic pathway). These parts invariably relate to transformations that require the simple addition or subtraction of two hydrogen atoms from the steroid molecule [e.g. pregnenolone (Pg) → progesterone (P); androstenedione (Ad) → T; T → 5α-dihydrotestosterone (5α-DHT] and are probably catalysed non-specifically by oxido-reductases that are unrelated to vertebrate steroid synthesis (reviewed by Scott, 2012). There are three specific P450 enzyme complexes in vertebrates that are absolutely critical for the biosynthesis of steroids (catalysing removal of the side chain of cholesterol, removal of the side-chain of P and, conversion of T to E_2). The genes that code for these enzymes in

vertebrates have not yet been found in the genomes of any of the thus far sequenced molluscan species.

In spite of the lack of evidence for endogenous steroid biosynthesis, the presence of receptors or of biological effects (see Chapter 1 for a critical review), the presence of vertebrate sex steroids (E_2, T and P especially) in tissues of gastropods and bivalves is indisputable. So where do they come from? The most likely possibility is that they come from outside the animals (i.e. by absorption from the environment or by ingestion). There are in fact several lines of evidence to support this theory, including a) EE_2 has been definitively identified in the tissues of molluscs caught in the wild (this is a man-made compound and it is inconceivable that the animals would be able to make it themselves) (Dévier et al., 2010; Goto et al., 2012; Lu et al., 2001); b) there is inevitably a linear increase (but never a decrease) over time in the amounts of steroid found in laboratory animals that are caged in the wild (Gust et al., 2010a; Gust et al., 2011a; Gust et al., 2014); c) it has been directly demonstrated that bivalve molluscs are able to take up radioactive T and / or E_2 from the water (Gooding and LeBlanc, 2001; Janer et al., 2004a; Janer et al., 2005b; Labadie et al., 2007; Peck et al., 2007); and d) it is now well known that steroids are widely distributed in the environment (coming not just from sewage treatment works, but from livestock, wild-living vertebrates and even human skin) (Matthiessen et al., 2006; Scott, 2012).

Until relatively recently, most studies on molluscs have quantified only those steroids that are 'free' (i.e. they are not covalently linked to any other compounds). It is well-known in vertebrates that steroids can be conjugated to sulphate and glucuronide groups which are far more water-soluble than free steroids (Scott and Vermeirssen, 1994). Enzymatic or chemical treatments are generally also used to turn these conjugated steroids into free steroids so they can subsequently be measured. It is known that such conjugates are present in molluscs, but they have been little-studied. Ronis & Mason (1996) found water-soluble steroid conjugates in the periwinkle, *Littorina littorea*. After injecting animals with radiolabelled T, they found a portion of the activity in the aqueous phase, and when they extracted and digested this phase with an enzyme

preparation containing ß-glucuronidase and sulphatase, 60 to 80 % of the radioactivity was converted to free steroid. Another study, on the sea slug, *Clione antartica*, found that exposing them to radiolabelled P led to the production of several water soluble conjugates (both sulphates and glucuronides; up to 9 % of the total activity) (Hines et al., 1996).

In their study, Hines *et al.* (1996) also identified small amounts of radioactive metabolites of Ad and P that were soluble in organic solvents but appeared to be conjugated to fatty acids (i.e. they appeared to be 'steroid esters'). Subsequent studies, on other species, found that steroid esters (especially of T and E_2) actually accounted for the majority of metabolites formed in some molluscs. Gooding & LeBlanc (2001), for example, exposed the eastern mudsnail, *Ilyanassa obsoleta,* to radiolabelled T over a period of 8 h. A high proportion of this steroid, (around 75 %) was taken up, and 70 % of this was transformed into highly non-polar metabolites. Enzymatic hydrolysis and then chromatography of these metabolites tentatively revealed the presence of T (i.e. the parent compound) and free fatty acids (identified using standards). A later study directly demonstrated the *in vitro* formation of E_2 and dehydroepiandrosterone fatty acid esters by digestive gland and gonad microsomes of the Eastern oyster *Crassostrea virginica* (Janer et al., 2004b). The same group also investigated metabolism of E_2 in the Mediterranean mussel *Mytilus galloprovincialis* at different concentrations over seven days. They found that over 78 % of E_2 had been esterified (Janer et al., 2004a). E_2 esters formed by bivalves were investigated further by Labadie *et al.* (2007) by exposing the blue mussel, *Mytilus edulis,* to [^{14}C]-E_2 for 13 days and [^{14}C]-oestrone ([^{14}C]-E1) for 8 days. High performance liquid chromatography (HPLC) revealed that the major [^{14}C]-E_2 metabolite had the same retention time as E_2 after hydrolysis. Exposure to [^{14}C]-E_1 yielded a steroid ester too. However, after hydrolysis it was revealed that the free steroid was not E_1 but E_2. They concluded that E_1 is taken up, reduced to E_2 and subsequently conjugated to fatty acids. The zebra mussel, *Dreissena polymorpha*, was proven to metabolise most (over 90 % of radioactivity) of E_2 into fatty acid ester(s) after exposure (Peck et al., 2007). On the basis of the above studies, it is evident that in order to obtain an idea on levels of vertebrate steroids in molluscs (or investigate

their uptake from the environment) measuring the concentrations of free steroids alone is not enough; levels of esterified steroids must be measured too. In this study, using blue mussels (where possible using just *M. edulis,* but sometimes including *M. galloprovincialis* or hybrids) as a test species and E_2 as the test steroid, the following questions have been addressed:

- What is the best way to carry out steroid uptake experiments? (how much water? how many animals? how much radioactive steroid? how long should the animals be exposed? do they need to be fed at the same time? how can it be done in order to minimise contamination of equipment and personnel?)

- What is the best way of extracting not just free but also esterified and, potentially glucuronidated and sulphated steroids from tissues?

- What is the best way to separate free from esterified steroids? (All previous studies involving immunoassay have quantified esterified steroids by splitting the extract into two, hydrolysing one half, assaying them both and then working out the amount of ester by subtracting the value in the hydrolysed half from that in the non-hydrolysed half; studies involving radioactive tracers have used some form of chromatography to separate free from esterified steroids; although chromatography works, it is time-consuming and expensive).

- Does metabolism (i.e. conversion to other steroids) play any role in the rate of uptake and esterification of vertebrate steroids by molluscs? (Previous studies make little or no mention of this possibility)

- Is the rate of uptake and esterification affected by the actual amount of steroid to which the animals are exposed?

2.2 Materials and methods

2.2.1 Chemicals

17β-[2,4,6,7,16,17-^{3}H]-oestradiol ([^{3}H]-E_2) was purchased from American Radiolabeled Chemicals, Inc. (101 ARC Dr. St. Louis, MO 63146 USA). 'Cold' 17β-oestradiol was obtained from Sigma-Aldrich Company Ltd (Dorset SP8 4XT, UK) and all other chemicals were purchased from Fisher-Scientific UK Ltd. (Loughborough LE11 5RG, UK).

Water used for laboratory exposures was filtered (50 µm) sea water and water used for all other purposes was reverse osmosis-treated water unless stated otherwise.

2.2.2 Laboratory exposures of *Mytilus* spp. to radiolabelled 17β-oestradiol

2.2.2.1 Study 1

2.2.2.1.1 Collection and acclimation

M. edulis were obtained from The Retreat, Brancaster Staithe, Norfolk – a catchment which holds a class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). The mussels were harvested in March 2014, transported in a cool-box overnight and immediately placed in a flow-through system of filtered sea water. Ninety animals (ranging between 42.92 to 52.88 mm, mean 48.7 mm) were selected and cleaned. Twenty of the animals were used for pre-study condition index analysis and the rest were glued to glass rods (five per rod; Figure 6). In order to acclimate the animals, individual rods were placed vertically in aerated cylindrical glass tanks with 13 L of filtered sea water at 16 ± 1 °C with a 16:8 h light:dark photoperiod for six days prior to exposure. The animals were fed Shellfish Diet® 1800 (a mix of *Isochrysis* spp., *Pavlova* spp., *Tetraselmis* spp., *Thalassiosira weissfloggi* and *Thalassiosira pseudonana*) following manufacturer's instructions and the water was changed daily.

2.2.2.1.2 Exposure

A total of 50 mussels (split between ten tanks) were exposed to a nominal concentration of 0.7 µCi L-1 (1.36 ng L-1) of [^{3}H]-E_2 for 48 h. The water was then changed and the radioactivity refreshed and the animals left for another 48 h. There were three sets of controls: a water control with 10 unexposed animals divided equally between two tanks, a solvent control with 10 animals in two tanks exposed to carrier only (100 µl ethanol) and a sorption control tank with radioactive steroid but no animals. In order to monitor uptake, water samples (1 mL) were taken at regular intervals (0, 3, 6, 10, 21, 33, 48; 0, 3, 19, 27, 43, 48 h) from all tanks and immediately mixed with 7 mL scintillation fluid (Prosafe+, Meridian Biotechnolgies, UK) for counting. Quench correction was carried out using an external radioactive source. At the end of the exposure, 10 mussels were removed and stored frozen at -20 °C for later analysis. All other mussels, including those from the solvent control, were placed in a shallow tank with a continuous flow of sea water.

2.2.2.2 Study 2 and 3

2.2.2.2.1 Collection and acclimation

M. edulis were obtained from 'Deepdock Mussels' in the Menai Strait, Wales – a catchment which holds a long term class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). The animals were harvested in April 2014, transported in a cool-box overnight and immediately placed in a flow-through system of filtered sea water. Ninety animals (ranging between 45.72-57.81 mm; mean 51.37 mm) were selected and cleaned. Twenty of the animals were used for condition index analysis and the rest were placed in individual nets as follows: 30 for Study 2, 30 animals for Study 3, 10 animals for solvent controls. In order to acclimate them, five animals were suspended in aerated glass tanks with 7 L (as opposed to 13 L in the first experiment) of filtered sea water at 16 ± 1 °C with a 16:8 h light:dark photoperiod for six days prior to exposure. The water was changed daily and the animals were fed a combination of live algae: *Isochrysis* spp. and *Tetraselmis* spp. were added three times a day at a concentration of 95 cells µl^{-1}. The amount of feed required was

calculated as the equivalent to 2.5 % of mean expected mussel dry weight. The concentration range of 50-100 cells μl^{-1} and particle size were deemed ideal for feeding with minimal production of pseudofaeces (Kiørboe and Møhlenberg, 1981; Sprung and Rose, 1988). The ratio *Isochrysis*:*Tetraselmis* was 1:3 in order to achieve the appropriate mass and concentration.

2.2.2.2.2 Exposure

Study 2 consisted of only one 48 h exposure to [^{3}H]-E_2. There were two treatments: 15 animals (i.e. three tanks) were exposed to a nominal concentration of 1.3 µCi L^{-1} (2.53 ng L^{-1}) of [^{3}H]-E_2 without feed (treatment A) and 15 animals were exposed to the same nominal concentration of [^{3}H]-E_2 but were fed daily (treatment B – see 2.2.2.2.1 for algal concentration). There were three sets of controls: a solvent control of 10 animals in two tanks exposed to the carrier (100 µL ethanol), a sorption control of one tank with empty nets for treatment A, and two sorption control tanks with empty nets and the same concentration of algae as treatment B. The latter had algae added at time 0 h and the equivalent amount of water was added when mussels were fed to achieve the same concentration of cells. Water (1 mL) samples were taken (0, 1, 3, 5, 8, 11.5, 21, 24, 27, 31, 34, 46 and 48 h) from all tanks and immediately mixed with scintillation fluid for counting. After exposure, the mussels were frozen at -20 °C for analysis. Data for Study 2 treatment B was volume adjusted to 7 L.

Study 3 consisted of a 48 h exposure of three treatments, all of which were exposed to the same nominal concentration of 1.3 µCi L^{-1} (2.53 ng L^{-1}) of [^{3}H]-E_2. Treatment A was as described above ([^{3}H]-E_2 exposure only); treatment C and D were exposed to 7.1 ng L^{-1} and 35.7 ng L^{-1} E_2 respectively as well as [^{3}H]-E_2. Water (1 mL) samples were taken (0, 1, 3, 5, 8, 11.5, 21, 24, 27, 31, 34, 46 and 48 h) from all tanks and immediately placed in scintillation fluid for counting. After exposure, the mussels were frozen at -20 °C, but were not analysed due to a lack of evidence that the addition of cold E_2 had had any noticeable effect on the rate of uptake of the radioactivity. The tissues from some of these animals were used instead for development and optimisation of the extraction and separation procedures.

2.2.2.3 Study 4

2.2.2.3.1 Collection and acclimation

Mussels from a mixed population of *M. edulis* and *M. galloprovincialis* were collected from Portland Harbour in May 2014. The nearby northeast Portland Harbour breakwater is a catchment holding a long term class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). They were transported to the lab in a cool-box and immediately placed in a flow-through system of filtered sea water and fed Shellfish Diet® 1800 following manufacturer's instructions. Forty animals were selected (ranging between 50.49 to 59.39 mm, mean 55.47 mm), cleaned, and placed in pairs in 1 L jugs lined with polyethylene bags (Figure 6) with 800 mL of aerated, filtered sea water (two animals jug^{-1}). Temperature was not controlled and ranged between 17.7 to 21.2 °C.

2.2.2.3.2 Exposure

This study was comprised of four treatments that all received the same amount of $[^3H]$-E_2 - 1.14 µCi L^{-1} (2.21 ng L^{-1}) - but varying amounts of unlabelled (cold) E_2 (Table 3). Each treatment had five replicates (with animals) and one sorption control (with steroid but no animals). All vessels were spiked with the same amount of carrier solvent (40 µL ethanol). During exposure, water samples were taken (0, 4, 8, 11, 24, 36 and 48 h) from each vessel and immediately placed in scintillation fluid for counting.

Table 3: Details of Study 4.

Treatment group	E_2 nominal concentration (ng L^{-1})	$[^3H]$-E_2 nominal concentration (ng L^{-1})
A	0	2.21
B	250	2.21
C	2500	2.21
D	25000	2.21

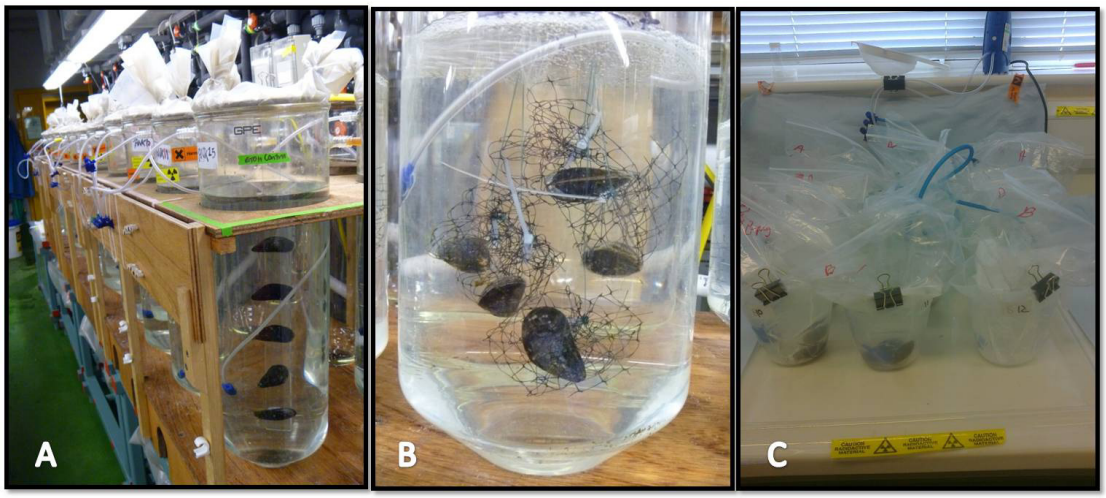

Figure 6: Experimental designs used for radioactive exposures. Mussels glued to rods for Study 1 (A), mussels suspended in individual nets for Studies 2 and 3 (B) and mussels in jugs lined with bags for Study 4 (C).

2.2.2.4 Study 5

2.2.2.4.1 Collection and acclimation

Mussels from a mixed population of *M. edulis* and *M. galloprovincialis* were collected from Portland Harbour in October 2014. The nearby northeast Portland Harbour breakwater is a catchment holding a long term class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). They were transported to the lab in a cool-box and immediately placed in a flow-through system of filtered sea water. Fifty animals were selected (ranging between 47.26 to 69.63 mm, mean 56.34 mm) and cleaned. Ten animals were used for pre-study condition index analysis. In order to acclimate the animals, five mussels were placed in an aerated bucket lined with a polyethylene bag and filled with 2 L of filtered sea water at 16 ± 1 °C with a 16:8 h light:dark photoperiod for five days prior to exposure. Water was changed daily and animals were fed Shellfish Diet® 1800 daily (following manufacturer´s instructions).

2.2.2.4.2 Exposure

Study 5 consisted of two exposures which served as positive controls for studies looking at the uptake of other steroids (see Chapters 4, 5 and 6). Exposure 1 took place first followed by exposure 2 fourteen days later – the

animals were kept under flow-through conditions until acclimation time. Both exposures lasted 24 h and the animals were dosed with a nominal concentration of 5 µCi L-1 (11.3 ng L^{-1}) of [^{3}H]-E_2. Each exposure had a solvent control of ten animals (divided between two bags) with 200 µL ethanol (carrier). As Study 5 was the positive uptake control for a larger study, there was no [^{3}H]-E_2 sorption control in place. Water (1 mL) samples were taken (0, 3, 6, 18, 24 h) from all bags and immediately placed in scintillation fluid for counting. After exposure, the mussels were frozen at -20 °C for analysis.

2.2.3 Condition index analysis

Animals within the study size range were sampled before (pre-study) and after (solvent and / or water control) exposure for condition index (CI) analysis. The animals were weighed immediately after removal from water (whole live weight, i.e. with water inside) before they were stored at -20 °C until analysis. The sampled animals were defrosted at room temperature and shucked to remove the soft tissue which was then blotted dry on absorbent tissue paper. The wet soft tissue was placed in an oven at 80 °C to dry; dry tissue weight was recorded routinely (over a period of up to two weeks) until the weight ceased to decrease. CI was calculated in the following manner:

CI = dry weight / whole live weight x 1000

2.2.4 Clearance rates

The rates at which individual mussels cleared steroids from water (i.e. clearance rates) were calculated for each study in the following manner:

($\triangle$R (%) - $\triangle$C (%)/ 100) / animals / t (h) * v (mL) = mL $animal^{-1}$ h^{-1}

Where:

- $\triangle$R = percentage of radiolabel that had disappeared from the water after 3 h (except for Study 4 where it was 4 h).

- △C = percentage of radiolabel that had disappeared (fitted data, linear model) from the sorption control after 3 h (except for Study 4 where it was 4 h).

- t = time of uptake (i.e. 3 or 4 h).

- v = total water volume.

- animals = number of mussels in the vessel.

2.2.5 Steroid extraction methods

2.2.5.1 Water extraction and extract clean-up

The mixture of E_2 and [^{3}H]-E_2 was extracted from small aqueous samples (up to 50 mL) using 360 mg C18 cartridges (Waters Sep-Pak, WAT020515) conditioned with 5 mL methanol and 5 mL water. Samples (other than water) were diluted with water to < 2 % solvent before extraction. Extracts (2.2.5.2) were washed with 5 mL water and eluted with 5 mL methanol. If required, extracts were concentrated by heating at 40 °C under a stream of nitrogen gas and re-suspended in the appropriate volume of ethanol or radioimmunoassay (RIA) buffer. Extracts were stored at -20 °C. For large exposure-water samples (> 1 L) a small subsample (5 mL) was extracted as described above (using C18 cartridges) in order to quantify tritiated water and the rest was extracted using solid phase C18 disks (Empore™ SPE Disks C18, Sigma-Aldrich). Solid phase extraction of [^{3}H] E_2 from water with C18 cartridges recovered, on average, 32.1 % more radioactivity than C18 disks The disks were conditioned with 20 mL methanol and 20 mL water in order to extract large water samples. Samples were extracted under vacuum and disks were eluted in methanol (10 mL and 5 mL washes) by bath sonication and decanted into a clean tube. Pooled extracts were concentrated by drying under a nitrogen stream at 40 °C. Extracts were stored at -20 °C.

2.2.5.2 Tissue extraction

Animals were defrosted at room temperature, shucked and weighed (without the shell) in pre-weighed 50 mL polypropylene centrifuge tubes (Fisher Scientific).

Extraction Method 1 consisted of homogenising the tissue in 10 mL methanol using a blender (Ultra Turrax). The homogenate was mixed for 10 min using a vortex and centrifuged at 2500 g for 10 min. The supernatant was decanted into a clean 50 mL polypropylene tube. Extraction was repeated three more times on the tissue – once again with 5 mL methanol and twice with 3 mL methanol:chloroform (1:2) or until the final recovery had fallen below 2 % of total activity. The supernatants were combined, adjusted to 20 mL with methanol and stored at -20 °C.

Extraction Method 2 consisted of homogenising the tissue in 3 mL methanol, using a blender. To this homogenate, 3 mL ethyl acetate was added before it was mixed for 10 min using a vortex and centrifuged at 2500 g for 10 min. The supernatant was decanted into a clean 50 mL polypropylene tube. Extraction was repeated with 3mL ethyl acetate. The supernatants were combined and a sample of each extract pool was taken for counting. They were then combined, adjusted to 15 mL with methanol and stored at -20 °C.

2.2.5.3 Procedure for separating free, esterified and sulphated steroids in extracts

Crude tissue extract (prepared in either of the two ways described above) was removed from the freezer, shaken and transferred (1 mL) to a microcentrifuge tube (Eppendorf). After centrifugation (to remove any solid fats that might have precipitated during storage), 800 µg of the supernatant was transferred to a borosilicate glass tube (Fisher Scientific). This was then dried down under a stream of nitrogen at 40 °C. After addition of 1.2 mL ethanol, 0.3 mL water and 3 mL heptane, the tube was capped and then shaken vigorously for 5 min. The tube was then centrifuged and the upper heptane layer removed and placed in a clean glass tube. The 80 % ethanol layer was shaken for another 5 min with a further 3 mL of heptane and centrifuged again. The two batches of heptane were combined (heptane contained > 96 % of esterified E_2; while the 80 % ethanol contained > 98 % of any free and sulphated E_2). To separate free from sulphated E_2, the 80 % ethanol phase was blown down under a stream of nitrogen gas at 40 °C, reconstituted in 100 µL water and 4 mL diethyl ether, shaken and centrifuged. Sulphates were in the water

and free steroids in the diethyl ether. To separate them, the aqueous layer was frozen in liquid nitrogen and the top layer decanted into a clean tube. Both were then dried under a stream of nitrogen at 40 °C and re-suspended in ethanol or RIA buffer.

As an extra precaution in the present study, the 80 % ethanol phase was diluted with water and passed through a C18 cartridge to check how much tritium may or may not have been removed from the steroid (and then presumably incorporated in water molecules). This represented activity that passed straight through the cartridges as opposed to free and sulphated steroids, which were retained in the cartridge. The cartridges were then eluted with 5 mL methanol that, after blowing down, was treated in the same way as above (i.e. mixed with water and diethyl ether).

2.2.6 Chromatography

2.2.6.1 Normal-phase High Performance Liquid Chromatography

Normal phase HPLC (np-HPLC) was used to separate free from esterified [^{3}H]-E_2 in a mussel tissue extract. It was carried out on a Luna 5 µm silica 100 Å, 250 x 20.2 mm preparative column fitted with a Guard Cartridge 15 x 21.2 mm (http://www.phenomenex.com). The column was developed isocratically with chloroform:methanol (98:2, v:v) at 2 mL min^{-1}. The dried sample (mixed with 20 µg of standard E_2) was dissolved in 2 mL of the same solvent. The eluate was monitored at 280 nm and fractions were collected every minute. A portion of each fraction was transferred into a scintillation vial for counting. The fractions containing the main radioactive peak were dried down under a stream of nitrogen gas at 40 °C, reconstituted in 500 µL methanol and stored at -20 °C to be used in further studies.

2.2.6.2 Reverse-phase High Performance Liquid Chromatography

Reverse-phase HPLC (rp-HPLC) (using an analytical column) was used to separate free and conjugated (i.e. sulphated or glucuronidated) metabolites of [^{3}H]-E_2 in water extracts and also the metabolites produced as the result of alkaline hydrolysis of esterified [^{3}H]-E_2. The procedure was as described previously (Arbuckle et al., 2005). Basically, the column was

developed with a gradient formed from two solvents (A, deionised water containing 0.01 % Trifluoroacetic acid (TFA) (Sigma-Aldrich) and B, 70 % acetonitrile in deionised water containing 0.01 % TFA) at a rate of 0.5 mL min^{-1} and one minute fractions were collected. The gradient ran from 20 % B to 100 % B over 60 min. The extract was mixed with 20 µg each of E_1 and E_2 and dissolved in 800 µL of Buffer A and 200 µL of Buffer B prior to loading. The eluate was monitored at 280 nm. A small portion of each fraction was counted and the tubes containing the main peaks were dried under a stream of nitrogen at 40 °C, re-suspended in ethanol and stored at -20 °C for later analysis.

2.2.7 Thin layer chromatography

2.2.7.1 Separation of free steroid and ester

A sample of crude mussel extract was mixed with 5 µg of Ad and E_2 standard (10 µl of 0.5 mg mL^{-1} standard stocks in ethanol) and dried under a stream of nitrogen at 40 °C. Samples were loaded onto TLC plates (catalog no. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) with 90 µL ethyl acetate and developed for 45 min with a mixture of chloroform:ethanol (50:2 v:v). After marking the positions of the standards using a UV lamp, the plate was sprayed with 10 % phosphomolybdic acid in ethanol and heated at 100 °C for 5 min. Lanes were then divided into 5 mm bands, and the silica gel from each band scraped off the plate. The scrapes were mixed with 500 µL ethanol, 500 µL water and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity.

2.2.7.2 Separation of free steroid and water soluble metabolites

After removal of esters using heptane (2.2.5.3), the radioactivity that remained in 80 % ethanol was dried down, mixed with 10 µg each cortisol (F), F sulphate and F glucuronide (as described previously; Scott et al., 2014) and loaded onto one lane of a TLC plate (catalog no. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) and developed for 45 min with a mixture of ethyl acetate:ethanol:ammonia solution (45:45:15, v:v:v), which enabled not just free, but also sulphated

and glucuronidated steroids to move on the chromatogram. This method was also applied to exposure-water extracts. After marking the positions of the standards using a UV lamp, the lane was divided into 5 mm bands, and the silica gel from each band scraped off the plate. The scrapes were mixed with 500 μL ethanol, 500 μL water and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity.

2.2.8 Statistics

Figures were produced in Sigmaplot (Systat Software Inc, TW4 6JQ, London, UK.) and statistical analyses were performed using R statistical software (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

Decay curves were transformed (square root or log transformation) in order to achieve a linear relationship before applying a linear regression model. Welsh's T test was used to compare condition indices observed between two treatments. Analysis of co-variance (ANCOVA) was used when comparing differences in steroid uptake over time between three or more treatments; when comparing uptake during the first three hours of exposure, time was treated as a factor.

2.2.9 Overall strategy

Exposure studies were designed to monitor steroid uptake (via radioactivity) in both water and animals. After steroid uptake had been accomplished, mussels were homogenised and extracted. The extracts were then separated broadly into free, sulphated (water-soluble) and esterified metabolites. Subsequently these fractions were elucidated further using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). The chromatographic methods were used individually or in conjunction, for example, the hydrolysed esters obtained during solvent partitioning were further separated using HPLC. After collecting all fractions, the radioactive peaks which eluted with known standards were then elucidated further using TLC. The differing matrices

and mobile phases allowed us to confirm the putative identity of the metabolites and ensure that they were in fact single chemicals.

Where possible, procedures were validated and accompanied by suitable controls: a pre-purified radioactive oestradiol ester was employed as a control during solvent partitioning; radioactivity of all fractions was quantified in every step of solid phase extraction; radioactivity of every extraction step was quantified (spiked controls were not employed as free steroid does not behave in the same manner as esters or sulphates, and was therefore deemed unsuitable) and extraction steps were carried out until the percentage recovery was > 5 % of the total activity.

2.3 Results

2.3.1 Condition index

Condition index was used as a means of monitoring the effects of exposure (particularly solvents and exposure conditions - when water controls were possible) on animal physiology. It was not possible to measure the condition index of animals from Study 4 as there were not enough spare animals in the relevant size range.

It should first be noted that there was no significant difference in condition between pre-study animals and solvent / water controls (pre-study, solvent and water control animals were treated as categories for all studies) in any of the studies (Figure 7: df = 2, f = 0.584, p = 0.563; Figure 8: Welsh's t-test: t = 1.2414, df = 20.557, p = 0.2284 and Figure 9: df = 2 , f = 1.106, p = 0.345), suggesting that exposure conditions (including exposure to the carrier) did not have a negative impact on the animals general health. There were, however, differences between studies. The mussels used for Study 5 had higher condition indices than those used in Study 1 and 2 / 3 (which in turn were relatively similar).

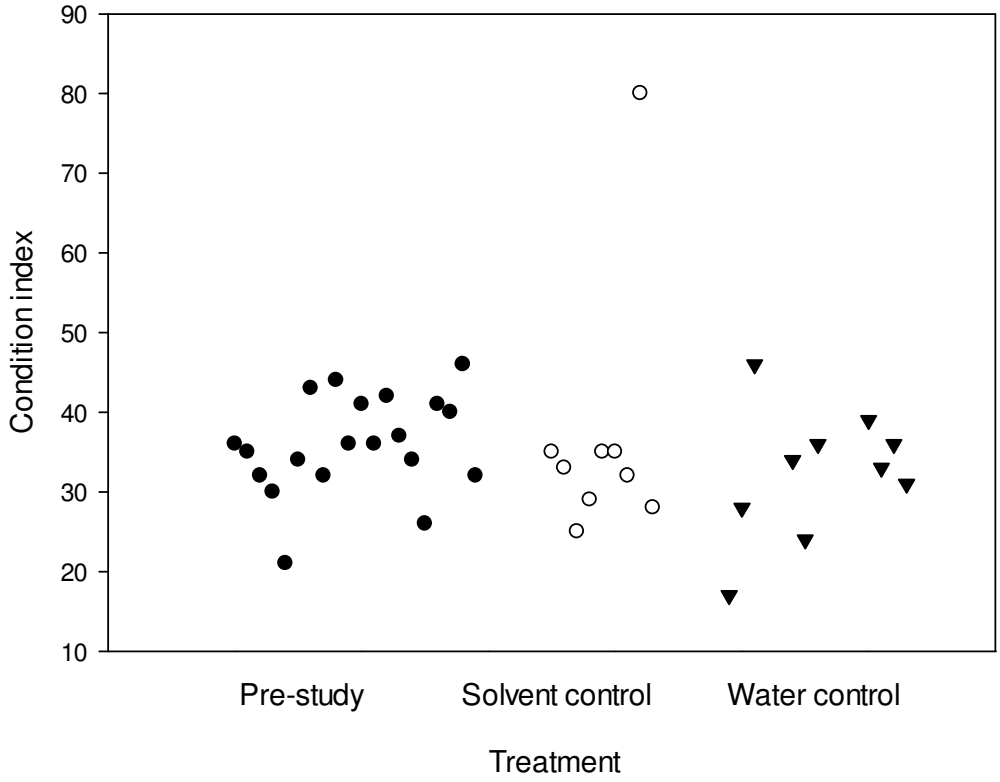

Figure 7: Condition index of animals before exposure (pre-study;●), solvent control (○) and water control (▼) animals of study 1. Data are presented as CI (n = 20 for pre-study; n = 9 and n = 10, split into two tanks for solvent control and water control respectively).

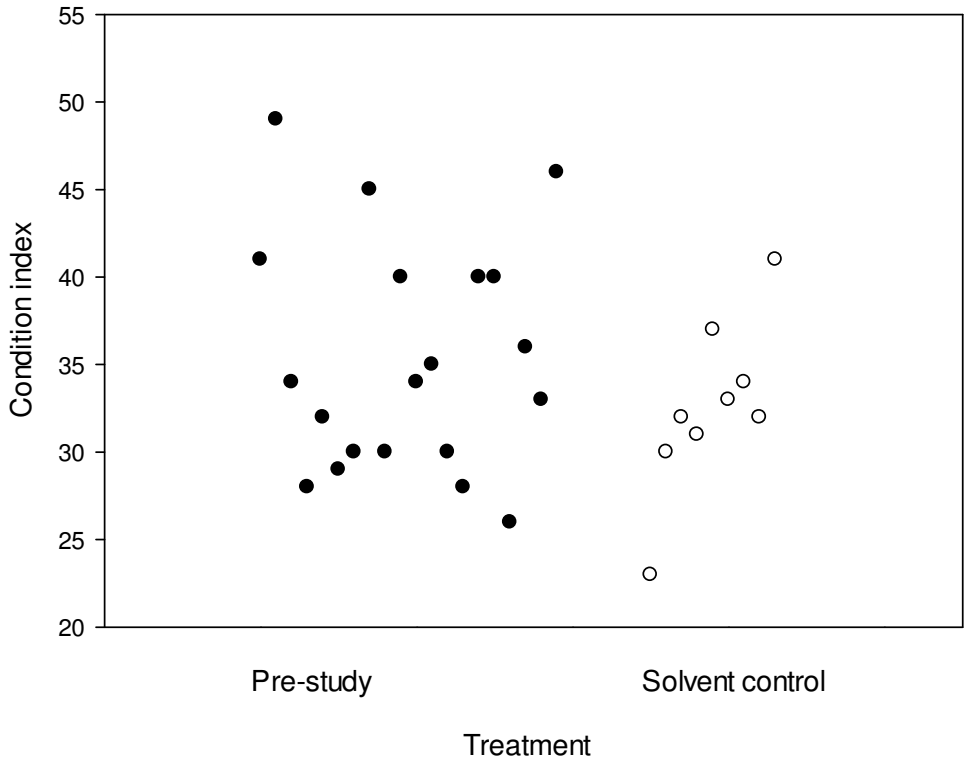

Figure 8: Condition index of animals before exposure (pre-study;●) and solvent control (○) during Study 2 and 3. Data are presented as CI (n = 20 for pre-study and n = 9, split into two tanks, for solvent control).

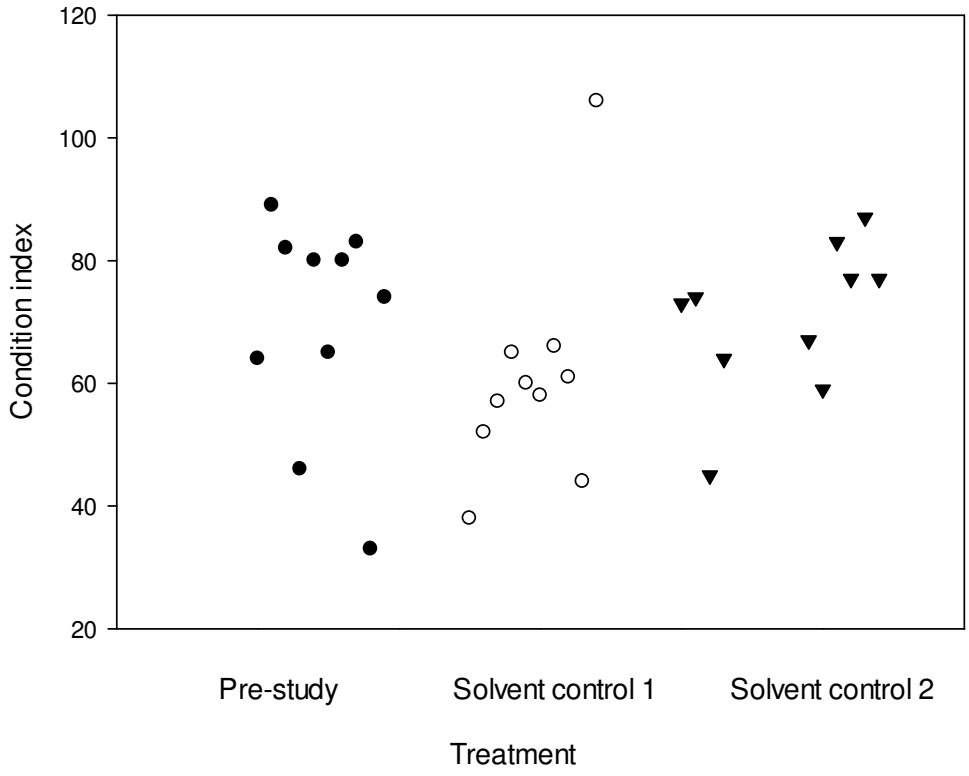

Figure 9: Condition index of animals before exposure (pre-study;●) and solvent controls 1 (○) and 2 (▼) of Study 5. Data are presented as CI (n = 10 split into two tanks).

2.3.2 Radiolabelled E_2 clearance from bath exposures

2.3.2.1 Study 1

In Study 1, the mean radioactivity (n = 10) in water was calculated to be 0.57 µCi L^{-1} at the start of the first exposure period and 0.51 µCi L^{-1} at the start of the second exposure period. By the end of the exposure periods, 37.1 and 27.5 % of the total [^{3}H]-E_2 that had been added had disappeared from the water (Figure 10). This decrease over time was significant (df = 1, f = 602.27, p < 0.0001), although it should be noted that in exposure 1 [^{3}H]-E_2 decayed faster than in exposure 2 (ß = -0.0083507, SE = 0.0003996, t = -20.898, p < 0.0001; and ß = 0.0029349, SE = 0.0005581, f = 5.259, p < 0.0001, respectively). A small amount of [^{3}H]-E_2 disappeared due to sorption (at an average rate of 0 and 0.14 % h^{-1} during exposure 1 and 2 respectively (fitted data, linear model). Clearance rates, based on the first 3 h of each exposure, are presented in Table 4. The rates were similar between exposures 1 and 2 (36 and 30 mL $animal^{-1}$ h^{-1} respectively). Although this experiment clearly demonstrated that the animals had

absorbed a significant amount of [^{3}H]-E_2 from the water column, it was decided that 13 L tank volume was excessive for the housing of five mussels (and thus wasteful of expensive radiolabel). It also demonstrated that a multiple-dosing procedure was unnecessary in order to carry out uptake experiments.

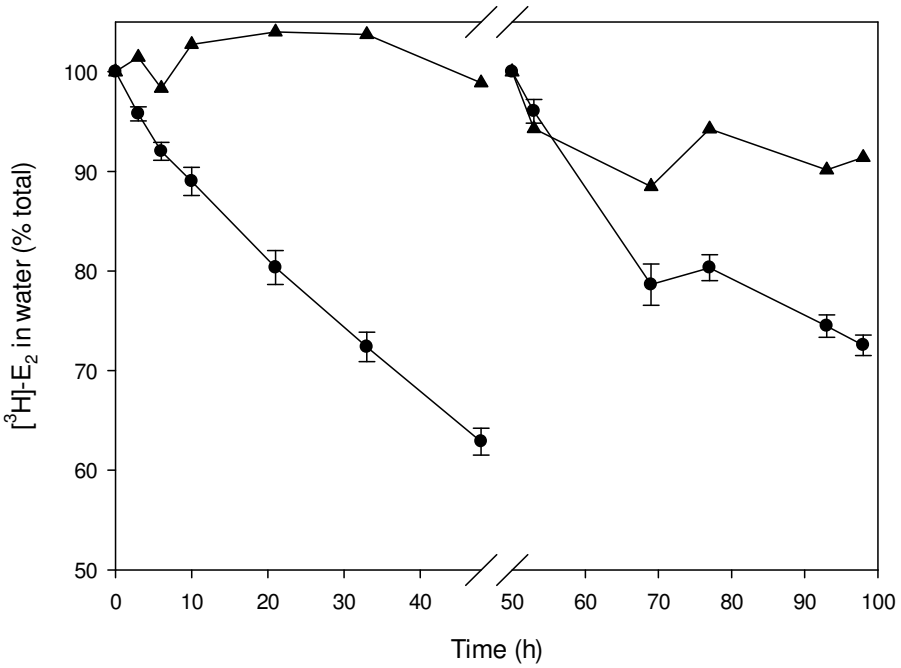

Figure 10: Radiolabelled E_2 removal from water by *M. edulis* (●). Mussels were exposed for two consecutive 48 h periods under semi-static conditions (water was changed and fresh label added; //) to 0.7 µCi L^{-1} (1.36 ng L^{-1}) [^{3}H]-E_2 nominal concentration per dose. Data are presented as mean % total of [^{3}H]-E_2 in water (n = 10 tanks per time point) and ± S.E.M. A sorption control (▲) tank with no animals was in place. Control data is presented as % total [^{3}H]-E_2 residues in water (n = 1).

2.3.2.2 Study 2

This experiment was carried out purely to check whether it was necessary to feed the animals during the exposure period (on the basis that the presence of food might increase the rate of filtering and thereby the amount of water that the animals process in a given time). Animals were exposed to only one dose of [^{3}H]-E_2 for 48 h in the same glass tanks, but using only 7 L of water (1.4 L $animal^{-1}$). The mean radioactivity (n = 3) in water at the time of dosing was 1.02 µCi L^{-1}. There was a significant decrease in [^{3}H]-E_2 concentration over time (square root transformation of time created a linear relationship between E_2 decay and time; df = 1, f = 1105.225, $p < 0.00001$); by the end of the exposure, 43.0 and 50.8 % of [^{3}H]-E_2 had been removed from the water column in the presence of food and the absence of food respectively (Figure 11). Feeding had no

significant impact on the decay of E_2 in water over time after 3 h (time was treated as a category; df = 1, f = 1.249, p = 0.286) or 48 h (squared root transformation of time was applied; df = 1, f = 0.510, p = 0.477). In other words, feeding during exposure had no impact on [^{3}H]-E_2 removal from the water column. The clearance rate of radiolabel was higher in this study than in the previous one (50 as opposed to 37 mL animal^{-1} h^{-1}).

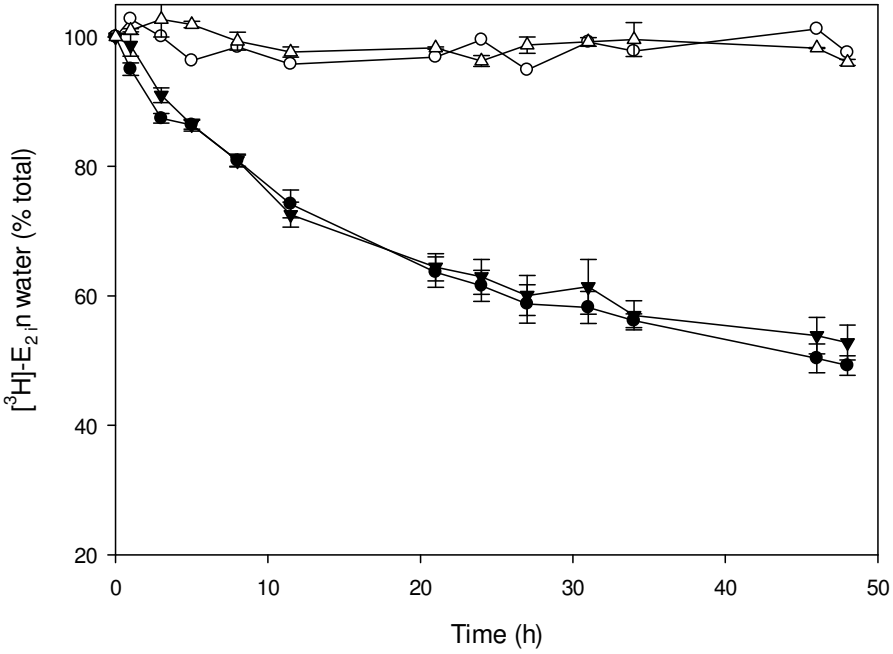

Figure 11: Radiolabelled E_2 removal from water by *M. edulis* in the presence (▼) and absence (●) of food during a 48 h exposure. Data are presented as mean % total of [^{3}H]-E_2 residues in water (n = 3 tanks per time point) and ± S.E.M. Sorption controls (no mussels in tank) were in place for each treatment, one [^{3}H]-E_2 (○) and two for [^{3}H]-E_2 with feed (△).

2.3.2.3 Study 3

Once it was confirmed that mussels readily remove [^{3}H]-E_2 from the water column and that the presence of food did not affect this process, the next problem to resolve was whether the rate of uptake was affected by the concentration of E_2 in the water (i.e. could the uptake of radiolabel be saturated?). Animals were thus exposed to [^{3}H]-E_2 in the presence of increasing amounts of non-radiolabelled ('cold') E_2 during Study 3. If saturation did exist, then it would be expected that there would be a decrease in the rate of uptake of radiolabel (i.e. a higher amount of [^{3}H]-E_2 should remain in the water due to a predominance of cold E_2). Figure 12 shows that over time there was a significant decrease (square root transformation of time was applied to achieve a linear relationship) in the

level of [^{3}H]-E_2 in the water (df = 1, f = 2144.922, p < 0.0001). Up to 50.8 %, 46.8 % and 50.6 % of total radioactivity was removed from each treatment over 24 h and sorption of [^{3}H]-E_2 only accounted for 0.9, 0.7 and 0 % (fitted data, linear model). Although treatment explained a lot of the variability between decay curves after 24 h (df = 2, f = 4.457, p = 0.0137), pairwise comparisons revealed that there was no significant difference between the decay curves of both low (7.1 ng L^{-1}) and high (35.7 ng L^{-1}) cold E_2 treatments and the 'hot only' regime (ß = -0.024917, SE = 0.039234, t = -0.635, p = 0.5267 and ß = -0.066870, SE = 0.039234, t = -1.704, p = 0.0911 respectively). In other words, there was no clear evidence of saturation of [^{3}H]-E_2 uptake after 48 h. Moreover, pairwise comparisons revealed there was no significant difference between treatments after 3 h of exposure when uptake was relatively linear (time was treated as a category; ß = -0.04667, SE = 0.03388, t = -1.377, p = 0.18533 and ß = -0.07000, SE = 0.03388, t = -2.066, p = 0.05355). The clearance rates (calculated from the first 3 h of uptake) were similar to Study 2 (50 %).

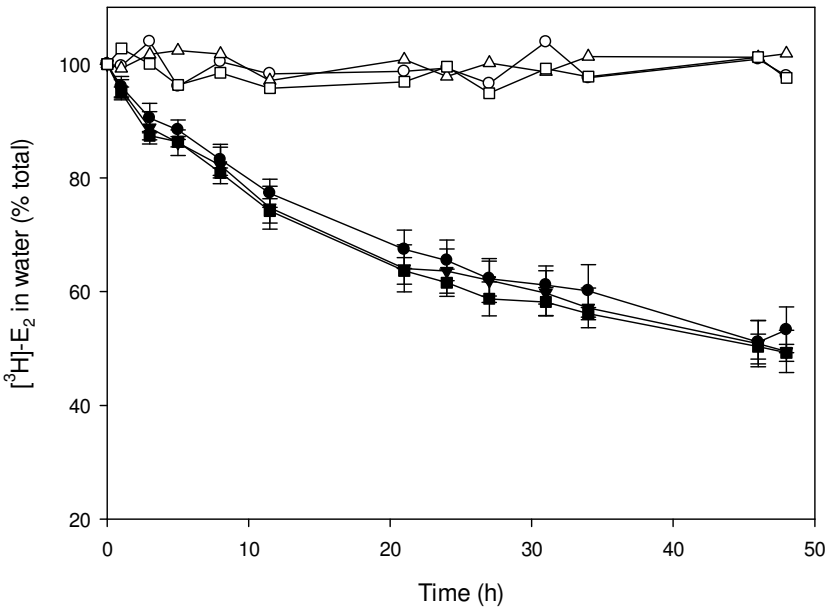

Figure 12: Radiolabelled E_2 removal from water by *M. edulis* in the presence of cold E_2 (no E_2: ■, 7.1 ng L^{-1} E_2:● and 35.7 ng L^{-1} E_2:▲) during a 48 h exposure. Data are presented as mean % total of [^{3}H]-E_2 residues in water (n = 3 tanks per time point) and ± S.E.M. Sorption controls (no animals in tank) were in place for each treatment (□, ○, △ respectively).

As no effect of added cold E_2 was detected in the uptake of radiolabelled E_2 during this study, it was decided to conduct a further experiment, using considerably higher doses of cold steroid. It was also decided that the

volume of water should be further lowered to 0.4 L animal^{-1} so that less radiolabel was needed and also the rate at which radioactivity disappeared from the water should be even steeper than in Studies 2 and 3. The use of less water also meant that the exposures could more easily be carried out in plastic bags as opposed to large glass tanks. The fact that the glass tanks had to be laboriously cleaned at the end of every experiment was also considered to pose a safety problem.

2.3.2.4 Study 4

Even though there was as much as 10,000 times more cold E_2 (25 µg L^{-1}) than [^{3}H]-E_2 in the highest concentration, treatment appeared not to have a significant difference on uptake after 24 h exposure (log transformation of time was applied which lead to a linear relationship; df = 3, f = 1.220, p = 0.30504) (Figure 13). It should be noted that pairwise comparisons showed a significant difference in uptake over time between treatment A and B (ß = 0.196630, SE = 0.064123, t = 3.066, p = 0.00263). This statistical difference, however, is not necessarily relevant as there was no difference between treatment A and the highest cold E_2 dose treatments (C and D; ß = -0.033463, SE = 0.064123, t = -0.522, p = 0.60264 and ß = 0.024927, SE = 0.064123, t = 0.389, p = 0.69809 respectively). Furthermore, although treatment appeared to explain some of difference between groups after 4 h of exposure (time was treated as a category ; df = 3, f = 3.408, p = 0.0292); pairwise comparisons showed no difference between treatment A and all other treatments (ß = 0.16000, SE = 0.08456, t = 1.892, p = 0.0675; ß = -0.04000, SE = 0.08456, t = -0.473, p = 0.6394; ß = -0.02000, SE = 0.08456, t = -0.237, p = 0.8145; B, C and D respectively). It should be noted that there were slightly lower clearance rates in those treatments that contained cold E_2 (Figure 13 and Table 4) but after 24 h, all bags looked to be similar (with between 55 % to 65 % of the radioactivity having disappeared from the water).

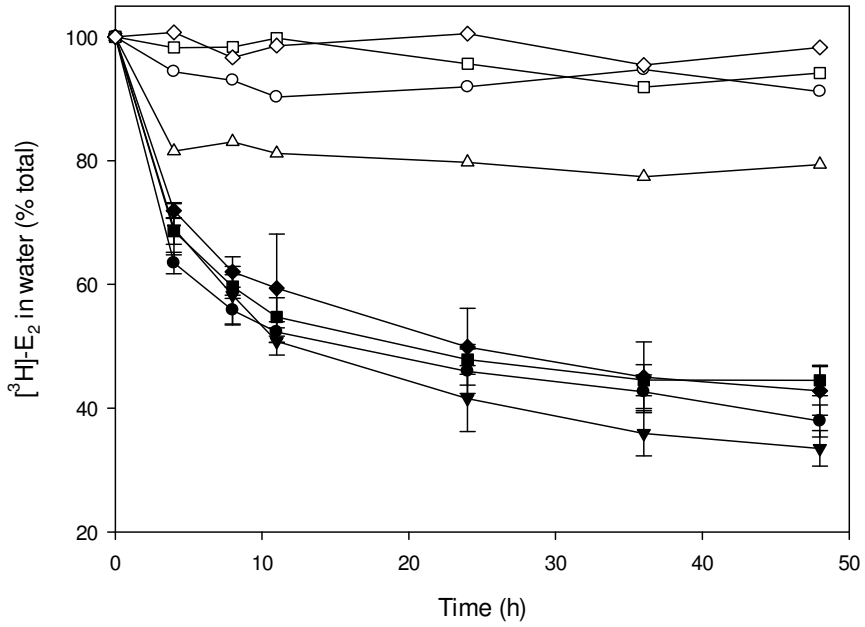

Figure 13: [^{3}H]-E_2 (2.21 ng L-1 nominal concentration) removal by *Mytilus spp.* in the presence of increasing amounts of cold E_2 ((0 µg L^{-1} :●, 0.25 µg L^{-1}:▼; 2.5 µg L^{-1}:■ and 25 µg L^{-1}:♦ nominal concentrations) over 48 h. Data are presented as mean % total (n = 5 tanks) and ± S.E.M. One sorption control (no mussels in tank) was in place for each treatment (○, △, □ and ◊ respectively).

2.3.2.5 Study 5

Regarding the experimental set up optimisation studies so far, it has been demonstrated that increasing the concentration of [^{3}H]-E_2 and reducing the volume of water successfully increases the total amount of radiolabel taken up by the mussels. However, since uptake levelled off at ca. 24 h, it was decided that it was unnecessary to actually keep the exposures going for 48 h. Therefore in the final two E_2 experiments that were carried out, animals were only exposed to the steroid for 24 h. The radiolabel concentrations were also increased to 2.74 µCi L^{-1} and 2.72 µCi L^{-1}. There was a significant decrease in [^{3}H]-E_2 over time (log transformation of time was applied which led to a linear relationship; df = 1, f = 216.492, p < 0.00001) with up to 56 % and 46 % of radioactivity removed after 24 h (

Figure 14). Although there was a significant difference between exposure 1 and 2 over time (df = 1, f = 4.703, p = 0.0455), pairwise comparisons revealed that these differences were not significant regarding the time the exposure was done (ß = -0.11402, SE = 0.29227, t = -0.390, p = 0.7016). Furthermore, there was no significant difference in [^{3}H]-E_2 removal

between exposures in the first 3 h of uptake (df = 1, f = 0.023, p = 0.886177) and the clearance rates were 40.4 and 41.4 mL animal^{-1} h^{-1}.

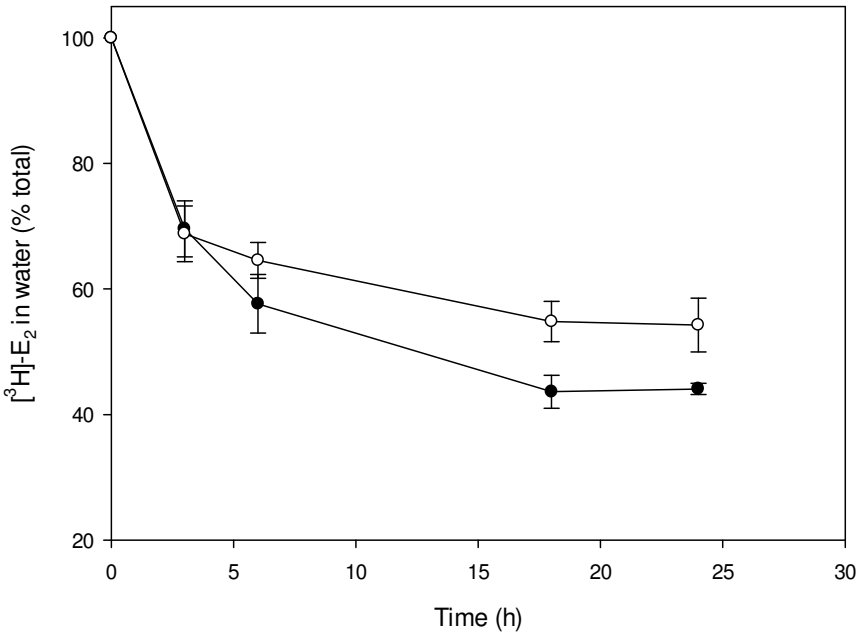

Figure 14: [^{3}H]-E_2 removal by *Mytilus* spp. during two 24 h exposures to a nominal concentration of 5 µCi L^{-1} (11.3 ng L^{-1}) of [^{3}H]-E_2. Exposure 1 (●) and 2 (○) data are presented as mean % total (n = 2 tanks) and ± S.E.M.

Table 4: Summary of exposure conditions and [^{3}H]-E_2 clearance rates from water by *Mytilus* spp.

Study	Water vol. (L)	N° animals	Nominal label conc. (µCi L^{-1})	Exposure time (h)	Removal at end of exposure (%)[a]	Fitted sorption after 3 h (%)	Removal after 3 h (%)	Clearance rate (mL $animal^{-1}$ h^{-1})[c]
1 Exp 1	13.0	5	0.70	48	37.1	0.0	4.2	36.6
1 Exp 2	13.0	5	0.70	48	27.5	0.4	4.0	30.9
2 feed	7.0	5	1.30	48	43.0	0.3	9.0	40.8
2/3 [^{3}H]-E_2	7.0	5	1.30	48	50.8	0.1	12.6	58.5
3 3x cold	7.0	5	1.30	48	46.8	0.0	9.5	44.0
3 15x cold	7.0	5	1.30	48	50.6	0.0	11.2	52.4
4 A	0.8	2	1.14	48	62.2	0.3[b]	36.5[b]	36.2
4 B	0.8	2	1.14	48	67.0	1.2[b]	31.0[b]	29.8
4 C	0.8	2	1.14	48	55.5	0.6[b]	31.4[b]	30.8
4 D	0.8	2	1.14	48	57.6	0.2[b]	28.1[b]	27.9
5 E_2 Exp 1	2.0	5	5.00	24	55.9	0.1	30.4	40.4
5 E_2 Exp 2	2.0	5	5.00	24	45.8	0.1	31.2	41.4

[a] Fitted sorption not accounted for in removal after exposure.

[b] Fitted sorption and removal after 4 h.

[c] Fitted sorption accounted for in clearance rates.

2.3.3 Evidence for metabolism of E_2

In theory, assuming a) an average clearance rate of E_2 of 40 mL animal^{-1} h^{-1}, b) 30 % of the label being removed from the water within the first 3 h and c) five animals being held in 2 L water, the radioactivity in the water in Study 5 should decay exponentially to reach < 5 % within 24 h (illustrated in Figure 25). Instead, as seen in

Figure 14, between 46 % and 56 % remained at this time. One possible explanation is that the mussels behave like a low affinity binding substrate - with E_2 being continuously exchanged between the water and the animal and eventually reaching an equilibrium (in this case to about 50:50, %:%, animal:water) by 24 h. However, knowing that much of the E_2 is bound covalently to fatty acids (and thus made water-insoluble) when it enters the animal, this explanation seems improbable. A much more likely explanation is that the E_2 label is being metabolised during the course of the exposure and is being converted to other compounds that cannot be either absorbed or esterified. In order to test this hypothesis, water samples (1 L) were taken at the end of the two exposures in Study 5. When the water was subjected to solid phase extraction, ca. 23 % of the activity passed straight through, suggesting the presence of tritiated water (i.e. labelled atoms released from the parent compound by unknown reactions). After the extracts had been eluted and dried down, a portion was dissolved in water and then shaken with diethyl ether. Study 5, exposure 1 extract retained 73 % of radioactivity in the water fraction (consistent with the presence of a water-soluble conjugates) and 27 % in the ether fraction (assumed to be free steroid). Study 5, exposure 2 water extract yielded 60 and 40 % respectively, i.e. both extracts had greater amount of water-soluble metabolites of [^{3}H]-E_2 than free steroid. Some of the extracts were then analysed by reverse-phase HPLC at 280 nm with added E_2 and E_1 standards. Figure 15 shows that the main radioactive peak from both exposures eluted at 38 min (with a smaller peak at 32 min), both at elution positions that are consistent with the presence of water-soluble steroid sulphates (Scott et al., 1991). A third small peak eluted at 52 min in the region of standard E_2. Extract 2 showed the presence of a further radioactive peak eluting at about 54 min. As water-soluble metabolites in

the animal had been tentatively identified as sulphates (and not glucuronides; see Figure 19) it was suspected that the same would be present in the water. To investigate this, some raw water extract and the HPLC peak collected at min 38 were both run on a TLC plate alongside E_2 3-sulphate and E_2 17β-sulphate standards (Figure 16). Indeed the radioactivity in the purified peak and the raw extract ran with E_2 3-sulphate; the raw extract had a second, smaller peak (26 % of the total radioactivity detected) which ran with the E_2 standard. So although the compounds in the water have only been tentatively identified, the results confirm that the radiolabel that is still in the water after 24 h is mostly either in the form of tritiated water or water-soluble steroids (probably E_2 3-sulphate). Sulphates are highly water-soluble and thus unlikely to be absorbed or retained by the animals. Only a minor fraction of (presumed) intact E_2 remains in the water after 24 h. In conclusion, E_2 uptake by the mussels seemed to cease by 24 h because the proportion of E_2 that remained in the water was converted into mainly water-soluble compounds.

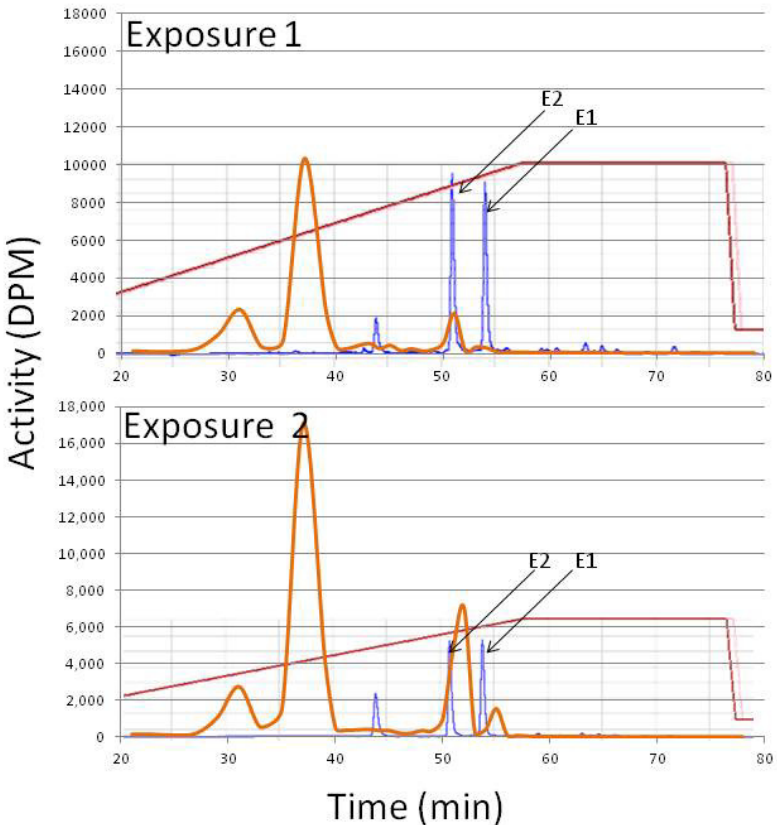

Figure 15: HPLC chromatogram of [^{3}H] E_2 water sample (Study 5) with E_2 and E_1 standards. Data are presented as UV absorbance (blue; 280 nm) and radioactivity peaks (orange).

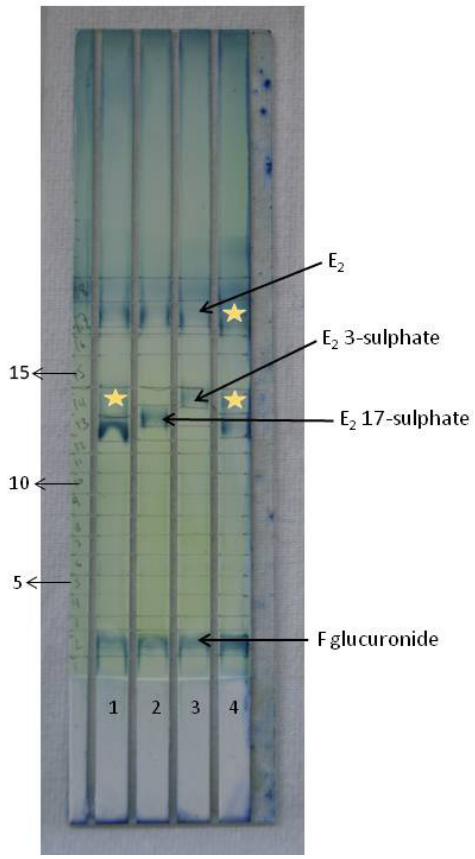

Figure 16: TLC plate of [^{3}H]-E_2 exposure- water extract with all standards. Lane 1: HPLC fraction 38 of water extract; Lane 2: F glucuronide, E_2 and E_2 17-sulphate standards; Lane 3: F glucuronide, E_2 and E_2 3-sulphate standards and Lane 4: raw water extract with all standards. Standards are labelled and golden stars indicate the position of radioactivity.

2.3.4 What happens to the E_2 that is absorbed by the mussels?

To confirm that the radioactivity lost from water during exposure had actually been absorbed by the animals, some mussels were sacrificed and their tissues extracted in either of two ways. Method 1 was based on that described by Peck et al. (2007), which involved homogenising the tissue and then mixing it several times with combinations of methanol and chloroform. After each step, a portion of the extract was counted; and the process was continued until the rate of recovery of radioactivity was < 2 % of the total. It was found that, when tissues were extracted five times, < 4 % of the total radioactivity was present in the last two extracts. Method 2, that involved a mixture of ethyl acetate and methanol, yielded 97 % of the total radioactivity in only two steps (see Table 5 for a summary and Table

22 for a detailed breakdown of how the extractions were developed). It must be pointed out that Method 1 did not work so well with some other steroids (e.g. T; unpublished data), and with Method 2, the extracts tended to separate into two phases during storage in the freezer.

Table 5: Summary of extraction method efficiency.

Extraction method	Efficiency of two extractions (%)	Efficiency of method (%)
1	77	96
2	97	97

Having established an efficient way to extract the radioactivity from the tissue, attention was turned to characterisation of the steroids. As mentioned in the Introduction, it is already known that E_2 is esterified by molluscs and that esters can be separated from free steroids using silica gel chromatography. It was confirmed that this was the case by running some pooled extracts from the first experiment on either TLC (Figure 17) or a preparative normal phase HPLC column (Figure 18). There was one very obvious peak of radioactivity coinciding with the area in which lipids elute (and where, on the basis of previous studies, one would expect E_2-ester to elute) and a small indistinct peak corresponding to the elution position of E_2 standard. Since chromatography is relatively time-consuming and expensive, it was decided to try and develop a solvent partition procedure for separating free and esterified E_2. This procedure involved shaking the extracts in water:ethanol:heptane in a ratio of 0.1:0.4:0.5 (v:v:v). This mixture formed two phases (heptane at the top and 80 % ethanol at the bottom). Lipids were expected to be mostly in the heptane phase and free steroids in the 80 % ethanol phase. When tested for the first time using [^{3}H]-E_2, it was confirmed that 99 % was recovered in the 80 % ethanol phase. However, when it was tested with esterified [^{3}H]-E_2 (using the first radioactive peak from np-HPLC; Figure 18), it was found that only ca. 70 % of the radioactivity was recovered in the heptane phase. However after a series of experiments, in which different volumes of heptane, and several repetitions of the phase separation step, were tried out, it was found possible to trap > 95 % of the ester in heptane (without removing much if

any of the free steroids from the 80 % ethanol phase) by using 2:1 (v:v) heptane:ethanol and doing the separation twice.

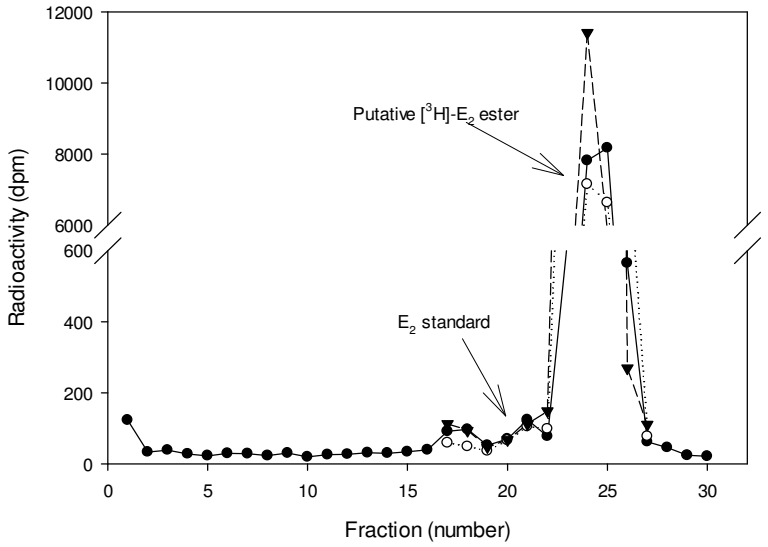

Figure 17: TLC chromatogram of mussel extracts 1 (●), 2 (○) and 3 (▼) from Study 1 (after exposure to [^{3}H]-E_2 for 96 h). Data are presented as total radioactivity fraction-1 (fraction = 5 mm silica). Mobile phase consisted of ethyl acetate and acetone (4:1) and E_2 standards were run in conjunction (5 µg each).

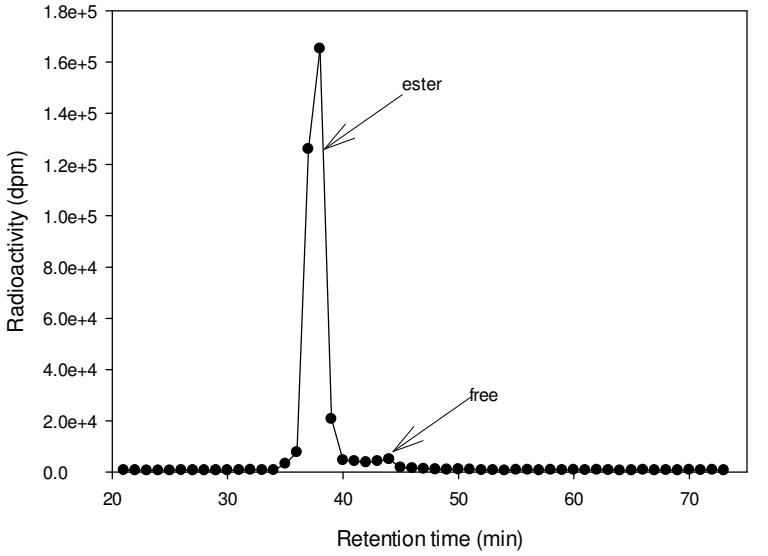

Figure 18: Normal phase HPLC chromatogram of tissue extract from mussels that had been exposed to [^{3}H]-E_2 for 96 h.

In order to measure the E_2 in the ester fraction by immunoassay (see Chapter 3), the fatty acids need to be removed from the steroid. Three hydrolysis methods were tested on radioactive extracts (see Table 6 for a summary of optimisation): heating to 40 °C for 3 h in a mixture of KOH and methanol (Gooding and LeBlanc, 2004), heating to 80 °C for 40 min (Peck et al., 2007) and digestion with subtilisin (personal communication from Dr

Katarzyna Smolarz, University of Gdansk, Institute of Oceanography, Poland). Subtilisin digestion showed no change (at either temperature) when compared with negative controls. Heating to 40 °C for 3 h was partially successful - with 86 % of the radioactivity being recovered in the ethanol fraction (i.e. free steroid) compared with heating to 80 °C for 40 min which resulted in 96 % transformation. Some of the radioactive ester (separated from free and sulphated radiolabel using heptane) was hydrolysed using the latter (most efficient) method and run on a reverse phase C18 column (Figure 20). The radioactive peak was found to have the exact same retention time as the E_2 standard, i.e. E_2 esters were comprised of E_2 only.

Table 6: Summary of conditions selected for metabolite separation method (0: no difference, ✓: improvement, ×: decline)

Method optimisation	Treatment	Result
Sample preparation	Heat	✓
	No heat	×
Number of heptane:80% ethanol extractions	1	✓
	2	✓
	3	0
	4	0
	5	0
Heptane extraction volume (mL)	1.5	×
	3.0	✓
	4.5	0
Ethanol (%)	80	✓
	90	×
Acidification	HCl	0
	No HCl	✓
Vortex time (min)	5	✓
	10	0
	15	0
Matrix volume (µl)	400	0
	600	0
	800	0
Hydrolysis	40°C, 3 h	×
	80°C, 40 min	✓
	Subtilisin	×

2.3.4.1 Comparison of the phase-separation procedure with TLC and / or HPLC

When the new separation method was tried out with a pooled mussel tissue extract (time zero in Study 1), it was found that the 80 % ethanol fraction (supposedly free steroid only) contained ca. 20 % of the total radioactivity. This contrasted with what had been found after np-HPLC of the extract (Figure 18), where the amount of free E_2 was very small (< 3 % of the total radioactivity). This implied either that the liquid / liquid separation method was affected by the presence of matrix or that there were water-soluble conjugates in the 80 % ethanol phase. The first possibility was dismissed after seeing no difference in efficiency when testing the method on [^{3}H]-E_2 with unexposed mussel extract as matrix (

Table 23). The second possibility was confirmed by drying down the ethanol and partitioning the extract between water and diethyl ether. In this case, the bulk of the radioactivity was found in the water phase. The reason why this probably has not been noticed before in the radioactive steroid uptake studies mentioned in the Introduction is that highly hydrophilic substances do not migrate on silica gel under the conditions used to separate free and esterified steroids (e.g. conditions used on TLC seen in Figure 17). It was shown that the bulk of the radioactivity in this fraction was most likely conjugated to sulphate groups, as there was little or no evidence for activity eluting in the position where steroid glucuronides would be expected to run (Figure 19). It was also possible to liberate most of the radioactivity by acid solvolysis (Scott et al., 2014) – a procedure that only works with sulphated, but not glucuronidated, steroids (see Chapter 3 for details of acid solvolysis). The TLC indicates that the sulphate fraction contains more than one compound. These metabolites remain to be identified.

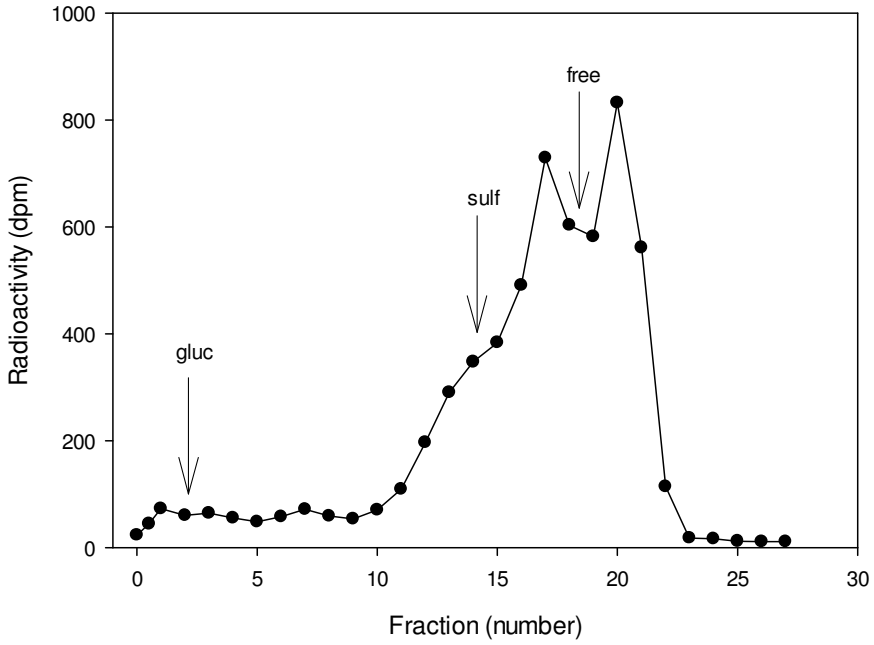

Figure 19: Representative TLC chromatogram of mussel extract (ethanol fraction of separation) after exposure to [^{3}H]-E_2 for 96 h. Data are presented as total radioactivity fraction^{-1} (fraction = 5 mm silica gel scrape). F glucuronide, F sulphate and free F standards were run in parallel (5 µg each).

After totalling the amount of radioactivity that had been absorbed from the water and the amounts that were present in the mussel extracts, it was discovered that, in most experiments, some of the radioactivity was unaccounted (between 0 % and 31 %; Table 7). There are several possible explanations (including counting error, sorption to the animal shells and incomplete extraction), but no experiments have yet been done to resolve this problem.

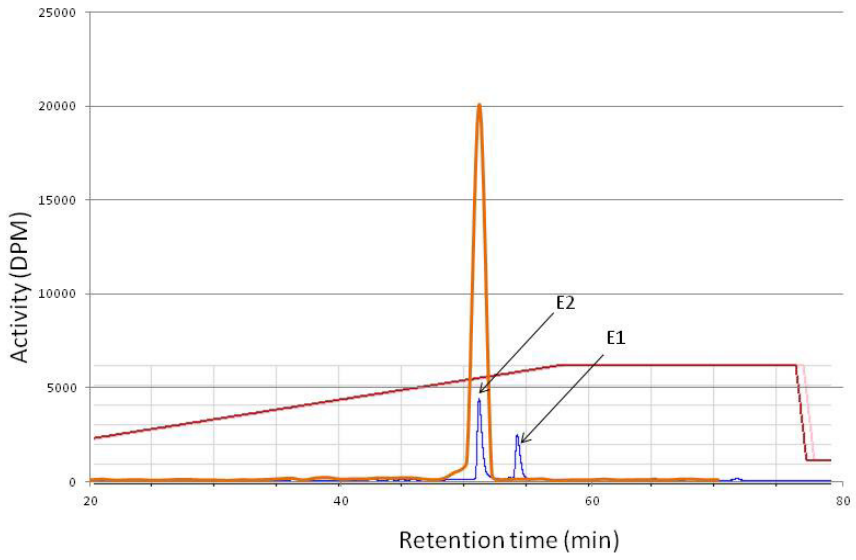

Figure 20: rp-HPLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-E_2 metabolites (Study 5) with E_2 and E_1 standards. Data are presented as UV absorption (blue; 280 nm) and radioactivity peaks (orange).

Table 7: Summary of E_2 equivalent distribution in all [^{3}H]-E_2 exposures. Data are presented as % total (per tank) at the end of each exposure (sorption data was fitted to linear model).

Study	Exposure time (h)	Activity in water (%)	Activity sorbed (%)	Activity in animals tank^{-1} (%)	Activity unaccounted (%)
1 Exp 1 &2	96	67.5	3.1	18.7	10.8
2 feed	48	52.8	4.1	22.5	20.5
2/3 [^{3}H]-E_2	48	49.2	0.9	21.0	28.9
3 3x cold	48	53.2	0.7	21.0[b]	25.1
3 15x cold	48	49.4	-0.5	21.0[b]	30.1
4 A	48	37.8	4.2	43.1	14.9
4 B	48	33.0	14.4	44.7	7.9
4 C	48	44.5	7.3	50.1	-1.8
4 D	48	42.4	2.3	39.5	15.8
5 E_2 Exp 1	24	44.1	2.8[a]	30.3	22.8
5 E_2 Exp 2	24	54.2	2.8[a]	30.5	12.4

[a] Value based on mean sorption at time 24 h in (similar) studies 1, 2 (no feed) and 4 (a).

[b] Value based on Study 2 (no feed) as animals were not processed.

2.4 Discussion

Laboratory exposures of *Mytilus* spp. to [^{3}H]-E_2 confirmed that molluscs remove the steroid from the water as already reported in other studies (Janer et al., 2004a; Labadie et al., 2007; Peck et al., 2007; Puinean et al., 2006).

2.4.1 Development of the Methodology

In this Chapter, procedures for studying the uptake and metabolism of vertebrate steroids by mussels were developed and optimised with the use of radialabelled E_2.

2.4.1.1 How much water, how many animals and how much radioactivity?

The studies were started off using a relatively large volume of water (13 L) and a low level of biomass (2.6 L animal^{-1}). Although this worked,

radiolabel was disappearing relatively slowly (only 30 % over 48 h) and potentially large amounts of expensive radiolabel were being wasted. Thus the water was reduced to 1.4 L animal^{-1} in the second experiment and to 0.4 L animal^{-1} in the final set of experiments. At each stage, there was an increase in the rate of disappearance of radiolabel from the water over the first 3 to 4 h (NB. this should not be confused with 'clearance rate'; see below). The concentration of label in the water is purely a matter of choice. As discussed below, the concentration of the radiolabel should not in theory have any effect on the rate of uptake or the clearance rate. The only reason the concentration of radioactivity in some of the later experiments was increased was purely in order to incorporate greater amounts of radioactivity in the animals, allowing easier identification of some of the metabolites.

2.4.1.2 For how long should the animals be exposed?

As can be seen from Table 8, there has been only one recent study that used an exposure period of < 24 h. That was the one by Gooding & LeBlanc (2001) who used 16 h. The widespread use of multiple dosing and exposure of animals for as long as 90 days, gives the false impression that it is difficult to get bivalves to absorb steroids from water. However, the results of the present study show, at least in the case of *Mytilus* spp., that multiple dosing is unnecessary and even a 24 h exposure period is probably excessive. In the later experiments, using 0.4 L animal^{-1}, there was a rapid reduction of radiolabel in the first three hours that then tailed off to reach a plateau between 12 and 18 h. The uptake of T by *I. obsoleta* exposed over a 16 h period reached a plateau after approximately 6 to 8 h (Gooding and LeBlanc, 2001). Le Curieux-Belfond et al. (2005) exposed Pacific oysters (*Crassotrea gigas*) to E_2 and found that radioactivity accumulation also levelled off at approximately 8 to 10 h. No-one has ever explained why the animals stop taking up radiolabelled steroids in this way. By examining the radioactivity remaining in the water, however, a plausible explanation arose. After a few hours of [^{3}H]-E_2 exposure, the radioactivity in the water consists mainly of sulphated metabolites and a small amount of free steroid that possibly is not actually intact E_2. In other words, what is left in the water probably can no longer be absorbed, let alone esterified

by the mussels. The capacity for steroid uptake by *Mytilus* spp. is extremely high (25 µg/ L did not saturate uptake) and has in fact no bearing in the slowing down of steroid removal from water.

Effectively, there are two major pathways competing for the biotransformation of [^{3}H]-E_2 by the mussels. These are esterification and sulphation. The relative activity of the sulphation pathways at any particular time and in any particular individual possibly explains why uptake tends towards an asymptote curve rather than following a typical decay curve. In order to visualise the effect that the accumulation of water-soluble metabolites of E_2 was having on overall uptake, predicted decay curves of [^{3}H]-E_2 if sulphation was not a factor (Figure 21 to Figure 25) together with actual data have been plotted. The predicted curves are based on extrapolating the relatively linear first three hours (or four hours in the case of Study 4) of exposure. The best example of the effect that sulphation has on uptake can be seen in Figure 25 - the difference between the amount of [^{3}H]-E_2 that was absorbed and what would have been taken in, had sulphation not not been a factor, is very high (ca. 50 % vs 95 % respectively). Since the differences become markedly significant only after around 5 h, it was concluded (from these studies) that the ideal exposure period for studying [^{3}H]-E_2 uptake and esterification in mussels is probably somewhere between 3 h and 6 h (i.e. experiments can be set up and completed within a working day).

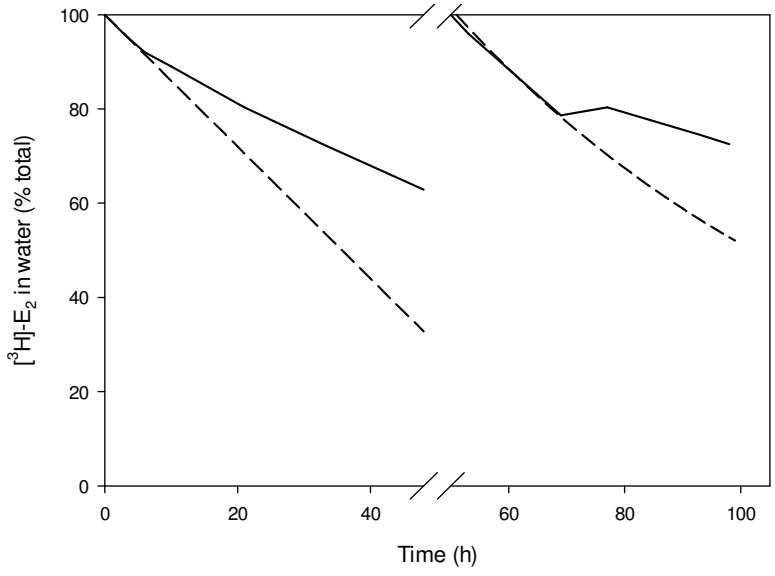

Figure 21: Predicted decay curves (based on the rate at 3 h being maintained for 48 h) (dashed lines) and actual decay curves (solid lines) of Study 1.

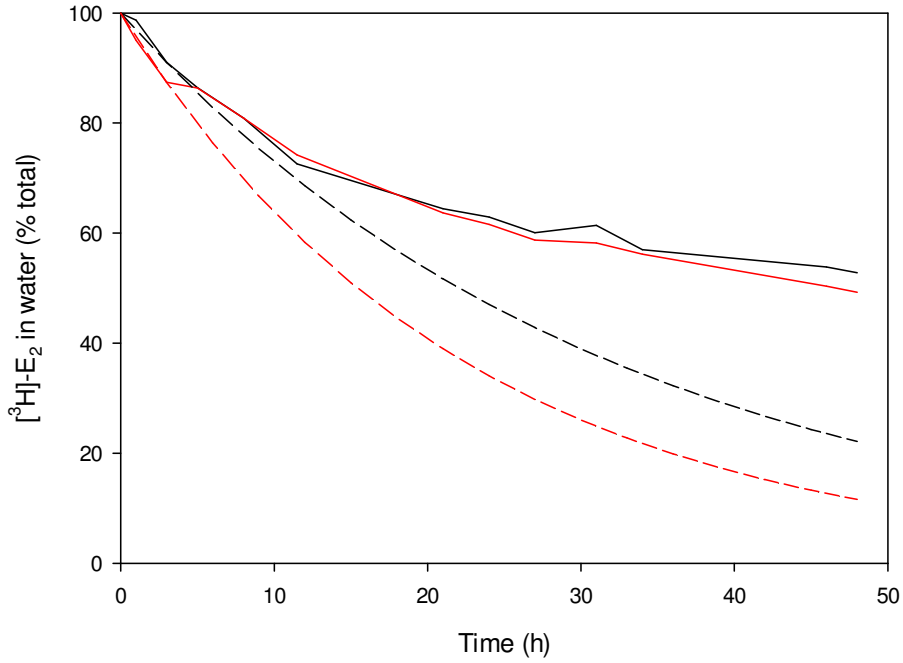

Figure 22: Predicted decay curves (based on the rate at 3 h being maintained for 48 h; dashed lines) and actual decay curves (solid lines) of Study 2 (feed: black and no feed: red).

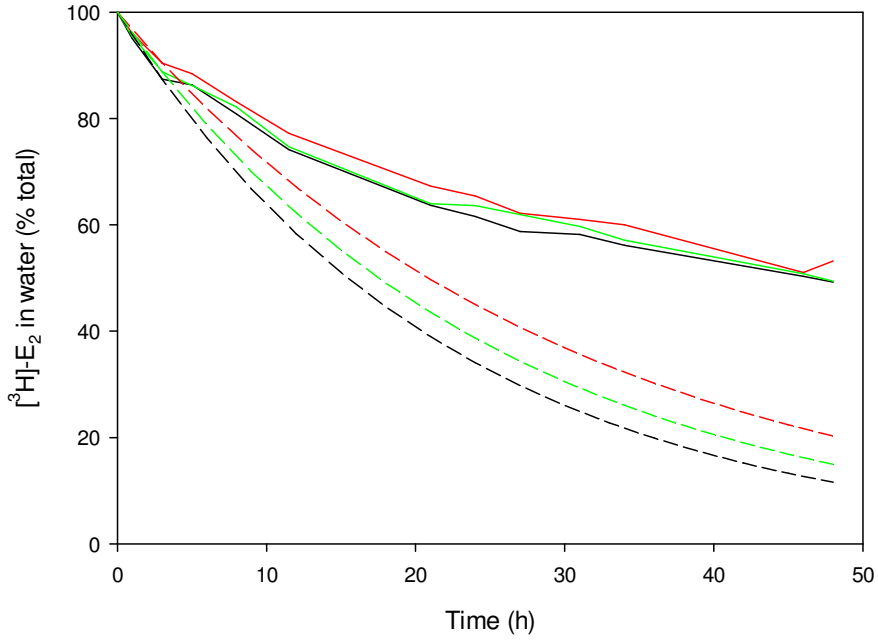

Figure 23: Predicted decay curve (based on the rate at 3 h being maintained for 48 h ; dashed lines and actual decay curves (solid lines) of Study 3 (no E_2: black; 7.1 ng L^{-1} E_2: red and 35.7 ng L^{-1} E_2: green).

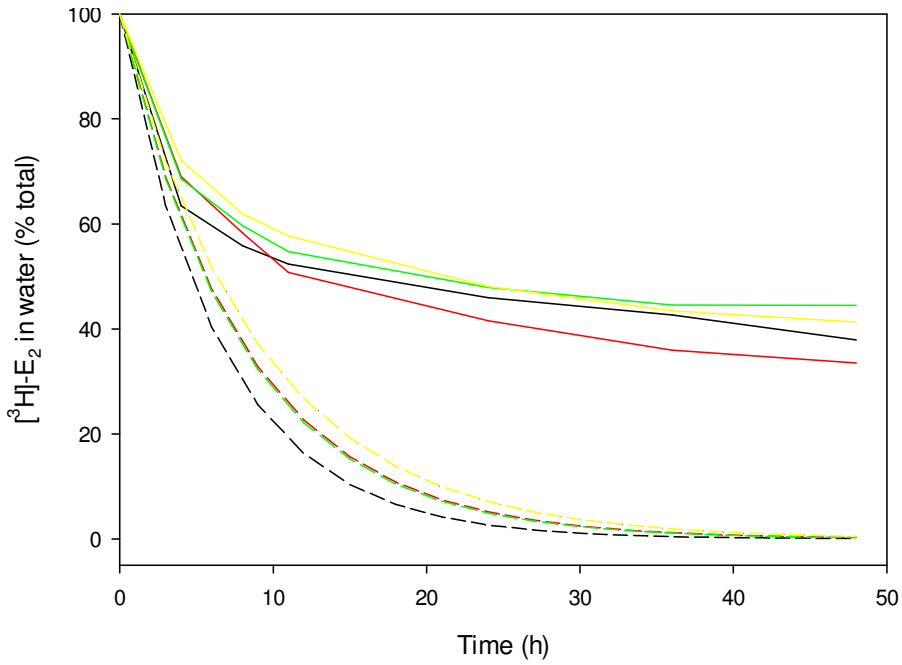

Figure 24: Predicted decay curves (based on based on the rate at 3 h being maintained for 48 h ; dashed lines) and actual decay curves (solid lines) of Study 4 (0 µg L^{-1} : black; 0.25 µg L^{-1}: red; 2.5 µg L^{-1}: green and 25 µg L^{-1}: yellow).

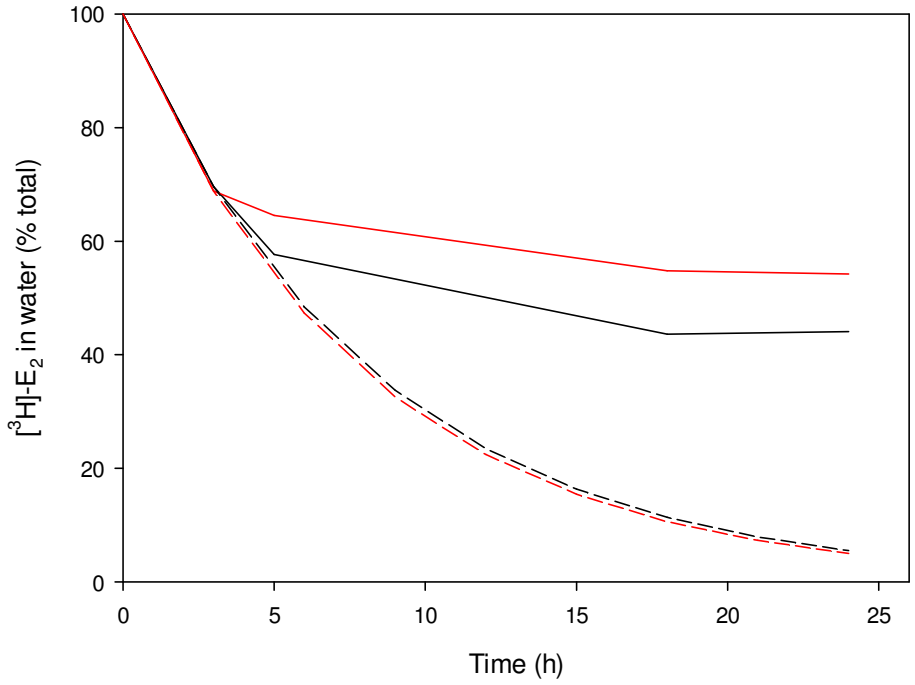

Figure 25: Predicted decay curves (based on the rate at 3 h being maintained for 24 h; dashed lines) and actual decay curves (solid lines) of Study 5 (exp. 1: black and exp 2: red).

Table 8: Summary of exposure, extraction and hydrolysis methods in the literature.

Species	Steroid	Exposure time	Depuration time	Extraction method	Number of steps	Method of hydrolysing esters	Study
Blue mussel, *Mytilus edulis*	E_2	10 days	-	Homogenised in 6 mL methanol, 7 mL dicloromethane:methanol (6:1 vv).	2	Methanol & KOH - 1 h , 80 °C	(Puinean et al., 2006)
Mediterranean mussel, *Mytilus galloprovincialis*	E_2	7 days	-	Homogenised in ethanol, 8 mL diethyl ether, 10 mL diethylether:methanol (4:1 vv) x 2.	3	Methanol & KOH - 3 h , 45 °C	(Janer et al., 2004a)
Blue mussel, *Mytilus edulis*	[^{14}C]-E_2 [^{14}C]-E_1	13 & 8 days	12 & 0 days	Homogenised in 5 mL methanol, 5mL dicloromethane:methanol (9:1 vv) x 2.	3	Methanol & KOH - 90 min , 80 °C	(Labadie et al., 2007)
Zebra mussel, *Dreissena polymorpha*	[^{14}C]-E_2	13 days	10 days	Homogenised in 5mL methanol x 2 and 3mL methanol:choloroform (1:2 vv).	3	Methanol & KOH - 40 min, 80 °C	(Peck et al., 2007)
Pacific oyster, *Crassostrea gigas*	[^{14}C]-E_2	2 days	-	Alkaline lysis with 3% sodium hydroxide and 4% alcohol for 3 h at 50 °C.	-	-	(Le Curieux-Belfond et al., 2005)
Mediterranean mussel, *Mytilus galloprovincialis*	T	5 days	-	Homogenised in ethanol, 2mL Ethyl acetate x 3.	3	Methanol & KOH - 3 h, 45 °C	(Fernandes et al., 2010)
Eastern mudsnail, *Ilyanassa obsoleta*	T	90 days	-	Homogenised in 1 mL ethanol, 1 mL ethyl acetate x3.	3	Methanol & KOH - 3 h , 45 °C	(Gooding and LeBlanc, 2004)
Eastern mudsnail, *Ilyanassa obsoleta*	[^{14}C]-T	16 hours	95 hours	Homogenised in ethanol, 3 mL ethyl acetate.	1	Enzymatic hydrolysis with Type H-1 *Helix pomatia* sulphatase	(Gooding and LeBlanc, 2001)
Sea slug, *Clione antartica*	[^{3}H]-P [^{3}H]-Ad	12 hours	-	Homogenised in 0.5 mL water, sonicated and incubated with HCl at 40 °C. After neutralisation with NaOH, steroids were extracted with 7 mL methylene chloride x3.	-	-	(Hines et al., 1996)

2.4.1.3 Do the animals need to be fed during [^{3}H]-E_2 uptake studies?

This question was set out to be answered very early in the study when it was still believed (on the basis of most previous studies; Table 8) that it was necessary to keep the mussels for long periods of time (possibly days) using multiple dosing. Obviously, if the exposure period is going to be < 6 h, then feeding is not an issue. Nevertheless, feeding during exposure to radioactive steroids could have enhanced the rate of uptake by increasing the filtration rates (reviewed by Jorgensen, 1996). Most other laboratory exposures of molluscs to steroids - E_2 and T - have been done in the absence of food (Gooding and LeBlanc, 2001; Peck et al., 2007) with a few exceptions, such as a study by Fernandes et al. (2010) where exposed animals were fed but there was no reference tank to compare them to. There was no significant difference in overall [^{3}H]-E_2 removal from water between treatments (Figure 11) and since these experiments were extremely short, concerns regarding weight loss and long term detrimental effects on health were not deemed important. Moreover, condition indices confirmed that indeed there was little detriment to animal health. The differences in CI between studies (Study 5 had a higher CI than Study 1-3) are likely due to differences in harvesting time (spring / autumn) or species differences (*M. edulis* vs mixed population of *M. edulis* / *M gallopronvinciallis*). All subsequent studies were performed without feeding during the exposure phase.

2.4.1.4 How can exposure experiments best be done in order to minimise contamination of equipment and personnel?

In order to minimise effort at the same time as optimising the results from the exposure experiments, several other changes in study design were made over the course of the study. The most important of these was the replacement of glass tanks by buckets lined with polythene bags. This did not seem to affect clearance rates nor non-specific adsorption of [^{3}H]-E_2. The biggest advantage was in not having to clean the tanks after every experiment. The bags could just be emptied and then safely disposed of.

2.4.1.5 'Rate of uptake' v. 'Clearance rate'

The rate of uptake is simply the rate at which the radioactivity disappears from the water. This is of course dependent on the number of animals and the amount of water. Thus the lowest rates were found in the first experiment in which the water volume was 2.8 L animal^{-1} and highest in the final two experiments in which the water volume was 0.4 L animal^{-1}. The clearance rate on the other hand (in the context of the present study) is the volume of water that is completely cleared of radiolabel in a given unit of time by a single animal. Hence, when it was stated that the clearance rate was 40 mL h^{-1} animal^{-1}, it was meant that, over the period of an hour, a single animal was able to absorb the amount of radiolabel that was originally present in 40 mL water. The actual concentration of radiolabel does not matter. It could be 1000 dpm mL^{-1} or 10,000 dpm mL^{-1}. In the latter situation, the animal will absorb ten times as much radiolabel, but in both cases, the clearance rate is the same. Similarly, clearance rate should be unaffected by the total volume of water. There was slight variation in the clearance rate between experiments. Since there was far less variation within experiments and there was no clear relationship to water volume or substrate concentration, it was speculated that this was due to inherent differences between the animals as evident from the differences in CI (that came from different sites and were tested at different times of the year) or possibly to differences in temperature. The experiment with the lowest clearance rate (Study 4) was the one that was carried out at the highest temperature. It must be noted that although clearance rates were based on the first 3 h of exposure, where radiolabel disappeared almost in a straight line, they are approximations as even within this short time period, the fall was curvilinear.

2.4.1.6 What is the best way of extracting not just free but also esterified and, potentially, glucuronidated and sulphated steroids from tissues?

There is a large range of procedures for extracting steroids from molluscan tissues (with some of them being mentioned in Table 8). One of the most popular procedures involves the homogenisation of tissue in dilute HCl followed by neutralisation with NaOH and then extraction of the steroids with the organic solvent dichloromethane (Ketata et al., 2007). However,

while probably suitable for free steroids, it was uncertain as to whether this would be suitable for esterified or sulphated steroids. Therefore only procedures that involved direct homogenisation of the tissues with organic solvents were looked at. In fact, extraction methods used by either Gooding & LeBlanc (2001) or Peck et al. (2007) were compared. Both yielded > 96 % efficiency, Method 2 recovered 97 % in two steps, while Method 1 only recovered 77 % in two steps and required further steps for satisfactory performance (Table 22). Extraction method efficiencies have been provided previously by quantifying the radioactivity in each extraction step, e.g. Gooding & LeBlanc (2001) recovered 95 % T radioactive residues using ethyl acetate and Labadie et al. (2007) recovered 97 % using dichloromethane. However, others have based efficiencies on spiked tissue homogenates using free steroid (labelled or cold) only, e.g. Gooding & LeBlanc (2004) recovered > 93 % of free radioactive T residues during extraction. It is now known that free, sulphated and esterified steroids fractionate differently as a result of distinct polarities and associations with tissue; these differences could in turn affect the performance of the method in question. On a note of caution, Method 1 did not work well for T or EE_2 extraction (see Chapter 4 and 6). Also, although Method 2 was effective for extracting E_2, P and 17,20ß-P, some but not all extracts tended to split into two phases during long-term storage in the freezer. It is not known yet if this might affect the long-term stability of the compounds.

2.4.1.7 What is the best way to separate free from esterified steroids?

It is in answering this question that it is felt the most important contribution to the study of steroid uptake and ester formation in molluscs has been made. As stated in the Introduction, most people do not attempt to separate free and esterified steroids prior to measuring them (they use subtraction, i.e. total minus free equals ester). In fact, some studies do not report even this difference and simply measure total steroid (i.e. free + esterified). Only a few researchers with a specific interest in radioactive steroids have attempted to separate them and in all such cases, the method of choice has been TLC. The major drawback of such methods is that they are time-consuming and expensive, limiting their value to studies

involving one steroid and / or a small number of samples. Furthermore, as shown, the chromatography conditions that are suitable for separation of free and esterified steroids are totally unsuitable for separating sulphated steroids. It was thus decided to investigate solvent partition by shaking the sample with equal amounts of heptane and 80 % ethanol; lipid removal was found to be relatively inefficient. However, if the amount of heptane was doubled and the shaking was carried out twice, then it was found that most if not all of the esterified E_2 could be separated from not just free E_2 but also from sulphated E_2. The procedure was much quicker than chromatography and there is no reason why over 50 samples could not be separated into free, ester and sulphate fractions in only one day.

Another area of uncertainty relates to the methods used to hydrolyse esters. Several are shown in Table 8. The commonest involves heating in KOH + methanol (though with a range of times and temperatures). One study used 'snail juice', which contains a range of different enzymes including not just sulphatase and glucuronidase activity but also esterase activity. For a while, it was also speculated that the enzyme subtilisin might be effective. This enzyme is used to break up the proteins in tissues so that small compounds can be more readily extracted. In a paper that had been sent to Dr Alex Scott for review in 2014, the authors had not only used this enzyme but also found unexpectedly large amounts of free T and E_2 (> 10 ng g^{-1} wet weight) in the tissues of the mussels they were working with. It was hypothesised that this was maybe because the enzyme was inadvertently hydrolysing steroid esters. Thus, with the authors permission (Hallmann et al., 2013; Zabrzańska et al., 2015), it was decided to test this. However, the results were negative (i.e. the subtilisin would not hydrolyse radioactive E_2 ester). The use of 'snail juice' was not investigated. However, it was shown that KOH / methanol hydrolysis at 80 °C (Table 23) was an efficient way of converting E_2-ester to E_2. The results of Peck et al. (2007) and Labadie et al. (2007) were also confirmed; that the radioactivity released by hydrolysis of the ester fraction had exactly the same elution position on HPLC as standard E_2.

2.4.1.8 Does metabolism (i.e. conversion to other steroids) play any role in the rate of uptake and esterification of vertebrate steroids by molluscs?

This has already been mentioned above in relation to the optimum time for carrying out exposure studies. It is now very obvious that any vertebrate steroids that animals come into contact with are going to be subject to metabolising enzymes. These need not necessarily be enzymes belonging to the animals but those belonging to bacteria, algae and other organisms that are associated with the animals (although there is no information on this). Although the existence of 'other pathways' has been acknowledged (e.g. bacterial degradation of steroids; reviewed by Ismail & Ru Chiang, 2012), their influence has to a great extent been disregarded in studies looking at the uptake and esterification of steroids.

Although the potential for steroid sulphate production by molluscs is already known (Janer et al., 2005b; Janer et al., 2005c), the scale of the formation in the present study was surprising. Not only were sulphates a significant proportion of the E_2 metabolites found in the tissue extracts, but also a large proportion of those found in the water after 24 h. Moreover, the water-soluble metabolite in the water was tentatively identified as E_2 sulphated via the 3-hydroxyl group, although it has to be made clear that the steroid attached to the sulphate group could be a metabolite of E_2 (e.g. E_1; this has not been investigated). The fact that some of the activity in the water did not stick to the C18 cartridges (assumed to be tritiated water) suggests that some reactions are going on and about the A ring, where the bulk of the tritium residues are attached. The fact that tritium residues can be removed from [^{3}H]-E_2 means that the some of the numbers calculated (e.g. clearance rate) are inherently inaccurate. If one needed to have more accurate measures of steroid dynamics, it would be better to use [^{14}C]-steroids. However, to answer the sorts of questions that have been asked in the present study, tritiated steroids were perfectly adequate at the same time as being safer and easier to work with.

2.5 Conclusion

This chapter presents a comprehensive method of studying the uptake (best exposure conditions) and metabolism of E_2 in mussels (chromatographic evidence of metabolites), including the separation of what are now distinct and well established metabolite fractions (i.e. esters, sulphates and free steroid). The novel separation method that was developed allows for integral quantification of all fractions, has high-throughput (> 50 samples a day) compared to chromatographic methods used before and grants the possibility of downstream applications (e.g. hydrolysis of the ester fraction).

Chapter 3

The fate of the radiolabelled 17β-oestradiol absorbed by the mussels.

3.1 Introduction

As argued in the previous two chapters, the presence of vertebrate steroids in molluscs is indisputable, furthermore, the fate of 17β-oestradiol (E_2) in mollusc tissue has been well characterised – at least in respect to its most abundant conjugated form, the lipophilic E2-fatty acid ester. Sulphation, on the other hand, which was far more abundant than the literature suggested, has been little investigated since it was first noted in the 1990s (Hines et al., 1996; Ronis and Mason, 1996), with the exception of a few recent *in vitro* studies that demonstrated that steroid sulphotransferase activity was present in the Giant Ramshorn snail, *Marissa cornuarietis* and the common mussel, *Mytilus edulis* (Janer et al., 2005b; Janer et al., 2005c; Lavado et al., 2006a). The method developed, described in Chapter 2, has provided definitive evidence of uptake of [^{3}H]-E_2 and a comprehensive analysis of the nature of all metabolites (sulphate, ester and free steroid) present in mussels.

Natural steroids are ubiquitous in nature (released by livestock, humans, fish and other vertebrates), and molluscs are able to absorb two of the most common of these, E_2 and testosterone (T), and convert them to lipophilic esters. So with this seemingly endless supply of steroids in the environment and the capacity of mussels to covalently bind to them, it appears important to ask: is there a limit to how much they can absorb and how much they can store? With definitive evidence that mussels readily pick up [^{3}H]-E_2 from water, another important question is, how long can they hold on to it? And if so, does it matter in what form the steroids are present? e.g. do esters persist in the tissue longer than free and sulphated steroids?

Labadie *et al.* (2007), is one of several studies that investigated the uptake and depuration of vertebrate steroids by molluscs, exposed *M. edulis* to radiolabelled [^{14}C]-E_2 for 13 days and reported that it was readily

bioaccumulated. When animals were placed in clean water (changed daily) for 10 days the E_2 burden decreased over time (half-life of 8.3 days). Peck *et al*. (2007) also investigated the uptake of [^{14}C]-E_2 – this time in the zebra mussel (*Dreissena polymorpha*) over 13 days. *D. polymorpha* picked up E_2 (> 550 bioconcentration factor) but there was no significant reduction in E_2 when animals were placed in clean water for 10 days. Gooding & LeBlanc (2001) exposed the eastern mudsnail, *Ilynassa obsoleta*, to radiolabelled T to elucidate its biotransformation within the tissue in order to identify possible sites by which TBT disruption could lead to imposex. They reported no detection of T excretion via polar metabolites. Instead the steroid was stored as five different highly lipid-soluble metabolites which showed only 4 % depuration over 96 h.

The few studies described above which have looked into depuration of E_2 and T present varying results from little or no depuration to quite a significant reduction. Regardless of these discrepancies (which could simply be down to the differences in experimental conditions and physiological state of the animals), the more pressing issue is the fact that all experiments have been carried out under semi-static conditions, i.e. the water was changed on a daily basis. Although insightful, these experimental conditions are not a true reflection of conditions in the industry, let alone nature. In the UK, bivalves for human consumption are categorised (Class A to D) based on their bacterial load (faecal coliforms and *Escherichia coli*). Bivalves from Class A waters can be harvested and sold for human consumption without treatment, Class B bivalves (the most common in England and Wales; http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf) however, must be purified (i.e. depuration in an approved plant), relayed (placed for two months in Class A waters) or undergo an approved heat-treatment process before being sold for human consumption. Depuration takes place under flow-through conditions in a recirculation system, where water passes through UV and organic matter is allowed to settle. The minimum depuration time for animals coming from Class B waters is 42 h. In this Chapter the fate of [^{3}H]-E_2 in mussels after exposure, the saturation limits - if any - and the effects of depuration, not only on the oestrogenic burden as a whole but on each of the steroid moieties (i.e. free, sulphated

and esterified), are investigated. A small study involving determination of E_2 concentrations in mussels collected from the wild – as measured by Radioimmunoassay (RIA) - is also included in this chapter. This was done in order to test the newly developed methodology for separating the three steroid moieties and to see whether the results matched (in terms of their ratios) the radiolabel uptake experiment findings.

3.2 Materials and Methods

3.2.1 Depuration

Following exposure to [^{3}H]-E_2 for two consecutive 48 h periods (see Chapter 2, section 2.2.2.1), mussels (*M. edulis)* from Study 1 were subjected to depuration in a flow-through system (1 L min^{-1}) for 20 days. Ten animals per day were sampled on day 0, 5, 10, 15 and 20 and stored at -20 °C immediately.

3.2.2 Extraction of steroids from tissue

Mussels from Studies 1, 2 and 4 were extracted using Method 1 (see Chapter 2, section 2.2.5.2).

3.2.3 Separation of free, sulphated and lipophilic [^{3}H] E_2 metabolites (optimised in Chapter 2)

Radioactive metabolites were separated as described in Chapter 2, section 2.2.7.1 and 2.2.7.2.

3.2.4 E_2 metabolite separation and quantification

3.2.4.1 Separation of lipophilic metabolites and chemical hydrolysis

Crude extracts were separated as described in 3.2.3. The heptane fraction was then dried at 40 °C under a stream of nitrogen and re-suspended in 1.8 mL methanol and 0.2 mL 3 M KOH for chemical hydrolysis. The sample was incubated in an oven at 80 °C for 40 min (mixed briefly with a vortex after 20 min) and the reaction was stopped by adding 40 µL 2.5 M HCl. The freed radiolabel was separated from any untransformed radiolabel by adding 0.21 mL water and 4.5 mL heptane to the hydrolysate. It was vortex-mixed for 5

min and centrifuged for 10 min at 2500 g. The top heptane layer was discarded and the process was repeated once more. A control sample was also used, consisting of [^{3}H]-E_2 ester that had been previously purified by heptane partitioning of a pool of extract from [^{3}H]-E_2-treated mussels. The ethanol phase (containing > 95 % of the now free E_2) was dried and re-suspended in RIA buffer and stored at -20 °C.

3.2.4.2 Separation of free and sulphated steroid

The ethanol fractions (presumed to contain free and sulphated E_2) from the crude extract separation (3.2.4.1) were dried under a stream of nitrogen at 40 °C and re-suspended in 100 µL of water before adding 4 mL diethyl ether. Samples were vortex-mixed for 5 min and centrifuged for 10 min at 2500 g. The water phase was frozen with liquid nitrogen and the ether was decanted into a clean tube. Both layers were dried at 40 °C under a stream of nitrogen – the ether fraction was re-suspended in RIA buffer and stored at -20 °C and the water fraction underwent acid solvolysis (see 3.2.4.3).

3.2.4.3 Acid solvolysis of sulphated steroid

The dry water fraction from the above water / ether partition (3.2.4.2) was re-suspended in 4 mL ethyl acetate:trifluoroacetic acid (100 mL:1.4 mL, v:v) and incubated at 40 °C overnight (Scott et al., 2014). The solvents were removed by drying at 40 °C under a stream of nitrogen. The now free steroids were removed by partitioning between 1 mL water and 4 mL diethyl ether (3.2.4.2). The ether fraction was dried overnight at room temperature, RIA buffer was then added and the sample was stored at -20 °C.

3.2.4.4 Quantification by RIA

All samples, re-suspended in RIA buffer, were diluted appropriately (based on expected values) and submitted to RIA.

The E_2 RIA is a well-established method (Scott et al., 1980) designed to detect low levels of E_2 in a highly specific manner. It makes use of the competition of radiolabelled E_2 with cold E_2 (standards and unknowns) for

limited binding sites on E_2 antibody. After an overnight incubation, unbound steroid is separated from bound steroid using dextran-coated charcoal, and the bound fraction is mixed with scintillation fluid and counted.

RIA buffer was comprised of 8 g L^{-1} NaCl, 5.8 g L^{-1} Na_2HPO_4, 2 g L^{-1} Bovine Serum Albumin, 1.3 g L^{-1} $NaH_2PO_4.H_2O$, 0.3 g L^{-1} EDTA and 0.1 g L^{-1} sodium azide (pH 7.6). The Charcoal separating solution was made by dissolving 0.5 g gelatin, 0.5 g dextran and 2.5 g charcoal in the same buffer as above but without the NaCl, BSA or sodium azide. RIA buffer and charcoal solution were made the day before the assay was set up and stored at 4 °C.

Borosilicate glass tubes were labelled in duplicate for all samples, controls and standard curve. Three tubes were labelled 1 to 3 for preparation of the standard curve. The dilution was performed as detailed in Table 9.

Table 9: Details for the serial dilution of RIA standard curve

	RIA Buffer (µL)	Transfer	Dilution	Concentration (pg μL^{-1})	Pg $tube^{-1}$
Stock		20 µL to 1		500000	
1	2000	20 µL to 2	0.01	4950	
2	2000	100 µL to 3	0.01	49	
3	400	100 µL to S1	0.2	9.8	
S1	100	100 µL to S2	0.5	4.9	490.1
S2	100	100 µL to S3	0.5	2.5	245.1
S3	100	100 µL to S4	0.5	1.2	122.5
S4	100	100 µL to S5	0.5	0.61	61.3
S5	100	100 µL to S6	0.5	0.31	30.6
S6	100	100 µL to S7	0.5	0.15	15.3
S7	100	100 µL to S8	0.5	0.077	7.7
S8	100	100 µL to S9	0.5	0.038	3.8
S9*	100	100 µL to S10	0.5	0.019	1.9
S10	100	100 µL to S11	0.5	0.0095	0.95
S11**	100	100 µL discard	0.5	0.00475	0.475

*** S9 for E_2 standard curve.**

**** S11 for EE_2 standard curve (see Chapter 4)**

Samples were diluted appropriately in RIA buffer - ensuring they were within the detection range of the standard curve – and distributed into tubes (100 µL per tube). To make the reagent master mix (cocktail), an

appropriate amount of RIA buffer (calculated on the basis of every tube receiving 100 µL) was mixed with enough [^{3}H]-E_2 and antibody to achieve approximately 6000 dpm 100 µL^{-1} and approximately 50 % binding of radiolabel in the absence of added cold steroid ('Maximum Binding'; M). The blank control (B) was spiked with 100 µL of the cocktail before the antibody was added. All other tubes were spiked with 100 µL of the full mixture, starting with the standard curve, total control (T) and M. A summary of the controls is detailed in Table 10. The tray was shaken gently and placed at 4 °C overnight.

After overnight incubation, 1 mL of charcoal was added to all tubes except T (1 mL RIA buffer was added instead). The tubes were incubated on ice for 28 min, before being centrifuged at 2500 g for 12 min. The contents of each tube were decanted into labelled scintillation vials and 7 mL scintillation fluid was added to each one. The vials were shaken, placed on a scintillation counter (Beckman LS 6500 multipurpose scintillation counter) and counted for 5 min each. The data was then analysed in a spreadsheet that compared the percent bound present in unknown samples with the percent bound present in standard tubes.

Table 10: Summary of the contents and purpose of the control tubes.

	Maximum (M)	**Total (T)**	**Blank (B)**
RIA buffer	100 µL	100 µL	100 µL
RIA Buffer & label	-	-	100 µL
RIA buffer, label & antibody	100 µL	100 µL	-
RIA buffer (day 2)	-	1 mL	-
Charcoal	1 mL	-	1 mL
Control information	Maximum binding of label in absence of any unlabelled steroid.	Total amount of label, because it all stays in tube due to lack of charcoal.	Label is removed since there is no antibody.

3.2.5 Field survey

3.2.5.1 Collection

Caged mussels (*Mytilus* spp.) from three assumed 'polluted' areas from the Thames (Gravesend, Southend-on-Sea and Chatham), a reference site at Norfolk (Morston, a shellfishery) and an offshore site (Wharp) (see Figure 26 for sites) were kindly caged *in situ* and collected six weeks later by Dr Tim Bean (Weymouth Laboratory) during different investigations. On the day the animals were harvested, a small sample of them was sent to Cefas laboratory in a cool-box. They were frozen at -20 °C upon arrival.

Figure 26: Map pinpointing the locations of the caged mussels.

3.2.6 Statistics

Figures were produced in Sigmaplot (Systat Software Inc, TW4 6JQ, London, UK.) and statistical analyses were performed using R statistical software (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.).

Decay curves were transformed (square root or log transformation) in order to achieve a linear relationship before applying a linear regression model. Welsh's T test was used to compare condition indices observed between two treatments. Analysis of co-variance (ANCOVA) was used when comparing differences in steroid uptake over time between three or more treatments; when comparing uptake during the first three hours of exposure, time was treated as a factor. To work out where the differences were after performing an analysis of variance (ANOVA) of steroid levels during depuration, pairwise comparisons were employed (t-test adjusted using Bonferroni correction).

3.3 Results

3.3.1 Metabolite profile of [^{3}H] E_2 and E_2 in mussels

In Chapter 2, a method to separate esters, sulphates and free E_2 was developed in order to better understand the distribution of metabolites in *Mytilus* spp. This method was applied to tissue extracts from studies 1 and 2 (Study 3 animals were not extracted as there was no evidence of E_2 saturation and Study 4 is discussed in section 3.3.1.1). Figure 27 and Figure 28 show the relative percentages of each of these metabolites. Fatty acid esters accounted for > 80 % of the total radioactivity in both studies and there was no significant difference in the percentage of esters between animals that were fed and those that were not (Study 2; Welsh's t-test: t = -1.542, df = 26.964, p = 0.135). Note that feeding had no effect on the [^{3}H]-E_2 uptake rate either (Figure 11 in Chapter 2). It was not possible to compare (statistically) the sulphated and free steroid portion between treatments as the extracts were pooled.

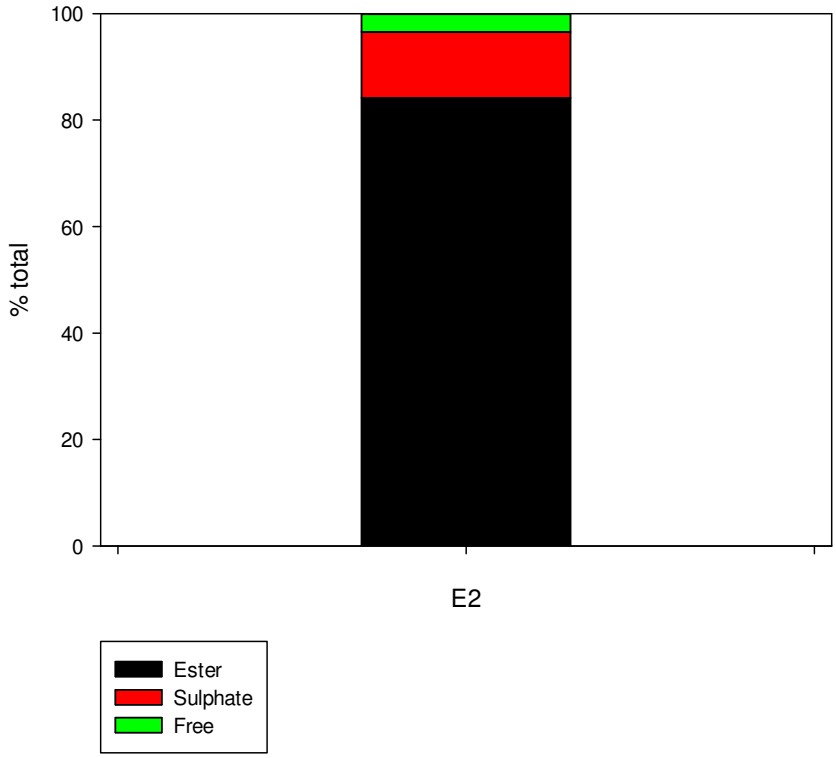

Figure 27: Proportion of radioactivity (as either free, sulphate or ester) in *Mytilus* spp. tissue extracts after exposure to [^{3}H]-E_2 for 96 h. Data are presented as mean percentage total (n = 10) for ester. Animals were pooled to estimate the proportion of sulphate and free.

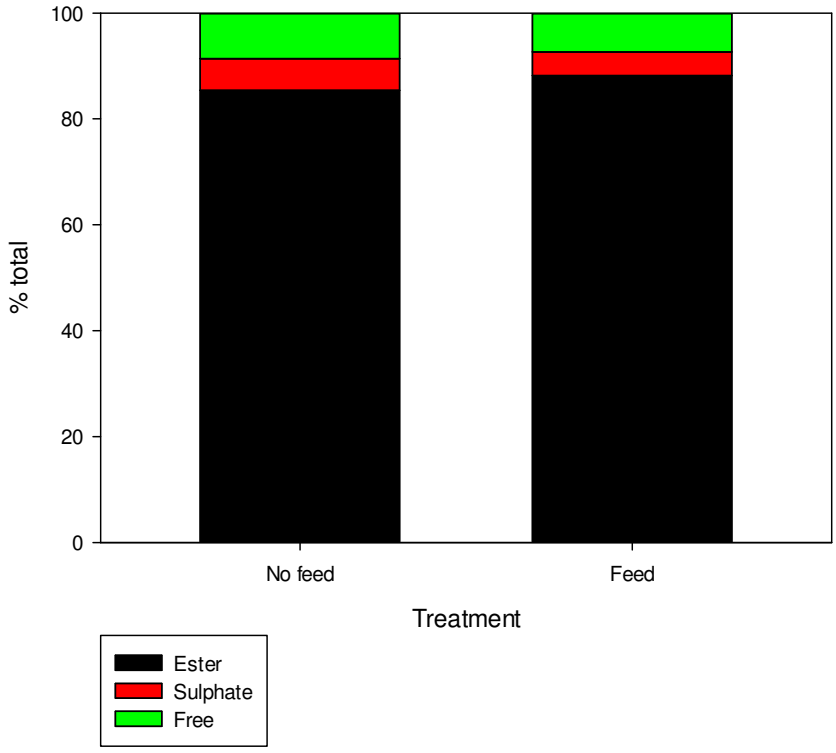

Figure 28: Proportion of radioactivity (as either free, sulphate or ester) in *Mytilus* spp. tissue extracts after exposure to [^{3}H]-E_2 for 48 h in the presence or absence of food. Data are presented as mean percentage total (n = 15). Animals were pooled to estimate the proportion of sulphate and free.

3.3.1.1 Can esterification and sulphation be saturated?

As discussed in Chapter 2, the rate of uptake of [^{3}H]-E_2 does not appear to be saturated even after the addition of micrograms of cold steroid to the water. However, the question to answer here is related to the metabolic pathway capacity within the animals; is there a saturation point at which esterification and sulphation of E_2 become overloaded? The extracts from mussels in Study 4 (that had been exposed to both hot and cold E_2) were all separated and radioactivity measured as described above. Treatment explained a significant amount of the variability observed in the proportion of E_2 esters between animals ($f = 5.028$, $df = 3$, $p = 0.006$). Pairwise comparisons showed treatment B to be significantly higher than A ($\beta = 11.0$, $SE = 3.424$, $t = 3.212$, $P = 0.003$); however, treatment C and D did not differ significantly from A ($\beta = -1.133$, $SE = 3.518$, $t = -0.322$, $P = 0.749$ and $\beta = 2.450$, $SE = 3.632$, $t = 0.675$, $P = 0.505$ respectively). So although B differed significantly from A, it does not appear to be physiologically relevant as C and D would be expected to differ too (and in fact have a greater effect, as they had higher doses of cold E_2). Like for Study 2, it was not possible to statistically compare the proportions of sulphated and free steroids as the extracts had been pooled. To summarise, there was no hard evidence that the free:sulphate:ester (F:S:E) ratio was affected by the amount of E_2 that had been added to the water. To establish the nature of the E_2 that had been either esterified or sulphated, some of the heptane phase was hydrolysed in order to release free steroid. A part of the 80 % ethanol phase, after removing any free steroid using water:diethyl ether, was also subjected to acid solvolysis. All three fractions and water samples (see section 3.3.1.2) were then radioimmunoassayed for E_2 (see Figure 29 for both E_2 and radiolabel residue esters). If all radioactivity represented the parent compound, then the steroid levels measured by RIA should show the same amounts as the radioactivity (see Figure 30 for both radioactive and immunoassay metabolite profiles). The discrepancy between metabolite profiles seen in Figure 30 implies that not all the radioactivity within the animal tissue represents intact E_2. For example, in treatment C, the radioactive metabolite profile indicates that approximately 20 % of [^{3}H]-E_2 should be sulphated. However, there was actually < 2 % E_2 when using immunoassay; suggesting that the 80 % ethanol phase contains

metabolites of E_2 rather than intact E_2 (with the metabolites being presumed to be sufficiently different in structure that they failed to cross-react with the E_2 antibody).

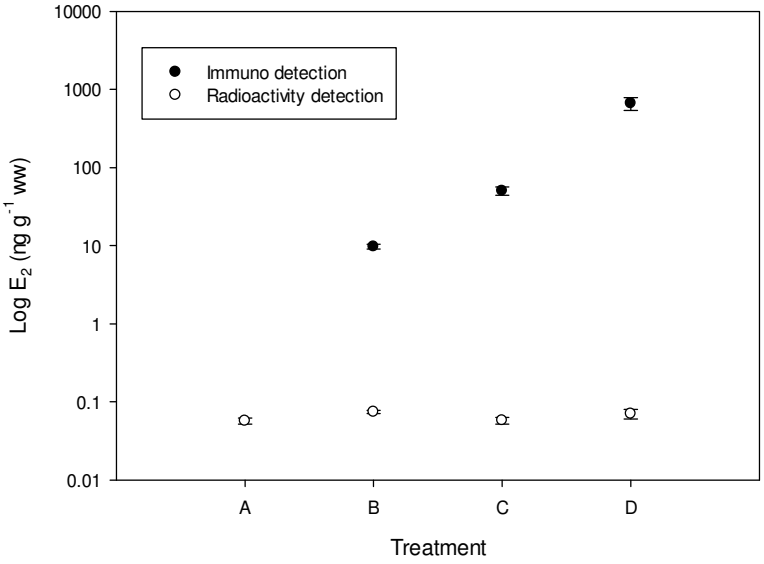

Figure 29: [^{3}H]-E_2 (○) and cold E_2 (●) esters in *Mytilus* spp. tissue after exposure to the same amounts of radiolabelled E_2 but differing amounts of cold E_2. Radiolabelled E_2 data are presented as mean ng g^{-1} wet weight E_2 ester equivalents ± S.E.M. Cold E_2 data are presented as mean ng g^{-1} wet weight E_2 esters ± S.E.M. For both data sets n = 10 animals (from 5 replicate tanks), except treatment D where n = 8 (from 4 tanks).

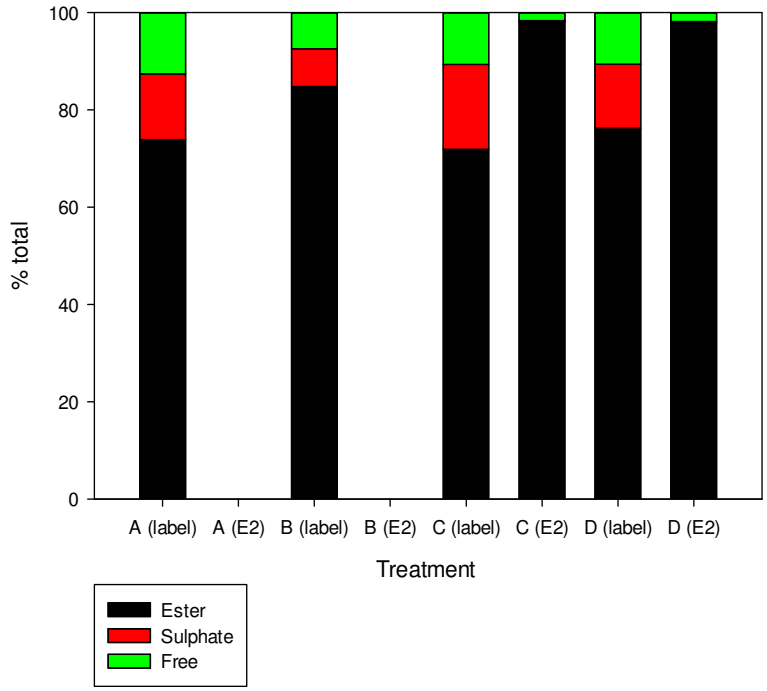

Figure 30: Metabolite profile of [^{3}H]-E_2 (label measured by scintillation counting) and E_2 (measured by RIA) in *Mytilus* spp. tissue after exposure to [^{3}H]-E_2 and increasing amounts of E_2 for 48 h (A: 0 µg L^{-1}: B: 0.25 µg L^{-1}; C: 2.5 µg L^{-1} and D: 25 µg L^{-1} nominal concentrations). Data are presented as mean percentage total (n = 10 for ester; n = 2 pools of five animals each for sulphated and free).

3.3.1.2 What is happening in the water?

In Chapter 2 it was noted that most of the radioactivity left in the water after 24 h was present as water soluble metabolites and there was very little, if any, intact E2 (Figure 15 in Chapter 2). Section 3.3.1.1 took this further, showing that, within the animal, E2 had been transformed into presumed sulphates (based on the radioactive metabolite profile) but that these were not made of intact E2 anymore (and therefore were not detectable by RIA). Table 11 presents an integral view of the differences between radioactivity and E2 in the study as a whole, i.e. how many nanograms of E2 were expected - based on radioactive proportions – and how many nanograms were actually found in the water or tissue. For example, according to the amount of radioactivity left in tank water at the end of the exposure in treatment C (44.5 % -see Table 7 in Chapter 2), one would expect to find 557 ng $tank^{-1}$ (expected value = nominal cold E_2 x percentage radioactivity left in tank / 100) but instead 109 ng $tank^{-1}$ was found by RIA. This amount just about matches the proportion (10 %) of radioactivity found in the elution position of E_2 after rp-HPLC of water extracts (Figure 15 in Chapter 2); suggesting that the unaccounted ~400 ng $tank^{-1}$ was present as sulphates of E_2 metabolites which the RIA antibody would be unlikely to cross-react with. It should be noted that the actual concentrations of E_2 found in the sorption control tanks (i.e. with no animals) matched very closely the expected values, i.e. nominal concentrations (Figure 31).

Table 11: Cold E_2 metabolite distribution from Study 4 and expected values based on radioactive profile. Data are presented as mean values.

Treatment	E_2 origin	E_2 in water (ng $tank^{-1}$)	E_2 in animals per tank (ng 2 $animals^{-1}$)	Unaccounted E_2 (ng $tank^{-1}$)[a]
A	Expected	-	-	-
	Actual	<LOD	11.0	-
B	Expected	51.9	70.2	35.0
	Actual	<LOD	71.6	83.3
C	Expected	556.9	627.3	68.5
	Actual	109.0	324.9	825.3
D	Expected	8138.9	7578.5	3468.0
	Actual	1119.8	4008.9	14141.1

[a] **Includes sorption.**

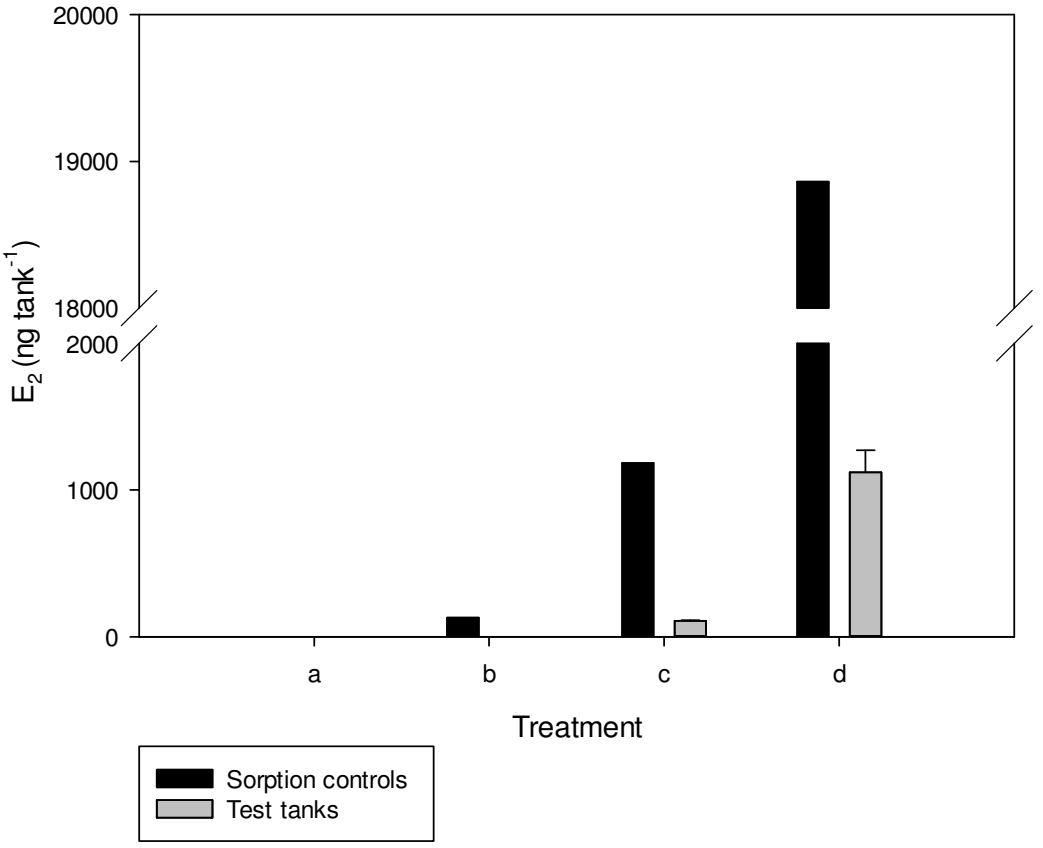

Figure 31: E_2 present in water in control tanks (black) and test tanks with *Mytilus* spp. (grey) after 48 hours exposure to [^{3}H]-E_2 in the presence of increasing amounts of cold E_2 (A: 0 µg L^{-1}; B: 0.25 µg L^{-1}; C: 2.5 µg L^{-1} and D: 25 µg L^{-1} nominal concentrations). Data are presented as mean total ng $tank^{-1}$ E_2 (n = 5).

3.3.2 Can mussels readily release the [^{3}H]-E_2 they have taken up?

So far it has been shown (in Chapter 2 and 3) that *Mytilus* spp. are able to pick up a large amount of E_2 and store most in the form of esters. The next question is whether mussels are able to release the [^{3}H]-E_2 that they have accumulated? Furthermore, does [^{3}H]-E_2 esterification and sulphation affect the rate of depuration? Do for example fatty acid esters persist longer than sulphates and / or free steroids? To investigate this, animals were placed in flow-through systems for up to 20 days after exposure to [^{3}H]-E_2 and sampled at regular intervals (every 5 days). Figure 32 shows that that there was a significant decrease ($f = 8.203$, $df = 4$, $p < 0.001$) in the total radioactivity over time (reduction of approximately two thirds). Pairwise comparisons of total [^{3}H]-E_2, using T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were significant differences between day 0 and all the other time points ($p = 0.014$, 0.007, < 0.001, < 0.001 respectively). This means that the largest

reduction in [^{3}H]-E_2 occurred between day 0 and 5, after which depuration slowed down. This was the case for both the ester and free / sulphate fractions, as seen in Figure 32. It is interesting to note that esters at time 0 showed the highest rate of variation (S.E.M) between animals, whereas the sulphate / free metabolites showed little to no variation.

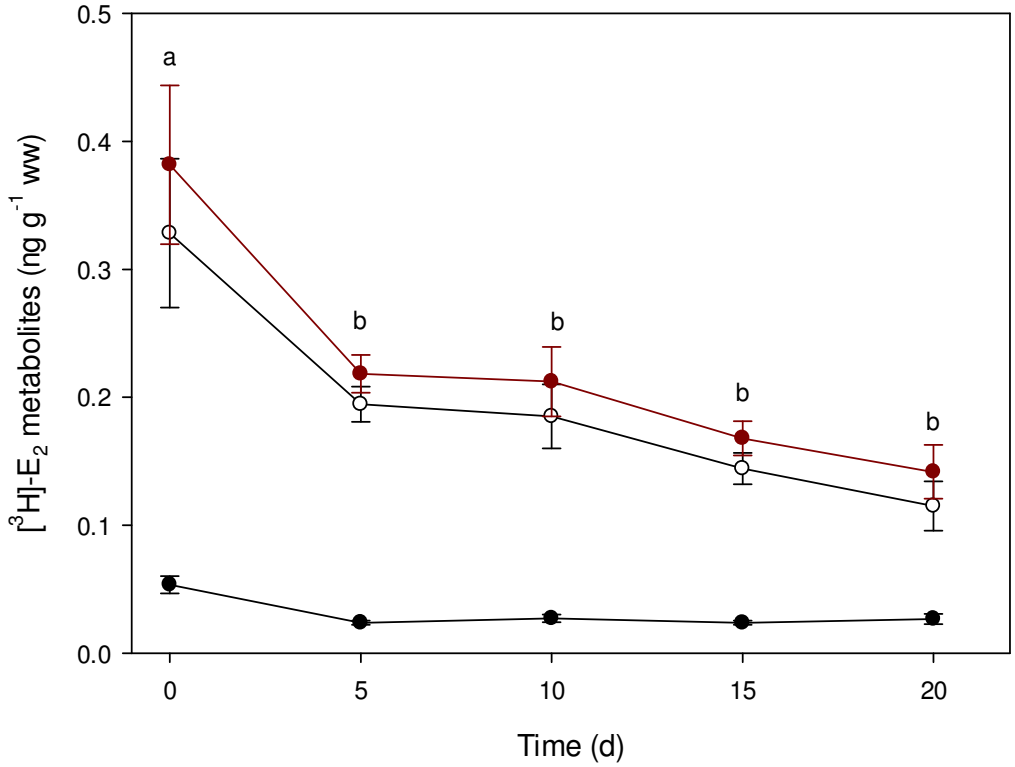

Figure 32: Free / sulphate fraction (●), ester fraction (○) and total [^{3}H]-E_2 (●) in mussel tissue during depuration after exposure to [^{3}H]-E_2 for 96 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-E_2 residues (n = 10); and points with a different letter ('a' or 'b') are statistically significantly different from each other ($p < 0.05$).

3.3.3 Field survey

It was important to test the applicability of the extraction and separation method developed in Chapter 2 and 3 using field-collected samples. Esters, sulphates and free E_2 were measured by RIA in four mussels from each cage deployment site (Figure 33). E_2 was easily quantifiable in the ester fractions (with most extracts needing to be diluted in order to fall on the steep part of the standard curve); satisfactory (with undiluted extracts falling on the steep part of the standard curve) in the free fractions; and 'barely detectable' (close to the detection limit of the standard curve of 1.95 pg) in the sulphate fraction (Figure 35). The amount of ester varied considerably between animals (cf. the variation in ester fraction of radioactive E_2 at time 0, Figure 32). There was no obvious sign of

differences between sites (Figure 34), although a much bigger sample size is needed to draw meaningful conclusions. These data show, however, that esterified E_2 is present ubiquitously at detectable levels in mussels regardless of location (e.g. E_2 is present in the reference site as well as 'polluted' sites).

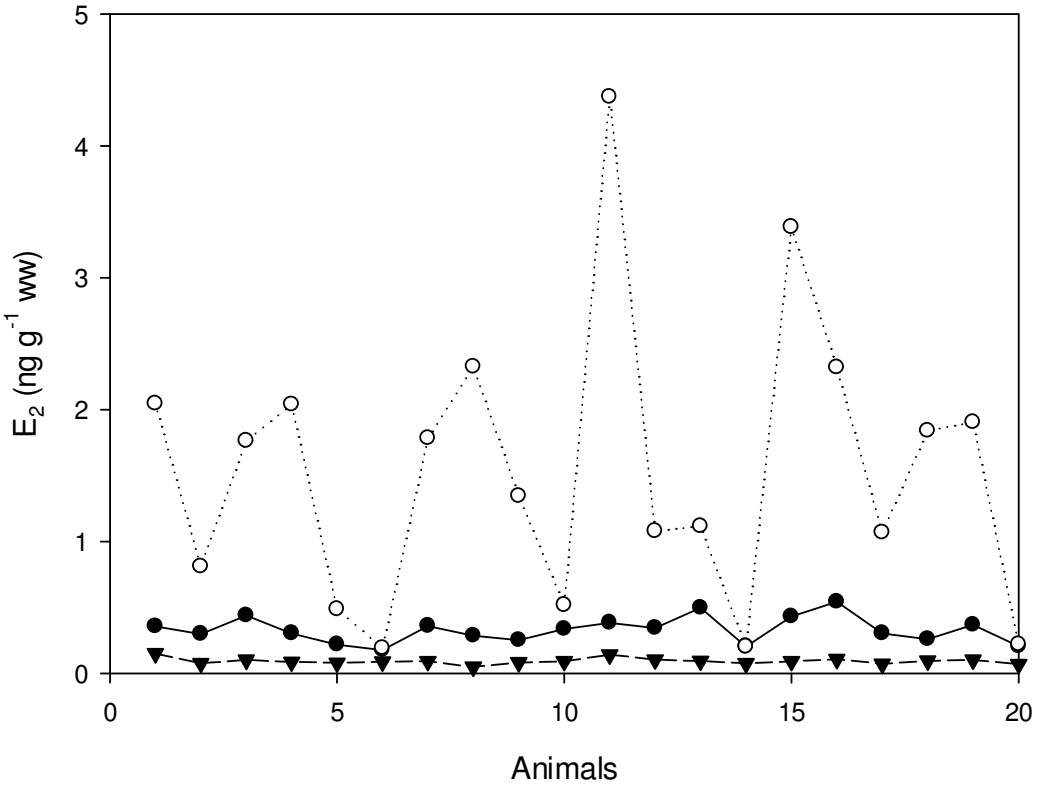

Figure 33: Immunoreactive E_2 distribution in *Mytilus* spp. held in cages in the wild. E_2 ester (○), free E_2 (●) and E_2 sulphate (▼) were measured separately in field mussel extracts taken from different locations. Data are presented as ng g^{-1} of E_2 ester, free or sulphated for each individual animal.

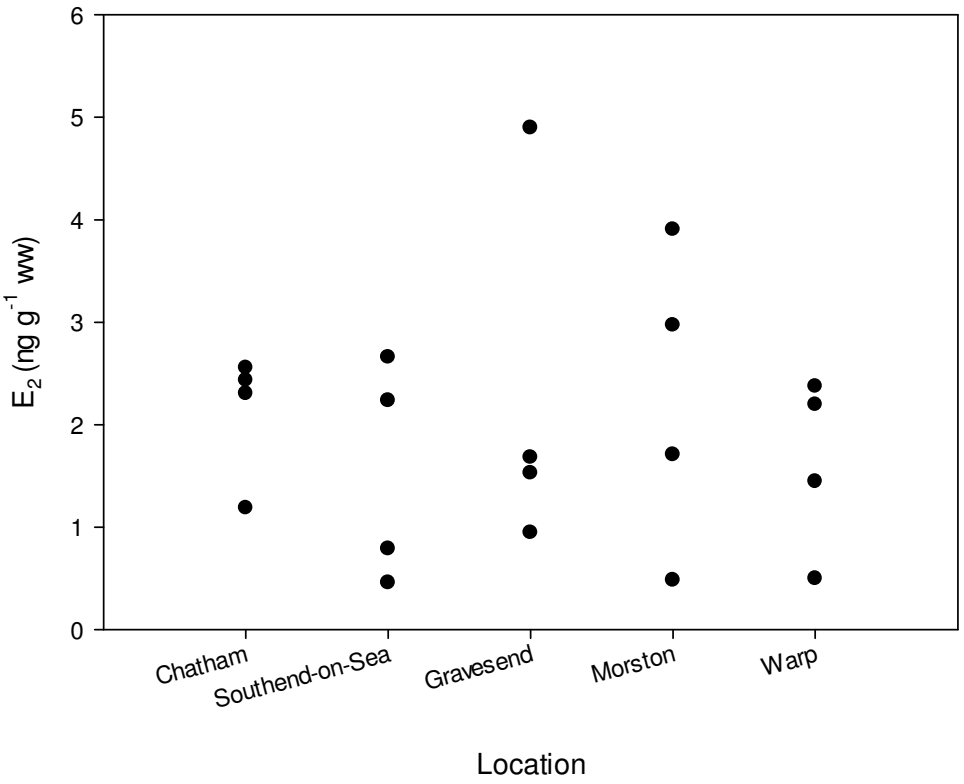

Figure 34: Total immunoreactive E_2 burden in field *Mytilus* spp. Tissue was extracted and submitted to three treatments (hydrolysis, solvolysis and extraction) before measuring E_2 via radioimmunoassay. Data are presented as total ng g^{-1} E_2 wet weight of four individual animals per location (n = 1).

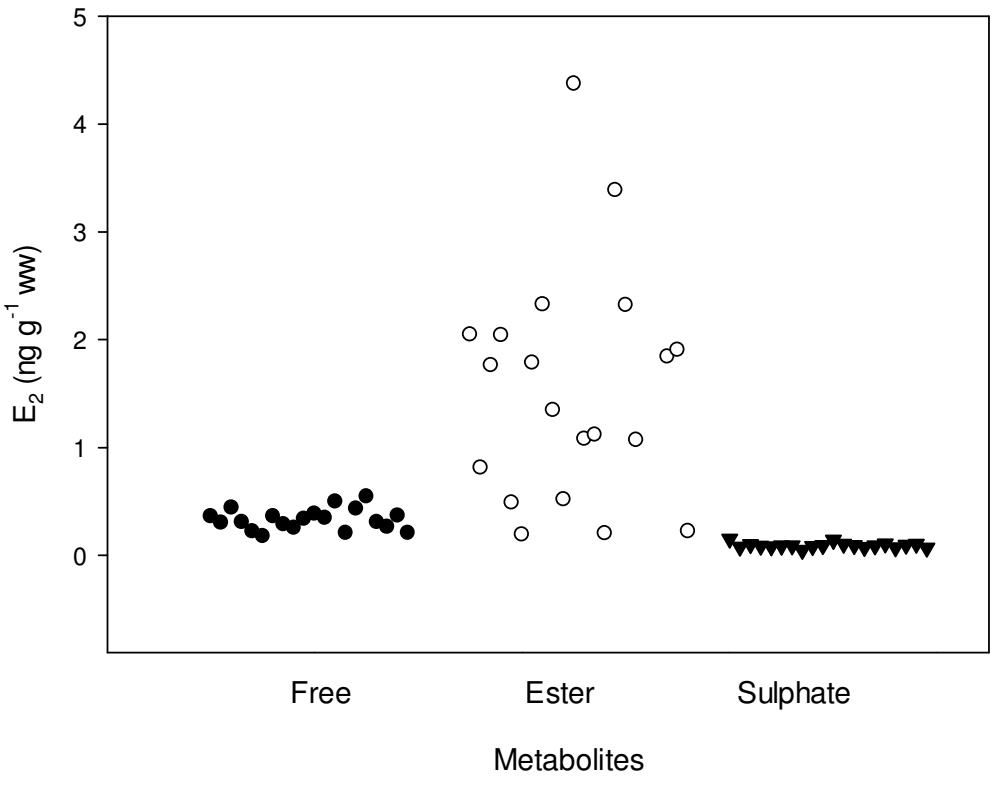

Figure 35: Different forms of immunoreactive E_2 in caged mussels from the Thames estuary (Chatham, Southend-on-Sea, Warp, Gravesend and Morston). Data are presented as ng g^{-1} ww of E_2 (n = 20).

3.4 Discussion

3.4.1 What happens to [^{3}H]-E_2 in *Mytilus* spp.?

The method developed in Chapter 2 allowed for definitive proof that [^{3}H]-E_2 was esterified by *Mytilus* spp. Over 70 % of radioactivity that was extracted from the flesh was found in the ester fraction; similar to results that have been reported in the literature. For example, Janer *et al. (*2004) found that most E_2 was present in the form of fatty acid esters (> 78 %) after *M. galloprovincialis* had been exposed to a range of concentrations of E_2 (20,200, and 2000 ng L^{-1}) for 7 days. It should be noted that esterification of E_2 increased in a concentration dependent manner but the proportion of free steroid did not. Several other studies report similar proportions of E_2 esterification (Fernandes et al., 2009; Lavado et al., 2006b; Peck et al., 2007). These studies, however, have only characterised the free and esterified E_2 by means of subtraction after hydrolysis or by using chromatography. No studies to date have characterised the fractions by separating the metabolites before quantifying them, let alone investigated the sulphated fraction, as done in these studies. In fact, after the identification of steroid sulphates in the 1990s, little to no work has been done regarding water soluble steroid metabolites formed in molluscs; apart from the demonstration that they contain the necessary enzymatic capability (Janer et al., 2005b; Janer et al., 2005c; Lavado et al., 2006a). As seen in Chapter 2, although they do not account for much of what is in the animal, they play an important role in the uptake of E_2 in a closed system (i.e. as sulphation of E_2 increases, it accumulates in the water and thus probably prevents any more E_2 being taken up and turned into ester).

Once the ratio of F:S:E was established, it was important to investigate whether any of these enzymatic processes could be altered by external factors. These findings suggested that availability of food made no difference to metabolite distribution. This reaffirmed that published results were not skewed by the lack of feed during exposures and that any differences among them were probably, instead, a result of the size / age, sex and the metabolic state of the animals used.

3.4.1.1 Is the rate of esterification affected by the actual amount of steroid to which the animals are exposed?

There is little doubt that molluscs have a large capacity for steroid ester formation. Gooding & LeBlanc (2004) exposed *I. obsoleta* to T at a concentration of 5.6 mg L^{-1} for 90 days and managed to get them to incorporate over 1000 times more in the form of immunoreactive T ester than untreated snails (27 µg g^{-1} v. 84 ng g^{-1}). In another study, Fernandes *et al.* (2010) exposed *M. galloprovincialis* to T at a concentration of 2 µg L^{-1} for five days and got the animals to incorporate ca. 300 ng g^{-1} in the form of immunoreactive T ester. Janer *et al.* (2005b) also exposed *M. galloprovincialis* to E_2 at a concentration of 2 µg L^{-1} for seven days and got them to incorporate ca. 300 ng g^{-1} in the form of immunoreactive E_2 ester. What has not been clear from these studies is whether there is a limit to the 'rate' of esterification of steroids. The fact that animals in these experiments were exposed for between five and ninety days implies that the animals needed a lot of time to accumulate microgram quantities of steroids. Thus, when the experiments with mixtures of cold and radioactive steroids were set up in the present study, strong evidence of saturation of the uptake of radioactive E_2 was expected. However, E_2 was incorporated in the ester fraction at a maximum concentration of 1.3 µg g^{-1} (with a mean of 0.7 µg g^{-1} ww, n = 10) in 48 h when using up to 25 µg L^{-1} of cold E_2 (an order of magnitude higher than in the experiment by Janer *et al.*, 2005b). There was no effect on the ratio of F:S:E. It should be mentioned here that, in the wild, the animals will be exposed for most if not all of their life to concentrations of steroids in the pg to low ng L^{-1} range. Thus the fact that they have the capacity to process steroids in the µg L^{-1} range serves to put the efficiency of this process into perspective.

Measuring the radioactive E_2 metabolites and the immunoreactive E_2 metabolites highlighted a discrepancy in the F:S:E ratios using the two different procedures. It turns out that the radioactivity measured in each fraction does not necessarily represent intact E_2, in fact most of the presumed sulphates and free steroid present in the 80 % ethanol fraction appeared to be something other than E_2. The cold E_2 metabolite ratio presented in Figure 29 is closer to that reported in the literature for unlabelled steroids. For example, Janer *et al.* (2006) found that 98 % of E_2

found in wild *M. cornuarietis* was esterified; and Abidli *et al.* (2012) reported that over 90 % of T and E_2 in two different gastropods (*Hexaplex trunculus* and *Bolinus brandaris*) were present as esters.

3.4.1.2 What is happening in the water?

It seems clear (from Chapter 2 investigations into stability of [^{3}H]-E_2 in water) that the main reason why [^{3}H]-E_2 uptake reached a plateau was as a result of heavy metabolism that led eventually to very little free parent compound remaining in the water. The analysis of cold E_2 left in water after 24 h in the present chapter further confirmed these findings; roughly 10 % of what was originally added to the tank water was still free and available after 48 h. Based on counting radioactivity only, the water was expected to have 45 % of the original amount added. This discrepancy suggests that the missing 35 % is likely present as water-soluble metabolites (detected in E_2 exposures in Chapter 2). This is highly plausible considering that not only have steroid sulphates been detected in exposure medium before (Hines et al., 1996) but several studies have reported the presence (transcriptional evidence) of sulphotransferases in molluscs (Janer et al., 2005d; Lavado et al., 2006b; Tian et al., 2013).

3.4.2 Does esterification affect depuration of [^{3}H]-E2?

Animals were placed in clean water, under flow-through conditions, after exposure. This reduced the [^{3}H]-E_2 burden by up to 60 % over 20 d. However, the bulk of the reduction in [^{3}H]-E_2 (40 %) occurred in the first five days. During the following 15 d, reduction was minimal (20 %). [^{3}H]-E_2 presents a half-life of about 12.5 d when fitted with a decay model. Animals depurated under semi-static conditions in other studies have shown a wide range of results, from no significant reduction in E_2 burden by *D. polymorpha* over 10 d (Peck et al., 2007) to a half-life of 8.3 d for E_2 by *M. edulis* (Labadie et al., 2007). The latter study is particularly interesting, as experiments were done on the same species as our studies; one would expect flow-through conditions to accelerate depuration but this was not the case. Other variables probably influence the rate of depuration more efficiently than the volume of clean water animals are exposed to (i.e. factors such as temperature and metabolic rate could explain the

differences between our data and those reported by Labadie *et al. (*2007). This strongly suggests that studies using semi-static conditions for depuration experiments are actually providing similar data to those obtained when using depuration conditions used in the industry. It would appear that depuration applied in the shellfish industry (a minimum of 42 h) would in fact have some impact on the E_2 burden, as although only 40 % of the total is purged, the vast majority of this is lost during the first five days. Another depuration experiment with more sampling points (e.g. day 1, 2 and 3) would be necessary to confirm exactly how many hours are necessary to achieve this 40 % loss of E_2 (and therefore find out the % depuration within 42 h).

It seems that esterification of steroids plays an important role in depuration (or the lack thereof) potential of *Mytilus* spp. Figure 32 shows that the ester fraction of [^{3}H]-E_2 is reduced by about 50 % in the first five days but remains relatively stable for the next 15 d, whereas the sulphated / free fraction is reduced by only 40 % in the first five days but continues to depurate throughout (up to 65 %) in a linear manner. As a result of the low contribution of the free / sulphated fraction to the overall [^{3}H]-E_2 burden this reduction is not very noticeable. This suggests that steroid esters are more persistent than the water-soluble free and sulphated steroids. Both studies mentioned earlier (Labadie et al., 2007; Peck et al., 2007) stated that all E_2 residues were present as lipophilic esters (though admittedly their methods would not have detected the presence of sulphates), which perhaps explains the low level of depuration that they reported.

A question mark hangs over the exact nature of the sulphate fraction in the flesh of the animals. Elsewhere it has been argued that sulphated steroids are highly water-soluble (viz. the putative E_2 3-sulphate that was identified in the water; see Chapter 2). Thus, in theory, they should undergo very rapid excretion and thus depuration. However, radioactive 'sulphates' were still present in the tissue after 20 days. One plausible hypothesis is that they might be 'mixed conjugates' that are simultaneously esterified at the 17ß-hydroxyl position and sulphated at the 3-hydroxyl position. The presence of the fatty acid group would probably be enough to retain them within the animal despite also having a sulphate group. Circumstantial

evidence to support this hypothesis includes a) the evidence favours sulphation occurring via the 3-hydroxyl group (Chapter 2) while esterification is likely via the 17ß-hydroxyl group (Labadie et al., 2007; Scott, 2012), b) the radioactive sulphates extracted from the tissue run very far up the TLC plate (close to the elution position of the free steroid) - a fact which would be in keeping with the presence of a lipophilic fatty acid group on the 17ß-hydroxyl position of these compounds and c) only low amounts of immunoreactive E_2 were detectable after acid solvolysis (e.g. for treatment D only 0.14 % of the 13 % expected sulphates– based on radioactivity – were detected) of the sulphate fraction in the tissue (indicating that there was very little pure E_2 3-sulphate or E_2 17ß-sulphate).

3.4.3 Field survey

No differences in oestrogen burden in the field samples collected from different sites were observed. However, the study was very limited as only four animals from each site could be spared for these investigations. However, a lack of difference in E_2 burden between sites should not be considered strange. Farmed animals like cows, pigs and horses excrete oestrogen within their urine (probably more so than humans). The Norfolk site for example is close to many agricultural operations and as such could be heavily 'impacted'. In addition, the 'offshore' site is very close to the Thames estuary (see Figure 34). A recent survey of estuarine and coastal waters in the UK (Dr Marion Sebire, 2010, unpublished results), showed no clear evidence of VTG induction in male fish, suggesting that the Thames estuary does not possess significant oestrogenic ED activity, and thus may not be the best area for studying potential 'vertebrate steroid pollution'.

Experiments discussed earlier showed that free E_2 levels were fairly stable throughout depuration (Figure 32) and presented little variation between individuals. It was interesting to note that free E_2 in caged animals was also very similar between individuals (Figure 33), regardless of location. However, E_2 esters were highly variable (Figure 33) and accounted for much of the variation seen in the total E_2 burden seen in Figure 34. The lack of activity in the sulphate fraction was expected. When the sulphated fraction of radioactive E_2 was run on a TLC plate (Figure 19 in Chapter 2), it

was noted that there were at least three separate peaks (implying that it was not just E_2 that was present in a sulphated form). Variation in ester levels, on the other hand, could be a result of any number of physiological differences such as age, sex or maturation stage. For example, Sternberg *et al.* (2008) reported differences in T and E_2 levels in *I. obsoleta* between males and females over a reproductive season. The fatty acid and lipid composition of molluscs is known to vary with sex and maturation (e.g. type and quantity changed over reproductive cycles in *M. galloprovincialis*; Martínez-Pita *et al.*, 2012) and could therefore, be responsible for the differences in steroid ester levels. Factors such as age might also contribute to differences in steroid levels in molluscs, particularly when considering the premise that the longer an animal is in the water the more time and opportunity it would have to pick up and accumulate steroids as esters. Gust *et al.* (2014), for example, found that the New Zealand mudsnail, *Potamopyrgus antipodarum*, caged up and downstream of a sewage treatment plant accumulated esterified E_2 and T as time progressed. Although all the above reasons for ester variation are plausible, it must be noted that none of these have yet been investigated experimentally and it is therefore mere speculation.

In general, levels of free E_2 in the wild mussel tissues were quite low (as the bulk of it is esterified) and our results are consistent with most, but not all, of those reported in the literature (see Table 2 in Introduction). Gauthier-Clerc *et al.*(2006) found between 0.2 and 0.4 ng g^{-1} ww in wild soft-shelled clams (*Mya arenaria);* it should be noted that they measured the gonad content only. Ni *et al.* (2013) harvested wild oysters (*Crassostrea angulata*) and kept them under semi-static conditions for 7 days. They measured free E_2 in the gonads by ELISA and found it ranged between 0.095 and 0.27 ng g^{-1} ww, despite the fact that the acclimation period could have reduced the amount of E_2 present as a result of inadvertent depuration. Another couple of studies reported free steroids to be under the limit of detection of their measurement techniques. For example, Dévier *et al.* (2010) were not able to detect free E_2 and T in *Mytilus* spp. when using GC / MS and E_1, E_2 and EE_2 in *Mytilus* spp. were also under the limit of detection when measured by tandem MS by Ronan & McHugh, (2013). These findings, however, could be a result of the

detection method employed, i.e. immunoassays are known to be more sensitive (though arguably less specific) than analytical chemistry methods (Gust et al., 2010b). Levels of esterified E_2 or even total levels of E_2 (both free and esterified steroid) have also been reported in studies measuring steroid levels in wild molluscs. Peck *et al.* (2007) measured the whole E_2 burden (free and esterified) in the *D. polymorpha* from four locations (two reference sites and two contaminated sites) with the yeast oestrogen screen. Results for esterified oestrogens (reported as ng g^{-1} E2 equivalents – EEQ) ranged between 1 and 300 ng g^{-1} with E_2 itself accounting for most of the activity (verified using rp-HPLC). Consistent with our results, they detected high variability of E_2 between individuals and found E_2 in animals from presumed 'contaminated' and 'reference' sites. A few studies have also reported high levels of variation in T esters in *I. obsoleta,* although the reported variability of free steroids was also considerably high (Gooding and LeBlanc, 2004; Sternberg et al., 2008).

3.5 Conclusion

This chapter consolidates the evidence for esterificaction and sulphation of E_2, and assesses the importance of these metabolic processes in the context of depuration. It was found that not only the esterification process is unaffected by the addition of large amounts of cold E_2 but the esters persist longer than free and sulphated steroids under depurating conditions, i.e. the industry's mandatory 42 h of depuration are not enough to purge E_2 (and / or its metabolites) fully. The separation method developed in Chapter 2, was employed to investigate steroid levels in mussels caged in the Thames estuary (following a pollution gradient). Although it was not possible to draw meaningful conclusions regarding pollution, the ratio of E_2 moieties found in wild animals matched our laboratory findings on the relative distribution of free, esterified and sulphated E_2.

Chapter 4

Uptake, metabolism and depuration of ethinyl-oestradiol.

4.1 Introduction

Ethinyl-oestradiol (EE_2) is a synthetic version of the natural steroid hormone oestradiol (E_2). It is one of the two ingredients of the contraceptive pill, and is chemically stabilised with an ethinyl group on Carbon 17 (Figure 36) in order to ensure slow release after oral administration. This chemical group not only stabilises the steroid but increases the hormone's potency as an oestrogenic ED (e.g. in fish; Thorpe *et al.*, 2003). Like E_2, EE_2 is excreted in human urine as a soluble metabolite and ends up in surface waters as a result of its incomplete removal and / or biodegradation in sewage treatment plants (Johnson and Sumpter, 2001; Petrovic et al., 2002; Yoshimoto et al., 2004).

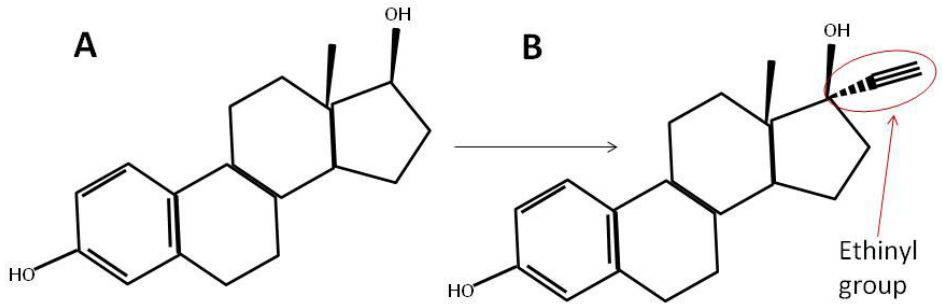

Figure 36: Diagram of chemical structures of E2 (A) and EE_2 (B).

The presence of EE_2 in mollusc tissue has not been as thoroughly investigated as that of E_2, T and P. In his review, Scott (2012) referred to two studies that confirmed the existence of EE_2 in mollusc soft tissue (and one of these - Liu *et al.* (2009) – included detection in species of abalone, clam, oyster, mussel and snail). Another as yet unpublished study reported the presence of EE_2 in blue mussel *Mytilus edulis trossulus* from the Baltic Sea (Hallmann *et al.*, unpublished) using high performance liquid chromatography coupled to electrospray ionisation and mass spectrometry (HPLC-ESI / MS). Although this supports the abovementioned findings (i.e.

EE_2 is present in some wild molluscs), it must be noted that this study detected unusually high levels of EE_2 in the tissue. This casts some doubt on the results, since others (Liscio et al., 2009; Ronan and McHugh, 2013) have reported EE_2 to be below the detection limit (using liquid chromatography coupled with tandem mass spectrometry; LC-MS / MS) in mussels (wild and caged) located near sewage treatment plant effluents (where EE_2 would be expected to be present). In other words, there are fewer reports on EE_2 presence in mollusc tissue (scarce positive data coupled with some negative findings) than for E_2, T and P. However, in contrast to E_2, T and P, the origin of EE_2 found in mollusc tissue (i.e. endogenous or exogenous) cannot be questioned – being a synthetic man-made hormone it is inconceivable that it comes from anywhere else other than the environment.

EE_2 is known to have endocrine disrupting effects on vertebrates (e.g. feminisation of fish; Kidd *et al.*, 2007) and, based on this, several studies have set out to investigate possible endocrine effect on molluscs (Andrew-Priestley et al., 2012; Hultin et al., 2014; Jobling et al., 2004; Langston et al., 2007a; Leonard et al., 2014b; Stange and Oehlmann, 2012; Stange et al., 2012); this, despite the lack of any solid evidence that vertebrate steroids have any hormonal role in molluscs (as discussed in Chapters 1, 2 and 3). Furthermore, one particular study that was intended to develop an invertebrate embryo test for endocrine disruption, reported no effects of EE_2 (or BPA – another vertebrate oestrogenic ED) at concentrations as high as 10 µg L^{-1} on the chosen biological endpoints in giant ramshorn snail, *Marisa conuarietis* (Schirling et al., 2006). Hallgren *et al.* (2012) also reported negative findings regarding similar biological endpoints (mortality, hatching success, etc.) in two gastropod species (*Radix balthica* and *Bithynia tentaculata*) after EE_2 exposure. There are however, other studies that claim that EE_2 has vertebrate-like endocrine disrupting effects on molluscs. Borysko & Ross (2014) reported contradictory findings (regarding reproductive endpoints) in two closely related snail species (*Nassarius burchardi* and *Nassarius jonasii*) exposed to EE_2 under the same conditions. Despite effect sizes being very low, there being an inconsistent effect between species and the authors themselves concluding that 'no consistent patterns between concentration of EE_2, length of adult exposure times and

observed effects were found', the authors nevertheless decided that EE_2 did have an endocrine disruptive effect on at least one of the species. In another example with 'positive spin', Andrew *et al*. (2010) reported an increase in a vitellogenin-like protein (putative identity of the protein was based on sequence homology) in the Sydney rock oyster, *Crassostrea angulata*, when exposed to EE_2 for up to 49 d. There are several problems with their findings: the protein in question had not yet been characterised and was only assumed to respond to EE_2 because a similar protein (i.e. vitellogenin; VTG) does so in vertebrates; the reported up-regulation of vitellogenin-like protein in a potentially concentration-responsive manner was inconsistent over time (e.g. it only happened in males at 4 d and 21 d but not at 49 d); and lastly, what they actually measured was not the protein but the amount of UV light absorption in a certain fraction eluting from an HPLC column that they only assumed to correspond to VTG; the wavelength (200 nm) of light that they were using is absorbed by any biological molecules that possess one or more unsaturated bonds (e.g. C=C or C=O). In other words, it is totally non-specific and could even have been detecting the EE_2 itself.

Having shown that E_2 – the natural counterpart of EE_2 - is readily absorbed by a number of species (see also Janer *et al.*, 2005; Peck *et al.*, 2007), one would expect EE_2 to be absorbed too. However, unlike E_2 and T, no one appears yet to have investigated the potential uptake (and / or metabolism) of EE_2 by molluscs. The only evidence for EE_2 uptake has been indirectly provided by those investigating biological endpoints. Cubero-Leon *et al. (*2010) exposed *Mytilus edulis* to EE_2 in the water for seven days, and although they did not measure the steroid in the tissue, they found the level in the water fell significantly over 24 h. This reduction in concentration suggests that EE_2 was taken up by the animals. Giusti *et al. (*2014) exposed the great pond snail, *Lymnaea stagnalis*, to a range of EE_2 concentrations (10 to 50,000 ng L^{-1}) for 21 d under semi-static conditions (water change every 48 h). EE_2 concentrations in water were presented as the mean value of samples taken at time 0 and 48 h after exposure and were approximately a third of the nominal concentrations for all levels used. This, again, suggests that EE_2 was taken up by the animals.

In this chapter, the uptake and depuration of [^{3}H]-EE_2 by mussels as well as its fate after exposure (i.e. can EE_2, like E_2, be esterified and sulphated?) are investigated. Saturation limits (if any) of [^{3}H]-EE_2 were also investigated. In order to confirm the scarce findings in the literature on EE_2 presence in wild mussel tissue, attempts to measure this steroid in tissues from mussels that had been caged in different locations in the Thames estuary (considered to be representative of a pollution gradient) were also made.

4.2 Materials and methods

4.2.1 Chemicals

Ethinyl-oestradiol, 17-[6,7-^{3}H(N)] ([^{3}H]-EE_2) was purchased from American Radiolabeled Chemicals, Inc. (101 ARC Dr. St. Louis, MO 63146 USA). 'Cold' 17α-ethinyl-oestradiol was obtained from Sigma-Aldrich Company Ltd (Dorset SP8 4XT, UK) and all other chemicals were purchased from Fisher-Scientific UK Ltd. (Loughborough LE11 5RG, UK).

Water used for laboratory exposures was filtered (50 µm) sea water and water used for all other purposes was reverse osmosis-treated water unless stated otherwise.

4.2.2 Laboratory exposures of *Mytilus spp.* to radiolabelled EE_2

To investigate the uptake rate of EE_2 in mussels, our first experiment was based on Peck *et al. (*2007). Our experimental design was simple yet effective. The bivalves were placed in containers supplied with filtered sea water. Then radioactive (tritiated) steroid was added and, at timed intervals, water samples (1 mL) were collected (in which the radioactivity was quantified by scintillation counting). There was a solvent control tank with mussels and carrier solvent (ethanol); and a sorption control tank with radioactivity but no mussels (to assess how much steroid might be sorbed to the container and / or the aerator). The experimental set up was refined and optimised over a series of exposures mostly involving [^{3}H]-E_2 (see Chapter 2).

4.2.2.1 Study 1

4.2.2.1.1 Collection and acclimation

As described in section 2.2.2.1.1 in Chapter 2.

4.2.2.1.2 Exposure and depuration

As described in section 2.2.2.1.2 in Chapter 2 but mussels were exposed to a nominal concentration of 0.7 µCi L^{-1} (3.45 ng L^{-1}) of [^{3}H]-EE_2. Following exposure to [^{3}H]-EE_2 for two consecutive 48 h periods, mussels from Study 1 were submitted to depuration in a flow-through system (1 L min^{-1}) for 20 d. Ten mussels were sampled at regular intervals (day 0, 5, 10, 15 and 20) and stored at -20 °C immediately.

4.2.2.2 Study 5

4.2.2.2.1 Collection and acclimation

As described in section 2.2.2.4.1 in Chapter 2.

4.2.2.2.2 Exposure and depuration

As described in section 2.2.2.4.2 in Chapter 2 but mussels in vessel 1 to 6 were exposed to a nominal concentration of 5 µCi L^{-1} (24.7ng L^{-1}) of [^{3}H]-EE_2 (hot only); vessels 7 to 8 also had 2.5 µg L^{-1} EE_2 (low) and vessels 9 to 10 had 25 µg L^{-1} EE_2 (high). Following exposure for 24 hours, mussels from Study 5 (vessels 1 to 6 only) were submitted to depuration in a flow-through system (1 L min^{-1}) for 10 days. Ten mussels were sampled on day 0 and five mussels on day 1, 2, 5 and 10; and stored at -20 °C immediately.

4.2.3 Condition index analysis

As described in section 2.2.3 in Chapter 2.

4.2.4 Clearance rates

The rates at which individual mussels cleared [^{3}H]-EE_2 from water (i.e. clearance rates) were calculated for each study as described in section 2.2.4 in Chapter 2.

4.2.5 Extraction of steroids from tissue

Mussels from Study 1 and 5 were extracted using Method 1 (see section 2.2.5.2 in Chapter 2). Note that chloroform was added to the first extraction step for mussels from Study 5.

4.2.6 Separation of free, sulphated and lipophilic EE_2 metabolites (hot and cold)

As described in section 2.2.5.3 in Chapter 2. Briefly, crude tissue extract (1 mL) was centrifuged in a microcentrifuge tube and 800 µL of the supernatant was transferred to a glass tube and dried down under a stream of nitrogen at 40 °C. After addition of 1.2 mL ethanol, 0.3 mL water and 3 mL heptane, the tube was shaken vigorously on a vortex for 5 min. The tube was then centrifuged and the upper heptane layer removed and placed in a clean glass tube. The 80 % ethanol layer was shaken for another 5 min with a further 3 mL of heptane and centrifuged again. The two batches of heptane (containing EE_2 esters) were combined. To separate free from sulphated EE_2, the 80 % ethanol phase was blown down under a stream of nitrogen gas at 40 °C, reconstituted in 100 µL water and 4 mL diethyl ether, shaken and centrifuged. Sulphates partitioned in the water phase and free steroids in the diethyl ether phase. To separate them, the aqueous layer was frozen in liquid nitrogen and the top solvent layer decanted into a clean tube. All three phases were then dried under a stream of nitrogen at 40 °C; the heptane and water fractions were re-suspended in ethanol or further processed, while the diethyl ether fraction was re-suspended in ethanol or RIA buffer (no further processing required as it was free, and therefore quantifiable, steroid).

As an extra precaution in the present study, the 80 % ethanol phase was diluted with water and passed through a C18 cartridge to check how much tritium may or may not have been removed from the steroid (and then presumably incorporated in water molecules). This represented activity that passed straight through the cartridges as opposed to free and sulphated steroids, which were retained. The cartridges were then eluted with 5 mL methanol that, after blowing down, was treated in the same way as above (i.e. mixing with water and diethyl ether).

4.2.6.1 Chemical hydrolysis of esterified steroid

Crude extracts were separated as described in section 4.2.5. The dried heptane fraction was then re-suspended in 1.8 mL methanol and 0.2 mL 3 M KOH for alkaline hydrolysis. The sample was incubated in an oven at 80 °C for 40 min (mixed briefly with a vortex after 20 min) and the reaction was stopped by adding 40 µl 2.5 M HCl . The now free hot or cold EE_2 was separated by adding 0.21 mL water and 4.5 mL heptane to the hydrolysate. It was vortex-mixed for 5 min and centrifuged for 10 min at 2500 g. The top heptane layer was discarded and the process was repeated once more. A control sample was in place, consisting of [^{3}H]-E_2 ester that had been previously purified by heptane partitioning of a pool of extract from [^{3}H]-E_2-treated mussels. The ethanol phase was dried and re-suspended in RIA buffer and stored at -20 °C.

4.2.6.2 Acid solvolysis of sulphated steroid

The dry water fraction from the above water / ether partition (section 4.2.6) was resuspended in 4 mL ethyl acetate:trifluoroacetic acid (100 mL:1.4 mL, v:v) and incubated at 40 °C overnight (Scott et al., 2014). The solvents were removed by drying at 40 °C under a stream of nitrogen. The now free steroids were removed by partitioning between 1 mL water and 4 mL diethyl ether (section 4.2.6). The ether fraction was dried overnight at room temperature, re-suspended in RIA buffer and stored at -20 °C.

4.2.6.3 Quantification by radioimmunoassay (RIA)

All samples, re-suspended in RIA buffer, were diluted appropriately (based on expected values) and submitted to radioimmunoassay (see section 3.2.4.4. in Chapter 3).

4.2.7 Chromatography

4.2.7.1 Normal-phase High Performance Liquid Chromatography

Normal phase HPLC (np-HPLC) was used to separate free from esterified [^{3}H]-EE_2 in mussel tissue extract. It was carried out as described in section 2.2.6.1 in Chapter 2 but with the appropriate standards.

4.2.7.2 Reverse-phase High Performance Liquid Chromatography

Reverse-phase HPLC (rp-HPLC) (using an analytical column) was used to separate free and conjugated (i.e. sulphated or glucuronidated) metabolites of [^{3}H]-EE_2 in water extracts and also the metabolites produced as the result of alkaline hydrolysis of esterified [^{3}H]-EE_2. The procedure was as described in section 2.2.6.2 in Chapter 2 but with the appropriate standards.

4.2.8 Thin layer chromatography

4.2.8.1 Separation of free steroid (after alkaline hydrolysis)

After separation of the alkaline hydrolysis products of esterified [^{3}H]-EE_2 by rp-HPLC, the main radioactive peak was collected, mixed with 5 µg of EE_2 standard (10 µl of 0.5 mg mL^{-1} standard stocks in ethanol) and dried under a stream of nitrogen at 40 °C. Samples were loaded onto thin layer chromatography (TLC) plates (catalog no. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) with 90 µL ethyl acetate and developed for 45 min with a mixture of chloroform:ethanol (50:2 v:v). After marking the positions of the standards using a UV lamp, the plate was sprayed with 10 % phosphomolybdic acid in ethanol and heated at 100 °C for 5 min. Lanes were then divided into 5 mm bands, and the silica gel from each band was scraped off the plate. The scrapes were mixed with 500 µL ethanol, 500 µL water and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity.

4.2.9 Statistics

As described in section 3.2.6 in Chapter 3.

4.2.10 Field survey

4.2.10.1 Collection

Caged mussels (*Mytilus spp.*) were collected as described in section 3.2.5.1 in Chapter 3.

4.3 Results

4.3.1 Condition index

Condition index was used as a means of monitoring the effects of exposure (particularly solvents and exposure conditions - when water controls were possible) on animal health. [^{3}H]-EE_2 and [^{3}H]-E_2 experiments were conducted at the same time (i.e. shared solvent and water controls) therefore a single batch of animals was used for condition index analysis. Figures for Study 1 and 5 can found in section 2.3.1 in Chapter 2. Results indicated there was no significant difference between pre-study animals and solvent / water controls, suggesting that exposure conditions (including exposure to the carrier) did not have a negative impact on the animals general health.

4.3.2 Removal of [^{3}H]-EE_2 from water by mussels during bath exposure

In Study 1, the mean radioactivity (n = 10) in water at the time of dosing was 0.84 µCi L^{-1} in the first 48 h and 0.86 µCi L^{-1} in the second 48 h exposure. The total radioactivity that disappeared during exposure 1 and 2 was 20 and 12.8 % respectively (compared to 37.1 and 27.5 % for [^{3}H]-E_2) (Figure 37). Over time there was a significant decrease (log transformation of time was applied to achieve a linear relationship) in the level of [^{3}H]-EE_2 in the water of exposure 1 (β = -0.20546, SE = 0.01258, t = -16.331, $p < 0.0001$) and exposure 2 (β = 0.06281, SE = 0.01739, t =3.612, p = 0.0004), however, the latter decreased at lower rate. Clearance rates, calculated from the first 3 h of exposure (taking into account the minor sorption that occurred, based on fitted data; linear model), are presented in Table 12. The rate did not differ greatly between exposures 1 and 2 (46 and 43.3 mL $animal^{-1}$ $hour^{-1}$ respectively). This experiment was conducted in parallel with the E_2 absorption experiment and the corresponding values for [^{3}H]-E_2 were 37 and 31 mL $animal^{-1}$ h^{-1}. The most obvious differences between the two steroids was that, after 3 h, the rate of [^{3}H]-EE_2 absorption levelled off more rapidly than that of [^{3}H]-E_2.

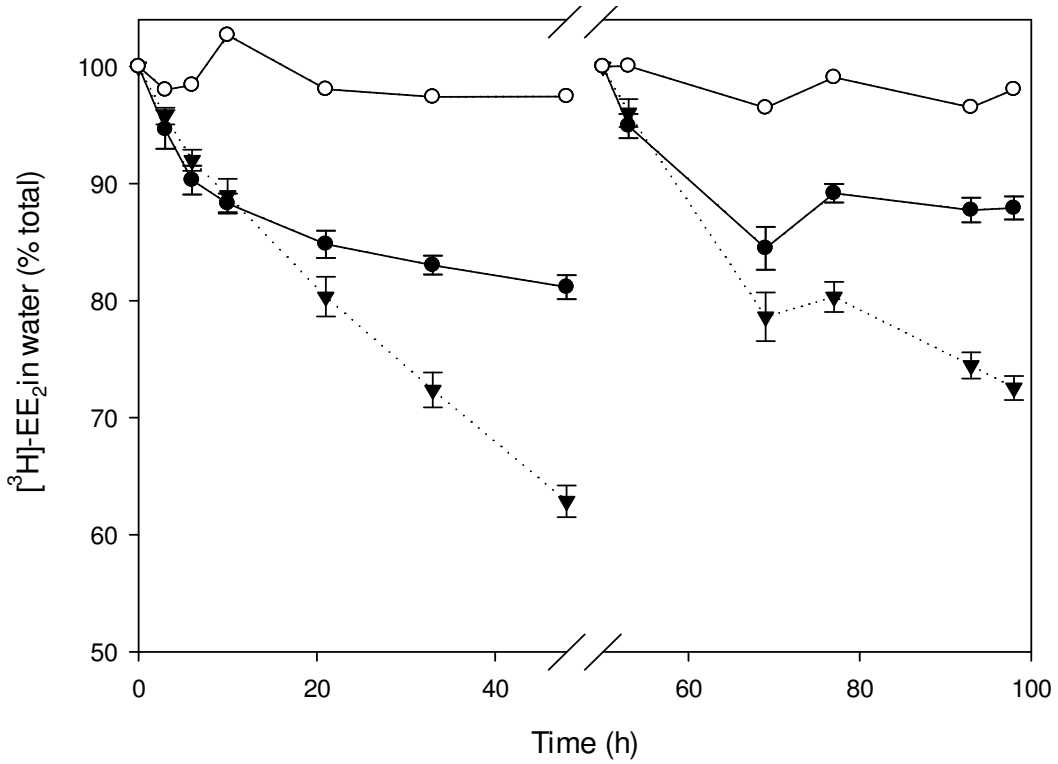

Figure 37: Removal of [^{3}H]-EE_2 (●) from water by *M. edulis*. Removal of [^{3}H]-E_2 (▼) is plotted in dotted lines for comparison purposes. Mussels were exposed for two consecutive 48 h periods under semi-static conditions (water was changed and fresh label added; //) to 0.7 µCi L^{-1} (3.45 ng L^{-1}) [^{3}H]-EE_2 nominal concentration per dose. Data are presented as mean % total of [^{3}H]-EE_2 in water (n = 10 tanks per time point) and ± S.E.M. A sorption control (○) tank with no animals. Control data is presented as % total [^{3}H]-EE_2 residues in water (n = 1).

4.3.2.1 Are there any saturation effects on [^{3}H]-EE_2 uptake?

A second [^{3}H]-EE_2 uptake experiment (Study 5) was done but this time the animals were exposed for only 24 h and were kept in a smaller amount of water (see Chapter 2 for study design changes). Cold EE_2 was also added to some of the tanks in order to investigate if there was a saturation limit for uptake of EE_2 by *Mytilus* spp. Concentrations of [^{3}H]-EE_2 were, on average (n = 6, 2 and 2), 2.9 µCi L^{-1}, 2.9 µCi L^{-1} and 2.8 µCi L^{-1} at the time of dosing for tanks with no added cold EE_2 ('hot only'), 2.5 µg L^{-1} EE_2 (low) and 25 µg L^{-1} EE_2 (high) respectively. Over time there was a significant decrease (log transformation of time was applied to achieve a linear relationship) in the level of [^{3}H]-EE_2 in the water ($df = 1$, $f = 571.147$, $p < 0.0001$), however, treatment had no significant effect on the decay curve ($df = 2$, $f = 1.333$, $p = 0.274$). After 24 h up to 50.0 %, 49.8 % and 47.2 % of total radioactivity was removed from each treatment (Figure 38). The calculated clearance rates (based on the first 3 h only) were 39.9, 35.3 and 31.0 mL $animal^{-1}$ h^{-1}

(Table 12; cf. 40 for the within-experiment [^{3}H]-E_2 control). In other words, although there appeared to be some indication of saturation within the first 3 h, this was not supported statistically (linear model with time points treated as factors; df = 2, f= 1.479, p = 0.261). Unlike in the first experiment, the rate of disappearance of [^{3}H]-EE_2 matched that of [^{3}H]-E_2 for the whole 24 h.

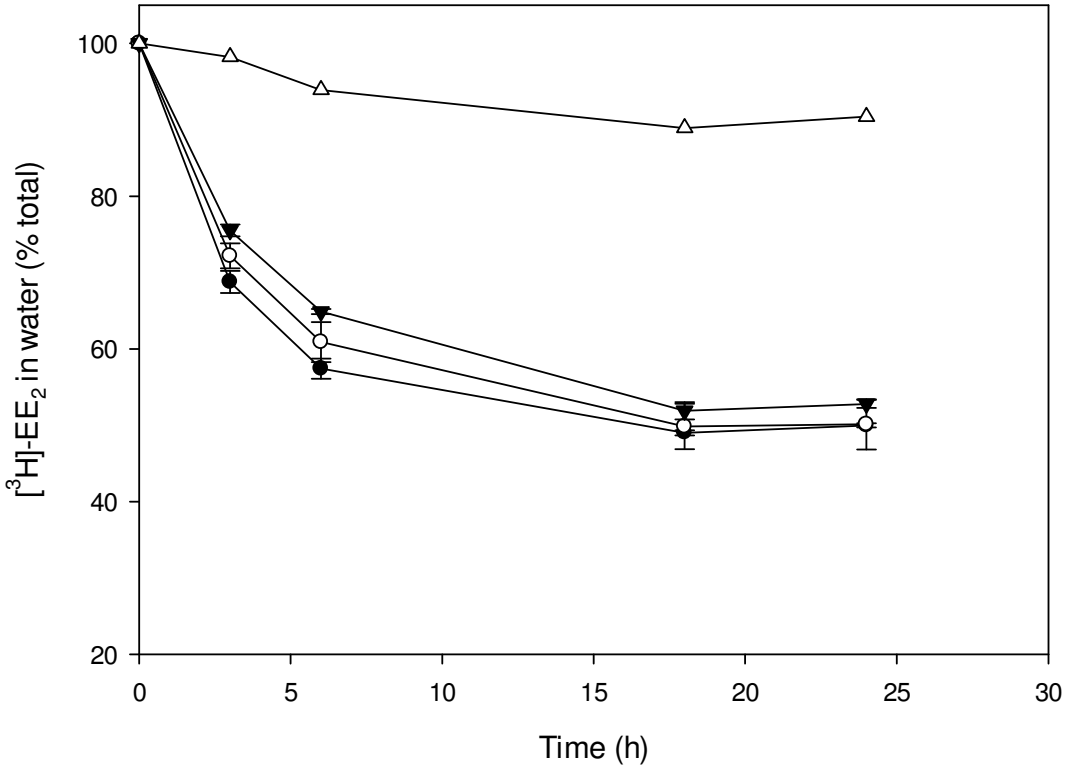

Figure 38: [^{3}H]-EE_2 (24.7 ng L^{-1} nominal concentration) removal by *Mytilus spp.* in the presence of increasing amounts of cold EE_2 (0 µg L^{-1}:●, 2.5 µg L^{-1}:○ and 25 µg L^{-1}:▼ nominal concentrations) over 24 h. Data are presented as mean % total (n = 6 for ● and n = 2 for ○ and▼) and ± S.E.M. Sorption control (no mussels in tank) (△; n = 1).

4.3.2.2 Are there any saturation effects on [^{3}H]-EE_2 accumulation in mussel soft-tissue?

[^{3}H]-EE_2 uptake was further supported by the accumulation of radioactivity in mussel soft tissue (Figure 39), in which case treatment did explain a significant amount of the variability observed between animals (f = 8.253, df = 2, p = 0.0016). Pairwise comparisons showed that both low and high amounts of cold EE_2 significantly increased the accumulation of labelled EE_2 compared to the hot only treatment (ß = 0.101, SE = 0.02960, t = 3.413, p =0.002 and ß = 0.107, t = 0.0296, df = 3.615, p = 0.001 respectively). These results, however, cannot be explained by saturation of uptake by cold EE_2, since that would have the opposite effect (i.e. the more cold EE_2,

the less hot EE_2 would be taken up). Note the difference in variability between treatments; the greater amount of variability in the 'hot only' regimen could be a result of the higher number of replicates this treatment had compared to the low and high EE_2 exposures (6 tanks vs 2 tanks).

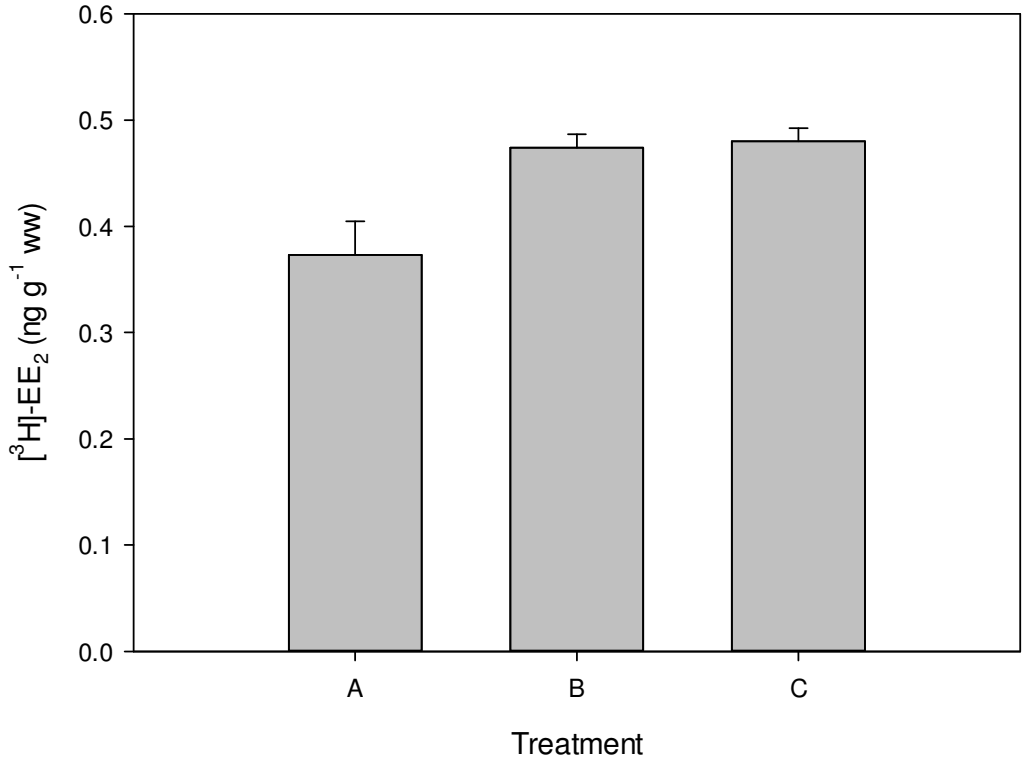

Figure 39: [^{3}H]-EE_2 residues present in *Mytilus* spp. soft tissue after 24 h exposure to [^{3}H]-EE_2 in the presence of increasing amounts of cold EE_2 (A: 0 µg L^{-1}; B: 2.5 µg L^{-1} and C: 25 µg L^{-1} nominal concentrations). Data are presented as mean total ng g^{-1} wet weight [^{3}H]-EE_2 equivalents (n = 10) ± SEM.

Table 12: Summary of exposure conditions and [^{3}H]-EE_2 clearance rates from water by *Mytilus* spp.

Study	Water vol. (L)	N° animals	Label conc. (µCi L^{-1})	Exposure time (h)	Removal after exposure (%)[c]	Fitted sorption after 3 h (%)	Removal after 3 h (%)	Clearance rate (mL $animal^{-1}$ h^{-1})[d]
1 [^{3}H]-EE_2	13.0	5	0.84	48	20.0	0.2	5.5	46.0
1 [^{3}H]-EE_2	13.0	5	0.86	48	12.8	0.2	5.2	43.3
5 [^{3}H]-EE_2	2.0	5	2.9	24	50.0	1.3	31.2	39.9
5 EE_2 low[a]	2.0	5	2.8	24	49.8	1.3	27.8	35.3
5 EE_2 high[b]	2.0	5	2.7	24	47.2	1.3	24.5	31.0

[a]2.5 µg L^{-1} cold steroid (nominal).

[b]25 µg L^{-1} cold steroid (nominal).

[c]Fitted sorption not accounted for in removal after exposure.

[d]Fitted sorption accounted for in clearance rates.

4.3.2.3 What was left in the water after exposure?

In Chapter 2, it was suggested that [^{3}H]-E_2 uptake (especially in the final experiment) was probably halted by the fact that [^{3}H]-E_2 was being sulphated as well as esterified and that, after 24 h, there was little, if any, intact [^{3}H]-E_2 left in the water that was available for uptake and esterification. To investigate whether the same applied to [^{3}H]-EE_2, water samples (1 L) were taken at the end of Study 5 from 'hot only' exposure vessels and the sorption control. When subjected to solid phase extraction, ca. 3.6 and 0.3 % of the activity from the exposure vessel and the sorption control, respectively, passed straight through. This probably represents tritiated water (released from the parent compound by unknown reactions). After the extracts had been eluted with methanol and then dried down, a portion was dissolved in water and then shaken with diethyl ether. The water extract from exposure vessels retained 24 % of radioactivity in the water fraction (assumed to be a water-soluble conjugate) and 76 % in the ether fraction (assumed to be free steroid). Sorption control extract yielded 2 and 98 % respectively, i.e. both extracts had greater amount of free steroid than water soluble metabolites.

The extracts were then analysed by reverse-phase HPLC at 280 nm with added EE_2 standard. Figure 40 shows that the main radioactive peak from both sorption control (no mussels) and test vessel (with mussels) exposures eluted at 53 min, which is also the retention time of the EE_2 standard. A smaller peak with a retention time of 39 min appeared in test vessel extract only - consistent with the presence of water-soluble metabolites. Although none of the compounds in the water have yet been definitively identified, the results suggest that a lot of intact radiolabel is still present in the water at 24 h.

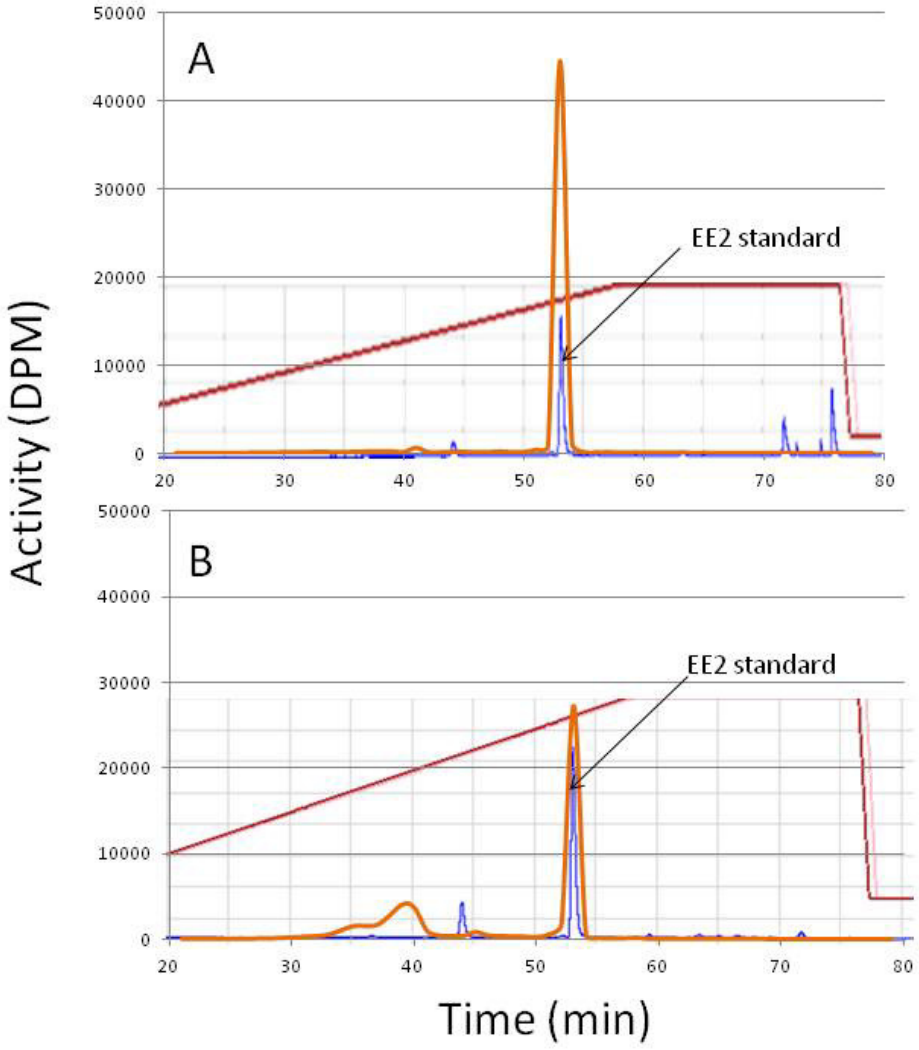

Figure 40: HPLC chromatogram of [^{3}H]-EE_2 water samples (sorption control, A and test vessels, B) with EE_2 standard. Data are presented as UV absorption (blue, 280nm) and radioactivity peaks (orange).

4.3.3 What happens to [^{3}H]-EE_2 that is taken up by mussels?

As a first step, the methods used to extract [^{3}H]-E_2 from tissue were tested and worked with similar efficiency on [^{3}H]-EE_2 (Table 13). The solvent procedure was also tested to make sure it was able to separate esterified label from free and any sulphated label.

Table 13: Method development of [^{3}H]-EE2 extraction (from tissue).

Method	Study	Labelled steroid	Extracts (% total)					Extracts 1 & 2 efficiency (%)	Method efficiency (%)
			1	2	3	4	5		
1[a]	5	EE_2	71	20	5	3	2	91	98
	5	EE_2	71	21	5	3	-	92	-
	5	EE_2	79	15	4	2	-	94	-
	5	EE_2	-	-	96	4	-	-	-
	5	EE_2	-	-	98	2	-	-	-
	5	EE_2	-	-	98	2	-	-	-
	5	EE_2	-	-	80	14	6	-	94
	5	EE_2	-	-	94	6	-	-	-
	5	EE_2	-	-	85	15	-	-	-
	5	EE_2	-	-	-	98	2	-	98

[a]**Method 1 with chloroform added in first extraction step.**

Radioactive residues from Study 1 (at time zero, i.e. before depuration) were separated into esters, sulphates and free steroid. Figure 41 shows that most of [^{3}H]-EE_2 was in the 'free' fraction of mussel tissue (> 70 %), while 30 % was distributed equally between the esterified and sulphated fractions. The distribution of [^{3}H]-E_2 is shown for comparison. It is interesting to note that though the [^{3}H]-EE_2 metabolite distribution differed greatly from [^{3}H]-E_2, the proportion of sulphates in both profiles was very similar (~10 to 15 %).

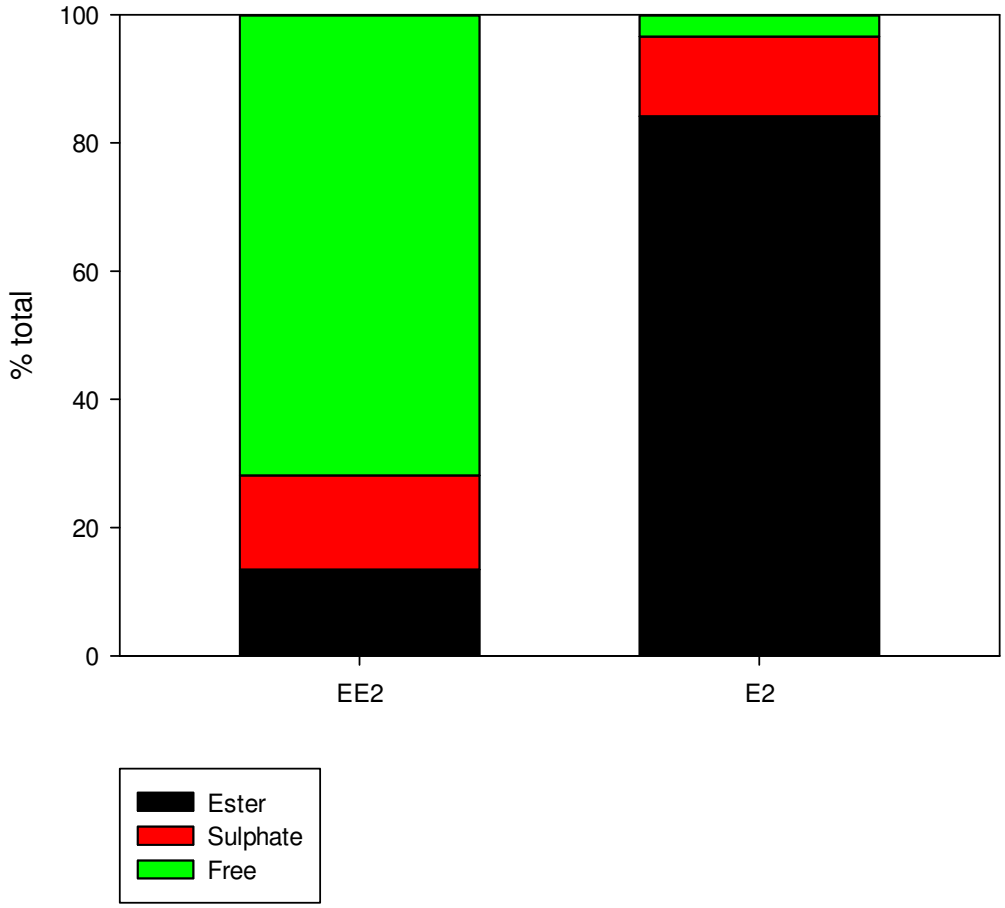

Figure 41: Radioactive metabolite profile in mussel tissue after exposure to [^{3}H]-EE_2 and [^{3}H]-E_2 for 96 h. Data are presented as mean percentage total (n = 10) for ester. Animals were pooled to estimate the proportion of sulphate and free.

Separation of the three main metabolite groups was based purely on this novel solvent portioning procedure, so it was important to further substantiate the evidence for the presence of esterified EE_2. Pooled extract from the first experiment was chromatographed on an np-HPLC column (Figure 42). There were two peaks of radioactivity – a large one coinciding with the retention time of free EE_2 and a smaller peak where the

EE_2 ester would be expected to elute. This matched the distribution of free and ester found using the solvent procedure. Some of the radioactive ester (separated from free and sulphated radiolabel using heptane) was hydrolysed using KOH / methanol and run on a reverse phase C18 column (Figure 43). The main radioactive peak was found to have the exact same retention time as the EE_2 standard. It was collected and further chromatographed by TLC (Figure 44) where it also ran with the EE_2 standard. However, there were other peaks, indicating that most (ca. 60 %), but not all the radioactivity in the ester fraction was otherwise unmetabolised [^{3}H]-EE_2.

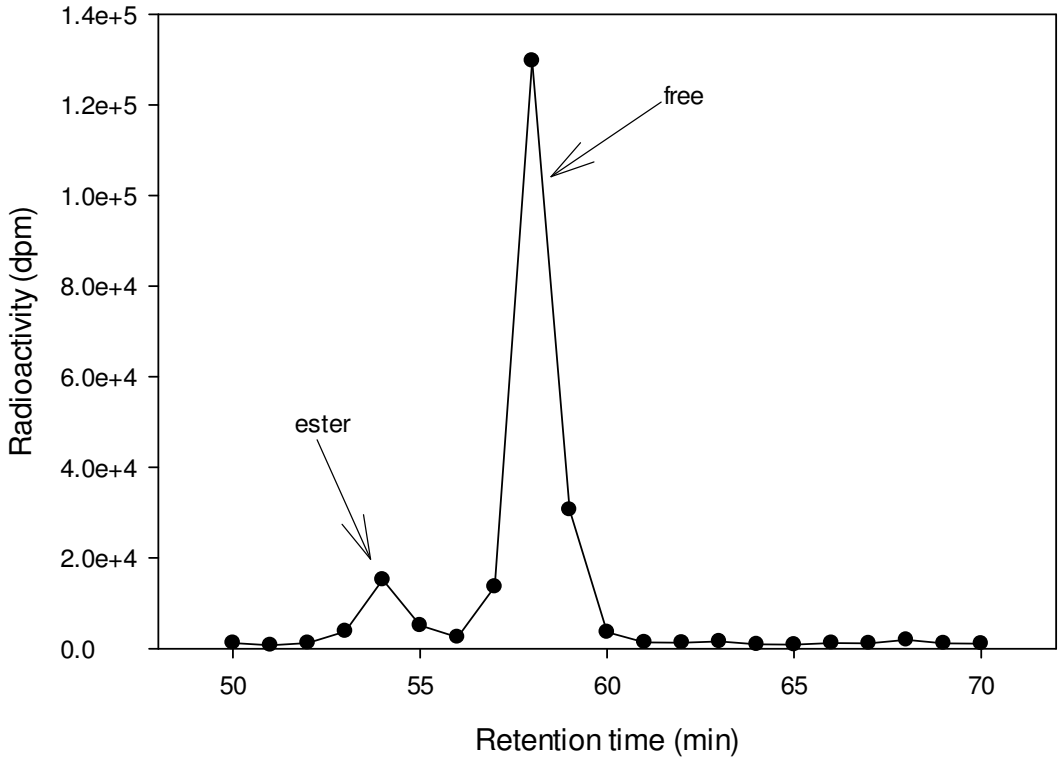

Figure 42: Representative normal phase HPLC chromatogram of radioactive residues in mussel soft tissue extract after exposure to [^{3}H]-EE_2 for 96 h. Data are presented as dpm fraction $^{-1}$ (1 mL min^{-1} fractions were collected).

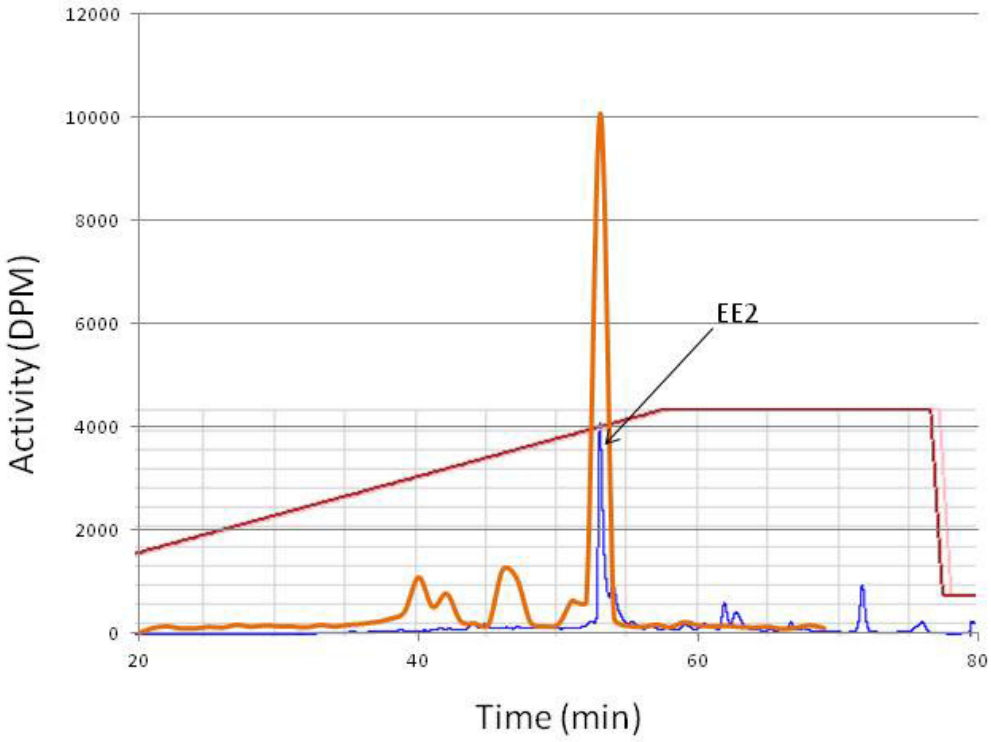

Figure 43: Representative HPLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-EE_2 metabolites (Study 5) with EE_2 standard. Data are presented as UV absorption (280 nm, blue) and radioactivity peaks (orange).

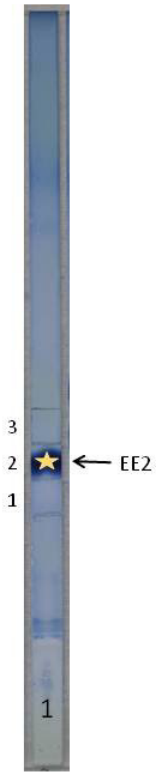

Figure 44: TLC plate of the HPLC-purified (minute 54, Figure 43), hydrolysed heptane phase (from tissue extract of [^{3}H]-EE2 exposure) with EE2 standard. Standards are labelled and golden stars indicate the position of sample radioactivity.

4.3.4 What happens to [^{3}H]-EE_2 when mussels undergo depuration?

So far it has been demonstrated that *Mytilus* spp. are able to absorb [^{3}H]-EE_2 from the water with a clearance rate similar to that of [^{3}H]-E_2. However, the ratio of free:sulphate:ester (F:S:E) in the tissue is very different from that of [^{3}H]-E_2. The question that this observation generates is how might this affect the rate of depuration of the radiolabel? To investigate this, some of the animals that had been exposed to [^{3}H]-EE_2 were placed in fresh running sea water and then a few removed at intervals and frozen for later extraction and separation into different fractions. Figure 45 shows that that there was a significant decrease ($df = 4$, $f = 43.53$, $p < 0.0001$) in the total radioactivity over time. Pairwise comparisons of total [^{3}H]-EE_2, using Student's T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were significant differences between day 0 and all the other time points ($p < 0.0001$ for all sample points); and between day 5 and 20 ($p = 0.016$). The abrupt decrease in total [^{3}H]-EE_2 in the first 5 days (reduction of ~ 80 %), appears to be due to losses of the free / sulphate fractions as the esterified fraction remained relatively stable over this time period and the rest of the experiment. [^{3}H]-EE_2 depuration was similar to [^{3}H]-E_2 depuration in the sense that the first five days show the largest reduction in steroid burden. However, [^{3}H]-EE_2 is subject to depuration to a larger extent overall (~80 % compared to ~40 %). Study 5 depuration results were consistent with Study 1. Figure 46 shows that there was a significant decrease ($df = 4$, $f = 48.71$, $p < 0.0001$) in the total radioactivity over time. Pairwise comparisons of total [^{3}H]-EE_2, using Student's T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were significant differences between day 0 and all the other time points ($p < 0.0001$ for all sample points). In other words, approximately 80 % of [^{3}H]-EE_2 was lost in the first 24 h of depuration. As the free and sulphated portion of [^{3}H]-EE_2 add up to approximately 85 %, and they are both water soluble (or more so than esters, in the case of free steroid) it would be logical to conclude that [^{3}H]-EE_2 is depurated more readily than [^{3}H]-E_2 as a result of a lower esterification rate than E_2. Essentially, it is only the esterified steroids that are retained for long periods of time.

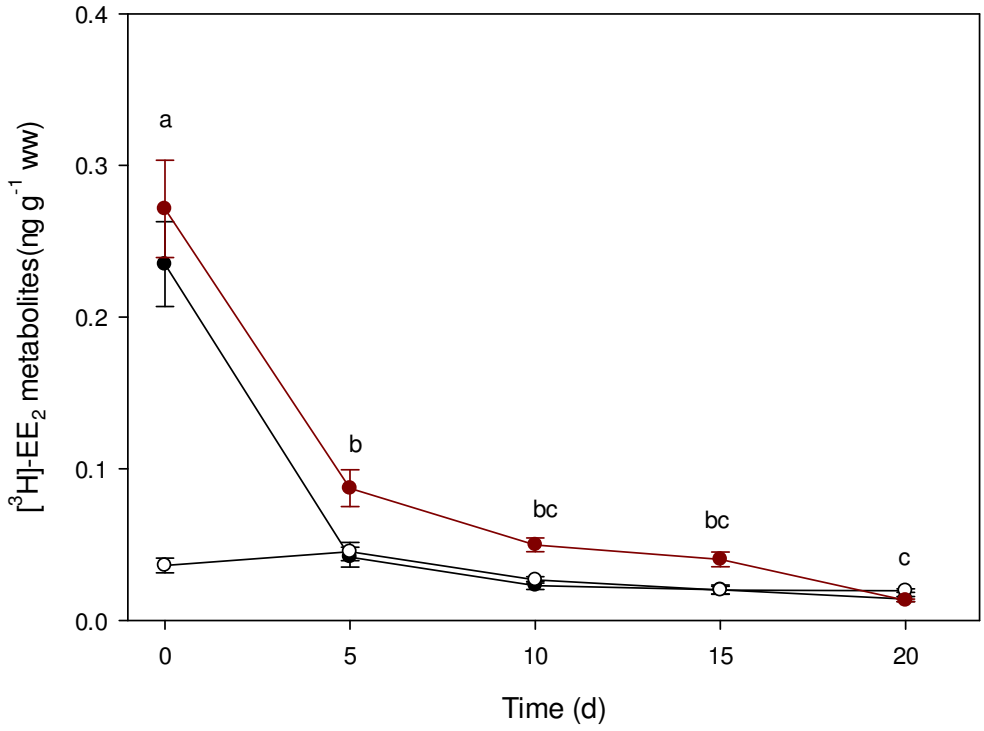

Figure 45: Free / sulphate fraction (●), ester fraction (○) and total [^{3}H]-EE$_2$ (●) in mussel tissue during depuration after exposure to [^{3}H]-EE$_2$ for 96 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-EE$_2$ residues (n = 10), where observations labelled with a different letter ('a', 'b' or 'c') are statistically significantly different from each other (p < 0.05).

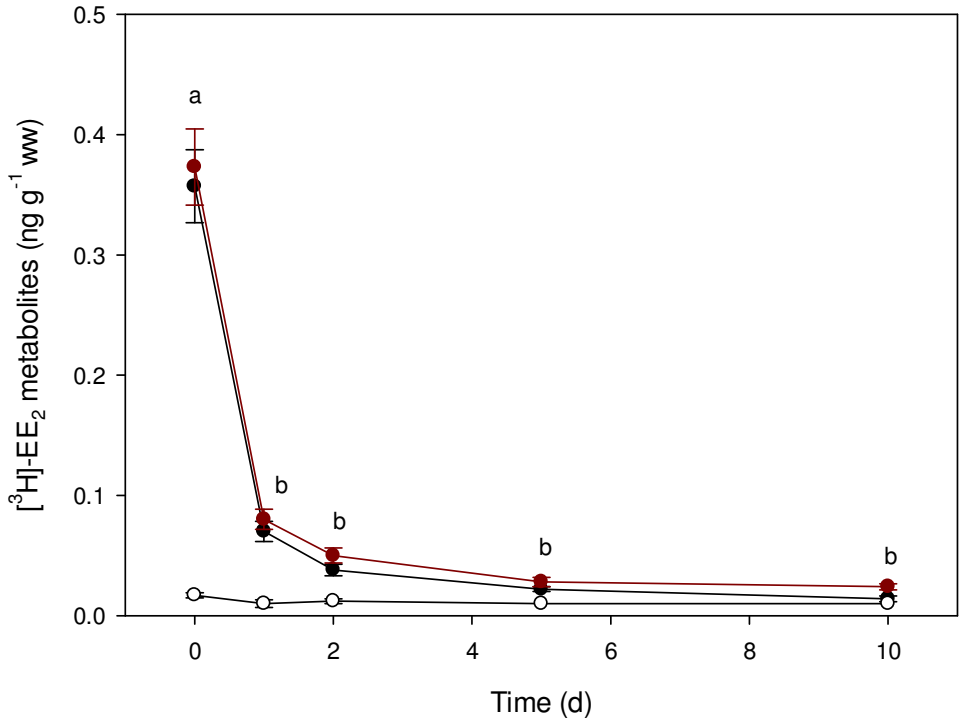

Figure 46: Free / sulphate fraction (●), ester fraction (○) and total [^{3}H]-EE$_2$ (●) in mussel tissue during depuration after exposure to [^{3}H]-EE$_2$ for 24 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-EE$_2$ residues (n = 5 except at time 0 where n = 10), where observations labelled with a different letter ('a' or 'b') are statistically significantly different from each other (p < 0.05).

4.3.5 Field Survey

Caged mussels (six weeks deployment) from three assumed polluted areas from the Thames (Gravesend, Southend-on-Sea and Chatham), a reference site at Norfolk (Morston, a shellfishery) and an offshore site (Wharp) were kindly collected by Tim Bean during different investigations. Esters, sulphates and free forms of EE_2 were measured in four mussels from each site (Figure 47). All EE_2 measurements were close to the detection limit of the assay (1.95 pg), which puts a question mark over their reliability (and will thus need further investigation). E_2 (see Figure 33 in Chapter 3), on the other hand, was easily quantifiable in the ester and free fractions. Like E_2, there was no obvious sign of differences between sites, but a much bigger sample size is needed to draw meaningful conclusions. It was interesting to note, though, that both free and esterified EE_2 in field mussels were variable among individuals (Figure 48 and Figure 49) suggesting that it is the steroid that is being measured and not non-specific compounds that cross-react with the antibody. The lack of activity in the sulphate fraction was consistent with E_2 data and was expected (i.e. several sulphate peaks were noted during TLC - implying that it was not EE_2 itself, but unknown metabolites, that were being sulphated.

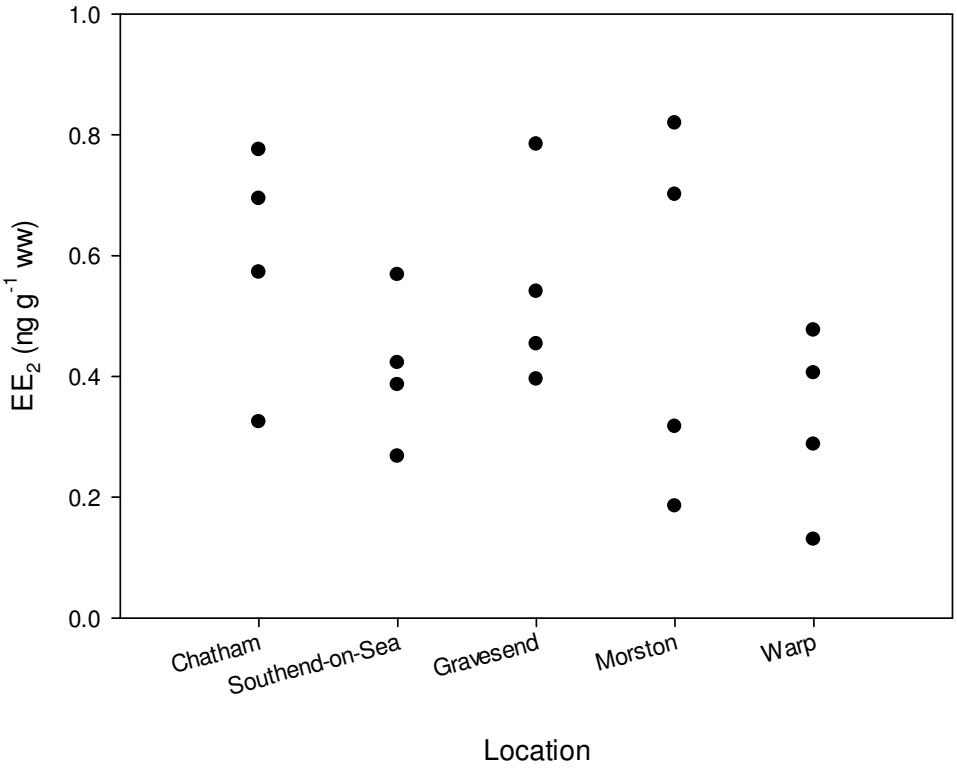

Figure 47: Total EE_2 burden in field mussels. Mussel tissue was extracted and submitted to three treatments (hydrolysis, solvolysis and extraction) before measuring EE_2 via radioimmunoassay. Data are presented as total ng g^{-1} EE_2 wet weight of four individual animals per location (n = 1).

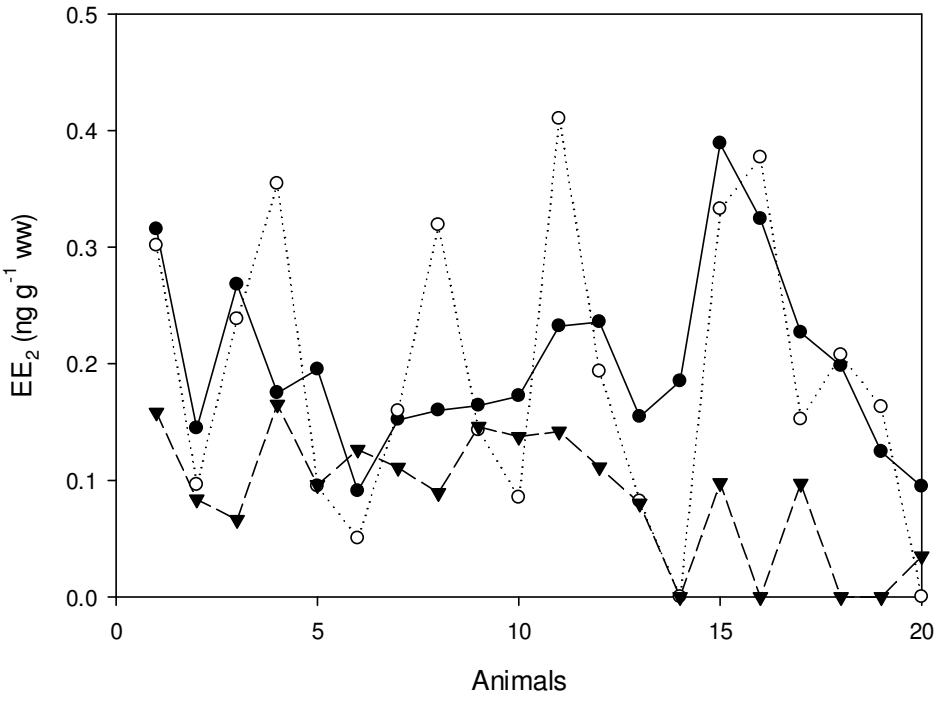

Figure 48: Immunoreactive EE_2 distribution in field mussels. EE_2 ester (○), free EE_2 (●) and EE_2 sulphate (▼) were measured separately in field mussel extracts taken from different locations. Data are presented as ng g^{-1} EE_2 ester, free or sulphated for each individual mussel (n = 1). Values of zero were assigned to samples below the limit of detection.

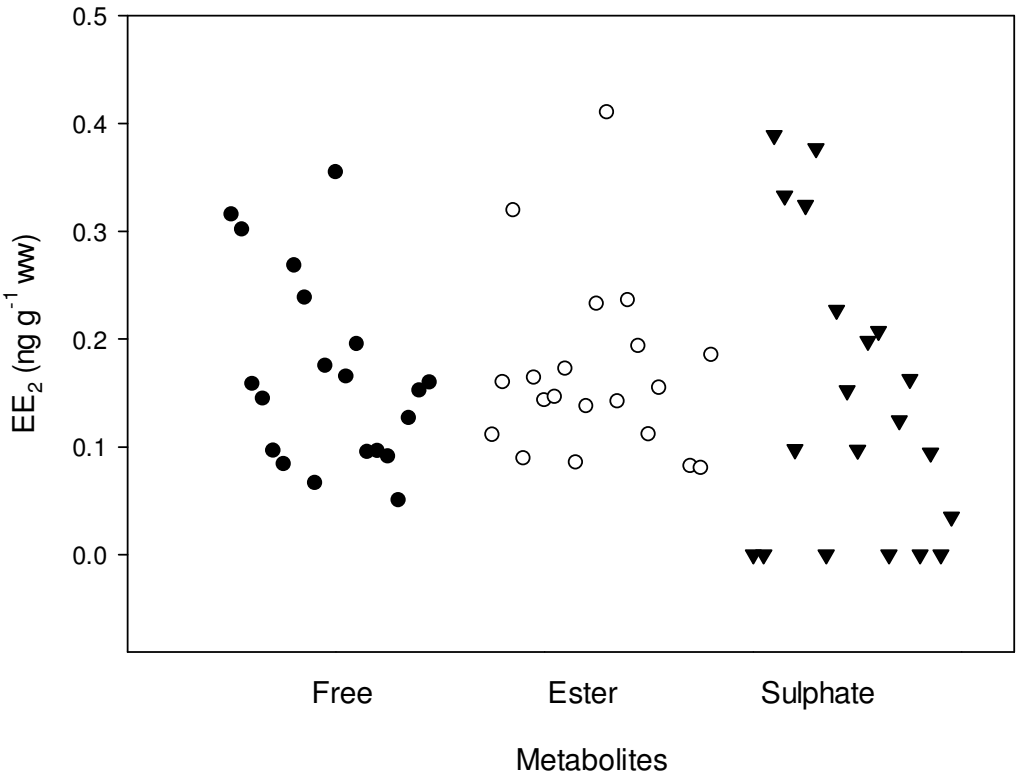

Figure 49: Different forms of immunoreactive EE_2 in caged mussels from from the Thames estuary (Chatham, Southend-on-Sea, Warp, Gravesend and Morston). Data are presented as ng g^{-1} ww of EE_2 (n = 20). Values of zero were assigned to samples below the limit of detection.

4.4 Discussion

4.4.1 *Mytilus spp.* pick up [^{3}H]-EE_2 from water.

Mussels exposed to [^{3}H]-EE_2 readily picked it up from the water. The literature had already hinted that this was possible when EE_2 was found in wild molluscs (Liu et al., 2009; Lu et al., 2001), since it is a man-made compound and does not exist naturally it therefore must have been picked up from the environment. EE_2 exposures, where the main objective was to assess vertebrate-like endocrine disruption, also gives a clue i.e. large amounts of EE_2 disappeared from the exposure water over time (Ciocan et al., 2010; Cubero-Leon et al., 2010; Giusti et al., 2014). To the best of our knowledge, this is the first time *Mytilus* spp. (or any other mollusc) have been exposed to radiolabelled EE_2, providing direct, solid evidence that bivalves can pick up [^{3}H]-EE_2 from water.

When comparing [^{3}H]-EE_2 to [^{3}H]-E_2 removal from water, the first thing that stands out is that although uptake of both steroids was very similar in Study 5, during Study 1 (exposures 1 and 2) [^{3}H]-EE_2 uptake halted (ca. 20 h) much earlier than [^{3}H]-E_2. The difference is also noticeable regarding clearance rates within the first 3 h – animals in Study 5 cleared around 40 mL animal^{-1} h^{-1} of both [^{3}H]-E_2 and [^{3}H]-EE_2; whereas in Study 1, [^{3}H]-EE_2 was cleared quicker than [^{3}H]-E_2 (ca. 46 v. 36 mL animal^{-1} h^{-1} respectively). Note that during Study 1, both [^{3}H]-E_2 and [^{3}H]-EE_2 clearance rates were lower during the second exposure period. There is no obvious reason for these differences other than, perhaps, the high variability between individuals (e.g. filtering rates) and differences in bacterial load / type which might affect steroid metabolism in the water. For example there is quite a difference (~10 %) between the first and second [^{3}H]-E_2 exposure of Study 5 (Figure 14 in Chapter 2), even though the animals were collected from the same place at the same time and were exposed under the same conditions. The lack of studies that have used radioactive material to investigate EE_2 uptake, means there are no data in the literature to compare clearance rates or even final uptake. The only conclusion that can be made is that, in general, E_2 and T uptake is usually higher overall (consistent with Study 1) than EE_2 uptake. Puinean *et al.* (2006) found that after 24 h ~70 % E_2 had disappeared from water (although this was based on immunoreactivity which would not have detected E_2 metabolites in the water and could, therefore, lead to an overestimation of uptake) and Gooding & LeBlanc, (2001) reported that 75 % of [^{14}C]-T was removed after 8 h.

4.4.2 Why does [^{3}H]-EE_2 uptake come to a halt?

4.4.2.1 What is happening in the water?

In Chapter 2 it was noted that [^{3}H]-E_2 uptake reached a plateau that appeared to be the result of [^{3}H]-E_2 metabolism. Basically, by 24 h, there was little to no intact [^{3}H]-E_2 left in the water. The bulk of what was left in the water was a sulphated compound (chromatography suggested it might be E_2 3-sulphate). Partitioning (between water and diethyl ether) of [^{3}H]-EE_2 water extracts after 24 h reveals a different scenario: 76 % of the radioactivity left in the water fractionated into the organic solvent, i.e.

most of the [^{3}H]-EE_2 left was still in its free form (presumably available for uptake). This finding was also confirmed chromatographically, where the largest radioactive peak (~71 %) co-eluted with the EE_2 standard and the retention time of the smaller peaks (8 and 21 %) were consistent with water soluble metabolites (Figure 40). This raises the question of why, if the bulk of the [^{3}H]-EE_2 in the water is still intact and its free form, does the rate of uptake still level off after 24 h. The experiment using different amounts of cold EE_2 (Study 5) showed that steroid concentration was not the limiting factor.

Based on the observations that, firstly, free [^{3}H]-EE_2 appears to be able to move freely not just from the water into the animal, but also from the animal into water (as indicated by the results of depuration experiments – see Figure 45 and Figure 46) and, secondly, that the rate of esterification of [^{3}H]-EE_2 is very obviously much slower than that of [^{3}H]-E_2, it was suggested that the uptake of [^{3}H]-EE_2 reaches a plateau because an equilibrium is reached in the exchange of free [^{3}H]-EE_2 between the two compartments (water and mussel tissue). Based on radiolabel specific activity it is estimated that at 24 h there was 0.007 ng mL^{-1} of free [^{3}H]-EE_2 in the water and 0.323 ng g^{-1} of free [^{3}H]-EE_2 in the tissue. This indicates that the steroid has more affinity for the tissue than the water. This is to be expected, as it will, firstly, be more soluble in fat than in water and, secondly, it is likely to be bound loosely to some of the proteins in the animal - in the same way that vertebrate steroids bind non-specifically to proteins such as albumin (Baker, 2002) and VTG (Yoshikuni et al., 1993). However, since this latter type of binding has a very low affinity (the affinity constant, K_d, of steroids is only between 10^{-4} M and 10^{-6} M for albumin), the percentages of free [^{3}H]-EE_2 would probably reach a fairly even balance between the animal and the water. Interestingly, these findings have many parallels with a study that looked at the *in vitro* uptake of radioactive 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) by oocytes of rainbow trout, *Oncorhynchus mykiss* (Scott et al., 1995). After an initial high rate of uptake by the oocytes (cf. the present study), the recovery of free 17,20β-P in the medium levelled off after 10 h at *c.* 50 % of the initial amount that was added (cf. the present study).

4.4.2.2 What is happening in the animal?

4.4.2.2.1 Extraction methodology

In order to investigate the fate of [^{3}H]-EE_2 in mussels, the extraction method developed with [^{3}H]-E_2 as a model (Method 1 in Chapter 2) was tested on other steroids ([^{3}H]-T and [^{3}H]-EE_2) and found to be significantly less effective. [^{3}H]-EE_2 extraction required a further step as well as the addition of chloroform to the methanol homogenisation step. It was decided that Method 2 should be tested for storage stability and used in future studies as it appeared to be consistently effective at extracting steroids from tissue.

4.4.2.2.2 Do *Mytilus* spp. esterify [^{3}H]-EE_2?

Research suggests the esterification of E_2 occurs preferentially via the hydroxyl group on C17 (Labadie et al., 2007; Scott, 2012). As [^{3}H]-EE_2 has an ethinyl group attached to the same carbon (and it has been placed there to make the molecule more recalcitrant) where esterification would in theory occur, it was initially thought that little or no [^{3}H]-EE_2 would be turned into a lipophilic ester in mussel tissue. To a certain extent this appears to be correct, since > 70 % of [^{3}H]-EE_2 was indeed free and only 15 % was esterified – almost the complete opposite to what happened to [^{3}H]-E_2 (see Figure 41 for comparison). However, the present study cannot confirm whether this small amount of esterification occurred at C17 or alternatively via the hydroxyl group on C3. Some of the [^{3}H]-EE_2 in the tissue (15 %) and possibly a small amount in the water, was also sulphated. In vertebrates, sulphation of EE_2 appears to occur exclusively via the C3 position (Han et al., 2010; Schrag et al., 2004), so perhaps, though this is only speculation, this is where sulphation also occurs in invertebrates. One argument against this is that, though the proportion of sulphates in tissue were the same for E_2 and EE_2 in mussels, in the water, sulphates (referring to any peak with a retention time under 40 min) accounted for > 50 % of the [^{3}H]-E_2 metabolites but < 20 % of [^{3}H]-EE_2 metabolites. If sulphation is purely occurring via the C3 position, one would not expect to see such a big difference between the two steroids. On the other hand, physical differences between the two steroids might make EE_2 less available to the sulphotransferase enzyme(s) in the tissues.

As these studies provided the first evidence of [^{3}H]-EE_2 esterification, it was deemed important to find out the proportion of [^{3}H]-EE_2 transformed into lipophilic metabolites and whether the esters were comprised of intact [^{3}H]-EE_2. Hydrolysis of the heptane fraction obtained from solvent partitioning followed by rp-HPLC revealed, surprisingly, that only 63 % of the total radioactivity corresponded to free [^{3}H]-EE_2 (by co-elution with EE_2 standard and further confirmed with co-migration with EE_2 on TLC) and four other peaks made up the other 37 %. The latter are unidentified metabolites of a lipophilic nature (hence their partitioning into the heptane phase). This means that not only was esterification of [^{3}H]-EE_2 a minor reaction (< 15 % of total radioactivity added to the water) but a third of this (~5.5 %) was actually esterification of unknown metabolites of [^{3}H]-EE_2. In the case of E_2, after hydrolysis of the ester, ca. 100 % of the radioactivity was intact E_2 (see Figure 20 in Chapter 2).

Characterisation of EE_2 metabolites in molluscs has not previously been reported. Studies that found EE_2 in molluscs in the wild have only measured free EE_2 (Liu et al., 2009; Lu et al., 2001) and exposure studies did not extract, let alone identify, EE_2 or its metabolites - most of them just measured reproductive endpoints (e.g. Andrew *et al.*, 2010; Giusti *et al.*, 2014; Leonard *et al.*, 2014; Langston *et al.*, 2007) or the effects of EE_2 on oestrogen receptor expression via quantification of mRNA (Ciocan et al., 2010; Hultin et al., 2014; Stange et al., 2012). These results show that, despite being relatively minor reactions, sulphation and esterification of EE_2 by mussels does occur and should be considered when quantifying any total oestrogen burden in wild mussels.

4.4.2.3 Are there any saturation effects?

Investigating [^{3}H]-EE_2 uptake was particularly interesting, as its natural counterpart – E_2 – was not only readily picked up but it was largely esterified - and its saturation limits, if any, where not reached (with a concentration of cold steroid as high as 25 µg L^{-1}). So, would [^{3}H]-EE_2 uptake be affected by the addition of cold EE_2 when most of what is taken up is free and not stored in the more permanent ester form? The answer was that, after the first three hours of exposure, there was only a hint of saturation (Figure 38) as shown by decreasing clearance rates of 39.9, 35.3

and 31.0 mL animal^{-1} h^{-1}. However, after 24 h, these differences were far less obvious, with total [^{3}H]-EE_2 removal reaching 50.0 %, 49.8 % and 47.2 %. These differences are extremely minor, reflecting most likely animal variability, when considering that it took 25 µg L^{-1} of cold EE_2 (a pharmacological concentration) to achieve them. Furthermore, exposure to increasing amounts of cold EE_2 proved to have the opposite effect on the accumulation of EE_2 residues in the animals. In other words, the mussels exposed to the highest amount of cold EE_2 were in fact the ones to accumulate the highest amount of radioactivity. This could potentially be due to reduced sorption to the shells of the animals or the exposure container, however, this is still only speculation.

4.4.3 Does a high proportion of free steroid increase the likelihood of depuration?

Placing the animals in clean water under flow-through conditions did indeed reduce the [^{3}H]-EE_2 burden by up to 80 %. The bulk of the reduction in [^{3}H]-EE_2 occurs within the first twenty four hours and after five days there appears to be no further depuration. This means that the rules of depuration applied in the shellfish industry (a minimum of 42 h depuration) would, in fact have the desired effect of purging any free EE_2. It would appear that esterification of steroids plays an important role in the depuration potential of a mussel. Figure 45 shows clearly that [^{3}H]-EE_2, stored mainly in its free form, is depurated more heavily than [^{3}H]-E_2 which is present in its majority as a lipophilic ester. This shows that steroid esters are more persistent than the more water-soluble free and sulphated steroids. The scarcity of published data on EE_2 presence in wild molluscs could perhaps be explained by the high level of depuration it undergoes. As most EE_2 is taken up as free steroid and animals are exposed to flowing water for a large part, if not the whole day, it is likely that EE_2 would not be retained for long. The amount of sulphated and esterified EE_2 (which might persist) would be very low, as suggested by these findings and, if the tissue extracts are not processed appropriately (i.e. hydrolysed), these metabolites would not be detected anyway.

4.4.4 Field survey

A small field survey was conducted in order to test the method developed in Chapter 2 on a different steroid and to corroborate these findings regarding esterification and depuration of free steroid. All three metabolites were close to the detection limit of the RIA (1.95 pg). Our findings differ greatly from one of the other few studies in the literature: Liu *et al.* (2009) reported an extraordinarily high (70 to 130 ng g^{-1} dry weight) level of free EE_2 in a variety of different molluscs, including mussels. In our experience, these levels seem improbable. It is not the presence of EE_2 that is in doubt (as the detection was done by mass spectrometry), but the levels. When similarly improbably large concentrations of steroids have been reported in the fish steroid literature, it has been shown that the most probable reason was calculation errors of one sort or another (Feswick et al., 2014). In fact, in their study (Feswick et al., 2014) found that three out of seven laboratories that carried out 'blind' steroid measurements on the same set of blood samples made calculation errors. In two cases, the calculation errors were as high as 1000-fold.

In all of the experiments involving EE_2, the readings in the RIA were so close to the limit of detection that they were not considered very reliable. The amounts were so low, in fact, that it is possible that immunoreactivity might be of non-specific nature. The only conclusions that could be drawn were that there appeared to be no difference between sampling sites and that as for E_2, EE_2 sulphates were close to the limits of detection. EE_2 esters tended to be slightly more abundant than free EE_2 (which would be expected if the esters are more persistent as they can accumulate over time). Ultimately, a larger sample size and more concentrated samples are necessary to draw more solid conclusions.

4.5 Conclusion

This Chapter has presented definitive evidence of the uptake, metabolism and depuration of [^{3}H]-EE_2 by *Mytilus* spp. It appears that the structural difference (the ethinyl group) between EE_2 and its natural counterpart, E_2, affects processes such as the rate of esterification and (as a downstream

effect) depuration. In other words, [^{3}H]-EE_2 was mostly (but not completely) unaffected by esterification and could therefore be easily depurated.

Chapter 5

Uptake, transformation and depuration of cortisol, progesterone and 17α,20β-dihydroxypregn-4-en-3-one

5.1 Introduction

Chapters 2 and 3 described the methods developed to expose mussels to radiolabelled 17ß-oestradiol ([^{3}H]-E_2) and then characterise the distribution of metabolites made in the animal and those present in the water. Chapter 4 followed on by applying this method to an extremely environmentally relevant synthetic oestrogen, 17α-ethinyl-oestradiol (EE_2). Results highlighted the importance of the hydroxyl group at position C17 of oestradiol (E_2) and EE_2 for esterification (specifically how the ethinyl group reduces the rate of esterification), and this, in turn, had a downstream effect on the rate of depuration. The differences between E_2 and EE_2 esterification (i.e. > 80 % vs < 30 %) - resulting from a small change in chemical structure - indicated that neither uptake nor metabolism of steroids by molluscs were subject to a universal law; instead these processes appear to be steroid-specific. This, together with the ubiquitous nature of natural steroids, led us to investigate the fate of three common vertebrate steroids with key structural differences: cortisol (F; as in Reichstein's substance F), progesterone (P) and 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P). F was chosen for the presence of a highly reactive hydroxyl group on C21; P for its lack of hydroxyl groups altogether and 17,20ß-P for its relatively reactive hydroxyl group on C20 (see Figure 50 for steroid structures). F also has hydroxyl groups at positions C11 and C17 (and 17,20ß-P at C17). However, these groups are not 'reactive' (i.e. they are in positions where neighbouring atoms prevent the attachment of conjugating groups).

F is very abundant in the aquatic environment. Fish studies have shown that it is heavily secreted by fish, particularly when stressed (Ellis et al., 2007). There have been very few uptake studies, even in fish. When exposed to [^{3}H]-F, tench (*Tinca tinca*) removed little to none (< 5 %) from

water in contrast to other steroids (e.g. E_2, 35 %) that were successfully removed from water and bioconcentrated (Scott et al., 2005). F, in fact was the only steroid in that study that was present at a higher concentration in water than in plasma at the end of the exposure period (i.e. it was not bioconcentrated at all) (Scott et al., 2005). Although F is very abundant in the aquatic environment, there is no data on its potential uptake and metabolism by molluscs.

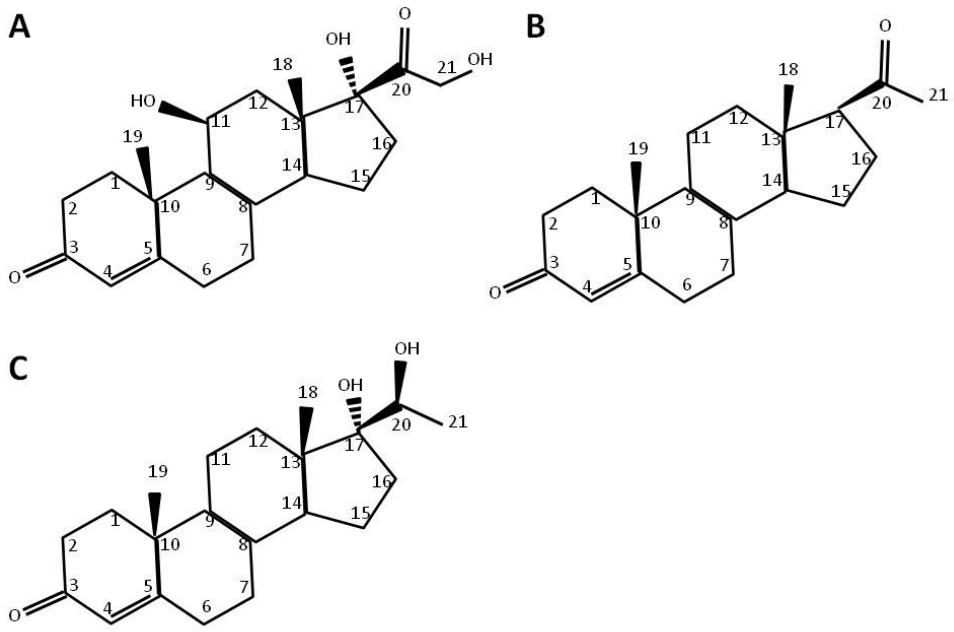

Figure 50: Chemical structure diagrams of F (A), P (B) and 17,20β-P (C).

P has been found in molluscan tissue, for example, Gust *et al. (*2010) detected low levels of P (total burden, i.e. free + esters) in caged New Zealand musdsnails, *Potamopyrgus antipodarum* (ca. 1 ng g^{-1}), by radioimmunoassay (RIA) and liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS); Tian *et al. (*2013) detected P (up to 0.25 ng g^{-1}) in Farrer's scallops, *Chlamys farreri*, using electro-chemoluminescence immunoassay after exposure to benzopyrene (B[a]P) and Dévier *et al.* (2010) found up 9 ng g^{-1} of free P in wild female blue mussels, *Mytilus* spp. These studies, amongst others, provide evidence of the presence of P in molluscs. However, as in the case of E_2, the presence of P in tissue has been ascribed in all cases (despite the lack of other evidence) to its endogenous production. Exposures of molluscs to P have been performed with the intent of investigating endocrine disruption (e.g.

effects on spawning) (Wang and Croll, 2006) or as a substrate to investigate enzymatic pathways pertaining to vertebrate steroidogenesis (Carreau and Drosdowsky, 1977; De Longcamp, 1974; Lehoux and Williams, 1971). Only two studies have exposed live molluscs to P to deliberately investigate uptake and metabolism. Hines *et al.* (1996) reported that after exposing the Antarctic pteropod, *Clione antarctica,* for 12 hours to tritiated P ([^{3}H]-P), ca. 70 % of [^{3}H]-P was turned into 5α-pregnane-3,20-dione (P5α), 3ß-hydroxy-5α-pregnan-20-one (3ß-P5α) and 3ß-hydroxy-4-pregnen-20-one, with some evidence of lipophilic ester production (1 %), although the latter was not identified. The authors also noted the presence of water soluble metabolites in the aqueous medium (also not identified). A more recent study exposed the Mediterranean mussel, *Mytilus galloprovincialis*, to unlabelled (i.e. 'cold') P *in vivo* as well as performing *in vitro* exposures of subcellular fractions using [^{3}H]-P (Dimastrogiovanni et al., 2015). Although the study measured the total immunoreactive P (free + ester) picked up by the mussels *in vivo*, only free steroids were investigated in the *in vitro* incubations. So there is some slight evidence of esterification in the literature but considering P does not possess any reactive hydroxyl groups, how does this occur?

Teleosts produce and use 17,20ß-P as a maturation-inducing hormone (i.e. oocyte final maturation) and pheromone (Sørensen and Scott, 1994). The latter role means it is released copiously into the water via the gills and as water soluble metabolites (sulphates and glucuronides) via urine during ovulation (Scott and Vermeirssen, 1994). Its presence in the water together with its particular distribution of hydroxyl groups made 17,20ß-P an environmentally relevant and structurally interesting candidate for this study.

The aim of this chapter was to gain further understanding of the requirements for uptake and esterification (and therefore long term storage and depuration potential) of vertebrate steroids by *Mytilus* spp.

5.2 Materials and methods

5.2.1 Chemicals

17α-Hydroxyprogesterone [1,2,6,7-^{3}H] ([^{3}H]-17α-P) and progesterone [1,2,6,7-^{3}H] were purchased from American Radiolabeled Chemicals, Inc. (101 ARC Dr. St. Louis, MO 63146 USA). Hydrocortisone, [1,2,6,7-^{3}H(N)] ([^{3}H]-F) was purchased from Perkin Elmer (Chalfont Road Buckinghamshire, Seer Green HP9 2FX, United Kingdom). Standard 'cold' P and F were purchased from Sigma-Aldrich Company Ltd. (Dorset SP8 4XT, UK). 17,20ß-P, P5α and 3ß-P5α were bought from Steraloids Inc. (PO Box 689, Newport, RI 02840,USA) and all other chemicals were purchased from Fisher-Scientific UK Ltd. (Loughborough LE11 5RG, UK).

Water used for laboratory exposures was filtered (50 µm) sea water and water used for all other purposes was reverse osmosis water unless stated otherwise.

5.2.1.1 Making radiolabelled 17,20β-P [1,2,6,7-^{3}H] ([^{3}H]-17,20β-P)

In order to make [^{3}H]-17,20ß-P, 250 µCi [^{3}H]-17α-P was dried under a stream of nitrogen at 40 °C. The dry label was then incubated on an orbital shaker at room temperature with 2 mg (2 units mg^{-1}) 20ß-hydroxysteroid dehydrogenase (20ß-HSD; Sigma Aldrich H2267; no longer manufactured); 2.3 mg reduced nicotinamide adenine dinucleotide (NADH; Sigma Aldrich 8129) and 1 mL tris(hydroxymethyl)aminomethane buffer (pH 7.6). After 2 h incubation, 3 mL of water were added to the vial and the radiolabel was extracted using 360 mg C18 cartridges (Waters Sep-Pak, WAT020515) conditioned with 5 mL methanol and 5 mL water. Extracts were washed with 5 mL water and eluted with 5 mL ethyl acetate. The extract was then dried at 40 °C under a stream of nitrogen, re-suspended in 1 mL ethanol and stored at -20 °C.

To confirm the enzymatic reaction had taken place, a small sample of the newly made [^{3}H]-17,20ß-P was mixed with 5 µg of 17α-hydroxyprogesterone (17α-P) and 17,20ß-P standards and run on a TLC place as described in section 5.2.5.1.

5.2.1.2 Study 5

5.2.1.2.1 Collection and acclimation

Mussels from a mixed population of *M. edulis* and *M. galloprovincialis* were collected from Portland Harbour in October 2014. The nearby northeast Portland Harbour breakwater is a catchment holding a long term class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). They were transported to the lab in a cool-box and immediately placed in a flow-through system of filtered sea water. Fifty animals were selected (ranging between 47.26 to 69.63 mm, mean 56.34 mm) and cleaned. Ten animals were used for pre-study condition index analysis. In order to acclimate the animals, five mussels were placed in an aerated bucket lined with a polyethylene bag and filled with 2 L of filtered sea water at 16 ± 1°C with a 16:8 h light:dark photoperiod for five days prior to exposure. Water was changed daily and animals were fed Shellfish Diet® 1800 daily (following manufacturer´s instructions).

5.2.1.2.2 Exposure and depuration

For Study 5, animals were acclimated under flow-through conditions for 14 d before exposing them to [^{3}H]-P, [^{3}H]-17,20B-P and [^{3}H]-F for 24 h. Six vessels contained hot (i.e. radioactive) steroid only and four vessels had hot and either of two concentrations of cold steroid (see Table 14 for nominal concentrations). Each exposure had a solvent control of ten animals (divided between two bags) with 200 µL ethanol (carrier) and one sorption control (no animals). Water samples (1mL) were taken (0, 3, 6, 18, 24 h) from all bags and immediately placed in scintillation fluid for counting. After exposure, the mussels were frozen at -20°C for analysis.

Following exposure for 24 h, mussels from Study 5 were placed in clean water in a flow-through system (1 L min^{-1}) for 10 days. Ten mussels were sampled on day 0 and five mussels on day 1, 2, 5 and 10 and immediately stored at -20 °C.

5.2.1.3 Condition index analysis

As described in section 2.2.3 in Chapter 2.

5.2.2 Clearance rates

The rates at which individual mussels cleared steroids from water over the first 3 h period (i.e. clearance rates) were calculated for as described in section 2.2.4 in Chapter 2.

5.2.3 Steroid extraction methods

5.2.3.1 Water extraction and extract clean-up

[^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20ß-P were extracted from water as described in section 2.2.5.1 Chapter 2,

5.2.3.2 Extraction of steroids from tissue

Radioactive residues were extracted from mussel tissue using Method 2 described in section 2.2.5.2 Chapter 2.

5.2.3.3 Separation of free, sulphated and lipophilic steroid metabolites

Mussel extracts were partitioned using the separation method developed in section 2.2.5.3 in Chapter 2. The method was first tested on three [^{3}H]-P and [^{3}H]-17,20ß-P mussel extracts. The heptane phase was collected and mixed with 800 µL of unexposed mussel extract before drying. They were then separated again to assess ester recovery into the heptane phase.

5.2.3.4 Alkaline hydrolysis

Crude extracts were separated as described in 3.2.3; the heptane fraction was then dried at 40 °C under a stream of nitrogen and submitted to alkaline hydrolysis as described in section 3.2.4.1 in Chapter 3.

5.2.4 Chromatography

5.2.4.1 Reverse-phase High Performance Liquid Chromatography

Reverse-phase HPLC (rp-HPLC) (using an analytical column) was used to separate free and conjugated (i.e. either sulphated or glucuronidated) metabolites of [^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20ß-P in water extracts and also the products of alkaline hydrolysis of the esters formed from [^{3}H]-P and

[^{3}H]-17,20ß-P. The procedure was as described in section 2.2.6.2 in Chapter 2 (with the appropriate standards).

5.2.5 Thin layer chromatography

5.2.5.1 Separation of steroid metabolites after alkaline hydrolysis

The heptane fraction of [^{3}H]-P was dried down, subjected to alkaline hydrolysis (3.2.4.1) and then partitioned again between heptane and 80 % ethanol. The 80 % ethanol fraction was dried down, mixed with 5 µg each of progesterone, P5α and 3ß-P5α (as described previously; Scott *et al.*, 2014) and loaded onto one lane of a thin layer chromatography (TLC) plate (catalog no. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) and developed for 45 min with a mixture of chloroform:methanol (50:2, v:v). After marking the positions of the standards using a UV lamp, the lane was divided into 5 mm bands, and the silica gel from each band was scraped off the plate. The scrapes were mixed with 1 mL 80 % ethanol and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity.

5.3 Results

5.3.1 Production of [^{3}H]-17,20β-P

When the new label was analysed by thin layer chromatography it revealed that all radioactivity co-migrated with the 17,20ß-P standard and none with the 17-P standard, proving that enzymatic conversion of [^{3}H]-17α-P → [^{3}H]-17,20ß-P was fully successful.

5.3.2 Condition index

Condition index was used as a means of monitoring the effects of exposure (particularly solvents) on animal health. [^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20ß-P experiments were conducted at the same time (i.e. shared solvent) as the [^{3}H]-E_2 (Study 5) exposure, therefore a single batch of animals was subjected to condition index analysis. Figures for Study 5 can be found in section 2.3.1 in Chapter 2. Briefly, results indicated there was no significant difference between pre-study animals and solvent controls,

suggesting that exposure conditions (including exposure to the carrier) did not have a negative impact on the animals' health.

5.3.3 Pattern of uptake of radiolabelled F, P and 17,20β-P from water

[^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20β-P uptake experiments were done by exposing mussels for 24 h in 2 L of water (five animals per vessel). Cold F, P and 17,20β-P were also added to some of the vessels in order to investigate if there was a saturation limit for uptake. Mean concentrations of [^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20β-P at the time of dosing of the vessels with no added cold steroid ('hot only'), low levels of cold steroid (low) and high levels of cold steroid (high) (n = 6, 2 and 2 respectively) are presented in Table 14.

Table 14: Summary of chemical concentrations during exposure studies.

Treatment	Radiolabel conc. (μCi L^{-1})[a]	Radiolabel conc. (ng L^{-1})	Nominal cold steroid conc. (ng L^{-1})
[^{3}H]-F	3.1	11.2	-
F low	3.0	11.1	2500
F high	3.1	11.3	25000
[^{3}H]-P	2.5	8.5	-
P low	2.3	7.8	2500
P high	2.4	8.1	25000
[^{3}H]-17,20β-P	3.2	21.4	-
17,20β-P low	3.4	22.4	2500
17,20β-P high	3.2	21.3	25000

[a]**Concentration of radiolabels is based on radioactivity in the water at time of dosing (i.e. time zero).**

5.3.3.1 [^{3}H]-F exposure

Over time there was a significant decrease (log transformation of time was applied to achieve a linear relationship) in the level of [^{3}H]-F in the water ($df = 1$, $f = 72.403$, $p < 0.0001$). Up to 8.8 %, 5.4 % and 6.7 % of total radioactivity disappeared from each treatment over 24 h (Figure 51). However, ca. 6.7 % activity also disappeared in the sorption control tanks over the same period. In other words, the loss of [^{3}H]-F from water was

likely a result of sorption only and was, therefore not a result of mussel uptake. This was also evident in the extremely low clearance rates (based on the first 3 h) of 5.4, 3.3 and 4.1 mL animal^{-1} h^{-1} (see Table 15 for comparison to other steroids). It should also be noted that treatment had no significant effect on the decay curves (df = 2, f = 1.586, p = 0.216).

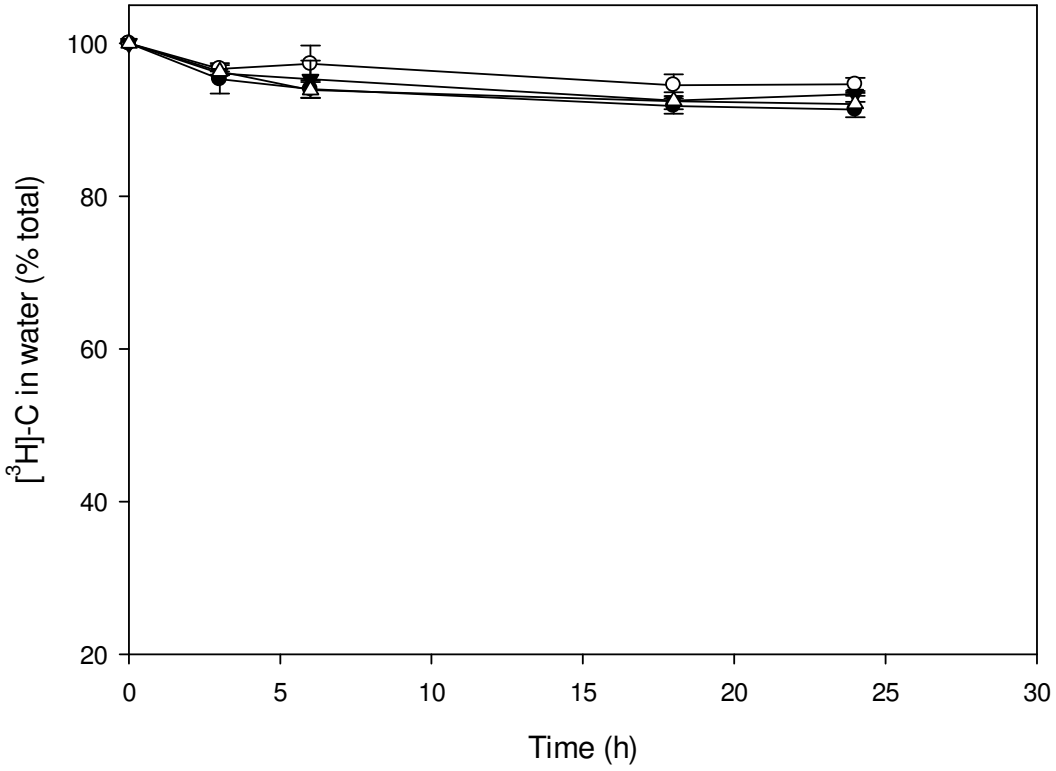

Figure 51: [^{3}H]-F (8.5 ng L^{-1}) nominal concentration) removal by *Mytilus* spp. in the presence of increasing amounts of cold F over 24 h (0 µg L^{-1}:●, 2.5 µg L^{-1}:○ and 25 µg L^{-1}:▼ nominal concentrations). Data are presented as mean % total (n = 6 tanks for ● and 2 for ○,▼) and ± S.E.M. And a sorption control (no mussels in tank) (△; n=1).

5.3.3.2 [^{3}H]-P exposure

Up to 68.0 %, 69.6% and 76.0 % of total radioactivity was removed from the water during each treatment over 24 h (Figure 52). However, unexpectedly, even larger amounts of activity were also lost to sorption (91.4 %; fitted data, linear model). Hence, the percentage loss of [^{3}H]-P from water at the end of the experiment was not a true reflection of what was accumulated by the animals. It was not possible to get a clear picture of the rates of uptake or saturation when comparing clearance rates either (63.7, 57.2 and 79.3 mL animal^{-1} h^{-1}, respectively - see Table 15), even though these were based on the first 3 h uptake. In order to see if the

addition of large amounts of cold P has any effects on uptake, the experiment would have to be repeated under conditions where the [^{3}H]-P is not subject to sorption (NB. a simple experiment with label and different types of containers showed that the aerator was the main cause of sorption).

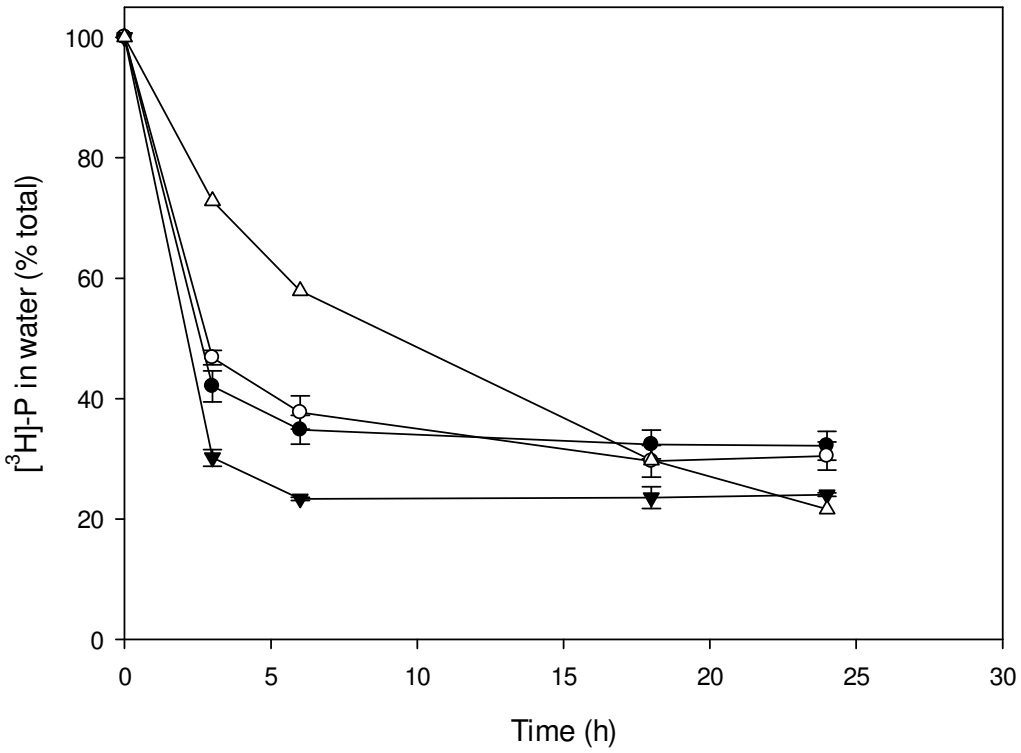

Figure 52: [^{3}H]-P (8.5 ng L^{-1} nominal concentration) removal by *Mytilus* spp. in the presence of increasing amounts of cold P over 24 h (0 µg L^{-1}:●, 2.5 µg L^{-1}:○ and 25 µg L^{-1}:▼ nominal concentrations). Data are presented as mean % total (n = 6 tanks for ● and 2 for ○,▼) and ± S.E.M.. And a sorption control (no mussels in tank) (n = 1).

5.3.3.3 [^{3}H]-17,20β-P exposure

Figure 53 shows that over time there was a significant decrease (log transformation of time was applied to achieve a linear relationship) in the level of [^{3}H]-17,20ß-P in the water (df = 1, f = 262.790, p < 0.0001). Up to 31.7 %, 33.7 % and 45.1 % of total radioactivity was removed from each treatment over 24 h and sorption of [^{3}H]-17,20ß-P only accounted for 6 % (fitted data, linear model). Although treatment explained a lot of the variability between decay curves after 24 h (df = 2, f = 14.221, p < 0.0001), pairwise comparisons revealed that there was no significant difference between the decay curves of both low and high cold 17,20ß-P treatments and the 'hot only' regime (ß = 1.4195, SE = 0.8310, t = 1.708, p = 0.0947

and ß = 0.2286, SE = 0.8310, t = -0.275, p = 0.7845 respectively). In other words, there was no clear evidence of saturation of [^{3}H]-17,20ß-P uptake after 24 h. Furthermore, clearance rates (based on the first 3 h; 27.8, 23.9 and 35.5 mL animal^{-1} h^{-1}) (Table 15) were not affected by the addition of large amounts of cold steroid either. A linear model analysis of the first three hours of uptake (where time was treated as a factor) revealed that treatment had a significant effect on the decay of [^{3}H]-17,20ß-P (df = 2, f = 3.774, p = 0.0489). However, pairwise comparisons showed that there was no significant difference in [^{3}H]-17,20ß-P decay between both the low and high cold steroid treatments and the 'hot only' regime (ß = 0.9500, SE = 0.8415, t = 1.129, p = 0.278 and ß = -0.2000, SE = 0.8415, t = -0.238, p = 0.816 respectively). In summary, there was little to no evidence of saturation throughout the experiment, even with addition of 25 µg L^{-1} of cold 17,20ß-P.

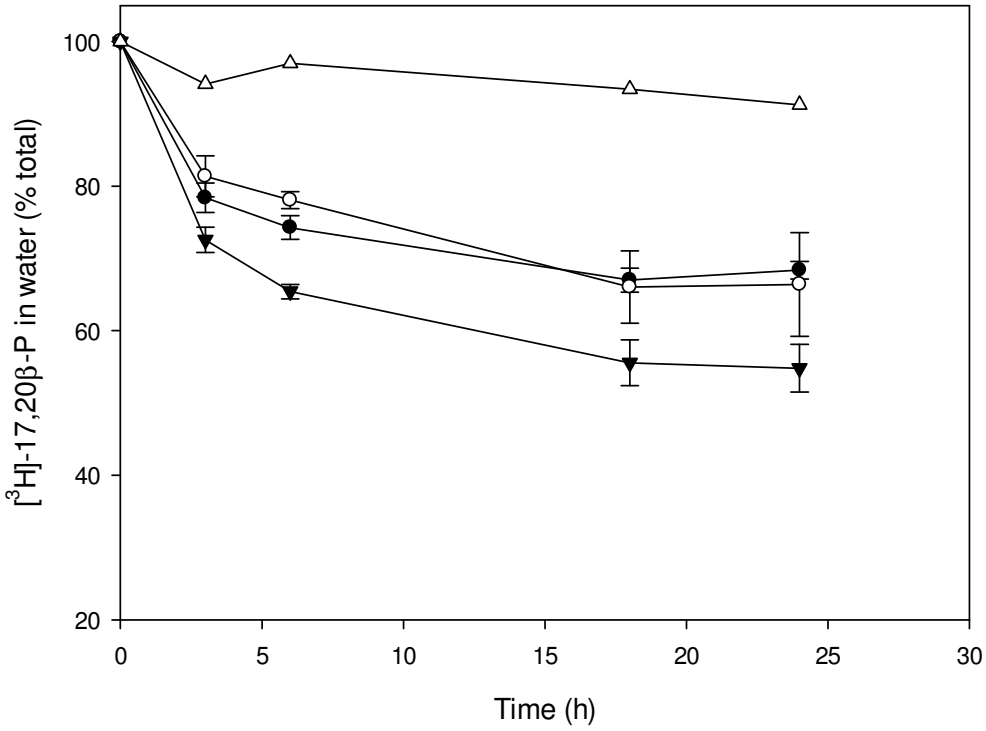

Figure 53: [^{3}H]-17,20β-P (8.5 ng L^{-1} nominal concentration) removal by *Mytilus* spp. in the presence of increasing amounts of cold 17,20β-P over 24 h (0 µg L^{-1}:●, 2.5 µg L^{-1}:○ and 25 µg L^{-1}:▼ nominal concentrations). Data are presented as mean % total (n = 6 tanks for ● and 2 for ○,▼) and ± S.E.M. And a sorption control (no mussels in tank) (n = 1)

Table 15: Summary of exposure conditions and radiolabelled steroid clearance rates from water by *Mytilus* spp.

Study	Water vol. (L)	N° animals	Label conc. (µCi L^{-1})	Exposure time (h)	Removal after exposure (%)[c]	Fitted sorption after 3 h (%)	Removal after 3 h (%)	Clearance rate (mL.animal^{-1}.h^{-1})[d]
5 [^{3}H]-F	2.0	5	3.1	24	8.8	0.8	4.9	5.4
5 F low[a]	2.0	5	3.0	24	5.4	0.8	3.3	3.3
5 F high[b]	2.0	5	3.1	24	6.7	0.8	3.9	4.1
5 [^{3}H]-P	2.0	5	2.5	24	68.0	10.3	58.0[e]	63.7[e]
5 P low[a]	2.0	5	2.3	24	69.6	10.3	53.2[e]	57.2[e]
5 P high[b]	2.0	5	2.4	24	76.0	10.3	69.7[e]	79.3[e]
5 17,20ß-[^{3}H]-P	2.0	5	3.2	24	31.7	0.7	21.6	27.8
5 17,20ß-P low[a]	2.0	5	3.4	24	33.7	0.7	18.7	23.9
5 17,20ß-P high[b]	2.0	5	3.2	24	45.1	0.7	27.4	35.5

[a]Low: 2.5 µg L^{-1} cold steroid.

[b]High: 25 µg L^{-1} cold steroid.

[c]Fitted sorption not accounted for in removal after exposure.

[d]Fitted sorption accounted for in clearance rates.

[e]Presented for comparison purposes only – data not reliable due to high sorption.

5.3.3.4 What happens to the steroids in the water?

5.3.3.4.1 [^{3}H]-F

Since there was no strong evidence that mussels picked up radiolabelled F, it was important to see what was happening to it in the water. Was a high level of sulphation (shown to impede uptake of [^{3}H]-E_2 - see Chapter 2) perhaps responsible for the lack of uptake? To investigate this, water samples were taken at the end of Study 5 from 'hot-only' exposure vessels and the sorption control. When subjected to solid phase extraction, ca. 11.1 and 1.5 % of the activity from the exposure vessel and the sorption control, respectively, passed straight through. This probably represented tritiated water (released from the parent compound by unknown reactions). After the extracts had been eluted from the solid phase extraction disks with methanol and then dried down, a portion was dissolved in water and then shaken with diethyl ether. The extract that had contained the mussels retained 28 % of radioactivity in the water fraction (assumed to be a water-soluble conjugate) and 72 % in the ether fraction (assumed to be free steroid). Sorption control extract yielded 21 % and 79 % respectively, i.e. both extracts had greater amount of free steroid than water soluble metabolites and there was very little difference between the test vessels and the control.

The extracts were then analysed by reverse-phase HPLC at 245 nm with added F standard. Figure 54 shows that the main radioactive peak from both sorption control (no mussels) and test vessel (with mussels) exposures eluted at 40 min, which was also the retention time of the F standard. A smaller peak with a retention time of 37 min appeared in the test vessel extract only - consistent with the presence of water-soluble metabolites. Although none of the compounds in the water have yet been identified, the results suggest that most of the intact radiolabel is still present in the water at 24 h, i.e. the inability to pick up [^{3}H]-F from water is not a result of [^{3}H]-F metabolism.

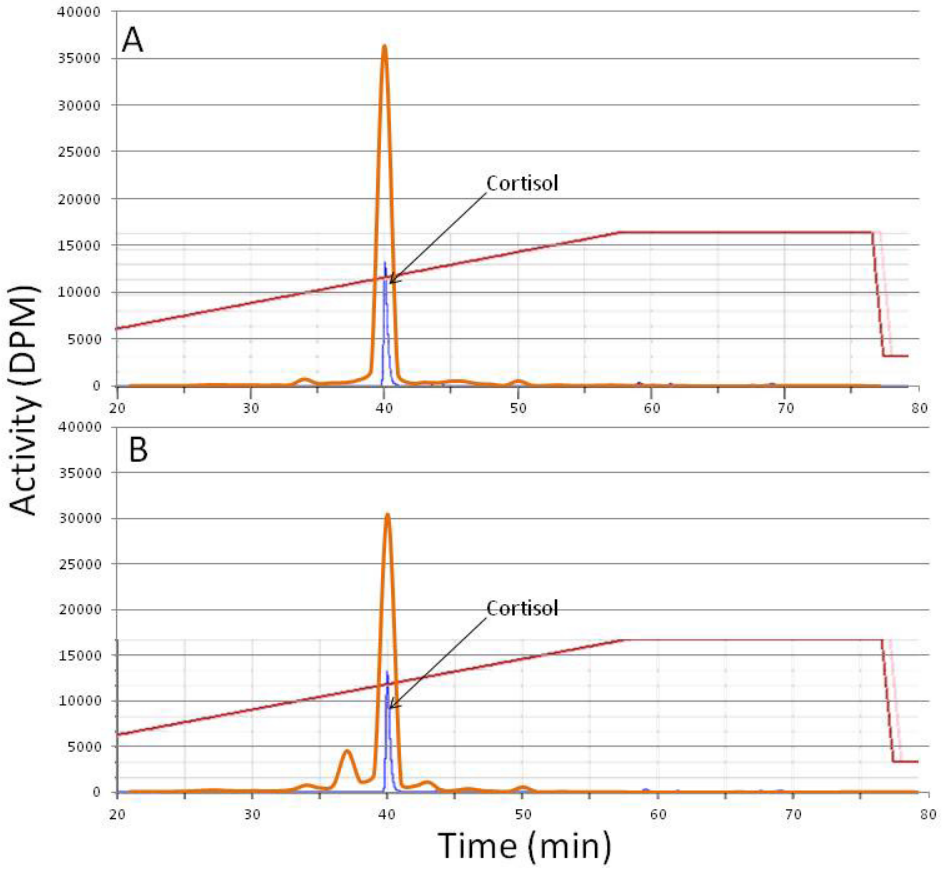

Figure 54: HPLC chromatogram of [^{3}H]-F exposure water; sorption control (A) and test vessel (B) (Study 5) with F standard. Data are presented as UV absorbance (245 nm; blue) and radioactivity (orange).

5.3.3.4.2 [^{3}H]-P

Water samples were taken at the end of Study 5 from 'hot-only' exposure vessels and the sorption control. When subjected to solid phase extraction, ca. 30.1 % and 51.1 % of the activity from the sorption control and exposure vessel, respectively, passed straight through (presumed to be tritiated water). The high proportion of tritiated water in both the sorption control and tests vessel was unusual. It could, however, be explained by the fact that after 24 h, there was actually very little radioactivity left in the water at all, the rest having been taken up by the mussels or adsorbed to the plastic bags and / or aerator. After the extracts had been eluted from the solid phase extraction disks with methanol and then dried down, a portion was dissolved in water and then shaken with diethyl ether. The test vessel (i.e. with mussels) water extract retained 15 % of radioactivity in the water fraction (assumed to be a water-soluble conjugate) and 85 % in the ether fraction (assumed to be free steroid). The sorption control extract yielded

2 and 98 % respectively, i.e. both extracts had greater amount of free steroid than water soluble metabolites.

The extracts were then analysed by reverse-phase HPLC at 245 nm with added standard. Figure 55 shows that the test vessel had no peak with the same retention time as the P standard and the sorption control had several radioactive peaks with only a very small one co-eluting with P standard at minute 65. Although none of the compounds in the water have yet been definitively identified, the results suggest that there is no intact [^{3}H]-P left in the water at 24 h in the test vessel but, most importantly, little to none in the sorption control tank either.

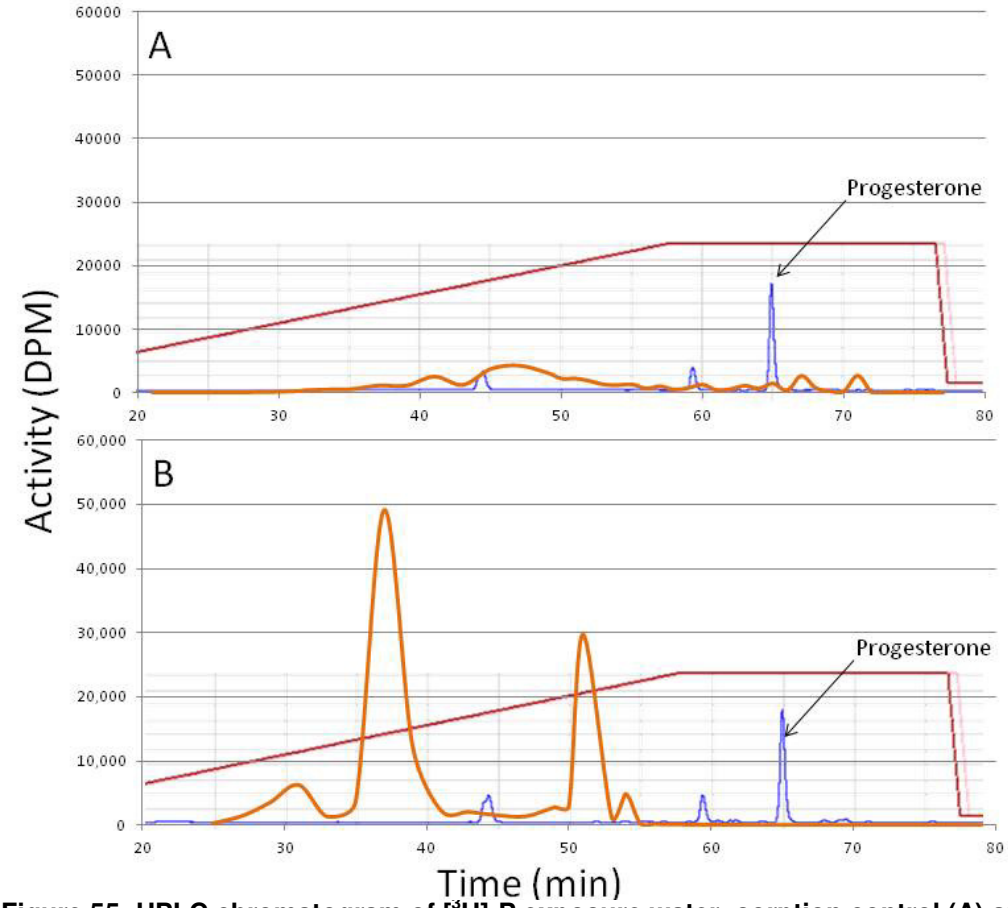

Figure 55: HPLC chromatogram of [^{3}H]-P exposure water; sorption control (A) and test vessel (B) (Study 5) with P standard. Data are presented as UV absorbance (245 nm; blue) and radioactivity peaks (orange).

5.3.3.4.3 [^{3}H]-17,20β-P

The uptake of [^{3}H]-17,20ß-P came to a halt after 5 to 10 h exposure roughly at the same time as it did for [^{3}H]-E_2. In order to see whether sulphation of [^{3}H]-17,20ß-P was taking place and hindering uptake, as was the case for

[^{3}H]-E_2, water samples were taken at the end of Study 5 from 'hot-only' exposure vessels and the sorption control. When subjected to solid phase extraction, ca. 6.8 and 31.7 % of the activity from the sorption control and exposure vessel, respectively, passed straight through. After the extracts had been eluted from the solid phase extraction disks with methanol and then dried down, a portion was dissolved in water and then shaken with diethyl ether. The test vessel (i.e. with mussels) water extract retained 10 % of radioactivity in the water fraction (assumed to be a water-soluble conjugate) and 90 % in the ether fraction (assumed to be free steroid). Sorption control extract yielded 2 and 98 % respectively, i.e. both extracts had greater amount of free steroid than water soluble metabolites and there was very little difference between the test vessel and the control.

The extracts were then analysed by reverse-phase HPLC at 245 nm with added 17,20β-P standard. Figure 56 shows that the main radioactive peak of the control extract co-eluted with the standard between minute 52 and 53. The same peak was also present in the test vessel extract, however, most of the radioactivity actually eluted at minute 57. The test vessel extract also presented several peaks with retention times < 40 min (~15 %, similar to the 10 % water-soluble metabolites found through fractionation), consistent with water soluble metabolites. Although none of the compounds in the water have yet been definitively identified, the results suggest that although there was some intact label after 24 h in the test vessel, most of it had been metabolised.

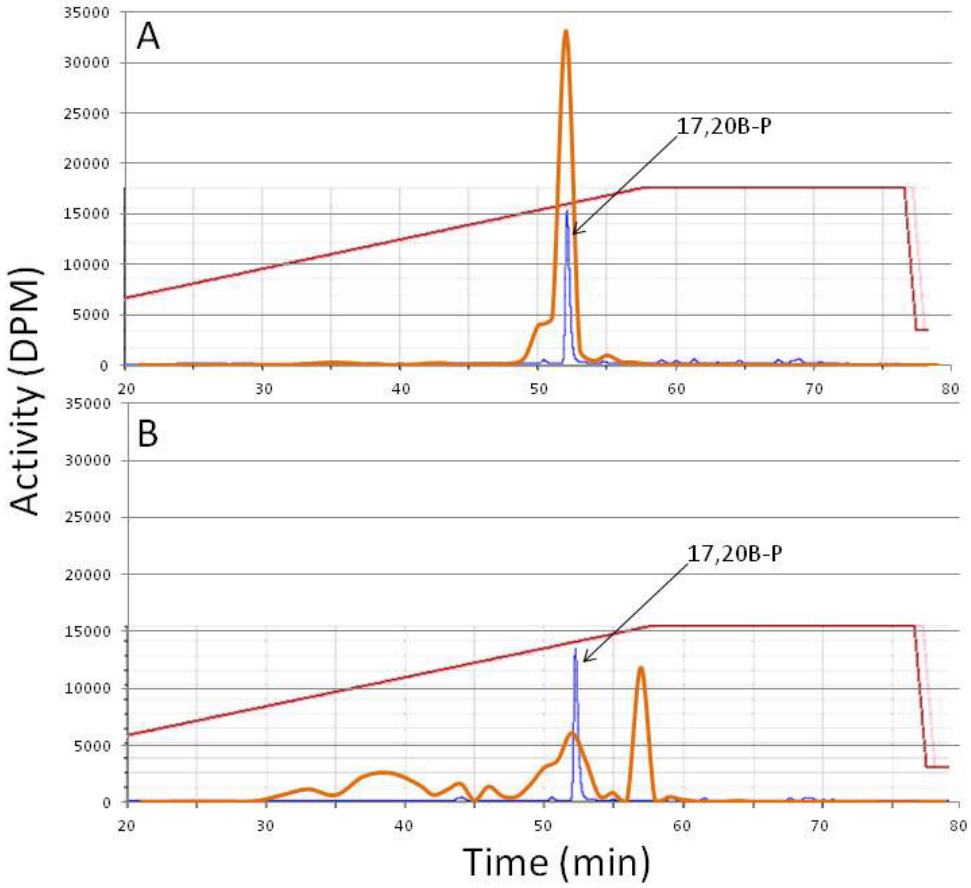

Figure 56: HPLC chromatogram of [^{3}H]-17,20β-P exposure water; sorption control (A) and test vessel (B) (Study 5) with 17,20β-P standard. Data are presented as UV absorbance (245 nm; blue) and radioactivity peaks (orange).

5.3.4 What happens to the steroids in the mussel?

In order to investigate the fate of [^{3}H]-P and [^{3}H]-17,20ß-P in mussels, extraction Method 2 (developed with [^{3}H]-E_2 as a model; see Chapter 2) was tested and found to be effective for both steroids (> 97 % recovery; Table 16). Mussels exposed to [^{3}H]-F were not extracted or further processed as there was no evidence of uptake.

The radioactive residues from time zero (i.e. before depuration) were then separated into free steroid, sulphate and ester (F:S:E) using the method developed in Chapter 2 (see Table 17). Figure 57 shows that the distribution of [^{3}H]-17,20ß-P was 45:7:49. The distribution of [^{3}H]-P was 62:3:35. Note that both progestagens had a very small proportion of sulphated metabolites (less than half of that found for [^{3}H]-E_2, see Chapter 3). It is also interesting that, even though [^{3}H]-17,20ß-P was largely free in

the mussel, the large amounts of cold steroid (25 µg L^{-1}) added to the water during exposure did not saturate uptake (see Figure 53).

Table 16: Method development of [^{3}H]-P and [^{3}H]-17,20β-P extraction (from tissue).

Method	Labelled steroid	Extracts (% total)				Extracts 1 & 2 efficiency (%)	Method efficiency (%)
		1	2	3	4		
2	17,20B-P	87	11	2		98	98
	17,20B-P	91	8	1		99	99
	17,20B-P	81	16	3		97	97
	17,20B-P	85	13	2		98	98
	17,20B-P	90	9	1		99	99
	17,20B-P	81	16	3		97	97
	17,20B-P	-	99	1		99	99
2	P	90	8	2		98	98
	P	85	13	2		98	98
	P	84	14	2		98	98
	P	-	99	1		99	99

Table 17: Summary of metabolite separation method efficiency.

Labelled sample[a]	Vortex time (min)	Mussel extract vol. (mL)[b]	Water vol. (mL)	Ethanol vol. (mL)	Heptane vol. (mL) (x2)	Ethanol fraction (% total)	Heptane fraction (% total)
17,20B-P ester	5	0.8	0.3	1.2	3	9	91
17,20B-P ester	5	0.8	0.3	1.2	3	6	94
17,20B-P ester	5	0.8	0.3	1.2	3	29	71
P ester	5	0.8	0.3	1.2	3	12	88
P ester	5	0.8	0.3	1.2	3	8	92
P ester	5	0.8	0.3	1.2	3	9	91

[a]**Heptane phase from a previous separation, i.e. ester only.**

[b]**Unexposed mussel extract was added to resemble real separation conditions.**

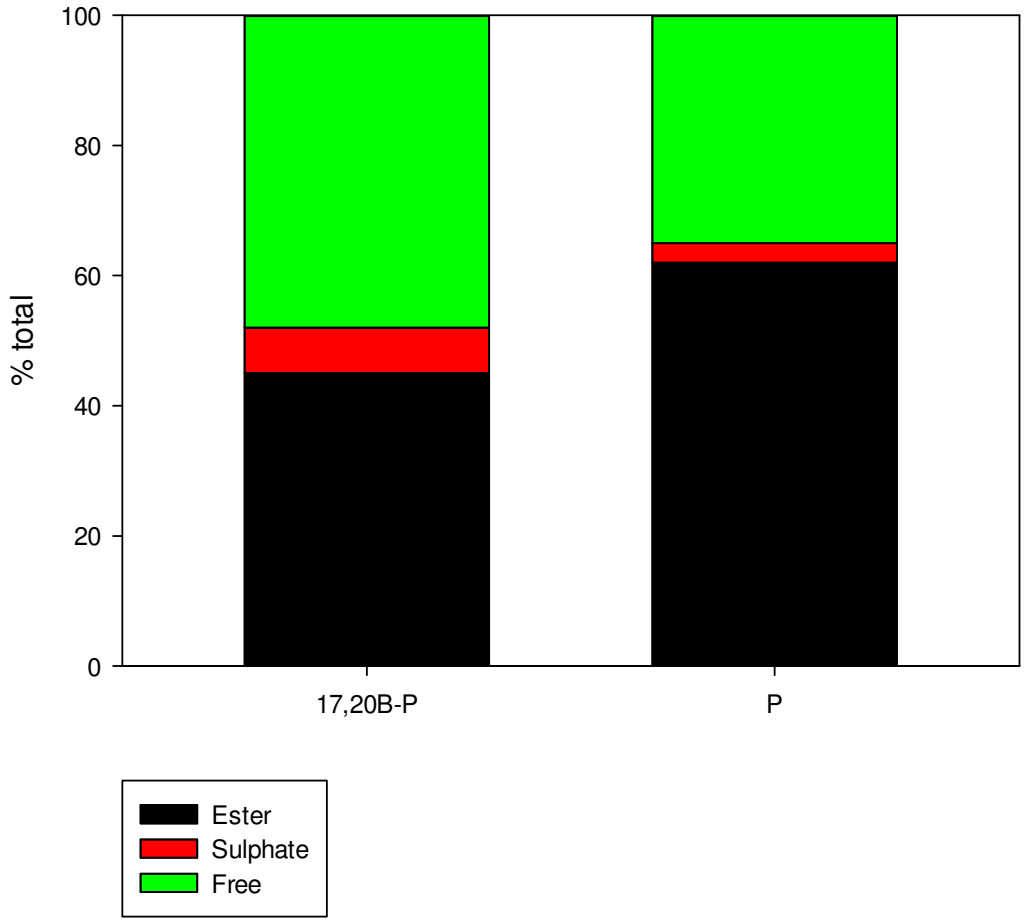

Figure 57: Radioactive metabolite profile in mussel tissue after exposure to [^{3}H]-17,20β-P and [^{3}H]-P for 24 h. Data are presented as mean percentage total (n = 10) of esters. Animals were pooled to estimate the proportion of sulphate and free.

Separation of the three main metabolite groups was based on the solvent partioning procedure, so it was important to further substantiate the evidence for the presence of esterified [^{3}H]-P and [^{3}H]-17,20β-P. To this effect, pooled extracts of each steroid at time 0 were hydrolysed and then submitted to a reverse phase C18 column (Figure 58 and Figure 60). The [^{3}H]-P chromatogram presented a large radioactive peak with a retention time of 67 min: two min after the P standard eluted. Since [^{3}H]-P does not possess any hydroxyl groups for fatty acid conjugation, the absence of any intact steroid was expected.

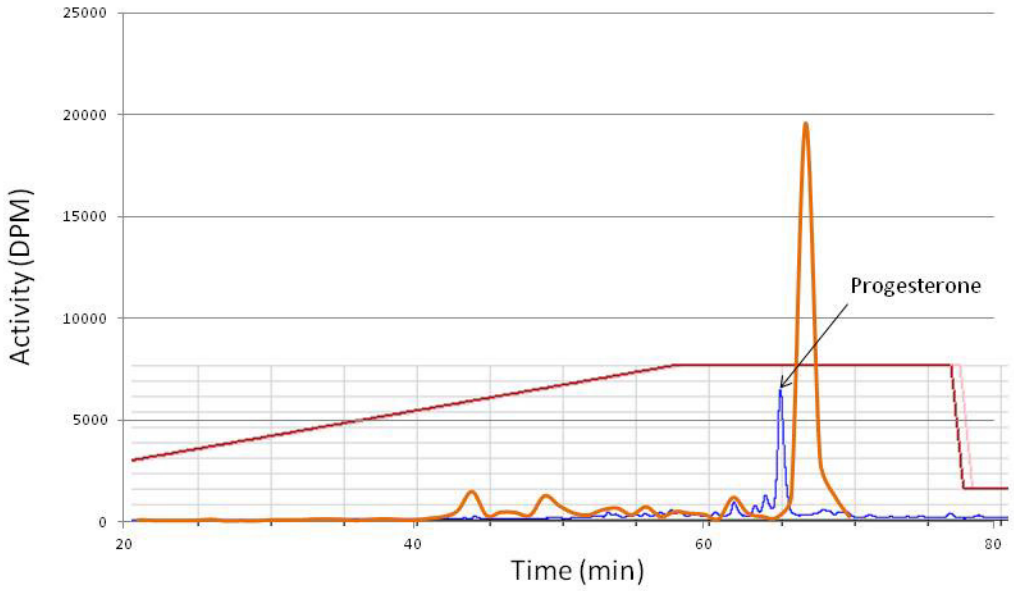

Figure 58: Representative HPLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-P metabolites with P standard. Data are presented as UVabsorbance (245 nm; blue) and radioactivity peaks (orange).

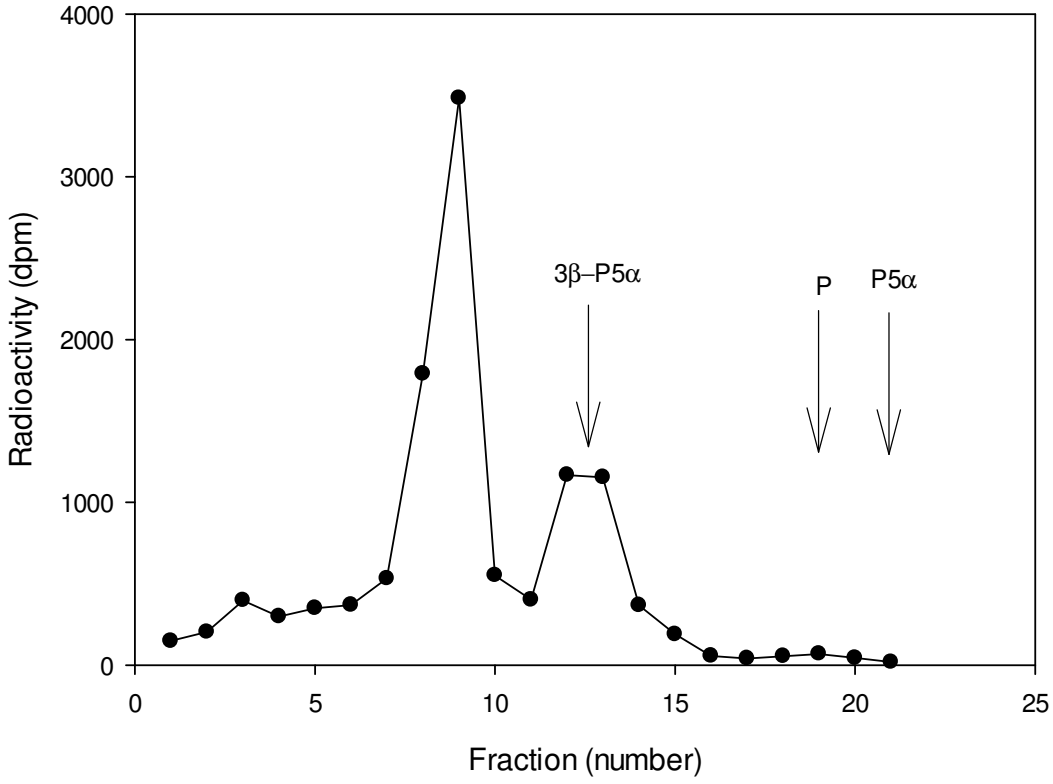

Figure 59: TLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-P metabolites with P, P5α and 3β-P5α standards (position of standards are indicated with arrows). Data are presented as total radioactivity fraction^{-1} (fraction = 5 mm silica)

When the major peak (with a 67 min retention time) was collected and analysed further by TLC it resolved into two peaks, which had obviously eluted on top of each other on the HPLC column (too close to discern). The major peak accounted for 87 % of the activity and the small peak for 13 %. On the TLC plate the large peak did not run with any of the standards, so it remains to be identified, but the small peak ran with the 3ß-P5α standard (Figure 59).

The [^{3}H]-17,20ß-P chromatogram presented a large peak with the same retention time as the 17,20ß-P standard (53 min); suggesting that most of the radioactivity found in the heptane layer was indeed [^{3}H]-17,20ß-P ester. There were, however, two other substantial peaks (51 and 59 min) which have not been identified yet (except that they were not 17,20ß-P or 17α-P). This means that a large amount (ca. 40 %, i.e. 18 % of the total radioactivity) of the radioactivity in the ester fraction was metabolised prior to esterification.

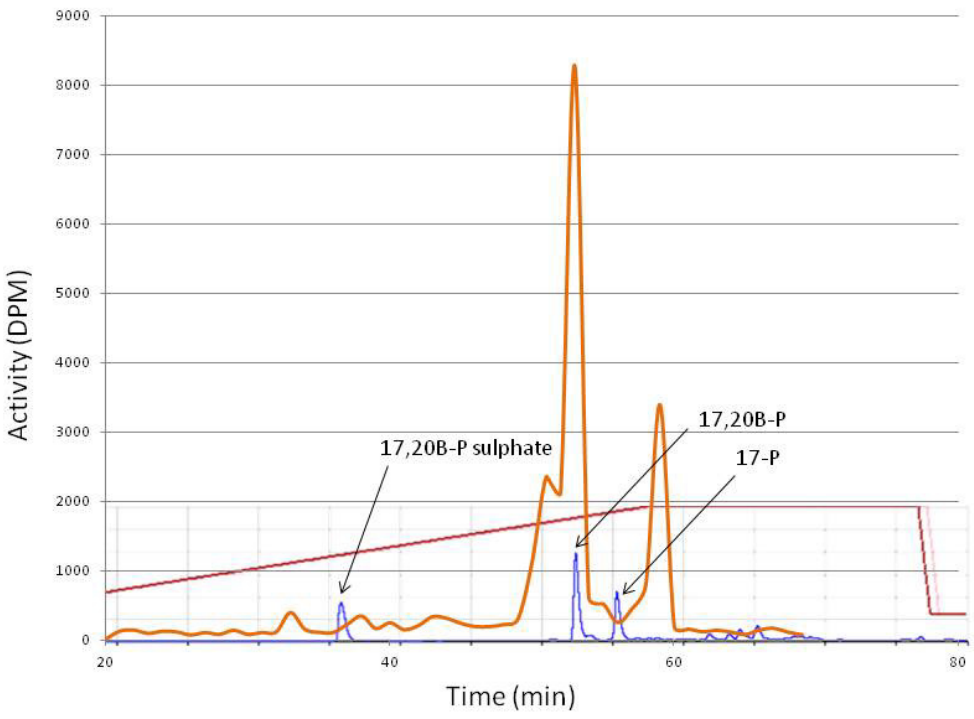

Figure 60: Representative rp-HPLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-17,20β-P metabolites with 17,20β-P, 17,20β-P sulphate and 17α-P standard. Data are presented as absorbance (245 nm; blue) and radioactivity peaks (orange).

5.3.5 What happens to [^{3}H]-P and [^{3}H]-17,20β-P when mussels undergo depuration

So far it has been demonstrated that *Mytilus* spp. absorb [^{3}H]-P and [^{3}H]-17,20ß-P (but not [^{3}H]-F) from the water and turn > 45 % of what was taken up into lipophilic metabolites. How might esterification affect the rate of depuration of the radiolabels? To investigate this, some of the animals that had been exposed to [^{3}H]-P, [^{3}H]-17,20ß-P and [^{3}H]-F were placed in fresh running sea water and then a few were removed at irregular intervals and frozen for later extraction and separation into different fractions. As stated earlier, mussels exposed to [^{3}H]-F were not extracted (before or after depuration) as there was no evidence of uptake.

Figure 61 shows that that there was a significant decrease (time was treated as a factor to achieve a linear relationship; df = 4, f = 6.197, p = 0.0013) in the total [^{3}H]-P over time. Pairwise comparisons of total [^{3}H]-P, using T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were significant differences between day 0 and days 2, 5 and 10 (p = 0.0023, 0.0215, 0.0443 respectively). It appears that there was an abrupt decrease in the free / sulphated fraction of [^{3}H]-P in the first 5 d (reduction of > 80 %) but the lipophilic fraction, on the other hand, remained relatively stable throughout the 10 d.

Figure 62 shows that that there was a significant decrease (time was treated as a factor to achieve a linear relationship; df = 4, f = 28.44, p < 0.0001) in the total [^{3}H]-17,20ß-P over time. Pairwise comparisons of total [^{3}H]-17,20ß-P using T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were significant differences between day 0 and all other sampling points (p < 0.0001 for all time points). This means that the largest reduction in [^{3}H]-17,20ß-P occurred between day 0 and 1, after which depuration slowed down. The free / sulphated portion of [^{3}H]-17,20ß-P was reduced by > 90 % in the first two days and the ester fraction shows some sign of depuration too, losing about 25 % over the first two days. It is interesting to note that even though both progestagens had relatively similar metabolite distribution, [^{3}H]-17,20ß-P was depurated much quicker and to a higher extent (> 70 % in two days compared to 40 % in five days). Logically, in both cases the more water-

soluble free / sulphated portion (which was mainly free steroid) was purged more readily than the more hydrophobic esterified portion. However, in the case of [^{3}H]-17,20β-P, the ester fraction underwent a relatively high amount of depuration (ca. 25 %) which could explain the differences between both progestagens.

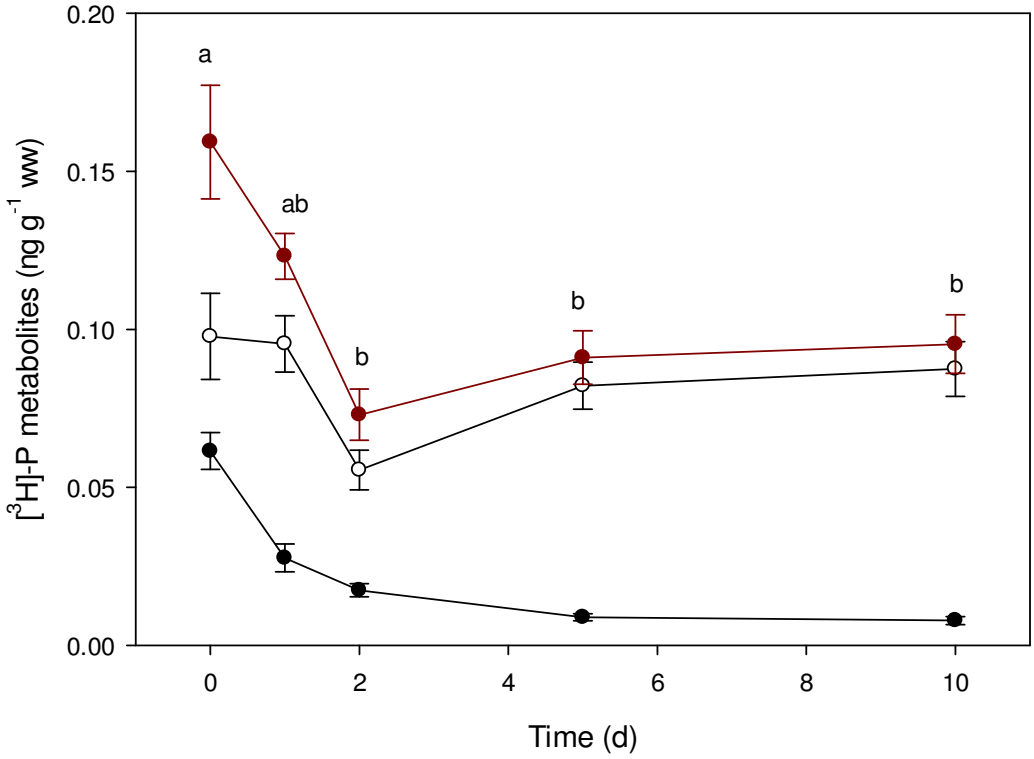

Figure 61: Free / sulphate fraction (●), ester fraction (○) and total [^{3}H]-P (●) in mussel tissue during depuration after exposure to [^{3}H]-P for 24 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-P residues (n = 10 for time 0 and n = 5 for all other sampling points), where observations labelled with a different letter ('a' or 'b') are statistically significantly different from each other ($p < 0.05$).

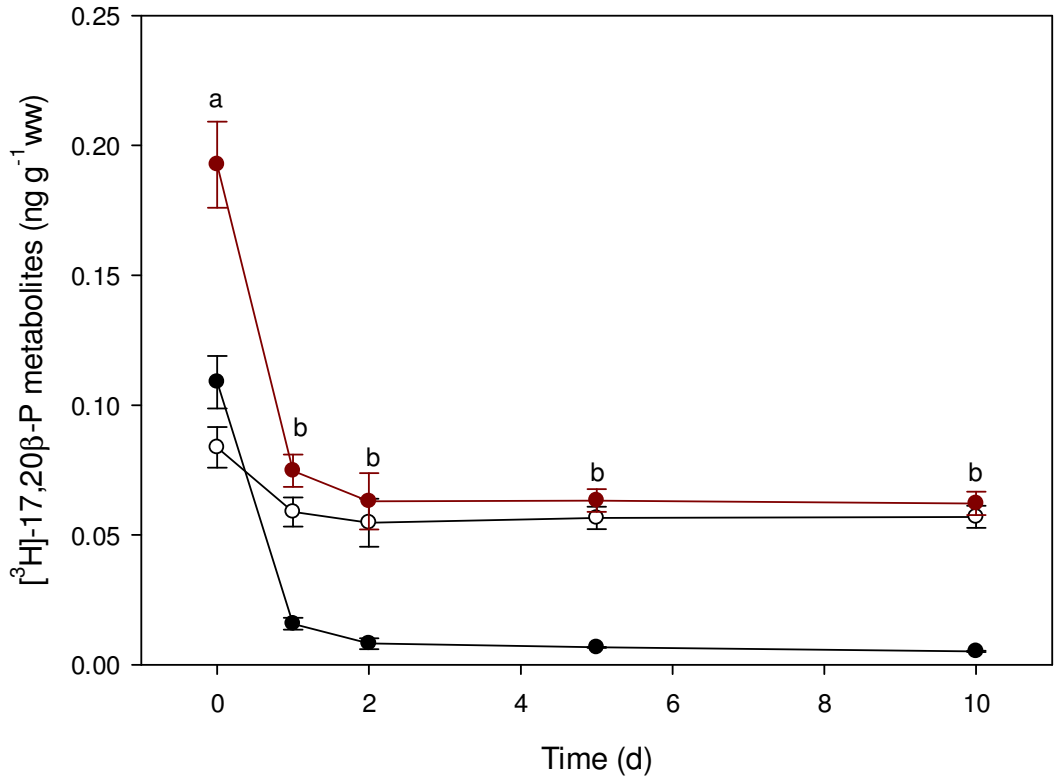

Figure 62: Free/ sulphate fraction (●), ester fraction (○) and total [^{3}H]-17,20β-P (●) in mussel tissue during depuration after exposure to [^{3}H]-17,20β-P for 24 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-17,20β-P residues (n = 10 for time 0 and n = 5 for all other sampling points) where observations labelled with a different letter ('a' or 'b') are statistically significantly different from each other (p < 0.05).

5.4 Discussion

5.4.1 Do mussels pick up [^{3}H]-F, [^{3}H]-P and [^{3}H]-17,20β-P from water?

5.4.1.1.1 [^{3}H]-F

Monitoring the radioactivity in the water of exposure studies showed that the mussels picked up little, if any [^{3}H]-F. This was an unexpected result as structurally, F would appear to be liable to esterification (a readily accessible hydroxyl group on C21). However, as mentioned in the Introduction, the only time this type of experiment has been carried out before, in a teleost fish, the tench (*Tinca tinca*), it was found that little if any [^{3}H]-F was removed from the water (Scott et al., 2005). This is despite the fact that fish in general have absolutely no problem in releasing F into the water via the gills and that the release appears to be a passive process (as there is a strong correlation between water release rates and plasma steroid concentrations (Ellis et al., 2005).

The loss of radioactivity in the test vessels was so similar to that lost in the single control vessel that it can probably all be explained by sorption. However, this is only an assumption (i.e. it was not possible to prove it statistically). Further studies with equal numbers of treatment and control groups would be required to do this. In the study by Scott *et al*. (2005), only two control tanks were used. In a *post hoc* examination of the original data from the F uptake experiment in that study (Scott, personal communication), one of the sorption controls had a similar rate of F loss to the treatment tanks (i.e. 5 % over 6 h) and the other showed little or none at all. Clearly, the low (possibly zero) uptake of F by fish needs to be re-examined as well with a greater number of replicates for both treated and control tanks.

Scott *et al*. *(*2005) speculated that the lack of uptake of F by the tench was because, in contrast to E_2 and T, there is no plasma steroid binding protein for F. However, the present results with the mussel indicate that this is perhaps not the right explanation and that there is some physical feature of F that allows it to diffuse freely in one direction (fish → water) but stops it diffusing in the other direction (water → animal). It is suggested that this feature is the oxygen atom on the C11 position and not necessarily the fact that F is more hydrophilic than E_2 or T. The reason for suggesting this is that tench are also unable to absorb little if any of the fish androgen, 11-ketotestosterone (which is not particularly hydrophilic). It is interesting that such evolutionary diverse organisms (i.e. bivalves and fish) are both apparently unable to pick up a steroid which, in vertebrates, is related to stress. Could the addition of an oxygen atom at C11 be the result of an evolutionary advantage in fish to prevent uptake of a steroid which could potentially lead to an unnecessary (potentially detrimental) stress response? Clearly more research is required to clarify these interesting observations.

5.4.1.1.2 [^{3}H]-P

Mussels exposed to [^{3}H]-P readily picked it up from the water, although the level of sorption was so high that it did not allow for rigorous monitoring of radioactivity in the water, i.e. clearance rates were not reliable and it was therefore not possible to make comparisons to the model steroid, E_2. The

literature had already hinted that uptake was possible when P was found in wild molluscs (e.g. Martínez-Pita *et al.*, 2012; Reis-Henriques & Coimbra, 1990; Siah *et al.*, 2002) and made definitive when radiolabelled P was shown to be taken up by *Clione antarctica* (Hines et al., 1996). Our hypothesis was that only a little (in its free form), if any [^{3}H]-P would be taken up and retained as it does not have any hydroxyl groups for esterification to take place. This was considered a potential limiting factor; interestingly this was not the case and the reasons why are discussed later (see section 5.4.1.3.2).

5.4.1.1.3 [^{3}H]-17,20β-P

Mussels exposed to [^{3}H]-17,20ß-P readily picked it up from the water, although at a lower rate than [^{3}H]-E2 (ca. 20 % vs 30 % removal after 3 h). No studies so far have investigated the presence of 17,20ß-P in molluscs, let alone exposed them to the steroid. [^{3}H]-17,20ß-P was chosen to investigate steroid uptake because it has a reactive hydroxyl group which suggested that uptake and esterification could be possible, although the same assumption was made for F and, as described above, it showed no evidence of uptake.

To the best of our knowledge, this is the first time *Mytilus* spp. have been exposed to either radiolabelled F, P or 17,20ß-P; providing direct, solid evidence that bivalves can pick up both [^{3}H]-P and [^{3}H]-17,20ß-P but not [^{3}H]-F from water.

5.4.1.2 What happens to the steroids left behind (or perhaps re-released) in the water?

5.4.1.2.1 [^{3}H]-F

F water extract partitioning (between water and diethyl ether) after 24 h revealed that 72 % of the radioactivity left in the water fractionated into the organic solvent, i.e. most of the [^{3}H]-F left was still in its free form (presumably available for uptake). This finding was also confirmed chromatographically, where the largest radioactive peak (ca. 87 %) co-eluted with the F standard and the retention time of the smaller peak (ca.13 %) was consistent with water soluble metabolites (Figure 54). This

observation at least ruled out the possibility that the lack of uptake of F was perhaps due to it all being converted to hydrophilic sulphates or other compounds.

5.4.1.2.2 [^{3}H]-P

P water extract partitioning (between water and diethyl ether) after 24 h revealed that 85 % of the radioactivity left in the water fractionated into the organic solvent, i.e. most of the [^{3}H]-P left was in its free form. However, when analysed chromatographically, the largest radioactive peak (ca. 58 %) was consistent with water soluble metabolites (e.g. sulphates) and the second largest peak (ca. 28 %) had a different retention time to the P standard: it eluted 14 minutes earlier. After 24 h, there was no un-metabolised [^{3}H]-P left in the water.

As already mentioned, the [^{3}H]-P exposure study was characterised by an extremely high level of sorption (> 90 % sorption in the control vessel). The presence of sulphates and the complete absence of free [^{3}H]-P in the water after 24 h suggested that during the first 5 h, mussels were able to pick up [^{3}H]-P (when it was still present in the water column, and not yet fully adsorbed). [^{3}H]-P uptake came to halt after 5 h as all remaining free [^{3}H]-P had been lost to sorption, and any water soluble sulphates produced in the same time period would have a greater affinity for water and therefore not be picked up (or adsorbed), let alone retained. Hines *et al*. (1996) exposed *C. antarctica* to radioactive P and reported that after 12 h, 69 % was found in the animals (i.e. had been taken up). In the present study, only 21.1% (mean, n = 10) of the total radioactivity added to the exposure vessel was found in the animals, although, as mentioned above, this was probably (mainly) a result of the great loss of [^{3}H]-P to sorption. Hines *et al*. (1996) also reported finding a small amount of water soluble metabolites in the animals (ca.13 % of total uptake) and as sorption did not seem to be an issue (although they did not monitor the radioactivity in the water, almost 70 % was accounted for in the animals) it is easy to speculate that the reason for uptake levelling off after approximately 3 and 6 h (like it did in this study) could be the production of sulphates and their tendency to accumulate in the exposure water.

5.4.1.2.3 [^{3}H]-17,20β-P

Water extract partitioning (between water and diethyl ether) after 24 h revealed that 90 % of the radioactivity left in the water fractionated into the organic solvent, i.e. most of the [^{3}H]-17,20β-P left was, presumably, still in its free form. However, when analysed chromatographically, the largest radioactive peak (ca. 38 %) had a different retention time to the 17,20β-P standard: it eluted 5 minutes later. The second largest peak (ca. 32 %) co-eluted with the 17,20β-P standard with a small peak (ca. < 10 %) just before it. The latter peak was also present in the sorption control chromatogram and was perhaps a result of incomplete enzymatic transformation when making the label. [^{3}H]-17,20β-P sulphates only represented a small fraction (ca. 21 %) of what was left in the water. So, why did [^{3}H]-17,20β-P uptake slow down after 5 h? Perhaps, as for EE_2, the ability of [^{3}H]-17,20β-P to move freely between compartments (water and animal) (Figure 62) meant that uptake reached an equilibrium after 5 h. In Figure 60, after hydrolysis of the esters, the peak co-eluting with the 17,20β-P standard accounts for ca. 60 % of the activity and the other peak (presumably a [^{3}H]-17,20β-P metabolite; retention time 59 min) only for ca. 24 %. This, coupled with the presence of the major peak in the water (minute 57), suggests that other reactions (beyond esterification and sulphation) are taking place and as [^{3}H]-17,20β-P metabolites accumulate, the uptake rate slows down, reducing the uptake and bioaccumulation until an equilibrium between animal and water is reached.

Scott *et al*. *(*2005) also exposed tench to [^{3}H]-17,20β-P and they reported that 29 % of the total was picked up after 6 h. This figure is similar to the uptake found in this study during the 'hot only' exposure (ca. 26 % after 6 h). It appears that there are factors (e.g. structure, solubility, metabolism, etc.) affecting the rate of uptake which apply to both fish and bivalves (see F uptake in section 5.4.1.1.1).

5.4.1.3 What happens to the steroids in the animal?

5.4.1.3.1 Extraction and separation methodology

Extraction Method 2 (developed with [^{3}H]-E_2 as a model; Chapter 2) was tested and found to be effective for both steroids. Raw extracts were then

separated into sulphates, esters and free steroid using the separation method developed in Chapter 2. The optimised method was tested on [^{3}H]-P and [^{3}H]-17,20ß-P and was found to be sufficiently effective, although not as effective as it was for E_2 metabolite separation (> 85 % for [^{3}H]-17,20ß-P and > 90 % for [^{3}H]-P vs > 95 % for [^{3}H]-E_2). The reason for the reduced partitioning efficiency is not clear, although it is plausible that differences in physical-chemical properties of the ester metabolites may be responsible. The method was optimised for use with [^{3}H]-E_2, for which esters are comprised solely of intact E_2. When separating [^{3}H]-17,20ß-P and [^{3}H]-P esters the method has to contend with at least two metabolites (or more, if fatty acids vary too) which, as seen when chromatographed as free steroids, have different properties (e.g. elution times). It must be noted that this is mere speculation; in order to find out why partitioning was less efficient for these steroids in comparison to E_2 or how to improve it, methodologies would have to be optimised for each steroid individually.

5.4.1.3.2 [^{3}H]-P metabolism by *Mytilus* spp.

Research suggests that the esterification of E_2 and T occurs (preferentially) via the hydroxyl group on C17 (Labadie et al., 2007). As [^{3}H]-P has no hydroxyl groups where esterification could in theory occur, it was initially thought unlikely that [^{3}H]-P would be turned into a lipophilic ester in mussel tissue. This assertion was wrong: 62 % of the [^{3}H]-P present in the flesh was in the ester fraction. So the question was, how is this possible, when the reaction requires a hydroxyl group in order to form the ester bond? To find out what the ester was comprised of, the heptane fraction obtained from partitioning was hydrolysed and submitted to rp-HPLC. Chromatography revealed that the retention time (67-68 min) of the major peak (ca. 87 %) was 2 minutes after that of the P standard and this was comprised of two steroids (only seen when separated by TLC). The only published study (Hines et al., 1996) which has investigated *in vivo* uptake and metabolism of radiolabelled P reported the production of P5α and 3ß-P5α. There is also another study (Dimastrogiovanni et al., 2015) that reported formation of the same two steroids by *M. galloprovincialis* from radioactive P *in vitro* (although as it has already been pointed out, the identification was purely done by elution position on HPLC). The presence of 5α-reductase in molluscs (mainly acting on T or androstenedione) has

also been reported frequently in other studies in the literature (De Longcamp, 1974; Fernandes et al., 2010; Lyssimachou et al., 2009; Ronis and Mason, 1996). Also, in the present studies, it was shown that T was converted to 5α-DHT and 3β,17β-A-5α (see Chapter 6). On the basis of these studies, it was thought highly likely that the main metabolite in the ester fraction of P would be 3β-P5α. It was thought highly unlikely that it would be P5α, as like P, this steroid does not have a hydroxyl group to which the fatty acids could be linked. The hypothesis seemed even more likely when it was established that cold 3β-P5α eluted at about 67-68 min on the HPLC. However, when the hydrolysed ester fraction was run on TLC, only 13% of the total activity ran in the same position as the standard 3β-P5α. The rest of the activity ran as a single band that was more hydrophilic than any of the P-related standards that were available in the laboratory. Although the identity of the major peak is still to be elucidated, the presence of at least some 3β-P5α ester (albeit only 13%) provided evidence that some esterification can occur on the 3β-hydroxyl group at position C3 of the 5α-reduced A ring.

Concerning the possible identity of the unknown P metabolite, one can only speculate. It is most likely a 5β-reduced steroid. It could be 3β-P5α that has been further reduced at the 20 position, although this would probably cause the steroid to elute on the HPLC column earlier than P. It could also be due to the insertion of an oxygen atom at some as yet unknown position on P, although again this would probably cause the steroid to elute earlier than P during HPLC. Some but not all of these possibilities could be tested by buying or making the appropriate standards. However, probably the best way would be to carry out an incorporation study with radioactive 17α-P. The presence of the 17-hydroxyl group would be unlikely to interfere with the metabolising or esterification reactions. However, unlike with P, any products could be readily converted into androgens by oxidising reagents such as chromium trioxide (De Longcamp, 1974). These radioactive androgens could then be compared with readily available androgen standards to test the nature of the A ring of the original metabolite (i.e. is it delta-4, 5α or 5β?) and the identity of any hydroxyl groups at the C3 position.

Partitioning of the 80 % ethanol fraction between water and ether revealed that only 3 % corresponded to sulphates and 35 % to free steroid (be it P or a known P metabolite). The identity of these steroids has not yet been studied.

5.4.1.3.3 [^{3}H]-17,20β-P metabolism by *Mytilus* spp.

Although, as mentioned earlier, it has been suggested that esterification of E_2 and T occurs preferentially at the hydroxyl group on C17 (Labadie et al., 2007) it has been shown that it can potentially also happen on the hydroxyl group at the C3 of 3ß-P5α (section 5.4.1.3.2). [^{3}H]-17,20ß-P was chosen to investigate uptake and esterification because it has another potentially available hydroxyl group on C20. Based on its structure, it was hypothesised that [^{3}H]-17,20ß-P would be taken up and promptly esterified via the C20 position. Esterification did indeed occur, however it only represented ca. 45 % of the total activity in the tissue, whereas almost 50 % was presumably free [^{3}H]-17,20ß-P (or a [^{3}H]-17,20ß-P metabolite). In order to find out if the ester was comprised of intact 17,20ß-P the heptane fraction obtained from partitioning was hydrolysed and submitted to rp-HPLC. The major metabolite (accounting for ca. 59 %) co-eluted with the 17,20ß-P standard at 53 minutes together with a small peak just before it (minute 51; 17 %). The latter was also present in the water - both in the test vessel and sorption control – and was therefore assumed not to be a result of any endogenous transformations in the mussel. There was also a second peak (59 min retention time; this accounted for 24 % of the radioactivity) that did not run with the 17α-P standard and differed from the major peak found in the water (retention time 57 min). Although the latter remains to be identified, the presence of intact 17,20ß-P after hydrolysis (if this can be confirmed) shows that the ester bond can be formed via the hydroxyl group on C20.

It should be noted that what was presumed to be intact [^{3}H]-17,20ß-P was only analysed using HPLC and in order to ascertain presumptive identity with more confidence (in order to rule out, for example, the presence of a 5α-reduced and / or 3ß-dehydrogenated metabolite – which might co-elute with the standard on the column) it would be insightful to run the radioactive peaks with 17,20ß-P standard on a TLC plate. This was not

possible during these investigations due to a lack of product (i.e. low esterification rates led to insufficient radioactivity); and up-scaling was not possible (to the levels of hot steroid required) as the high fat content of the extracts caused problems during clean-up. Some of the [^{3}H]-17,20ß-P in the tissue (ca. 7 %) and a small amount in the water (ca. 20 %, for peaks with a retention time < 40 min) also appeared to have been sulphated. However, no further identification studies have yet been carried out on this material either due to the low yields.

Even though 17,20ß-P is a highly ubiquitous vertebrate progestagen involved in oocyte maturation in fish, the real value of investigating the uptake of 17,20ß-P by mussels lay in its particular structure. Our results show that although esterification can take place on the hydroxyl group at C20, the rate of esterification is not as vigorous as that of T or E_2; and the rate of sulphation not as effective as that on E_2.

5.4.1.4 Were there any saturation effects?

5.4.1.4.1 [^{3}H]-P

The present study, unexpectedly, presented a large amount of sorption which made it difficult to establish reliable clearance rates and final uptake. This, in turn, meant that it was not possible to verify whether or not [^{3}H]-P uptake was saturated by the addition of cold P. This study needs to be repeated (using a different material to which P does not present such affinity) as it appears that P undergoes at least five different enzymatic reactions (not just esterification and sulphation) and it would be very interesting to see whether any of these stages can be considered a rate limiting step in the process of uptake and storage.

5.4.1.4.2 [^{3}H]-17,20β-P

Like EE_2, the majority of [^{3}H]-17,20ß-P present in the mussel was in its free form (49 %). Would [^{3}H]-17,20ß-P uptake be affected by the addition of cold 17,20ß-P? The answer was that there was no clear evidence of saturation after the first three hours of exposure (i.e. clearance rates) or in the final removal (31.7 %, 33.7 % and 45.1 % for 'hot only', low and high treatments respectively). The presence of cold steroid appeared to

actually enhance the total that was removed over 24 h (although this was not supported statistically and the experiment would need to be repeated to find out whether this was a genuine result or just chance).

5.4.2 Was there evidence for depuration; and were metabolites differentially depurated?

5.4.2.1.1 [^{3}H]-P

Placing the animals in clean water under flow-through conditions did indeed reduce the overall [^{3}H]-P burden by up to 40 %. This reduction, however, consisted only of the free / sulphated fraction. The main esterified fraction was hardly affected after 10 d. The bulk of the reduction in free [^{3}H]-P activity occurred within the first two days and there was no further depuration after five days. This means that depuration periods applied in the shellfish industry (a minimum of 42 h depuration) would, in fact have the effect of purging only free / sulphated P.

What is puzzling about our results is that there are numerous studies in the literature (see Table 2 in Chapter 1) in which P has been detected in the flesh of molluscs. How is this possible, if the animals do not actually hold on to it (under depurating conditions) in a free form? There are several possibilities. Those studies that use immunoassays (the majority) might probably have been detecting other cross-reacting compounds (that might not even be steroids!). Contamination in the laboratory, or the presence of P in the water when the animals were collected, might be another reason. Yet one further reason that cannot yet be dismissed is that P actually is being produced endogenously and that, despite leaking from the animals, is being continuously replenished within the animals. In support of this possibility, pregnenolone (Pn), which is the immediate precursor of P in vertebrates has been detected in the flesh of mussels in concentrations similar to those of P (Dévier et al., 2010). Also, the enzyme activity (3ß-HSD) necessary for conversion of Pn to P has been shown to be present in mussels (de Jong-Brink et al., 1981; Dimastrogiovanni et al., 2015). The main question that arises from this scenario is: is Pn itself formed endogenously or has it also been absorbed from the environment?

5.4.2.1.2 [^{3}H]-17,20β-P

Placing the animals in clean water for ten days reduced the overall [^{3}H]-17,20ß-P burden by up to 66 %. The free / sulphated portion of [^{3}H]-17,20ß-P was reduced by up to 90 % in the first 24 h. Interestingly, the esterified fraction was also reduced in the first day, although to a lesser extent (ca.25, %). The [^{3}H]-17,20ß-P ester fraction is comprised of (presumably) intact 17,20ß-P (76 %) and an unidentified 17,20ß-P metabolite (24 %). This raises the question as to whether the ca. 25 % reduction in the ester fraction could be the result of the loss of the unknown metabolite ester only.

As 50 % of [^{3}H]-17,20ß-P was present in its free form in the animal and up to a quarter of the esters seem to be susceptible to depuration, it is safe to assume that the 42 h depuration period enforced on the shellfish industry would mean that little if any of this steroid would be present in mussels going for human consumption. In any case there is no evidence that this steroid poses a risk for humans.

5.5 Conclusion

This Chapter has presented definitive evidence of the uptake, metabolism and depuration of [^{3}H]-P and [^{3}H]-17,20ß-P by *Mytilus* spp. There was no evidence however, for the uptake of [^{3}H]-F. Although the latter findings remain inconclusive as to why, it was interesting to note that this was also the case in a teleost fish (Scott et al., 1991); hinting that lack of F uptake is based on structural differences and is conserved amongst species. This Chapter has also shown that esterification can take place on the reactive hydroxyl groups of C3 (of the 5α-reduced A ring) and possibly of C20 (albeit at a lower rate). As described in Chapter 3 and 4 (for E_2 and EE_2), esters persisted longer than free and sulphated steroids under depurating conditions, although a considerable amount of [^{3}H]-17,20ß-P esters were in fact purged. It appears that the industry's mandatory 42 h of depuration is suitable for 17,20ß-P but not for P metabolite(s).

Chapter 6

Uptake, transformation, depuration and metabolite identification of T.

6.1 Introduction

Testosterone (T) is the major vertebrate androgen. It is exuded and excreted, reaching surface waters via sewage treatment plant effluents and agricultural runoffs (Johnson et al., 2006; Leusch et al., 2014). Although highly susceptible to biodegradation, it is ubiquitously detected in the aquatic environment (Backe et al., 2011; Liu et al., 2011). T has been measured in the tissues of numerous molluscan species (Table 2 in Chapter 1). Among the more recent studies, for example, Zheng *et al*. (2014) detected T (free only) in the scallop *Chlamys farreri* by ultra performance liquid chromatography and tandem mass spectrometry (UPLC-MS / MS); Martínez-Pita *et al*. (2012) reported ca. 4 to 7 pg ml^{-1} T (using enzyme-linked immunosorbent assay; ELISA kits) in the haemolymph of the Mediterranean mussel, *Mytilus galloprovincialis*; and Cheour *et al*. (2014) measured free T in the gonads of a gastropod, *Osilinus articulates* by radioimmunoassay (RIA). Several studies have also shown that T can be taken up by molluscs and turned into a lipophilic ester, just like E_2. Gooding & LeBlanc, (2001) exposed the eastern mudsnail, *Ilyanassa obsoleta,* to radiolabelled T and found that not only did they pick up > 70 % in eight hours but most of it was esterified. Another study exposed the great pond snail, *Lymnaea stagnalis,* to several vertebrate endocrine disrupting compounds and T at four different concentrations - they reported a dose dependent increase of esterified T in the flesh (Giusti et al., 2013). Gust *et al*.(2010) measured total T (free and esterified) in the New Zealand mudsnail, *Potamopyrgus antipodarum,* by RIA and LC-MS / MS after conducting an exposure up and downstream of waste water effluents.

The presence of T (and other steroids) in the tissues of molluscs has contributed to the ongoing belief that they synthesise their own vertebrate-like steroids. This theory has been further supported by evidence of certain enzymatic reactions which take place in the steroid

biosynthesis pathway (as well as in numerous other pathways). Most of this evidence comes in the form of production of particular metabolites from known precursors. For example, *in vitro* exposure of radiolabelled androstenedione (Ad) and T yielded, among other things, the 5α-reduced metabolites: 5α-androstane-3,20-dione and 5α-dihydrotestosterone (5α-DHT;) (Janer et al., 2005a). The presence of 5α-reductase in molluscs has been reported in approximately twelve studies since the late 1960s; these are summarised in Scott, (2012). Since then, Dimastrogiovanni *et al*. (2015) have reported the activity of 5α-reductase in molluscs by demonstrating the conversion of P to 5α-reduced P (P5α) by *M. galloprovincialis* microsomal fractions. There are examples of other enzymatic reactions (that are involved in steroid synthesis in vertebrates) which have been suggested to occur in molluscs (e.g. 3α / 17β-hydroxysteroid dehydrogenase – 3 / 17β-HSD - and C17-20-lyase activities) (Carreau and Drosdowsky, 1977; Janer et al., 2005a; Ronis and Mason, 1996). However, most published data is based on the conversion of substrates, which only provides information regarding a chemical reaction, not the identity of an enzyme. So although it is be possible that molluscs have enzymes which can perform certain reactions, it does not necessarily mean they are the same as those used by vertebrates or that steroids are their intended substrate. For example, Zhou *et al*. (2011) cloned 17β-hydroxysteroid dehydrogenase 12 (HSD17B12) from the abalone *Haliotis diversicolor supertexta* and transiently expressed it in eukaryotic cells. When transfected cells were exposed to E_1, the precursor was converted to E_2 (i.e. it underwent dehydrogenation). This, however, does not mean that E_1 (or any steroid for that matter) is its intended substrate, in fact it has been shown that HSD17B12 from the dog whelk *Nucella lapillus* is more likely involved in the metabolism of lipids (Lima et al., 2013).

T was used in this study because it has been shown to undergo esterification and other types of metabolism (e.g. reduction at position C5 – see Figure 63 for chemical structure). However, the metabolites observed during *in vitro* conversions and the production of esters *in vivo* have never been studied as a whole, i.e. are these metabolites esterified too? If so, which ones and in what proportions? Understanding testosterone uptake,

metabolism and depuration is key in figuring out the relationship between molluscs and vertebrate steroids.

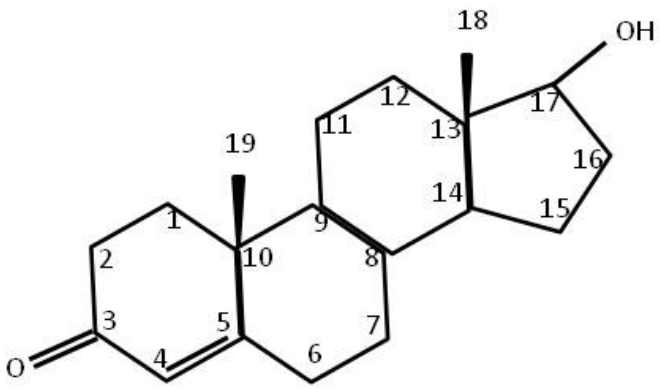

Figure 63: Testosterone chemical structure diagram.

6.2 Materials and methods

6.2.1 Chemicals

Testosterone - [1,2,6,7-^{3}H] was purchased from American Radiolabeled Chemicals, Inc. (101 ARC Dr. St. Louis, MO 63146 USA). 'Cold' progesterone (P), cortisol (F), Ad and T were purchased from Sigma-Aldrich Company Ltd. (Dorset SP8 4XT, UK). 5α-androstan-3Β,17β-diol (3Β,17β-A5α) and 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone; 5α-DHT; 17β-A5α) were bought from Steraloids Inc. (PO Box 689, Newport, RI 02840,USA) and all other chemicals were purchased from Fisher-Scientific UK Ltd. (Loughborough, LE11 5RG, UK).

Water used for laboratory exposures was filtered (50 μm) sea water and water used for all other purposes was reverse osmosis water unless stated otherwise.

6.2.2 Laboratory exposures of *Mytilus* spp. to radiolabelled testosterone.

6.2.2.1 Study 5

6.2.2.1.1 Collection and acclimation

Mussels from a mixed population of *Mytilus edulis* and *M. galloprovincialis* were collected from Portland Harbour in October 2014. The nearby northeast Portland Harbour breakwater is a catchment holding a long term class B shellfish harvesting classification (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/shc2014.pdf). They were transported to the lab in a cool-box and immediately placed in a flow-through system of filtered sea water. Fifty animals were selected (ranging between 47.26 to 69.63 mm, mean 56.34 mm) and cleaned. Ten animals were used for pre-study condition index analysis. In order to acclimate the animals, five mussels were placed in an aerated bucket lined with a polyethylene bag and filled with 2 L of filtered sea water at 16 ± 1°C with a 16:8 h light:dark photoperiod for five days prior to exposure. Water was changed daily and animals were fed Shellfish Diet® 1800 daily (following manufacturer´s instructions).

6.2.2.1.2 Exposure and depuration

As described in section 2.2.2.4.1 in Chapter 2 but mussels in vessels 1 to 6 were exposed to a nominal concentration of 5 µCi L^{-1} (13.1 ng L^{-1}) of [^{3}H]-T; vessels 7 to 8 also had 2.5 µg L^{-1} T and vessels 9 to 10 had 25 µg L^{-1} T. Following exposure for 24 hours, mussels from vessels 1 to 6 were submitted to depuration in a flow-through system (1 L min^{-1}) for 10 days. Ten mussels were sampled on day 0 and five mussels on day 1, 2, 5 and 10 and stored at -20 °C immediately.

6.2.3 Condition index analysis

The mussels overall condition was determined using condition index analysis as described in section 2.2.3 in Chapter 2.

6.2.4 Clearance rates

The rates at which individual mussels cleared [^{3}H]-T from water (i.e. clearance rates) were calculated as described in section 2.2.4 in Chapter 2

6.2.5 Steroid extraction methods

6.2.5.1 Water extraction and extract clean-up

[^{3}H]-T was extracted from water as described in section 2.2.5.1 in Chapter 2.

6.2.5.2 Extraction of steroids from tissue

Radioactive residues were extracted from mussel tissue using a modification of Method 1 described in section 2.2.5.2 in Chapter 2. Two more chloroform:methanol (2:1 v/v) extraction steps were added in order to recover > 90 % radioactivity.

6.2.5.3 Separation of free, sulphated and lipophilic steroid metabolites

Mussel extracts were partitioned using the separation method developed in section 2.2.5.3 in Chapter 2. The method was first tested on three [^{3}H]-T mussel extracts; the heptane phase was collected and mixed with 800 µl of unexposed mussel extract (in order to simulate conditions) before drying it. It was then separated again to assess ester recovery into the heptane phase.

6.2.5.4 Alkaline hydrolysis

Crude extracts were separated as described in 3.2.3. and the heptane fraction was then submitted to alkaline hydrolysis as described in section 3.2.4.1 in Chapter 3.

6.2.6 Chromatography

6.2.6.1 Reverse-phase High Performance Liquid Chromatography

Reverse-phase HPLC (rp-HPLC) (using an analytical column) was used to separate free and conjugated (i.e. sulphated or glucuronidated)

metabolites of [^{3}H]-T in water extracts and also the metabolites produced as the result of alkaline hydrolysis of esterified [^{3}H]-T. The procedure was as described in section 2.2.6.2 in Chapter 2 with the appropriate standards (described in the results section).

6.2.7 Thin layer chromatography

6.2.7.1 Separation of free steroid metabolites after alkaline hydrolysis

After alkaline hydrolysis of esters (section 3.2.4.1 in Chapter 3) in the heptane fraction (obtained from partitioning, see section 3.2.3 in Chapter 3) and a second separation, the radioactivity that remained in 80 % ethanol was dried down, mixed with 5 µg each T, 3ß,17ß-A5α and 5α-DHT as described previously (Scott *et al.*, 2014) and loaded onto one lane of a TLC plate (catalog no. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) and developed for 45 min with a mixture of chloroform:methanol (50:2, v:v), which enables free steroids to move on the chromatogram. After marking the positions of the standards using a UV lamp, the lane was divided into 5 mm bands, and the silica gel from each band scraped off the plate. The scrapes were mixed with 1 mL 80 % ethanol and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity. Single peaks collected from rp-HPLC separation of hydrolysed esters were also further analysed using the TLC method (using the same standards) described above.

6.3 Results

6.3.1 Removal of [^{3}H]-T from water by mussels during bath exposure

[^{3}H]-T uptake experiments were done by exposing five mussels (10 replicates) for 24 h in 2 L of water. Cold T was also added to some of the tanks in order to investigate whether there was a saturation limit for uptake of T by mussels. Concentrations of [^{3}H]-T and cold T are summarised in Table 18.

Figure 64 shows that over time there was a significant decrease (log transformation of time was applied to achieve a linear relationship) in the

level of [^{3}H]-T in the water (df = 1, f = 1397.636, p < 0.0001). Up to 64.7 %, 65.3 % and 73.6 % of total radioactivity was removed from each treatment over 24 h and sorption of [^{3}H]-T only accounted for 9 % (fitted data, linear model). Although treatment explained a lot of the variability between decay curves after 24 h (df = 2, f = 10.006, p = 0.000262), pairwise comparisons revealed that there was no significant difference between the slopes of the decay curves of both low and high cold T treatments and the 'hot only' regime (ß = 0.12810, SE = 0.35959, t = 0.356, p = 0.723 and ß = -0.45148, SE = 0.35959, t = -1.256, p = 0.216 respectively). The calculated clearance rates (based on the first 3 h) were 38.3, 37.9 and 44.8 mL animal^{-1} h^{-1} (Table 19; cf. 40 for the within-experiment [^{3}H]-E_2 control). A linear model analysis of the first three hours of uptake (where time was treated as a factor) revealed that treatment had a significant effect on the decay of [^{3}H]-T (df = 2, f = 4.975, p = 0.0233). However, pairwise comparisons showed that indeed there was no significant difference in [^{3}H]-T decay between both the low and high cold steroid treatments and the 'hot only' regime (ß = 0.06667, SE = 0.28605, t = 0.233, p = 0.819 and ß = -0.43333, SE = 0.28605, t = -1.515, p = 0.152 respectively). Hence, there was no evidence of saturation throughout the experiment, even with addition of 25 µg L^{-1} of cold T; and the rate of disappearance of [^{3}H]-T followed closely that of [^{3}H]-E_2.

Table 18: Summary of chemical concentrations during exposure studies.

Treatment	Radiolabel conc. (µCi L^{-1})[a]	Radiolabel conc. (ng L^{-1})	Nominal cold steroid conc. (ng L^{-1})
[^{3}H]-T	4.2	11.1	-
T low	4.3	11.2	2500
T high	4.1	10.7	25000

[a]Concentration of radiolabels is based on radioactivity in the water. Data are presented as mean concentration of T (n= 6, 2 and 2) at time of dosing (i.e. time 0).

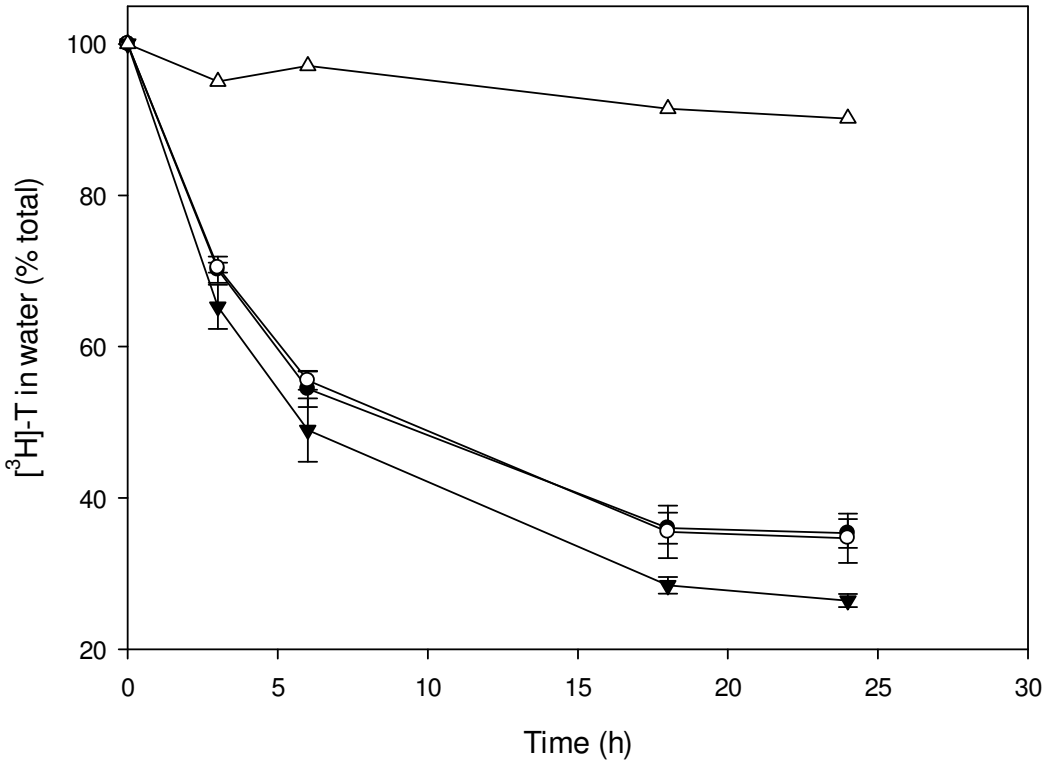

Figure 64: [^{3}H]-T (13.11 ng L^{-1} nominal concentration) removal by *Mytilus spp.* in the presence of increasing amounts of cold T over 24 h (0 µg L^{-1}:●, 2.5 µg L^{-1}:○ and 25 µg L^{-1}:▼ nominal concentrations). Data are presented as mean % total (n = 6 for ● and n = 2 for ○ and▼) and ± S.E.M. And a sorption control (no mussels in tank) (△; n = 1).

Table 19: [^{3}H]-T clearance rates from water by *Mytilus* spp..

Study & treatment	Water vol. (L)	N° animals	nominal label conc. (µCi L^{-1})	Exposure time (h)	Time of first sample (h)	Sorption after 3 h (fitted %)	Removal after 3 h (%)	Clearance rate (mL animal^{-1} h^{-1})
5 [^{3}H]-T	2.0	5	5.00	24	3	1.1	29.9	38.3
5 T low	2.0	5	5.00	24	3	1.1	29.6	37.9
5 T high	2.0	5	5.00	24	3	1.1	34.7	44.8

6.3.1.1 What was left in the water after exposure?

In Chapter 2, it was suggested that [^{3}H]-E_2 uptake (especially in the final experiment) was probably halted by the fact that E_2 was being sulphated as well as esterified and that, after 24 h, there was little if any intact E_2 left in the water that was available for uptake. To investigate whether the same applied to [^{3}H]-T, water samples (1 L) were taken at the end of Study 5 from 'hot-only' exposure vessels and the sorption control. When subjected to solid phase extraction, ca. 58.9 and 7.7 % of the activity from the exposure vessel and the sorption control, respectively, passed straight through. This most likely represents tritiated water (released from the parent compound by unknown reactions). After the extracts were eluted from the solid phase disks with methanol and then dried down, a portion was dissolved in water and then shaken with diethyl ether. The mussel-containing water extract retained 13 % of radioactivity in the water fraction (assumed to be a water-soluble conjugate) and 87 % in the ether fraction (assumed to be free steroid). Sorption control extract yielded 1 and 99 % respectively, i.e. both extracts had greater amount of free steroid than water soluble metabolites.

The extracts were then analysed by reverse-phase HPLC at 280 nm with added T, F, Ad and P standards. The sorption control has a single radioactive peak with the same retention time as the T standard (Figure 65). The test vessel water extract presents multiple radioactive peaks and it is not possible to discern whether or not there is any [^{3}H]-T left after 24 h. Radioactive peaks with a retention time lower than 40 min (presumed to be water soluble) account for ca. 9.5 % of the total activity. This is consistent with the proportion of water soluble metabolites (13 %) determined during partitioning (ether and water) of the extract. Although none of the compounds in the water have yet been definitively identified, the results suggest that there is little intact radiolabel still present in the water at 24 h.

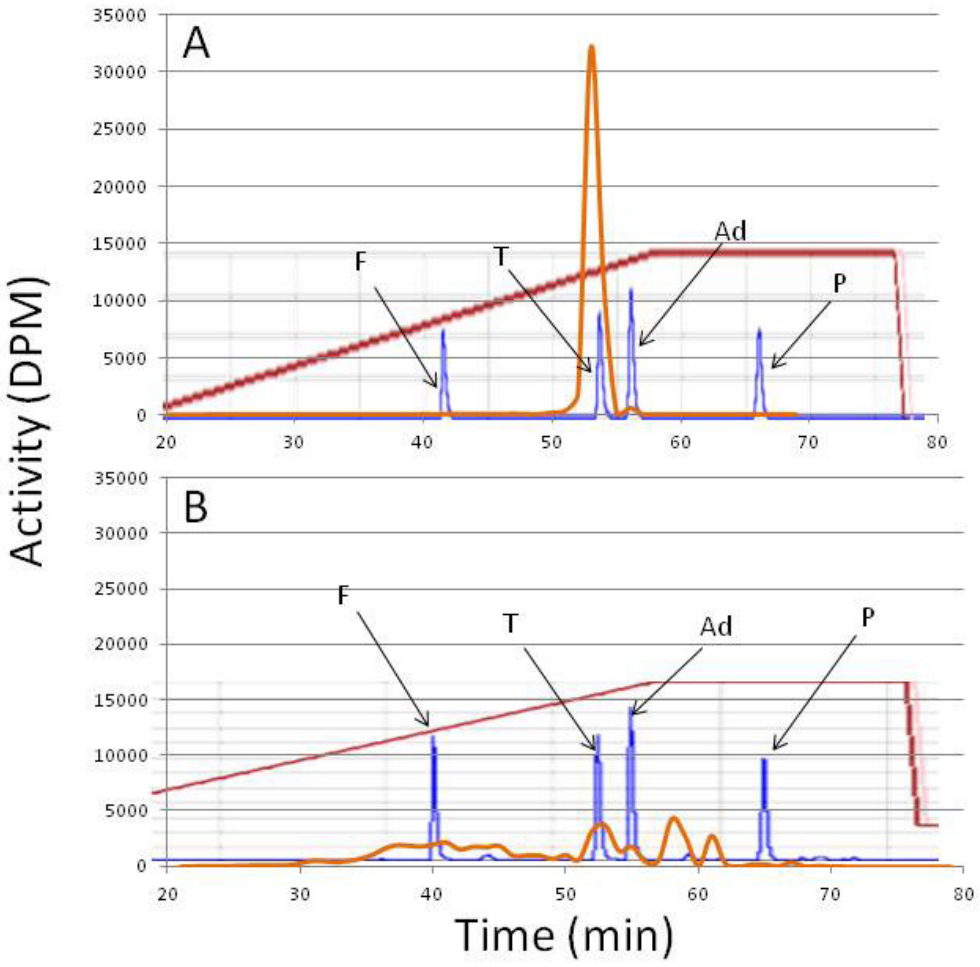

Figure 65: HPLC chromatogram of [^{3}H]-T exposure water; sorption control (A) and test vessels (B) with T, F, A and P standards. Data are presented as UV absorption (245nm; blue) and radioactivity (orange).

6.3.2 What happens to [^{3}H]-T that is taken up by mussels?

The first step involved investigations on extraction method efficiency (Table 20). Extraction Method 1 required the addition of two more extraction steps in order to achieve the desired recovery of radioactive residues. It was also confirmed that the solvent procedure was able to separate esterified label from free and any sulphated label (93 % mean efficiency; Table 21).

Table 20: Method development of [^{3}H]-T extraction (from tissue).

Extraction method	Study	Steroid	Extracts (% total)							Extracts 1 & 2 (%)	Method efficiency (%)
			1	2	3	4	5	6	7		
1[a]	5	T	5	9	24	29	20	14		14	-
	5	T	9	12	10	33	23	13		21	-
	5	T	12	31	29	16	8	4		43	-
	5	T	16	21	29	21	8	5		38	-
	5	T	11	33	20	23	8	5		43	-
	5	T	-	-	-	-	82	12	6	-	94
	5	T	-	-	-	-	89	11		-	-
	5	T	-	-	-	-	95	5		-	-
	5	T	-	-	-	-	-	96	4	-	96
	5	T	-	-	-	-	-	95	5	-	95

[a]**Modified by addition of two more extraction steps.**

Table 21: Summary of metabolite separation method efficiency.

Sample[a]	Vortex time (min)	Mussel extract vol. (mL)[b]	Water vol. (mL)	Ethanol vol. (mL)	Heptane vol. (mL) (x2)	Ethanol fraction(% total)	Heptane fraction (% total)
T ester	5	0.8	0.3	1.2	3	4	96
T ester	5	0.8	0.3	1.2	3	12	88
T ester	5	0.8	0.3	1.2	3	6	94

[a]**Heptane phase from a previous separation, i.e. ester only.**

[b]**Unexposed mussel extract was added to resemble real separation conditions.**

Radioactive residues (at time 0, i.e. no depuration) were separated into esters, sulphates and free steroid. Figure 66 shows that most of [^{3}H]-T was esterified (87 %), while 11 % was in the 'free' fraction and only 2 % was in the sulphated fraction. Note that the high proportion of esters was also characteristic of [^{3}H]-E_2 metabolite distribution (see Chapter 3).

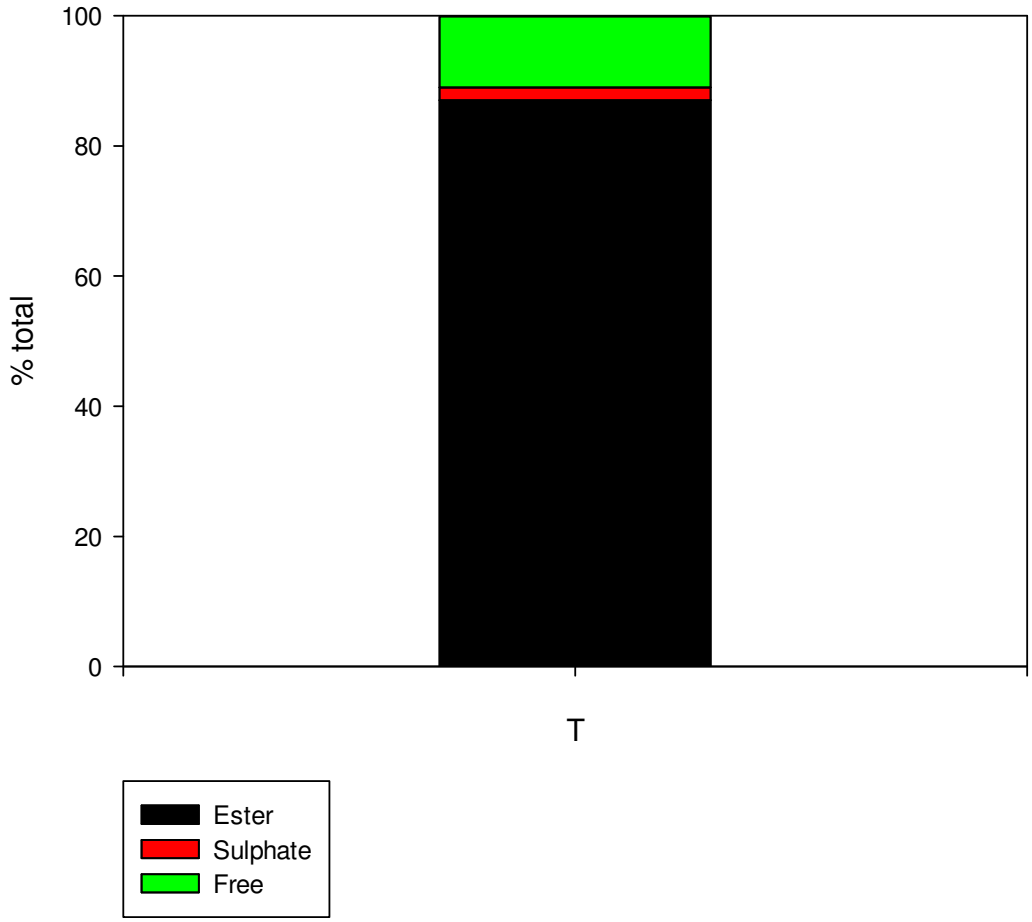

Figure 66: Radioactive metabolite profile in mussel tissue after exposure to [^{3}H]-T for 24 h. Data are presented as mean percentage total (n=10) of esters. Animals had to be pooled to estimate the proportion of sulphate and free.

Some of the radioactive ester (separated from free and sulphated radiolabel using heptane) was hydrolysed using KOH / methanol and run on a reverse phase C18 column (Figure 67). There were four radioactive peaks: the smallest peak had a retention time of 44 min and accounted for 7 % of the activity; another minor peak accounting for ca.9 % of the activity (retention time 52 min) co-eluted with the T standard; the majority of the activity was in peak 54 and 58 min with 37 and 47 % of the total activity respectively. As the chromatogram showed that most of the esters were in fact not made of intact [^{3}H]-T, the radioactive peaks were collected and

separated further (Figure 68). Three peaks (referred to as peak 52, 54 and 58) were run on a separate lane with 5 µg each of T, 5α-DHT and 3β,17β-A5α as standards. These metabolite standards were chosen based on their retention times and on evidence presented in the literature of 5α-reductase and 3β-HSD enzymatic activity in molluscs (Fernandes et al., 2010; Hines et al., 1996). Peak 52 ran with the T standard (as it did on the C18 column); peak 54 ran with the 3β,17β-A5α standard and peak 58 ran with the 5α-DHT standard (Figure 68). Peak 44 was not investigated on this occasion as not only was activity very low, but the retention time indicated a more hydrophilic metabolite which did not match the properties of any of the suspected structures.

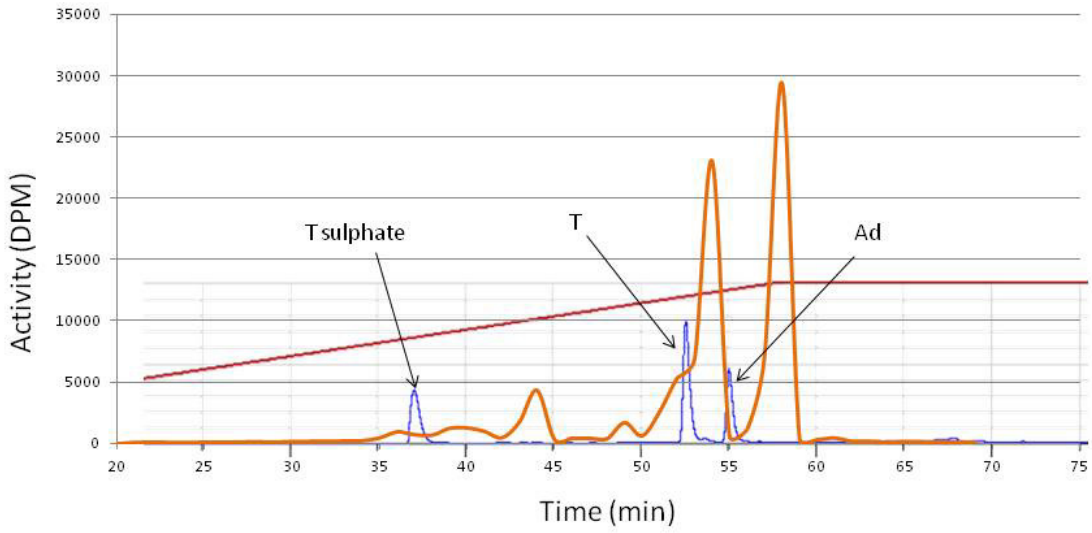

Figure 67: Representative HPLC chromatogram of tissue extract after hydrolysis of lipophilic [^{3}H]-T metabolites with standards. Data are presented as UV absorption (245 nm; blue) and radioactivity (orange).

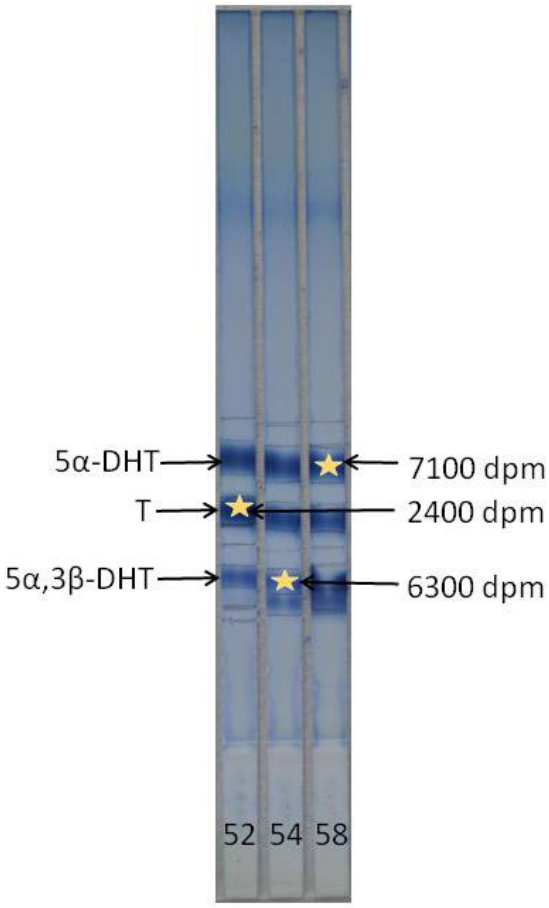

Figure 68: TLC plate of [^{3}H]-T metabolites separated by HPLC (peaks 52, 54 and 58 min) with T, 5α-DHT and 3β,17β-A5α standards (labelled dark blue bands). The delimitation of scrapes are in pencil, and the position (★) and radioactivity values (dpm) are given.

6.3.2.1 Does the ester metabolite profile vary under different conditions?

After tentatively identifying the ester metabolites it was thought important to investigate: a) whether the proportion of T, 3ß,17ß-A5α and 5α-DHT changed during depuration, and b) whether any of these enzymatic reactions was saturated with the addition of cold T. The hydrolysed heptane fraction of pooled extracts from time zero (no depuration), time 10 (10 d of depuration) and high treatment (highest cold dose; 25 µg L^{-1}) extracts were analysed on the C18 column. There were virtually no differences in the proportions of each metabolite (Figure 69), providing evidence that [^{3}H]-T metabolism was not saturated with the large amount of cold T added and the esterified metabolite distribution was unaffected by depuration.

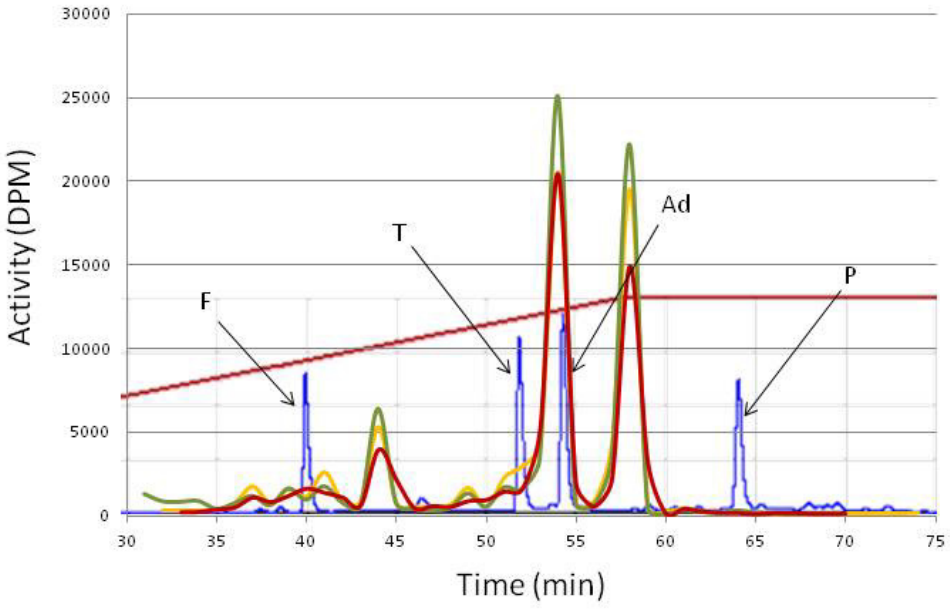

Figure 69: HPLC chromatogram of tissue extracts after hydrolysis of lipophilic [^{3}H]-T metabolites (Study 5) from day 0 (yellow) and 10 (green) of depuration and high cold dose (red) with standards. Data are presented as UV absorption (245 nm; blue) and radioactivity (yellow, red and green).

6.3.3 What happens to [^{3}H]-T when mussels undergo depuration?

So far it has been shown that *Mytilus* spp. are able to absorb [^{3}H]-T from the water with a clearance rate similar to that of [^{3}H]-E_2. However, not only was the esterification rate of [^{3}H]-T higher than that of [^{3}H]-E_2 but [^{3}H]-T was heavily metabolised. How might this metabolism and high rate of esterification affect the rate of depuration of the radiolabel? To investigate this, some of the animals that had been exposed to [^{3}H]-T were placed in fresh running sea water and a few were removed at irregular intervals and frozen for later extraction and separation into different fractions. Figure 70 shows that there was no significant decrease ($df = 4$, $f = 0.563$, $p = 0.692$) in total radioactivity over time. Furthermore, pairwise comparisons of total [^{3}H]-T, using T tests adjusted for multiple comparisons using a Bonferroni correction, demonstrated that there were no significant differences between day 0 and all the other time points ($p = 1$ for all time points). In other words, although 86 % of the sulphated / free portion of [^{3}H]-T was lost over 10 days (75 % in the first 24 h); the stability of the

ester fraction meant that this reduction (in free / sulphate [^{3}H]-T) was not noticeable overall.

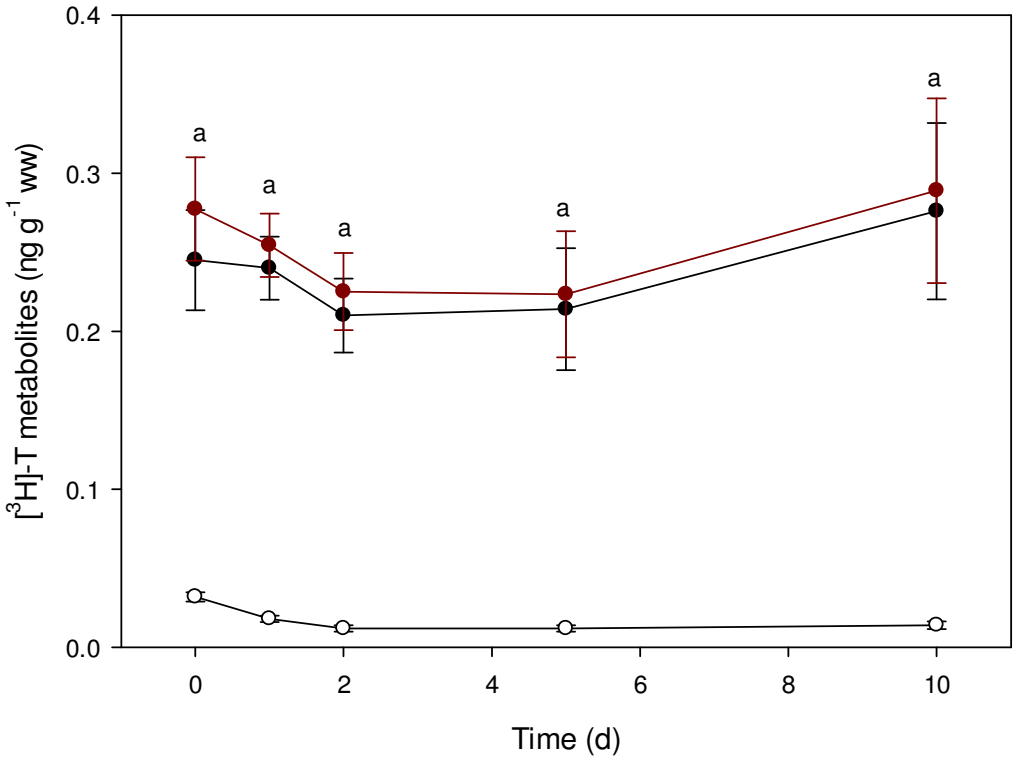

Figure 70: Free / sulphate fraction (○), ester fraction (●) and total [^{3}H]-T (●) in mussel tissue during depuration after exposure to [^{3}H]-T for 24 h. Data are presented as mean ng g^{-1} wet weight [^{3}H]-T residues (n = 10 for time 0 and n = 5 for all other sampling points), where observations labelled with the same letter ('a') are not significantly different from each other (p > 0.05).

6.4 Discussion

6.4.1 *Mytilus* spp. pick up [^{3}H]-T from water.

Mussels exposed to [^{3}H]-T readily picked it up from the exposure water. A few studies, mentioned in the Introduction, had already demonstrated that molluscs were able to pick up T. For example, a simple 21 day *in situ* exposure of snails (*P. antipodarum*) upstream and downstream of sewage effluent showed that the animals were able to accumulate esterified T (< 0.5 ng animal^{-1} on day 0 and > 2 ng animal^{-1} by day 21) (Gust et al., 2010b). Gooding & LeBlanc, (2001) reported 75 % uptake of radioactive testosterone by *I. obsoleta*. This figure was similar to the final uptake in the present study (65 %) but there is a large difference in the number of hours it took to reach these values. They reported a plateau (and therefore maximum uptake) after 8 h (similar to E_2), whereas in this study

it was found it took up to 18 h to reach peak uptake. However, this difference can easily be explained by differences in the amount of water in which the animals were placed (the snails were in just 3 ml water) and the fact that, one being a bivalve and the other a snail, clearance rates probably differ. Giusti *et al.* (2013) reported uptake of T by *L. stagnalis* in a concentration responsive manner. T in water was monitored; however data presentation precluded direct comparisons regarding uptake rates. Another exposure study, the only one thus far involving bivalves, found that *M. galloprovincialis* bioaccumulated immunoreactive T 100-fold in the form of fatty acid esters (Fernandes et al., 2009). Our study provides further evidence of T uptake by bivalves - it is the first study, to our knowledge, to investigate the accumulation of [^{3}H]-T in the animals at the same time as monitoring the decrease in the exposure water.

6.4.2 Why does [^{3}H]-T uptake come to a halt?

6.4.2.1 What is happening in the water?

When comparing [^{3}H]-T uptake to [^{3}H]-E_2 (during Study 5 exposure, i.e. under the same conditions), it appears that the clearance rates were very similar (38 % T vs 40 % E_2). However, the final uptake of [^{3}H]-T was higher than that of [^{3}H]-E_2 (ca. 65 % vs 50 % respectively). When inspected visually, plots of radioactivity removal from water show that [^{3}H]-E_2 uptake reached a plateau after ca. 6 h (Figure 14 in Chapter 2) and [^{3}H]-T after ca. 18 h (Figure 37). This difference is likely a result of the fact that [^{3}H]-E_2 was subject to extensive sulphation via the 3-hydroxyl group on C3 (preventing it from being re-absorbed by the animals). [^{3}H]-T, on the other hand, although it was heavily metabolised (as seen in Figure 40), was only partially converted into water soluble compounds (ca. 10 % compared to > 50 % for [^{3}H]-E_2). Partitioning (between water and diethyl ether) of [^{3}H]-T water extracts after 24 h confirmed this finding when 87 % of the radioactivity left in the water fractionated into the organic solvent and only 13 % (matching the ca.10 % found chromatographically) corresponded to water soluble metabolites. With little [^{3}H]-T sulphation (which was expected, as T does not possess a hydroxyl group on the C3 position), uptake continued for longer and the subsequent slowing down was a result of the accumulation of non-esterifiable breakdown products.

6.4.2.2 What is happening in the animal?

6.4.2.2.1 Extraction and separation methodology

In order to investigate the fate of the radioactive residues in mussels, the extraction method developed with [^{3}H]-E_2 as a model (Method 1 in Chapter 2) was tested for [^{3}H]-T. It required two further extraction steps (chloroform:ethanol 2:1; v:v) to achieve the desired efficacy (> 95 %). It was decided that, for future studies, it would be best to adopt Method 2.

Raw extracts were then separated into sulphates, esters and free steroid using the separation method developed in Chapter 2. The optimised method was tested on [^{3}H]-T and was found to be sufficiently effective (> 88 %, mean 92 %, n = 3), although not as efficient as it was for [^{3}H]-E_2 metabolite separation (mean 98 %). This poorer efficiency could account for some (possibly all) of the apparently 'free' T that was measured in the tissues.

6.4.2.2.2 Do *Mytilus* spp. esterify [^{3}H]-T?

As [^{3}H]-T has only one reactive hydroxyl group on C17, which a study by Labadie *et al.* (2007) suggests is the preferred site where esterification of E_2 occurs, it was hypothesised that T would be picked up and turned into a lipophilic ester. This assertion was to a certain extent correct as > 80 % of the radioactive residues found in the flesh were bound to fatty acid(s). Esterification of T in several molluscs had already been reported in the literature (Fernandes et al., 2009; Giusti et al., 2013; Gooding and LeBlanc, 2001; Gust et al., 2010b; Janer et al., 2006b). However, when the heptane fraction obtained from partitioning (mussels exposed to [^{3}H]-T only and without depuration) was hydrolysed and submitted to rp-HPLC; chromatography revealed that there were four distinct peaks. Only a small peak co-eluted with the T standard (retention time of 52 min) which accounted for 9 %. The rest of the radioactivity was distributed between a small peak at 44 min (7 %), and two larger peaks with retention times of 54 and 58 min (37 and 47 %). This means that although [^{3}H]-T had a reactive hydroxyl group on the C17, the majority of it was metabolised before the addition of a fatty acid. This finding is similar to the study by Gooding & LeBlanc, (2001) where a gastropod (*I. obsoleta)* transformed radioactive

testosterone into five distinct T metabolite esters (separated by TLC; but not identified).

Hines *et al.* (1996) investigated *in vivo* uptake (ca. 50 % over 12 h) and metabolism of radioactive Ad by the sea slug *Clione antartica*. They reported that the majority of the label had been turned into a 5α-reduced metabolite: 5α-androstane-3,20-dione (i.e. 5α-DHT). The rest of the precursor radioactivity corresponded to two metabolites of the 5α-reductase and 3α / 3β-HSD pathway: 3α-hydroxy-5α-androstan-17-one (androsterone) and 3β-hydroxy-5α-androstan-17-one (epiandrosterone). The presence of these enzymes (5α-reductase and 3α / 3β-HSD) in molluscs has been reported in several studies concerning a variety of species (De Longcamp, 1974; Fernandes *et al.*, 2010; Lyssimachou *et al.*, 2009; Ronis & Mason, 1996). On the basis of these studies (and the fact that the hydroxyl group at position C17 was probably being used for fatty acid conjugation), it was thought likely that the two main metabolites in the ester fraction of T (retention time 54 and 58 min) would be products of the 5α-reductase and 3α / 3β-HSD pathway. This possibility was investigated using TLC with T, 5α-DHT and 3β,17β-A5α standards. All three peaks ran with the standards – this, together with their positions on the C18 column, gave us a provisional ester metabolite profile: 9 % T, 43 % 5α-DHT, 37 % 3β,17β-A5α and 7 % unidentified (more hydrophilic) metabolite. At the moment, one can only speculate regarding the nature of the last unidentified peak (as for the unknown P metabolite). Any number of reactions could be responsible for the presence of a significantly more hydrophilic metabolite. For example the insertion of an oxygen atom (at some, as yet, unknown position) would account for the shift in elution time. For example, 11-keto testosterone (which has an =O group at the C11 position) elutes several minutes before T (Blasco et al., 2009). Another possibility is that T underwent 3β-HSD reduction (without 5α / 5β-reduction) converting it into 4-androstene-3β,17β-diol. This metabolite has been tentatively identified as a major product of T metabolism *in vitro* by an echinoderm (*Paracentrotus lividus*)(Janer et al., 2005a), and would perhaps have a shorter retention time than T. Most of these possibilities could be tested by buying or making the appropriate standards. Despite the incomplete T metabolite profile, the discovery that the ester fraction had very little

intact T, and was made up of mainly reduced metabolites, sparked a new question: does the T ester metabolite profile change over time or is it skewed as a result of saturation? To investigate this, the heptane fractions of the partitioned extracts of animals from day 10 of depuration and from the high 'cold' exposure were pooled, hydrolysed and submitted to rp-HPLC. These results are discussed in the following two sections.

6.4.2.3 Are there any saturation effects?

[^{3}H]-T was readily picked up by mussels and promptly esterified. Note that the ester portion was predominantly comprised of 5α-reduced [^{3}H]-T metabolites and only a little parent compound. With this in mind, would the addition of cold T affect: a) [^{3}H]-T uptake and b) ester metabolite distribution. a) Uptake saturation limits, if any, where not reached (with a dose as high as 25 µg L^{-1}). As seen in Figure 37 there was no difference in removal of [^{3}H]-T from water between treatments after 24 h or clearance rates (38.3, 37.9 and 44.8 mL $animal^{-1}$ h^{-1}). It should be mentioned that the clearance rate of the high dose treatment was slightly elevated (44.8 mL $animal^{-1}$ h^{-1}), however this could be due to differences in physiology or increased availability of [^{3}H]-T as a result of saturation of sorption sites (in the bags, aerators, shells) by cold T. In any case, the high dose would have the lowest uptake of [^{3}H]-T if indeed there was saturation of uptake. b) Ester metabolite distribution was investigated by comparing HPLC profiles (of the hydrolysed heptane fraction from partitioning, see Figure 69 for chromatograms) of animals exposed to no cold T (i.e. 'hot only') and those in the high dose group (25 µg L^{-1}). There was no difference between profiles regarding both retention times (i.e. both profiles had the same peaks) and radioactivity distribution (i.e. both profiles had a similar proportion of radioactivity between peaks). This suggests that none of the reactions that T underwent (5α-reduction, 3β-hydrogenation, esterification, etc.) after uptake reached saturation.

6.4.3 Does a high proportion of free steroid increase the likelihood of depuration?

Placing the animals in clean water under flow-through conditions did not reduce the [^{3}H]-T burden in any way. Although up to 86 % of the free /

sulphated fraction was purged after 10 d, this had little or no impact on the overall [^{3}H]-T burden as it accounted for < 20 % of total radioactive steroid. The esterified fraction remained stable throughout the depuration period. This means that the rules of depuration applied in the shellfish industry (a minimum of 42 h depuration) would likely have no effect on the levels of T (and its metabolites) in mussels. This shows that T (and T metabolite) esters are very persistent; even more so than E_2 esters, where a small amount was purged in the first five days. Gooding & LeBlanc, (2001) reported similar results regarding the lack of depuration of T esters by a snail (< 4 % in 96 h). Although the more water-soluble free and sulphated steroids were extremely susceptible to depuration, with 75 % lost in the first 24 h, this is of little importance as their contribution to the total T burden is very low.

The metabolite profile of the esters and the resistance of these to depuration raise another question: does depuration (or time, for that matter) affect the metabolite distribution of esters? To investigate this, the HPLC ester metabolite profile of animals that were sampled before depuration was compared to those that underwent the whole depuration period, i.e.10 days. As for the high dose metabolite distribution described above, there was no difference in both type of metabolite or proportion. This is further evidence of the stable and persistent nature of steroid esters.

6.5 Conclusion

This Chapter has presented definitive evidence of the uptake, metabolism and depuration (or the lack thereof) of [^{3}H]-T by *Mytilus* spp. [^{3}H]-T was not prone to sulphation (< 2 %; probably due to lack of a C3 reactive hydroxyl group), which in turn meant that uptake lasted longer, i.e. [^{3}H]-T removal from water reached a plateau ca. 8 h after that of [^{3}H]-E_2. The proportion of [^{3}H]-T esters was very high (> 80 %) and similar to that of [^{3}H]-E_2. However, > 85 % of those esters were comprised of [^{3}H]-T metabolites (mainly 5α-DHT and 3ß,17ß-A5α), whereas 100 % of [^{3}H]-E_2 esters were comprised of intact [^{3}H]-E_2 only. This Chapter also showed that there was no evidence that uptake or esterification could be saturated

(even with the addition of 25 µg L^{-1} cold T). Furthermore, the [^{3}H]-T metabolite esters proved to be incredibly persistent, i.e. the industry's mandatory 42 h of depuration would like have no effect on the overall T and 5α-DHT burden in *Mytilus* spp. It should be pointed out that 5α-DHT is the main androgenic signal in humans and arguably more potent than T.

Chapter 7

General discussion

7.1 Key findings

7.1.1 Method development

Using the blue mussel (*Mytilus* spp.) as a test organism, procedures were developed and refined for studying *in vivo* uptake of vertebrate steroids from the water and their subsequent metabolism, storage and depuration. This, in summary, is what has been accomplished so far and what still remains to be done:

- An efficient way has been devised to carry out radioactive steroid uptake experiments (plastic bags, 2 L of water, 5 animals per bag, maximum 24 h exposure with no feeding required). However, there is an aspect of our exposure studies that must be investigated before repeating any experiments (whether with one or five animals per bag), and that is the unaccounted loss of radioactivity (the difference between what was removed from the water and what was found in the flesh of the animals). These losses are likely to be the result of a number of compounding factors (e.g. pipetting error), however the most likely to have a significant effect is quenching during scintillation counting (due mainly to colour in the extracts). This will be investigated by comparing samples before and after bleaching.

- Two published procedures have been tested for extracting free and conjugated steroids from mussel flesh and both have shown to be > 95 % efficient for oestradiol (E_2). However, one procedure (Method 2) was effective on all steroids, i.e. it extracted all steroids with similar efficiency without the need of additional steps. Although this method did cause some downstream problems regarding storage (phase separation and precipitation), it was deemed more suitable and should be used in future studies after assessing whether or not long term storage is viable or indeed needed.

- An efficient and rapid solvent-based procedure has been developed for separating free, sulphated and esterified E_2. This novel procedure will have a strong impact in the field as there is currently no high-throughput way of separating these three metabolite fractions. The commonly employed chromatographic methods can only separate two out of three metabolite groups at a time (i.e. different columns and conditions are required to separate esters from free steroid; and free steroid from sulphates) and are a labour-intensive not to mention expensive. Our method was developed and optimised with tissue extracts from [^{3}H]-E_2 exposed mussels; when tested on other steroids it was found to be slightly less efficient (although deemed sufficient for the purposes of these studies). In future studies, the procedure should be refined for steroids individually; possibly by increasing the volume or the number of heptane washes. It should be mentioned that this method was developed with radiolabelled steroids but was then adapted for measuring any immunoreactive steroids too; it can therefore be used in laboratory exposures and in the field. A problem which arose both when processing tissue samples and water samples for immunoreactive identification, was the presence of what is referred to as 'tritiated water' (radioactivity that does not bind the C18 matrix of solid phase extraction cartridges). The identity of this radioactive fraction and the reasons for its presence are still unknown and need to be investigated.

7.1.2 Oestradiol

In terms of the ability of mussels to pick up and metabolise E_2, it has been:

- Confirmed that mussels readily absorb E_2 from water.

- Confirmed that mussels readily esterify E_2.

- Shown for the first time that mussels have a great capacity to absorb and esterify E_2 (it was found impossible to saturate the uptake of radiolabel with even 25 μg L^{-1} of cold steroid; i.e. 10,000 times higher than levels commonly found in the environment).

- Shown for the first time that mussels metabolise E_2 and that, within 12 h, there is effectively no intact steroid left in the water (i.e. any steroid which has not been esterified has been turned into metabolites that are no longer available for esterification). This evidence explains the cessation of E_2 uptake observed in some published studies (Gooding and LeBlanc, 2001; Hines et al., 1996), but which has never before been investigated.

- Shown for the first time that a large proportion of the E_2 that is not esterified is turned into a water-soluble sulphate conjugate. The presence of sulphates had been suspected before but had not been fully investigated (Janer et al., 2005b; Lavado et al., 2006a). This is the first time that the water-soluble metabolite has been putatively identified as E_2 3-sulphate (by HPLC and TLC co-migration) and will be soon confirmed by liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS).

- Demonstrated that the new separation procedure can be used to quantify immunoreactive free and esterified E_2 in mussels (sulphates of intact E_2 were too low in environmental samples to quantify definitively) collected from the wild. However, a survey with a larger number of samples from potentially heavily impacted sites needs to be done.

- Shown that mussels depurate E_2 esters very slowly (half-life > 10 d); in other words, E_2 could potentially remain in the fat stores of the animals for many months. An experiment with a longer depuration time (several months) is required to confirm this.

The procedures developed in the E_2 study were then used to investigate the uptake, metabolism, storage and depuration of several other steroids with key structural differences which would reveal any underlying mechanisms.

7.1.3 Testosterone (T)

In studies involving T it has been:

- Confirmed that mussels readily absorb T from water.

- Confirmed that mussels readily esterify T.

- Confirmed that mussels have great capacity to absorb and esterify T (it was found impossible to saturate the uptake of radiolabel with even 25 μg L^{-1} of cold steroid; i.e. 10,000 times higher than levels commonly found in the environment).

- Shown for the first time that T is also metabolised during the exposure period and that, within 18 h (under the conditions used in the present study) there was effectively no intact steroid left in the water (i.e. any steroid which had not been esterified had been turned into metabolites that were no longer available for esterification).

- Shown for the first time that the ester fraction is not just composed of intact T, in fact it accounts for < 10 % of the radioactivity. There are three additional metabolites of T that can nevertheless still be esterified. Two out of three metabolites have been tentatively identified as 5α-dihydrotestosterone (5α-DHT; ca. 45 %) and 5α-androstan-3ß,17ß-diol (3ß,17ß-A5α; ca. 40 %). The identity of these 5α-reduced metabolites needs to be confirmed by mass spectrometry.

- Shown that mussels do not depurate T esters (which include T metabolite esters); in other words, they remain intact in the fat stores of the animals for at least 10 d, and presumably longer. An experiment with a depuration period of several months is required to confirm this.

7.1.4 Ethinyl-oestradiol (EE_2)

The studies involving EE_2 have:

- Shown for the first time that mussels can absorb EE_2 from the water (at a similar rate to E_2).

- Shown for the first time that the rate of uptake is little affected by the amount of EE_2 in the water (i.e. the animals have a very high capacity just like E_2 and T).

- Shown for the first time that only some of the EE_2 is esterified (i.e. a noticeably smaller proportion than E_2).

- Shown that EE_2 remains mostly intact in the water, even after 24 h exposure, i.e. metabolism of EE_2 is very low (for both esterification and sulphate formation) and thus probably continues to be esterified long after E_2 has ceased to be picked up. The metabolism of EE_2 was not the reason for the slowing down of uptake (like it was for E_2 and T), instead it was likely a result of EE_2 exchange between water and animal reaching an equilibrium.

- Shown that EE_2 depurates faster than E_2 (half-life < 5 days) probably because most of the radioactivity in the tissue is in a free rather than esterified form.

7.1.5 Cortisol (F)

The studies involving F have:

- Shown that mussels are likely unable to pick up (let alone esterify) F. However, the exposure should be repeated with a higher number of replicates (with individual animals) and equal numbers of sorption controls to find out whether the small amount of radioactivity lost was actually a result of sorption (as was suspected) and not of uptake. In addition to this, as was speculated in Chapter 5, exposing mussels to 11-ketotestosterone and 11-deoxycortisol (which, as its name implies, has the same structure as F but without the oxygen atom on C11) would tell us whether or not the oxygen atom on C11 is the reason for the lack of uptake (or is it perhaps due to something, such as solubility?).

7.1.6 Progesterone (P)

The studies involving P have:

- Confirmed that mussels can absorb P.

- Shown that P, unlike any of the other steroids, is strongly adsorbed to the plastic bags and / or aerators. The P exposure study will have to be repeated with aerators of a different material (a preliminary experiment has suggested that these were the main problem) to determine the rate of uptake and metabolism.

- Shown for the first time that, despite the absence of a reactive hydroxyl group, some of the P radioactivity ends up in the ester fraction (though slightly less than for T and E_2). The presence of P esters has been reported before (Dimastrogiovanni et al., 2015; Gust et al., 2010b) but their *in vivo* metabolism has never been investigated with radiolabelled P.

- Shown for the first time that the steroid in the ester fraction is definitely not intact P (for fatty acid conjugation to occur it must be a metabolite with a reactive hydroxyl group). On the basis of a recent paper from Dimastrogiovanni *et al.* (2015) and the large number of reports of 5α-reductase and 3β-HSD activity (e.g. Janer *et al.*, 2005; Lavado *et al.*, 2006; Lyssimachou *et al.*, 2009), it was predicted that this metabolite would be 3β-hydroxy-5α-pregnan-20-one (3β-P5α). However, the HPLC peak turned out to be two metabolites, with the tentatively identified 3β-P5α accounting for only 13 % of the total activity. The definitive identity of both metabolites has to be confirmed by chromatography and mass spectrometry.

- Shown that mussels hardly depurate P esters (which are in fact P metabolite esters); in other words, they remain intact in the fat stores of the animals for at least 10 d, and presumably longer. An experiment with a depuration period of several months is required to confirm this. The free / sulphated fraction is almost completely

purged after 5 days, however as this accounts for < 40 % it has no major impact on overall P burden.

7.1.7 17,20β-dihydroxypregn-4-en-3-one (17,20β-P)

The studies involving 17,20β-P have:

- Shown for the first time that 17,20β-P can be absorbed by mussels.
- Shown that some of the radioactivity can be esterified (but not to the same extent as E_2, T and P).
- Shown that 17,20β-P is heavily transformed into presumably un-esterifiable metabolites (including, although not majorly, some water-soluble ones). These metabolites remain to be identified.
- Shown that the ester fraction is not solely comprised of the intact parent compound but also of a 17,20β-P metabolite (different to the main metabolite found in the water). The latter remains to be identified.
- Shown that mussels depurate over 75 % of the total 17,20β-P burden in a few days; depuration is highly effective because most of the steroid is present in its free form. Although it is a positive finding regarding the shellfish industry's regulations, 17,20β-P is a steroid used by fish and thus is irrelevant to humans.

7.2 Implications

One of the larger implications of our findings for public health concerns the production of esterified steroid metabolites. It was already known that measuring only the free fraction of E_2 and T was not representative of the whole steroid burden in mussels. It is now known that this applies to P too (as > 60 % was esterified) and to a certain extent EE_2. Furthermore, it is now known that measuring the ester content by quantifying the parent compound after hydrolysis is not enough either for some steroids; there are a number of esterifiable metabolites which need measuring too. This is

extremely relevant in the case of T and P (and 17,20ß-P if anyone chose to measure it), especially when measuring by immunoassay. This is because it is difficult to distinguish, without carrying out chromatography, how much of the T or P being measured is actually due to intact steroid and how much is due to cross-reaction with their metabolites? In fact, a couple of studies (Dimastrogiovanni et al., 2015; Gust et al., 2010b) have inadvertently proved that this has already happened (i.e. cross-reactivity) by reporting detection of P by RIA after hydrolysis, even though this is not possible, as 100 % of the ester fraction is made up of P metabolites. The authors should have inferred this (despite the lack of published evidence at the time) since P has no hydroxyl groups for fatty acid conjugation and must therefore be a hydroxylated metabolite. Given the knowledge produced in this thesis, this means that the studies listed in Table 2 in Chapter 1 that measured the free fraction only, or indeed both fractions (free and esterified) of the parent compound, only tell part of the story. The only exceptions to this are those studies that measured the free and ester fraction of E_2, since the ester fraction is solely made up of intact E_2 in mussels. It should be mentioned, though, that water-soluble E_2 metabolites also accounted for a reasonable proportion of the absorbed steroid. However, when measuring immunoreactive E_2 in mussels it was found to be largely made up of steroids other than E_2 which were unidentified due to small yields. In other words, one cannot expect this fraction to be quantified, it is simply not feasible.

The lack of depuration of steroid esters (particularly for those steroids presenting high rates of esterification) also has potential implications for the shellfish industry; according to our results, the mandatory minimum of 42 h depuration would not significantly reduce the ester burden of any of the steroids herein investigated. On a positive note, steroids that are not heavily esterified are rapidly purged. This was the case for the potent synthetic oestrogen, EE_2. However, in the case of the highly esterified T and P, even 10 d under depurating conditions did not manage to reduce the load in the slightest. This is particularly important for T esters, since the two main ester metabolites (5α-DHT and 3ß,17ß-A5α) are known to be biologically active in vertebrates. 5α-DHT is a more potent androgenic steroid than T in vertebrates (e.g. in rats; Wright *et al.*, 1996) and 3ß,17ß-

A5α has been shown to be estrogenic in rats by activating the ERβ (Oliveira et al., 2007). If mussels are consumed it is likely that steroid esters would be hydrolysed during digestion, releasing the biologically active compounds. As an example, algal toxins that are present as esters in bivalves (Elgarch et al., 2008; Rossignoli et al., 2011; These et al., 2009) are able to cause disease when contaminated shellfish are consumed (Hossen et al., 2011). This implies that the fatty acid conjugates must be hydrolysed during digestion, or the toxins (e.g. okadaic acid) would not be biologically active. It should be noted, however, that although ingesting metabolically-active steroids via shellfish consumption is possible; it is not actually known yet what the total steroid burden in animals destined for consumption is. In fact, it is not known yet what the steroid burden is in wild molluscs either, as all surveys so far (Alvarez-Muñoz et al., 2014; Yan et al., 2011) have failed to measure either steroid esters or their metabolites. It is encouraging, though, that most of the steroids that do bioconcetrate as esters (i.e. E_2, T and P) are all highly biodegradable (which reduces their availability via the water) (Fujii et al., 2003; Ismail and Chiang, 2011; Yang et al., 2011); and the most recalcitrant steroid investigated, EE_2, was in fact readily purged.

Although it is likely that steroid exposure via shellfish is minor; chronic, low-level endocrine disruption in communities with high seafood consumption cannot be discounted. A study by Sjafaraenan (2013) investigated oestrogen levels in women before and after consumption of the clam *Semele* sp., the local staple food. Despite the study not being very rigorous or scientifically sound, it showed a link between regular *Semele* sp. consumption and later onset of menopause in women. This idea, that shellfish consumption might produce ED effects, is not entirely far-fetched; steroids are known to be present in molluscs, and this study has highlighted that it is likely that the burden is higher than first thought (with some potent hormonal steroids that have not been measured before, e.g. 5α-DHT). For example, if on average a commercially harvested mussel has approximately 8 g of wet weight and the average portion of a serving consists of 10 shellfish then the total oestrogen could be as high as 2 ng g^{-1} (concentration found in our survey) x 8 g x 10 animals = 160 ng E_2 per serving.

The discovery of the extent of E_2 sulphation sheds a light on the uptake dynamics of E_2; both in experimental set ups and in nature. Now it is known why E_2 uptake comes to a halt after ca. 8 h in laboratory exposures (also seen in the literature): sulphation competes with esterification. And even if the reaction occurs on different hydroxyl groups (the literature points to esterification preferably taking place on C17 and this study has tentatively identified the reactive hydroxyl on C3 to be the preferred site for sulphation), the conjugation of a fatty acid or a sulphate group would presumably sequester the steroid and make it unavailable for the other reaction to occur (i.e. sulphates accumulate in water and esters in fatty tissues). This could perhaps have implications on steroid uptake in nature. For example, the potentially higher levels of bacterial sulphatases in sediments could mean that any E_2 that is sulphated and then excreted will be converted back to E_2 in the sediment and then made available for re-absorption. This means that molluscs that inhabit sediments could potentially accumulate higher levels of E_2 than those that live on rocks or other surfaces suspended in water where there is a lower concentration of bacteria. Interestingly, Langston *et al*. (2007) found that, when exposed to E_2 in the presence of sediment, the clam *Scrobicularia plana* continued to absorb E_2. In tanks with just water, however, E_2 uptake reached a plateau after about 12 h just as found in the present study.

7.2.1 How does the evidence of uptake and bioaccumulation affect the endogenous biosynthesis theory?

So, how do the new findings fit in with the dispute over the origin of steroids? i.e. endogenous vs exogenous origin. Clearly, none of the results in the present study can actually disprove the hypothesis that steroids are biosynthesised by molluscs. However, while the evidence for endogenous production (Chapter 1) is rather weak, that for uptake, metabolism and long term storage of steroids is strong (at least in *Mytilus* spp.). In fact, the evidence provided in this thesis makes several of the arguments for biosynthesis obsolete. For example, it has been shown that P and T and / or their metabolites are retained as esters for over 10 days under flow-through conditions. If they are hardly affected after 10 days of depuration, what reason is there to believe this would not be the case after 6 months?

This ability to hold onto steroids 'indefinitely' means that one can **never** discount the possibility that whatever steroid one measures in mollusc tissue has not been picked up from the environment. This inference dismantles one of the main arguments for the endogenous origin theory: that the presence of steroids (even in apparently clean environments – see below) is evidence of endogenous production.

If it is accepted that one can never discern whether steroids found in mollusc tissue are of endogenous or exogenous origin, how can one assume that any changes in concentration are a result of 'hormonal' changes associated to reproductive development (i.e. seasonality)? Indeed, in view of the evidence presented in this study, these changes could well be the result of numerous other (far more plausible) reasons. Here is a list, by no means exhaustive, of reasons (of which several have been evidenced) why differences in steroid concentrations in molluscs could occur:

- differences in rate of uptake from the water
- differences in rates of release into the water (i.e. depuration)
- differences in rates of hydrolysis of steroid ester
- differences in rates of formation of steroid ester
- differences in the availability of steroids in the water or food (that could be caused by presence or absence of reproductively mature fish, presence of waste effluents, changes in currents and tides, changes in rainfall, agricultural run-off, etc.)
- differences in the age of the animals (on the basis that older animals probably will have had a lot more time to accumulate esters than younger animals)
- differences in the condition of the animals (e.g. if feeding is poor, they might metabolise their steroid esters and / or reduce filtration rates)

- differences in the living conditions (e.g. mussels tend to clump together; those in the middle might be less exposed to steroids)

- differences in the condition factor and hence water content of the animals (this would influence steroid 'concentration' – most often quoted as ng g^{-1} wet weight – without there actually being any difference in steroid 'content' between animals)

- differences in the content of lipids and proteins (e.g. due to different reproductive stage) that potentially influence the ability of the animals to retain free steroids(i.e. non-specific binding)

- differences in the presence of chemical compounds and / or conditions (e.g. pH, protein and salt content) that could cause interference in immunoassays (and thereby yield incorrect values for steroid concentrations)

- calculation errors

In order to explain why some studies (see 'seasonality' column of Table 2 in Chapter 1) show an apparent strong relationship between sex steroid concentrations and the reproductive cycle, it is suggested that any one (or indeed combination) of these factors could easily occur, at certain times, in a way that would give the illusion of there being a 'cycle' in steroid concentrations that was linked to a stage of reproduction.

7.2.1.1 Where do steroids in molluscs from clean environments come from?

Although the uptake levels investigated in this work are considerably higher than any concentration a wild animal would ever encounter, it highlights the fact that mussels essentially have a 'vast' capacity for uptake and storage (especially of E_2 and T metabolites). This, coupled with the fact that no evidence was found for depuration of T and P (and to a lesser extent E_2) esters over 10 days, questions the claim in some studies that the steroids must be of endogenous origin because the animals were collected from clean sites (i.e. sites where steroids in the water were under the

detection limit at the time of sampling). In other words, the steroids that have been found could have been picked up by the animals some weeks to months before the measurements were made. It should also be pointed out that the denomination 'clean' is often a subjective term; there are, for example, no class A shellfish sites (i.e. free of human-derived pathogens) anywhere in England. In addition, steroids (such as E_2, T and P) do not solely originate from human waste; they are excreted by farm animals and fish (and indeed by all vertebrates). In summary, not detecting steroids in water at the time of sampling does not mean that small (probably immeasurable) amounts of free steroid are or have been in the water and could easily build up in the animal to large (measurable) levels of esterified steroids (i.e. they will 'bioconcentrate') over a matter of weeks or months.

The same reasoning applies to studies that claim that they have kept the animals for several weeks in clean laboratory tanks to depurate (Santos et al., 2005; Zhu et al., 2003). As it has been shown in the present study, esterified steroids can remain in the animals potentially for months (i.e. any steroids that the animals picked up from the environment prior to their capture will still be there when any experiments are carried out). To add to this, no one has yet considered the possibility that any free steroids that are found in molluscan tissue extracts might actually have been formed from esterified steroids during the extraction process. Scott *et al.* (2014) for example, induced unintentional acid solvolysis of steroid sulphates during extraction of fish bile. It is not unreasonable to suggest that under certain conditions (e.g. high pH), saponification (i.e. alkaline hydrolysis) could also occur during extraction. It would only need a very small amount of hydrolysis of the stored steroid esters to produce the picogram amounts of free steroids in tissues that are reported in most studies. Furthermore, as pointed out by Scott (2012), humans in the laboratory are also a potential source of steroids (i.e. there is always the potential for fresh contamination even when the animals are kept in clean laboratory conditions).

The notion that one can never discount the origin of a steroid as exogenous should also be considered when thinking of using molluscs for vertebrate endocrine disruption tests. Even if molluscs did produce and use

vertebrate steroids as hormones, the fact that they actually pick them up and store them at all would invalidate any results.

7.3 Why do molluscs esterify steroids?

The present study has led to a better understanding of the underlying mechanisms of steroid uptake, metabolism and depuration by the blue mussels. It has not, however, provided any insight into why mussels (or any other mollusc for that matter) esterify steroids in the first place, particularly E_2 and T. First, it should be emphasised that the process of conjugating a fatty acid to a hydroxylated compound is not unique to steroids. The same chemical reaction takes place when organisms form fats (e.g. fatty acids + glycerol → triglyceride). Retinol (Gesto et al., 2012) and cholesterol (de Souza and de Oliveira, 1976) esterification has also been shown in molluscs; and as mentioned earlier, many algal toxins are known to be esterified after being picked up by filter-feeding molluscs (Rossignoli et al., 2011; Torgersen et al., 2008). Does this last finding mean that molluscs use esterification to inactivate vertebrate steroids? Although this is a possibility, it seems unlikely; why make a compound more hydrophobic when the ultimate goal is to get rid of it (surely it should be made more hydrophilic)? Also, why store it potentially for months? Moreover, it is now known that mussels quite readily sulphate E_2, in other words why would they perform both reactions that lead to different outcomes (i.e. storage and excretion)? It has been suggested, by those that believe that steroids are biosynthesised by molluscs, that esterification controls the levels of free (and therefore biologically active) hormones, much like steroid-binding proteins do in vertebrates. Following that, it has also been proposed that esterification controls the availability of precursors for hormone production, e.g. T aromatization. Both these suggestions assume that vertebrate steroids are produced and used as hormones by molluscs; a theory that has been heavily outweighed by evidence for the exogenous origin of steroids. Another possible option is that the molluscs treat the steroids (and indeed any compounds with a suitable hydroxyl group) as a potential food source. In other words, steroids (and cholesterol and even toxins) are treated as a valuable source of soluble food and are therefore esterified for long-term storage along with the triglycerides. So

far, there is no solid evidence to favour any of these suggestions over any other.

7.4 Further work

The findings of the present study have posed new questions that remain to be answered. First of all, if molluscs indeed only have vertebrate steroids because they pick them up from their environment; then what compounds do they use to regulate reproduction and development? Perhaps the easiest way to even begin to answer this question would be to look at full genome sequences to look for candidates genes to investigate (Vogeler et al., 2014). Another pertinent question is, do all molluscs esterify and sulphate steroids? There is a lot of evidence to suggest gastropods and neogastropods (snails) do; but what about cephalopods? This is the only molluscan class in which esterification has not been investigated. What about other invertebrates (e.g. crustaceans)? Do they process steroids in the same manner? Are there any differences between male and female uptake and metabolism? In which tissues are steroids preferentially stored? Does spawning affect steroid load (e.g. as a result of fatty acid mobilisation)? Do steroid esters get mobilised if the animals are starved? Does this influence the free steroid levels? What about other synthetic steroids (e.g. progestins)? Are they picked up and processed in the same manner? Can any of the metabolites found during these investigations (e.g. 5α-DHT) be picked up directly from the water? And can the formation of this compound from T be inhibited by 5α-reductase inhibitors?

7.5 Conclusion

The findings presented in this thesis have built a solid case for the uptake and bioaccumulation of steroids from the environment; far stronger than the evidence for endogenous biosynthesis of steroids by molluscs. Although the evidence of uptake, metabolism and lack of ester depuration does not disprove endogenous steroid biosynthesis; it does mean it is impossible to tell whether steroids in molluscan tissues are of endogenous or exogenous origin. The implications of this are very important: any studies investigating the presence of steroids in relation to reproductive cycles or hormonal roles in molluscs are rather pointless; and the potential use of

molluscs as models for vertebrate endocrine disrupting tests (in a laboratory or as a sentinel species) can be completely discounted.

Appendices

Table 22: Steroid extraction (from tissue) method development.

Extraction method	Study	Steroid	Extracts (% total) 1	2	3	4	5	6	7	Extracts 1 & 2 efficiency (%)	Method efficiency (%)
1	1	E_2	75	15	7	3				90	97
	1	E_2	50	27	17	6				76	94
	1	E_2	80	15	3	1				95	99
	3	E_2	14	34	31	15	6			48	94
	3	E_2	79	17	5					95	95
2	5	E_2	88	11	1					99	99
	5	E_2	76	20	4					96	96
	5	E_2	93	6	1					99	99
1[a]	5	T	5	9	24	29	20	14		14	-
	5	T	9	12	10	33	23	13		21	-
	5	T	12	31	29	16	8	4		43	-
	5	T	16	21	29	21	8	5		38	-
	5	T	11	33	20	23	8	5		43	-
	5	T	-	-	-	-	82	12	6	-	94
	5	T	-	-	-	-	89	11		-	-
	5	T	-	-	-	-	95	5		-	-
	5	T	-	-	-	-	-	96	4	-	96
	5	T	-	-	-	-	-	95	5	-	95
1[b]	5	EE_2	71	20	5	3	2			91	98
	5	EE_2	71	21	5	3				92	-

Extraction method	Study	Steroid	Extracts (% total) 1	2	3	4	5	6	7	Extracts 1 & 2 efficiency (%)	Method efficiency (%)
	5	EE_2	79	15	4	2				94	-
	5	EE_2	-	-	96	4				-	-
	5	EE_2	-	-	98	2				-	-
	5	EE_2	-	-	98	2				-	-
	5	EE_2	-	-	80	14	6			-	94
	5	EE_2	-	-	94	6				-	-
	5	EE_2	-	-	85	15				-	-
	5	EE_2	-	-	-	98	2			-	98
2	5	17,20B-P	87	11	2					98	98
	5	17,20B-P	91	8	1					99	99
	5	17,20B-P	81	16	3					97	97
	5	17,20B-P	85	13	2					98	98
	5	17,20B-P	90	9	1					99	99
	5	17,20B-P	81	16	3					97	97
	5	17,20B-P	-	99	1					99	99
2	5	P	90	8	2					98	98
	5	P	85	13	2					98	98
	5	P	84	14	2					98	98
	5	P	-	99	1					99	99
2	5	C	84	12	3					97	97
	5	C	88	9	3					97	97
	5	C	87	10	3					97	97

[a/b] Variations of Method 1; details can be found in section 2.2.5.2 in Chapter 2.

Table 23: Summary of metabolite separation method development.

Method optimisation steps	Sample	Mixing time (min)	Mussel extract (µl)[a]	Water (mL)	Ethanol (mL)	Heptane (mL)	Ethanol activity (% total)	Heptane activity (% total)
Separation of esters and free steroids using standards	E_2 label	5	400	0.3	1.2	1.5	98.6	1.4
	EE_2 label	5	400	0.3	1.2	1.5	99.0	1.0
	free EE_2 (HPLC)	5	400	0.3	1.2	1.5	99.0	1.0
	EE_2 ester (HPLC)	5	400	0.3	1.2	1.5	43.2	56.8
	E_2 ester (HPLC)	5	400	0.3	1.2	1.5	21.8	78.2
Extract vol. and heptane volume	E_2 mussel extract	5	100	0.3	1.2	1.5	28.9	71.1
	E_2 mussel extract	5	100	0.3	1.2	3	22.0	78.0
	E_2 mussel extract	5	100	0.3	1.2	4.5	21.0	79.0
	E_2 mussel extract	5	200	0.3	1.2	1.5	28.9	71.1
	E_2 mussel extract	5	200	0.3	1.2	3	21.1	78.9
	E_2 mussel extract	5	200	0.3	1.2	4.5	20.8	79.2
	E_2 mussel extract	5	400	0.3	1.2	1.5	27.7	72.3
	E_2 mussel extract	5	400	0.3	1.2	3	21.7	78.3
	E_2 mussel extract	5	400	0.3	1.2	4.5	19.9	80.1
Number of heptane extractions	E_2 mussel extract	5	200	0.3	1.2	1.5	13.8	63.4
						1.5		15.9
						1.5		4.6
						1.5		1.5
						1.5		0.7
Use of heat during sample preparation	E_2 mussel extract	5	100	0.3	1.2	1.5	34.5	65.5
	E_2 mussel extract	5	100	0.3	1.2	3	25.2	74.8
	E_2 mussel extract	5	100	0.3	1.2	4.5	22.0	78.0
	E_2 mussel extract	5	100	0.3	1.2	1.5	13.2	58.9
						1.5		18.9

Method optimisation steps	Sample	Mixing time (min)	Mussel extract (µl)[a]	Water (mL)	Ethanol (mL)	Heptane (mL)	Ethanol activity (% total)	Heptane activity (% total)
						1.5		6.5
						1.5		2.6
Ethanol (%)	E_2 mussel extract	5	200	0.3	1.2	1.5	14.8	72.8
						1.5		8.8
						1.5		3.6
	E_2 mussel extract	5	200	0.15	1.35	1.5	19.3	57.8
						1.5		14.6
						1.5		8.3
Mixing time and acidification	E_2 heptane	5	200	0.3	1.2	3	4.2	82.7
						3		13.1
	E_2 heptane	10	200	0.3	1.2	3	4.3	81.2
						3		14.4
	E_2 heptane	15	200	0.3	1.2	3	4.0	87.7
						3		8.3
	E_2 heptane	5	200	0.3	1.2	3	4.0	80.6
						3		15.3
Testing conditions on pure E_2 ester from heptane fraction.	E_2 heptane	5	200	0.3	1.2	1.5	1.2	85.1
						1.5		9.3
						1.5		2.7
						1.5		1.0
						1.5		0.7
Extract volume	E_2 heptane	5	400	0.3	1.2	3	2.6	84.9
						3		12.5
	E_2 heptane	5	600	0.3	1.2	3	2.2	85.5
						3		12.3
	E_2 heptane	5	800	0.3	1.2	3	2.2	86.2
						3		11.6
Separation after hydrolysis: vol. of	E_2 heptane -	5	150	0.45[b]	1.8[c]	4.5	85.5	5.9

Method optimisation steps	Sample	Mixing time (min)	Mussel extract (µl)[a]	Water (mL)	Ethanol (mL)	Heptane (mL)	Ethanol activity (% total)	Heptane activity (% total)
water and heptane adjusted to the vol. of methanol used for hydrolysis.	hydrolysis 40°C, 3 h							
						4.5		4.6
						4.5		4.0
	E_2 heptane - no hydrolysis	5	150	0.45[b]	1.8[c]	4.5	10.1	59.9
						4.5		21.2
						4.5		8.7
	E_2 heptane - hydrolysis 80°C, 40 min	5	150	0.45[b]	1.8[c]	4.5	95.5	1.8
						4.5		1.4
						4.5		1.2
	E_2 heptane - no hydrolysis	5	150	0.45[b]	1.8[c]	4.5	11.7	60.7
						4.5		19.5
						4.5		8.1
	[^{3}H]-E_2 exposed mussel tissue - subtilisin 4°C	5	500	0.3	1.5	3	18.4	69.5
						3		12.1
	[^{3}H]-E_2 exposed mussel tissue - subtilisin (RT)	5	500	0.3	1.5	3	19.1	69.0
						3		11.9
	[^{3}H]-E_2 exposed mussel tissue - no subtilisin (RT)	5	500	0.3	1.5	3	19.8	68.3
						3		12.0

Method optimisation steps	Sample	Mixing time (min)	Mussel extract (µl)[a]	Water (mL)	Ethanol (mL)	Heptane (mL)	Ethanol activity (% total)	Heptane activity (% total)
	[^{3}H]-E_2 heptane from separation - subtilisin (RT)	5	-	0.3	1.5	3	4.2	82.2
						3		13.6
	[^{3}H]-E_2 heptane from separation - no subtilisin (RT)	5	-	0.3	1.5	3	4.1	83.2
						3		12.7

[a] **If the sample was not in mussel extract (e.g. HPLC standards), unexposed mussel extract was added to simulate conditions.**

[b] **Constituted of 0.2 mL 3M KOH, 0.04 mL 2.5M HCl and 0.21 mL water (from hydrolytic reaction).**

[c] **Methanol (from hydrolytic reaction).**

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