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Steroid hormones from agroecosystems: A review

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Steroid hormones from agroecosystems: A review

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Executive Summary

In recognition of the potential risks that steroid hormones pose to aquatic ecosystems, DPI provided funding for this project, designed to review current understanding of the sources, transport and ecosystem risks posed by agriculturally derived steroid hormones.

The review has addressed such questions of broad interest to the community and land, water and farm managers as:

- What is the likelihood that Victorian agricultural effluent is hormonally active?
- Will fish and other organisms be affected by the discharge? How much exposure is required before an effect is observed and is it reversible?

These questions remain, since there have been few studies of steroid transport from agriculture in Australia, with most work in this area, i.e. looking at the occurrence of steroid hormones in agricultural watersheds, occurring in the United States. Overseas studies have, for the most part, focussed on estrogenic steroids derived from confined feeding operations, and the land application of their manures and/or discharges from waste storage lagoons, with some limited assessment of the contribution from extensive livestock farming systems.

Endogenous and therapeutic steroidal hormones may not be consistently detected in the vicinity of feedlot operations or grazing systems, in part because these compounds can strongly sorb to soils and sediments. Although sorption to organic matter in soils and sediments provides a sink for these compounds, there is also evidence that these compounds can be released from the sediments and re-enter the water. Moreover, although limited in number, scale and scope, the work that has been reported on steroid transport from agroecosystems clearly shows that there is estrogen and androgen contamination of receiving waters from grazing systems and land onto which CFO manures and effluents from storage lagoons are applied. There is also some direct evidence from the field to suggest that the hormonal contamination is having physiological impacts on fish in the receiving environments.

Almost half of Australia's dairy farms are located in Victoria, with most of the farms located in the higher rainfall areas of southern and north-east Victoria. At any one time, some 1.3 million cows are in milk in Victoria, producing some 6 billion litres of milk annually, or some $2 billion to farmers at factory paid prices (2004/05). Given that livestock manure and dairy farm effluents contain high levels of steroid hormones, recycling of these resources onto land creates the potential for contamination of surface waters by hormones through run-off.

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1. Introduction

"There's more to water than salt and nutrients"

Victoria has a diverse range of agricultural landscapes, from rolling alpine hills to flat river plains, but despite this diversity, most of these landscapes are home to beef and dairy production. No agricultural operations are chemical free, since all living things are made from, and use a wide range of chemicals for energy production, growth, maintenance of bodily structure, and reproduction. Many of these chemicals are excreted by organisms naturally as a function of being alive. It is well known that chemicals (natural or otherwise) can be transported off-site via air, soil, and water to surface and groundwater in turn potentially causing adverse impacts on aquatic ecosystems as a result of toxic effects, both lethal and sub-lethal, to aquatic species. [1,2] The migration of chemicals to aquatic environments and effects on water quality has been studied in many regions of the world. However, despite the potential risks, in comparison there have been relatively few studies in Australia, in particular Victoria. [2]

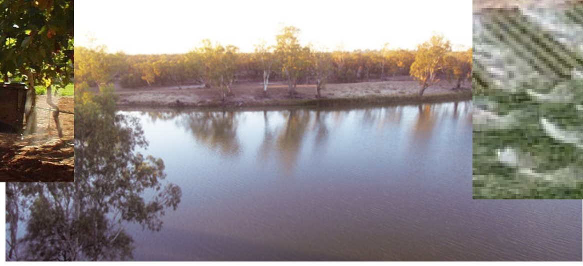
The need to protect Victoria's water resources and to address emerging water quality issues is highlighted in various Victorian government documents [3-6] including the White Paper "Securing our water future together", the "Victorian River Health Strategy" and the policy "Water: Growing Sustainable Primary Industries".

Managing the effects of trace organic chemical contaminants in Victorian waterways requires far more information than we (as scientists and/or managers) can afford to directly measure for all the places at all of the times, and for all the chemicals of interest. Strategies and/or tools are therefore required to focus monitoring and risk assessment programs in a cost-effective manner, and to predict agrochemical concentrations and effects in locations that have never been directly assessed (or inadequately assessed).

To assess the risk of agriculturally derived chemicals to aquatic ecosystems, information is required on the environmental fate of particular chemicals, their concentrations in the environment (exposures) and toxicity to aquatic organisms. The overall ecological risk can then be determined based on the general principle that risk is a function of toxicity and exposure (or likelihood of an adverse effect).

In recognition of the potential risks that agriculturally derived naturally occurring steroid hormones may pose to water quality, DPI provided funding for this review, the specific objectives of which are:

- To identify and review previous studies relating to steroid hormone transport from agroecosystems.
- To identify knowledge gaps for future research.



DPI projects are investigating ways to improve the integration of farming systems into landscapes, including understanding threats to water quality from chemical transport

2. Steroid hormones from agroecosystems

2.1 Introduction

Almost half of Australia's dairy farms are located in Victoria, with most of the farms located in the higher rainfall areas of southern and irrigation regions of north-east Victoria. At any one time, some 1.3 million cows are in milk in Victoria, producing some 6 billion litres of milk annually, or some $2 billion to farmers at factory paid prices (2004/05).

Dairy manure and dairy farm effluents contain high levels of estradiol and its breakdown product, estrone (combined load up to 4000 ng/L in effluents). Recycling of these resources onto land creates the potential for contamination of surface waters by hormones through run-off.

Recent reports from the UK and NZ have identified substantial estradiol inputs from dairy farming into streams and rivers (up to 25% of total hormone load in UK). There is other limited evidence from Ireland and Denmark suggesting that reference sites (streams and lakes) chosen for their supposedly pristine character, have waters which estrogenic activity. This estrogenic activity has been linked to agricultural activity, including livestock rearing and land application of manure. There are, of course, differences between the Victorian dairy industry and that of New Zealand, the UK, Ireland and Denmark, in terms of scale, intensity, and the landscape, soils and climate in which it is conducted, but there are also similarities, e.g. manure treatment of fields.

Nash et al [6] found a wide range of compounds, including the physico-chemically similar cholesterol (precursor of all the steroid hormones) in overland flow from dairy pasture in southern Victoria. This suggests that despite the apparent differences in dairy practices between Australia and overseas jurisdictions, steroid hormones may also be entering waterways from Victorian agriculture. While the focus of this section so far has been on dairy, Matthiessen et al [7] did find estrogenic inputs from beef, sheep and pig production in the UK, albeit at lower levels.

Global concern exists about the occurrence of endocrine disrupting compounds (EDCs), including naturally occurring hormones such as estradiol and testosterone in the freshwater environment and their effects on indigenous fauna. This public concern, and the research underpinning the scientific concern that EDCs have had adverse effects on aquatic wildlife, has more than been adequately been reviewed by the World Health Organisation, and it is from their 2002 review ("Global assessment of the state-of-the-science of endocrine disruptors," edited by Damstra et al) that the following summary of EDCs and their impact on vertebrates is primarily taken. [8]

The occurrence of endocrine disrupting chemicals (EDCs) in the aquatic environment, and their impact on indigenous fauna has generated a significant amount of scientific and public interest since the publication of the book *Our Stolen Future.* [9] Since then, substantial evidence has emerged that many chemicals induce hormone-like effects in wildlife and humans, [8] at the concentrations observed in the environment, i.e. at concentrations much lower than those used in toxicity tests designed to see if the chemicals cause cancer. Chemicals with hormonal activity, i.e. potential EDCs, include:

- Natural hormones. These can be from any animal, and once released into the environment, chemicals produced by one species can exert hormonal actions on other animals, e.g. human hormones unintentionally reactivated during the processing of human waste in sewage effluent, may result in physiological changes to fish.

- Natural chemicals, including toxins produced by algae, and components of plants (phytoestrogens, such as genistein or coumestrol), and certain fungi.
- Synthetically produced pharmaceuticals that are intended to be highly hormonally active, e.g. components of the contraceptive pill, treatments for hormone-responsive cancers, and therapeutic agents used in agriculture (e.g. trenbolone acetate).
- Man-made chemicals and by-products released into the environment (e.g. phthalates leached from plastics).

Estrogen mimics are not the only class of endocrine disruptors. Some chemicals antagonise male hormones, i.e. they are androgenic. Indeed, perversely, some compounds may be both estrogen agonists and androgen antagonists, e.g. DDE. [10]

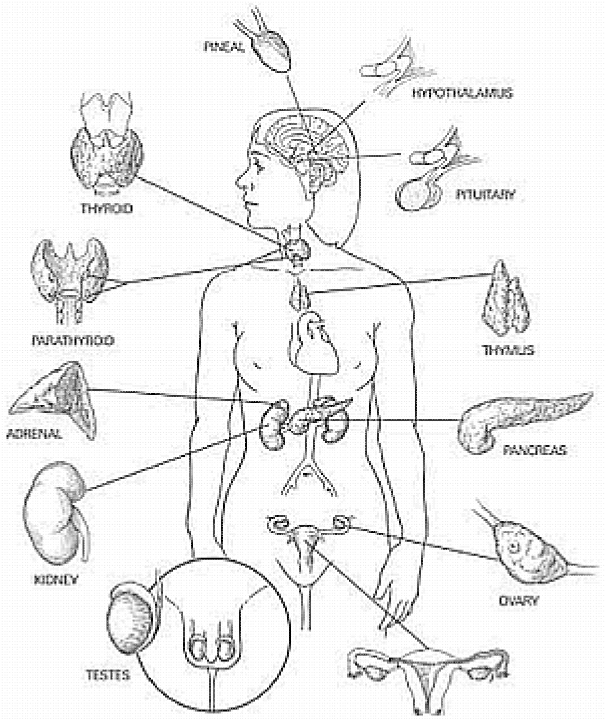
The endocrine and reproductive effects of EDCs are thought to be due to their ability to (1) mimic, (2) antagonise, or (3) disrupt the synthesis and metabolism of hormones, or (4) disrupt the synthesis and metabolism of hormone receptors. The discovery of this hormone-like activity occurred long after the release of chemicals into the environment, [10] yet for most associations between exposure to EDCs and subsequent biological outcomes, the mechanism(s) of action are still poorly understood. [8] This can make it difficult to distinguish between both direct and indirect effects of exposure to EDCs, and primary versus secondary effects.

- Some studies assume that EDCs only produce effects on development rate, growth and reproduction by disrupting the sex hormones controlling reproduction and maturation, but interference with other hormonally controlled systems, e.g. thyroid and retinoid axes of development, can also produce undesirable physiological changes in the developing organism. Life-history traits may also be the result of toxic effects, or sex-related differences in sensitivity (i.e. survival, condition).
- To truly assess the endocrine disrupting effects of chemicals in fish, for instance, one would need to ensure that one discriminates between the sub-lethal effects resulting from changed behaviour or energy intake, or those resulting in changed condition, from those related to endocrine disruption *per se*.

The risk to wildlife of exposure to environmental estrogens has been demonstrated in both field and laboratory. [8, 11] Reports of adverse effects (from subtle changes in physiology to permanently altered sexual differentiation) have come from Europe and North America, although in many cases the causal link between endocrine disruption and effect is unclear. There are, however, a number of remarkable, well-publicised and accepted examples of demonstrated endocrine disruption in the field:

- Male alligators reared in Florida's Lake Apopka have very small penises, an effect linked to chemical residues in their tissues caused by an accident at a chemical plant on the lake's shores. [12]
- Birds of prey exposed to DDT laid eggs with unnaturally thin eggshells, resulting in breakage during laying or incubation, reduced chick survival and ultimately population decline. [13]
- Embryonic abnormalities have been observed in fish-eating birds, e.g. in the Laurentian Great Lakes, which can be ascribed to PCB exposure. [14]

Endocrinology 101: The endocrine system



Human endocrine system

It is beyond the scope of this section to describe the entire endocrine system of even a single organisms (e.g. humans), but it is fair to say that while the precise structures and roles of the various organs and hormones differ between different vertebrates, particularly in relation to the different life cycle and development stages in different species, the endocrine systems of all vertebrates are similar, and similar enough to that of humans that our understanding of our endocrine systems can shed light on potential affects in other species.

Non-mammalian vertebrates differ from mammals, and each other, in terms of their reproductive strategies. These include sequential or simultaneous hermaphro-ditism, parthenogenesis, viviparity, ovo-viviparity and gonochorism. Breeding frequency may also be more limited, and includes the semelparous (breed only once) as well as iteroparous (breed two or more times). However, the endocrine axes are similar to that of mammals in their operation, in the pattern of feedback mechanisms and in the hormones involved. For instance, in general, testosterone and estradiol are the major androgen and estrogen produced by vertebrates, although in many male teleosts (bony fishes) 11-ketotestosterone is the main circulating androgen. Female teleosts also produce testosterone, at times circulating levels may be as high as that of estradiol. Telesosts also produce a range of progesterone-like molecules that cause final oocyte maturation and ovulation, although in some teleosts the corticosteroid deoxycorticosterone has the same function. Female amphibians also have high levels of circulating androgens as well as estrogens during the reproductive portions of their lives. One major difference is found in teleosts (bony fishes), which have a third estrogen receptor (ER-γ; cf. the two found in mammals (ER-α and ER-β)).

The endocrine system plays an essential and all-pervasive role in the regulation of metabolic processes. In other words, this is a system that works with the nervous system, reproductive system, kidneys, gut, liver and fat to help maintain and control body energy levels, and the processes of reproduction, growth and development, homeostasis, and responses to stress, surroundings, and injury. To function normally, the body needs glands that work correctly, a blood supply that works well to move hormones through the body to their target points, receptor places on the target cells for the hormones to do their work, and a system for controlling how hormones are produced and used.

The fundamental role of all endocrine systems is to enable the coordinated response of one tissue to signals originating in either another organ or, in some cases, cues originating outside the body. For most endocrine systems, the primary objective is to maintain homeostasis, avoiding wild swings in hormone levels or responses (e.g. the maintenance of blood glucose levels by insulin). To a large extent all endocrine systems operate on a ‘seesaw’ principle, in which target cells send feedback signals to the regulating cells. If the feedback is negative, secretion of the hormone is altered (usually reduced). However, there are usually refinements to this simple scenario that enable all the body’s endocrine systems to be integrated such that organism age, reproductive status, nutritional status, and stress levels are able to override other endocrine systems when danger threatens. This is vital for maintenance of good health.

The homeostatic balance must be set (or programmed) before the system will work correctly. The setting up of the endocrine axes takes place largely during foetal/neonatal development, during which time feedback sensitivity of the hypothalamus and pituitary gonadotropes to steroids from the gonads is established. At the same time, the male and female feedback centres are established. The "wiring" of the male and female hypothalamus must be induced during development to ensure the pituitary gland of an adult responds the way it should, i.e. female as in a female, not as in a male. Testosterone produced during the foetal or neonatal stage plays a role in programming the development of a 'male' hypothalamus and brain, and administration of testosterone to a female during this critical programming period may result in masculinisation of the hypothalamus within knock-on effects in later life.

It is important to note that the ecotoxicological paradigm for effects due to natural steroid hormones as pollutants of waterways is not one of bioaccumulation or bioconcentration through food chains (as has historically been the concern with most pollutants), rather one of inappropriate exposure, i.e. exposure to higher than required concentrations of hormone at critical life stages. This has been seen in humans with some pharmaceuticals, e.g. thalidomide, desethylstilbestrol, and appears to be true in the wild in fish and reptiles. This concept can be further explained by looking at sex differentiation in mammals.

Before sex differentiation, the mammalian embryo has the potential to develop into a male or female phenotype. Following gonadal sex differentiation, perinatal testosterone secretion by the testis is responsible for masculinisation of the body in general. Females avoid developing as a male by not switching on secretion of testosterone in the ovary. The central role of testosterone in masculinsation has two important implications: [8]

1. If a genotypic male fails to secrete sufficient testosterone, it will not masculinise and may develop as a phenotypic female (but with testes).
2. If a genotypic female is exposed to sufficient testosterone (or other androgen), it will masculinise and may develop as a phenotypic male (but with ovaries).

These are not always all or nothing scenarios. Partial masculinisation, or partial failure of masculinisation, can also occur. Perhaps the most important aspects of these, and other imprinting/programming changes is their irreversibility, leading to perhaps the greatest concern about environmental endocrine disruptors, namely that exposure to an agent during perinatal life can result in permanent adverse or abnormal change.

- The exposure does not need to be long term (chronic), simply sufficient, i.e. short-term sub-lethal exposure at a critical time during development.

While vertebrates have the ability to both produce and metabolise the natural steroid hormones, complete or partial sex reversal has been observed when the eggs, larvae or juveniles of non-mammalian vertebrates are exposed to androgens or estrogens. Androgens usually inhibit female duct (Mullerian) development while enhancing male duct (Wolffian) development, while estrogens typically do the reverse. Estrogens stimulate the synthesis of ovalbumin protein in birds, and the synthesis of vitellogenins in adult female vertebrates that produce yolky eggs, i.e. reptiles, birds, amphibians and fish. If adult male fish are exposed to estrogens, they can be induced to produce vitellogenins. Thus, plasma vitellogenins can be used as a biomarker for exposure to environmental estrogens.

Adapted from:

WHO/IPCS (2002). Global assessment of the state-of-the-science of endocrine disruptors. Edited by: Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. World Health Organisation / International Program on Chemical Safety. Available on-line: www.who.int/ipcs/publications/new issues/endocrine_disruptors/ en/index.html

Endo 101 (2006). Endo 101: the endocrine system. The Hormone Foundation. Available on-line: www.hormone.org/endo101/

Stryer L (1995). Biochemistry. 4th Ed. W.H. freeman & Co., New York.

- Exposure of marine gastropods to TBT from marine anti-fouling paints causes a masculinsation of female gastropods, and ultimately population decline. [15]
- The observation of feminised male fish near sewage outlets in several UK and German rivers. [16, 17]

The effluent from municipal wastewater treatment plants (WWTPs) was at first considered the source of much of the EDC input into aquatic environments. [18, 19] However, more recently attention has turned towards other sources of hormonally active contaminants, including run off from confined animal feeding operations (CFOs), and extensive dairy and beef agriculture (as a source of naturally occurring hormones such as estradiol and testosterone, as well as hormonally active veterinary medicines (VetMeds)). [20, 21, 22]

Endocrine disruption is a very contentious issue for waterway managers, as science plays catch-up with community concern. Estrogens, such as the estradiol found in dairy effluent, can affect fish development and behaviour at extremely low concentrations (ng/L, i.e. a million times lower than the concentrations of nutrients that cause eutrophication, and a thousand times lower than the levels at which pesticides exert their effects), but as yet there have been no studies of hormonal inputs into creeks and rivers from Victorian agricultural systems.

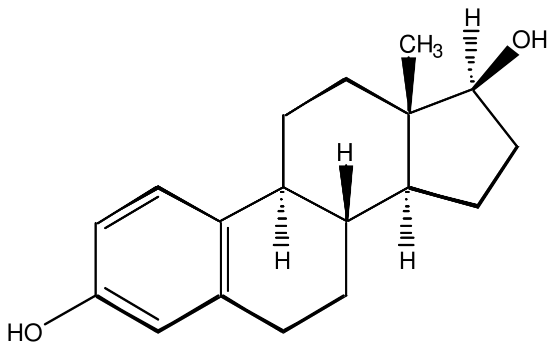
This review builds on a statewide investigation of hormones in recycled water funded by the Victorian Water Trust (Project# 33V-4000), which highlighted the need to investigate other sources of hormones entering the environment as a prerequisite to any ecological study of the effect of WWTP discharges on fish in Victorian aquatic environments. [23]

2.2 Steroid hormones from livestock

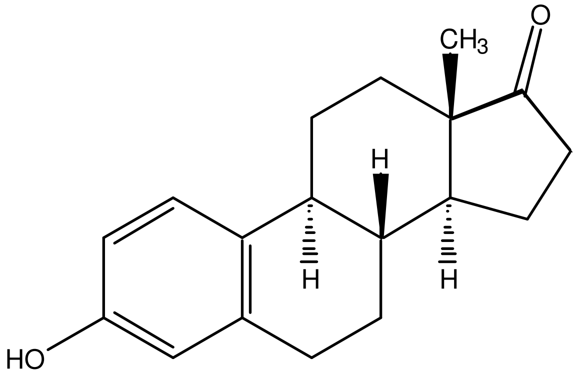
Hanselman et al produced the first major review of manure-borne estrogens as potential environmental contaminants, concluding that steroidal estrogen hormones (i.e. estradiol, estrone, and estriol) are a particular concern because of the evidence that low ng/L concentrations of estrogens in water can adversely affect the reproductive biology of fish and other aquatic vertebrate species. [20] Since then Johnson et al and Khan et al have also reviewed this topic. These papers provide the foundation for this review, updated to include recent studies not covered by those authors. [21, 22]

Hanselman et al, Johnson et al, and Khan et al all report that steroidal estrogen hormones are excreted in the urine and faeces of all livestock species, but that different estrogens (chemical species) are excreted by different livestock species. [20, 21, 22] For instance, Hanselman et al describes how cattle (*Bos taurus*) excrete more than 90% of their estrogens as both free and conjugated metabolites of estradiol (both 17α-estradiol and 17β-estradiol; ratio ~ 3:2) and estrone, whereas pigs (*Sus scrofa*) and poultry (*Gallus domesticus*) primarily excrete 17β-estradiol, estrone, and estriol (again as both free and conjugated metabolites). [20]

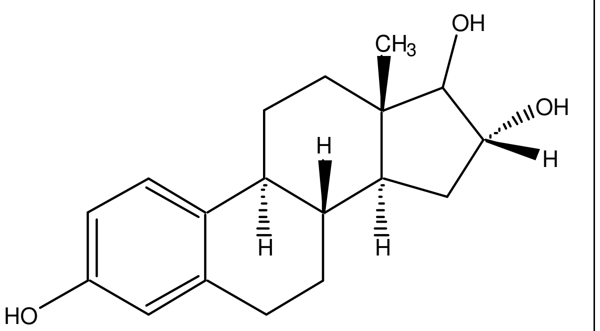
- The stereochemical distinction between α and β of estradiol provides a mechanism for identifying the livestock species contributing to contamination of water ways (i.e. cattle *vs.* poultry *vs.* swine). [20, 22]
- Conjugated estrogens are analogous in structure to estradiol, estrone, or estriol, except that a sulphate or glucuronide group is substituted onto parent compound (e.g. 17β-estradiol-3-sulphate, 17 β -estradiol-17-sulphate, 17 β -estradiol-3,17 disulphate).



17β-estradiol (E2)

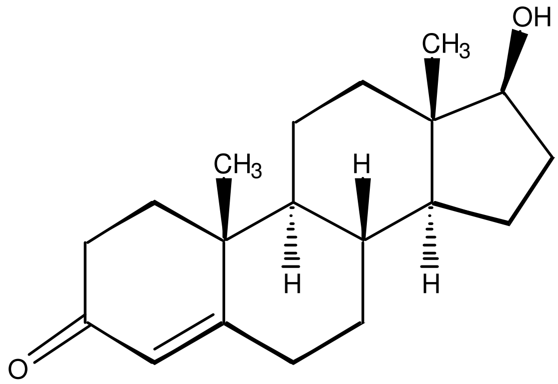


estrone (E1)

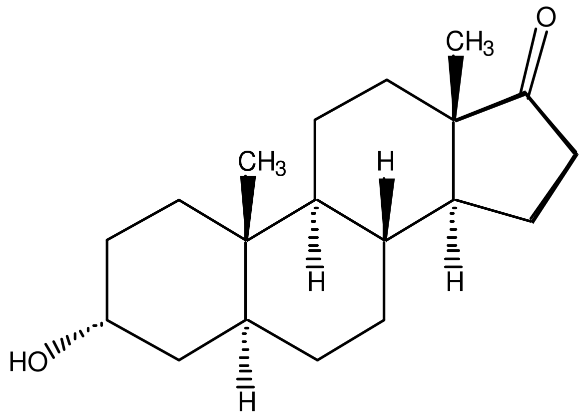


estriol (E3)

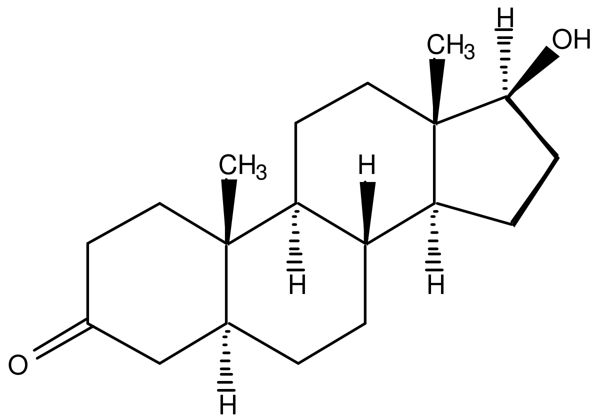
Figure 2.1 Chemical structures of major female sex hormones (estradiol, estrone and estriol).



testosterone



androsterone



dihydrotestosterone

Figure 2.2 Chemical structures of major male sex hormone (testosterone), and its major metabolites (androsterone, dihydro-testosterone).

An in depth discussion of conjugation is beyond the scope of this review, but in short conjugation typically makes the molecule more water soluble (and hence easier to excrete in the urine).

Hanselman et al and Johnson et al both report that different species excrete estrogens by different routes. [20, 21] For instance, cattle excrete estrogens mostly in their faeces (~60%), whereas swine and poultry excrete estrogens mostly in urine (96% and 69%, respectively). At first glance, the route of excretion may not seem an important environmental consideration since urine and faeces are not usually handled separately in commercial animal production systems. However, in the urine estrogens are mostly observed as conjugates, whereas faecal estrogens are excreted as unconjugated 'free' steroids.

- Conjugated estrogens are considered to be relatively biologically inactive (cf. free estrogens). However, deconjugation can release the more highly biologically active free estrogens.
- At present, the environmental significance of conjugated *vs*. unconjugated estrogens in aquatic ecosystems is unclear because of a lack of information regarding conjugate fate in soils and water.

Hanselman et al [20] suggested that the data compiled for their review could not be used to accurately calculate the total mass flux of estrogens to the environment from whole populations of cattle, swine, or poultry because the data had been produced for other reasons, e.g. for identifying pregnancy in cows, for describing the patterns of hormonal changes that occur during estrus and pregnancy as part of programs designed to produce new ways to control these processes, and because ambiguous quantification methods had been used across the three decades over which information had been produced.

Species	USA		
	head (million)	estrogens (tons)	androgens
cattle	98	26	1.9
pigs	59	3.0	0.35
sheep	7.7	1.3	-
chickens	1816	2.8	2.1

Species	European Union		
	head (million)	estrogens (tons)	androgens
cattle	82	26	4.6
pigs	122	3.0	1.0
sheep	112	1.3	-
chickens	1002	2.8	1.6

5.1 Estimated yearly steroid hormone excretion by farm animals in the USA (top) and European Union (bottom; 2000 figures); from Lange et al. [24]

Hanselman et al did, however, use their collated contaminant information to report that the concentration of 17β-estradiol ranged from below detectable limits (BDL) to 239 ± 30, BDL to 1215 ± 275, and up to 904 µg/kg in dairy, swine, and poultry wastes, respectively. [20]

Lange et al used essentially the same data as Hanselman et al to calculate estrogen excretion for various livestock species, suggesting that cattle, pigs, sheep and chickens contribute 49 tons of estrogens per year in the USA (2000 figures; Table 5.1). [24]

Shore and Shemesh reported that slurry from milk cows contains up to 500 times more estrogens than slurry from bulls (up to 1230 cf. <2 µg/kg 17β-estradiol, and up to 640 cf. <2 µg/kg estrone, respectively).[25]

- Pig slurry contains up to 64 µg/kg 17β-estradiol (similar amounts of estrone).
- Chicken manure contains up to 533 and up to 670 µg/kg estrogen and testosterone, respectively.

Irwin et al suggested that manure production in the USA is ~112 million tons each year, and that the presence of estrogenic compounds in ponds located on beef breeding farms is likely given the large numbers of pregnant cattle maintained at such facilities.[26]

The distinction between pregnant and non-pregnant animals is as important when assessing steroid hormone transport from agriculture. Shore and Shemesh noted that pregnant cattle excrete higher amounts of steroid hormone levels than non-pregnant female or male cattle with pregnant cattle showing a gradual increase in daily excreted estrogens (from 9.0 µg/kg in fresh manure in the 100 days before parturition, to ~19 µg/kg in fresh manure 30 days before parturition, to ~60 µg/kg in fresh manure 5 days before parturition).[25]

Johnson et al modelled the potential contribution of livestock to steroid hormone concentrations in the UK's rivers, concluding that the combined farm animal population in the UK is considerably larger than the human one, and consequently livestock are an important contributor to the environmental load of steroid hormones entering the UK's water bodies.[21] To make comparisons on the amount of steroid hormones produced by the different livestock, Johnson et al gathered information on the structure of the UK's farm animal populations, and the amount of hormones excreted by animals at each of their life stages. They reported that:[21]

- The biggest contributor on the animal side is the relatively small dairy cow population (2.2. million head (2004) at ~ 365 kg/year 17β-estradiol; Table 5.2).
- The UK's pig population (~ 5 million head) contributes a lesser amount of 17β-estradiol (~180 kg/year; Table 5.2).

Dairy cows	E1	βE2
	(µg/cow/d)	
Faecal excretion	51	148
Urine excretion	787	236
Combined total	838	384
	(kg/d)	
UK dairy herd	1.9	1.0

Pigs	E1	βE2
	(µg/pig/d)	
Cycling faecal excretion	12	9
Cycling urinary excretion	82	<DL
Cycling combined value	94	9
Pregnant faecal excretion	33	28
Pregnant urinary excretion	1400	ND
Pregnant combined value	1433	28
	(kg/d)	
UK pig herd	1.0	0.05

Organism	ΣE1 + E2 kg/y	(%)	βE2 kg/y
Humans	365	(17)	146
Dairy cattle	1058	(49)	365
Pigs	386	(18)	19
Broiler chicken	49	(2)	34
Laying hens	260	(12)	-
Breeding ewes	25	(1.9)	6
Non-breeding sheep	2	(0.1)	0.4
Total farm animals	1780	(83)	424
Total (all)	2145	(100)	570

Table 5.2 Estimated rates of excretion of estrone and estradiol by dairy cattle (top) and pigs (middle) and other organisms (including humans; bottom) in the UK (2004); DL, less than detection limits; ND, not detected; adapted from Johnson et al.[21]

- The UK's chicken population (broilers and laying-hens) generates 260 kg total estrogens per year.
- The UK's sheep population (~8 million head, including 6.5 million breeding ewes and 1.5 million rams and non-breeding females) generate 136 kg total estrogens per year

Johnson et al suggest that in terms of excretion, the combined farm animal population (1780 million head, including cows, pigs, poultry and sheep) probably generates around four times more estrogens than the U.K.'s human population (59 million; Table 5.2). [21]

- An individual normalised dairy cow excretes two orders of magnitude more steroid estrogens than a normalised human.

Johnson et al concluded that if the steroid estrogens behave like herbicides, for which in the UK a worst case loss to surface waters is considered to be around 1%, then it could be argued that farm animals are responsible for 15% of all the estrogens in UK waters. [21]

Hanselman et al, Johnson et al, and Khan et al all reviewed the environmental fate of free and conjugated steroid hormones in manures and the environment, reaching similar conclusions. [20, 21, 22]

- The biological activity of free (unconjugated) estradiol is rapidly dissipated in soils, primarily as a function of 17β-estradiol being rapidly converted to estrone by a wide range of micro-organisms.
- Estrogens, being non-volatile, slightly hydrophobic compounds that do not ionize at typical environmental pH, should be extensively sorbed by soils and sediments. [20]

Laboratory based experiments generally confirm these conclusions, but since they have typically been undertaken using pure compounds in the absence of the wide range of other compounds present in manures, may underestimate the risk of contamination of water adjacent to agricultural soils treated livestock wastes. [20, 21, 22]

Hanselman et al, Johnson et al, and Khan et al also note that field studies with manure have demonstrated that estrogens are sufficiently mobile to impact surface and groundwater quality. [20, 21, 22] These reviewers suggest that because sorption is typically evaluated without additions of manure, the information gained does not (a) allow assessment of the effects of the chemical, physical, and microbiological changes that can occur in a soil following manure application, nor (b) allow that natural surfactants might increase the mobility of estrogens in soils (which together with erosion and preferential flow mechanisms could lead to the transport of manure borne estrogens to waterways).

Further conclusions of Hanselman et al, Johnson et al, and Khan et al's reviews are:

- Rapid biodegradation of estrogens occurs in river water. Jurgens et al report that the half-lives of estradiol and estrone at 20 °C are reported in the range 0.2-9 and 0.1-11 days, respectively. [27]
- Some microorganisms metabolise steroidal estrogens. For instance, Lai et al report that estradiol and estrone are readily transformed via oxidative and reductive pathways by 17β-hydroxy-steroid dehydrogenase in a range of microorganisms, including those that do not produce estrogens, such as fungi and bacteria. Moreover, the common freshwater algae *Chlorella vulgaris* is capable of oxidizing 17β-estradiol to estrone. [28]
- Unlike unconjugated (free) estrogens, the fate of estrogen conjugates is not

clearly known, in either manures or natural waters. [20, 21, 22]

2.3.1 Confined animal feeding operations

The concentration of androgens (e.g. testosterone), estrogens (e.g. estradiol) or both in agricultural runoff from confined animal feeding operations (CFOs) has been little studied (cf. those in WWTP effluents). Finlay-Moore et al suggest that there were only three studies prior to 2000. These included: [29]

- Shore et al who measured estrogens and androgens in ponds and streams receiving runoff from fields fertilized with chicken litter. [30] Pond concentrations reached 28 and 34 μg/L of estrogens testosterone, respectively, while stream concentrations reportedly reached 5 and 28 ng/L of estrogens and testosterone, respectively. [30]
- Nichols et al suggest that the concentration of estradiol in runoff following broiler litter is application rate dependent, but can be as high as 1,280 ng/L. [31]
- Nichols et al, in a follow up study, suggested that an 18 m grass buffer strip can reduce the concentration of estradiol in runoff from tall fescue (*Festuca arundinacea* Schreb.) plots amended with poultry litter by 94%. [32]

Finlay-Moore et al [29] studied the effect of grazing by cattle on runoff from broiler litter amended grasslands. Finlay-Moore et al noted that grazing animals did not appear to contribute hormones to the runoff (no significant difference between grazed and non-grazed paddocks), finding up to 150 ng/L estradiol, and up to 125 ng/L testosterone in background runoff, respectively. When broiler litter was applied to the pasture, Finlay-Moore et al found up to 2530 ng/L estradiol, and up to 1830 ng/L testosterone in runoff, respectively. [29]

Peterson et al reported that 17β-estradiol had been observed in spring water from mantled karst aquifers in agricultural areas of northwest Arkansas, USA, following winter recharge. [33] Estradiol concentrations were in the range 6 – 66 ng/L, and correlated well with changes in *E. coli* in the springs, suggesting that contamination of aquatic ecosystems with endogenous hormones is not limited to surface waters.

Irwin et al assessed the levels of xenoestrogens in ponds at Clemson University Simpson Agricultural Station, near Pendleton, South Carolina, receiving effluent from beef cattle farms, and found up to 7.4 ng/L free estradiol in the ponds. [26]

Soto et al tested the hypothesis that CFOs release significant amounts of natural hormones, anabolic steroids, and their metabolites into the environment by measuring total androgenic and estrogenic activity (using the A-SCREEN and E-SCREEN bioassays), and the concentrations of anabolic agents (using GC-MS and ELISA) in water samples collected over a 3 year period from six sites confluent with the Elkhorn River, Nebraska, USA. [34] These sites included a feedlot retention pond, a site downstream from the feedlot retention pond, a stream with intermediate livestock impact, three sites with no observable livestock impact, and two sources of potable water. The estrogenic activity of the site immediately downstream of the effluent holding pond had significantly higher androgenic and estrogenic activity than the non-impacted sites (e.g. ~4 cf. ~2 pM androgen equivalents (AeQ), and ~2 cf. <1 pM estradiol equivalents (EEQ), respectively). The potable (tap) water had no hormonal activity. [34]

Raman et al quantified estrogen concentrations in swine and dairy waste treatment and storage structures considered typical of dairy facilities in the eastern United States and swine facilities in the south-eastern and western United States. [35] Estrogen (estrone, 17α-estradiol and 17β-estradiol) concentrations ranged from below detection levels to over 140 μg/kg (wet weight basis; Table 5.3(a)), and varied with animal type, with storage structure (Table 5.3(b)), and measurement basis. For instance, on a wet weight basis the highest estradiol concentrations were observed in swine finishing hoop structures, with the highest estrone concentrations observed in swine finishing hoop structures, in swine farrowing pit slurries, and in dairy dry-stack semisolids. The highest concentrations of 17α-estradiol, were observed in dairy dry-stack semisolids. In contrast, on a dry weight basis the highest estradiol and estrone concentrations were observed in swine farrowing pits, reflecting the relatively low total solids content of the swine waste in the pits (~1-3%). [27]

Kolodziej et al measured a range of estrogens, androgens and progestins in samples from dairy farms, aquaculture facilities, and surface waters with actively spawning fish to assess the potential importance of these sources of steroid hormones to surface waters. [36] The authors included aquaculture facilities because they considered that in the USA aquaculture represents another rapidly growing intensive agricultural operation that could serve as a source of steroid hormones to surface waters.

- Fish excrete free and conjugated steroids, and the wastewater treatment practices employed by most aquaculture facilities are unlikely to remove steroid hormones.

Facility	Estrogen		
	17β	estrone	17α
	μg/kg (wet weight basis)		
SFIL	4	10	4
SFIH	40	55	4
SFAL	5	8	3
SFAP	18	58	8
DDM	22	48	84
DDS	12	30	28
DHP	2	8	4

5.3(a) Estrogen concentrations in manure at dairy and swine facilities in the USA (estimated from author figures) [35]

- SFIL, swine finishing lagoon; SFIH, swine finishing hoop structure; SFAL, swine farrowing lagoon; SFAP, swine farrowing pit; DDM, dairy dry-stack semisolid; DDS, dairy dry-stack solid; DHP, dairy holding pond.

Facility	Emission factors		
	17β	estrone	17α
	mg/day/ (1000 kg live animal)		
SFIL	3.5	14	<BDL
SFIH	2.5	3.4	<BDL
SFAL	9.7	16	<BDL
SFAP	8.2	28	<2.0
DDM	2.9	7.0	8.7
DDS	1.2	2.6	1.9
DHP	1.1	3.3	2.5

5.3(b) Estrogen emissions from dairy and swine facilities in the USA; adapted from Raman et al. [35]

Kolodziej et al suggest that if, after taking into account difference in size, the excretion rates of steroids from fish are comparable to excretion rates for livestock or humans.

Consequently, the steroid discharge from a typical US aquaculture operation (i.e., 50-200 tonne of fish) might resemble the steroid production of a cattle herd of several hundred animals or a wastewater treatment plant serving several thousand people. [36]

The hypotheses put forward by Kolodziej et al are supported by Barel-Cohen et al, who found evidence of elevated estrogen (2.4 ng/L), and testosterone (4.8 ng/L) in a stretch of the Lower Jordan River which received effluent discharges from aquaculture ponds. [37] Clearly, if fish in aquaculture facilities are releasing steroid hormones, then it is not unreasonable to think that steroid hormones will be released by wild fish in surface waters, especially before and during periods of reproduction. Indeed, Kolodziej et al suggest that in many rivers along the west coast of North America during low flow conditions, concentrations of steroid hormones may have been due solely to the presence of the spawning salmon. The authors suggest that such releases of steroid hormones, which are natural parts of the reproductive cycles of certain species of fish, may not necessarily be problematic for aquatic organisms, but they could complicate efforts to assess contributions and effects of steroid hormones from agriculture and wastewater discharges. [36]

Kolodziej et al found 17β-estradiol (~10 ng/L), estrone (650 ng/L), testosterone (~100 ng/L), androstenedione (~110 ng/L), in a dairy waste lagoon at a farm in the north-eastern San Joaquin Valley of central California. Steroid hormones were detected in ~25% of shallow groundwater samples, although at much lower concentrations than in the waste lagoon. [36] Steroids were sporadically observed in the surface waters, usually at concentrations near or below 1 ng/L. The authors reported 17β-estradiol, estrone, testosterone, and androstenedione in the raceways and effluents of three fish hatcheries that discharge to the Mokelumne and Nimbus Rivers, but at concentrations near 1 ng/L. Similar concentrations were detected in the American River near a sand bar containing spawning adult Chinook salmon. [36]

- Kolodziej et al suggest that these results highlight that even spawning fish can lead to detectable concentrations of steroid hormones in surface waters. Moreover, the concentrations of these compounds in dairy wastewater exhibit, considerable temporal and spatial variation. [36]

Stuer-Lauridsen et al found that the water in half of the samples they collected in drains from Danish fields where manure had been applied in the spring of 2004 was estrogenic, although the levels of total estrogens were low (up to 1.1 ng/L EEQ, and attributed primarily to estrone). [38]

- Stuer-Lauridsen et al also observed slightly elevated levels of estrogenic activity downstream of fish farms (~0.9 ng/L EEQ cf. upstream ~ 0.6 ng/L EEQ). [38]

Little is known concerning the chemical make-up and potential ecological effects of androgenic substances associated with discharges from animal feeding operations. Durhan et al suggest that insights may be gained through consideration of chemicals used for livestock production. [39] For instance, in the USA, much of the beef production openly utilizes anabolic androgens to promote production of muscle mass in the animals. Trenbolone acetate is one of the most commonly used chemicals for this purpose. Durhan et al suggest that two metabolites, 17α- and 17β-trenbolone, are relatively stable in animal waste and in the environment. Both are relatively potent androgens in fish and mammals. [39]

Durhan et al evaluated the occurrence of 17α- and 17β-trenbolone in river water upstream and downstream of a beef cattle feedlot discharge, compared with the discharge itself. Whole-water samples from the discharge contained measurable concentrations of 17α- and 17β-trenbolone (up to 120, and 20 ng/L, respectively). The river water samples also contained measurable concentrations of 17α- and 17β-trenbolone, but at much lower concentrations. [39]

Shappell et al measured the hormonal activity of the effluent from the swine farrowing facility at North Carolina Agricultural & Technical State University, Greensboro, North Carolina, and the effectiveness of a lagoon-based constructed wetland in reducing the concentrations of steroid hormones. [40] At the time of the study (2004-05), the NCU swine farrowing system housed ~100 sows and 15 boars. The manure-handling system comprised a manure pit, primary, secondary lagoon, and anaerobic lagoons, four parallel 40 × 11 m constructed wetland cells, and a storage pond. Manure was flushed through the barns with "gray" water to the lagoons and thence to the wetland cells. Influent and effluent samples were collected weekly in July 2005, and estrogenic activity and the concentration determined by E-screen and LC-MS/MS. [40]

Shappell et al report that estrogenic activity in the manure pit averaged 850 pM (~ 255 ng/L) EEQ, whereas the activity in the primary lagoon was only 9 pM (~2.7 ng/L) EEQ, or ~1% of the pit activity. By the time the wastewater reached the wetlands estrogenic activity had decreased further to 3 pM EEQ (~ 0.7 ng/L). Mean estrogenic activity of wetland effluent was 7% of the influent activity. [40]

Shapell et al report that during a time of higher than usual influent estrogenic activity (~ 100 ng/L) the wetlands were able to reduce the estrogenic concentration to 8.7 ng/L E2, highlighting the potential of constructed wetlands, as part of a properly functioning treatment train, to polish effluents to below the lowest estradiol concentration at which intersex has been observed in fish (medaka), [41] although not below the lowest level of estradiol at which physiological effects have been observed in fish (1 ng/L reduced semen volume, sperm density and sperm fertility in rainbow trout). [42]

In a follow up study to that of Soto et al, [34] Kolok et al recently deployed polar organic chemical integrative samplers (POCIS passive samplers) in two waterways considered likely to be impacted by runoff from cattle feeding operations, namely Fisher Creek and Sand Creek at their confluence with the Elkhorn River in Nebraska, USA. [43] The POCIS extracts contained a number of estrogens and androgens, and their metabolites. For instance, up to 8.8 and 12.9 ng/L of estradiol and estrone, respectively. Moreover, of the androgens, up to 1.8 and 21 ng/L of testosterone and androstenedione, were observed respectively, and of the progestins, up to 4.2 and 2.6 ng/L of progesterone and melengestrol acetate (a growth-promoting gestagin), respectively. [43]

- Chemicals extracted from passive samplers provide a semi-quantitative time-weighted average concentration indicative of the relative levels of substances that aquatic organisms may be exposed to. Passive samplers can be calibrated in the laboratory to provide quantitative measurements, although there are many physico-chemical variables that can affect actual recovery in the field. That said, passive samplers are a useful tool when evaluating the occurrence of trace organic compounds in natural waters, and the potential for exposure (e.g. Matthieson et al [7]).

Zheng et al measured a range of steroid hormones (17α- and 17β-estradiol, estriol, estrone, progesterone, medroxy-progesterone) in wastes from a 3000 head dairy farm located in San Jacinto, California. [44] Three estrogens were detected in the dairy wastewater and waste lagoon water (17α-estradiol (~2750 ng/L), 17β-estradiol (~2250 ng/L), and estrone). Levels of these compounds in the dairy's treatment lagoons were several orders of magnitude lower, highlighting the attenuation capacity of lagoons. The authors suggest this attenuation is attributable to dilution, biodegradation, photodegradation, sorption, residence time, and settling of hormone-associated manure particles. [44]

Australian and New Zealand studies assessing hormonal contaminants in CFO effluent are somewhat lacking, with only two papers specifically discussing this issue.

Sarmah et al measured the concentration of natural estrogens (17β-estradiol, 17α-estradiol, estrone, and estriol) in representative animal wastes in the Waikato region of New Zealand. [45] The dairy farm effluents had high levels of estradiol (19–1360 ng/L) and breakdown product estrone (41–3123 ng/L). The piggery effluent had lower total estrogen load (46 ng/L), with estrone accounting for nearly 60% of the estrogenicity. [45]

Khan et al's review provides something of a ready introduction to the feedlot industry in Australia. For instance, Khan et al suggest: [22]

- Commercial feedlots are a major method of finishing beef cattle in preparation for slaughter in Australia. Cattle entering feedlots at 12–24 months of age, where, depending on their intended use, they may be fed for 60 to 400 days while gaining about 100–350 kg in weight.
- The main by-products from cattle feedlots are manure (harvested from the surface of the pens), and liquid effluent (collected during rainfall runoff events).
- A typical animal entering a feedlot initially produces ~20 kg of manure per day, increasing to up to ~36 kg manure per day (for a heavy finished animal of 600 kg).
- Fresh manure, which comprises of faeces and urine, is normally composed of around 90% water and 10% solids.
- From the point of manure deposition on the pad, through stockpiling, to eventual removal from the feedlot site, there are opportunities for the transport and/or transformation of the chemical components of the manure. Transport routes include runoff (e.g. from the impermeable surfaces of pads) to drains and streams,, and leaching through soil to groundwater.

Khan et al identified a number of important classes of trace chemicals present in beef feedlot effluents by including steroidal hormones, antibiotics, ectoparasiticides, and mycotoxins. Of these compounds, Khan et al concluded that: [22]

- Few of these chemical contaminants have been thoroughly investigated in terms of concentrations, effects and attenuation in Australian feedlot wastes.
- When assessing steroid hormone transport from Australian feedlot operations, natural and synthetic androgens are a more important target than the estrogens (because the feedlots primarily stock male cattle rather than lactating cows).
- Natural and synthetic androgenic hormones, such as testosterone and

trenbolone, are significantly active in feedlot wastes, but they are poorly understood in terms of environmental fate and impact.

- The careful management of antibiotic residues is prudent, to minimise the risk of potential public health impacts from resistant strains of bacteria.
- Good management of ectoparasiticides is important for the prevention of potential ecological implications (e.g. towards dung beetles).

Khan et al essentially reached the same conclusions as Hanselman et al four years earlier, and Allinson et al in 2007, i.e. that livestock wastes are a potentially important, but poorly understood, source of estrogenic and androgenic compounds in the environment that requires further investigation. [20, 22, 23] For instance, dairy wastes are strongly estrogenic due to the presence of a number of the same estrogenic steroidal hormones as WWTP effluents, e.g. estradiol. Conversely, discharges from beef cattle feedlots are typically androgenic in nature.

2.3.2 Extensive agriculture – dairy and beef

There have been few studies of steroid transport from non-intensive beef and dairy farming systems, with most work in this area, i.e. looking at the occurrence of steroid hormones in agricultural watersheds, focussing on estrogenic steroids derived from CFOs, and the land application of their manures and/or discharges from waste storage lagoons. This is perhaps surprising, since there are a small number of studies that suggest estrogen contamination from extensive grazing systems may be widespread, with direct discharges of animal wastes to receiving waters a significant source of contamination (e.g. see Johnson et al). [21]

Stuer-Lauridsen et al reported the free and total estrogenic activity of various sub-categories of the aquatic environment in Denmark, including streams and rivers (general), streams and rivers in husbandry areas (areas with high densities of pigs and/or cattle), and reference streams (un-impacted streams considered to reflect background conditions). [38] Stuer-Lauridsen et al noted that the estrogenic activity in streams and rivers in husbandry areas was low, but slightly more than streams and rivers in general (~ 0.3 ng/L total estrogens cf. 0.2 ng/L), but 5 × the estrogenic activity of reference sites (~0.3 ng/L total estrogens cf. ~0.06 ng/L). [38]

Tarrant et al assessed the risks posed by environmental estrogens to the Irish freshwater environment by quantifying the levels of estrogenic activity at sites on the Liffy, Lee and Brandon Rivers and various lakes in Ireland using the YES *in vitro* bioassay. [46] The work was focussed on estrogens from WWTPs, with effluent, upstream and downstream measurements being taken. Receiving waters upstream of WWTPs were estrogenic, with activity in the range of 0.9–2.9 ng/L EEQ. The background levels of estrogens reported for Lee headwaters and Lough Barfinnihy were up to 1.1 ng/L, highlighting that low levels of estrogenic activity may often be present in surface waters in isolated regions where anthropogenic activity is minimal. [46]

When Tarrant et al used a chemical-based deterministic model to predict the estrogen levels of WWTP effluents the model worked well (good correlation between observed and predicted estrogenic activity), but the model under-estimated estrogenic activity in receiving waters. The under-estimation was greatest for those rivers with catchments dominated by intensive livestock agriculture, and Tarrant et al suggest that the differences could be accounted for by the estrogenic input (runoff) from livestock (dairy) agriculture.[46]

In 2006, Matthiessen et al investigated the contamination of headwater streams in the UK by estrogenic hormones from livestock farms. Most of the 10 sites selected (from south-west, north-west and central southern England, and south-east Wales) were streams running through dairy farms, although some sites had beef, sheep and pigs.[7] Matthiessen et al deployed passive samplers (specifically the POCIS system) up-stream and down-stream of the farms for up to 10 weeks in the late Autumn-early Winter of 2004/05. Average estrogenic activity (YES assay) across all sites was in the range 0–26.5 ng/EEQ (with one exception, a site not specifically linked with livestock agriculture with an activity 292 ng/L EEQ). Estrogenic activity and the specific concentration of steroid hormones were higher downstream of farms in 50 and 60% of cases, respectively. Estrone and 17β-estradiol were almost ubiquitous in the streams (estrone, 0.1-9.31 ng/L; 17β-estradiol <BDL – 0.89 ng/L).[7]

Kolodziej and Sedlak assessed the contribution of rangeland grazing areas in the western United States to steroids in associated surface waters.[47] Their samples were collected from 30 sites in Stanislaus, Marin, and Sonoma counties in central California, with stream sites considered representative of the many small headwater creeks found in Californian watersheds, where cattle grazing is the predominant land use. Steroids were detected in 86% of samples from creeks where cattle had direct access to the water.[47]

Kolodziej and Sedlak reported that estrone was the most frequently detected estrogen (78% samples, up to 38 ng/L), followed by 17α-estradiol (31% samples, up to 25 ng/L), then 17β-estradiol (18% samples, up to 1.7 ng/L). Androgens, were also detected, but less frequently e.g. testosterone (11% samples, up to 2.3 ng/L) and androstenedione (18% samples, up to 44 ng/L). Progesterone was detected in 5% of stream samples (up to ~ 28 ng/L), with medroxyprogesterone detected only once (but below quantification limit).[47]

Kolodziej and Sedlak suggest that in 10-20% of their samples estrogens were present at concentrations above the predicted no-effect concentrations for fish, and androstenedione was detected at concentrations higher than response thresholds for pheromonal communication in fish.[47]

- Kolodziej and Sedlak conclude that simple measures, such as fencing in rangeland areas to limit direct access of stock to streams (and hence discharge of animal wastes to surface waters), or better manure management practices could, in certain cases protect ecosystem health.

In 2007, Williams et al undertook a pilot study on estrogenic compounds in South Australia, the ACT, and Queensland environments, including in streams and drains associated with dairy farming, stock grazing and horticulture.[48] The concentrations of estrone and 17β-estradiol observed were in the low ng/L range. For instance, estrone concentrations ranged from 0.17 ng/L in a national park to 38.5 ng/L in the effluent from a dairy milking shed. Similarly, 17β-estradiol concen-trations ranged from 0.52 ng/L in the national park to 8.6 ng/L in the milking shed effluent.

- Wiliams et al concluded that livestock sources contribute to the estrogenic load in Australian rural streams.[48]

2.4 Risk

One of the first questions asked by land and water ways managers, policy makers and the community when scientists report water quality data is, "*so what*?" This question can be less colloquially put as, *"what is the likely environmental impact of the contaminant (in this case, current / future discharges of hormones in CFO) effluent run-off in receiving waters?"* This question is often asked when discussing organic micro-contaminants, such as pesticides and the steroid hormones discussed above, in part because stakeholders often believe they understand the impacts of changes in common water quality parameters (e.g. nutrients, salinity), or are at least familiar with these parameters, but are unsure of the effects of very low levels of unfamiliar contaminants. In part the question reflects a reluctance to investigate issues with unknown outcomes (and hence unknown reactions and investment needs).

The *"so what?"* question is a difficult question, but can be addressed through risk assessment exercises, and field monitoring (with the former not an excuse not to do the latter).

Typically, in a first-pass environmental risk assessment one first looks to agreed national or international guideline or trigger values for the type of waters being assessed. Currently, there are no guideline or trigger values for steroid hormones in natural waters. Moreover, one confounding factor when considering the risk to aquatic organisms from transport of natural and synthetic steroid hormones from agricultural systems to receiving waters is that vertebrates (such as cattle) excrete a mixture of free and conjugated hormones with different relative potencies. For instance, Hoffmann et al noted that cows excrete large quantities of 17α-estradiol (αE2) and 17β-estradiol (βE2) in the ratio ~ 2:1, as well as estrone (E2:E1, ~2:3). [49] However, αE2 and βE2 are not equipotent. For instance, Hoogenboom et al [50] report that αE2 has only 1% of the potency of βE2, and as such its contribution to the estrogenic activity of freshwaters is likely to be negligible unless this hormone is converted to βE2 in the environment (something that has been observed in sheep metabolism (Adams et al [51])).

One way to overcome this issue (the effect of mixtures) is to measure the concentration of as many different compounds as possible, and, knowing their relative potency, express hormonal activity as the sum of potencies normalised to an accepted standard compounds (in this case, 17β-estradiol). Measurements of estrogenic activity are then presented as estradiol equivalents (EEQ).

Alternatively, one can use *in vitro* assays. Several in vitro assays have been developed to screen the hormonal (usually estrogenic) activity of compounds in freshwaters or waste-water treatment plant effluents. and, with the exception of ELISA, the assays do not seek to identify individual compounds in a mixture, rather they look to provide a broad-brush indication of the level of hormonal activity of a sample, again expressing hormonal activity normalised to an accepted standard compounds (e.g. 17β-estradiol).

Both approaches have been used to assess the hormonal activity of waste water treatment plant effluents, yet, despite the level of understanding of this source of EDCs to the natural environment, at the time of writing, there are still no guideline values for the hormonal activity of natural waters, although the UK Environment Agency is apparently considering a trigger value of 1 ng/L EEQ in river water (Pers. Comm., Dr. Sue Jobling, Brunel University, London).

Without guideline values to drive the assessment, one can compare a chemical's concentration in a sample (in this case, a feedlot effluent or river water) with data obtained from toxicological experiments in which the concentration known to elicit a specific effect has been determined. For instance:

- The levels of 17β-estradiol reported by Soto et al [34] (~2 pM or ~ 0.55 ng/L) were below the lowest reported level to induce the production of female-only proteins in male fish (plasma vitellogen; 1 ng/L), and consequently it might not unreasonable to assume there were unlikely to be site specific risks to fish in that case.
- The levels of 17β-estradiol reported by Kolodziej and Sedlak [47] (up to 1.7 ng/L) were above the lowest reported level to induce the production of female-only proteins in male fish (plasma vitellogen; 1 ng/L), and consequently there may have been some risks to those aquatic receiving environments.
- The levels of 17β-estradiol reported by Williams et al [48] (up to 8.6 ng/L) were above the lowest reported level to induce the production of female-only proteins in male fish (plasma vitellogen; 1 ng/L), and in some cases close to the lowest level at which intersex has been observed in fish (8.7 ng/L; medaka, *Oryzias latipes*) [41], and consequently there may again have been some risks to organisms in the receiving environments.

The natural steroids 17β-estradiol (E2) and estrone (E1) have been measured in waters receiving run-off from agricultural enterprises. These, and other natural and synthetic compounds, bind to vertebrate estrogen receptor(s) to elicit a range of physiological responses in fish at environmentally relevant concentrations. Thorpe et al used vitellogenin induction in a 14-day in vivo juvenile rainbow trout screening assay to derive relative potency estimates for the most common steroidal estrogens observed in European waterways (E2, E1, and EE2). [52] They determined that EE2 was consistently the most potent steroid tested, with an estrogenic potency between 11 and 27 times greater than that of E2 and 33-66 times greater than that of E1. From the perspective of this review (which is focussed on endogenous steroids from cattle), knowing this is important because assertions often made (to this author) are that cattle only excrete estrone and its conjugated metabolites, or that the highly biologically active estradiol (or its conjugate) is rapidly converted to the less potent estrone in soils, sediments and natural waters, and/or that estrone is '*nothing to worry about*.' The first assertion is not true, even if we were to exclude cows in the definition of cattle (because, as noted earlier, all vertebrates, even the male of the species, excrete estradiol. Cattle are no exception). The second assertion is broadly true, but does not preclude impact during the time taken for the material to breakdown. The final assertion is most problematic since it assumes that estrone has no biological activity or potential impact.

Estrone is typically seen at higher (2-3 ×) levels than estradiol in and around agroecosystems, but is a hormone in its own right, and consequently estrone is by no means inactive. Thorpe et al report that E2 was only 2-3 × more potent than E1 (or, to put it another way, the potency of E1 is ~ ⅓ that of E2). [52] Consequently it is not unreasonable to assume the higher levels of estrone contribute a similar amount to the hormonal activity (and risk) as the lower concentrations of estradiol.

Little is known concerning the physiological impacts on fish and potential ecological effects of androgenic substances (cf. what is understood about the estrogens) associated with discharges from animal feeding operations. That said, natural androgens (e.g. testosterone, andro-stenedione), synthetic androgens (e.g. trenbolone), and industrial chemicals (e.g. some phthalates) are known to disrupt androgen actions. [53] Very little is known about the biodegradation rates of natural androgens in rivers, nor have natural androgen levels in rivers been determined. This lack of knowledge of actual concentrations of natural androgens in the aquatic environment, combined with a lack of understanding of the (possible) effects of androgens on fish, make it difficult to assess whether or not these trace organic pollutants pose a hazard to aquatic organisms. The situation is a little clearer with synthetic androgens such as trenbolone. There are well conducted laboratory studies that have shown that trenbolone 'masculinises' female fish at very low ng/L concentrations. [54] Moreover, Bovee et al have reported the relative potencies of a range of androgens in their yeast-based in vitro assay, suggesting that some synthetic androgens are considerably more potent than testosterone and its metabolites, e.g. 5α-dihydrotestosterone (relative potency, 2.3) > 19-nortestosterone (nandrolone, 1.7) > 17β-trenbolone (1.5) > 17β-testosterone (1) = methyl testosterone (1) » androstenedione (0.011) » androsterone (0). [55] Again, this highlights the need to assess the potency of all components of mixtures, and the benefit (when assessing risk) of reporting hormonal activity relative to a normalised standard (a process comparable to the well established principle of using Toxic Equivalents (TEQ) when assessing dioxin and PCB toxicity).

Another assumption commonly made when assessing chemicals in effluents or drainage discharges is that they will be diluted significantly in the receiving environment, bringing their hormone (e.g. estradiol) concentrations below the lowest reported level to induce physiological changes in fish, and perhaps leading to the conclusion that in such cases there may be minimal risk of endocrine disruption caused by the steroid hormones.

- To assume significant dilution may not be appropriate in some circumstances, e.g. where the discharge represents all, or most of, the environmental flow in a waterway, or where discharges are to enclosed water bodies (e.g. lakes). In such cases, there may be significant risks to aquatic wildlife.

No instrument can measure toxicity or other chemical impacts on organisms, and consequently risk assessments based on analytical determinations of chemical concentrations in water are necessarily limited (even if often used). Moreover, the *in vitro* assays used in many studies of hormonal activity are useful as screening tools for monitoring studies, but they are a simplification of the *in vivo* situation, i.e. although they are living systems *in vitro* tests do not have the complexity (in scale or scope) of more complex organisms, and thus do not take into account processes such as bioavailability, metabolism and excretion, or cross-talk between biological pathways, nor address effects that result from multiple mechanisms. Consequently, to truly assess the risk (hormonal impact) of these farm effluents, *in vivo* testing needs to be undertaken, ideally with a representative native species but failing that with a 'standard' species such as the fathead minnow.

- For some in the community (and some scientists) the *only* acceptable way to be sure that an effluent or water body is not causing an impact on aquatic organisms, is to prove that the water elicits no effects in a whole-organism assay, e.g. using a fish reproduction test.

A more robust, although more difficult, way to assess potential impacts from contaminants in effluents and drainage water is to assess changes in the field. From the field, there is some evidence that there may be some significant impacts from agriculturally derived hormones. For instance, Irwin et al. reported that natural hormones in ponds below cattle holding facilities were associated with elevated plasma concentrations of the yolk precursor protein vitellogenin in female painted turtles (*Chrysemys picta*). [26]

Orlando et al reported that wild fish (fathead minnow (*Pimephales promelas*)) collected below a feedlot on the Elkhorn River in eastern Nebraska, USA, exhibited altered reproductive biology, including decreased testosterone synthesis, altered physiology (including smaller testis size in males) and decreased estrogen : androgen ratio in female fish (cf. fish collected from upstream feeder creeks with no obvious feedlots). [56] Orlando et al did not, however, observe any changes in either male or female fish suggestive of environmental exposure to estrogens, but rather their data demonstrated androgenic activity from water obtained below feedlots. The methods used in their study could not identify the causal agents, and consequently the androgenic activity reported could be due to natural androgens found in faecal material or androgenic pharmaceuticals routinely used in growth implants in the USA. [56] Orlando et al, however, consider that the androgenic activity is due to the therapeutic agent trenbolone acetate, in part because natural androgens have relatively short half-lives in faeces and in the open water of retaining ponds [56] compared to the metabolites of synthetic androgens used as growth stimulants (e.g., trenbolone-β from trenbolone acetate).

In their follow up study (to that of Orlando et al, and Soto et al [56,34]) , Kolok et al [43] deployed caged fish in two waterways considered likely to be impacted by runoff from cattle feeding operations, namely Fisher Creek and Sand Creek at their confluence with the Elkhorn River in Nebraska, USA. Fish deployed in Sand Creek has significantly elevated levels of gene expression for two genes (StAR and P450scc) relative to those deployed in Fisher Creek. What this means is unclear, since the authors have no functional explanation for the differences observed

2.5 Conclusions

The review has addressed such questions of broad interest to the community and land, water and farm managers as:

- What is the likelihood that Victorian agricultural effluent is hormonally active?
- Will fish and other organisms be affected by the discharge? How much exposure is required before an effect is observed and is it reversible?

These questions remain, since there have been few studies of steroid transport from agriculture in Australia, with most work in this area, i.e. looking at the occurrence of steroid hormones in agricultural watersheds, occurring in the United States. Overseas studies have, for the most part, focussed on estrogenic steroids derived from CFOs, and the land application of their manures and/or discharges from waste storage lagoons, with some limited assessment of the contribution from extensive livestock farming systems.

Endogenous and therapeutic steroidal hormones may not be consistently detected in the vicinity of feedlot operations or grazing systems, in part because these compounds can strongly sorb to soils and sediments. Although sorption to organic matter in soils and sediments provides a sink for these compounds, there is also evidence that these compounds can be released from the sediments and re-enter the water. Moreover, although limited in number, scale and scope, the work that has been reported on steroid transport from agroecosystems clearly shows that there is estrogen and androgen contamination of receiving waters from grazing systems and land onto which CFO manures and effluents from storage lagoons are applied.

There is also some direct evidence from the field to suggest that the hormonal contamination is having physiological impacts on fish in the receiving environments.

Almost half of Australia's dairy farms are located in Victoria, with most of the farms located in the higher rainfall areas of southern and north-east Victoria. At any one time, some 1.3 million cows are in milk in Victoria, producing some 6 billion litres of milk annually, or some $2 billion to farmers at factory paid prices (2004/05). Given that livestock manure and dairy farm effluents contain high levels of steroid hormones, recycling of these resources onto land creates the potential for contamination of surface waters by hormones through run-off.

Based on the information collected for this review, there are a number of significant knowledge gaps in the Victorian context. For instance:

- Cost-effective manure treatment and recycling strategies to reduce or eliminate manure-borne endocrine disruption hazards are required. Consequently, more information is needed about the types and amounts of hormones that exist in fresh and aged manure, and in particular more work needs to be done regarding the fate of conjugated hormones (especially estrogen sulphates) and un-conjugated hormones in manure, Victorian soils, and water.
- More field studies are needed to determine the mechanisms of steroid hormone transport (surface runoff *vs.* leaching) to waterways, including a study of the environmental fate of steroid hormones in field soils, *in situ* in the presence of co-contaminants (to assess risks to soil health and the potential transport risks from enhanced mobility associated with fertigation).
- Surveys of surface and groundwater resources in Victoria are required (to assess if contamination is a widespread phenomenon or is localized to intensive livestock production areas).
- Surveys of Victorian wildlife are required (to look for evidence of exposure and reproductive abnormalities). These should include:
    - A study of Victoria's riverine environments to ascertain whether there have already been exposure and impacts on fish (native and for some exotics (e.g. trout, redfin perch)) in Victorian waterways.
    - A study evaluating fish development when grown in dairy effluent ponds compared and contrasted to fish developing in natural waters. Although exploring a 'worst-case exposure scenario,' dairy effluent ponds have been identified as a reasonably secure water source for aquaculture development in Victoria, which in turn could pose an ecological risk if native fish are grown for restocking purposes (through stocking of developmentally impacted fish)..

- Development of an *in vivo* test system using a representative native fish, including exploring androgen concentrations in effluent and measurement of impact (cause-effect) using an *in vivo* system.

When this review was initiated, the 'watching brief,' being held by the livestock industry on the topic of endocrine disrupting chemicals and their potential effects on aquatic wildlife was considered too passive by many. It still is, by some. The review provides no reassurance for the livestock industry, and consequently there is still a need for further extensive on-ground, reassurance research to provide data for higher-level risk assessment by industry and government agencies.

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