Steroid Metabolism in Racing Greyhounds

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A Doctoral Thesis

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ABSTRACT

The metabolism of androgenic anabolic steroids has been studied in the racing greyhound. Various drug preparations have been investigated utilising different derivatisation techniques, coupled with gas chromatographic analysis, to enable the identification of key metabolites in canine post administration samples. This has led to an increased understanding of some of the generic routes of steroid metabolism that take place in the greyhound. This valuable information can help to support metabolism studies in the future. The identification of specific metabolites for each compound investigated, has provided a means for controlling the misuse of these compounds, and contributed valuable enhancements to screening protocols utilised in the canine sports drug testing industry.

Utilisation of the techniques described, resulted in the identification of specific major metabolites of the anabolic steroid methyltestosterone, namely 17α-methyl-5β-androstan-3α-17β-diol and 17α-methyl-5β-androstan-3α,16α,17β-triol. 16α-hydroxylation was shown to be a major phase I metabolic pathway in the canine along with phase II conjugation with glucuronic acid. Similar results were obtained during the metabolism study of the progestatgenic steroid norethisterone. Several di- and tri-hydroxy metabolites were detected in the glucuronic acid fraction of the post administration urines from this study. The norethisterone metabolism study also provided some insight, into the area of trace contaminants of pharmaceutical preparations. Low levels of nandrolone metabolites were also detected in the norethisterone post administration urine samples, leading to the discovery that the administered pharmaceutical tablets contained small quantities of nandrolone and 19-norandrostenedione, albeit below FDA approved contaminant levels. Modern methods of drug screening employ such highly sensitive techniques, that they allow for the detection of metabolites of such trace contaminants, following administration of the drug preparation to the greyhound. It is therefore important to have a broad understanding of the metabolism of various drug preparations, both banned and permitted substances alike; as detection of a trace amount of a banned substance metabolite, arising from the administration of a permitted medication, whose

metabolite profile is unknown, and therefore potentially not detected, could present an interesting case.

In conjunction with research into controlling the use of banned substances for the purposes of suppressing oestrus in the greyhound bitch, an investigation into normal/reference levels of endogenous hormones has been carried out. The endogenous steroid levels in a population of 212 greyhound bitches have been studied with a view to establishing a method for the detection of the exogenous administration of the endogenous anabolic steroid testosterone. The major urinary metabolites investigated were epiandrosterone, 5β-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. Statistical evaluations have been carried out to support the implementation of a suitable threshold for the key testosterone metabolites, namely 5β-androstane-3α,17β-diol and epiandrosterone. The detection of 5β-androstane-3α,17β-diol was found to be a very good indicator of the exogenous administration of testosterone to the greyhound bitch, when compared with the reference population data for this metabolite. However, further statistical/analytical data evaluation was deemed necessary before an absolute threshold could be implemented for this analyte, for the purposes of controlling the misuse of testosterone in the racing greyhound bitch.

To support the understanding of endogenous steroid levels in the female greyhound, yet further, the endogenous reproductive steroid profiles were measured throughout the entire oestrus cycle of a cohort of 33 racing bitches. The results of the study clearly indicate a surge in androgen metabolites during the first 7-10 days of the oestrus cycle, in particular epiandrosterone and 5α-androstane-3β,17β-diol. This unique set of data has provided detailed information regarding the fluctuating concentrations of androgen and progesterone metabolites (following ovulation), at key stages of the canine oestrus cycle. The information obtained from this research can be used to support regulatory decisions regarding the misuse of testosterone in the racing greyhound bitch.

Keywords: Greyhound, oestrus cycle, testosterone, anabolic steroid metabolism, gas chromatography, mass spectrometry, methyltestosterone, norethisterone, nandrolone.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
BaB-Diol	5β-androstane-3α,17β-diol
DC	Direct current
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
ELISA	Enzyme linked immunosorbent assay
FSH	Follicle Stimulating Hormone
GC	Gas Chromatography
GCMS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-mass spectrometry/mass spectrometry
GBGB	Greyhound Board of Great Britain
HFL	Horseracing Forensic Laboratory
HP	Helix pomatia
HPA	Hypothalamic pituitary axis
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LH	Luteinising Hormone
LLOQ	Lowest limit of quantitation
LOD	Limit of detection
MO-TMS	Methoxyoxime-trimethylsilyl
MSTFA	N-Methyl-N-(trimethylsilyl)-trifluoroacetamide
NGRC	National Greyhound Racing Club
NMR	Nuclear Magnetic Resonance
OFN	Oxygen Free Nitrogen

PA Sample	Post Administration sample
RF	Radio frequency
RIA	Radioimmunoassay
SD	Standard Deviation
SG	Specific Gravity
SHBG	Sex Hormone Binding Globulin
SIM	Selected Ion Monitoring
SPE	Solid phase extraction
TBDMS	Tertiary-butyl-dimethylsilyl
TBME	Tertiarybutylmethylether
THG	Tetrahydrogestrinone
TIC	Total ion current
TMS	Trimethylsilyl
WADA	World Anti Doping Organisation

CHAPTER

INTRODUCTION

1. INTRODUCTION

1.1 GREYHOUNDS: HISTORY, RACING AND REGULATION

1.1.1 The Greyhound

Greyhounds are one of a number of breeds of dog that hunt using a combination of sight and speed as opposed to scent and endurance. They are therefore categorised and commonly referred to as sighthounds. They originate from ancient Europe and are believed to be early relatives of herding dogs. The modern day racing greyhound can be traced back to 18th and 19th century studbooks which were ultimately registered with coursing and racing authorities in the United Kingdom. Regular competitions were held in Great Britain to see which breeder owned the most agile dog in the country; such competitions and indeed animal stock, being the predecessors of historical hare coursing and the modern, track based greyhound racing industry we know today.

The greyhound has been revered for centuries both for its aesthetic looks (the first ever Best in Show at Crufts was given to a greyhound), but moreover for its athletic ability and speed. The greyhound is the second fastest land mammal from a standing start (the Cheetah being the fastest) and can reach up to 45 mph in a race, just a few strides after leaving the starting traps. The physique of the greyhound makes it perfectly suited for sprint racing, as it has a high proportion of fast twitch muscle surrounding a relatively light frame. Male dogs stand shoulder height at about 28-30in and are 4.5 - 6 stone in weight. The female ‘bitch’ is usually smaller; 27-28in at the shoulder and 3.5 - 5 stone in weight. Despite this difference in stature, greyhound racing is one of very few sports and possibly the only unaccompanied, individual athletic sport, where females compete against males on a ‘level playing field’, with no concession allocated to the female.

1.1.2 Greyhound Racing

Greyhound racing was first introduced in Great Britain in the 1920s and soon increased in popularity becoming the second highest spectator sport after football. Unlike the historical hare coursing, greyhounds now follow a mechanical hare or lure that circuits the perimeter of the track and provides them with the stimulus to chase ‘potential prey’ at top speed for a short period of time. Various venues have come and gone over time resulting in 26 present day tracks currently operating in Great Britain. During the early

years of greyhound racing it soon became apparent that a governing body was required to oversee proceedings, due to the increase in race prize funds and money changing hands, including 'wagers' between owners, and the ever increasing threat of foul play. In 1928 the National Greyhound Racing Club (NGRC) was set up to control the industry and enforce a new set of rules to ensure fair practice by all interested parties. Bookmaking, previously confined to the racetrack by law since the mid 1800s, became legal on British high streets in 1961, although various betting outlets had already been operating illegally for many years prior to this. The temptation to cheat for monetary gain has been apparent across numerous sporting disciplines for many years (animal and human) and with an increase in the outlets and opportunity for wagering on the outcome of greyhound races, drug testing was a required part of the developing infrastructure of the sport.

The Greyhound Board of Great Britain (GBGB) was set up in 2009 to oversee all aspects of greyhound racing. This involves track promotion and regulatory affairs, as well as the stewardship at the tracks along with integrity and welfare measures, including drug testing, much of which was previously controlled by the NGRC.

1.1.3 Drug testing

In 1963 the Horseracing Forensic Laboratory (HFL) was established in Newmarket to provide drug testing services to the British horseracing industry. In 1991 the laboratory took on the role of providing drug testing to the greyhound racing industry as well, and continues to provide this service today. The work of the laboratory involves the detection of veterinary medications (subject to the rules of greyhound racing[1]) as well as such substances that would be considered doping agents used in an attempt to alter the performance of a racing greyhound, either positively (e.g. anabolic steroids) or negatively (e.g. sedatives).

The rules of racing include strict controls on the use of medications in racing greyhounds and one area of significant debate for many years has been the use of steroidal preparations for the purposes of suppressing the oestrus cycle in racing greyhound bitches. To suppress or not to suppress?....that is the question. For every trainer that believes it is necessary to suppress the season of a bitch in order to stabilize her form and enable her to be trained uniformly all year round, there is another who is happy to allow the bitch to come into season and use the potential fluctuations in her

form to their possible advantage (although this is by no means guaranteed). Whilst both of these approaches to training bitches are equally acceptable to the GBGB (racecards are required to provide information regarding suppression and season dates) it is the nature of some of the compounds that have been used for suppression of oestrus, in particular testosterone, that have caused the industry the greatest concern. Regular and continued use of testosterone to suppress oestrus in greyhound bitches could be performance enhancing and also potentially have a negative impact on fertility in those animals destined for breeding after racing (although this has not been proved). Hence, the use of testosterone as an oestrus suppressant is no longer permitted by the GBGB.

Testosterone has been one of the most widely abused anabolic steroids in a variety of sports for many decades but is also a naturally occurring endogenous steroid and therefore its qualitative detection in a racing animal does not necessarily constitute a breach of the rules of racing. Therefore, controlling the abuse of testosterone in greyhound racing requires a quantitative approach.

1.1.4 Steroids and Doping in sport

The term ‘doping’ dates back to the 1800s in England, apparently defining a mixture of opium and narcotics given to horses. Prior to that, it originated as a Dutch word used in South Africa to define the taking of strong alcoholic drink at religious worship and was also used to define sedating or ‘nobbling’ of either animals or indeed individuals[2].

Nowadays, the term “doping offence” would probably be interpreted by most people as the use of ‘drugs in sport’, animal or human, whatever the pharmacological effects.

Anabolic steroids are clearly used in an attempt to enhance performance, as opposed to ‘nobbling’ and the history of their use in humans dates back to the ancient Greek Olympics where athletes of the time are thought to have explored different means of improving their strength, involving the consumption of various organs of animals, including the testicles, which were thought to impart strength.

Historical studies within the last hundred years have indeed shown that testosterone can be extracted from such tissues in milligram quantities[3]; work which ultimately provided the building blocks for the early pharmaceutical industry in the 1930s to develop synthetic testosterone and eventually other synthetic mimics with structurally specific alkyl substituent groups, designed to inhibit first pass metabolism and hence make them orally active.

Current knowledge of *in vivo* first pass metabolism indicates that testosterone is predominantly metabolised when taken orally and its beneficial activity therefore reduced[4]. Interestingly, the androstenedione (a precursor of testosterone) content of testicular tissue may have been of more benefit to the ancient Greek athlete than testosterone itself. Androstenedione is one of the many precursor 'pro-hormones' available through the Internet for the past decade or more, that are marketed with a knowledge of first pass metabolic mechanisms, to try and introduce as much of the related parent steroid (in this case testosterone) into the circulation as possible, despite the compound being taken orally. Many of these compounds have some anabolic activity in their own right, but large quantities need to be regularly ingested in order for them to be really effective; the relative androgenicity of androstenedione being about 15% of that of testosterone[5] and increases in serum free testosterone levels (i.e. biologically active), following androstenedione administration, although elevated, have been shown to be relatively modest[6].

So for many years, human athletes have chosen to take a variety of compounds to try and enhance their sporting performance. The establishment of international routine dope testing involving sophisticated analytical techniques has introduced an almost 'secondary competition' for some athletes, who try and beat the ever evolving system. Doping control programmes are therefore vital in supporting the integrity of sport and to an extent the health education of athletes, as regular abuse of anabolic steroids can cause harmful side effects such as liver damage and endocrine system disruption. They can also cause high blood pressure and actual anatomical changes, including, amongst others, alteration of the physical structure of the heart that can lead to severe cardiac complications[7,8].

1.1.5 Drug testing and the rules of Greyhound Racing

It is by and large the choice of a human athlete whether they take performance enhancing substances that could also cause them significant health risks; in some cases life-threatening; although the German Democratic Republic government controlled athlete doping programme, in the 1980s, is a well-documented exception to this premise[9]. Nevertheless, despite understanding the risks, many athletes have expressed a much greater desire for victory and short term prestige over health and longevity of life[10]. However, for an animal athlete the choice still lies with humans and, as such,

routine dope testing in this sphere is not only vital to the integrity of the sport but also to the welfare of the racing animals as well.

The rules of the Greyhound Board of Great Britain (GBGB) regarding the use of drugs and medications in greyhound racing are as follows:-

Rule 217: A greyhound when taking part in a Race or Trial must at that time be free of medicines, tonics or substances that could affect its performance or well-being, the origin of which could not be traced to normal and ordinary feeding. The only permitted exceptions to this rule are:-

i) Medicinal products which have been authorised by the Veterinary Medicines directorate for the suppression of a bitch's season, prescribed by a Veterinary surgeon.

ii) Medicinal products which have been authorised by the Veterinary Medicines Directorate as anti-parasitic drugs (for internal/external) parasites or as vaccines.

The drugs permitted for use for the suppression of oestrus, under the current exceptions of Rule 217(i) are four progestins, megestrol acetate, proligestone, medroxyprogesterone acetate and more recently norethisterone. The use of any other steroid hormone for oestrus suppression is currently prohibited.

Therefore, the use of anabolic steroids for performance enhancement in either male or female greyhounds is strictly prohibited and methods for detecting the administration of such compounds to greyhounds are necessary for an effective doping control programme. Knowledge of the metabolism of steroids in the greyhound is therefore vital to support such a programme.

1.2 STEROID STRUCTURE AND NOMENCLATURE

1.2.1 Steroids

Steroids are naturally occurring lipid compounds involved in a wide range of homeostatic and physiological mechanisms in living organisms and their biochemical activity has been extensively studied in a variety of mammalian species. They are synthesised in various tissues of the body, e.g. adrenal gland, liver, testis and ovary.

The structures of all steroids are based on a 4-ring perhydrocyclopentanophenanthrene nucleus (Figure 1.1).

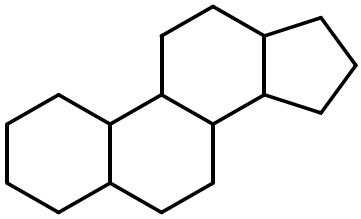


Figure 1.1: Perhydrocyclopentanophenanthrene nucleus (gonane)

Each hydrocarbon ring is assigned a letter (A-D) which is often referred to when discussing Phase I metabolism of steroids. Each carbon atom is sequentially numbered to assist with the chemical naming of steroid compounds that belong to the various different classes. The parent compound from which all the steroid hormones are biosynthetically derived is cholesterol (Figure 1.2), which is believed to have been first identified in the 18th century when it was isolated from gallstones (Greek names chole=bile and stereos=solid).

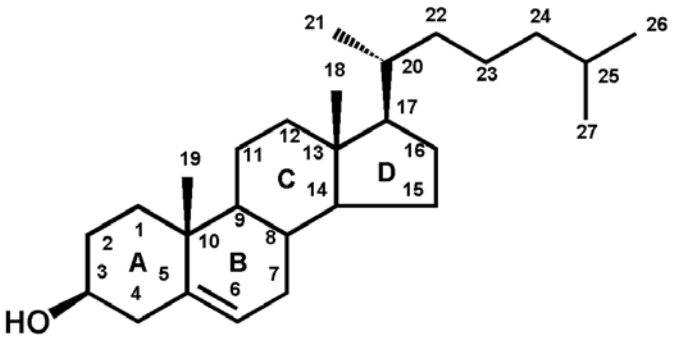
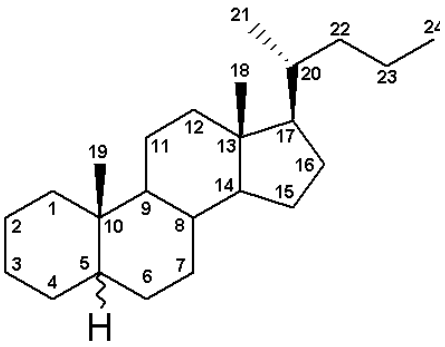


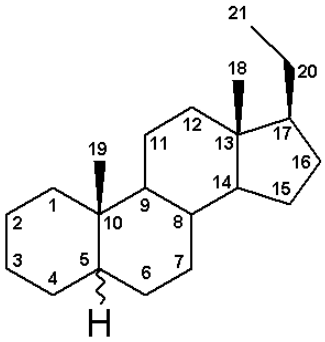
Figure 1.2: Structure and nomenclature of cholesterol

Important groups of physiological steroids are formed *in vivo* initially by the action of various lyase enzymes on the cholesterol structure. This results in a range of different subclasses (see Figure 1.3) being produced within or near to the tissue locations in which they are required to elicit their activity. Conversely, as is also the case for a lot of endogenous steroids, they originate in tissues that act as a site for production and are then distributed around the body to remote target organs where they then exert their effect.

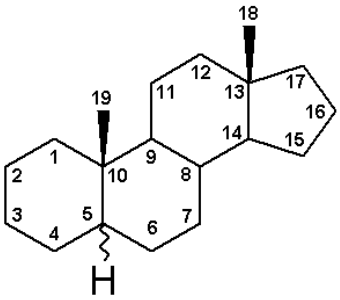
Enzymatic cleavage of cholesterol between C24 and C25 produces the C24 hydrocarbon cholane, to which the bile acids are related (e.g. cholic acid). Cleavage between C20 and C22 results in the formation of the C21 series, the pregnanes (e.g. progesterone). Cleavage between C17 and C20 results in the formation of the C19 series, the androstanes (e.g. testosterone) and subsequent removal of the C-19 methyl group from C10 (a process of aromatisation) produces the C18 series, the estranes.



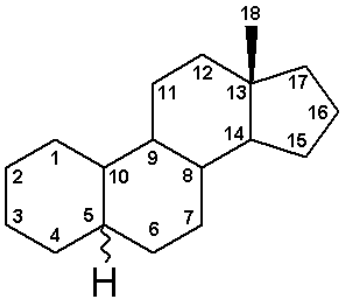
Cholane (C24)



Pregnane (C21)



Androstane (C19)



Estrane (C18)

Fig 1.3: Hydrocarbon nuclei used in steroid nomenclature

Officially, steroids are probably most appropriately named using the International Union of Pure and Applied Chemistry (IUPAC) nomenclature[11] (e.g. pregn-4-ene-3,20-dione; Figure 1.4). In brief summary, this naming process involves choosing the category of steroid depending upon its hydrocarbon nucleus (Figure 1.3), adding in any sites of unsaturation within the 4-ring nucleus; listing as prefixes the key functional groups attached to the chosen base structure nucleus, followed by adding a final single suffix for the functional group which holds the highest rank according to the IUPAC recommendations, which for a large number of -keto steroids in the pregnane, androstane and estrane class, invariably results in the suffix -one or -dione, for di-keto compounds. For ease of interpretation in scientific texts, steroids are also often referred to by their trivial names as well (e.g. progesterone, Figure 1.4). Either is considered appropriate depending upon the required level of detail and/or context.

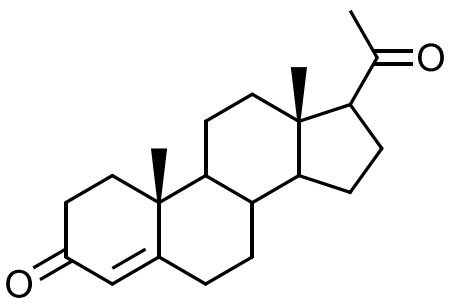


Figure 1.4: Structure of progesterone; pregn-4-ene-3,20-dione (note double bond at C4=C5)

1.2.2 Steroid conformation

The cyclohexane rings of the steroid nucleus can adopt one of two conformations; either the ‘chair’ or ‘boat’ form (Figure 1.5), although the boat form is not sterically favoured. When the A, B and C rings are all in the chair form the molecule is effectively planar in structure. The valencies of the carbon atoms of the ring structure are of two types, those at right angles to the ring structure are the ‘axial’ bonds and those in the general plane of the ring are the ‘equatorial’ bonds (Figure 1.5). By convention it is agreed that the methyl groups at C18 and C19 project upwards from the upper face of the molecule (with their free chemical rotation, they are effectively analogous to two helicopter propellers, albeit tetrahedral in conformation).

The upper side of the molecule is known as the beta-side; the underside being the alpha. Any substituent functional groups projecting upwards are therefore assigned the β-configuration and those projecting downwards the α-configuration. When structures are drawn in scientific texts β-bonds are usually represented by a solid black notation and α-bonds by a dotted line notation. Bonds where the stereochemistry is unknown are represented by a wavy line.

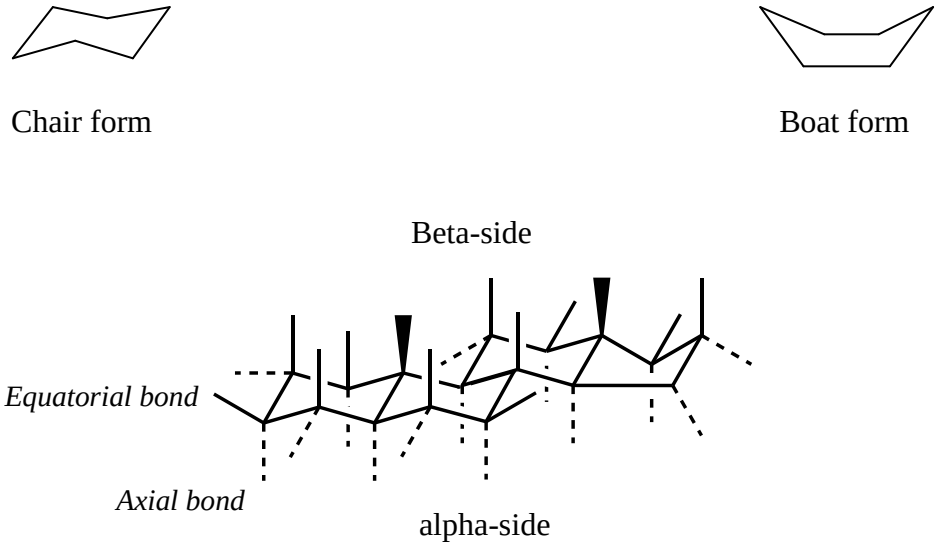


Figure 1.5: Steroid configuration

Reduction of the double bond at C4=C5 (see Figure 1.4) i.e. 4-pregnene to 5-pregnane can occur from either the alpha- or beta- side of the molecule depending on the enzyme involved (e.g. 5α or 5β reductase). 5α reduction results in the incorporation of hydrogen in the α-configuration (below the plane of the molecule) at C5, which keeps the chair conformation of the A-ring intact and therefore the molecule remains planar in structure. 5β reduction incorporates hydrogen in the β-configuration (albeit virtually equatorial in orientation) which produces a significant change in the confirmation of the A-ring in relation to the rest of the molecule, the A-ring being effectively positioned at right angles to the planar structure of the B-ring (see Figure 1.6).

In 5α-androstane the angular methyl group (C19) and the hydrogen atom at C5 are on opposite sides of the plane of the steroid molecule forming an AB-ring 'trans' union. In 5β-androstane, the angular methyl group and the hydrogen atom project from the B-side of the steroid molecule producing a 'cis' AB-ring union. Both -cis and -trans AB-

ring unions are prevalent in steroid hormone structures, but the BC and CD ring unions are always in the trans configuration.

This confirmation difference is quite significant when considering steroid receptor binding *in vivo*, as the 5β-reduced androgenic steroids for example, are often markedly reduced in activity and effect due to a poor 'fit' with receptors, compared with their 5α-reduced counterparts[12]. The difference in confirmation is also of significance for a number of compounds when considering their separation on an analytical column during gas chromatographic steroid analysis.

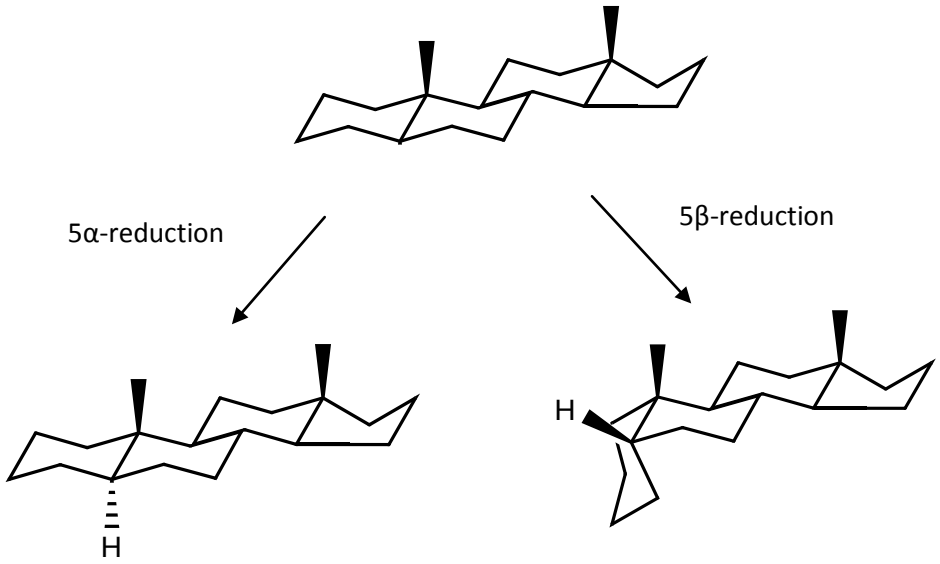


Figure 1.6: Steroid conformation

The different steroid classes and structures define the biochemical and pharmacological action of individual steroids within the body. Substituent groups attached at the different numbered carbon locations, provide the structural variation necessary for the cellular activities and receptor interactions required for each steroid to exert its effect *in vivo*.

Synthetic steroids are chemical analogues of natural steroids. In the case of synthetic androgens these are manufactured and produced by making modifications or adding substituent groups to the structure of testosterone, in order to alter the ratio of androgenic to anabolic activity of the molecule, depending upon the desired pharmacological action. Synthetic progestagens tend to be based on the structure of progesterone, with structural alterations enhancing the mode of action and reducing potential side effects.

1.3 STEROID BIOCHEMISTRY AND BIOSYNTHESIS

The majority of cholesterol used for steroid biosynthesis comes from the blood, originating from hepatic synthesis. A relatively small contribution comes from intestinally absorbed cholesterol from the diet[12]. Any cholesterol that is surplus to the requirements of the body is stored, lipid bound, to low density lipoprotein (LDL), on the inside wall of blood vessels (hence the need to control excessive dietary intake to avoid exacerbating certain cardiovascular diseases). Cholesterol is transported into steroid producing cells bound to lipoprotein. Once in the cell it is disassociated from the lipid and initially converted into pregnenolone. At this stage, endogenous steroid synthesis is under control from the hypothalamic-pituitary axis (HP axis).

The hypothalamus produces hormone 'releasing factors' that act on the pituitary gland causing it in turn to secrete protein hormones that act on target organs such as the adrenal gland, testis and ovary. These tissues are the primary producers of the array of endogenous steroids required for the maintenance of normal mammalian homeostasis. The activities of these tissues are discussed below, with greater emphasis on the reproductive tissues and their associated steroids, as they form the basis of the research in this thesis.

1.3.1 Adrenal gland

Adrenocorticotrophic hormone (ACTH) is secreted by the pituitary to act on the adrenal gland, which in turn, produces a range of corticosteroids (C_{21} steroids) , these being divided into the subclasses of glucocorticoids and mineralocorticoids. Cortisol is the primary glucocorticoid secreted from the adrenal gland in mammals, usually in response to stress and is responsible for mobilising glucose into the bloodstream from muscle stores, regulating the immune system and controlling inflammation in certain tissues. Its production increases markedly during the classical fear, fight or flight response in mammalian species. Mineralocorticoids, e.g. aldosterone help to maintain blood pressure via control of kidney function and the reabsorption of salt and water.

Under ACTH control the adrenal gland also produces the weak androgens dehydroepiandrosterone (DHEA) and androstenedione, the latter of which is the precursor to the production of relatively small quantities of testosterone and oestrogens (oestrone / oestradiol). In female dogs and humans this is the major source of

physiological testosterone production, whereas in the male, much greater quantities are produced directly by the testis.

1.3.2 Testis

Testosterone is one of the C_{19} steroids which make up the androgen class of compounds. Testosterone and other androgenic compounds are synthesised primarily in the testis in the male (and to a much lesser extent in the ovaries in the female), where they are responsible for maintenance of sexual characteristics and the hormonal control of reproduction. The anabolic effect of endogenous testosterone is most noticeable during male puberty where increases in bone density and overall muscle mass are observed.

The production of testosterone in the testis is under the control of follicle stimulating hormone (FSH) and luteinising hormone (LH), both of which are released by the pituitary gland. In humans, testosterone is released into the circulation and bound to two major transport proteins, albumin and sex hormone binding globulin (SHBG). Only approximately 2% of circulating testosterone is present in the unbound/free form in human males and 1% in females[12]. The free form testosterone is the biologically active form which can be transported into the relevant cells for androgen receptor binding, or further conversion to 5a-dihydrotestosterone (DHT) which is the active and indeed more potent androgen in various target tissues[5]. Under normal homeostatic conditions the level of unbound testosterone is in dynamic equilibrium with the protein bound form, ensuring that biologically active levels are maintained. The levels of total testosterone in the bloodstream are controlled by a negative feedback mechanism through the HP axis whereby increased testosterone levels exert a direct negative effect on the production of LH, which in turn reduces the production of testosterone.

Negative feedback mechanisms are prevalent in nature particularly in relation to the regulation of hormones via the HP axis. When testosterone is abused and administered at high doses for prolonged periods in males, the activity of the HP axis can be disturbed to the point where little to no LH is secreted, leading to testicular atrophy, although this is considered to be reversible some weeks/months after cessation of use. In the female, regular abuse of a high level of testosterone would interfere with normal hormonal balance and cause potentially irreversible side effects due to masculinisation (i.e. virilisation.)

Figure 1.7 shows some of the key biosynthetic pathways of steroidogenesis that take place in the adrenal gland, testis and ovary. The cellular enzymes involved in these processes are also highlighted. The abundance and activity of these enzymes varies from each tissue location depending upon the required terminal products[13].

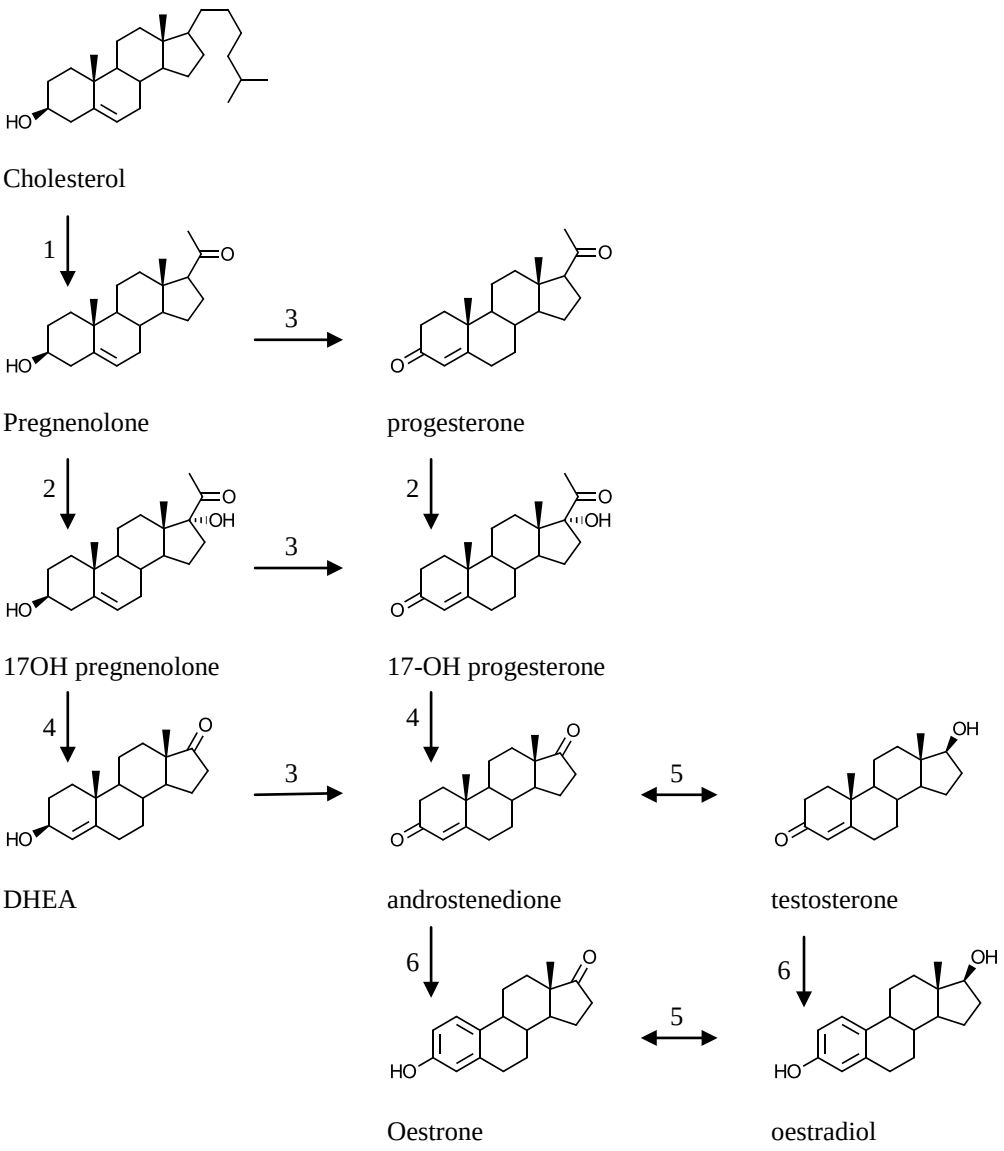


Figure 1.7: Biosynthesis of reproductive steroids: Enzymes involved: 1. C20-22 desmolase and 20- and 22-hydroxylases; 2. 17-hydroxylase; 3. 3β-hydroxysteroid oxidoreductase and $\Delta_{5\rightarrow4}$-3-oxosteroid isomerase; 4. C20-17-desmolase; 5. 17B-hydroxysteroid oxidoreductase. 6. Aromatase cascade: 19-hydroxylase; 19hydroxysteroidoxidoreductase; C10-19-desmolase.

1.3.3 Ovary

Steroidogenesis in the ovary is also under the control of FSH and LH[14]. Demethylation of androgens at C-19 and aromatisation of the A-ring results in the formation of oestrogens (C_{18} steroids), which are produced in large quantities in the female and are responsible, in conjunction with progesterone and related compounds (C_{21} steroids), for the normal control of the oestrus cycle in the female. At certain stages of the oestrus cycle and during pregnancy there are marked alterations in the levels of hormones produced by the ovary. Progesterone (and pregnenolone) is a precursor to the production of androgens and subsequently oestrogens, and as such is not secreted into the bloodstream in significant quantities until key stages of the oestrus cycle. This is also true of the androgens up to the point of ovulation. The production of steroids in the ovary is therefore carefully compartmentalised and controlled by various interactions of the pituitary hormones and the ovarian steroid products alike. It is a finely balanced mechanism, which to this day is not completely understood in detail at the cellular level. The complex balance of oestrogen and progesterone in the normal oestrus cycle can, however, be disrupted and indeed controlled, by exogenous administration of androgenic, oestrogenic or progestagenic hormones.

1.4 THE OESTRUS CYCLE

The greyhound bitch, unlike the human, is essentially a seasonally mono-oestrus animal. Before highlighting the key events in the canine oestrus cycle, a brief summary of events in the human ovarian cycle is given to provide a comparison with that of the bitch as well as outlining features that are common to both.

1.4.1 Human ovarian/menstrual cycle

The human ovarian cycle occurs on average once a month and begins on day one with about 4 days of menstrual bleeding[14]. As oestrogen levels begin to rise the follicular phase begins with a number of follicles being prepared for potential ovulation. Under control by FSH and LH the follicles develop and increase in size with the proliferation of various cell types (these will be discussed in more detail for the greyhound in chapter 5). As ovulation approaches one of the more dominant follicles is chosen and continues to develop while the other follicles atrophy. Around 2 weeks into the cycle, oestrogen levels, having reached a maximum, suddenly drop away. This maximum

level is achieved *via* one of nature's few and rather interesting positive feedback mechanisms; where once a critical oestrogen level is reached, rather than negative regulation of production taking place as before, a dramatic further increase occurs. This is followed by a surge in LH. This stimulates ovulation via rupture of the dominant follicle, releasing the egg into the fallopian tube for potential fertilization, which usually needs to occur with 24 hours of ovulation (the egg can remain viable for up to a week in the canine[15]). In the ovary, the residual ruptured follicle, or corpus luteum (Latin for 'yellow body' – due to the high lipid content of the cells) starts to secrete progesterone in preparation of the maintenance of the uterus lining for a possible implantation and ultimately pregnancy. If no fertilization takes place the corpus luteum degenerates through a process called luteolysis (luteal phase of the cycle). Luteolysis occurs when there is no luteotrophic hormonal support for the developing corpus luteum and can last up to 2 weeks resulting in the shedding of the uterine lining and the egg (menstruation). The cycle then begins again. In summary the cycle has three main phases; the follicular phase, ovulation and the luteal phase.

1.4.2 The oestrus cycle of the greyhound bitch

The oestrus cycle of the bitch has been characterised by as many as six phases but for simplicity there are four clearly defined phases of significant duration that will be discussed here. These comprise Pro-oestrus, Oestrus, Di-oestrus and Anoestrus[16].

Pro-oestrus: lasts approximately 9 days, during which time the bitch will exhibit both anatomical and behavioural changes including the commencement of bleeding, increased urination and attractiveness to male dogs (but generally unreceptive).

Oestrus: which again lasts on average for 9 days, at the outset of which the bitch will normally ovulate and be receptive to males; this being a major reason for not allowing bitches to race during this time.

Di-oestrus: (80-90 days) during which post-ovulatory levels of progesterone rise (produced from the developing corpus luteum) and ultimately fall back to basal after around 3 months.

Anoestrus: the final stage, following luteal regression, which will last for around 4 months in the average bitch. The bitch will remain sexually inactive during this time until the commencement of pro-oestrus returns.

Therefore the average oestrus cycle in the greyhound bitch lasts for 8 or 9 months[17]. One notable significant difference in the greyhound cycle compared with the human is the maintenance of the corpus luteum in the absence of fertilization and pregnancy. The levels of progesterone produced in pregnancy are on average higher than those produced in the normal oestrus cycle, but levels will be variable from one animal to another, as will be the lifespans of the corpora lutea. In fact, the only reliable hormonal indicator of pregnancy in the greyhound is the protein relaxin[18,19], which is produced in part to prepare the pelvic ligaments for parturition. It appears in plasma at about 25 days after ovulation with levels peaking at around day 50. The prolonged luteal phase in di-oestrus means that the bitch is under the influence of progesterone for a prolonged period and in the latter luteal stages, prolactin as well, which continues to maintain the corpus luteum. The basis of regression of the corpus luteum in the greyhound bitch is poorly understood as it does not involve secretion of prostglandin F2a (a luteolytic fatty acid produced by the ovary or uterus in other animals, including the mare[20]) or the withdrawal of luteotrophic support as in the human.

The hormonal and pheromonal changes in the canine resulting in ‘oestrus’ receptivity are believed to have evolved out of the human, hence the terminology ‘ovarian’ or ‘menstrual’ cycle. However, there is a body of research that might suggest evidence to the contrary[21]. The canine oestrus cycle is discussed in further detail in chapter 5.

1.5 CONTROL OF THE OESTRUS CYCLE

1.5.1 Synthetic steroids used to control the oestrus cycle in the greyhound

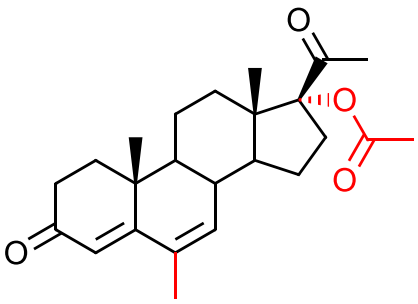
The prolonged luteal phase is the reason given by many trainers for poor performance in the bitch for several weeks towards the end of di-oestrus and the beginning of anoestrus. Such trainers prefer to suppress or postpone the oestrus cycle in the bitch to maintain a consistent level of form for racing.

The following compounds are examples of the types of steroid preparations used and their proposed mode of action to prevent the racing bitch coming into season. The compound used and the dosing regimen will vary depending upon the stage of the cycle that the bitch is currently at before treatment.

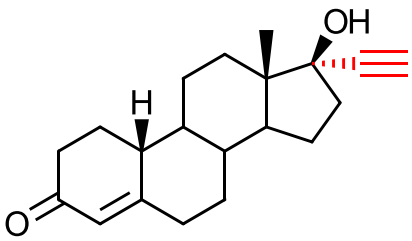
1.5.1.1 Synthetic progestagens

Megestrol acetate (see Figure 1.8) is a progestagen that is structurally related to and mimics the action of progesterone, putting the bitch in a 'luteal' state. This drug is considered effective when given as a short course in pro-oestrus and for a longer duration in anoestrus[21,23]. Its actions involve the suppression of follicle development and ovulation via modulation of the secretion of FSH and LH.

Another progestagen, norethisterone (see Figure 1.8) is a human contraceptive that acts by thickening the uterine wall to prevent implantation of the egg following ovulation. It can prevent ovulation as well, but not consistently. It is not known whether these modes of action are similar in the greyhound bitch, but nevertheless from recent anecdotal evidence as well as previous research[24,25] it does appear to be an effective oestrus suppressant, although it may need to be administered on a daily basis (as in humans) to be fully effective. One study has shown no ill effect of the long term usage of norethisterone in the greyhound bitch[26]. Due to their substituent groups present at C-17 both megestrol acetate and norethisterone are orally active and therefore available as tablet preparations.



Megestrol acetate



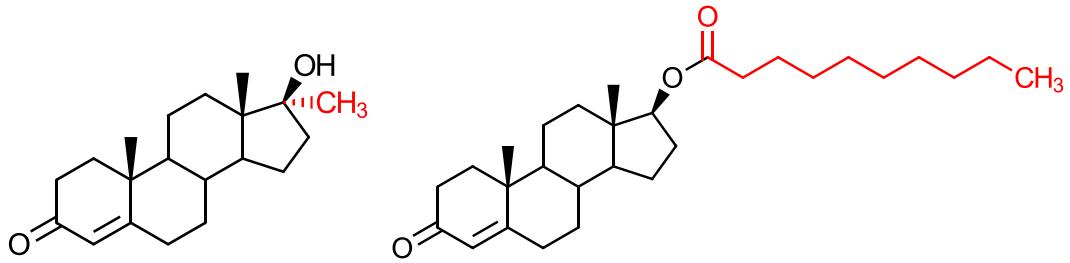
Norethisterone

Figure 1.8: Structures of synthetic progestagens: the functional groups shown in red represent the synthetic additions to the structure of progesterone (in the case of megestrol acetate) and to the structure of nandrolone (in the case of norethisterone)

1.5.1.2 Synthetic androgens

The main reproductive effect of exogenous androgen administration is to cause suppression of ovarian activity via negative feedback on the pituitary which decreases gonadotrophin secretion. The ovaries still contain primary and secondary follicles but

these are unable to mature to ovulatory size. Androgens are not as effective as progestagens when given in short timespan doses at key stages of the oestrus cycle[16,27]. They therefore tend to be used on a semi-regular basis. For example methyltestosterone is known to have been given in tablet form on an almost daily basis to suppress oestrus in racing greyhounds (based on intelligence from international drug testing authorities). Nandrolone and testosterone are usually given in the form of injectable esters, providing a slow release of the drug into the circulation over a period of many weeks. Dosing is therefore only required once or twice a month. Structures of some of these compounds (including the ester form) are shown in Figure 1.9.



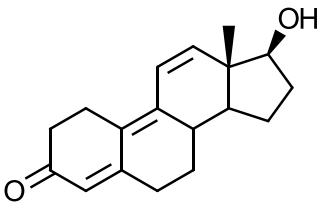
Methyltestosterone Testosterone decanoate ester

Figure 1.9: Structures of synthetic androgens: the functional groups shown in red represent the synthetic additions to the structure of testosterone.

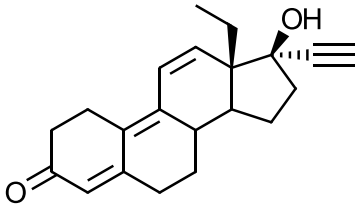
1.5.2 Structure /Activity relationship of synthetic steroids

It is interesting to note the structure/receptor binding activity relationship for various compounds depending upon the functional groups that are present in the C-17α position. In general terms, fully saturated alkyl groups are present at this position in the structure of androgenic-anabolic steroids whereas unsaturated groups are present in progestagens. There is some crossover of androgen and progesterone receptor binding activity for various compounds *in vivo*, i.e. neither group is 100% selective for its intended pharmacological receptor activity[12,28]. The 3-keto-4-ene moiety of the A-ring is extremely important for efficient androgenic activity *in vivo*[5], but clearly this is also in conjunction with a suitable associated structure at C-17 in the D-ring. In fact epimerisation of the 17β-hydroxy function of testosterone to 17α-hydroxy testosterone or 'epitestosterone' results in this compound having virtually no androgenic/anabolic activity at all[5].

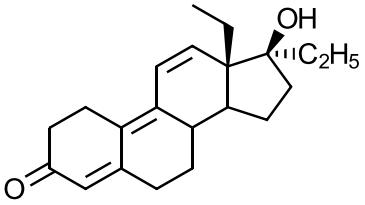
Some examples of related steroid structures and their pharmacological action are shown in Figure 1.10. All of these compounds have been produced in their time by the pharmaceutical industry for human or veterinary use with the exception of tetrahydrogestrinone (THG). THG was one of the first 'designer' steroids to be discovered in 2003[29]. Based on its structure and knowledge of steroid biochemistry it was clearly predicted by its makers to have androgenic activity and be effective when taken orally. It has been shown to be a potent androgenic compound some 10x stronger than trenbolone[30,31]. It was also expected to be potentially difficult to detect (see derivatisation techniques).



Trenbolone (1,2)

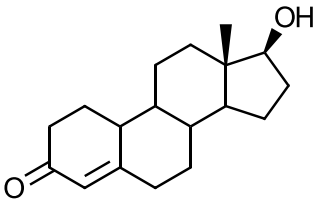


Gestrinone (1,2,3)

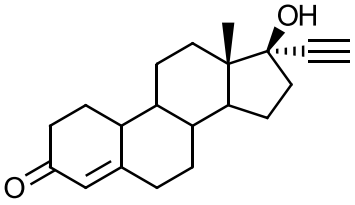


THG (1,2)

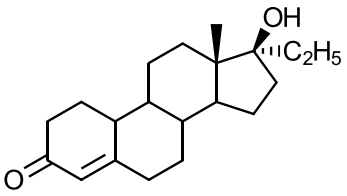
(1) Binds androgen receptors
(2) Binds progesterone receptors
(3) Binds oestrogen receptors



Nandrolone (anabolic)



Norethisterone (progestagen)



Norethandrolone (anabolic)

Figure 1.10: Structurally related anabolic and progestagenic steroids

Gestrinone, THG, norethandrolone and norethisterone are orally active compounds.

Nandrolone and trenbolone are normally given as ester linked preparations to avoid first pass metabolism of the unprotected hydroxyl group at C-17 by liver enzymes.

1.6 STEROID METABOLISM

The metabolism of anabolic steroids has been studied and reviewed in various mammalian species, including the cow[32], the horse[33], human[34], and the dog[35], although to date studies have not been extensive in the latter. Knowledge of metabolism is clearly important in the sports testing industry in order to be able to identify key marker analytes for the detection of drugs of abuse.

1.6.1 Phase I metabolism

Structural alterations to the steroid, as well as activating it for receptor activity, can also result in rendering the molecule relatively inactive and thus prepare it for excretion from the body. Such structural alterations are termed Phase I metabolic conversions, the majority of which are performed by liver enzymes. Steroids enter the liver *via* the circulating blood vessels. Endogenous steroids that enter the circulation are therefore constantly being metabolised. Exogenous drug administrations via injection routes (e.g. intramuscular or intra-articular) introduce the proprietary preparation into the bloodstream, and in the case of steroid esters (following de-esterification by hydrolase enzymes present in the blood), the parent compound is able to exert its effect before reaching the liver and being metabolised. Oral steroid administration results in the drug being absorbed from the intestine and transported directly through the liver *via* the hepatic portal vein. This results in what is termed extensive first pass metabolism of the drug, meaning that a much smaller percentage of the active parent compound reaches the bloodstream. However, as previously highlighted, many of the orally active steroids have 17a-alkyl substituent groups that block enzyme activity on the D-ring and allow more of the intact drug to reach the circulation, thereby increasing its bioavailability.

The primary role of Phase I metabolism is to reduce the pharmacological activity of drug a molecule and increase its polarity to aid excretion from the body, (e.g. addition of hydroxyl functional groups). Various enzymes perform a range of Phase I transformations including oxidation of 17-hydroxy functions to -keto groups (this significantly reduces the pharmacological activity of anabolic steroids[5]); reduction of the 4-ene double bond (5α/β reductases); reduction of the 3-keto function to 3α or 3β hydroxy groups and various hydroxylases that incorporate hydroxy functions at

different carbon locations of the steroid ring structure. Some common sites of steroid metabolism are shown in Figure 1.11.

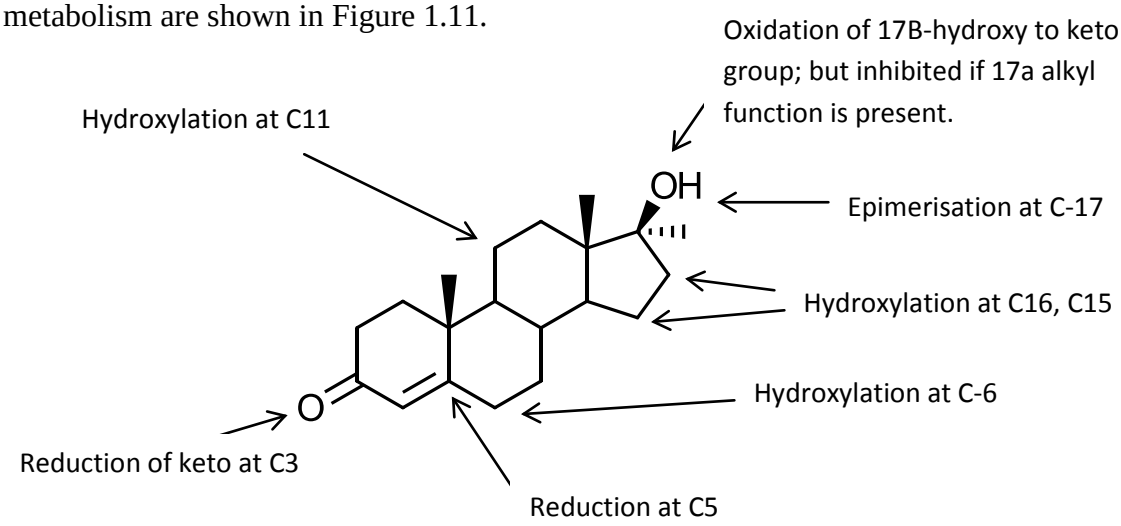
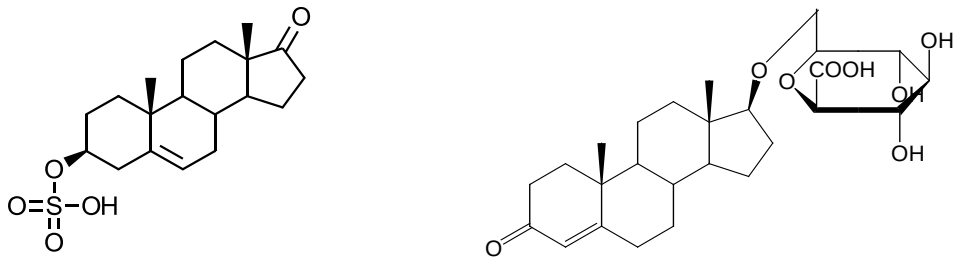


Figure 1.11: Common sites of metabolism for anabolic steroids

1.6.2 Phase II metabolism

Liver enzymes are also responsible for Phase II metabolism which increases the polarity of a steroid molecule still further by conjugating it with various water soluble molecules such as sulphate groups or glucuronic acid. This aids the urinary excretion process for steroidal compounds. In fact for some compounds, whose structures are unable to be altered by Phase I enzymes, conjugation may be the only means by which the body can increase their polarity to aid excretion. Such compounds tend to be quite toxic to the liver and include the 17α-alkyl steroids and compounds whose structures have a high degree of A,B,C ring unsaturation, (e.g. trenbolone related), making them a poor 'fit' in the active site for many of the Phase I liver enzymes[34]. Some examples of steroid conjugate structures are shown in Fig 1.12.



DHEA sulphate

Testosterone glucuronide

Figure 1.12: Structures of Phase II conjugates

1.7 METHODS OF STEROID DETECTION

Steroids can be analysed in a variety of biological matrices including blood, saliva, urine, bile, hair and tissue. Various methods have been described over many years and reviewed for various mammalian species including the cow[32], and in particular in relation to sports drug testing in the horse[33],[36] and human[37],[38].

The analytical approaches to steroid detection have developed and adapted over time to improve selectivity and sensitivity. Radioimmunoassay (RIA) was a popular analytical technique in the 1970s and developments for application to the analysis of anabolic steroids were implemented for use at the Olympic games around that time[39]. This technique was accompanied by enzyme linked immunosorbent assays (ELISA) in the 1980s. Unwanted cross reactivity in immunoassays can be an issue with complex matrices (providing 'false' positives) and analyte identification is not necessarily possible, (requiring confirmation by an alternative technique, e.g. gas chromatography-mass spectrometry [GC-MS]) although rapid throughput is advantageous and some of these assays are still used for certain applications today, particularly if they are analyte specific or limited to a minimum number of analytes (i.e. drug group specific).

However, alongside these techniques gas chromatography (GC) and liquid chromatography (LC) were developing fast, ultimately to be combined with mass spectrometry to provide highly selective methods for detection of whole drug groups such as anabolic steroids as well as stimulants, beta blockers and non-steroidal anti-inflammatory drugs (NSAIDS), all of which are of interest in the anti-doping sector. In the 2000s, particularly with the introduction of atmospheric pressure chemical ionization (APCI), liquid chromatography-mass spectrometry (LC-MS) became the analytical approach of choice for most drug groups in various sectors of the drug testing industry and is suited to the analysis of plasma as a matrix. This technique can provide relatively comprehensive drug testing coverage for anabolic steroids[40] since the parent drug is detectable in plasma for a lot of compounds following intramuscular administration. The presence of a 4-ene moiety adjacent to the the 3-keto function, which is present in a large number of anabolic agents, allows these compounds to be ionised effectively for analysis by LC-MS.

However, as previously described earlier in this chapter, the oral route of drug administration results in extensive first pass metabolism of certain anabolic steroids,

with little or no parent drug being excreted in some cases, and reduction of structural double bonds produces relatively poorly-ionisable, fully saturated steroid metabolites, which are unsuitable for effective LC-MS analysis[33,36]. Therefore, GC-MS and now the recently revitalised GC-MS/MS market still plays a significant role in sports drug testing labs for the analysis of androgenic-anabolic steroid metabolites, particularly if more informative mass spectra are required.

The choice of analytical method will largely depend upon the matrix being analysed, the chemical nature of the analytes of interest in that matrix and the required context for the results obtained. In recent years blood analysis has made its way into mainstream animal sports testing, providing the sports regulator with a means to take a large number of samples from numerous animals in a relatively short space of time. This can be a useful approach depending upon the requirements of such testing and the actual timing of the sampling. The British Horseracing Authority (BHA) currently operates a system of testing horses in their training stables to detect potential steroid abuse that may be occurring during the lead up to the actual race day.

However, at present (analogous to GC-MS vs LC-MS), urine still represents the main matrix of choice over blood for regular routine drug testing in sport (both animal and human) and therefore the vast majority of research over the years has focussed on developing methods to provide routine detection drug capability in this matrix. Urine samples will usually contain a greater concentration of analytes available for analysis and will also provide a greater retrospective window of detection for compounds that may have been administered many days or even weeks prior to the test sample collection date.

The process of successful steroid analysis and data interpretation can involve various stages, including sample preparation, extraction, derivatisation and instrumental analysis. Focussing on analysis of urine by GC-MS the various stages are summarised as follows:-

1.7.1 Sample preparation

The conjugated status of the analytes needs to be taken into consideration when developing methods for the analysis and detection of anabolic steroids in biological matrices.

Removal of phase II conjugate groups is necessary for analysis prior to GC-MS. Intact conjugates can be analysed by LC-MS/MS for certain compounds, but this is still a complex analysis for a multi residue screening approach, with reduced detection capability for some analytes, although progress is being made in this area[41].

Hydrolysis of steroid glucuronides usually involves the use of β-glucuronidase from E.coli, which is relatively pure and provides a clean glucuronide fraction analysis. The process usually involves incubation at a suitable pH and temperature for a number of hours. An enzyme preparation from Helix Pomatia (HP) also cleaves glucuronides but contains aryl sulphatase activity as well, allowing it to cleave primarily aryl sulphates and to a much lesser extent, some alkyl sulphates (but this is stereochemistry dependant), which could lead to miss-assignment of a metabolite to the wrong Phase II fraction. HP has also been shown to produce steroid analyte artefacts under certain conditions[42]. However, with knowledge of its enzyme activity HP has been used successfully for specific applications[43].

Hydrolysis of sulphates can be achieved by mild acid hydrolysis or solvolysis. Strong acid hydrolysis or methanolysis techniques can remove both glucuronide and sulphate conjugates in one step, which can be advantageous to speed up analysis under certain circumstances. However, this process can result in artefact formation and degradation of certain steroids[44,45] so is not recommended for initial research purposes.

Hydrolysis of conjugates can take place as the first step in an analytical procedure (known as direct hydrolysis) but this can have implications if samples have any potential bacterial contamination/activity associated with them. If so, some bacterial degradation may already have occurred following sample collection, although this will be significantly exacerbated by incubating the sample at 37^{o}C for 12-16 hours (a standard process for hydrolysis with β-glucuronidase from E.Coli.). Therefore it is recommended to pre-extract samples prior to hydrolysis to remove any potential bacteria and their enzymes. Solid phase extraction is the best way to achieve this.

1.7.2 Extraction methods

Liquid liquid solvent extraction methods are simple approaches to the preparation of unconjugated steroid extracts from urine samples that have been utilised for many years and simply involve maximising the partition co-efficient between the chosen organic extracting solvent phase and the aqueous sample phase. Diethyl ether is a good example of an extraction solvent for this purpose.

Solid phase extraction involves the use of solid material (sorbent) appropriately supported, to which the sample is applied to allow interaction of the drug molecules with the solid material. The residual matrix (urine) is rinsed away before choosing an appropriate solvent to use for elution of the analytes from the surface of the sorbent. Early studies of solid phase extraction involved the use of resins such as amberlite XAD2[46] and sephadex LH20[47], but perhaps the most important and influential breakthrough came with the introduction of solid phase in cartridge form, most notably the C18 Sep-pak This phase was extensively studied for use in steroid extraction, including a thorough evaluation of analyte recoveries which were shown to be equal to or better than other methods of extraction[48]. In the 1980s there was a marked increase in the development of solid phase extraction (SPE) cartridges that provided a more selective and cleaner extract prior to derivatisation and analysis by mass spectrometry. C18 cartridges are essentially a silica backbone coated with silanol residues, which can then be substituted with functional groups such as C18 chains providing a hydrophobic structure with which the 4-ring steroid structures readily associate through Van der Waals interactions. SPE generally produces cleaner sample extracts than liquid–liquid, allowing for lower limits of detection and improved longevity of response from the analytical instrumentation before routine maintenance is required, which maintains instrument efficiency for longer.

More modern SPE phases have been developed in the last decade using polymer technology including various substituted styrene-divinyl benzenes (SDVB)[49]. These cartridges give a wide range of flexibility of analytical approach and some novel methods have been developed for steroid analysis using these cartridges to separate different conjugates[35].

Pure neutral extracts can also be obtained by utilising a sequential combination of SPE approaches in one procedure using mixed mode anion exchange cartridges. This can produce very clean extracts allowing for sub-nanogram steroid detection capability when coupled with a selected ion monitoring (SIM) GC-MS procedure[50]. Modern polymer phases have become invaluable in the routine screening environment due to their chemical robustness compared with silica based sorbents[49]. Silica based cartridges usually require activation of the sorbent/conditioning prior to use, and the extraction should be a continuous process to avoid any possibility of the sorbent drying out, resulting in loss of phase viability. This is in contrast with polymer phases that are not prone to phase collapse during the extraction process.

Unsubstituted silica phase cartridges are also of significant value to clean up extracts of steroids further still. Such methods can remove very polar steroids and can help with removal of a number of adrenal corticosteroid metabolites to improve the chromatographic quality and analyte co-elution issues. Nevertheless a final steroid extract that has been through a number of different sample preparation stages will still contain a complex mixture of endogenous steroids and their metabolites. Therefore, suitable chromatographic methods are still required to assist with the detection of, and if required, accurate quantitation of anabolic steroid metabolites.

Despite essentially being a group of neutral non-polar analytes the steroids have quite a variety of extraction behaviours even within that group and therefore the choice of extraction is vital, particularly when exploring all potential analytes available in a metabolism study. Many of the steroid recoveries originally established for C18 cartridges (80-90% for most analytes) are applicable today and this classical approach is a cornerstone for steroid extraction, despite the development of more novel approaches.

Solvent choice is also important in these methods to ensure that analytes are not wrongly assigned to different conjugate fractions. For C18 cartridges this can be overcome by eluting the entire fraction with methanol, splitting the eluent in half and only performing enzyme hydrolysis on one portion of eluent, then comparing the results and yields of the very polar metabolites obtained after analysis of the two separate extracts. Identical yields for a particular metabolite would indicate that it is excreted in the unconjugated form. Due to their complexity such analytical approaches are unlikely to be used in routine screening environments when being applied to large numbers of samples on a daily basis. However, this knowledge could be important if the highly polar metabolite in question appears to be the most abundant in the overall profile and potentially the most useful for detecting abuse of the parent drug.

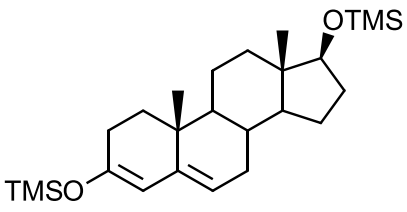
To assist with monitoring extraction processes, deuterated steroid standards have become available for inclusion into extraction processes, and in more recent years certain conjugates of deuterated standards as well. These can be quite expensive but will no doubt increase in availability and reduce in price over time.

1.7.3 Derivatisation techniques

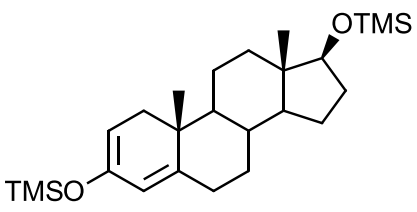
Steroids will normally require derivatisation prior to GC-MS analysis as they exhibit poor gas chromatographic properties. Steroid metabolites containing keto and hydroxyl

functions are poorly resolved on GC columns and can also undergo degradation in the injection port when underivatised. The most common approach to the derivatisation of steroids is the formation of trimethylsilyl (TMS) derivatives using N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) and this approach was first introduced in 1969[51] Hydroxy groups react quantitatively with MSTFA to produce stable TMS ethers. Sample extracts can be dissolved in MSTFA and injected directly onto the column. This will result in spontaneous formation of the TMS ether. However for more sterically hindered hydroxyl functions a catalyst is also required to force the derivatisation such as trimethylsilylimidazole (TMSI) [52,53]. Steroids containing keto functions require a different approach. One such method is condensation with amines such as hydroxyl amine or methoxyamine to form oximes[54]. The hydroxyl functions are then derivatised with MSTFA as a second reaction step. For most compounds this results in the formation of two derivative isomers and often two chromatographic peaks in a 75:25% ratio. This can be very useful as a diagnostic aid, particularly as the oxime group is stable during the second TMS ether derivatisation step, resultimg in an MO-TMS derivative.

A one step derivatisation process for both keto and hydroxyl functions has been developed[53] that produces TMS ether TMS enol ether derivatives. More than one product can be formed but in practice the shift is often 95-99% in favour of the most stable form. These derivatives are stable under GC-MS conditions. Due to handling issues with TMSI reagent, derivatisations are often performed with a reaction mixture comprising MSTFA/NH_4I/ethanethiol (2000:1:3)(v/w/v), from which the reactive TMSI is formed in situ. Like MSTFA this mixture can be injected directly onto the GC-MS system. Example structures of the derivatised products are shown in Figure 1.13.



Enol-tms product 1 (99%)



Enol-tms product 2 (1%)

Figure 1.13: Derivatised structures of testosterone enol-bis-tms[53].

The enol-tms derivatisation results in products with a greater degree of unsaturation within the steroid ring structure. As such compounds whose molecular structures already have a significantly high degree of unsaturation throughout the ring A,B,C rings (e.g trenbolone and THG) generally will not form stable enol-tms for analysis. These compounds are therefore best detected by LC-MS techniques.

A similar derivative to the TMS derivative that can also be used for steroid analysis is the tertiarybutyldimethylsilyl (TBDMS) derivative[55]. This is a more bulky derivative and requires a catalyst to assist with its formation. Enol-TBDMS derivatives can also be formed using a similar procedure to enol-tms derivatives, but the reaction mixture is not volatile enough to allow it to be directly injected on to the GC-MS system like the enol-tms mixture. Therefore a back extraction to remove excess reagent is required, which although is a more time consuming analytical process, results in a final extract that can be cleaner than the enol-tms final extract and also more stable for a longer period of time if subsequent injections of the extract are required. Unlike TMS derivatives the more bulky TBDMS derivatives are stable to hydrolysis increasing the practicality of their use in the laboratory. The spectra obtained with TBDMS derivatives exhibit intense, relatively stable, M^+ - 57 ions[55] (loss of the tertiary butyl group) and this can be particularly useful when analysing fully saturated –diol and -triol compounds whose mass spectra are often dominated by lower mass ions, making them less suitable for GC-MS/MS analysis if sensitive detection methods are required.

Other derivatives of interest are those that can assist with structural elucidation, e.g. in relation to stereochemistry of particular functional groups. Examples include acetonide derivatives[56] and cyclic boronates which will form across adjacent hydroxyl functions (1-2 diols) and in the case of the boronates across non-adjacent hydroxyl functions (1-3 diols) as well[57].

1.7.4 Gas Chromatography

Gas chromatography consists of the introduction of a small volume of volatile solvent, which contains a proportion of the derivatised sample extract, into a heated glass liner where the analytes are vaporised and swept by carrier gas (usually helium) onto a microbore capillary column. The column is coated on the inside surface with a liquid/stationary phase. The analytes in the extraction mixture are then separated based on their volatility and differing interactions with the column phase. Elution in gas chromatography is based upon the effective vapour pressure of the analyte, which is a

function of its latent vapour pressure, reduced by its solubility in the stationary phase and by the number of interactions the analyte undergoes with the stationary phase. All of these are affected by temperature and, combined, they determine the equilibrium distribution of the analyte between the two phases.

The open tubular column was invented by Golay in 1957 and since that time important advances have been made in the development of GC columns. The most significant advancement in analysis of steroids by GC has been the development of fused silica capillary columns, particularly those with deactivated surfaces and cross linked liquid/stationary phases. Common examples in use today are dimethyl polysiloxane (100% phase) or various substitutions of this phase with more polar embedded groups to alter column selectivity and the potential separation / elution order of a group of analytes (e.g. 5% phenyl, 50% phenyl). Non-polar phases tend to exhibit greater thermal stability than polar phases.

However, novel column chemistries and the incorporation of silphenylene into the column backbone has increased thermal stability of columns such as the cross linked BPX5 (5% phenyl polysilphenylene-siloxane), allowing for increased operating temperatures whilst reducing column bleed. All of these modifications serve to enhance detection sensitivity by reducing the bleed and background noise detected by the mass spectrometer compared with the signal from the eluting analytes.

With complex mixtures of steroids in the urinary extract it is important to ensure that all the analytes have eluted from the column before the end of the analytical run. Any compounds not eluted may end up being eluted during subsequent runs at much earlier retention times than is appropriate for their derivatised structure, molecular weight, boiling point etc. causing confusion in interpretation of data and, in the case of SIM data, co-elution with a key target analyte could cause quantitative interpretation errors. In practice (unpublished) some of the latest eluting urinary analytes are those from the adrenal gland such as allo-tetrahydrocortisol, the retention time and elution of which has been used as a reasonable marker for determining the appropriate end to a GC oven cycle for many of the methods used for analysis of steroid extracts in this thesis. Subsequent to this work, backflushing techniques have become relatively mainstream, meaning once the last analyte of interest has entered the mass spectrometer, the carrier gas flow can be reversed to allow rapid backflushing of the residual analytes off the column via an oulet through a switching valve. This results in shorter run times and a cleaner mass spectrometer which can be advantageous for large batches of samples.

1.7.5 Mass Spectrometry

A mass spectrometer is a sensitive analytical instrument that provides information about a chemical sample. The sample is converted into ions by interaction with high energy electrons (usually 70eV). High energy electron ionisation (EI) results in fragmentation of the molecular ion ($M^{+\cdot}$). Fragment ions are then separated according to their mass-to-charge ratios. The ions formed and their relative abundance under a specific set of conditions serves as a 'fingerprint' for analyte identification. There are various types of mass spectrometer and each includes a device to introduce the sample; an ionization source in which the sample is ionized; a mass analyser in which the charged particles are separated according to their mass ratios; ion collection, amplification and detection devices; and finally a data system for data interpretation[58]. Different instruments are capable of different degrees of discrimination between two components of similar mass or resolving power. Magnetic sector instruments have high resolution capability >10,000 (e.g. the ability to distinguish between two components of mass 100.000 and 100.010). Steroid analysis is generally carried out on quadrupole analysers or ion traps, both of which have unit resolution capability (i.e. the ability to distinguish mass 100.0 from 101.0).

1.7.5.1 Quadrupole Mass filters

A quadrupole is a mass analyser comprising four rods (coll. 'quads') arranged equidistantly in a parallel rectangle (see Figure 1.14). The ion beam from the ion source travels through the quadrupole in the z-axis. A high DC voltage in combination with a

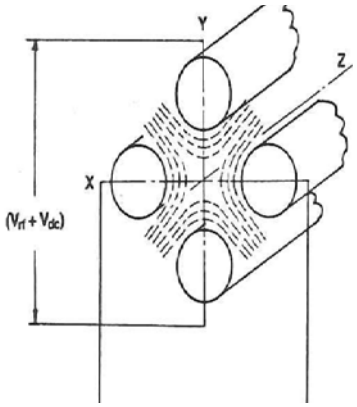


Figure 1.14 – Cross section of a quadrupole mass analyser
(reproduced from Finnigan corporation manual 1990)

radio frequency (RF) applied to the rods creates a 3D time variable field within the quadrupole. Ions travel through this field following an oscillating path. By controlling the RF and DC voltages it is possible to select only ions of interest to be transferred to the detector. This can be in the form a large mass range for full scan analysis, or for greater sensitivity, only a handful of ions of certain mass to charge ratio can be selected (selected ion mode or SIM). The mass analyser is therefore operating as a mass filter.

In a single quadrupole there is only one analyser, but a triple quadrupole has three sets effectively comprising two mass analysers with a collision cell between them (see Figure 1.15). Ions from Q1, selected for entry into the collision cell (Q2) on the basis of their m/z can be further fragmented (usually using collision with a high energy inert gas e.g. argon) and the resulting fragments can be separated in the third quadrupole (Q3) and detected. As with single quad analysis, Q3 can scan over a large range to produce a product-scan mass spectrum or for just a handful of product ions of selected mass to charge ratios (known as selected reaction monitoring [SRM] transitions).

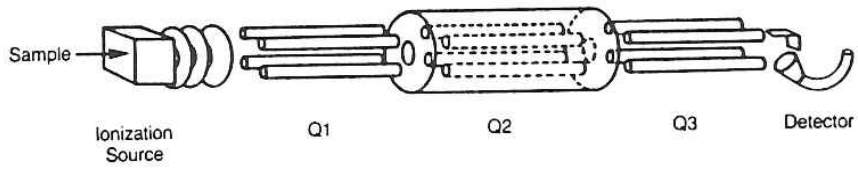


Figure 1.15: Schematic of a triple quadrupole mass analyser
(reproduced from Finnigan corporation manual 1990)

1.7.5.2 Ion Trap mass spectrometer

Mass spectrometers based on ion trap technology are different in design from other MS systems. Ion traps combine the functions of an ion source and mass analyser in one location. This is achieved in a cylindrical ion trap by a simple three electrode assembly, consisting of a ring electrode and two end caps (see Figure 1.16). Electrons from a heated filament are pulsed into the central cavity by a gate electrode, where they ionize the molecules. The ion trap is then scanned from low to high mass by increasing the RF potential on the ring electrode. The unique feature of ion trap mass spectrometers is that they trap and store ions generated over time within the trap cavity. Applying RF voltage to the central ring electrode causes ions of interest to be trapped and

accumulated in the ion source. The trapped ions are sorted by changing the electric field inside the trap by manipulating the RF field and then sequentially ejecting the ions from the trap according to their mass-to-charge ratio (from low to high mass) into the electron multiplier where they are detected. The effective 'trapping' and accumulation of ions results in the concentration of the ions of interest and consequently in the very high sensitivities obtainable with the ion trap detector in full-scan mode. A high mass adjust facility for the tuning parameters also allows for accumulation of much greater quantities of molecular ions, even for some compounds that are normally significantly fragmented in quadrupole analysers. This provides molecular ion information in full scan mode and a suitable precursor ion for MS/MS analyses.

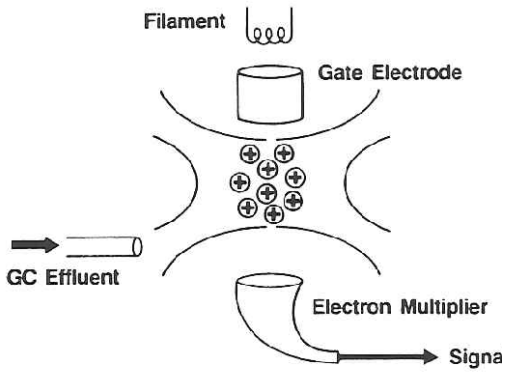


Figure 1.16: Schematic of the ion trap mass spectrometer

(reproduced from Finnigan corporation manual 1990)

1.7.6 Technical challenges and alternative techniques

Despite the major advances in technology over the years there are still technical challenges that arise during routine analysis of anabolic steroids. These include the continued use of endogenous steroids such as testosterone. In the human testing field testosterone is detected by comparing the ratio of testosterone to epitestosterone (T:E ratio). If this exceeds 4:1 it is deemed suspicious. Significantly elevated levels of testosterone are also suspicious (e.g. >200 ng/ml urine) even if the T:E ratio is less than 4:1 and in particular, if in conjunction, the epitetosterone level is also greater than 200 ng/ml. Such a scenario indicates the possible administration of both testosterone and epitestosterone, the latter being used to 'mask' the former. However, the technique of

isotope ratio mass spectrometry (IRMS) is now employed in the human testing area allowing an assessment of the $\delta^{13}C$ to $\delta^{12}C$ ratio of certain key metabolites to indicate whether the origin of the testosterone is from an exogenous source, as synthetic drug preparations contain a slightly higher proportion of the ^{13}C isotope[13,59,60]. The technique is relatively complex and certainly not mainstream in all laboratories but has been used to successfully confirm the use of endogenous steroids of a synthetic origin. However, this is not without its challenges though, as it has been recently suggested that underground labs are now using different plant based materials for their synthetic production, such that the isotopic signature of the resulting compounds is more closely aligned to the endogenous ratio, which reduces the ability of the test to discriminate a difference. To date the technique has only been applied with minimal use for anabolic steroid detection in the animal sport testing industry[61] but it may have further applications in the future.

1.8 PROJECT OVERVIEW

The aim of this project is to apply extraction, derivatisation and GC-MS techniques to the structure elucidation and quantitation of metabolites of anabolic and progestagenic steroids in the greyhound. The outcomes of these studies will support drug testing programmes in the greyhound racing industry. Knowledge of steroid metabolism is essential for the identification of key analytes to underpin a successful drug screening programme for both synthetic and endogenous compounds in animal sports. To date there is relatively little published information on the metabolism and detection of anabolic steroids in the racing greyhound and there are no established population based reference ranges for endogenous steroids, to support methods to control the abuse of testosterone; one of the major challenges of the sports drug testing industry as a whole. The thesis contains four experimental chapters:-

Chapter 2 describes an investigation into the metabolism of the anabolic steroid methyltestosterone, using reference material synthesis coupled with derivatisation techniques and mass spectrometry to elucidate the major metabolites excreted in the greyhound.

Chapter 3 describes an investigation into the metabolism of the progestagenic steroid norethisterone, using the procedures outlined for the methyltestosterone investigation, along with an analysis of the proprietary preparation of norethisterone to provide some insight into the metabolic profile observed.

Chapter 4 describes an investigation into the metabolism of the endogenous steroid testosterone to identify key marker analytes for quantitation in a population of blank greyhound bitches: thereby establishing an understanding of the ‘normal’ levels of these metabolites in female greyhound urine. This data would support the implementation of a threshold for these metabolites to allow the administration of testosterone to be controlled by the greyhound racing industry.

Chapter 5 describes the development of a quantitative extraction procedure for determining the levels of testosterone and progesterone metabolites in greyhound urine. The methodology was used to produce longitudinal urinary profiles of these key endogenous metabolites, throughout the oestrus cycle in a population of greyhound bitches.

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CHAPTER

METABOLISM OF METHYLTESTOSTERONE

2. SYNTHETIC STEROID METABOLISM: METHYLTESTOSTERONE

2.1 INTRODUCTION

The abuse of anabolic steroids for performance enhancement in sports is well documented and has been discussed in chapter 1. A number of metabolism studies have been undertaken in various species other than the greyhound, in order to identify the key metabolites which enable the detection of the administration of steroids for regulatory drug testing procedures.[1-5]. The use of anabolic steroids for performance enhancement both in training and during competition is strictly prohibited by the Greyhound Board of Great Britain (GBGB). The use of specifically licensed steroidal oestrus suppressants is currently permitted for racing greyhound bitches, both in Great Britain[6] and internationally. Veterinary preparations of both progestagens and androgens are available for such use, but the androgenic compounds are currently banned in Great Britain under the GBGB rules of racing (see chapter 1). Repeated use of certain anabolic agents to suppress the oestrus cycle may have unwanted side effects on the animals, which is of concern to the GBGB, whose aim is to try and eradicate the use of androgenic anabolic steroids from greyhound racing in Great Britain. Methyltestosterone is an anabolic agent that has been widely used as an effective oestrus suppressant for many years particularly in America, but racing greyhound bitches may also benefit substantially from the primary pharmacological effect of the drug, namely its anabolic activity[7]. As an orally active compound of the 17α-alkyl group of steroids it may also cause liver damage if used regularly for long periods. Therefore its use as an oestrus suppressant is now prohibited and so its potential abuse requires strict control.

Anabolic steroids tend to be extensively metabolised in mammalian species and the development of effective control procedures in sport requires knowledge of the major urinary metabolites to target, for drug screening and confirmatory analysis. The metabolism of methyltestosterone has previously been investigated in detail in human[4,8] and horse[3,9,10] but to a lesser extent in the greyhound. An early study by Mosbach et al[11] into the faecal metabolism in the dog indicated that a large proportion of an oral dose was excreted by this route; an observation also supported by Yamamoto et al[12]. Williams et al studied both the *in vivo*[13] and *in vitro*[14] metabolism of methyltestosterone in the dog using liquid chromatography-mass spectrometry (LC-

MS) techniques. However, due to the limitations of this analytical technique for steroid metabolite detection, only metabolites containing a keto function were observed, since fully reduced metabolites tend to show a poor response under electrospray ionisation conditions[15,16]. As highlighted in chapter 1 the preferred technique for the analysis of anabolic steroids in animal sports drug surveillance is gas chromatography-mass spectrometry (GC-MS). This chapter represents the first GC-MS study of the metabolism and detection of methyltestosterone in the greyhound.

2.2 MATERIALS AND METHODS

2.2.1 Solvents and chemicals

C18 Sep-Pak Vac SPE cartridges (500 mg, 6 cc, end capped) were obtained from Waters Ltd (Hertfordshire, UK). Methanol, hexane, chloroform, acetone, toluene and diethylether, Analar or HPLC grade, were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Sodium hydroxide, disodium hydrogen orthophosphate dihydrate, sodium dihydrogen orthophosphate monohydrate (AnalaR grade) and para-toluenesulphonic acid were supplied by BDH (Poole, UK). Sodium sulphate, anhydrous calcium chloride, ammonium iodide, pyridine, lipophilic sephadex, and methoxylamine hydrochloride, tert-butyl methyl ether (TBME), methylmagnesium bromide in ether and β-Glucuronidase from *Escherichia.coli* were supplied by Sigma Chemical Co. Ltd (Poole, UK). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). Ethanethiol was supplied by Aldrich Chemical Co. Ltd (Poole, UK). [$^{3}H_2$]-16,16,17-5α-androstan-3α-17β-diol (D3-androstanediol internal standard) was prepared at HFL Sport Science, Fordham, Camb., UK.

16α-hydroxy-5α-androstane-3α-ol-17-one, 16α-hydroxy-5α-androstane-3β-ol-17-one, 16α-hydroxy-5β-androstane-3α-ol-17-one, 16α-hydroxy-5β-androstane-3β-ol-17-one, 17α-methyl-5α-androstan-3α-17β-diol, 17α-methyl-5α-androstan-3β-17β-diol and 16β-hydroxytestosterone were all obtained from Steraloids Inc. (Newport, Rhode Island, USA)17α-methyl-5β-androstan-3β-17β-diol was obtained from Research Plus Inc. (Bayonne, New Jersey, USA) and 17α-methyl-5β-androstan-3α-17β-diol from Cerilliant (Austin, Texas, USA.)

2.2.2 Drug administration to animals: sample collection

Administration studies were carried out at Racing Chemistry, Iowa, U.S.A. (see Appendix II regarding ethical considerations). Pre-administration urine samples were collected from two greyhound bitches and the drug preparation Orandrone™ (Intervet UK Ltd) was administered orally to both animals at a dose of 5mg per day for 7 days. Naturally voided urine samples were collected at +2, 4, 8, 24, 36, 48, 72 hours and +4, 5, 6 and 7 days after the final dose. The urines were stored at -20^{o}C awaiting analysis.

2.2.3 Sample extraction

Aliquots of canine urine (4 mL) were spiked with D3-androstanediol (internal standard) at a level of 25 ng mL^{-1}. Extraction cartridges (C18 Sep-Pak Vac, 6 cc, 500 mg), were conditioned with methanol (5 mL) and water (reagent grade, 5 mL). The urine samples were then passed through the cartridges followed by water (5 mL). The column was washed with hexane (5 mL) and then dried with air under vacuum for 5 min. Free fraction extracts were then eluted with diethyl ether (4 mL). The glucuronide fraction was eluted with methanol (5 mL). The ether was evaporated to dryness under oxygen free nitrogen (OFN) at 40ºC in GC vials. The methanolic eluates were evaporated using OFN at 80ºC, the residue dissolved in phosphate buffer (1 mL, pH 6.8, 0.1 M) containing β-glucuronidase *E. coli* (250 µL, 10 units µL^{-1}) and the solution incubated overnight at 37ºC.

After incubation, sodium hydroxide (500 µl, 2 M) was added to each sample and the sample mixed using a Whirlimixer. Liquid-liquid extraction was carried using tertiary butyl methyl ether (TBME, 4 mL). The aqueous layer was removed and the organic solvent washed with sodium hydroxide (2 x 500 µL, 2 M). The TBME was dried over anhydrous sodium sulphate and then evaporated to dryness under OFN at 40ºC in GC vials.

2.2.4 Derivatisation procedures

2.2.4.1 Enol-tms derivatives

The Enol-tms derivatisation reagent was prepared from MSTFA (1 mL), ammonium iodide (3 mg) and ethanethiol (6 µL); the mixture being heated at 80ºC for 20 mins to dissolve solid material. The derivatising reagent (30 µL) was added to standards and

urinary extracts and the solution heated at 80°C for 1.5 – 2 hours, and a 1 µL aliquot injected on to GC-MS for analysis.

2.2.4.2 O-Methyloxime-TMS derivatives

O-methyloxime-TMS derivatives (MO-TMS) were also formed with separate extracts of post administration urines. Following solid phase extraction and evaporation to dryness under OFN, MO-TMS derivatives were formed by adding 'MO reagent' (8% methoxylamine HCl in pyridine; 100 µL) to the urinary extract and heating at 80ºC for 30 mins. The extracts were then evaporated to dryness under OFN. Trimethylsilylimidazole (50 µL) was added and the solution heated at 80°C for 2 hours. The reaction mixture was then dissolved in chloroform:hexane (1:1, 1 mL) and passed through a 20 mm Sephadex LH20 mini-column [prepared in-house]. A further aliquot of chloroform:hexane (1:1, 1 mL) was used to rinse the vial and then added to the Sephadex column. The extracts were then evaporated to dryness, reconstituted in MSTFA and analysed by GC-MS.

2.2.4.3 Acetonide-tms derivatives

Acetonide-tms derivatives were prepared using a modified version of the procedure described by Houghton et al[2]. 16β-hydroxy testosterone internal standard was added to each sample prior to derivatisation in order to demonstrate the efficiency of acetonide derivative formation. Dried urine extracts or synthesised reference standards (as applicable) were reconstituted in dried acetone (2mL) containing ρ-toluenesulphonic acid (~2 mg). Anhydrous $CaCl_2$ (~50 mg) was added and the solution was rotary mixed for 4-5 hr. Following centrifugation the supernatant was removed and evaporated to dryness in a clean glass tube under OFN at 60ºC. The dried residue was dissolved in toluene and washed with 2 M sodium hydroxide (1 mL) followed by reagent grade water (1 mL). The final aqueous layer was removed and the organic solvent dried over anhydrous sodium sulphate. The organic solvent was then evaporated to dryness. Trimethylsilyl derivatisation was performed using the Enol-tms derivatisation procedure previously described and samples analysed by Gas Chromatography-Mass Spectrometry (GC-MS). To check the potential formation of acetonide derivatives for the more polar tetrol metabolites, toluene was substituted with TBME during the washing stage of the procedure to ensure solubility and efficiency of extraction for these analytes.

2.2.5 Gas chromatography/Mass spectrometry analysis

Full Scan GC-MS analysis was carried out in positive ion electron ionisation mode, using a Thermo Finnigan (Austin, Texas, USA) DSQII bench top mass analyser instrument fitted with an SGE (SGE Europe Ltd, Milton Keynes, U.K.) BPX5 column, (approx. 29m, 0.25 mm id, 0.25 μm film thickness). The temperature of the split/splitless injector was 260ºC. Injections were made in the splitless mode which was maintained for 1.0 minute. The interface temperature was 260ºC. Injection volume was 1 μl. The GC temperature programme was started at 180 ºC and held for 0.5 min. Initial ramp 180ºC to 290ºC at 3ºC min^{-1} and then further from 290 ºC to 330ºC at 40 ºC min^{-1}. The final temp was held at 330ºC for 3 min. Data was acquired from 8 to 41 min.

2.2.6 Synthesis of 17-methyl-5-androstane-3,16α,17-triol stereoisomers

5α-androstane-3α,16α-diol-17-one, 5α-androstane-3β,16α-diol-17-one, 5β-androstane-3α,16α-diol-17-one and 5β-androstane-3β,16α-diol-17-one (2 mg) were dissolved separately in dried ether (1 mL) to which was added methylmagnesiumbromide in dried ether (1 mL; 3M: Grignard reagent.) The solution was left overnight at room temperature. Isopropyl alcohol was then carefully added to destroy the residual Grignard reagent. Ethyl acetate (4 mL) was added to the mixture followed by 1 M hydrochloric acid (4 mL) and the mixture shaken. Following brief centrifugation, the aqueous layer was discarded and the organic layer dried with anhydrous sodium sulphate for 15 min. The organic layer was then evaporated to dryness under OFN at 80ºC. The residue was analysed by GC-MS as the TMS and acetonide-TMS derivatives.

2.2.7 NMR Spectroscopy of synthetic starting material

NMR spectroscopy experiments were carried out to determine the authenticity of the starting material (5β-androstane-3α,16α-diol-17-one) used to synthesise the major metabolite identified in this study. Two experiments were performed using a Bruker (Coventry, UK) Advance DRX500 spectrophotometer to determine both the purity and verify the structure of the compound.

Purity was ascertained by the acquisition of a C13 spectrum. A single set of signals demonstrated the presence of only one compound. An NOESY (Nuclear Overhauser

Effect) experiment showed that the 18-methyl group was on the same face of the molecule as the proton at C-16, thereby indicating that the hydroxyl function at C-16 was in the alpha configuration.

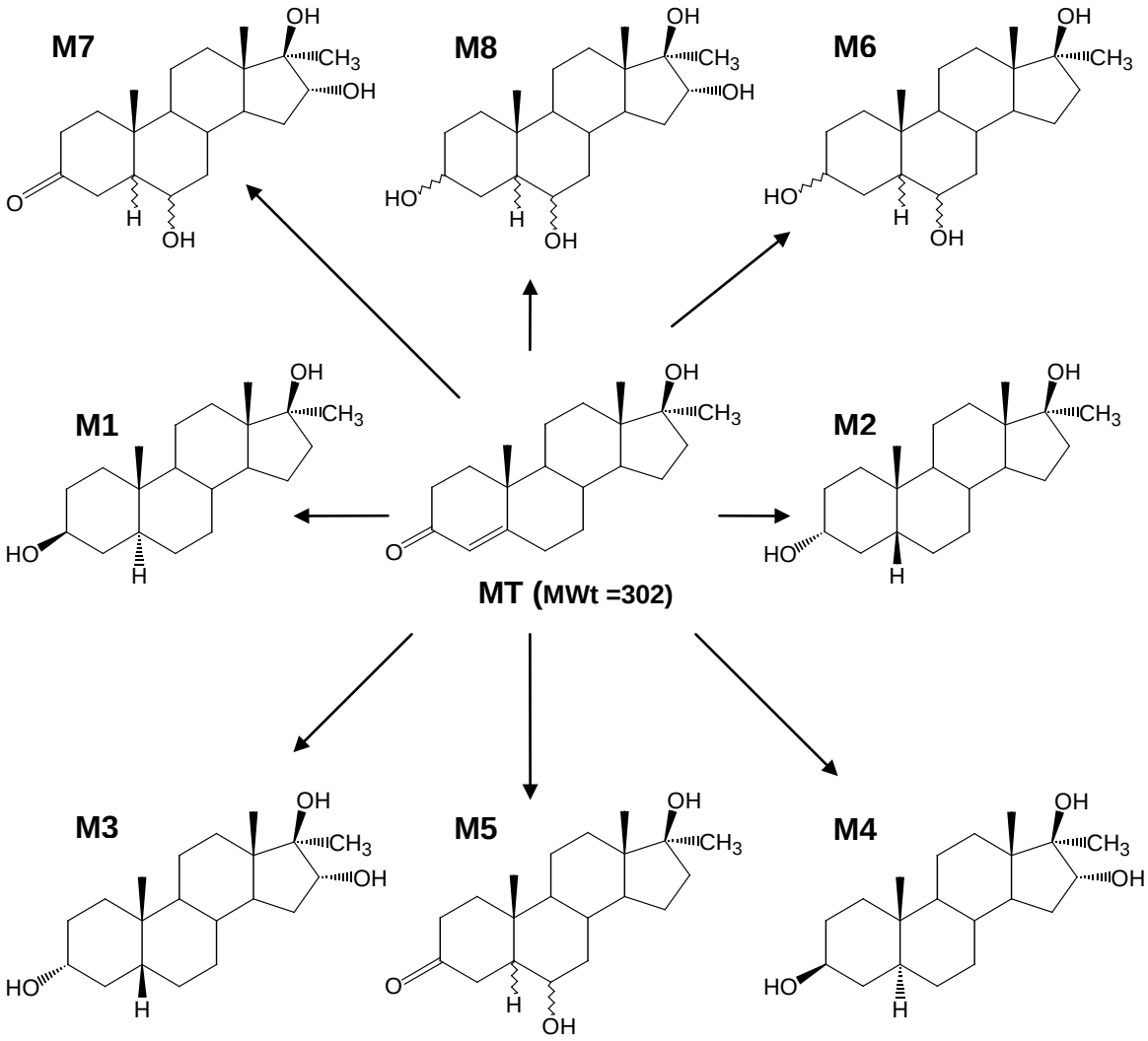
2.3 RESULTS AND DISCUSSION

The GC-MS analysis of enol-tms derivatised urinary extracts of the post-administration urine samples, resulted in the identification of several different metabolites of methyltestosterone: no parent drug was observed. Phase one transformations involved reduction of the 4-ene group, either individually or in conjunction with the keto group at C-3 along with hydroxylations at C-16 and possibly at C-6. The mass spectra of the C16 hydroxylated di-hydroxy metabolites indicate that the second hydroxylation is not associated with the D-ring. The metabolites that have been identified or tentatively identified are listed in Table 1. The sulphate fraction was not analysed as previous studies by the authors (unpublished data) and other workers[12,13] have indicated that sulphate conjugation does not appear to be a significant phase II metabolic pathway in the canine.

Table 2.1: GC retention time (RT) data, relative abundance and conjugated status of urinary methyltestosterone metabolites as TMS derivatives.

Urinary metabolites of methyltestosterone	RT mins	MWt (tms)	Base peak (*m/z*)	% of total*	Conj. status
17α-methyl-5α-androstan-3β,17β-diol (M1)	25.94	450	143	25%	G
17α-methyl-5β-androstan-3α,17β-diol (M2)	23.99	450	143	2%	G
17α-methyl-5β-androstan-3α,16α,17β-triol (M3)	27.95	538	218	45%	G
17α-methyl-5α-androstan-3β,16α,17β-triol (M4)	30.06	538	218	5%	G
?17α-methyl-5z-androstan-6z,17β-diol-3one (M5)	28.87	536	143	15%	G
?17α-methyl-5z-androstan-3z,6z,17β-triol (M6)	26.48	538	143	3%	G
?17α-methyl-5z-androstan-6z,16α,17β-triol-3one (M7)	31.47	624	218	2%	**F:G
?17α-methyl-5z-androstan-3z,6z,16α,17β-tetrol (M8)	30.79	626	218	3%	**F:G

***total excreted** based on TIC areas of these eight metabolites in the +4hr and +8hr urines from both animals. **F- represents analytes detected in the ether fraction (non-hydrolysed). G – represents analytes detected in the methanol fraction (enzyme hydrolysed) MWt (tms): Derivatised molecular weight.



Scheme 2.1: Structures of methyltestosterone (MT) and its canine urinary metabolites: 17α-methyl-5α-androstan-3β,17β-diol (M1); 17α-methyl-5β-androstan-3α,17β-diol (M2); 17α-methyl-5β-androstan-3α,16α,17β-triol (M3); 17α-methyl-5α-androstan-3β,16α,17β-triol (M4); ?17α-methyl-5z-androstan-6z,17β-diol-3one (M5); ?17α-methyl-5z-androstan-3z,6z,17β-triol (M6); ?17α-methyl-5z-androstan-6z,16α,17β-triol-3one(M7); ?17α-methyl-5z-androstan-3z,6z,16α,17β-tetrol(M8).
(? = tentative assignment)

2.3.1 17-methylandrostane-3,17-diols

Two fully reduced 17α-methylandrostanediol metabolites were detected by comparison with authentic reference standards (Scheme 2.1); 17α-methyl-5α-androstan-3β-17β-diol (M1) and 17α-methyl-5β-androstan-3α,17β-diol (M2). The mass spectra of these two compounds (see figures 2.1 and 2.2) were dominated by the well documented fragment *m/z* 143, assigned to the unaltered D-ring of a 17α-methyl steroid[3,17], (see

scheme 2.2a), with molecular ions of *m/z* 450 and fragments ions of *m/z* 435 $[M\text{-}CH_3]^+$, *m/z* 345 $[M\text{-}CH_3\text{-}TMSiOH]^+$ and *m/z* 255 $[M\text{-}CH_3\text{-}2xTMSiOH]^+$.

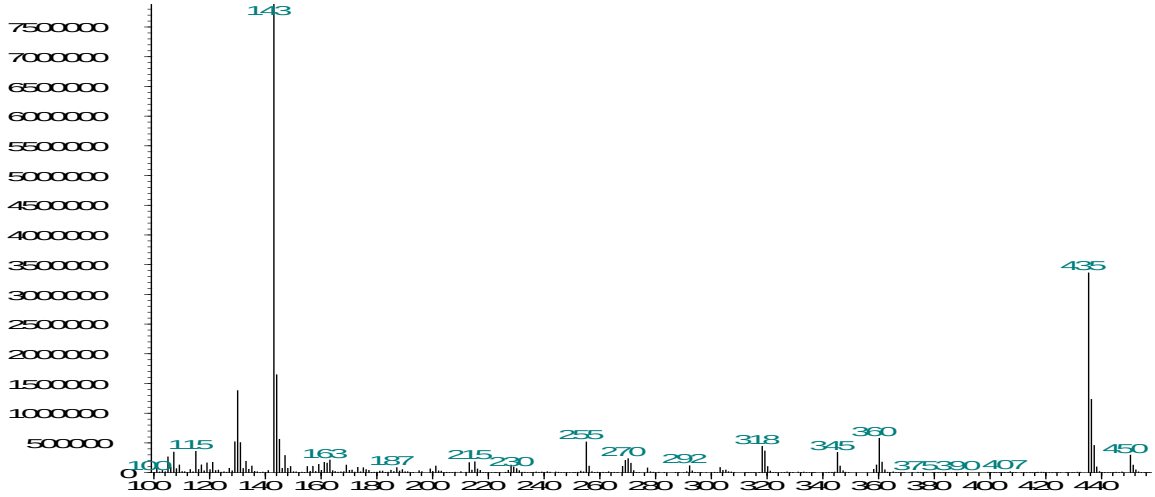


Figure 2.1: Full scan mass spectrum of 17α-methyl-5α-androstan-3β-17β-diol (M1) - enol-tms derivative

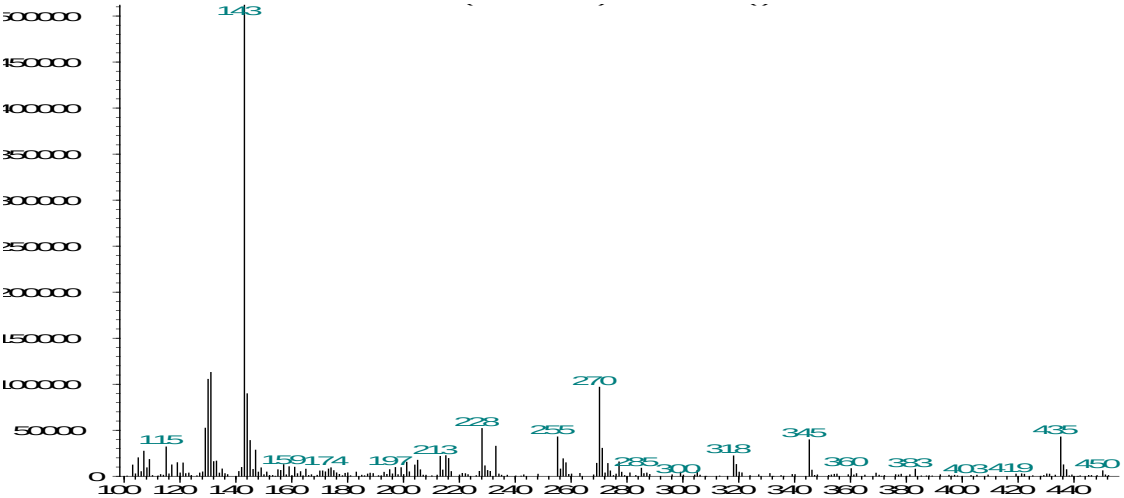
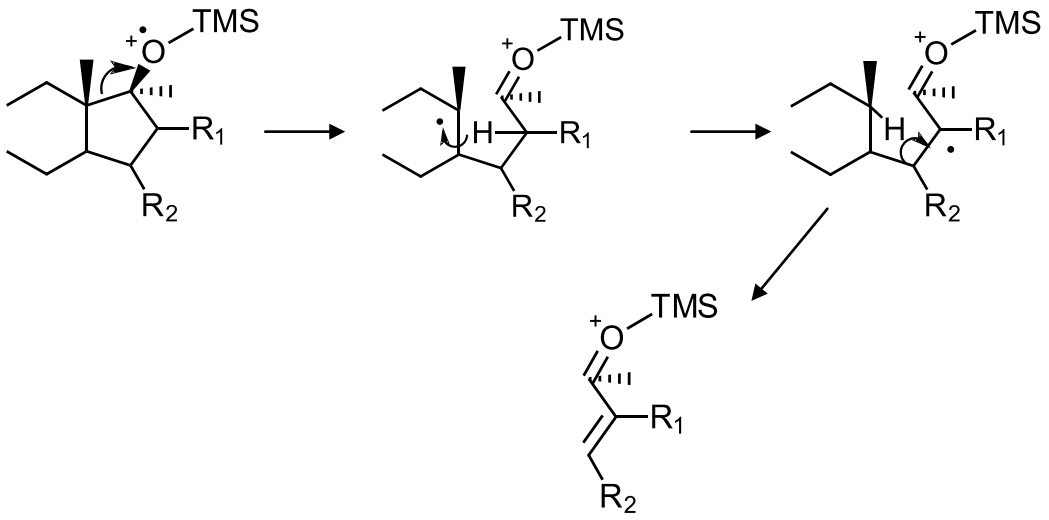
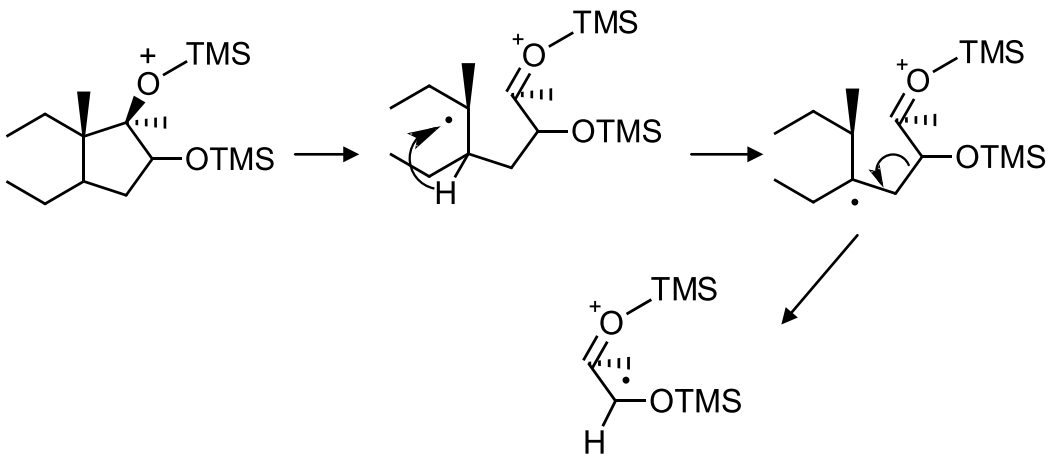


Figure 2.2: Full scan mass spectrum of 17α-methyl-5β-androstan-3α,17β-diol (M2) - enol-tms derivative

Both metabolites were detected exclusively in the glucuronide fraction with the 17α-methyl-5α-androstan-3β-17β-diol isomer excreted in significant quantities and being one of the major metabolites observed in this study (see Table 2.1).



Scheme 2.2a: D ring fragmentation mechanism to produce *m/z* 143 (R1 and R2 = H) and 231 (R1= O-TMS and R2 = H)



Scheme 2.2b: D ring fragmentation mechanism of 17α-methyl 16-hydroxy metabolite to produce *m/z* 218.

The presence of both *m/z* 218 and *m/z* 231 ions, indicates the presence of a 16-hydroxy group in the D-ring. A 15-hydroxy group would produce only the *m/z* 231 ion.

2.3.2 17-methylandrostane-3,16,17-triols.

The most abundant (based on signal intensity in the TIC) metabolite (M3) present in the post administration urine was excreted in such large quantities (see Table 2.1), that it was desirable to try and elucidate its structure in order to understand more about the major phase I metabolic pathways in the greyhound. The metabolite showed a molecular ion of *m/z* 538 with major fragment ions at *m/z* 218 and *m/z* 231. This indicated a bis-reduced mono-hydroxy metabolite and the major fragment ions have previously been identified as the characteristic D-ring fragments from 16-hydroxy metabolites of 17α-methyl anabolic steroids[3] (see Scheme 2.2b). The full scan mass spectrum of the enol-tms derivative of this compound is shown in Figure 2.3.

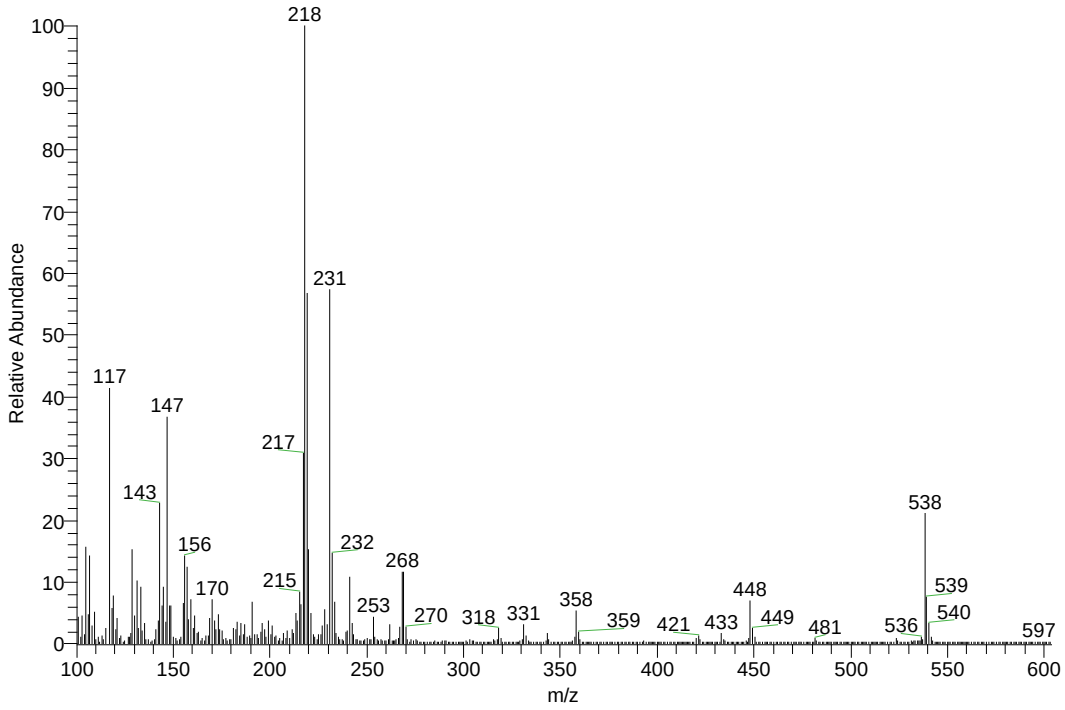


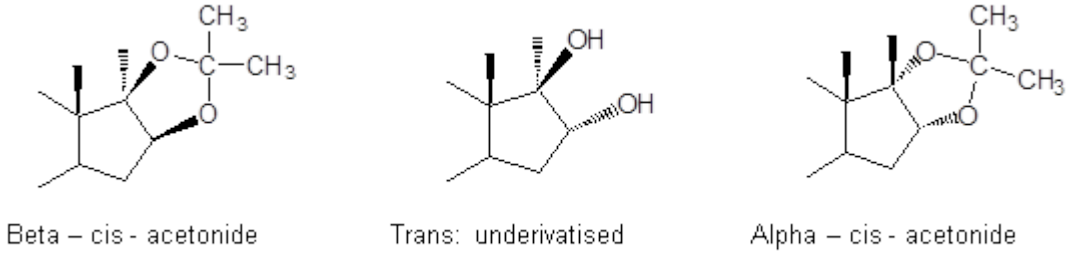
Figure 2.3: Full scan mass spectrum of M3 enol-tms derivative

The other major fragment ions of *m/z* 448, *m/z* 358 and *m/z* 268 represent three sequential losses of 90Da (3xTMSiOH groups), indicating three hydroxy functions are present in the metabolite structure. This metabolite was therefore identified as an isomer of 17-methylandrostane-3,16,17-triol (M3).

A second metabolite with the same mass spectrum and different GC retention time was also detected but was present in much smaller quantities than the major metabolite and was believed to be a second stereoisomer of 17-methyl-5-androstane-3,16,17-triol. An

initial experiment to elucidate the stereochemistry of the D-ring was performed, which involved the formation of an acetonide derivative. The GC-MS analysis of urine extracts subjected to this derivatisation procedure resulted in the same tris-tms derivative formation and mass spectrum ($M^{+\cdot}$ 538) for this analyte, thereby indicating that no acetonide derivative had been produced. Acetonide derivatives will only form across adjacent cis hydroxy functions and are not observed for trans isomers (see Scheme 2.3). The internal marker, 16β-hydroxytestosterone (a cis-diol) added to all the extracts analysed, formed an acetonide under the conditions used. The administered drug, 17α-methyltestosterone has the 17β-hydroxy configuration and it was therefore concluded that the 16-hydroxylated metabolites had the 16α-configuration.

Scheme 2.3: Acetonide derivatisation for 16,17-diol moieties:



Beta – cis - acetonide Trans: underivatised Alpha – cis - acetonide

To establish the stereochemistry of the major metabolite (M3) at C5 and C3 a Grignard synthesis was performed on four stereoisomers of 16α-hydroxy-5-androstane-3-ol-17-one. The reaction produced one major and one minor product in each case, both producing full scan mass spectra similar to that shown in Figure 2.3 when subjected to the enol-tms derivatisation procedure. The products were then derivatised using the acetonide-tms procedure, which resulted in the major synthetic products forming acetonide derivatives with $M^{+\cdot}$ ions of *m/z* 434 and fragment ions *m/z* 419 $[M - CH_3]^+$, *m/z* 359 $[M -(CH_3)_2CO_2H]^{\cdot+}$ and 269 $[M - CH_3]^+$. The minor products produced tris–tms derivatives as before. Since the Grignard reaction can only produce two products it was concluded that the minor product in each case was the 17α-methyl-17β-hydroxy-stereoisomer; the major products being the 17β-methyl-17α-hydroxy-stereoisomers. The GC retention times for each of the reference standards produced are shown in Table 2.2 The chromatographic conditions used enabled separation of all eight stereoisomers allowing a relative retention time comparison with the metabolite peaks in the post administration urine extract. When compared with the retentions times of

the urinary metabolites (Table 2.1) the major excreted metabolite was identified as 17α-methyl-5β-androstan-3α,16α,17β-triol (M3), with the minor metabolite being 17α-methyl-5α-androstan-3β,16α,17β-triol (M4). The stereochemistry of these metabolites at C5 and C3 are the same as that for the 17-methylandrostane-3,17-diol metabolites identified, although the quantities excreted are reversed (Table 2.1), perhaps suggesting that the 5β3α A-ring configuration is more amenable to subsequent 16-hydroxylation than the 5α3β; possibly due to steric 'fit' with the metabolic enzyme.

Table 2.2: GC retention time data for the TMS derivatives of the 17α-methylandrostane-3,17-diol and 17α-methylandrostane-3,16α,17-triol reference standards

Reference standards	Derivatised MWt (tms)	Retention time of tms derivative (mins)
17α-methyl-5α-androstan-3β,17β-diol	450	25.93
17α-methyl-5β-androstan-3α,17β-diol	450	23.98
17α-methyl-5α-androstan-3α,17β-diol	450	23.81
17α-methyl-5β-androstan-3β,17β-diol	450	23.40
17α-methyl-5β-androstan-3α,16α,17β-triol	538	27.94
17α-methyl-5β-androstan-3β,16α,17β-triol	538	27.59
17α-methyl-5α-androstan-3α,16α,17β-triol	538	28.04
17α-methyl-5α-androstan-3β,16α,17β-triol	538	30.06
17β-methyl-5β-androstan-3α,16α,17α-triol	538	24.52
17β-methyl-5β-androstan-3β,16α,17α-triol	538	24.74
17β-methyl-5α-androstan-3β,16α,17α-triol	538	28.38
17β-methyl-5α-androstan-3α,16α,17α-triol	538	25.08

There is the possibility that the major 16-hydroxy metabolites detected could be 16β-hydroxy-17-epimers (17α-hydroxy-17β-methyl-), analytes which also would not form acetonide derivatives. However, epimerisation of 17α-methyl steroids in human and horse has been shown to be associated with hydrolytic removal of sulphate conjugate groups, resulting in analytes being present in the free urinary fraction as well as the glucuronide fraction[4,18,19]. Previous studies have also indicated that sulphate conjugation is not a major pathway in the greyhound[12,13] and the major 16-hydroxy metabolites observed in this study were all exclusively detected as glucuronides. The

presence of 17-epimeric metabolites in the glucuronide fraction has been observed to an extent in humans and is believed to be partly due to enterohepatic circulation of metabolites[18], but this process has previously been reported as relatively minor in the dog despite significant biliary excretion[12].

2.3.3 Additional $C_{20}O_3$ metabolites

Two further metabolites with base peaks of *m/z* 143 were also detected in the post administration urine. The enol-tms derivative full scan mass spectrum of the first of these is shown in Figure 2.4. The spectrum shows an $M^{+\cdot}$ ion of *m/z* 536 with fragments ions of *m/z* 521 $[M - CH_3]^+$, *m/z* 446 $[M - TMSiOH]^{\cdot+}$, *m/z* 431 $[M - CH_3 - TMSiOH]^+$, *m/z* 356 $[M - 2xTMSiOH]^{\cdot+}$ and *m/z* 341 $[M - CH_3 - 2xTMSiOH]^{\cdot+}$. These data indicated a mono-reduced, mono-hydroxy metabolite.

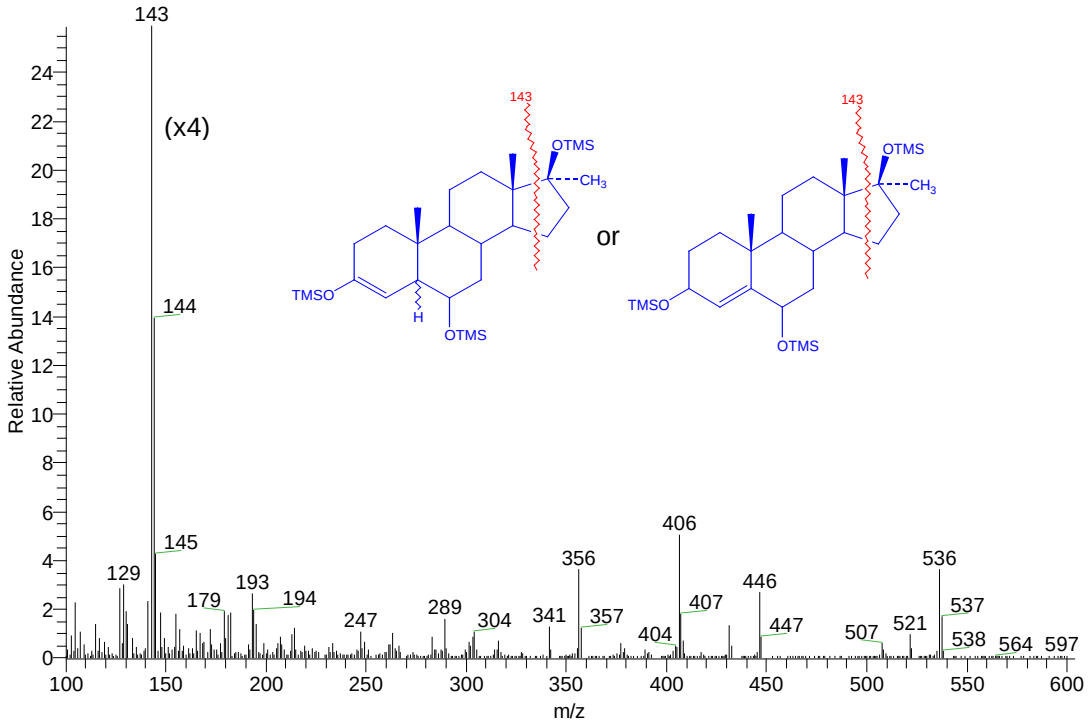


Fig 2.4: Mass spectrum of M5-enol-tms derivative (*m/z* 143 base peak is scaled to 25%)

The presence of the *m/z* 143 ion shows that the D-ring was unaltered and the site of oxidation is suggested as possibly C6, in keeping with other mammalian species such as human[2] and horse[9]. The presence of a 3-keto function was demonstrated for this metabolite by the formation of an O-methyloxime-trimethylsilyl ether derivative, which

produced a full scan mono-methoxime-bis-tms mass spectrum (see Figure 2.5) showing a relatively weak molecular ion of *m/z* 493 and fragment ions at *m/z* 478 $[M - CH_3]^{\cdot+}$, *m/z* 462 $[M - OCH_3]^{\cdot+}$, *m/z* 403 $[M - TMSiOH]^{\cdot+}$, *m/z* 388 $[M - CH_3 - TMSiOH]^{+}$, *m/z* 313 $[M - 2xTMSiOH]^{\cdot+}$ and *m/z* 282 $[M - OCH_3 - 2xTMSiOH]^{\cdot+}$. This metabolite was therefore postulated to be 17α-methyl-5z-androstan-6z,17β-diol-3-one (M5).

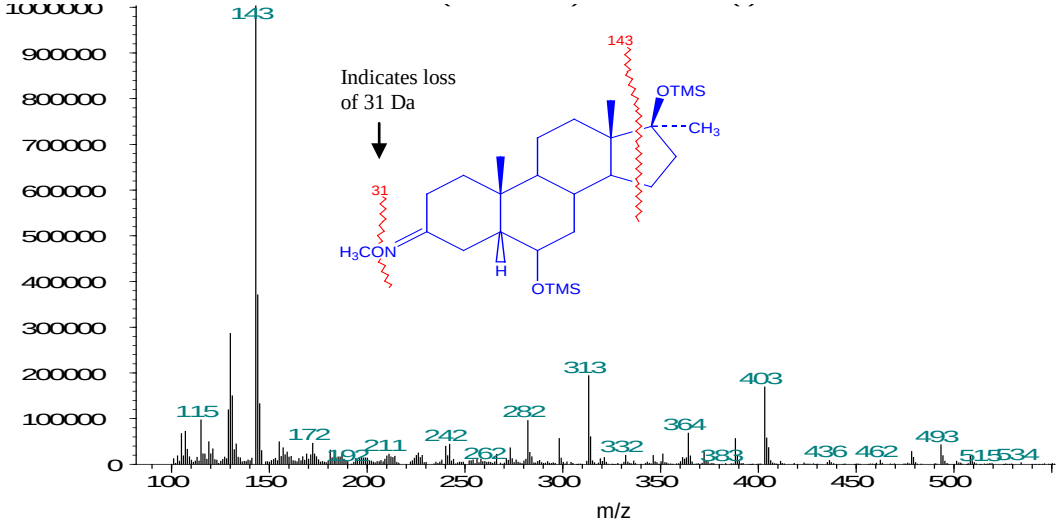


Figure 2.5: Full scan spectrum of M5 – O-methyloxime-tms ether derivative

M5 was the third most abundant metabolite observed in this study and was excreted exclusively in the glucuronide conjugate fraction. The second of the additional $C_{20}O_3$ metabolites observed showed a molecular ion of *m/z* 538, indicating a bis-reduced-mono-hydroxy metabolite. The full scan TMS-derivative mass spectrum of this metabolite is shown in Figure 2.6. As with M5 the *m/z* 143 ion was still present indicating the most likely site of oxidation to be at C-6, along with full reduction of the 4-ene-3 keto function. Similar to M3 and M4, the other major fragment ions of *m/z* 448, *m/z* 358 and *m/z* 268 represent three sequential losses of 90Da (3xTMSiOH groups), indicating three hydroxy functions are present in the metabolite structure. This metabolite was therefore postulated as a fully saturated 17α-methyl-5z-androstan-3z,6z,17β-triol (M6) and was also excreted in the glucuronide fraction. A smaller quantity of an additional stereoisomer exhibiting the same mass spectrum as M6 but with different GC retention properties was also detected suggesting the presence of another stereoisomer of 17α-methyl-5z-androstan-3z,6z,17β-triol

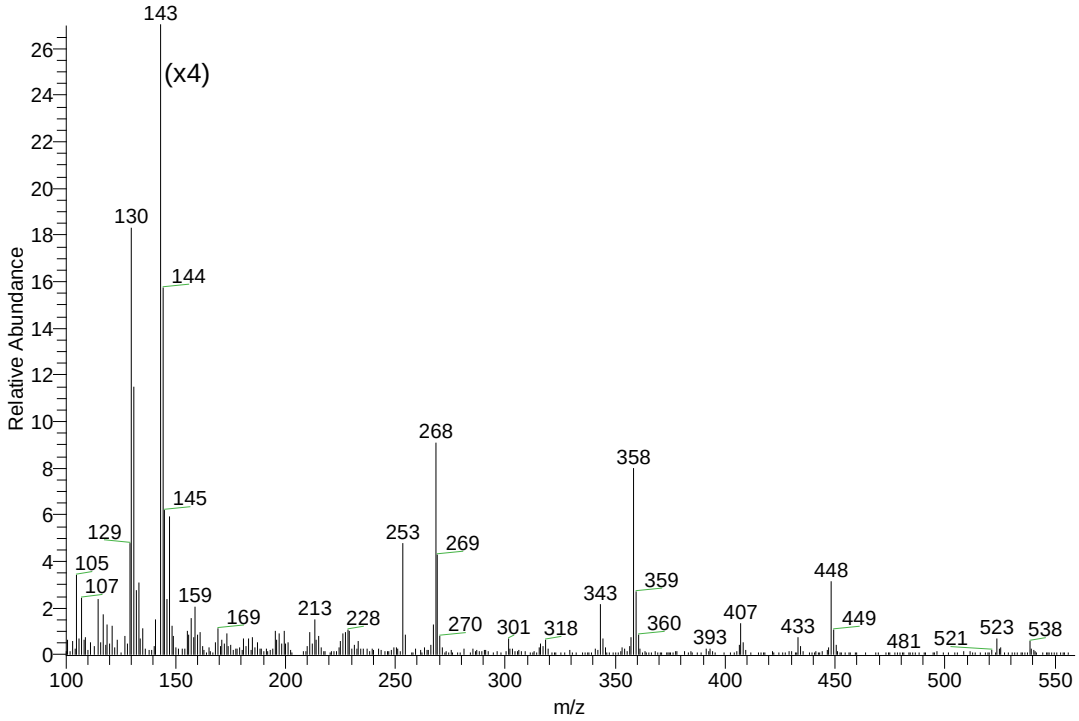


Figure 2.6: Mass spectrum of M6–enol-tms derivative (*m/z* 143 base peak is scaled to 25%)

2.3.4 $C_{20}O_4$ metabolites

Figure 2.7 shows the enol-tms derivative full scan mass spectrum of a metabolite with a molecular ion of *m/z* 624 and fragment ions at *m/z* 534 $[M - TMSiOH]^+$, *m/z* 444 $[M - 2xTMSiOH]^+$. The major ions in the mass spectrum are the diagnostic D-ring fragments for a 16-hydroxylated metabolite (*m/z* 218 and *m/z* 231). These data indicated a mono-reduced, bis-hydroxy metabolite. As with metabolite M5, this compound had only one site of reduction. This was again identified as C4/C5 by generating an O-methyloxime-trimethylsilyl ether derivative which produced a derivatised product with a molecular ion of *m/z* 581 (Figure 2.8), indicating that the 3-keto function remained intact.

The mass spectrum of this derivative showed fragments at *m/z* 550 $[M - OCH_3]^{\cdot+}$, *m/z* 491 $[M - TMSiOH]^+$, *m/z* 476 $[M – CH_3 - TMSiOH]^{\cdot+}$, *m/z* 401 $[M – 2xTMSiOH)]^+$ and *m/z* 311 $[M – 3xTMSiOH)]^+$. This metabolite was therefore postulated as 17α-methyl-5z-androstan-6z,16α,17β-triol-3-one (M7).

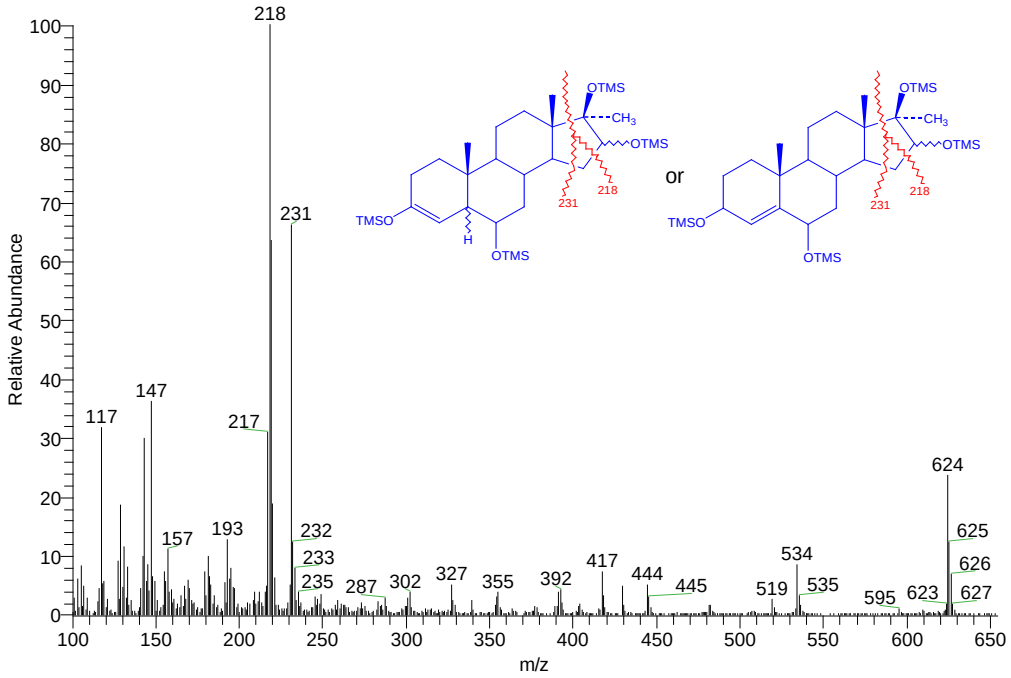


Figure 2.7: Full scan spectrum of M7 enol-tms derivative

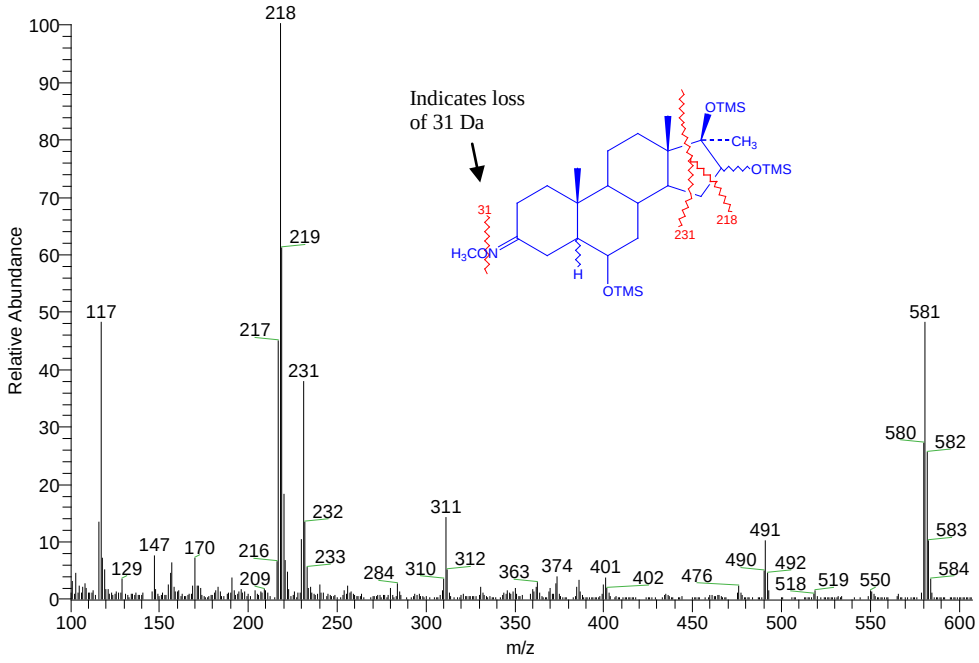


Figure 2.8: Full scan spectrum of M7 – O-methyloxime-tms ether derivative

Figure 2.9 shows the tetra-tms mass spectrum of a fully A-ring reduced tetrol metabolite with a molecular ion at *m/z* 626 and fragment ions at *m/z* 536, *m/z* 446 and *m/z* 356 representing three sequential losses of 90Da (3xTMSiOH groups), indicating

three hydroxy functions are present in the metabolite structure. The characteristic D-ring fragments *m/z* 218 and *m/z* 231 are also present. This metabolite was therefore postulated as a fully saturated 17α-methyl-5z-androstane-3z,6z,16α,17β-tetrol (M8).

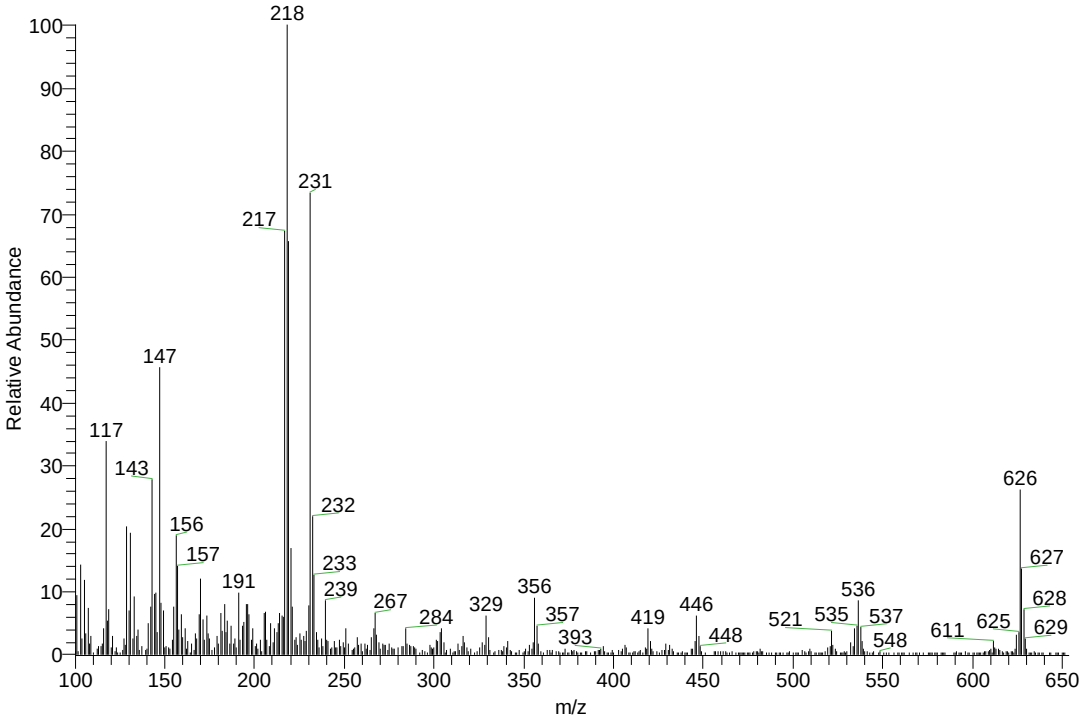


Figure 2.9: Full scan spectrum of M8 enol-tms derivative

Smaller quantities of two additional stereoisomers exhibiting the same mass spectra as M8, but with different GC retention properties were also detected. All of the $C_{20}O_4$ metabolites detected in the post administration urine were present in approximately equal amounts in both the free and glucuronide conjugate fractions. The hydroxyl functions at C-16 and C-17 for all of these compounds were all concluded to be in the trans configuration as none of these metabolites formed acetonide derivatives when subjected to that derivatisation procedure. It was therefore concluded that all the 16-hydroxylated metabolites detected had the 16α-configuration. The wash stage of the procedure was performed using TBME, as initial use of toluene resulted in the apparent 'loss' of the metabolites prior to GC-MS analysis, most probably due to the poor partitioning of these highly polar metabolites between the sodium hydroxide and toluene.

2.4 CONCLUSION

The urinary metabolites identified in this study have provided an increased understanding of the *in vivo* phase I metabolism of 17α-methyltestosterone in the greyhound. The metabolic pathways include reduction of the 4-ene-3-one group of the A-ring, and oxidations at C16 and possibly C6. The 5α3β and 5β3α stereochemistry of the fully A-ring reduced metabolites is comparable to the stereochemistry of the major metabolites observed in the metabolism of testosterone in the greyhound[20,21] (chapter 4).

The structures of the urinary metabolites reported in this study are shown in Scheme 1 and stereochemical configurations have been assigned where possible by comparison with suitable reference standards. 16α-hydroxylation would appear to be a major phase I metabolic pathway in the greyhound based upon both the prevalence and quantity of metabolites identified with that substituent group. The apparent lack of 16-beta hydroxylated metabolites is interesting in relation to other mammalian species where 16-beta hydroxylation has been shown to be a prevalent phase one metabolic transformation for many anabolic steroids[4,5,13].

A 16β-hydroxy structural configuration could be possible for the greyhound in conjunction with 17-epimerisation, but this would seem less likely for metabolites excreted exclusively in the glucuronide fraction. Considering this in relation to the $C_{20}O_4$ metabolites that were apparently present in both the free and conjugated fractions in equal amounts, it is concluded that their partitioned excretion may be based upon their polarity as opposed to hydrolytic desulphation, which has been reported in both human and equine metabolism studies. However, it may also be possible that such highly polar, polyhydroxylated metabolites are not completely eluted from C18 with ether during the extraction procedure described and that a residual amount of unconjugated metabolite may be eluted with methanol, resulting in it being potentially, erroneously assigned as a glucuronide, after hydrolysis. Future work is necessary to clarify the conjugated status of the more polar metabolites and determine whether 17-epimerisation exists as a systemic metabolic process in the greyhound.

The results of this study have supported the drug surveillance control of the administration of methyltestosterone, having been readily included in a generic extraction and GC-MS steroid screen at HFL Sport Science. The data should also

support future metabolic studies of other anabolic steroids in the greyhound. The structures of the major metabolites 17α-methyl-5β-androstan-3α-17β-diol and 17α-methyl-5β-androstan-3α,16α,17β-triol have been fully elucidated and their detection and identification in urinary extracts by mass spectrometric techniques will provide an effective approach to screening and confirmatory analysis for the control of 17α-methyltestosterone use in the greyhound.

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CHAPTER

METABOLISM OF NORETHISTERONE

3. SYNTHETIC STEROID METABOLISM: NORETHISTERONE

3.1 INTRODUCTION

The greyhound industry in Great Britain has, for a number of years, been reviewing some of the options regarding the use of steroidal preparations for oestrus suppression in the greyhound bitch and seeking an effective solution in line with a policy of banning the use of performance enhancing agents under the rules of racing[1]. As outlined in chapter 1 both anabolic agents and progestagens are available for use to suppress oestrus, but those available for use are the subject of differing concern for greyhound trainers and racing regulators.

Most of the available progestins (e.g. proligestone and medroxyprogesterone acetate) are considered to have a number of unwanted negative side effects related to racing performance in the bitch such as weight gain, lethargy and diabetes, as well as under certain circumstances potentiating the incidence of clinical conditions such as cyctic endometrial hyperplasia and pyometra. These effects can be minimised depending upon the duration of treatment and the point in the cycle at which treatment commences. The anabolic agent testosterone (licensed for oestrus suppression for many years in the industry) has no adverse performance related side effects but is clearly an androgenic anabolic agent and is therefore considered performance enhancing in the racing greyhound.

In March 2011 the Greyhound Board of Great Britain (GBGB) following consultation with the Veterinary Medicines Directorate (VMD) permitted the use of norethisterone via the cascade system (allowing a licensed human drug preparation to be used in greyhounds under certain circumstances[2]).

Norethisterone (a progestagen) has a wide variety of human uses, including the postponement of menstruation and the treatment of menstrual disorders such as endometriosis. One of the major uses is as an ingredient of oral contraceptives where it inhibits ovulation by suppressing the mid cycle surge of luteinising hormone and rendering the endometrium unreceptive to implantation. In the mid 1970's, Prole demonstrated that norethisterone could be used as an effective oestrus suppressant in the greyhound bitch[3,4] with no apparent significant side effects. Its use has therefore become popular amongst certain greyhound trainers, who wish to suppress the season of a racing greyhound bitch.

Metabolism of norethisterone has been reported in human[5] and rat[6]. There have also been a number of studies in humans relating to the detection of nandrolone metabolites following the administration of pharmaceutical preparations of norethisterone[7-10]. This has had implications when considering that nandrolone use is prohibited by the World Anti Doping Agency (WADA).

Metabolites of nandrolone have been shown to be endogenous in humans[11-19] and threshold levels are therefore in place to control its misuse in human sports. To date nandrolone metabolites have not been shown to be endogenous in the greyhound and as such there is no threshold in place for these substances. The administration of nandrolone is prohibited in UK greyhound racing.

The metabolism of norethisterone has not been previously investigated in the greyhound and this paper represents the first such study. It has been noted in previous greyhound steroid metabolism studies that liquid chromatography-mass spectrometry (LC-MS) is not suitable for the analysis of saturated steroid metabolites[20]. Gas chromatography-mass spectrometry (GC-MS) and selective derivatisation techniques have therefore been used to identify urinary metabolites of norethisterone following oral administration to the greyhound. An investigation of the pharmaceutical preparation itself has also been carried out to try and establish the origins of some of the metabolites observed. The metabolites identified in this study have been used in the routine drug surveillance screens performed at HFL Sport Science to monitor the use of norethisterone in greyhound racing and provide insight into the overall urinary profile observed for certain animals.

3.2 MATERIALS AND METHODS

3.2.1 Solvents and chemicals

C18 Sep-Pak Vac SPE cartridges (500 mg, 6 cc, end capped) were obtained from Waters Ltd (Hertfordshire, UK). Methanol, hexane, chloroform, acetone, toluene and diethylether, Analar or HPLC grade, were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Sodium hydroxide, disodium hydrogen orthophosphate dihydrate, sodium dihydrogen orthophosphate monohydrate (AnalaR grade) and para-toluenesulphonic acid were supplied by BDH (Poole, UK). Sodium sulphate, anhydrous calcium chloride, ammonium iodide, pyridine, lipophilic sephadex, and methoxylamine

hydrochloride, tertiary-butyl methyl ether (TBME), β-Glucuronidase from *Escherichia.coli* , nandrolone, 19-norandrost-4-ene-3,17-dione and norethisterone were supplied by Sigma Chemical Co. Ltd (Poole, UK). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). Ethanethiol was supplied by Aldrich Chemical Co. Ltd (Poole, UK).

$^{2}H_3$-16,16,17-5α-androstan-3α,17β-diol (d3-androstanediol internal standard) was prepared at HFL Sport Science, Fordham, Camb., UK. $^{2}H_5$-etiocholanolone (d5-etiocholanolone internal standard) and 19-noretiocholanolone glucuronide were obtained from NMI, Sydney, New South Wales 2073, Australia. 17α-ethynyl-5α-estran-3β,17β-diol, 17α-ethynyl-5β-estran-3α,17β-diol, 19-norepiandrosterone, 19-noretiocholanolone and 16β-hydroxytestosterone were all obtained from Steraloids Inc. (Newport, Rhode Island, USA).

3.2.2 Drug administration to animals: sample collection

Administration studies were carried out at Racing Chemistry, Iowa, U.S.A. Following ethics approval (Appendix II) pre-administration urine samples were collected from two greyhound bitches and the drug preparation Utovlan® (Pharmacia, Milton Keynes, UK) was administered orally to both animals at a dose of 5mg per day for 8 days. Urine samples were collected at +2, 4, 8, 24, 36, 48, 72 hours and +4, 5, 6, 7 and 8 days after the final dose. The urines were stored at -20^{o}C awaiting analysis.

3.2.3 Sample extraction

Aliquots of canine urine (4 mL) were spiked with internal markers d3-androstanediol and d5-etiocholanonlone at a level of 25 ng mL^{-1}. Extraction cartridges (C18 Sep-Pak Vac, 6 cc, 500 mg, Waters Ltd., Hertfordshire, UK) were conditioned with methanol (5 mL) and water (reagent grade, 5 mL). The urine samples were then passed through the cartridges followed by water (5 mL). The column was washed with hexane (5 mL) and then dried with air under vacuum for 5 min. Free fraction extracts were then eluted with diethyl ether (4 mL). The glucuronide fraction was eluted with methanol (5 mL). The ether was evaporated to dryness under oxygen free nitrogen (OFN) at 40ºC in GC vials. The methanolic eluates were evaporated using OFN at 80ºC, the residue dissolved in phosphate buffer (1 mL, pH 6.8, 0.1 M) containing β-glucuronidase *E. coli* (250 µl, 10 units µL^{-1}) and the solution incubated overnight at 37ºC.

After incubation, sodium hydroxide (500 µL, 2 M) was added to each sample and the sample mixed using a Whirlimixer. Liquid-liquid extraction was carried out using tertiary butyl methyl ether (TBME, 4 mL). The aqueous layer was removed and the organic solvent washed with sodium hydroxide (2 x 500 µL, 2 M). The TBME was dried over anhydrous sodium sulphate and then evaporated to dryness under OFN at 40ºC in GC vials.

3.2.4 Derivatisation procedures

3.2.4.1 Enol-tms derivatives

Enol-tms derivatives were prepared as described in chapter 2 section 2.2.4.1.

3.2.4.2 Methoxylamine-tms derivatives

Methoxylamine-tms derivatives were prepared as described in chapter 2 section 2.2.4.2

3.2.4.3 Acetonide-tms derivatives

Acetonide-tms derivatives were prepared using a previously described procedure[20] as outlined in chapter 2 section 2.2.4.3. 16β-hydroxy testosterone internal standard was added to each sample prior to derivatisation in order to demonstrate the efficiency of acetonide derivative formation. Dried urine extracts or synthesised reference standards (as applicable) were reconstituted in dried acetone (2mL) containing ρ-toluenesulphonic acid (~2 mg). Anhydrous $CaCl_2$ (~50 mg) was added and the solution was rotary mixed for 4-5 hr. Following centrifugation the supernatant was removed and evaporated to dryness in a clean glass tube under OFN at 60ºC. The dried residue was dissolved in toluene and washed with 2 M sodium hydroxide (1 mL) followed by reagent grade water (1 mL). The final aqueous layer was removed and the organic solvent dried over anhydrous sodium sulphate. The organic solvent was then evaporated to dryness. Trimethylsilyl derivatisation was performed using the Enol-tms derivatisation procedure previously described and samples analysed by GC-MS. To check the potential formation of acetonide derivatives for the more polar tetrol metabolites, toluene was substituted with TBME during the washing stage of the procedure to ensure solubility and efficiency of extraction for these analytes.

3.2.5 GC-MS analysis

Full Scan GC-MS analysis was carried out in positive ion electron ionisation mode, using a Thermo Finnigan (Austin, Texas, USA) Voyager single quadrupole bench top instrument fitted with an SGE (SGE Europe Ltd, Milton Keynes, U.K.) BPX5 column, (approx. 25 m, 0.22 mm id, 0.25 µm film thickness). The temperature of the split/splitless injector was 260ºC. Injections were made in the splitless mode which was maintained for 1.0 minute. The interface temperature was 280ºC. Injection volume was 1 µl. The GC temperature programme was started at 180 ºC and held for 0.5 min, followed by an initial ramp 180 ºC to 290 ºC at 5ºC min^{-1} and then a further ramp from 290 ºC to 330 ºC at 40 ºC min^{-1}. The final temp was held at 330ºC for 3 min.

3.2.6 GC-MS/MS analysis for confirmation of the identity of 19-norepiandrosterone in norethisterone post administration urine.

GC-MS/MS analysis was carried out in positive ion electron ionisation mode, using a Thermo Finnigan GCQ ion trap bench top instrument with an SGE BPX50 column (approx. 25m, 0.25mm id, 0.25um film thickness).

The temperature of the split/splitless injector was 275 ºC. Injections were made in the splitless mode which was maintained for 1.0 minute. The interface temperature was 280 ºC. Injection volume was 1 µl. The GC temperature programme was started at 125 ºC and held for 1.0 min, followed by an initial ramp 125 ºC to 190 ºC at 25 ºC min^{-1} and then a further ramp from 190 ºC to 240 ºC at 6 ºC min^{-1} and finally from 240 ºC to 310 ºC at 35 ºC min^{-1}. The final temp was held at 310 ºC for 1 min. GC-MS/MS acquisition was performed using a precursor ion of *m/z* 405 (representing M^+ -15 [CH3] for 19-norandrosterone-enol-bis-tms) with a collision energy of 1.0v, notch width 1.0amu, isolation time 8.0ms, collision time 15ms and a q factor of 0.45, scanning over the mass range *m/z* 100 to *m/z* 410. The source temperature was set at 220 ^oC.

3.2.7 Analysis of norethisterone (Utovlan®) tablets

One 5mg tablet was crushed and mixed with 2 mL of reagent grade water. The mix was centrifuged (3000 rpm, 5 min) and the water layer removed for extraction. A liquid/liquid extraction with 4mL chloroform:ethyl acetate (1:1) was performed for on a

rotary mixer (20 min). The mixture was centrifuged (3000 rpm, 5 mins), the organic layer removed, evaporated to dryness under nitrogen (60 ^{o}C) and reconstituted in 1 mL reagent grade water for solid phase extraction. A SPE cartridge (C18 Sep-Pak Vac, 6cc, 500 mg) was conditioned with methanol (5 mL) and water (5 mL). The tablet extract was passed through the cartridge followed by water (5 mL). The column was washed with hexane (5 mL), dried (5 min) and then eluted with diethylether (4 mL). The ether was dried over anhydrous sodium sulphate and then evaporated to dryness under nitrogen. An enol-tms derivative of the extract was prepared for GC-MS analysis as previously described. GC-MS analysis was performed in full scan mode using the GC conditions described for the confirmation analysis of 19-norepiandrosterone in post administration urine.

3.3 RESULTS AND DISCUSSION

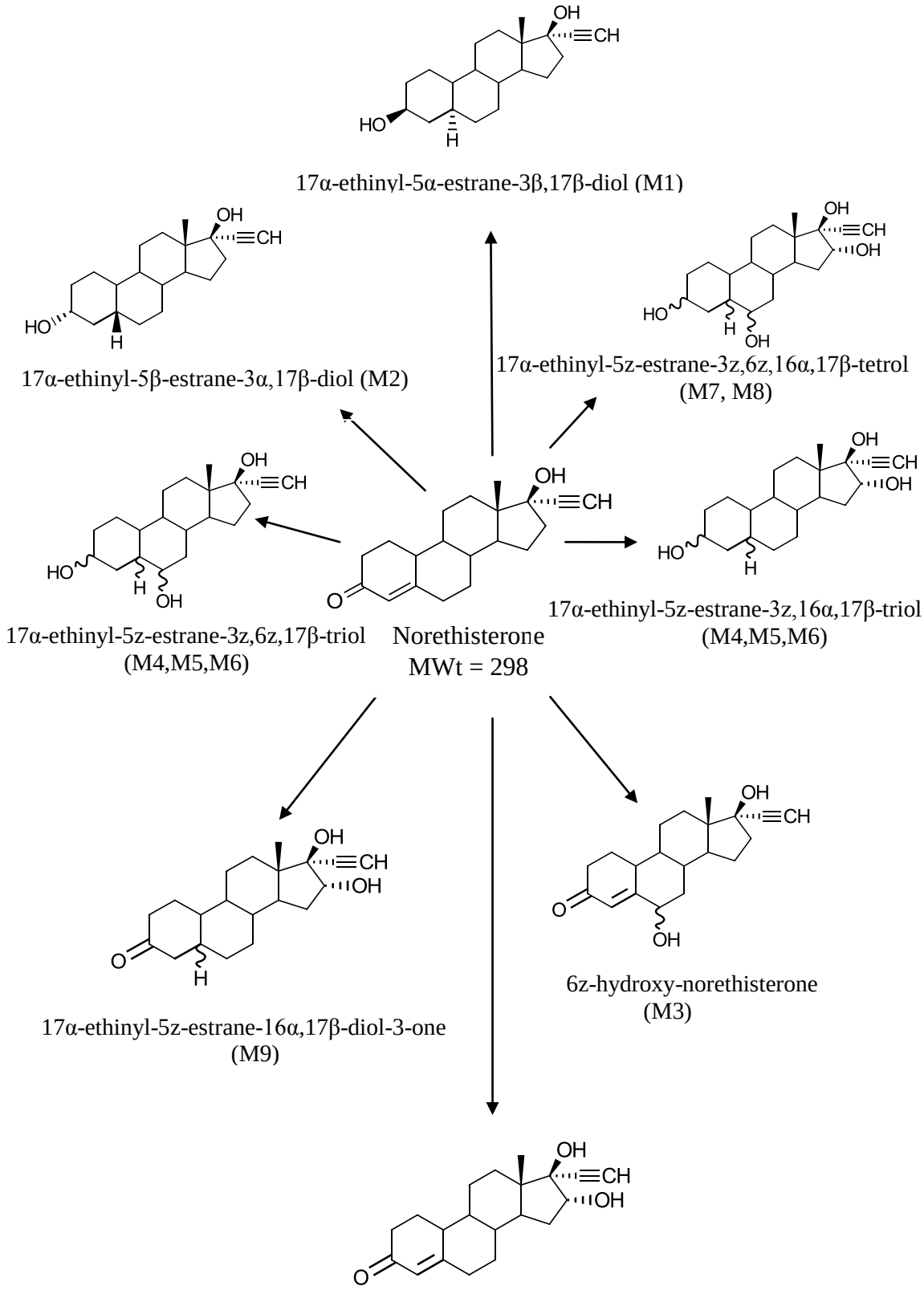
The GC-MS analysis of enol-tms derivatised urinary extracts of the post administration urine samples resulted in the identification of the parent drug norethisterone and several metabolites of norethisterone, summarised in Scheme 3.1. Phase one transformations involved reduction of the 4-ene group, either alone or, more frequently, in conjunction with the keto group at C-3. Hydroxylations presumed to be at C-6 and C-16, although it was not possible to establish the precise locations of the hydroxyl functions. The metabolites that have been either identified or tentatively suggested are listed in Table 3.1.

Selective enzymatic hydrolysis procedures indicated that the major metabolites were predominantly excreted as glucuronic acid conjugates and detection of the administration of norethisterone was possible for up to 8 days post dose (based upon detection of metabolites M4 and M5) using the methods described in this manuscript. The sulphate fraction was not analysed as previous studies by the authors (unpublished data) and others[21,22] have indicated that sulphate conjugation does not appear to be a significant phase II metabolic pathway in the canine.

Table 3.1: Summary of norethisterone metabolites with estimated distribution in free and conjugated fractions and analytical nomenclature

Urinary metabolites of norethisterone (?= tentative assignment)	Derivatised MWt (TMS)	Base Peak (*m/z*)	RT Mins	Total* Excreted	Conjugated status
Norethisterone	442	442	18.52	5%	G
17α-ethinyl-5β-estrane-3α,17β-diol (M2)	446	431	16.03	10%	G
17α-ethinyl-5α-estrane-3β,17β-diol (M1)	446	431	16.91	10%	G
?16-hydroxynorethisterone or 6-hydroxynorethisterone (M3)	530	515	19.43	10%	G
17α-ethinyl-5-estrane-triol (M4)	534	519	17.95	15%	G
17α-ethinyl-5-estrane-triol (M5)	534	519	18.66	15%	G
17α-ethinyl-5-estrane-triol (M6)	534	519	19.08	10%	G
?17α-ethinyl-5-estrane-3,6,16,17-tetrol (M7)	622	607	19.20	10%	F**,G
?17α-ethinyl-5-estrane-3,6,16,17-tetrol (M8)	622	607	19.77	5%	F**,G
17α-ethinyl-5-estrane-diol-3-one (M9)	532	517	19.06	10%	G

*based on TIC areas of these eight metabolites. **F- represents analytes detected in the ether fraction (non-hydrolysed). G – represents analytes detected in the methanol fraction (enzyme hydrolysed)



Scheme 3.1 – Identified and postulated metabolites of norethisterone in the greyhound

3.3.1 17-ethynyl-estrane-3,17-diols

Two fully reduced 17α-ethynylestranediol metabolites were detected by comparison with authentic reference standards; 17α-ethynyl-5α-estran-3β,17β-diol (M1) and 17α-ethynyl-5β-estran-3α,17β-diol (M2). The mass spectra of the TMS derivatives of these two compounds were dominated by the fragment ion *m/z* 431, $[M\text{-}CH_3]^+$ resulting from the loss of a methyl group from the molecular ion of *m/z* 446 and fragment ions of *m/z* 341 $[M\text{-}CH_3\text{-}TMSiOH]^+$. The full scan mass spectrum of M2 is shown in Figure 3.1. The 17α-ethynyl function appears to impart a significant degree of stability to the structure resulting in the intense *m/z* 431 ion, in contrast to the fully saturated '-diol' metabolites which normally exhibit a considerably greater degree of fragmentation than that observed here[20,23,24]. Both metabolites were detected exclusively in the glucuronide fraction. (see Table 3.1).

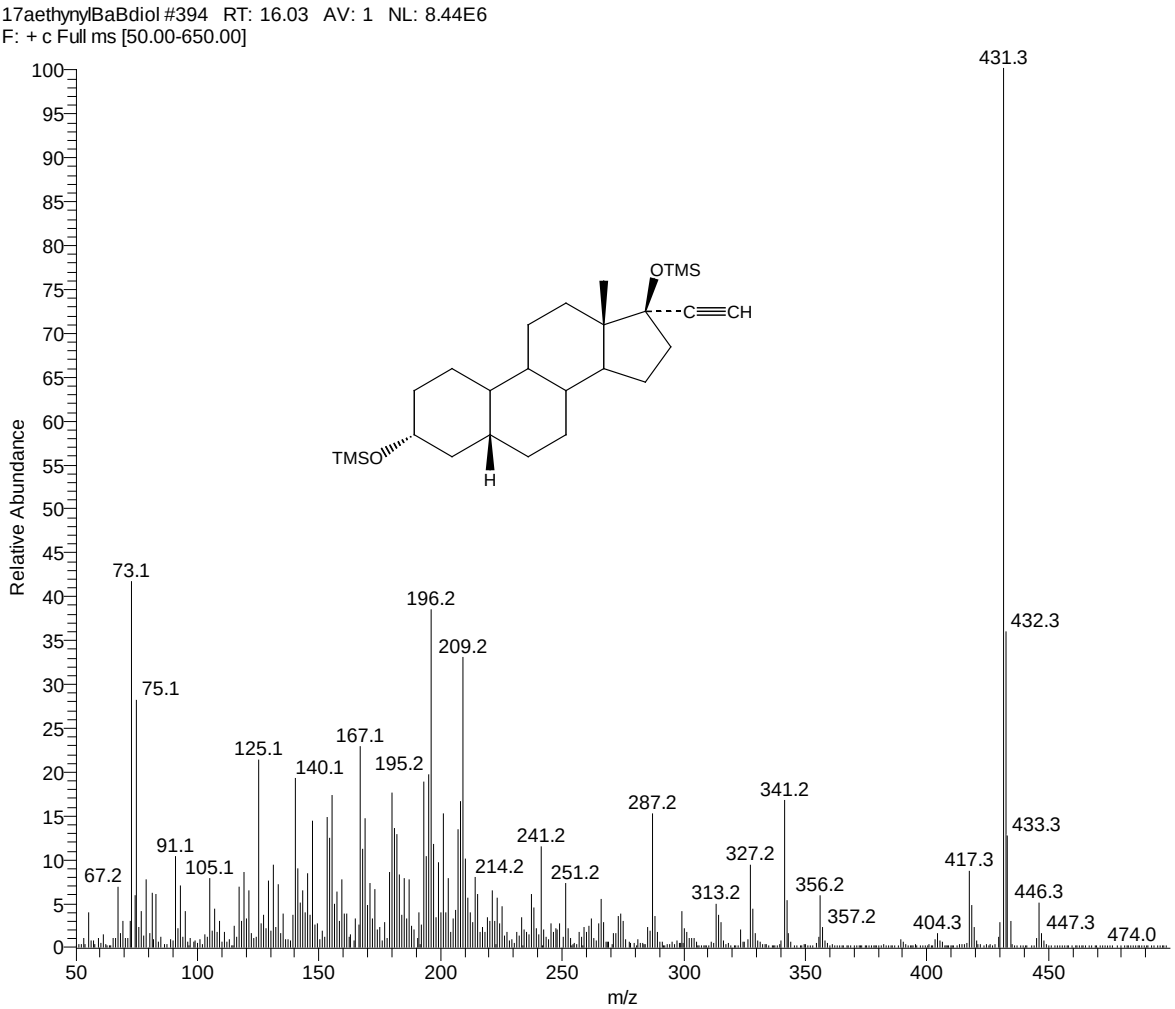


Figure 3.1: Mass spectrum and derivatised chemical structure of 17α-ethinyl-5β-estrane-3α,17β-diol (M2)

3.3.2 17-ethynyl-dihydroxy-estran-3-one

A single metabolite with an intense molecular ion of *m/z* 530 was observed representing a mono-hydroxy norethisterone (M3, Figure 3.2). This metabolite was postulated as either 6z-hydroxy norethisterone or 16α-hydroxy norethisterone, C6 and C16 being the most likely sites of phase I metabolic hydroxylation based on studies performed in the greyhound to date[20,23,25]. Following acetonide derivatisation and subsequent TMS derivatisation of a post administration urine extract, the mass spectrum of this metabolite remained unchanged as an enol-tris tms deriviative. Therefore, if the hydroxyl function was present at C-16 it would be in the 16α configuration, as a -cis acetonide derivative would be formed across a 16β,17β-diol moiety[20]. This metabolite (M3) was excreted in the glucuronide fraction.

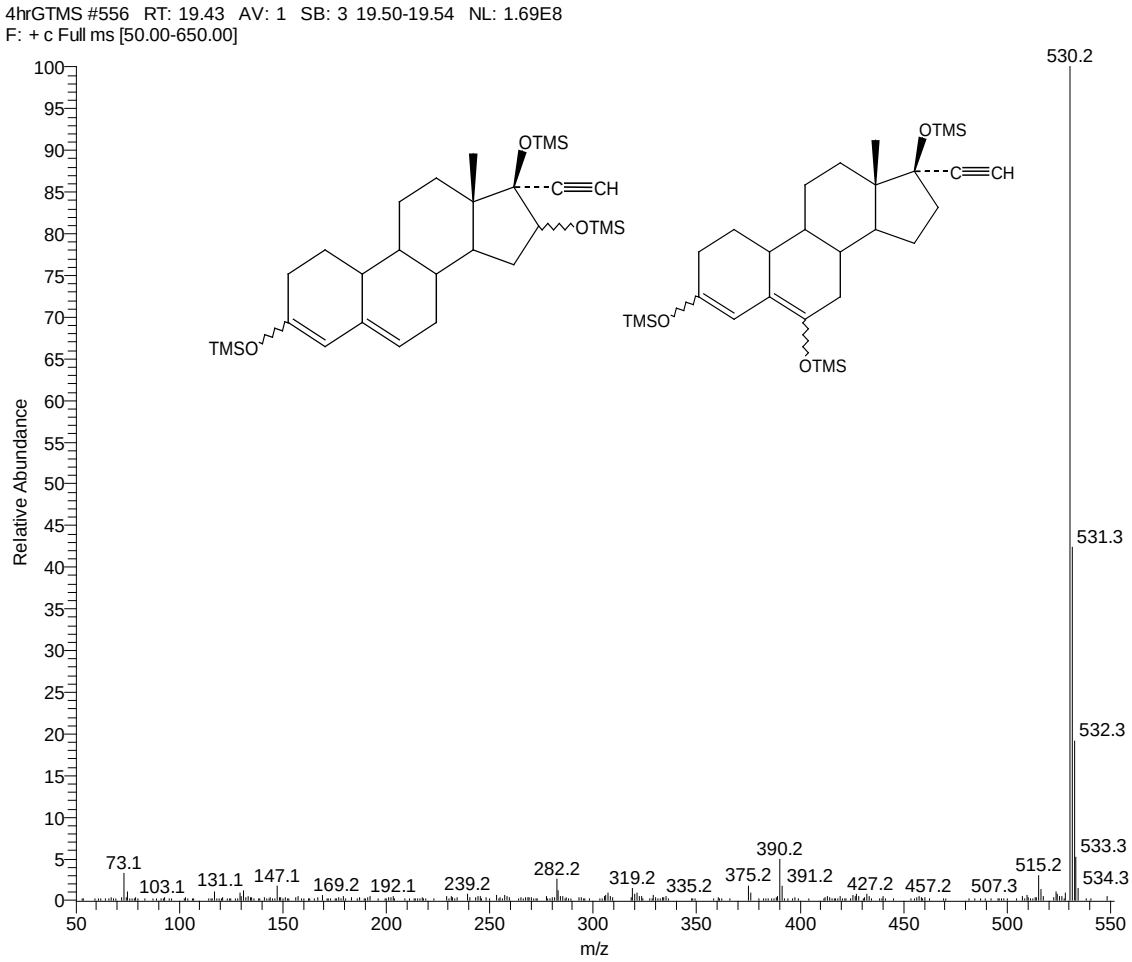


Figure 3.2: Mass spectrum and proposed derivatised chemical structures of 16-hydroxynorethisterone or 6-hydroxynorethisterone (M3)

3.3.3 17-ethynyl-estranetriols

A number of metabolites (3 major, 2minor) were observed exhibiting characteristics of mono-hydroxy, bis-reduced phase I metabolic transformations. These metabolites showed a molecular ion of *m/z* 534 with a major fragment ion at *m/z* 519 $[M\text{-}CH_3]^+$. An example full scan mass spectrum of the enol-tms derivative of one of these metabolites is shown in Figure 3.3. The other major fragment ions of *m/z* 429, 339 and 249 represent three sequential losses of 90Da (3xTMSiOH groups), indicating that three hydroxyl functions are present in the metabolite structure. These metabolites were therefore postulated as either 17α-ethynyl-estrane-3,6,17-triols or 17α-ethynyl-estrane-3,16α,17-triols, but there was insufficient fragmentation information to distinguish 6-hydroxy- from 16-hydroxy- metabolites. The major triol metabolites are likely to have the same stereochemistry at C3 and C5 as the 17α-ethynyl-estranediols, namely 5α,3β and 5β,3α, which would also be in keeping with previously identified major metabolites of methyltestosterone and testosterone in the greyhound[20,23]. The triol metabolites were excreted almost exclusively in the glucuronide fraction.

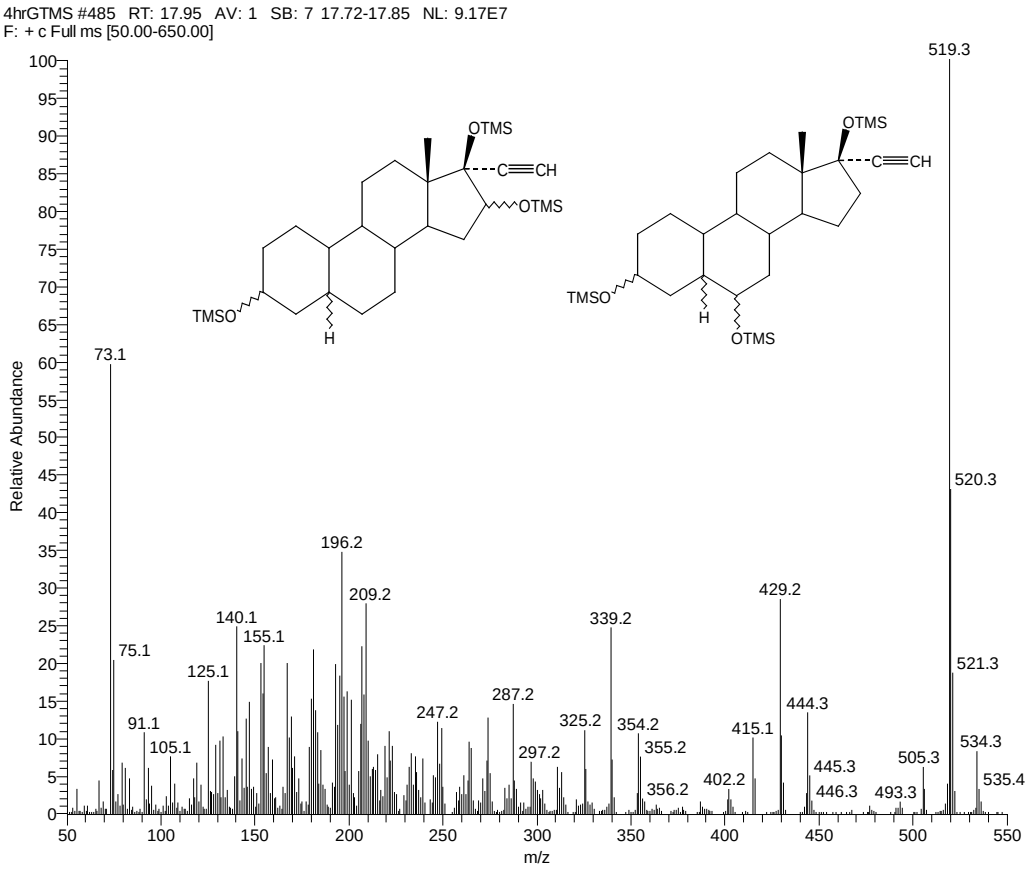


Figure 3.3: Mass spectrum and proposed derivatised chemical structures of 17α-ethinyl-5-estrane-triol (M4).

3.3.4 17-ethynyl-estranetetrols

Two major metabolites were observed exhibiting characteristics of di-hydroxy, bis-reduced phase I metabolic transformations. These metabolites showed a molecular ion of *m/z* 622 with a major fragment ion at *m/z* 607 $[M\text{-}CH_3]^+$. An example full scan mass spectrum of the enol-tms derivative of one of these metabolites is shown in Figure 3.4. The other major fragment ions of *m/z* 517, 427, 337 and 247 represent four sequential losses of 90Da (4xTMSiOH groups), indicating that four hydroxyl functions are present in the metabolite structure. These metabolites were therefore postulated as 17α-ethynyl-estrane-3,6,16,17-tetrols.

None of the triol or tetrol metabolites produced acetonide derivatives leading to the conclusion that any 16-hydroxylation would be in the alpha configuration, which is in keeping with previous greyhound steroid metabolism studies[20]. The tetrol metabolites were excreted in both the free and glucuronide fractions.

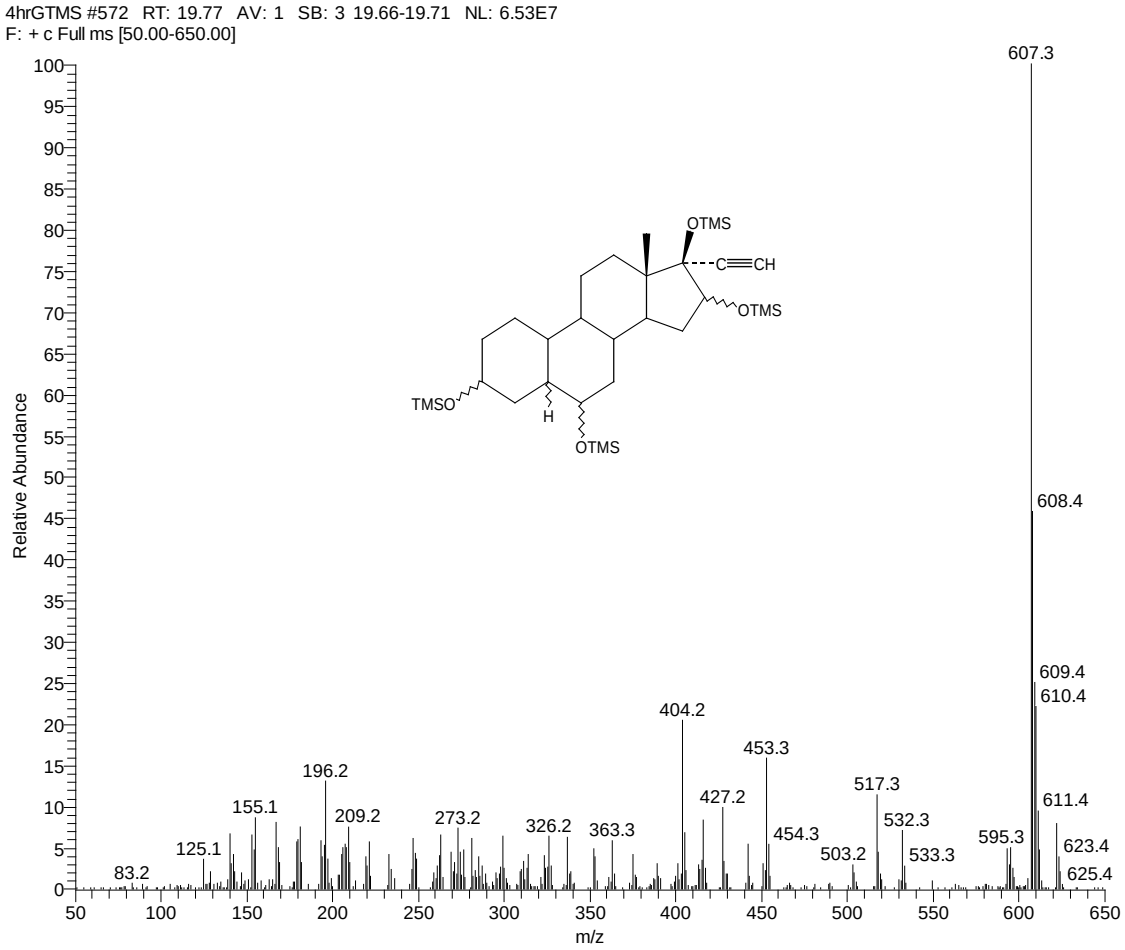


Figure 3.4: Mass spectrum and proposed derivatised chemical structures of 17α-ethinyl-5-estrane-3,6,16,17-tetrol (M8)

3.3.5 17-ethynyl-trihydroxy-estran-3-one

A single metabolite with a base peak of *m/z* 517 was also detected in the glucuronide fraction of the post administration urine. The enol-tms derivative full scan mass spectrum of this metabolite is shown in Figure 3.5a. The spectrum shows an $M^{+\cdot}$ ion of *m/z* 532 with fragment ions of *m/z* 517 $[M - CH_3]^+$, *m/z* 442 $[M - TMSiOH]^{\cdot+}$, *m/z* 427 $[M - CH_3 - TMSiOH]^+$, *m/z* 352 $[M – 2xTMSiOH]^{\cdot+}$ and *m/z* 337 $[M - CH_3 - 2xTMSiOH]^+$. These data indicate a mono-reduced, monohydroxy metabolite. The presence of a 3-keto function was demonstrated for this metabolite by the formation of an O-methyloxime-trimethylsilyl ether derivative, which produced a full scan mono-methoxime-bis-tms mass spectrum (Figure 3.5b) showing a molecular ion of *m/z* 489 and an intense fragment ion at *m/z* 474 $[M - CH_3]^+$; other fragment ions included *m/z* 399 $[M - TMSiOH]^{\cdot+}$, *m/z* 384 $[M - CH_3 - TMSiOH]^+$, *m/z* 368 $[M- TMSiOH - OCH_3]^{\cdot+}$, *m/z* 309 $[M - 2xTMSiOH]^{\cdot+}$ and *m/z* 278 $[M - OCH_3 - 2xTMSiOH]^+$. This metabolite was therefore postulated to be either 17α-ethynyl-6z,17β-dihydroxy-5z-estran-3-one or 17α-ethynyl-16α,17β-dihydroxy-5z-estran-3-one (M9), the 16α-configuration proposed as no acetonide derivative was formed with this metabolite.

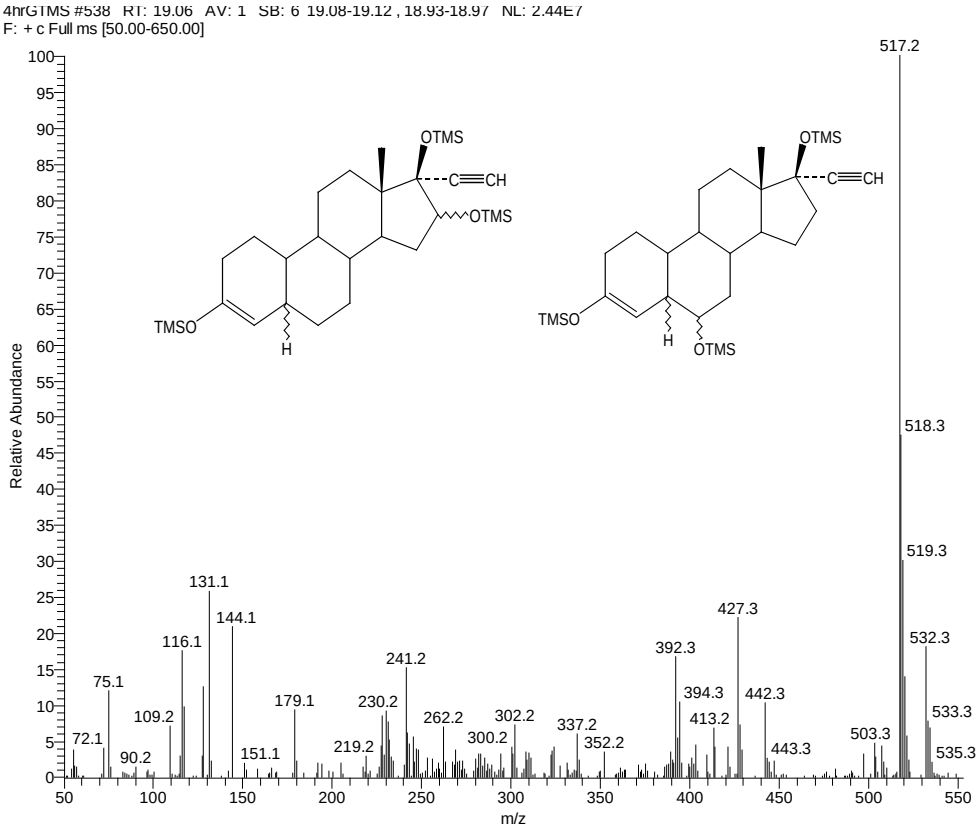


Figure 3.5a: Mass spectrum and proposed derivatised chemical structures of the mono-hydroxy, mono-reduced norethisterone metabolite (M9)

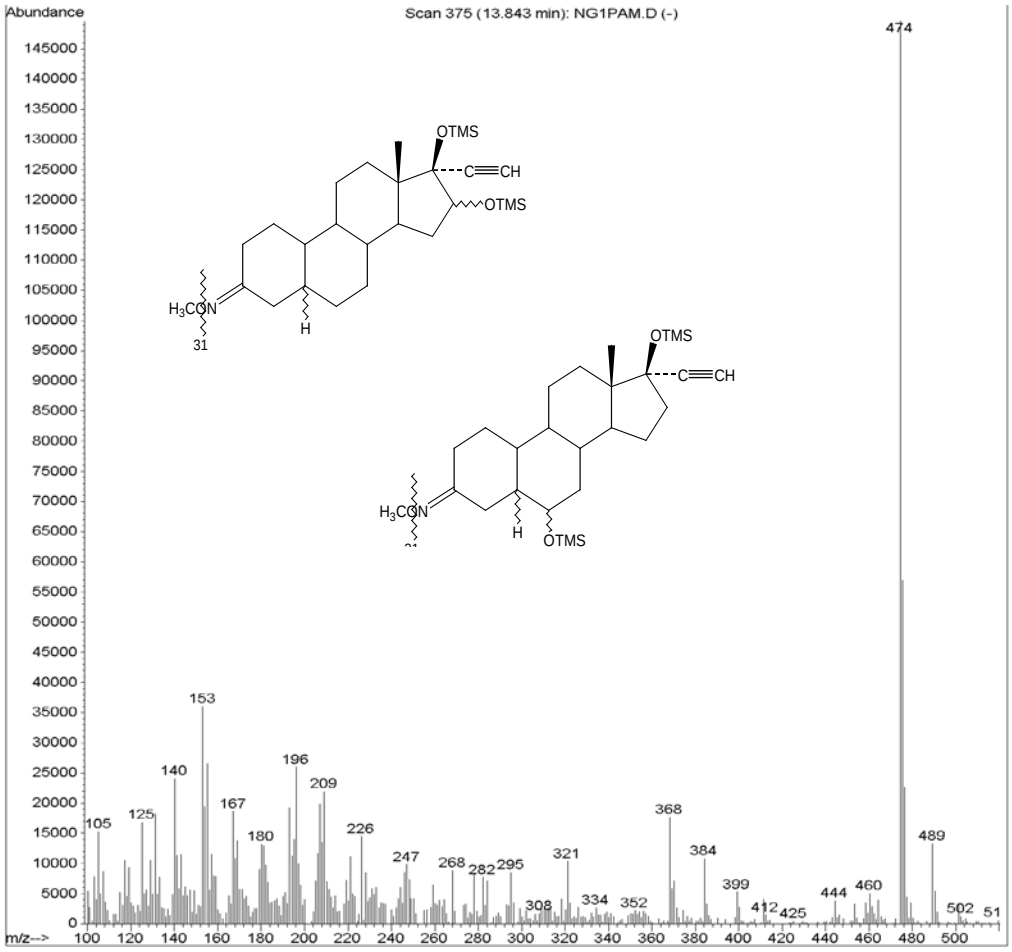


Figure 3.5b: Mass spectrum and proposed derivatised chemical structures of MO-TMS derivative of 17α-ethinyl-5-estrane-diol-3-one (M9)

3.3.6 19-norepiandrosterone and 19-noretiocholanolone

Nandrolone metabolites (19-norepiandrosterone and 19-noretiocholanolone) were also observed in the early post administration urine samples at low levels. Confirmation of the presence of 19-norepiandrosterone was perfomed using GC-MS/MS (Figure 3.6) The identification of nandrolone metabolites following the administration of norethisterone was considered to be relatively unusual bearing in mind they represent C-dealkylation as a phase I metabolic transformation; dealkylation of hetero atoms N,O and S would appear to be a more common metabolic process[26]. However, C-dealkylation has been suggested as a minor metabolic pathway for other species[10,27,28]. The electron withdrawing nature of the ethynyl function compared to the electron donating nature of aliphatic alkyl C-17 substituent groups present on orally active anabolic steroids may, in part, facilitate this C-dealkylation. Nevertheless an analysis of the Utovlan® tablets was performed to investigate their composition.

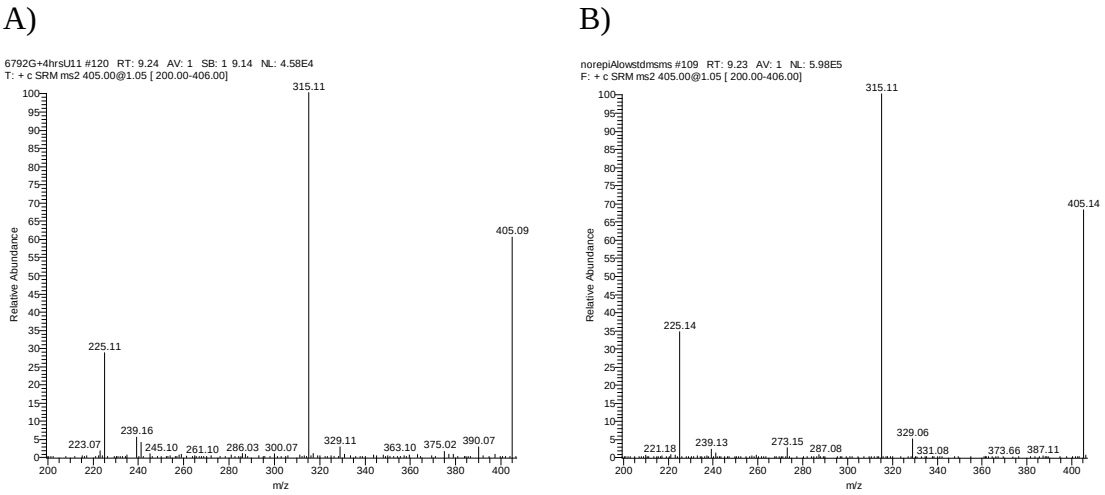


Figure 3.6: Comparison of GC-MS/MS mass spectra (enol-tms derivatives) of 19-norepiandrosterone from a +4hrs norethisterone post administration urine (A) and a 19-norepiandrosterone reference standard (B)

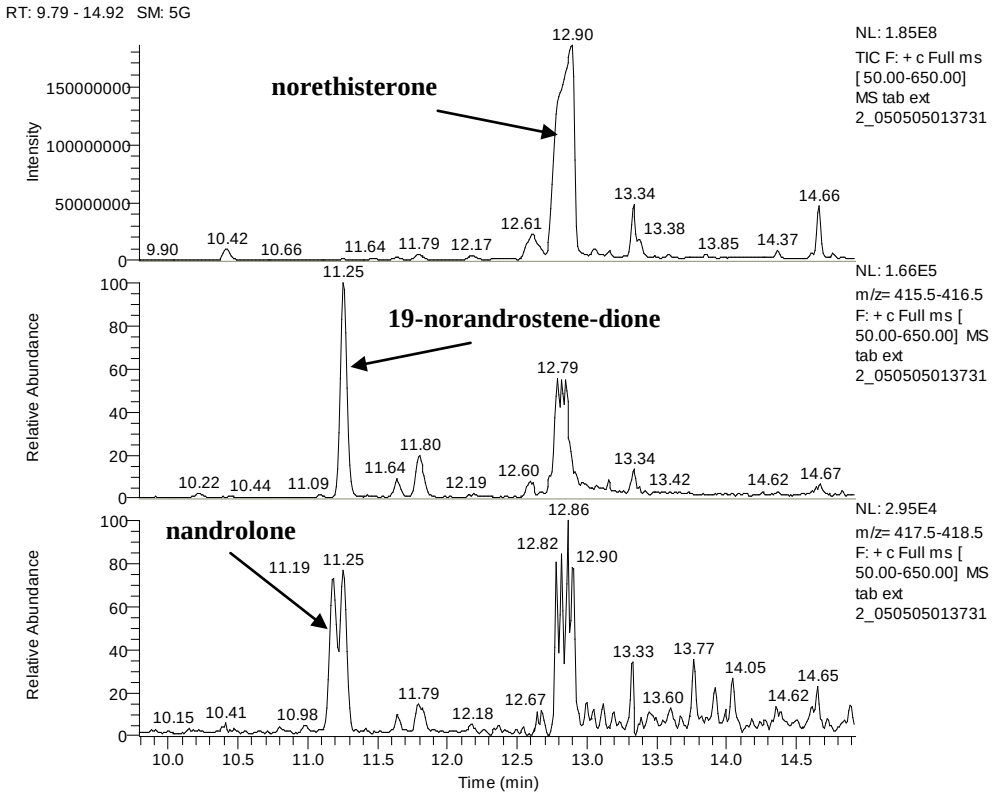


Figure 3.7: GC-MS analysis of norethisterone tablet extract (TIC and derivatised molecular ion chromatograms of key contaminants)

The tablet analysis revealed the presence of both nandrolone and 19-norandrostenedione (see Figure 3.7). These compounds were identified following comparison to known reference standards (see Figures 3.8 and 3.9) and were estimated to be present at approximately 15 ug per tablet for 19-norandrostenedione and 2 ug per tablet for nandrolone. The levels are well below the industry approved level for contaminants in pharmaceutical grade preparations of 0.1% [29], and are not considered to be likely to have any anabolic effect at these concentrations.

Clearly, the qualitative administration of both of these compounds would give rise to the related metabolites observed in the post administration urine samples. These metabolites (or their analogous stereoisomers) have been observed in the human following the administration of 19-norandrostenedione[30]. The administration of low level amounts of these compounds in humans, comparable to the amounts administered to the greyhound in this study, have been shown to produce detectable concentrations of nandrolone metabolites similar to those observed here[31] and this appears to be the most likely source. However, the presence of 19-norandrosterone has also been shown to be present in human urine following the administration of a purified form of northisterone[10], and a contribution from the metabolic C-de-ethylynation route suggested in this work, although not proven in the greyhound, cannot be ruled out.

It is interesting to note that the ratio of 19-norepiandrosterone to 19-noretiocholanone was in the order of 10:1 suggesting quite a significant bias towards 5α- reduction vs. 5β- reduction. This was not entirely consistent with nandrolone administration to the greyhound where the levels of 5α and 5β reduced metabolites appeared to be more comparable (unpublished data) but such a bias has been observed following the administration of 19-norandrost-4-ene-3,17-dione in the human[30]. It may therefore in theory be possible to distinguish the nandrolone metabolite profile arising from a nandrolone administration from that of a norethisterone administration. Nevertheless, in the human drug testing arena, where norethisterone has been used by a female athlete, this issue is dealt with by WADA, by means of a reporting level for the major nandrolone metabolite, 19-norandrosterone[32]. This ensures that no adverse analytical finding is reported for levels of nandrolone metabolites that have most likely arisen from the ingestion of norethisterone. In this greyhound study, the amounts of 19-norandrosterone and 19-noretiocholanolone observed were always significantly less than the major metabolites of norethisterone. Additionally, in those samples where the

presence of 19-norepiandrosterone and 19-noretiocholanolone were observed, the estimated concentrations of these substances were always below 2 $ngmL^{-1}$. The concentrations were estimated by comparison with a 2 $ngmL^{-1}$ extract of 19-noretiocholanolone glucuronide which was prepared and analysed alongside the post administration urine samples.

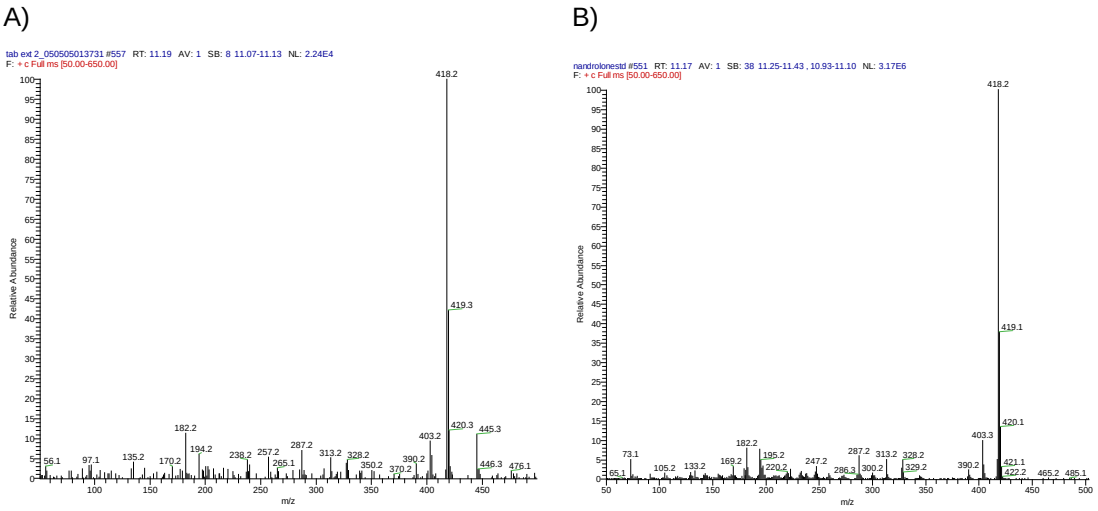


Figure 3.8: Comparison of GC-MS mass spectra of nandrolone from a norethisterone tablet extract (A) and a nandrolone reference standard (B)

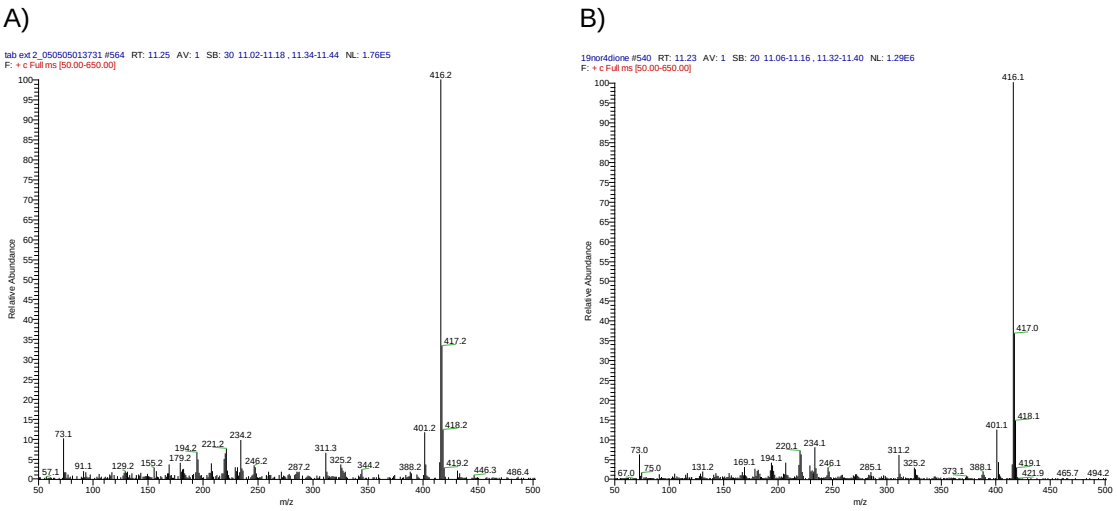


Figure 3.9: Comparison of GC-MS mass spectra of 19-norandrostenedione from a norethisterone tablet extract (A) and a 19-norandrostenedione reference standard (B)

3.4 CONCLUSIONS

The urinary metabolites identified in this study have provided an understanding of the *in vivo* phase I and phase II metabolism of norethisterone in the greyhound and have further increased the overall knowledge of canine steroid metabolism. Only a limited number of reference compounds were available for unequivocal identification of the proposed structures of the metabolites, but a number of suitable marker analytes have been established to provide effective detection of the administration of norethisterone in the routine drug testing environment. One of the major metabolic pathways was reduction of the 4-ene-3-one group of the A-ring to produce two –diol metabolites, 17α-ethynyl-5β-estrane-3α,17β-diol and 17α-ethynyl-5α-estrane-3β,17β-diol. These structures were confirmed by comparison of chromatographic and mass spectral data with reference compounds. Reduction of the 4-ene-3-oxo group of the A-ring has been noted in the metabolism of both testosterone[23] and methyltestosterone[20] in the greyhound, with the major stereoisomers being present in the 5α3β and 5β3α configuration. The structures of a number of other metabolites have been tentatively assigned on the basis of interpretation of mass spectral data and proposed metabolic pathways. In addition to reduction of the 4-ene-3-keto moiety, these include oxidation at C-16 and C-6 to produce a series of triols, tetrols and hydroxy-keto steroids (see Scheme 3.1). The proposed assignment of the stereochemistry at C16 in the alpha configuration was based on the lack of acetonide derivative formation, as discussed for the metabolites of methyltestosterone in chapter 2. In relation to the nandrolone metabolites detected in this study, future in-vitro metabolism studies using canine liver microsomes with purified norethisterone, may serve to provide further insight into the possibility of C-dealkylation as a minor metabolic pathway in the greyhound.

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CHAPTER

METABOLISM OF TESTOSTERONE

A POPULATION STUDY

4. ENDOGENOUS STEROID METABOLISM: TESTOSTERONE
Determination of endogenous metabolite levels in a population of greyhound bitches.

4.1 INTRODUCTION

Various steroid preparations have been used to delay or suppress oestrus in racing greyhound bitches. As highlighted in previous chapters, of particular concern in this area is the use, and possible abuse, of androgenic anabolic steroids due to their potential for enhancing performance. Durateston™ (a testosterone ester mix for intramuscular administration) has been permitted in the past by some greyhound racing jurisdictions for the suppression of oestrus in the greyhound bitch, as it is a licensed veterinary preparation for veterinary clinical use. However, following a ban on the use of testosterone in UK racing there is a requirement for methods to detect its potential abuse. This particular piece of research addresses the metabolism and detection of testosterone in the greyhound.

Testosterone is a naturally occurring (endogenous) androgenic/anabolic steroid in both greyhound dogs and bitches. Therefore research was needed to determine normal endogenous concentrations of the parent steroid and/or its major metabolites in order to try and establish a threshold level that could potentially be implemented to detect the administration of testosterone to the racing greyhound. Knowledge in other species such as the horse[1] has shown the levels in male animals to be both high and extremely variable, making detection of the use of testosterone in this gender more challenging. This is also true for metabolites of testosterone in the male greyhound[2]. Therefore, the main focus of this research work was to provide a means for detecting the use of testosterone in the female greyhound, following the proposed ban on the use of testosterone as an oestrus suppressant.

Previous studies have reported extensive metabolism of testosterone in the greyhound bitch following an administration of 50mg of Durateston™ [3]. The urinary concentration of testosterone reached a maximum of only 14 $ngmL^{-1}$, 26 hours after administration. However, the concentrations of the characterised testosterone metabolites were significantly higher. The major testosterone metabolite was epiandrosterone, which peaked at 1240 $ngmL^{-1}$, also at 26 hours. Four major androstanediol isomers were identified, with one peaking at 266 $ngmL^{-1}$, again at 26

hours. The last testosterone metabolite identified was etiocholanolone, which peaked at 50 $ngmL^{-1}$, again at 26 hours. Testosterone and its various metabolites returned to their pre-dose concentrations within 4 days. Williams *et al*. reported the findings of additional testosterone metabolites in greyhound urine after oral doses of 1mg/kg bwt testosterone to 3 greyhound bitches[4]. The metabolites found were in addition to the metabolites described by Brockwell *et al.*[3]. They were not characterised, although mass spectrometry suggested that a hydroxy- and a reduced hydroxy- testosterone were present. Studies using incubations of testosterone with microsomal preparations from beagle liver, also suggested a hydroxytestosterone metabolite[5]. This was characterised as 6β-hydroxytestosterone and accounted for approximately 41% of the total testosterone metabolites from the liver incubation. Other significant characterised testosterone metabolites from this study were 16α-hydroxytestosterone and androstenedione.

4.2 MATERIALS and METHODS

4.2.1 Solvents and Chemicals

C18 Sep-Pak Vac SPE cartridges (500mg, 6cc, end capped) and Silica Sep-Pak Vac SPE cartridges (500mg, 6cc) were obtained from Waters Ltd (Hertfordshire, UK). Methanol, hexane, diethyl ether, chloroform and ethyl acetate, Analar or HPLC grade, were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Sodium hydroxide, disodium hydrogen orthophosphate 2-hydrate and sodium dihydrogen orthophosphate 1-hydrate, analar grade, were supplied by BDH (Dorset, UK). Sigma Chemical Co. Ltd (Dorset, UK) supplied sodium sulphate, ammonium iodide β-Glucuronidase from *Eschericia.coli* and authentic androstanediol reference standards. N-Methyl-n-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Machery-nagel (Düren, Germany). Ethanethiol was supplied by Aldrich Chemical Co. Ltd (Dorset, UK). Epiandrosterone was obtained from Steraloids Inc. PO Box 689, Newport, RI 02840, USA and $^{2}[H_5]$-etiocholanolone was obtained from NARL, Sydney, NSW 2073, Australia. $^{2}[H_3]$-androstanediol was prepared at HFL, Camb , UK.

4.2.2 Animal administration studies

Animal administration studies were performed at Racing Chemistry, Iowa State University, U.S.A. (see Appendix II regarding ethical considerations). Durateston™ (50mg) was administered intramuscularly to two greyhound bitches. Naturally voided urine samples were collected at 0, 2, 4, 8, 24, 32, 48, 56, 72, 79, 96, 104hrs and then daily until +18 days after administration. The urines were stored at –20°C awaiting analysis.

4.2.3 Collection of 'normal' blank urine samples

Greyhound bitch urine samples were collected by representatives of the NGRC from racing kennels. HFL's request, where possible, was for samples from bitches not being suppressed at the time of sampling or from those that had recently been suppressed. Many of the bitches had never been suppressed and details of last season date were also available for some of the animals. The population comprised 212 samples.

4.2.4 Extraction Method

Aliquots of canine urine (4mL) were spiked with $^{2}[H_5]$-etiocholanolone and $^{2}[H_3]$-androstanediol (internal standards) at levels of 25 $ngmL^{-1}$ each. Extraction cartridges (C18 Sep-Pak Vac, 6cc, 500mg) were conditioned with methanol (5mL) and water (reagent grade, 5 mL). Each sample was then passed through a cartridge followed by water (5 mL). The column was washed with hexane (5mL) and then dried with air under vacuum for 5 min. Extracts were then eluted with methanol (4 mL). The eluates were evaporated using oxygen free nitrogen at 80ºC and dissolved in phosphate buffer (1mL, pH6.8, 0.1M) containing β-glucuronidase *E. coli* (250µl, 10units/µL). The extracts were hydrolysed overnight at 37ºC.

After incubation, sodium hydroxide (50µL, 2M) was added to each sample and the sample mixed using a Whirlimixer. Liquid-liquid extraction was carried using diethyl ether (4mL). The ether was removed and washed with sodium hydroxide (2 x 500µl, 2M). The ether was dried over anhydrous sodium sulphate and then evaporated to dryness under OFN at 40ºC. The samples were then reconstituted in ether: hexane (1 : 1; 1mL) and thoroughly mixed.

A clean up extraction was performed using Silica Sep-Pak Vac cartridges (6cc, 500mg). These were pre conditioned using chloroform (5mL) and ether: hexane (1:1)

5mL. The reconstituted samples were loaded onto the cartridges and drawn through. The extracts were eluted with chloroform: ethyl acetate (1:1) 3mL + 1mL. The eluants were combined and evaporated to dryness under OFN at 80ºC in GC vials. Enol-tms derivatisation was performed by adding 30µl of 'enol-tms reagent' {MSTFA 1mL: Ammonium Iodide 3mg: Ethanethiol 6ul, prepared by heating at 80°C for 20mins to dissolve solid material}, capping and heating at 80°C for 1.5 – 2 hours.

4.2.5 GC-MS Method

Selective Ion Monitoring Gas Chromatography-Mass Spectrometry analysis of each batch was carried out in positive ion electron ionisation mode, using a Finnigan Voyager bench top instrument with an SGE BPX5 column (approx. 25m, 0.22mm id, 0.25µm film thickness). Injector temperature was 260°C, splitless for 1.0 minute, and the interface temperature was 280°C. Injection volume was 1µL. The GC temperature programme was started at 180°C and held for 0.5 minute. Initial ramp 180°C to 290°C at 5°C min^{-1} and then further from 290°C to 330°C at 40°Cmin^{-1}. The final temp was held at 330°C for 3 minutes. Data was acquired from 6 to 26 minutes. The ions used to monitor the derivatised parent steroid and its major metabolites are shown in Table 4.1.

Table 4.1: $^{m}/_{z}$ ions used in Selected Ion Monitoring (SIM) mode to detect the enol-TMS derivatives of testosterone and related metabolites.

$^{m}/_{z}$ IONS monitored	**Analytes**
346.30, 421.40	Androstanediols
349.30	D3 Androstanediol
417.40, 432.40	Testosterone
419.40, 434.40	Epiandrosterone
424.40, 439.40	D5 Etiocholanolone

4.2.6 Quantitative GC-MS analysis of the major urinary metabolites

For the analysis of the population of blank samples, calibration standards were prepared in steroid stripped canine urine for the three major metabolites epiandrosterone, 5α-androstane-3β,17β-diol and 5β-androstane-3α,17β-diol at concentrations 250 $pgmL^{-1}$, 500 $pgmL^{-1}$, 1, 2, 4, 8 and 16 $ngmL^{-1}$ with quality control samples at 0.375, 3 and 12 $ngmL^{-1}$. For the analysis of the post administration samples, calibrants were prepared at concentrations 4, 8, 16, 32, 64 and 128 $ngmL^{-1}$ with quality control samples at 6 and

48ngmL^{-1}.The internal standards $^{2}[H_3]$-androstanediol and $^{2}[H_5]$-etiocholanolone were added to all samples , quality controls and calibration standards at a concentration of 25 ngmL^{-1}. The samples, calibration standards and quality control samples were then prepared using the method described for quantitative GC-MS analysis in the SIM mode.

4.3 RESULTS AND DISCUSSION

4.3.1 Qualitative analysis results

The major metabolites identified in the post administration urine extracts were 5α-androstane-3β,17β-diol (αββ-Adiol), 5β-androstane-3α,17β-diol (βαβ-Adiol) and epiandrosterone. Figure 4.1 shows the mass chromatogram of a post administration urine extract monitoring the major ions, *m/z* 241 and *m/z* 434 for the enol-TMS derivative of these major metabolites respectively. The stereochemistry of the androstane-3,17-diol isomers was confirmed by the comparison of the retention times and mass spectral data with authentic reference standards.

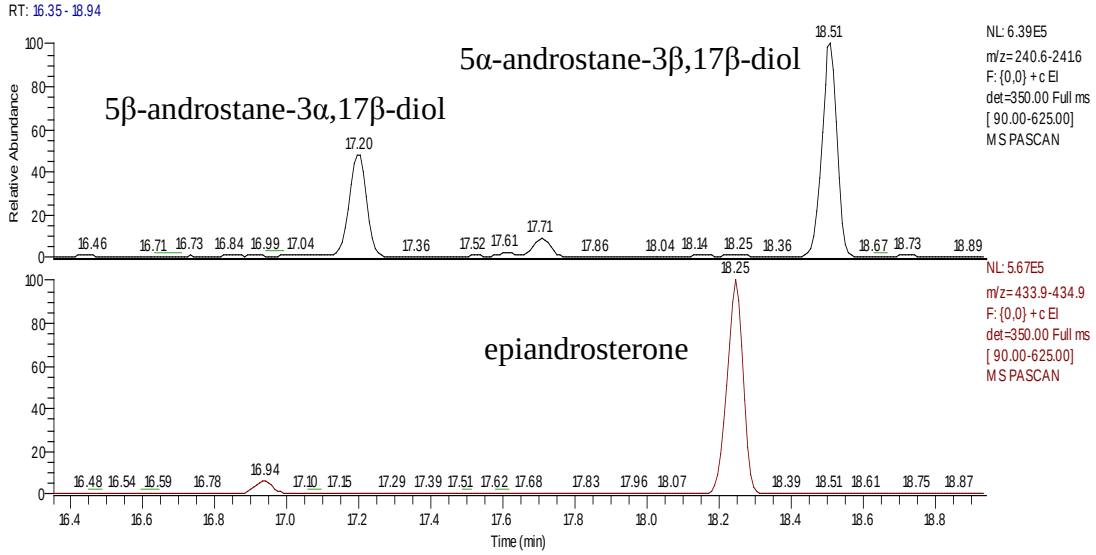


Figure 4.1: GC-MS analysis of Durateston™ (testosterone esters) post administration sample.

The full scan mass spectra of 5β-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol are shown in figures 4.2 and 4.3 respectively. It is worth noting that the intensity of the *m/z* 241 is less than that of the *m/z* 256 ion in the 5β reduced androstane-3,17-diols (4 stereoisomers); the opposite being true of the 5α reduced androstane-3,17-diols (4

stereoisomers), a phenomenon previously observed for estranediol isomers[6]. The full scan mass spectrum of epiandrosterone is shown in figure 4.4. The structures of the major metabolites identified are shown in figure 4.5.

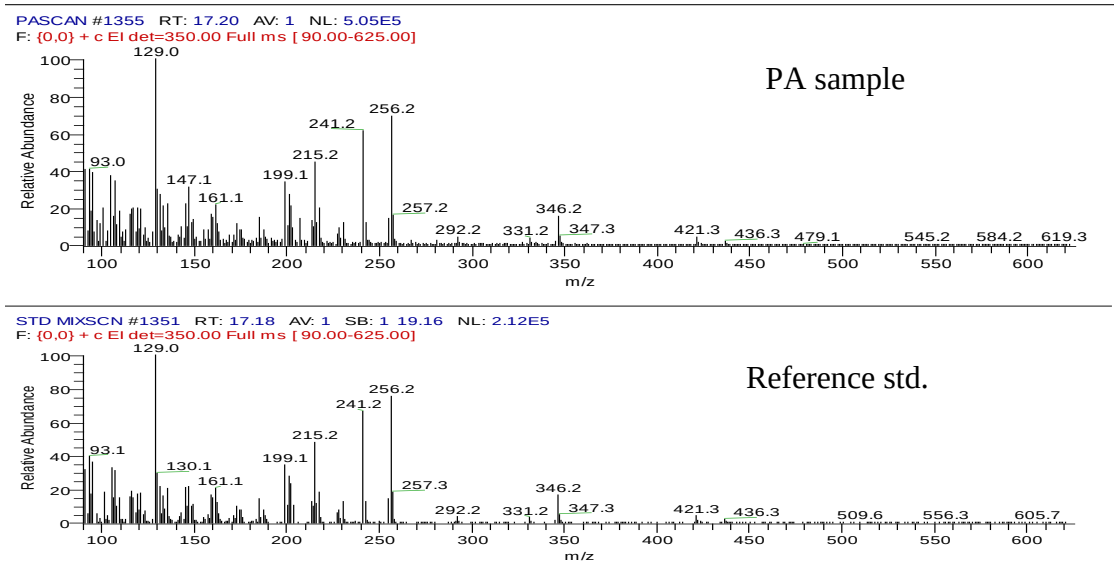


Figure 4.2: Mass spectrum of 5β-androstane-3α,17β-diol - bis TMS derivative

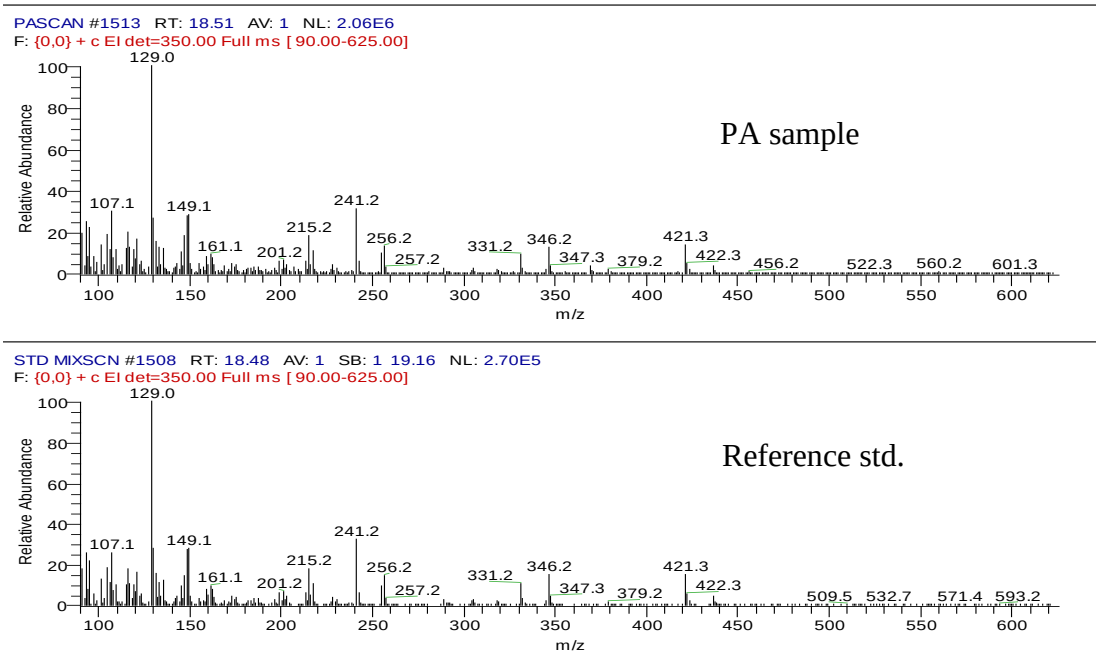


Figure 4.3: Mass spectrum of 5α-androstane-3α,17β-diol - bis TMS derivative

The mass spectra of the androstanediols show a base peak at m/z 129 which represents the D-ring fragment, analogous to the *m/z* 143 ion observed for 17α-methyl-androstanediols in the methyltestosterone administration study. The TMS derivatives of

fully saturated metabolites are clearly prone to much greater fragmentation than the metabolites that retain a degree of unsaturation following derivatisation, where M^+ - 15 and $M^{+\cdot}$ ions are quite stable (see figure 4.4).

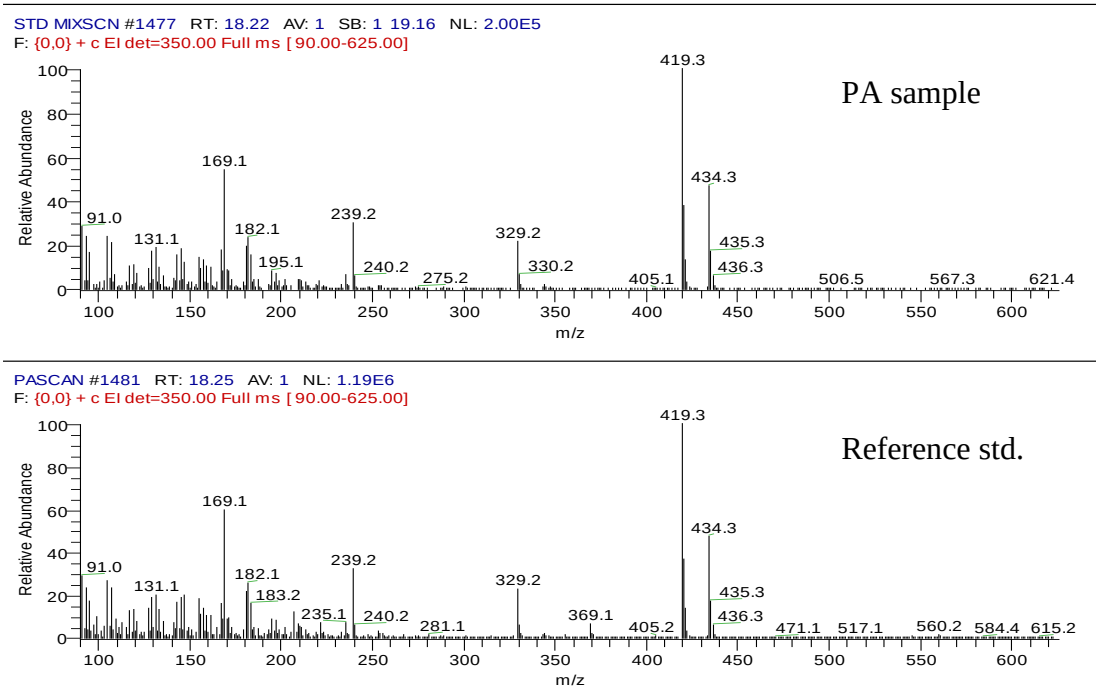


Figure 4.4: Mass spectrum of epiandrosterone enol - bis TMS derivative

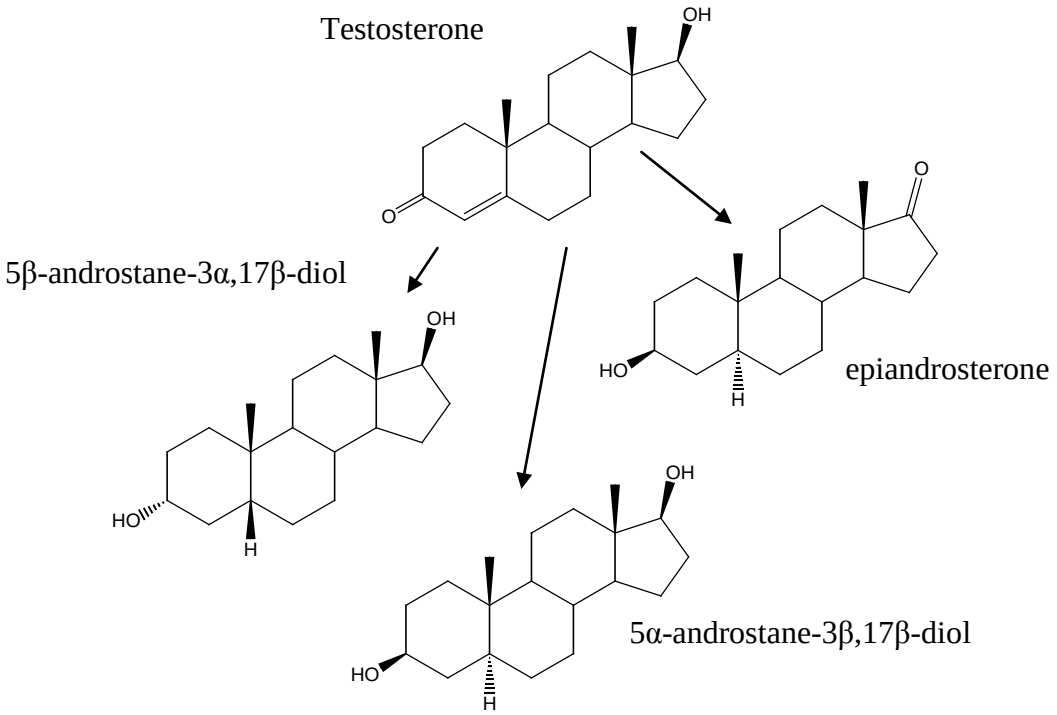
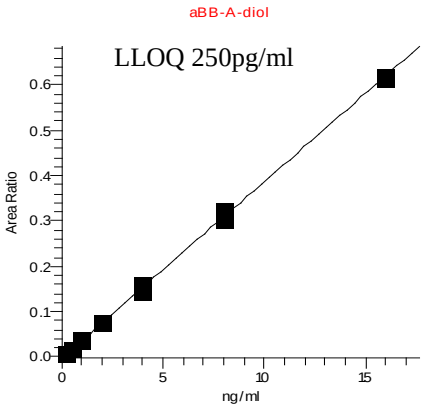


Figure 4.5: Structures of testosterone and the major metabolites identified

The levels of etiocholanolone were very low in the post administration samples and only those analytes with the most significant change from resting level were selected for quantitative analysis.

4.3.2 Quantitative analysis results

The method allowed for the quantification of the three major metabolites in a single analysis. Quality control samples were analysed in duplicate with each batch of samples. A typical calibration line with quality control data for one of the metabolites, 5α-androstane-3β,17β-diol, is shown in figure 4.6. Based upon replicate analysis of the calibration standards, the lower limit of quantification (LLOQ) of the assay for all three steroids was 250 pgmL^{-1}. Precision and accuracy data at the LLOQ for each analyte is shown in Table 4.2. The remaining precision and accuracy and quality control data for the developed assay are shown in Appendix I.



Sample	Specified Amount ng/ml	Calculated Amount ng/ml	%Diff
LOW A	0.375	0.374	-0.22
LOW B	0.375	0.376	0.26
MED A	3.000	3.059	1.96
MED B	3.000	3.070	2.32
HIGH A	12.000	12.411	3.42
HIGH B	12.000	12.943	7.86

Equation: Y = -0.0022+0.0389*X R^2 = 0.9987

Figure 4.6: Example calibration line and quality control data for 5α-androstan-3β,17β-diol

Table 4.2: Summary of calibration sample data at the assay LLOQ

Batch	**βαβ-Androstanediol (ngmL^{-1})**		**Epiandrosterone (ngmL^{-1})**		**αββ-Androstanediol (ngmL^{-1})**	
1	0.311	0.292	0.260	0.276	0.254	0.259
2	0.245	0.266	0.178	0.200	0.277	0.266
3	0.268	0.273	0.265	0.266	0.207	0.267
4	0.199	0.179	0.198	0.191	0.231	0.257
Mean	0.254		0.230		0.252	
Std dev	0.045		0.041		0.023	
%CV	17.676		17.819		8.977	
%RE	1.650		-8.300		0.900	

4.3.2.1 Durateston™ (testosterone esters) administration results

Figures 4.7 and 4.8 show the urinary metabolite excretion profiles for the two bitches following administration of Durateston™. All three metabolites reached maximum concentrations at approximately 24h post dose. For bitch 7321 5α-androstane-3β,17β-diol levels peaked at approximately 135 ngmL^{-1} and returned to resting levels after 14 days with small amounts still being sporadically excreted for several days until the final collection on day 18. Both epiandrosterone and 5β-androstane-3α,17β-diol followed a similar trend with the exception that 5β-androstane-3α,17β-diol appeared to return to basal levels after 11 days.

For bitch 7335, 5α-androstane-3β,17β-diol levels peaked much higher at approx. 220ngmL^{-1} and did not return to resting levels for the duration of the sample collection. Both epiandrosterone and 5β-androstane-3α,17β-diol followed a similar trend with 5β-androstane-3α,17β-diol appearing to return to basal levels during the final days of sample collection.

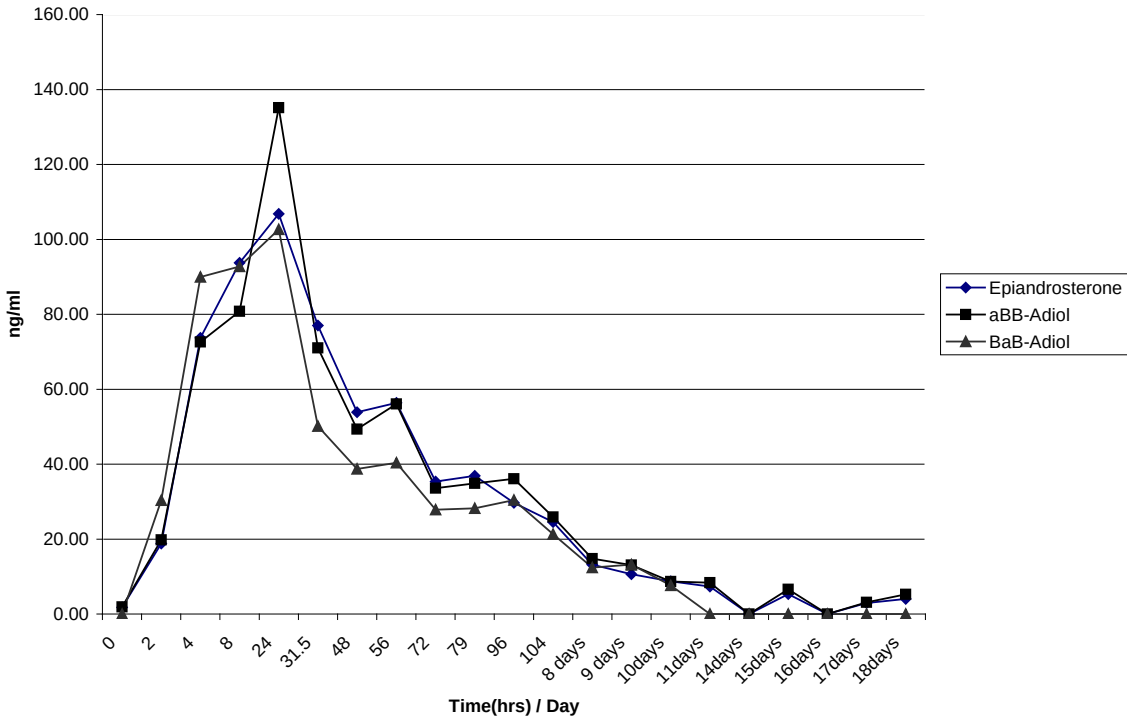


Figure 4.7: Urinary excretion profiles of major testosterone metabolites following the administration of Durateston™ (50mg IM) to greyhound bitch 7321

Key: aBB-Adiol = 5α-androstane-3β,17β-diol; BaB-Adiol = 5β-androstane-3α,17β-diol

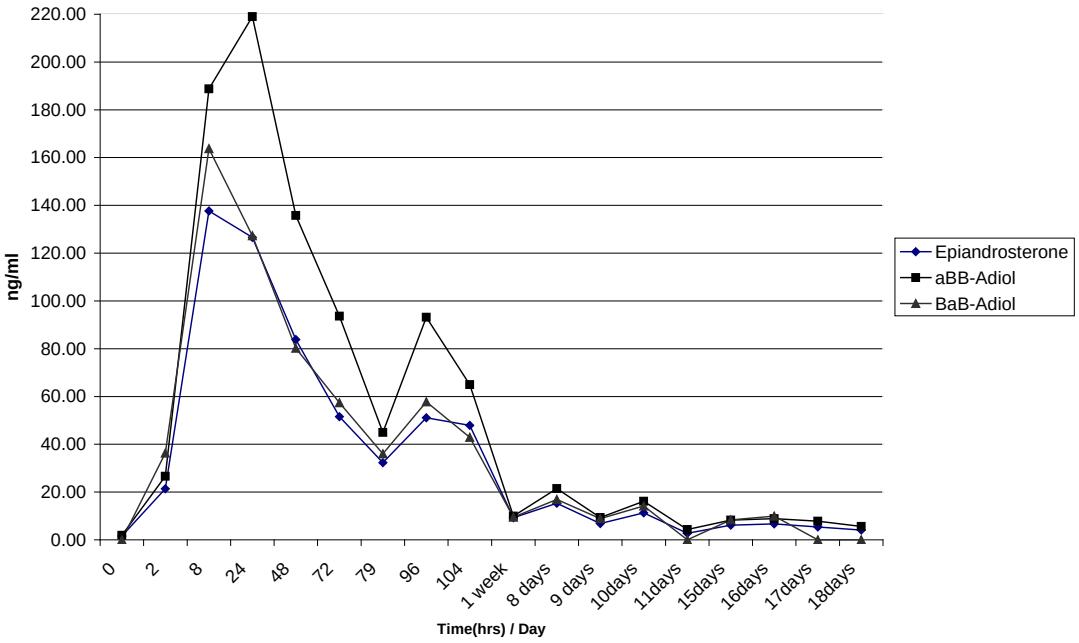


Figure 4.8: Urinary excretion profiles of major testosterone metabolites following the administration of Durateston^{TM} (50mg IM) to greyhound bitch 7335

4.3.2.2 Population Study results

The concentrations of the three metabolites were also determined in 212 "normal" samples from untreated bitches. The concentration for epiandrosterone ranged from undetectable (i.e. below the LLOQ of 250 pgmL^{-1} for the assay) to 13 ngmL^{-1} and from undetectable to 22 ngmL^{-1} for 5α-androstane-3β,17β-diol. The distribution of the concentrations of 5α-androstane-3β,17β-diol and epiandrosterone in the blank sample population are shown in figures 4.9 and 4.10 respectively. With the exception of two highly significant outliers at 21 ngmL^{-1} and 22 ngmL^{-1} for 5α-androstane-3β,17β-diol, the distribution of the two populations is very similar. The stereochemistry of epiandrosterone and 5α-androstane-3β,17β-diol is the same at C3 and C5 and the two compounds are linked through enzymatic metabolic pathways.

The distribution of the concentrations of 5β-androstane-3α,17β-diol is shown in Figure 4.11 and is heavily positively skewed towards the undetectable/low concentrations.

5β-androstan-3α,17β-diol was detected in only 23 of the 212 samples analysed and in only one sample (7.5 ngmL^{-1}) was the concentration greater than 2 ngmL^{-1}. The low excretion of 5β-androstane-3α,17β-diol ties in with the qualitatively observed low levels of etiocholanolone, as the stereochemistry of these two compounds is the same at C3 and C5. An interesting point to note here is the significant excretion of 5β-

androstane-3α,17β-diol following administration of testosterone without the concomitant excretion of etiocholanolone in similar quantities as these two compounds are also expected to be linked through enzymatic pathways.

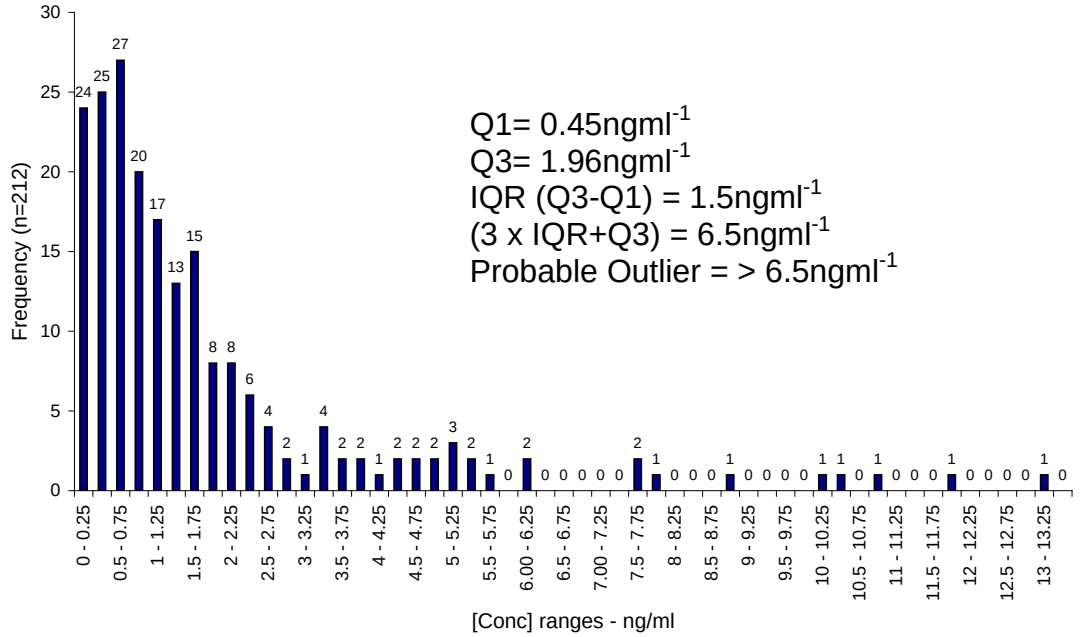


Figure 4.9: Urinary levels of epiandrosterone in a 'normal' population of untreated greyhound bitches

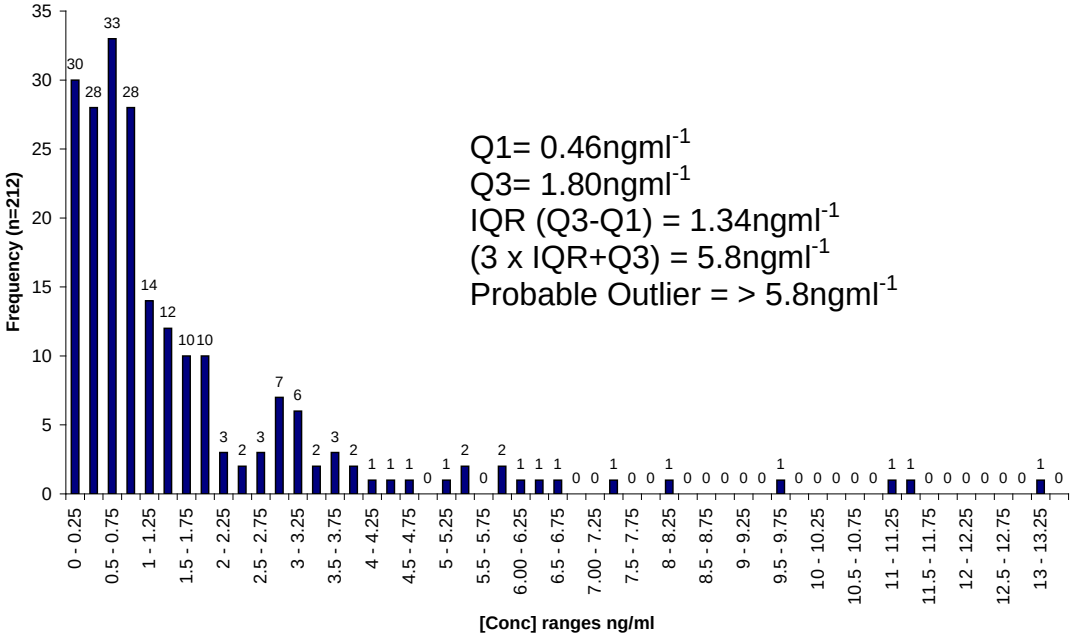


Figure 4.10: Urinary levels of 5α-androstan-3β,17β-diol in a 'normal' population of untreated greyhound bitches*****

***N.B.** There were two further significant outliers in the population of values for 5α-androstan-3β,17β-diol at 21.2 and 22.2 ngmL^{-1}. These have been removed from figure 8 to allow direct comparison with the other analyte populations over the same range of concentrations.

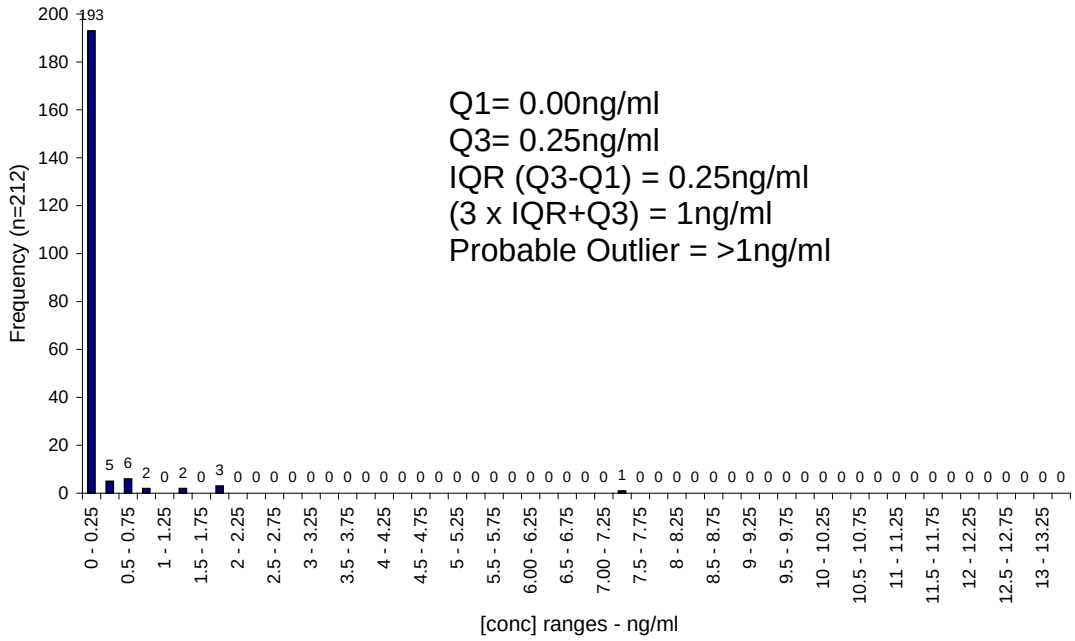


Figure 4.11: Urinary levels of 5β-androstan-3α,17β-diol in a normal population of greyhound bitches

The concentration values of the testosterone metabolites calculated for each individual bitch are shown in table 4.5. The results for certain bitches are highlighted where they are known to have been in season. Some other samples are also highlighted where the values appear elevated and accompanying notes were available from the trainer at the time of collection. These notes are highlighted in table 4.6.

Table 4.5: Concentrations of major testosterone metabolites in the population of blank bitch urines ($ngmL^{-1}$)

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
1	2072es	0.206	0	0
5	2070es	1.038	0.798	0
7	1909es	0.135	0	0
9	1873es	1.115	0.908	0
12	1907es	1.281	0.852	0
14	1984es	0.363	0.577	0
16	1982es	1.047	0.897	0
18	1983es	0.381	0.54	0
19	1979es	2.351	1.577	0.26
20	1901es	4.793	3.618	0
23	1765es	1.083	0.93	0
24	1744es	4.039	4.455	0
25	1754es	1.596	1.198	0
26	1710es	0.834	1.381	0

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
98	2549es	1.59	1.14	0
99	2543es	5.066	5.448	0
100	2556es	0.358	0.385	0
102	2534es	3.4	2.887	0
103	4099es	0.068	0	0
104	4085es	0.293	0.325	0
105	3731es	5.327	4.627	0
106	3729es	7.511	6.051	0
107	3718es	6.204	7.263	0.594
108	4142es	0.949	0.713	0
109	4144es	0.688	0.608	0
111	4150es	0.958	0.923	0
112	4152es	2.852	2.968	0.262
113	3722es	0.927	0.92	0

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
27	1748es	0.383	0.408	0
30	1711es	7.918	11.2	1.417
31	1830es	0.329	0.432	0
32	1760es	2.462	3.104	0.527
33	1810es	1.089	1.095	0
34	1955es	0.952	1.297	0
36	1735es	0.754	0.388	0
37	1750es	0.544	0.564	0
39	1764es	1.586	1.621	0
40	1758es	0.403	0.407	0
41	1827es	1.021	0.796	0
42	1819es	0.465	0.396	0
45	1824es	0.363	0.281	0
48	1968es	0.597	0.556	0
49	1730es	0.524	0.628	0
50	1741es	0.665	0.504	0
51	1728es	1.036	1.309	0
52	1809es	1.569	1.124	0
53	1969es	0.551	0.493	0
60	1726es	1.631	1.028	0
62	1708es	0.831	0.875	0
63	1731es	0.55	0.657	0
65	1715es	1.879	1.751	0
66	1721es	1.35	1.226	0
68	1713es	0.849	0.852	0
75	1790es	0.114	0	0
86	1751es	0.477	0.457	0
89	1707es	4.333	5.49	0.623
95	1953es	3.891	5.889	0.507
Bitch No.	**External code**	**Epiandro**	**αββ – Adiol**	**βαβ – Adiol**
97	2525es	4.594	3.934	0.455
152	3702es	1.43	1.31	0
153	3957es	0.137	0	0
154	3963es	0.485	0.714	0
155	3937es	0.186	0	0
156	3959es	0.35	0.441	0
157	3967es	0.26	0	0
158	3946es	0.841	0.541	0
159	3958es	0.391	0	0
160	3944es	5.683	3.561	1.88
161	3949es	3.358	3.156	0.729
162	3962es	0.343	0.311	0
164	3942es	1.833	1.948	0
166	3994es	1.34	0.998	0
167	3991es	1.423	0.873	0
168	3993es	1.355	0.907	0
169	4003es	1.388	1.208	0
170	3998es	0.783	1.053	0
171	3990es	0.713	0.395	0
172	4006es	0.839	0.696	0

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
115	4145es	1.649	1.189	0
116	4096es	3.094	2.696	0
117	4083es	5.198	6.609	0.284
118	4084es	0.802	0.526	0
119	4109es	0.846	0.897	0
122	4154es	0.213	0	0
123	4147es	0.083	0	0
124	4139es	1.845	1.873	0
125	4148es	0.33	0.332	0
126	4146es	0.201	0	0
127	4125es	1.493	1.646	0
128	4140es	0.694	0.785	0
129	4137es	0.78	0.393	0
130	4138es	0.203	0	0
131	4141es	0.534	0.541	0
133	4153es	1.038	1.371	0
136	3966es	0.128	0	0
137	3520es	1.576	2.784	1.971
138	3941es	0.351	0.551	0
139	3955es	0.628	0.46	0
140	3533es	1.398	0.752	0
141	3514es	1.267	1.484	0
142	3714es	0.692	0.615	0
143	3511es	2.484	2.838	0
144	3526es	4.415	3.085	0
145	3517es	2.598	3.166	0
147	3953es	0.575	0.443	0
148	3877es	0.083	0	0
150	3950es	0.352	0.642	0
Bitch No.	**External code**	**Epiandro**	**αββ – Adiol**	**βαβ – Adiol**
151	3951es	1.159	0.63	0
206	4531es	2.035	1.777	0
209	4850es	1.086	0.96	0
211	4404es	3.493	2.876	0
213	4527es	2.512	2.014	0
214	4861es	1.526	1.844	0
216	4844es	2.252	4.167	0
217	4542es	0.688	0.916	0
218	4372es	2.544	2.599	0
219	4528es	1.542	1.159	0
220	4835es	0.583	0.594	0
221	4521es	1.069	1.598	0
222	4505es	1.042	1.976	0
223	4808es	11.886	11.377	0
224	4866es	10.955	9.694	**7.458**
225	4385es	1.07	1.254	0
226	4526es	2.021	2.776	0.101
228	4975es	0.173	0.284	0
229	4532es	0.337	0.491	0
230	4511es	0.686	0.931	0

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
173	4017es	2.589	2.106	0
174	3996es	0.688	0.514	0
175	4031es	0.873	0.666	0
176	3992es	1.158	0.582	0
177	4005es	6.025	3.939	0
178	4007es	1.445	1.372	0
179	4001es	1.804	1.21	0
180	4013es	0.688	0.596	0
181	3997es	1.819	1.774	0
182	4004es	2.453	1.801	0.928
183	4002es	5.287	3.715	0
184	4023es	0.771	0.578	0
185	3945es	1.852	1.628	0
186	4113es	0.637	0.53	0
187	3926es	2.022	1.654	0
188	3907es	0.535	0.298	0
189	4114es	0.383	0	0
190	3889es	0.432	0.296	0
191	3888es	2.992	2.144	0
192	3887es	1.954	0.984	0
193	3921es	0.425	0	0
194	3913es	2.151	1.733	0
195	3903es	1.594	1.144	0
196	3880es	1.281	0.653	0
197	3584es	2.028	1.326	0
198	3560es	1.607	1.509	0
199	2259es	4.535	5.871	0
201	4549es	0.292	0	0
202	4417es	1.656	1.391	0
203	4512es	0.878	0.763	0
204	4513es	8.923	13.25	1.319
265	2026es	0.439	0.979	0
266	5088es	0.452	0.511	0
267	5094es	4.987	1.118	0.815
268	4998es	0.209	0.344	0
269	5023es	0.093	0	0
270	5039es	0.083	0.223	0
271	5087es	0.142	0.193	0
272	5053es	1.046	1.162	0
274	4379es	0.268	0.223	0
275	4565es	0.439	0.402	0
276	4568es	0.434	0.313	0

Bitch No.	External code	Epiandro	αββ – Adiol	βαβ – Adiol
232	4541es	13.22	21.191	0.731
233	4961es	0.662	0.909	0
234	4953es	3.346	3.346	0.235
235	4955es	2.037	1.71	0
236	4964es	7.519	22.165	0
237	4957es	0.45	0.48	0
238	4978es	2.135	1.926	0
239	4947es	0.409	0.605	0
240	4380es	0.457	0.654	0
242	4544es	1.963	2.481	0
244	4504es	1.741	2.321	0
245	4959es	10.118	8.123	0
246	4972es	0.406	0.733	0
247	4810es	3.664	3.146	0.215
248	4807es	2.216	2.558	0
249	4811es	0.19	0	0
250	4830es	2.481	2.946	0.219
251	4857es	1.537	1.351	0
252	4813es	0.591	0.413	0
253	4846es	0.683	0.714	0
254	5049es	0.564	0.957	0
255	5045es	0.033	0	0
256	5032es	0.567	0	0
257	5034es	0.038	0.155	0
258	509es	5.234	5.206	1.988
259	5091es	1.148	1.359	0
260	5001es	0.692	0.615	0
261	5029es	0.183	0	0
262	5017es	0.155	0.207	0
263	5010es	1.347	1.964	0
264	5021es	0.964	0.968	0
277	4560es	0.47	0.466	0
278	4383es	10.354	6.454	0
279	4583es	0.713	0.545	0
280	4563es	0.399	0.467	0
281	4554es	0.102	0.157	0
282	4378es	1.197	0.998	0
283	4553es	3.509	3.358	0
284	4550es	3.867	3.022	0.261
285	4573es	1.646	1.625	0
286	4572es	0.81	0.868	0
287	4382es	0.422	0.228	0

(Key: Epiandro = epiandrosterone, αββ-Adiol = 5α-androstane-3β, 17β-diol, βαβ-Adiol = 5β-androstane-3$^{\alpha}$, 17β-diol)

Bitch known to be in season

Values elevated / notes available

Table 4.6 - Notable samples with available sample collection comments

Bitch no.	Sample collection notes
30	+6 months after last season (Never Suppressed)
32	IN SEASON +22 days
89	IN SEASON +1 day
95	+3-4 months after last season (Never Suppressed)
99	+11months after last season (Never Suppressed)
106	Orandrone +3months
107	+13months after last season (Never Suppressed)
117	Comment – Due to 'break' in 7 days
144	IN SEASON +13 days
160	No info. (Never Suppressed)
177	No info. (Never Suppressed)
199	+5 months after last season
204	+6months after last season (Comment: Never Suppressed)
223	No season date info. (Comment: Never Suppressed)
224	No season date info. (Comment: Never Suppressed)
232	IN SEASON +2 days
236	IN SEASON +4 days
245	IN SEASON +4 days
258	No season date info. (Comment: Never Suppressed)
278	IN SEASON +5 days
284	IN SEASON same day

4.3.3 Statistical evaluation of population data

When dealing with non-normally distributed data one approach is to transform the data using a variety of mathematical conversions e.g. $\log_{10}X$ or the cube root of X and test the transformed data for normality. However, some data sets are so positively skewed that this is not always possible.

The three metabolites detected in the present study were also below the assay LLOQ of 250 $pgmL^{-1}$ in a significant number of "normal" samples. A non-parametric statistical model provides an approach whereby the data from these samples can be included in the statistical analysis. This non-parametric assessment of the data predicts probable outliers using a boxplot and whisker approach[7]. Taking 3x the interquartile range of the numerically ranked data and adding the 75^{th} percentile (75^{th}P) gives an approximation to 3 sigma or 1/1000 probability that a value exceeding this level would be part of the real population. Previous thresholds for other endogenous compounds in animal sports testing have been set using 1/10,000 probability where the data showed a normal distribution following transformation by various mathematical conversions[8].

This might be considered fairly conservative, but carries weight in an area where data might need to withstand legal scrutiny. Using the non-parametric approach the cut-off values for probable outliers for epiandrosterone, 5α-androstane-3β,17β-diol and 5β-androstan-3α,17β-diol are shown in figures 4.9, 4.10 and 4.11 respectively. The calculation for 5β-androstan-3α,17β-diol in figure 4.11 assumes the 75thP to be 250pgmL^{-1} (the LLOQ of the assay) in order to produce a cut-off value for a probable outlier. The 75thP is in fact zero for this population (i.e. 5β-androstan-3α,17β-diol was undetectable in greater than 90% of the samples analysed.)

The single value at 7.5 ngmL^{-1} for 5β-androstan-3α,17β-diol appears to be a significant outlier using the non-parametric approach. It is worth noting that values of 5β-androstan-3α,17β-diol continue to be above 10 ngmL^{-1} for up to 10 days post dose in both animals administered with Durateston.

To establish thresholds using the non-parametric approach various multiplications of the "far outside value" have been proposed[9,10]. It is obviously desirable when using a non-parametric approach for the sample population to be a large number. It may therefore be desirable to collect further data for a greater number of 'normal' samples in order to allow the future setting of an action level (to trigger further investigation of a particular test sample) for testosterone metabolites with greater confidence. Figures 4.12 to 4.14 show the "far outside values" from the non-parametric treatment of the population study data, applied to the excretion profiles for the major testosterone metabolites following the administration of Durateston™ to bitch 7335 (see figure 4.8).

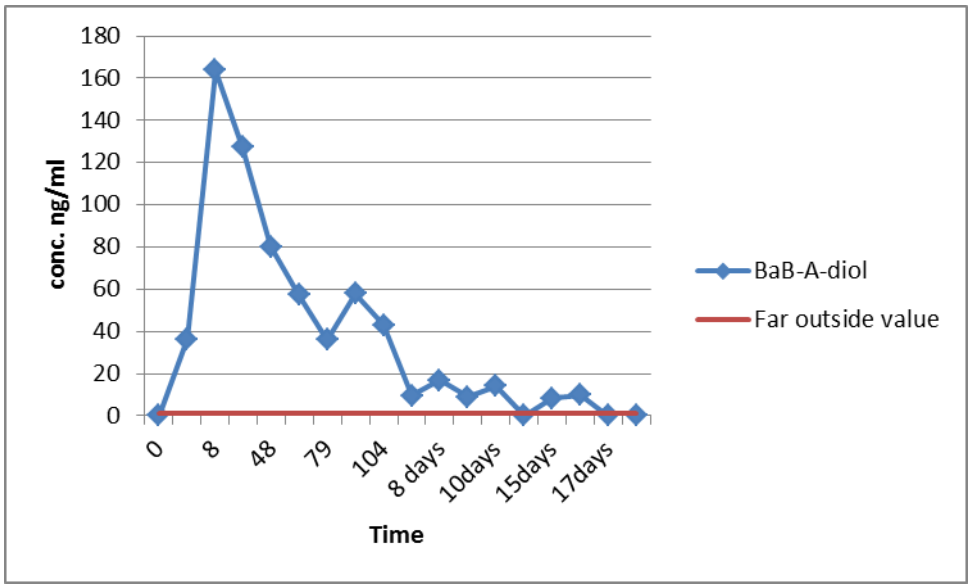


Figure 4.12: Urinary excretion profile of 5β-androstane-3α,17β-diol from bitch 7335 showing far outside value from population study.

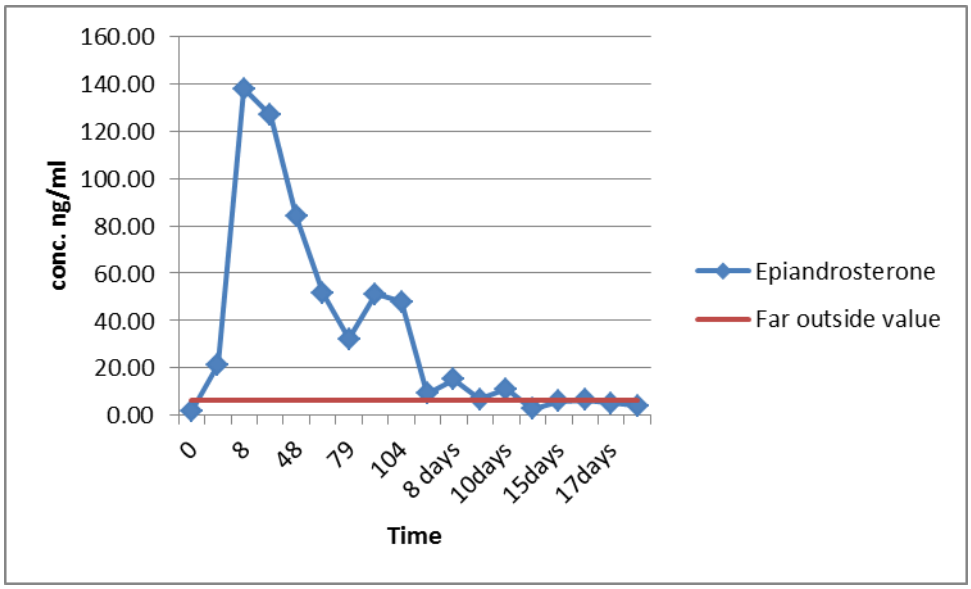


Figure 4.13: Urinary excretion profile of epiandrostserone from bitch 7335 showing far outside value from population study.

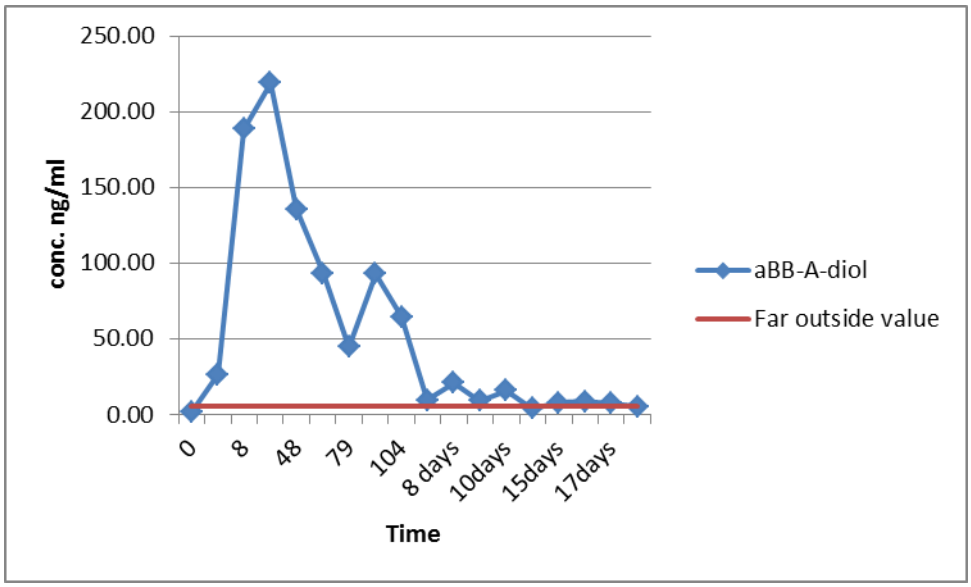


Figure 4.14: Urinary excretion profile of 5α-androstane-3β,17β-diol from bitch 7335 showing far outside value from population study.

It was possible to produce a $\log_{10}X$ distribution of the epiandrosterone data, as all the samples tested gave a detectable value for this analyte that approximated to normal (see Figure 4.15). The mean and SD could then be used to estimate a 1 in 10,000 threshold for a one tailed test (1.71 log) that equals approximately 52 $ngmL^{-1}$. The conservative degree of 'confidence' provides a potential threshold value with a relatively small window of 'functionality' (see Figures 4.7 and 4.8).

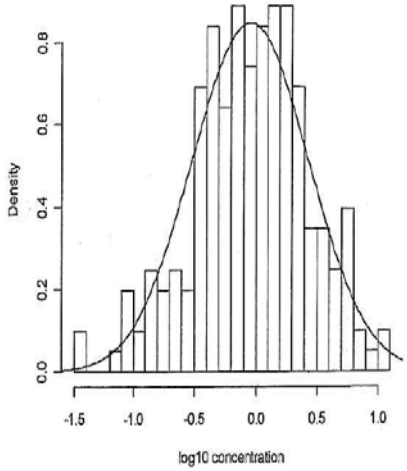


Figure 4.15: Normal distribution fit of $Log_{10}X$ epiandrosterone concentrations

Of significant interest was an observation that several of the 'probable outliers' from the blank population were bitches that had recently come into season (details of known season related occurrences were noted on accompanying sample collection paperwork – see table 4.6). It became apparent that further investigations would therefore be needed to determine the androgen profile throughout the normal oestrus cycle of the bitch in order to establish the possibility of variations in testosterone metabolite concentrations throughout this period. Such findings may have implications in relation to setting final threshold values for the key testosterone metabolites.

4.3.3.1 'βαβ-diol'

The analyte of greatest interest due to the distinct lack of its presence in over 80% of the samples analysed was 5β-androstane-3α,17β-diol (βαβ-diol), suggesting that this metabolite is either excreted in very small quantities or not at all (based on assay LOD) in greyhound bitches. However, it is not possible to define a threshold value for this analyte using routine statistical approaches in the absence of a good fit to a known distribution. With such positively skewed data, as well as 80% non-detects or 'zero-values', the only approach (other than the analysis of 10,000 samples with well-known provenance) is to apply a statistical formula called the Chebyshev inequality.

A one-tailed Chebyshev inequality is $p <_ 1/(1 + k^2)$ which defines the maximum probability p of observing a value, k standard deviations above the mean.

Following the removal of samples from the population known to be in oestrus, based upon the current 21 day layoff rule (see chapter 5), the data was processed using the statistical formula as follows:-

General Chebyshev formula for an asymmetric distribution:

$$t_g \leq \mu + \sigma\sqrt{\frac{1-p}{p}}$$

μ= mean, σ= S.D., t_u = proposed threshold value

mean = 0.109182 , s.d. = 0.596395, p=0.0001 (1/10,000 probability of a normal sample exceeding the threshold value (99.99% confidence)

$0.109182 + 0.596395 \times \sqrt{(1 - 0.0001) / 0.0001} = 60 ngmL^{-1}$

This is clearly a very conservative estimate if used as a threshold value, as the variation in SD is enhanced by the large number of zero values present in the population and a threshold value of 60ngmL^{-1} would only provide a short window of detection for the administration of testosterone (see Figures 4.7 and 4.8).

The same statistic applied for p1/1000 (99.9% confidence) gives a result of 19ngmL^{-1}.

It is interesting to note that none of the 'normal' cycling and original 212 population samples displayed a level anywhere near 19 ngmL^{-1} or more, for βaβ-diol. However, this level was exceeded for up to a week or more in the both of the excretion profiles depicted in figures 4.7 and 4.8, as well as in a large number of animals treated with Durateston™ in a Short Term Suppression project accompanying the work carried out in chapter 5 (not recorded in this thesis).

Depending upon the level of 'confidence' required this value could, at the very least, be used as an 'action level', to focus on 'intelligence' based future testing of certain animals. A full statistical review of this data was carried out by a professional statistician to check the validity of the approaches used and interpret the data in relation to setting threshold/action levels for use by the GBGB to control the use of testosterone in the greyhound bitch[11]. The content of this review was discussed in conjunction with the GBGB and the outcomes resulted in setting a threshold for epiandrosterone at 50ngmL^{-1}. The βαβ-diol analyte was suggested for use as action level analyte at 10ngmL^{-1}. This level was based on an overview of the population data and the fact that the highest value observed for βαβ-diol was a potential outlier at 7.5 ngmL^{-1}.

It may be possible to reanalyse the population of samples using a more sensitive GC-MS/MS approach. This might involve the use of a TBDMS derivative to enhance the available precuror ion for a relevant MS/MS transition. If an increase in sensitivity was observed by using this approach this might result in establishing actual values for a

number of the original non-detects for the βαβ-diol metabolite. This could serve to reduce the variance of the data set based on SD values and produce a less conservative threshold value if the data was then applied to the Chebyshev formula or another potentially suitable statistical approach.

Further approaches and evaluations of the population data may be required following an assessment of the steroid profile during the oestrus cycle of the bitch.

Other approaches, such as the ratio of key analytes (an approach used for testosterone detection in human sports testing) could also be investigated, along with Isotope Ratio Mass Spectrometry, should this technique prove suitable for greyhound urine analysis in the future.

4.4 CONCLUSIONS

The metabolite profiles determined following administration of a single dose of Durateston™ to two bitches were similar to those reported previously with respect to the metabolites identified and their relative abundances[3]. Epiandrosterone, 5α-androstane-3β,17β-diol and 5β-androstane-3α,17β-diol were identified as the key metabolites. Urinary concentrations of these steroids were determined by a validated, quantitative GC-MS method in both the post administration samples and a population of urine samples from greyhound bitches. Data from this population has been treated using a non-parametric statistical approach to provide a cut-off level for probable outliers to highlight test samples of potential interest. Some conservative threshold levels for the key metabolites have also been calculated and may be implemented if desired, although further work may be necessary.

Some of the 'outlier' samples from this population study clearly showed elevated levels of testosterone metabolites in relation to the timing of their recorded oestrus. The levels of metabolites observed in these samples, despite not breaching either of the tentatively proposed and albeit conservative threshold levels, would certainly be of interest as potentially originating from 'testosterone treated' animals, without any information to the contrary.

Based on the data obtained from this study, longitudinal steroid profiling throughout the oestrus cycle of the greyhound bitch could provide valuable information about potentially significant variations in urinary androgen levels at different stages of the cycle.

4.5 REFERENCES

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CHAPTER

THE OESTRUS CYCLE

5. STEROID PROFILING THROUGHOUT THE OESTRUS CYCLE OF THE GREYHOUND BITCH

5.1 INTRODUCTION

The oestrus cycle of the greyhound bitch has been the subject of a considerable amount of discussion and indeed research for many years. The main focus of attention on the cycle primarily surrounds two key areas, namely that of the racing performance of the animal at different stages of her cycle and a knowledge of the cycle in relation to effective breeding from a bitch, particularly if she has been a high class performer on the racetrack. The two factors are therefore, to a degree, intrinsically linked.

Greyhound bitches are known to be essentially mono-oestrus animals as they ovulate on average only once per breeding season. The average length of time between cycles is around 8 months[1].

The oestrus cycle is classified into four main phases as previously outlined in chapter 1.

Pro-oestrus: lasts approximately 9 days, during which time the bitch will exhibit both anatomical and behavioural changes including the commencement of bleeding, increased urination and attractiveness to male dogs (but generally unreceptive).

Oestrus: which again lasts on average for 9 days, at the outset of which the bitch will normally ovulate and be receptive to males; this being a major reason for not allowing bitches to race during this time.

Di-oestrus: (80-90 days) during which post-ovulatory levels of progesterone rise (produced from the developing corpus luteum) and ultimately fall back to basal after around 3 months. (Note: this stage, or parts of it, is also referred to as Metoestrus by some clinicians)

Anoestrus: the final stage, following luteal regression, which will last for around 4 months in the average bitch. The bitch will remain sexually inactive during this time until the commencement of pro-oestrus returns.

The timeframes for each phase of the cycle are variable, not only from animal to animal but also from one cycle to the next for a particular animal as well[1].

The greyhound bitch is also recognised as a breed that suffers from having what are termed split heats or even silent heats. A split heat is characterised by the bitch going

into oestrus but not ovulating. She will then return into oestrus two to four weeks later and ovulate normally; upon which progesterone levels should start to rise[1].A silent heat is characterised by essentially very little. The bitch will come into season without showing any physical external signs to either, the trainer, the vet or indeed any attractiveness to male dogs[2]. Her general behaviour can also remain relatively normal[1].

Research into hormone levels throughout the bitch's cycle has been achieved over the years *via* the analysis of blood samples[3,4,5]. A schematic hormone profile is shown in in figure 5.1 (reproduced from Concannon 2011[6].)

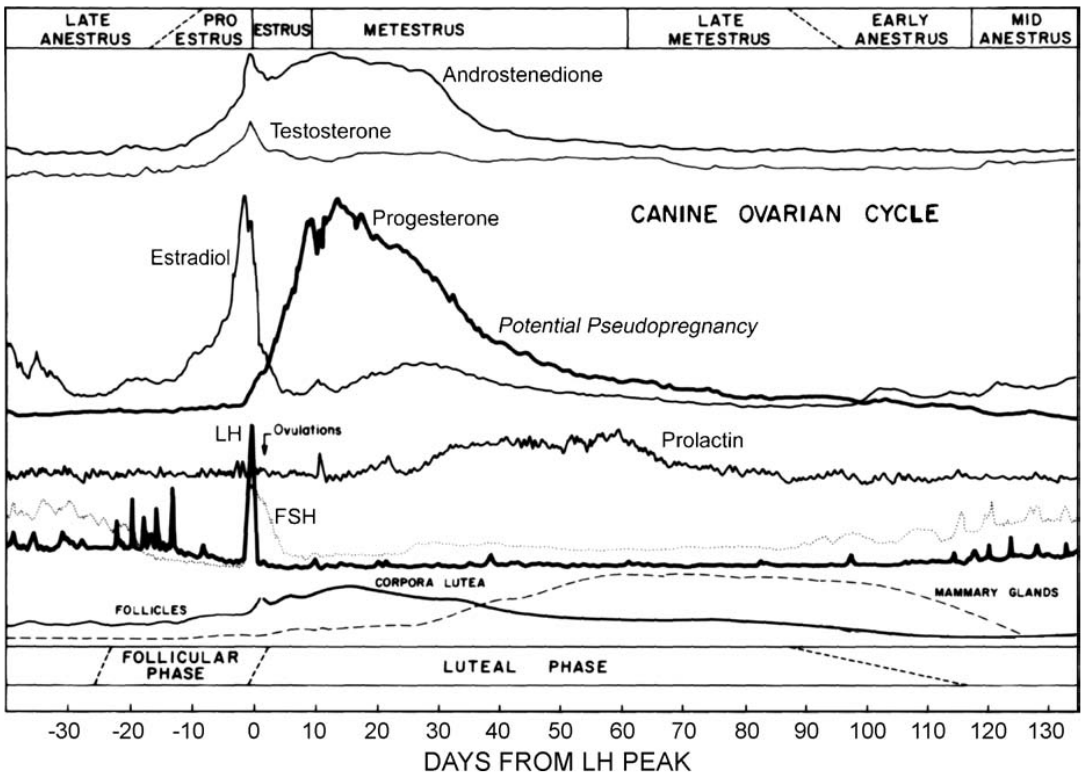


Figure 5.1: Schematic plasma profile of oestrus cycle hormones

The results of many of these studies are based on RIA and ELISA technology and very little, if any data appears to be available from mass spectrometry analysis.

Over many years, a body of anecdotal evidence has also developed amongst owners and trainers, relating the racing performance of greyhound bitches to certain stages of the oestrus cycle. Such evidence has suggested that unsuppressed bitches run well immediately at the end of their oestrus. However, it is believed that their performance declines from about day 70 and remains poor for a number of weeks before returning to normal. Prior to 2004, bitches were also banned from running for 70 days after

commencement of their season. An NGRC trial was organised which allowed unsuppressed bitches to run during that phase. While the knowledge gained from that was not as wide-ranging and full as might have been hoped for, it did indicate that one widespread belief was false; this being that the injury rate during that 70 days would be significantly elevated. Following this trial, the NGRC altered the rules, allowing bitches to run following a now reduced '21-day layoff' period, after they came into heat (this rule is still in place today). One point worth considering here is that by not running in regular races for 70 days the animals may have returned less fit after that lay-off, which could have contributed to the reduced performance assessment for some animals on their return to the track.

The information surrounding the oestrus cycle gathered over the years has therefore led to a desire amongst many trainers to suppress the cycle of the bitch thereby minimising the variability of her racing performance and allowing her to compete all year round. The types of compounds available to suppress oestrus have been discussed in previous chapters. The main focus for this research is based on the detection of the administration of the endogenous hormone testosterone and research into the hormone profiles of bitches throughout their reproductive cycles. The latter has been mainly performed on blood samples, urine being primarily used for clinical assessment of LH and progesterone by immunoassay[7]. However, the matrix of choice for routine drug screening is currently urine. To date, no research has been carried out to determine longitudinal androgenic steroid profiles throughout the reproductive cycle of the greyhound bitch, using urine as the analytical matrix, coupled with mass spectrometry as the analytical technique.

The generic phases of the cycle have been outlined in this thesis and reviewed extensively in relation to clinical aspects of reproduction[8,9,10]. The main analytes of interest in relation to routine drug testing are the androgens, along with a possible requirement to understand the likelihood of ovulation *via* detection of progesterone.

Oestrogens are required for follicular development prior to ovulation and at a key point during that development oestrogens are produced at a rapid rate *via* a positive mechanism before production is then rapidly switched off just prior to and triggering the LH surge that ultimately stimulates ovulation. A schematic of the overall regulation of the oestrus cycle by gonadotrophins *via* the hypothalamic pituitary axis (HPA) is shown in Figure 5.2. Evidence has been provided indicating that in dogs the LH surge is activated by a relatively rapid decrease in circulating oestradiol[11].

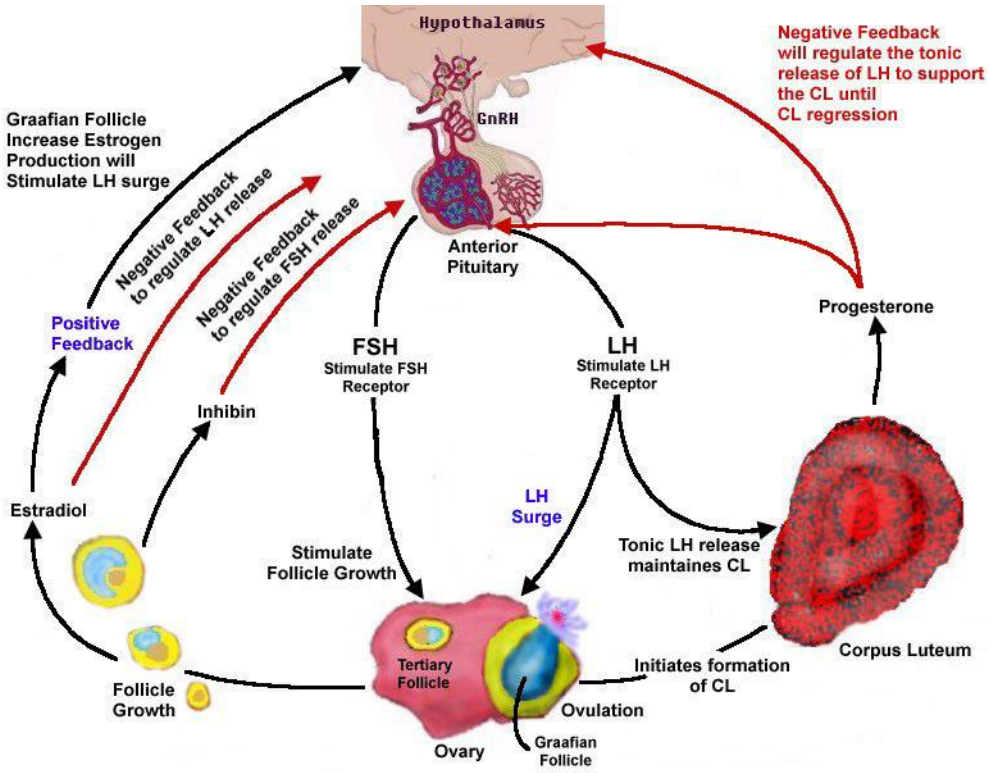
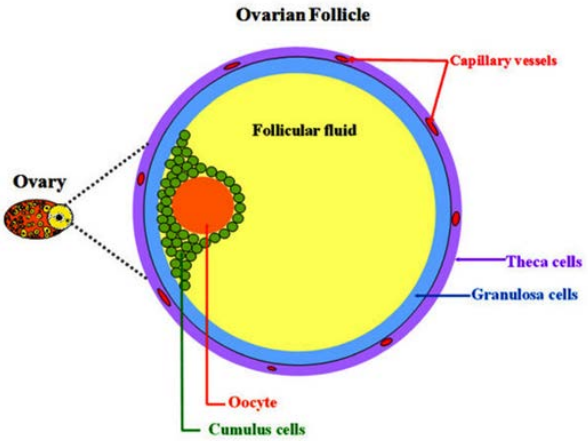


Figure 5.2: Schematic generic overview of the HPA and gonadotrophic control of the oestrus cycle, (reproduced from Essential Reproduction, Johnson M. 1995)

The production of oestrogen is 'fuelled' by androgens acting as substrates. Androgens are produced in developing theca cells, as these cells contain the necessary enzymes required to convert cholesterol through to androgens[12]. Theca cells do not have the ability to convert androgens to oestrogens, i.e do not contain aromatase enzymes. Therefore the androgens are transported to the granulosa cells which do contain aromatase and can therefore convert the androgens substrates into oestrogens. The oestrogens are transported to the developing oocyte and also to the bloodstream for self-regulation of production *via* the HPA axis.

Figure 5.3 shows a schematic of the structure of the developing follicle and the theca/granulosa cell interplay of steroidogenesis resulting in production of oestradiol. It is not known in what form the androgens are transported from theca cells to granulosa cells but since the theca is vascularised these androgens may enter the circulation. Elevations in testosterone and androstenedione concentrations have been observed in canine serum using RIA techniques[3,4].



(Reproduced from Fahiminiya S. et al. **Proteomic analysis of mare follicular fluid during late follicle development.** *Proteome Science* 2011, **9**:54.)

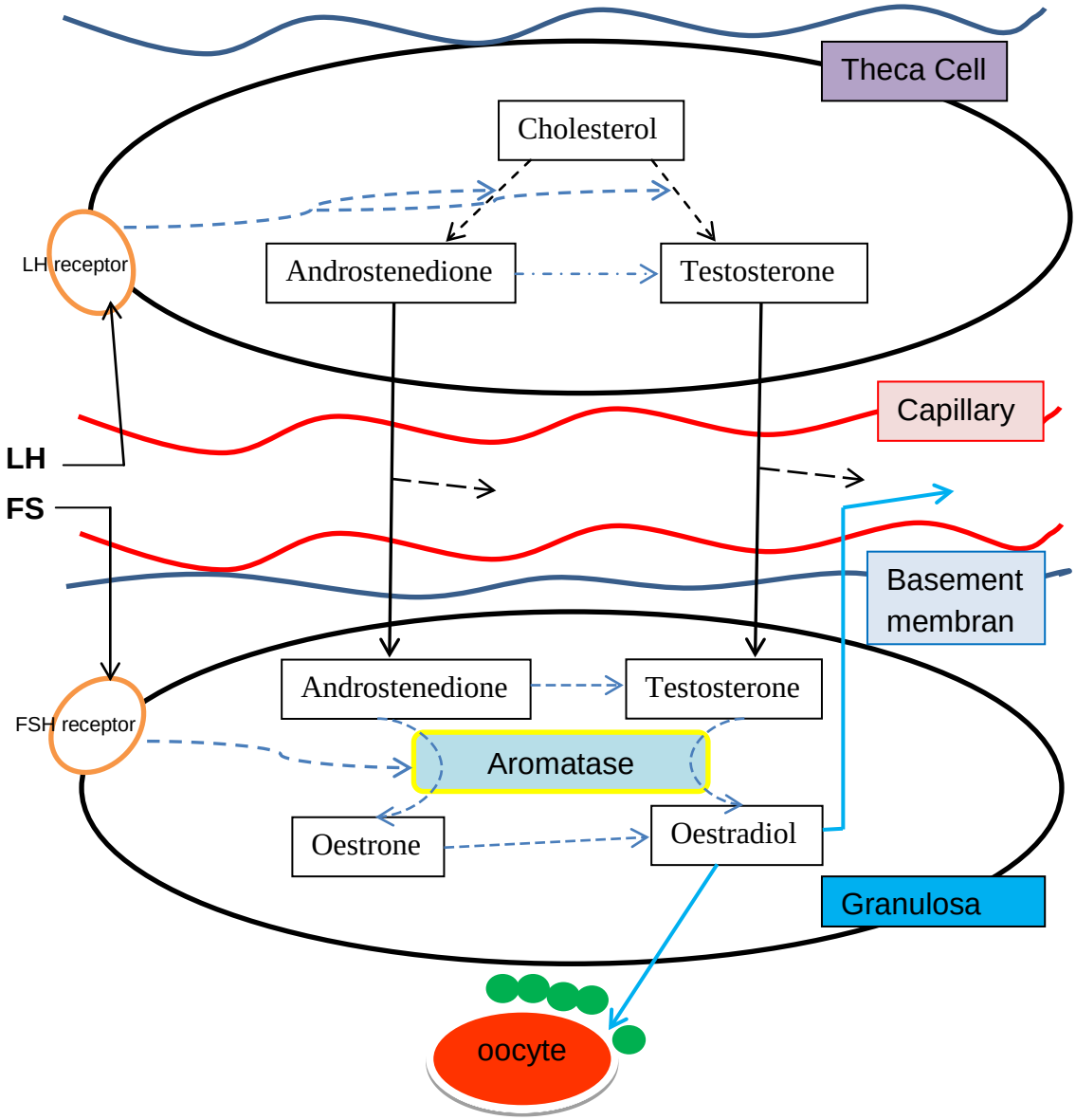


Figure 5.3: Schematic diagrams of the developing follicle and the associated production of androgens as substrates for the production of oestrogens.

Previous studies into the ‘normal’ androgen concentrations in a population of greyhound bitches (described in chapter 4) had indicated that there may be some variation in urinary testosterone metabolite concentrations at different stages of a bitch’s oestrus cycle.

Therefore in order to provide information to support setting a threshold, to enable the detection of the administration of testosterone to racing bitches, knowledge of the normal androgen profiles throughout the entire oestrus cycle was required. A population of greyhound bitches was therefore needed to support such a study, to provide an understanding of potential variation between animals, and present a more generic understanding of the androgen levels in the ‘normal’ animal. Clearly, the more animals the better for research of this type, but establishing a suitable population presented its challenges. Following evaluation of a number of possible sources of animals for the study, it was decided to set up a quorum of volunteer trainers to support the research, who would submit animals in their care into the scheme for the duration of the project.

5.2 MATERIALS AND METHODS

5.2.1 Solvents and Chemicals

C18 Sep-Pak Vac SPE cartridges (500mg, 6cc, end capped) and Silica Sep-Pak Vac SPE cartridges (500mg, 6cc) were obtained from Waters Ltd (Hertfordshire, UK). Methanol, hexane, diethyl ether, chloroform and ethyl acetate, Analar or HPLC grade, were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Sodium hydroxide, disodium hydrogen orthophosphate 2-hydrate and sodium dihydrogen orthophosphate 1-hydrate, analar grade, were supplied by BDH (Dorset, UK). Sigma Chemical Co. Ltd (Dorset, UK) supplied sodium sulphate, ammonium iodide β-Glucuronidase from *Eschericia.coli* and reference standards, 5α-androstane-3β,17β-diol, 5β-androstane-3α,17β-diol, testosterone, [$^2H_3$]-testosterone and etiocholanolone. N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Machery-nagel (Düren, Germany). Ethanethiol was supplied by Aldrich Chemical Co. Ltd (Dorset, UK). Epiandrosterone was obtained from Steraloids Inc. PO Box 689, Newport, RI 02840, USA and [$^2H_5$]-Etiocholanolone, [$^2H_5$]-5β-androstane-3α,17β-diol, [$^2H_4$]-19-norandrosterone, [$^2H_4$]-19-noretiocholanolone and [$^2H_4$]-Androsterone were obtained from NARL, Sydney, NSW 2073, Australia. [$^2H_3$]-16,16,17-5α-androstan-3α,17β-diol ([$^2H_3$]-androstanediol internal standard) was prepared at HFL Sport Science, Fordham, Camb., UK. HPLC column ACE C18-HL (150x4.6mm id, 3um) was obtained from Advanced Chromatography Technologies, Aberdeen, Scotland.

5.2.2 Urine Sample Collections for cycling bitches

Urine samples were collected from greyhound bitches throughout their entire oestrus cycle. Animals were provided by trainers who stated that they would not be using oestrus suppressants in the animals volunteered by them for the scheme. These trainers were in the cohort that preferred not to suppress the cycles of their racing animals. Sample collection began on the first day of the animals 'season'. This was based upon visual recognition of the cycle status of the bitch, by the trainer concerned, i.e. visible and behavioural signs of developing pro-oestrus.

From this point onwards, urine samples were collected twice a week for 14 weeks and then once a week thereafter, until the animal was deemed to be starting its next oestrus

cycle (i.e. commencement of pro-oestrus again), at which time urine sample collection then ceased.

This procedure therefore provided samples for each animal from pro-oestrus through oestrus and di-oestrus, back into anoestrus and ultimately into pro-oestrus again. In general, pro-oestrus bleeding was used as the indicator to trainers that the bitch was beginning her seasonal cycle and that sample collection should therefore begin. In the case of the return to pro-oestrus, bleeding would indicate that sampling should then cease.

Animals were volunteered into the scheme by trainers who then needed to wait until the animal came into season before starting sample collection. Precise knowledge of each animals 'due' date was not available in many cases. This is not an uncommon situation for trainers of racing bitches. Therefore, in a number of instances this resulted in several months passing before the first sample could be collected. The average oestrus cycle of the greyhound bitch is around 8-9 months. However, many racing bitches will have significantly different timespans between their individual cycles, which therefore meant that completion of the monitoring process and sample collection throughout the cycle, could take up to one year for each animal.

Longitudinal sample collections were ultimately obtained for 33 bitches over the course of several years. More animals started the scheme, but for various reasons, not all of them were able to complete the entire sampling programme. Several different trainers volunteered to help with the scheme and whilst they were each remunerated for their efforts, their willingness to provide such a unique set of urine samples over a long period of time was still nevertheless, highly commendable.

One trainer in particular collected daily urine samples for one animal leading up to the start of the bitch's oestrus and for several weeks thereafter, to try and help provide even more detailed information on the urinary hormonal status of the animal during that phase of the cycle.

Trainers were required to fill out forms with the relevant information and return them to HFL Sport Science along with the samples upon completion. Urine sample kits were provided for each trainer in the scheme, allowing provision for a maximum sample volume of 50ml. Trainers were required to add both the date of sampling and the animals ear-tag number to pre-printed labels provided for each bitch submitted for the cycling study. Sample kits were provided with pre-paid first class postage to assist the trainers with returning the samples to the laboratory through the normal postal service,

thereby enhancing the efficiency of the process. On arrival at the laboratory samples were recorded on a database, dispensed and stored frozen at -20^{o}C until required for analysis. The process was extremely efficient and samples were received in good time with all the relevant information associated with them.

5.2.3 Animal Administration - progesterone

The animal administration was performed at the Racing Chemistry Veterinary Diagnostic Laboratory, Iowa State University, U.S.A. Following ethical approval, one bitch (7335, Weight 28kg) was administered an intramuscular injection (3mg/kg, total 84.5mgs) of progesterone in sesame oil. The animal was selected at a time when she was believed to be in anoestrus to try and provide a greater distinction between the steroid levels in the pre-dose and post dose samples. A naturally voided blank urine sample was obtained prior to administration. Post administration urine samples were collected at +8, 24hrs and +2, 3, 4, 21 and 28 days after the dose. The urine samples were stored at -20^{o}C awaiting analysis.

5.2.4 Qualitative Sample Analysis

5.2.4.1 Progesterone metabolism

To assist with the identification of suitable marker metabolites of progesterone in post ovulatory greyhound urine, samples from the intramuscular progesterone administration study were analysed alongside urine samples from some of the normal cycling bitches. Urine samples from the cycling bitches were chosen at time points that were considered likely to present the highest possible levels of progesterone metabolites, in order to try and identify suitable markers for monitoring throughout the oestrus cycle. These were selected at approximately 3-4 weeks after the onset of pro-oestrus.

5.2.4.2 Sample extraction

Aliquots of canine urine (4 mL) were spiked with internal markers [$^{2}H_{3}$]-androstanediol and [$^{2}H_{5}$]-etiocholanonlone at a level of 25 ng mL^{-1}. Extraction cartridges (C18 Sep-Pak Vac, 6 cc, 500 mg) were conditioned with methanol (5 mL) and water (reagent grade, 5 mL). The urine samples were then passed through the cartridges followed by water (5 mL). The column was washed with hexane (5 mL) and then dried with air

under vacuum for 5 min. The cartridge was eluted with methanol (5 mL). The methanol was mixed and split into 2 equal portions. The eluates were evaporated using OFN at 80ºC, the residue dissolved in phosphate buffer (1 mL, pH 6.8, 0.1 M). β-glucuronidase *E. coli* (250 µl, 10 units μL^{-1}) was added to one of the extracts and the solution incubated overnight at 37ºC.

After incubation, sodium hydroxide (500 µl, 2 M) was added to all sample extracts and mixed using a whirlimixer. Liquid-liquid extraction was carried out using tertiary butyl methyl ether (TBME, 4 mL). The aqueous layer was removed and the organic solvent washed with sodium hydroxide (2 x 500 µL, 2 M). The TBME was dried over anhydrous sodium sulphate and then evaporated to dryness under OFN at 40ºC in a low volume vial.

5.2.4.3 Derivatisation

The enol-tms derivatisation reagent was prepared from MSTFA (1 mL), ammonium iodide (3 mg) and ethanethiol (6 µL); the mixture being heated at 80ºC for 20 mins to dissolve solid material. The derivatising reagent (30 µL) was added to the urinary extracts and the solution heated at 80ºC for 1.5 – 2 hours, and a 1 µl aliquot injected on to GC-MS for analysis.

5.2.4.4 GC-MS analysis

Full Scan GC-MS analysis was carried out in positive ion electron ionisation mode, using a Thermo Finnigan (Austin, Texas, USA) DSQII bench top mass analyser instrument fitted with an SGE (SGE Europe Ltd, Milton Keynes, U.K.) 5% phenyl polysilphenylene-siloxane (BPX5) column, (approx. 29 m, 0.25 mm id, 0.25 µm film thickness). The temperature of the split/splitless injector was 260ºC. Injections were made in the splitless mode which was maintained for 1.0 minute. The interface temperature was 260ºC. Injection volume was 1 µL. The GC temperature programme was started at 180ºC and held for 0.5 min. Initial ramp 180ºC to 290ºC at 5ºC min^{-1} and then further from 290ºC to 330ºC at 40 ºC min^{-1}. The final temp was held at 330ºC for 4 min.

5.2.4.5 Evaluation of C18 / Silica Sep-Pak extraction for the cycle sample analyses

Samples were extracted using the original procedure developed for the testosterone population study in chapter 4 (section 4.2.4) to establish whether this method was

suitable for the analysis of the progesterone metabolites. This method involves the use of C18 and silica SPE. The C18 Sep-Pak methodology has historically shown high, stable and reproducible recoveries (80-90%) for all the various androgenic anabolic steroid metabolites, including 17-hydroxy-3-keto steroids, 3-hydroxy-17-keto steroids, androstanediols, estranediols and various -triols of differing stereochemistries[13,14]. However, analysis of the progesterone metabolites observed in the administration study had not been previously investigated.

5.2.4.6 HPLC Fractionation

Sample extracts were also purified using an LC fractionation procedure utilising an Agilent 1100 series HPLC system with Diode Array coupled to a Gilson FC204 Fraction Collector, as an alternative approach to providing clean sample extracts for quantitative analysis of all the analytes of interest.

Following sample extraction using C18 Sep-pak/liquid liquid preparation only, extracts were reconstituted in methanol:water 80:20 (100ul) and injected onto an HPLC system utilising an ACE C18-HL column (150x4.6mm ID, 3 um), flow rate 500 ul/min with mobile phase start conditions 80:20 methanol:water, isocratic for 5 mins, rising to 100% methanol at 5.1 mins held to 17 mins, before re-equilibrating to start conditions (total cycle time 24 mins).

The fraction between 5-11 mins was collected for each sample. The collected fraction was evaporated to dryness in a GC vial and the extract derivatised as detailed in section 5.2.4.3.

5.2.5 Quantitative Sample Analysis

Previous research (see chapter 4) had indicated that the major observed metabolites following the administration of testosterone to the greyhound were 5α-androstane-3β,17β-diol, epiandrosterone and 5β-androstane-3β,17β-diol. These metabolites were therefore selected for quantitation in the oestrus cycle urine samples, along with some other analytes of interest, namely testosterone, etiocholanolone and epitestosterone.

An appropriate metabolite of progesterone (M1 – see section 5.3.1) was selected for detection alongside the androgens, but no reference standard was available for quantitation.

Steroid stripped bitch urine was prepared and used for calibration and quality control samples. Calibration standards were prepared for testosterone, epitestosterone,

etiocholanolone, epiandrosterone, 5α-androstane-3β,17β-diol and 5β-androstane-3α,17β-diol at concentrations 125 $pgml^{-1}$, 250 $pgml^{-1}$, 500 $pgml^{-1}$, 1, 2, 4, and 8 $ngml^{-1}$ with quality control samples at 0.375 and 3 $ngml^{-1}$

Repeat analyses were performed on urine samples that had concentrations of analytes above the top point of the calibration curve. These samples were re-analysed alongside a higher range calibration curve to ensure that higher concentrations obtained for each analyte at certain timepoints, were not derived from extrapolation. The calibration range used was that applied to the analysis of the Durateston™ post administration samples (chapter 4, section 4.2.6). Details of the batch acceptance data are shown in appendix III. Samples with apparently 'unusual' results, following data interpretation, were also reanalysed to ensure that no inappropriate conclusions were drawn regarding these findings.

5.2.5.1 Sample extraction

All urine samples, calibration and QC samples were spiked with internal markers [$^2H_4$]-androsterone, [$^2H_3$]-testosterone and [$^2H_5$]-5β-androstane-3α,17β-diol at a concentration of 25ng/mL. Extraction cartridges (C18 Sep-Pak Vac, 6cc, 500mg) were conditioned with methanol (5 mL) and water (reagent grade, 5 mL). Each sample (4 mL) was then passed through a cartridge followed by water (5 mL). The cartridge phase was washed with hexane (5 mL) and then dried with air under vacuum for 5 min. Extracts were then eluted with methanol (4 mL). The eluates were evaporated using oxygen free nitrogen at 80ºC and dissolved in phosphate buffer (1mL, pH6.8, 0.1M) containing β-glucuronidase *E. coli* (250µl, 10units/µl). The extracts were hydrolysed overnight at 37ºC.

After incubation, sodium hydroxide (500 µL, 2 M) was added to all sample extracts and mixed using a whirlimixer. Liquid-liquid extraction was carried out using tertiary butyl methyl ether (TBME, 4 mL). The aqueous layer was removed and the organic solvent washed with sodium hydroxide (2 x 500 µl, 2 M). The TBME was dried over anhydrous sodium sulphate and then evaporated to dryness under OFN at 40ºC in a low volume vial. The resulting extracts were reconstituted in mobile phase (100 uL methanol:water, 80:20) and further purified using the LC fractionation procedure described in section 5.2.4.6. The final collected fractions were then evaporated and

enol-tms derivatives were formed prior to GC-MS analysis as described in section 5.2.4.3.

5.2.5.2 GC-MS analysis

Data was acquired in the Selected Ion Monitoring mode for the quantitative analysis of testosterone metabolites in the oestrus cycle urine sample extracts. From 18 minutes onwards until the end of the run, data was acquired in full scan mode to monitor the progesterone related metabolites.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of each batch was carried out in positive ion electron ionisation mode, using a Thermo DSQII bench top mass analyser instrument, fitted with an SGE BPX5 column (approx. 29m, 0.25mm id, 0.25µm film thickness). The injector temperature was 260^{o}C, splitless for 1.0 minute, and the interface temperature was set at 330^{o}C. Injection volume was 1µl. The GC temperature programme was as follows: initial temperature 180^{o}C, held for 0.5 minute, then ramping from 180^{o}C to 290^{o}C at 5^{o}Cmin^{-1} followed by 40^{o}Cmin^{-1} from 290^{o}C to 330^{o}C, with a final hold time of 3 minutes. The ions acquired in Selected Ion Monitoring (SIM) mode for the relevant analytes are shown in Table 5.1.

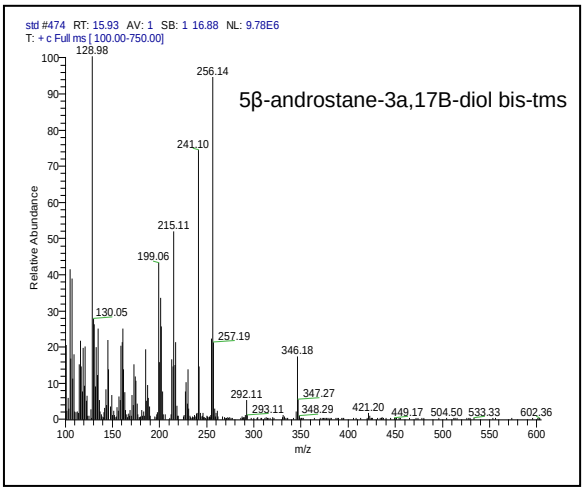
Table 5.1: GC-MS (SIM) method acquisition ions

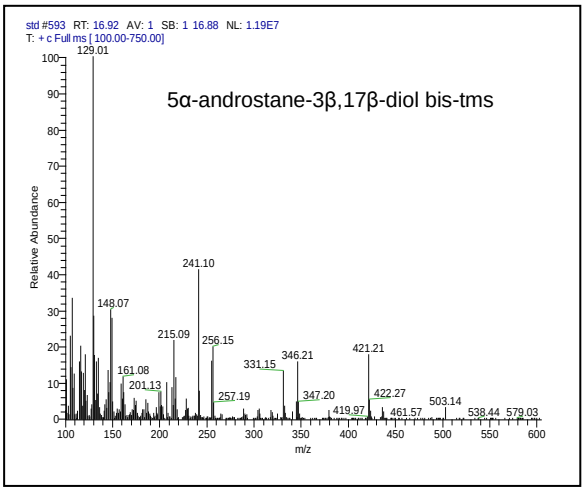
Ions	**Monitoring**
256.1, 346.2, 421.3	Androstanediols
351.2, 426.3	[$^{2}H_5$]-Androstanediol
417.3, 432.3	Testosterone, epitestosterone
419.3, 434.3	Epiandrosterone
420.3, 435.3	[$^{2}H_3$]-Testosterone
423.3, 438.3	[$^{2}H_4$]-Androsterone

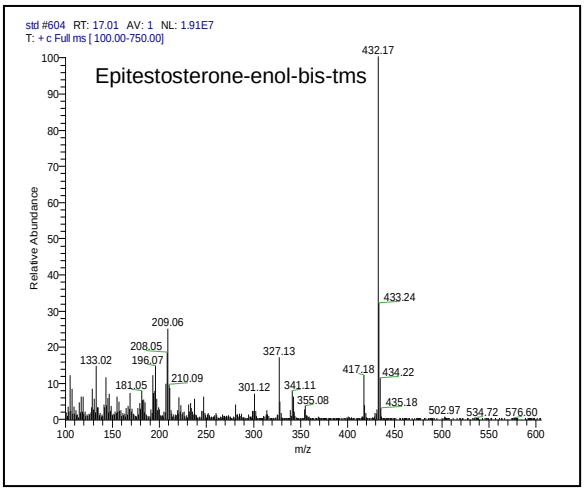
Each analyte had two appropriate fragment ions selected for analysis, one as the main ion for quantitation and the other as a qualifier ion to support the identification of each compound, so that the correct analyte was being quantified at all times. Ions were chosen based on optimum sensitivity and selectivity for the assay.

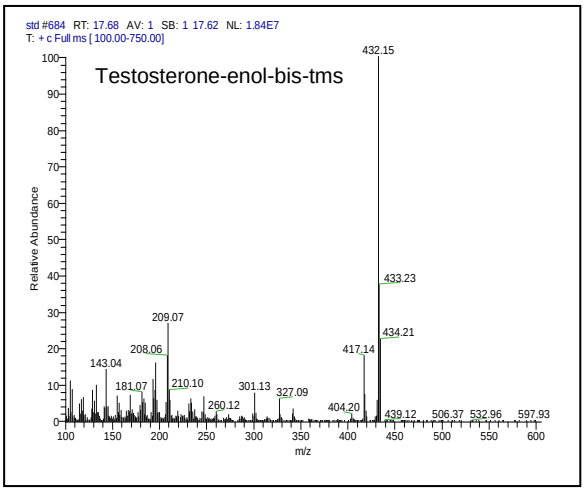
Full scan spectra for the trimethylsilyl derivatives of the major testosterone metabolites in the greyhound as well as those of testosterone itself, epitestosterone and etiocholanolone showing the relevant fragment ions, are presented in Figure 5.4.

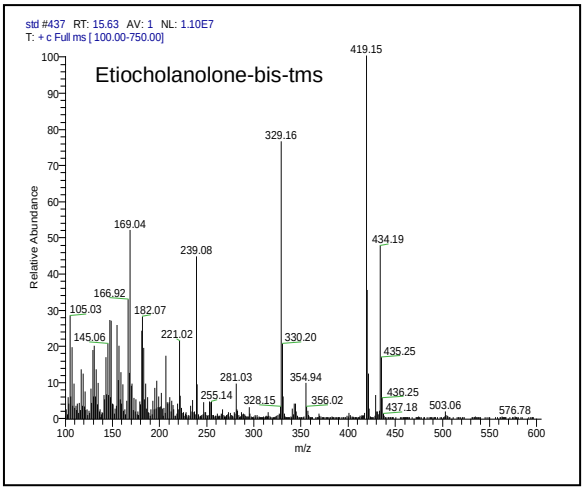
Figure 5.4: Trimethylsilyl derivative mass spectra of the major androgens quantified throughout the oestrus cycle of the greyhound bitch

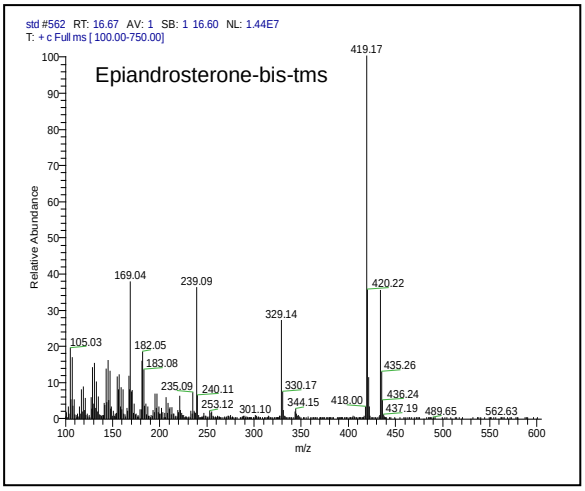












5.3 RESULTS AND DISCUSSION

5.3.1 Metabolism of progesterone

Figures 5.5 and 5.6 show pre-dose and post-dose ion chromatograms proposed major metabolites observed following the administration of progesterone to the greyhound The pre-dose urine sample clearly contained small quantities of the progesterone metabolites as they are naturally occurring endogenous compounds in the greyhound bitch. The amounts observed in the pre-dose sample are likely to represent the latter stages of dioestrus/early anoestrus as they were collected at around 90 days post season for the bitch used in the study. They are probably not absolute basal anoestrus amounts but nevertheless allowed for discrimination between pre and post dose samples. Four key metabolites were detected (assigned M1-M4). The first metabolite (M1) has a mass spectrum dominated by the base ion *m/z* 117 which is a characteristic fragment ion generated from trimethylsilyl derivatised pregnanes with a reduced keto function at C-20 (see figure 5.7). Figure 5.8 shows a zoomed in spectrum to display the lower intensity, higher mass, fragment ions to aid structural elucidation. . The metabolite was proposed as a pregnanetriol (see scheme 5.1) with a suspected derivatised molecular weight of 552 mass units. Fragment ions were observed at *m/z* 537 (M^+-15), 462 ($M^{+\cdot}$-90), 447 (M^+-15+90), 435 (M^+-117), 372 ($M^{+\cdot}$-90+90) and 282 ($M^{+\cdot}$-90+90+90).

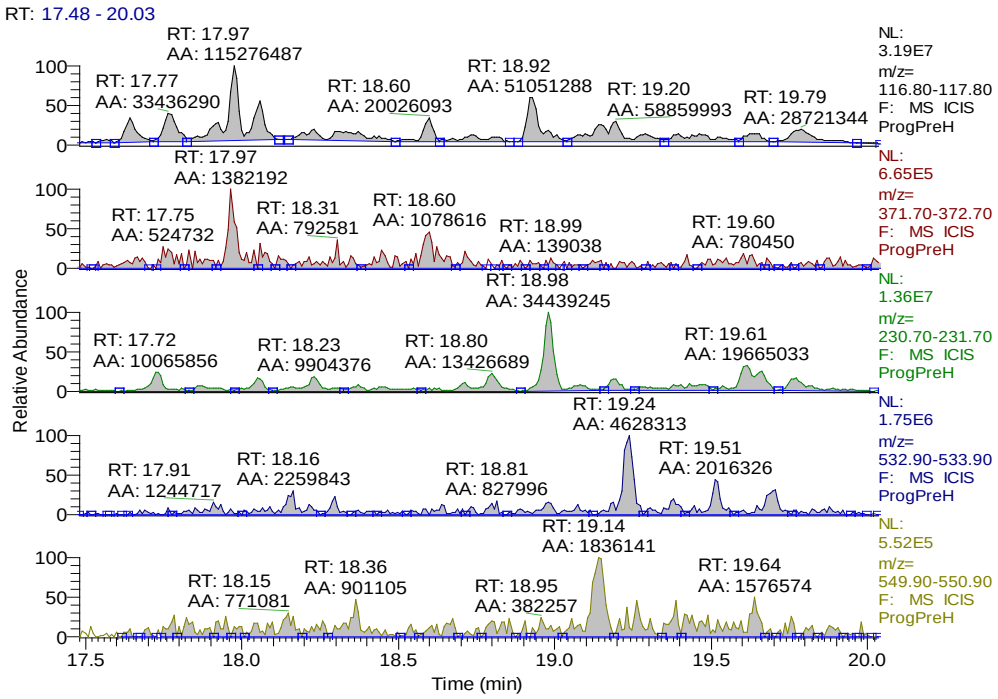


Figure 5.5: Ion chromatograms for progesterone pre-dose urine (glucuronide fraction)

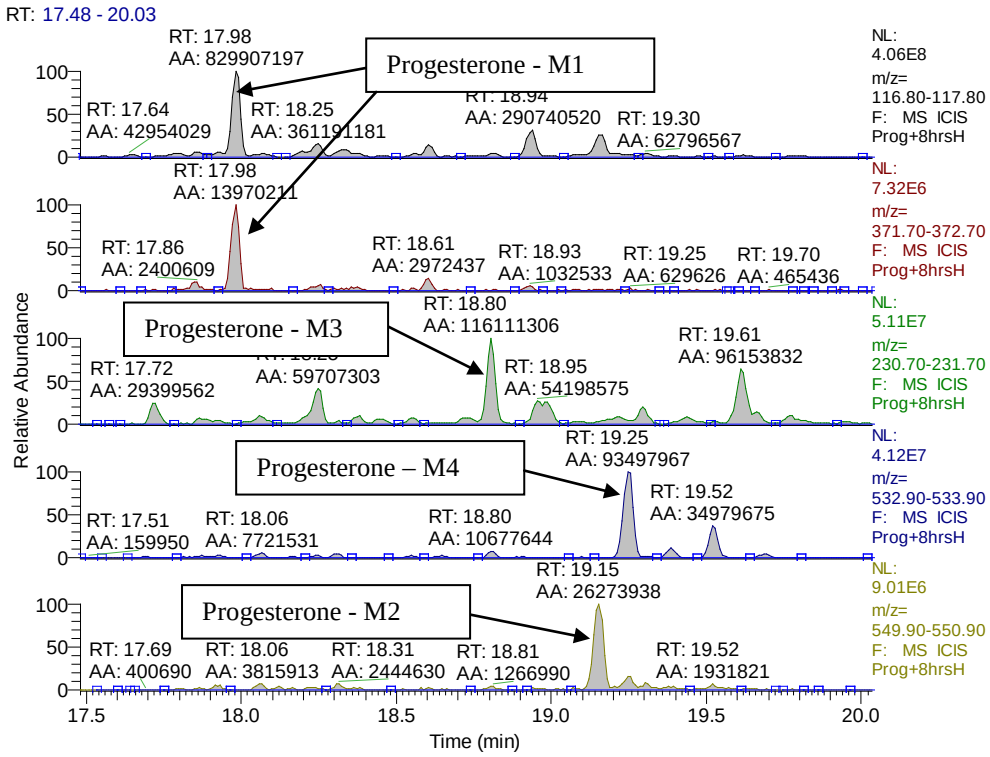


Figure 5.6: Ion chromatograms for progesterone post-dose (+8hrs) urine (glucuronide)

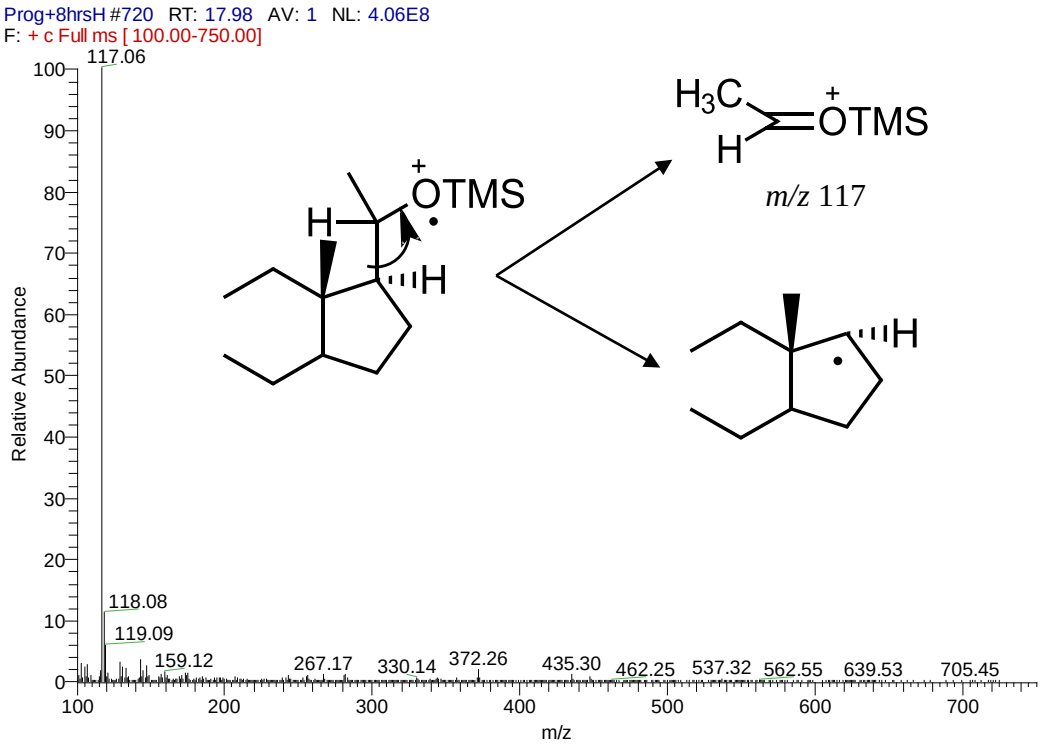


Figure 5.7: Mass spectrum of ?pregnanetriol metabolite (M1) of progesterone post-dose (+8hrs) urine (glucuronide fraction)

The loss of three TMSiOH groups (M-90) indicated the presence of three aliphatic hydroxyl functions in the molecule suggesting reduction at C3 in addition to reduction

at C20 and mono-hydroxylation at possibly C6. The intensity of the 357 ion (M^{+}-15+90+90) is less than that of the 372 ($M^{+\cdot}$-90+90). This might suggest that this is a 5β-reduced metabolite, analogous to the same losses for the androstanediol metabolites described in chapter 4.

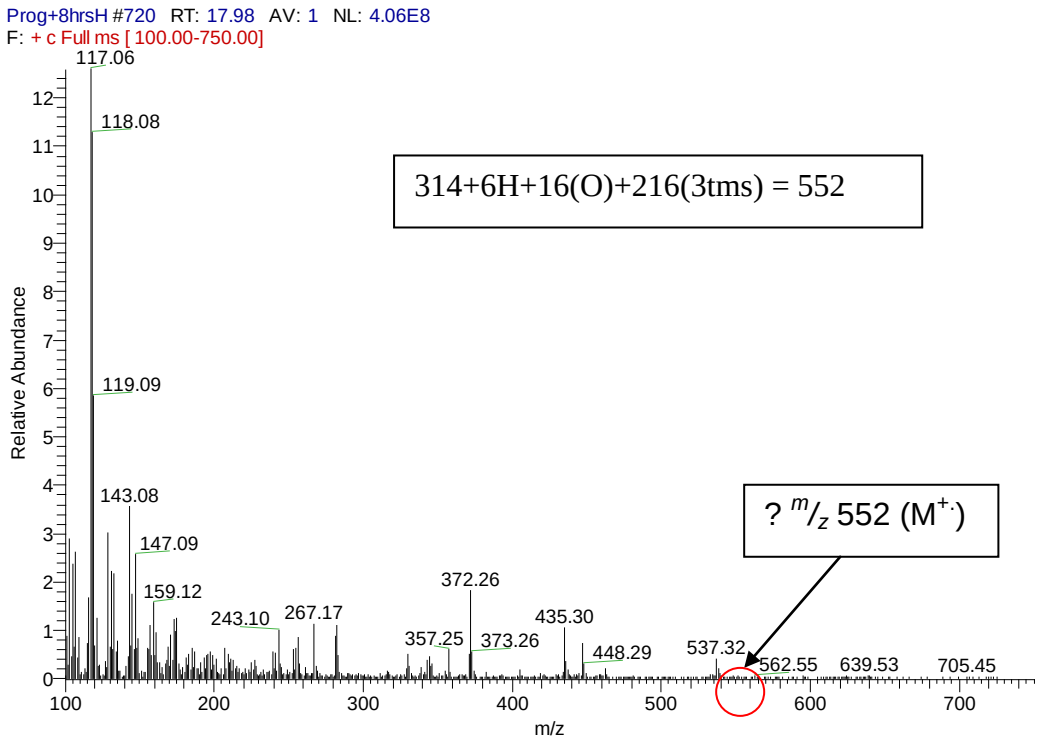


Figure 5.8: 'Zoomed in' mass spectrum of putative pregnanetriol metabolite (M1) of progesterone post-dose (+8hrs) urine extract (glucuronide fraction)

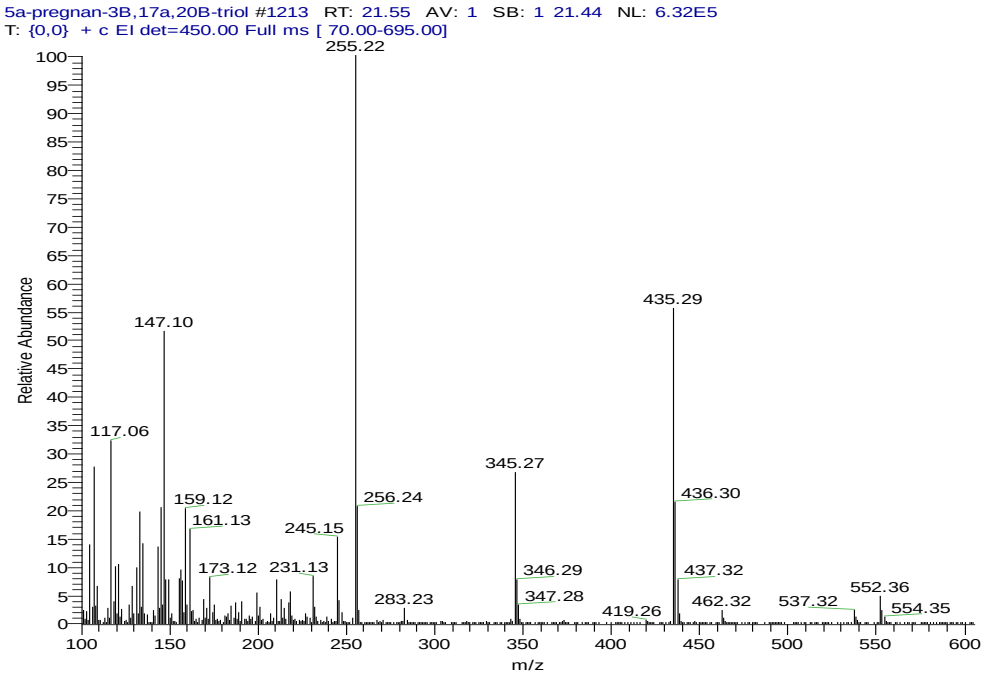


Figure 5.8a: Mass spectrum of 17α-hydroxy-5α-pregnane-3β,20B-triol reference std.

The M1 metabolite was not considered to be a 17-hydroxy compound as such analytes are known to give qualitatively different mass spectra as TMS derivatives than that observed for M1, with major fragment ions at 255, 345 and 435. See figure 5.8a.

Figure 5.9 shows the mass spectrum of M2 with a derivatised molecular weight of 550 mass units. The fragment ion of m/z 117 again indicated that the keto function at C-20 had been reduced. The compound was postulated as a mono-hydroxy bis reduced metabolite. No reference standards were available for comparison. Figure 5.10 shows the mass spectrum of M3 indicating a derivatised molecular weight of 638 mass units. This represents a possible bis-hydroxy-bis-reduced metabolite. The fragment ion at m/z 231 suggests possible hydroxylation of the D-ring, probably at C-16. Figure 5.11 shows the mass spectrum of M4 indicating a mono-hydroxy, mono-reduced metabolite, with a derivatised molecular weight of 548 mass units. The fragment ion at m/z 157 shows that the D-ring is unaltered. Suggested structures for M1–M4 are shown in scheme 5.1. An excretion profile for M1-M4 is also shown in figure 5.12. The profile indicates that progesterone is cleared relatively quickly following intramuscular administration. This has previously been observed by during a pharmacokinetic study in canine serum[15].

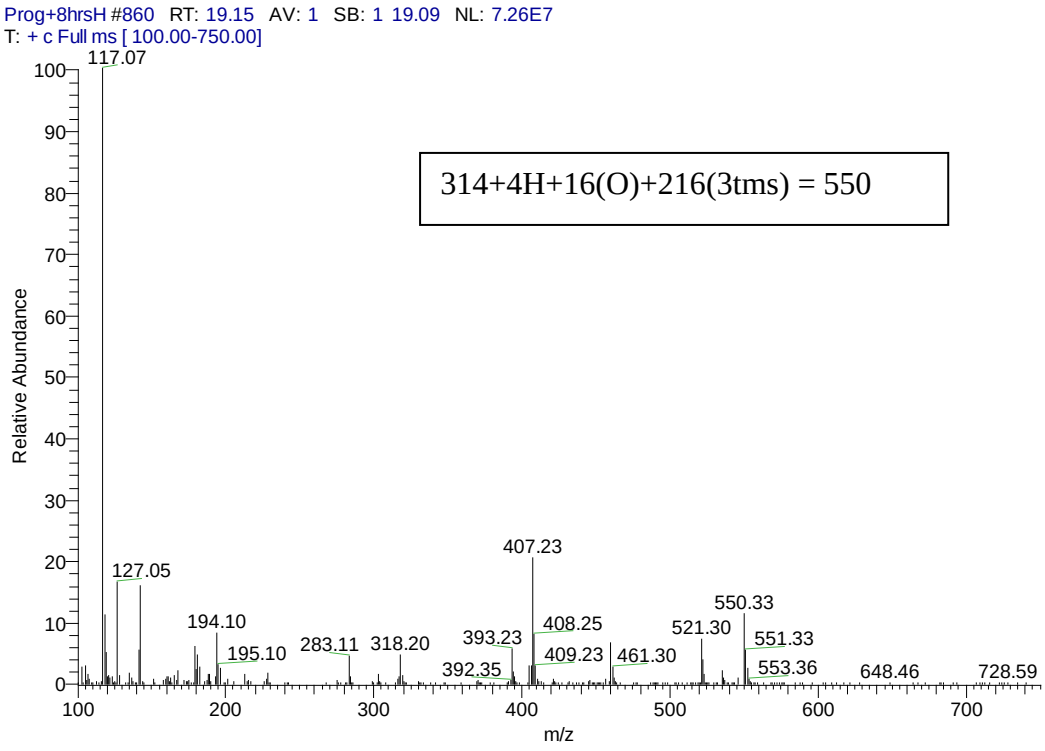


Figure 5.9: Mass spectrum of proposed progesterone metabolite (M2) (glucuronide fraction)

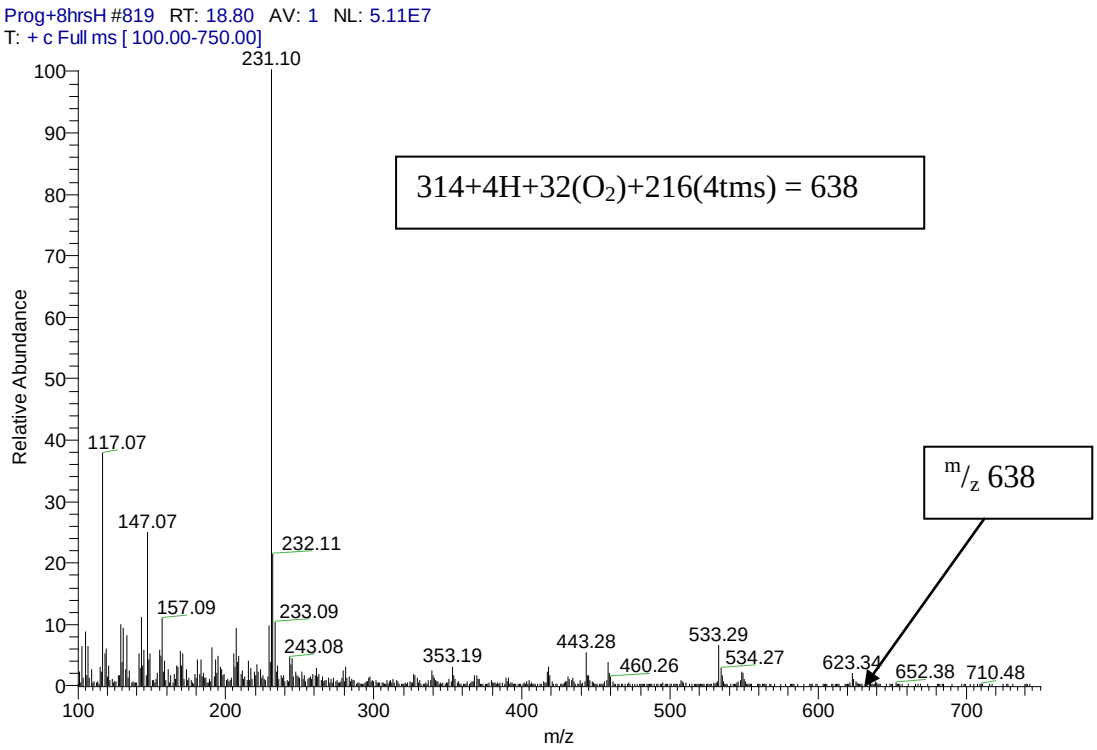


Figure 5.10: Mass spectrum of proposed progesterone metabolite (M3) (glucuronide fraction)

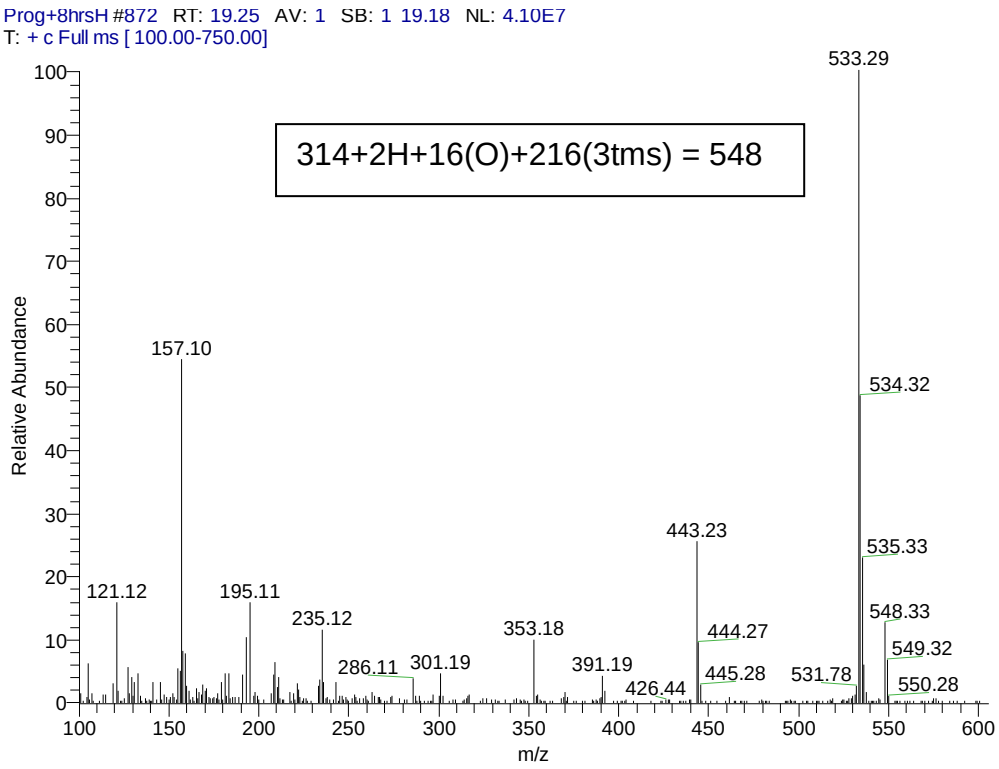
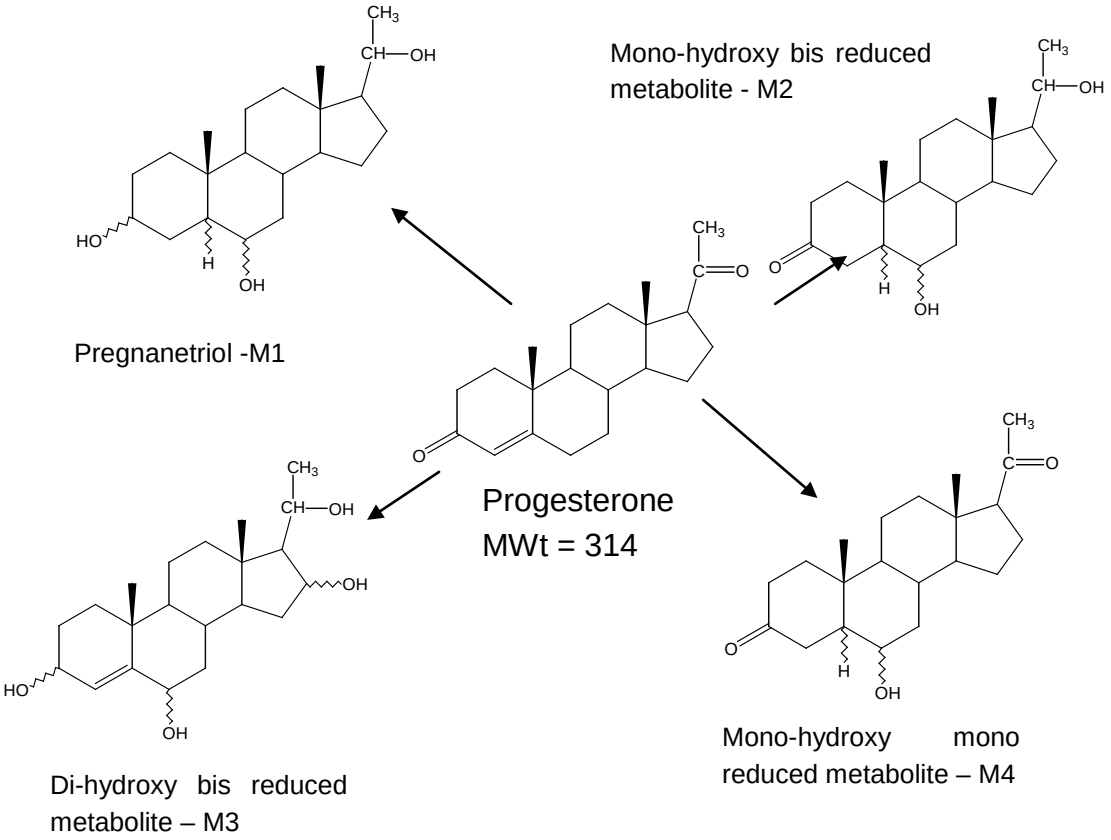


Figure 5.11: Mass spectrum of progesterone metabolite (M4) (glucuronide fraction)



Scheme 5.1: Summary of proposed major progesterone metabolites

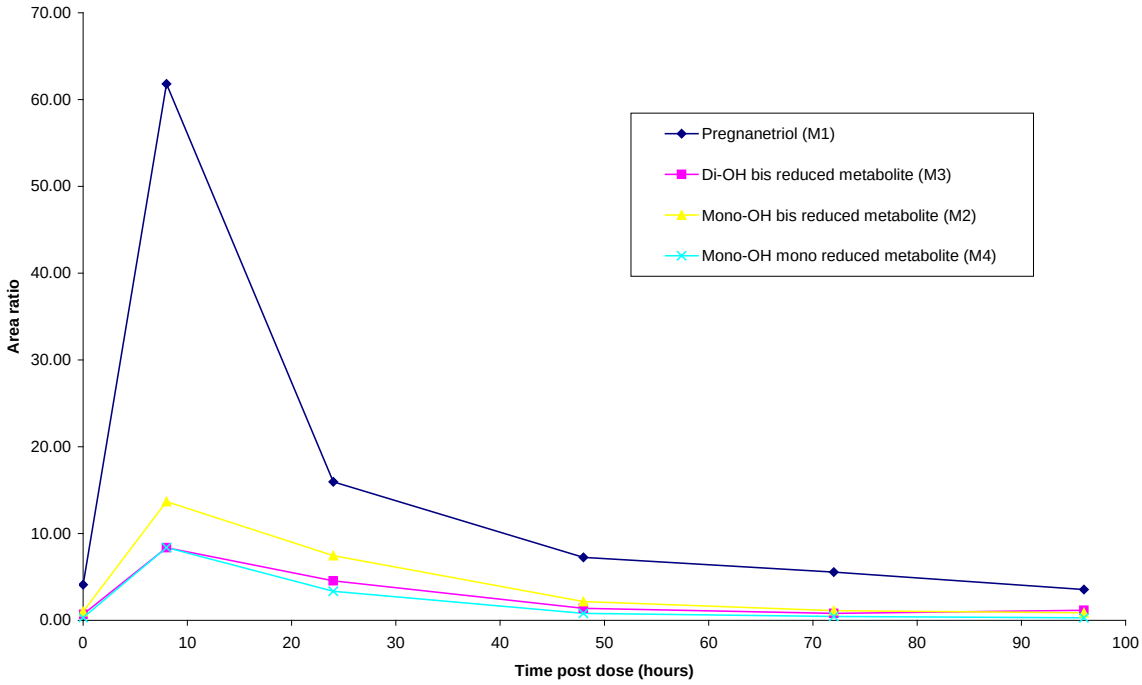


Figure 5.12: Urinary excretion profile of proposed progesterone metabolites following intramuscular administration to the greyhound

All of the metabolites were detected in the hydrolysed fraction and were therefore considered to be glucuronic acid conjugates. No significant quantities of these metabolites were observed in the urinary free fraction.

5.3.1.1 Analysis of oestrus cycle samples for progesterone metabolites.

Several of the urine samples analysed from bitches at 3-4 weeks into their oestrus cycles gave indications for the presence of all of the major progesterone metabolites to a lesser or greater extent. The most significant marker metabolite present in all of the bitches was the proposed pregnanetriol (M1). Figure 5.13 shows an example of extracted ion chromatograms for the major progesterone metabolites in the +26days post season urine sample from Queen of Stout. The chromatograms indicate the M1 metabolite as the most abundant marker metabolite. This pregnanetriol metabolite was therefore selected as the analyte to monitor in the cycling bitches in order to determine their progesterone production throughout the cycle and provide indication that they had ovulated during oestrus and subsequently formed a corpus luteum.

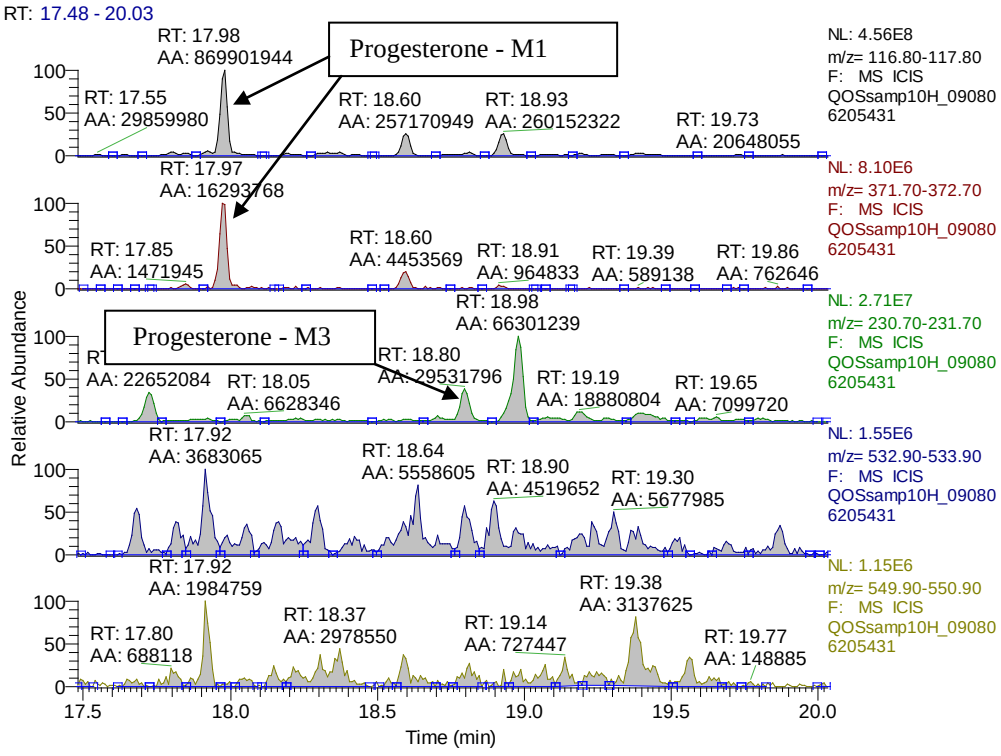


Figure 5.13: Ion chromatograms for Queen of Stout (+26 days in season) urine (glucuronide fraction).

5.3.2 Results of C18/Silica Sep-Pak extraction evaluation (cycle samples)

The *m/z* 117 ion chromatogram in figure 5.14 shows the detection of the major pregnanetriol (M1) urinary progesterone metabolite, following the C18/liquid-liquid extraction procedure only. Metabolite M1 is clearly present.

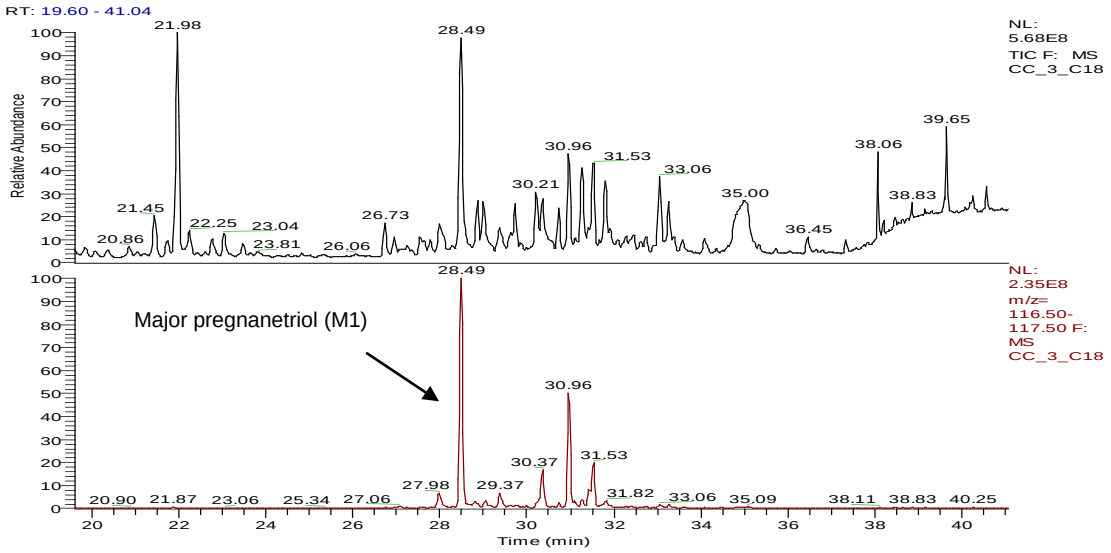


Figure 5.14: Urine extract using solid phase/liquid-liquid extraction only

The *m/z* 117 ion chromatogram in Figure 5.15 shows that following further clean-up of the C18/liquid-liquid extract using the silica clean-up procedure, the major progesterone metabolite was not recovered. This silica clean-up method was therefore not suitable to recover all the compounds of interest in the oestrus cycle profile analysis

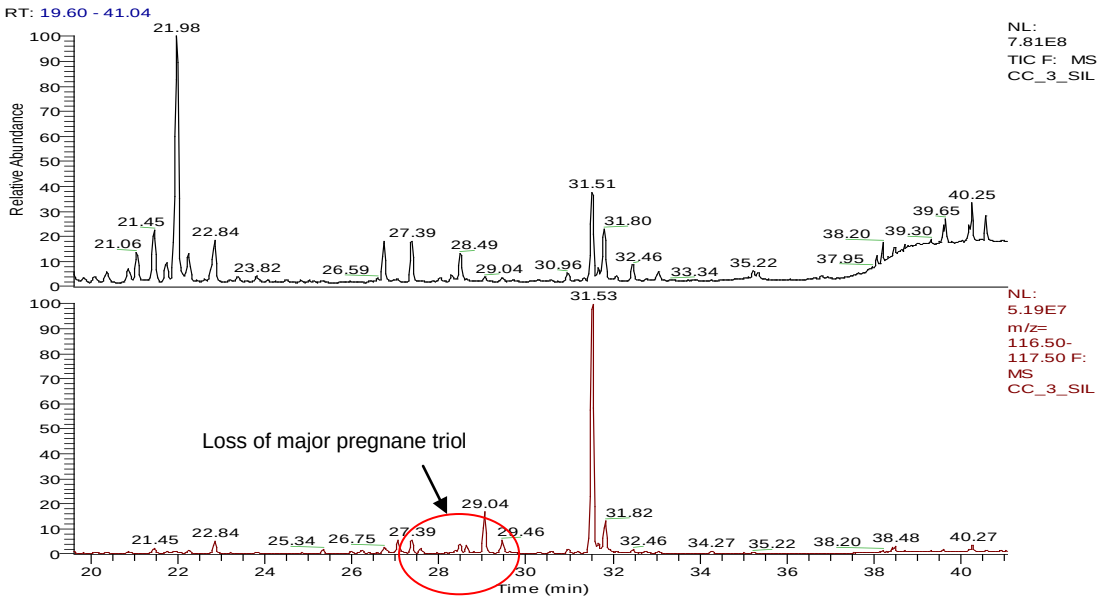


Figure 5.15: Urine extract using solid phase/liquid-liquid extraction and silica clean-up

It is interesting to note that previous experience with 6-hydroxy steroids has shown these compounds to bind to silica quite readily, even for di-hydroxy 3-keto structures, whereas 3,16,17-triols are recovered using the silica clean up. This may be due to an increased 'coverage' of the ring structure leading to an increase in polar behaviour of the analyte under these extraction conditions.

5.3.3 C18 Sep-Pak / liquid-liquid method re-evaluation

Having established that the major progesterone metabolite to be monitored was not recovered using the silica-clean-up procedure, further development work was needed to establish a clean-up method for the extracts that recovered all the analytes of interest. The data in Figures 5.16-5.19 shows that the combination of C18 and liquid/liquid extraction, for sample extract preparation, did not appear to be sufficient for accurate quantitation of some of the key analytes of interest. On inspection of the full scan data, it was shown that there were co-eluting analytes for both epiandrosterone (Figures 5.16 and 5.17) and 5α-androstane-3β,17β-diol (Figures 5.18-5.19). For epiandrosterone, an isotope ion from the co-eluting analyte could give rise to falsely elevated values. For the analysis of 5α-androstane-3β,17β-diol, the co-eluting compound appears to contain the same ion that is monitored for this particular androstanediol isomer resulting in even greater potential for false elevation of the concentration of this analyte. Therefore, a further clean-up method was investigated involving HPLC fractionation prior to GC-MS quantitation, in order to aid the quantitation of low level concentrations of analytes in untreated animals.

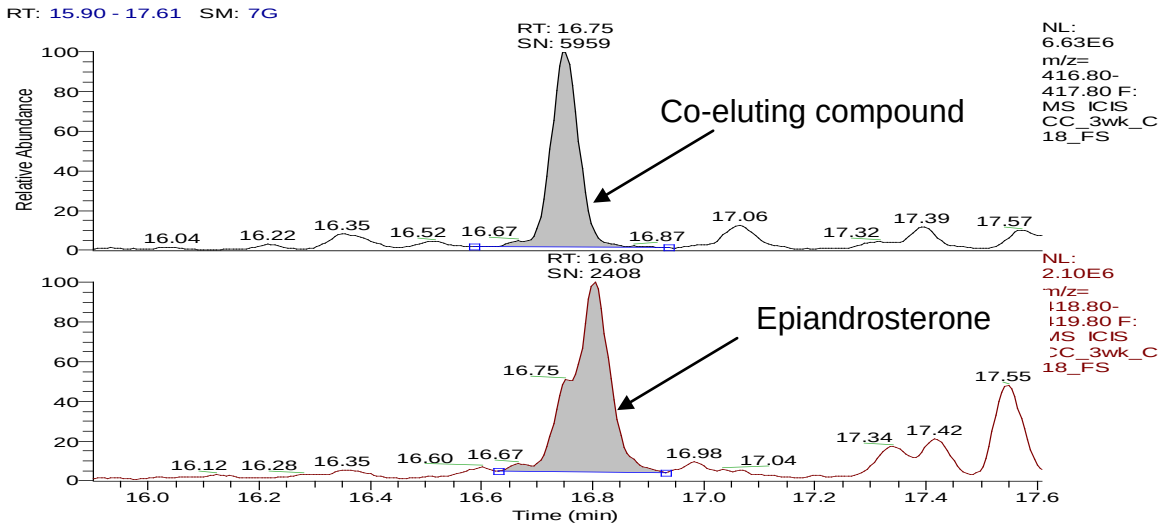


Figure 5.16: Ion chromatograms for epiandrosterone and co-eluting compound

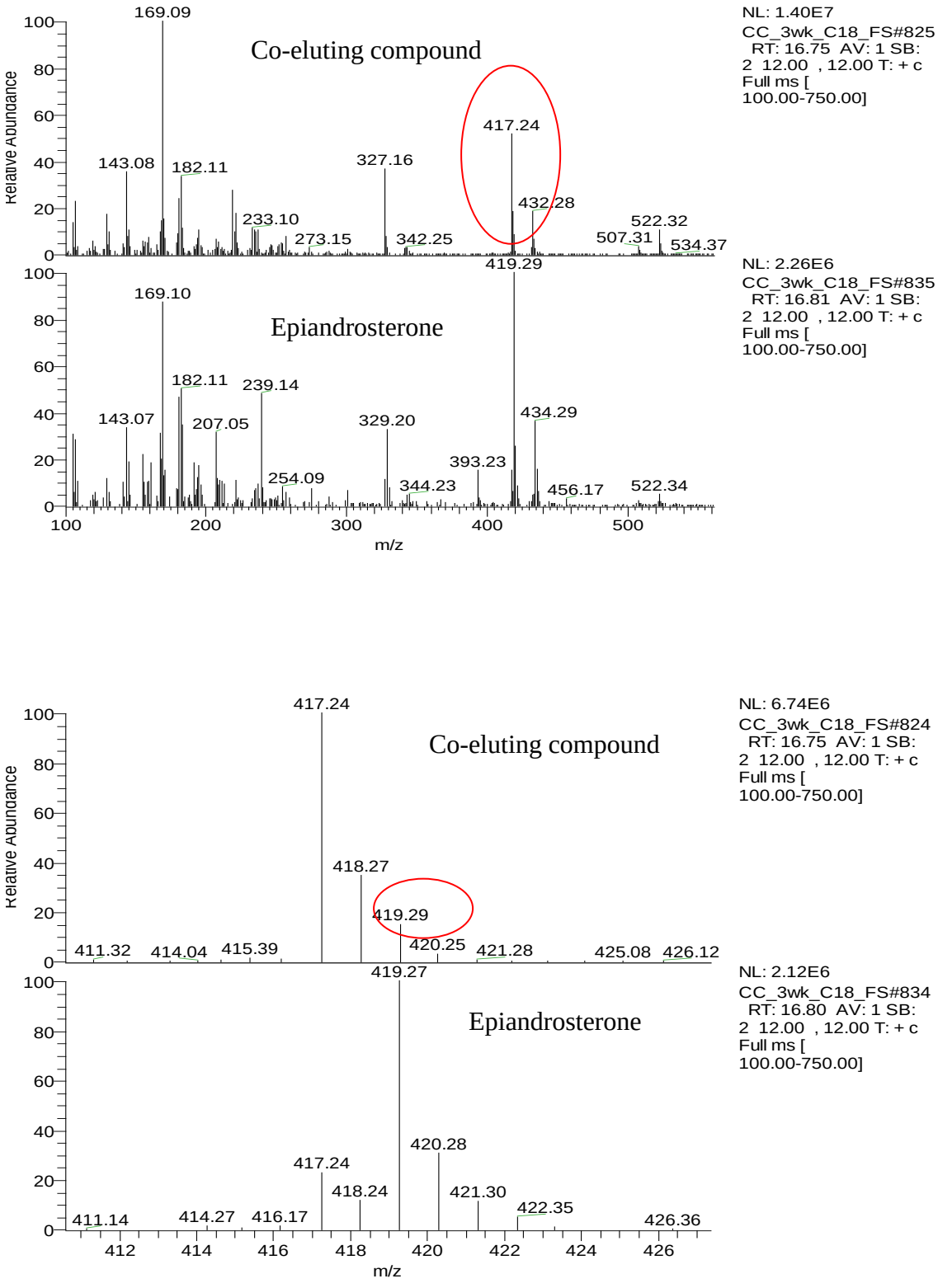


Figure 5.17: Mass spectra for epiandrosterone and co-eluting compound

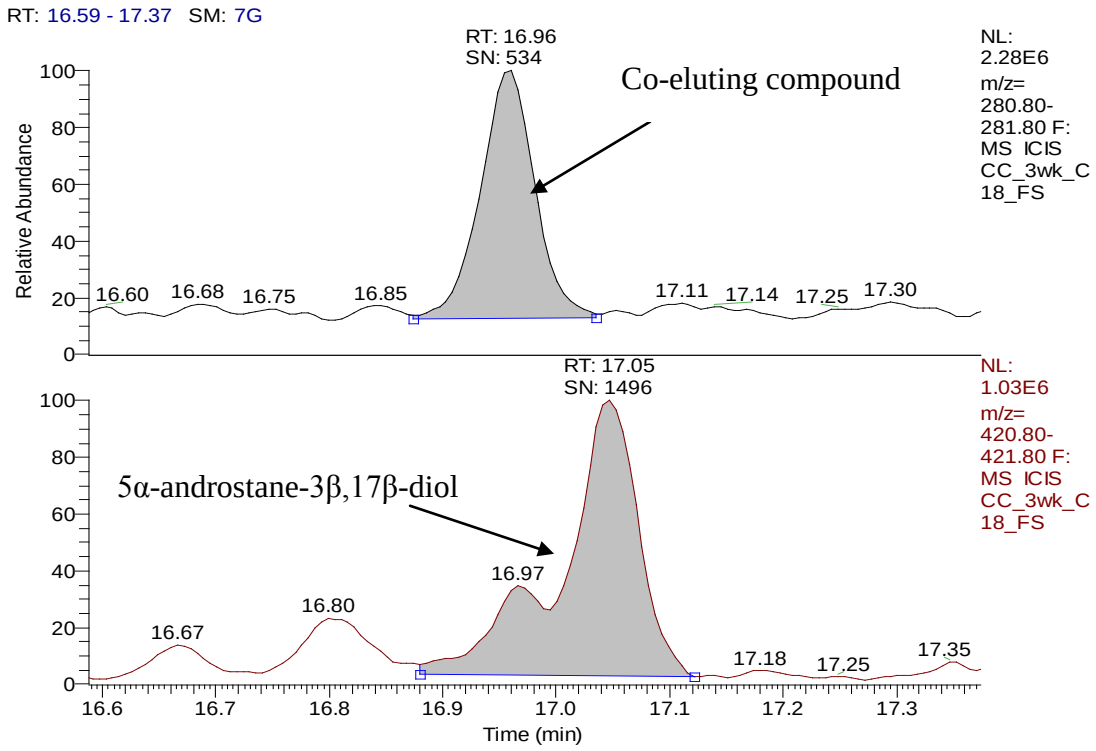


Figure 5.18: Ion chromatograms for 5α-androstane-3β,17β-diol and co-eluting compound

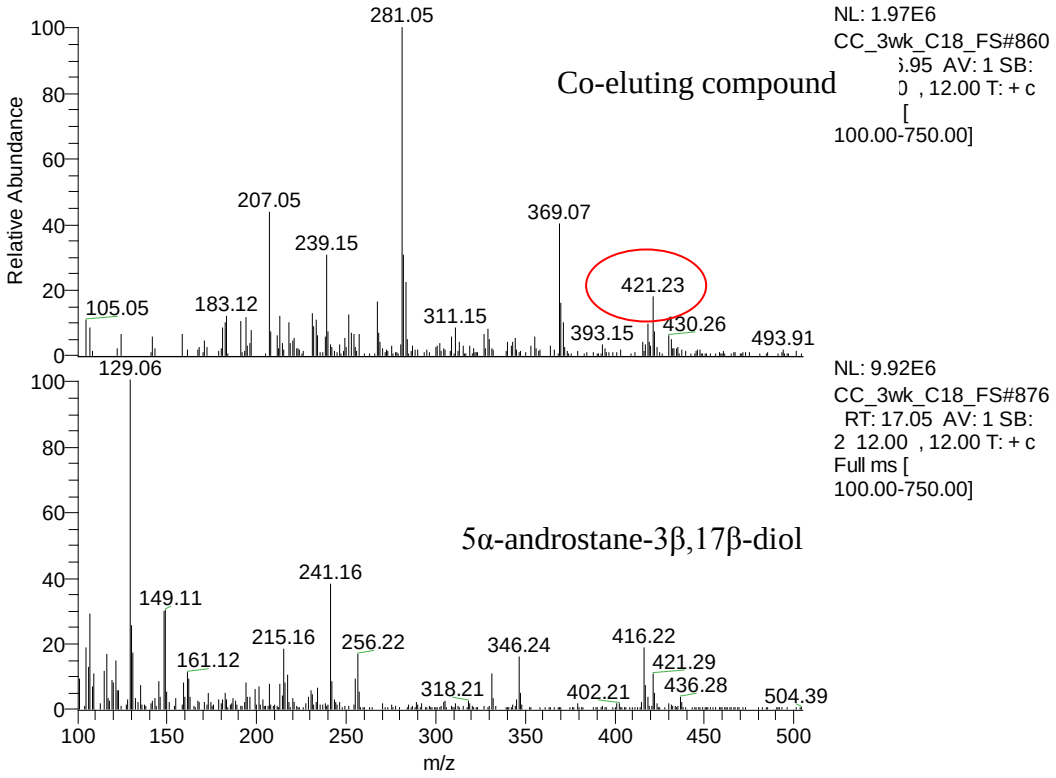


Figure 5.19: Mass spectra for 5α-androstane-3β,17β-diol and co-eluting compound

5.3.4 HPLC fractionation sample clean-up

Figures 5.20 and 5.21 show the HPLC chromatograms generated from a urine extract and a reference standard respectively. By collecting the fraction between 5-11 minutes all the steroids of interest were able to be analysed in one single quantitative GCMS run. The co-eluting compounds were removed in the fractionation process. Example chromatograms following the HPLC clean-up are shown in figures 5.22 and 5.23.

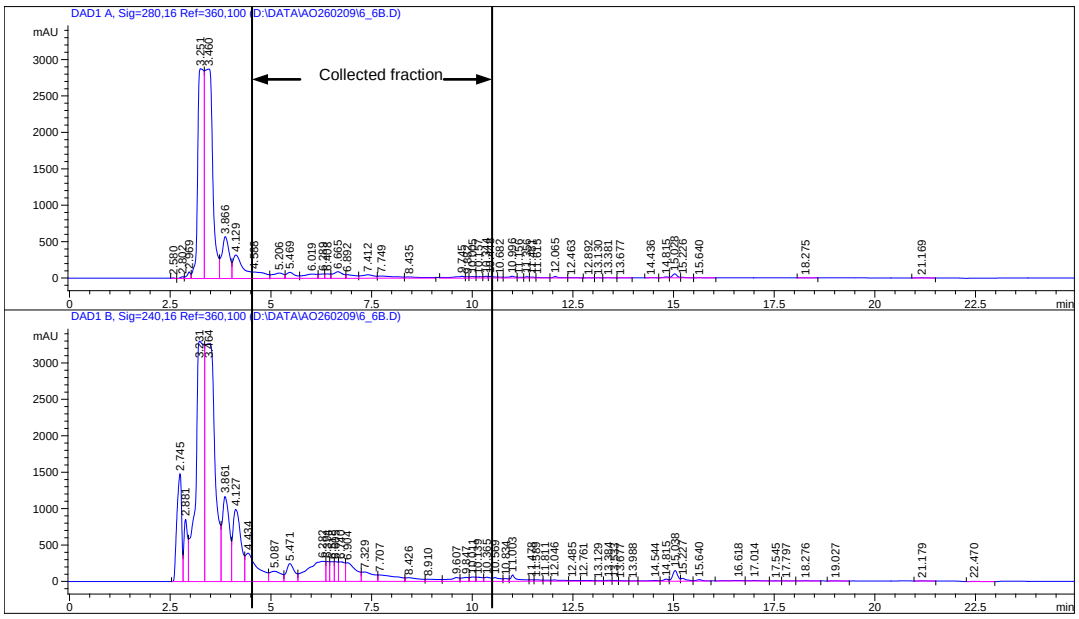


Figure 5.20: Chromatogram from HPLC fractionation clean up analysis – urine extract

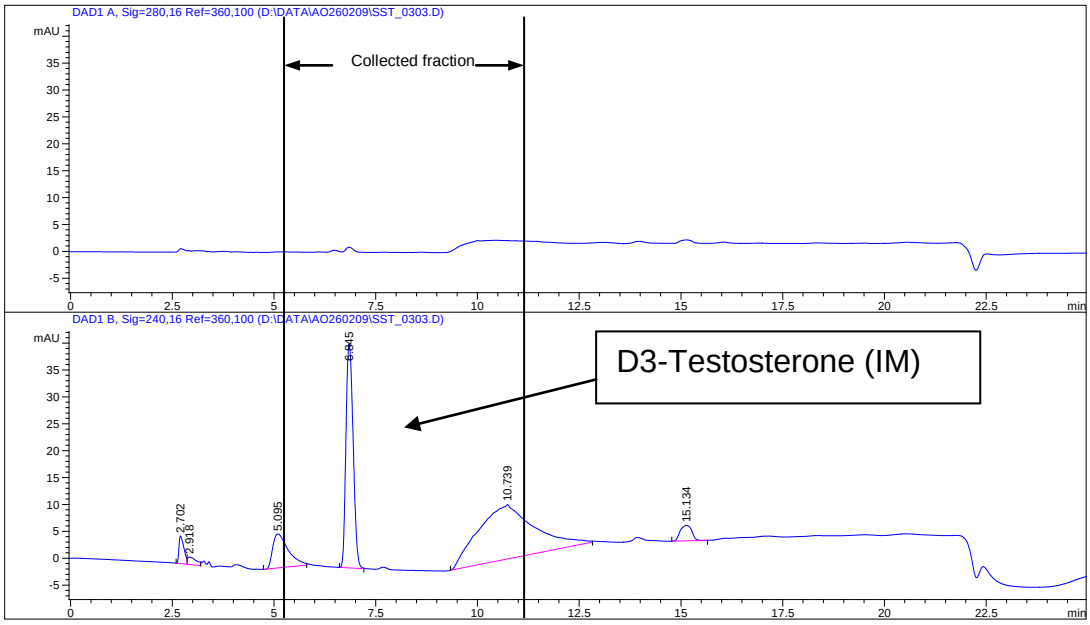


Figure 5.21: Chromatogram from HPLC fractionation clean-up process – reference standard

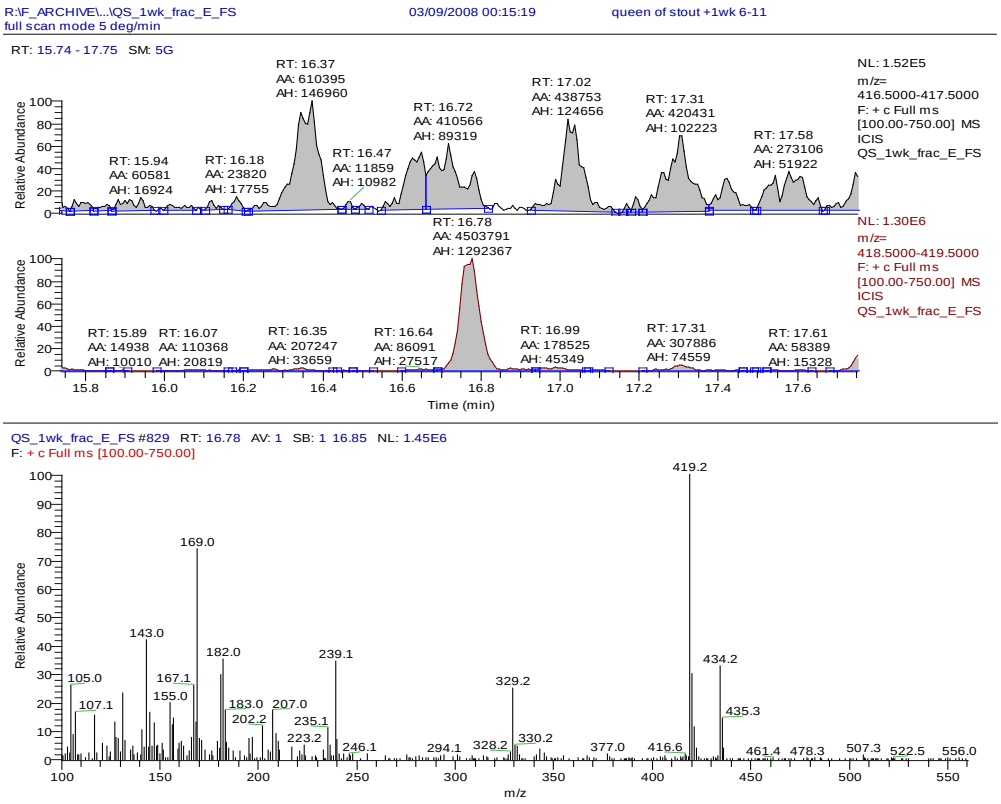


Figure 5.22: Ion chromatogram and mass spectrum for epiandrosterone and ion chromatogram at m/z 417 for originally co-eluting compound.

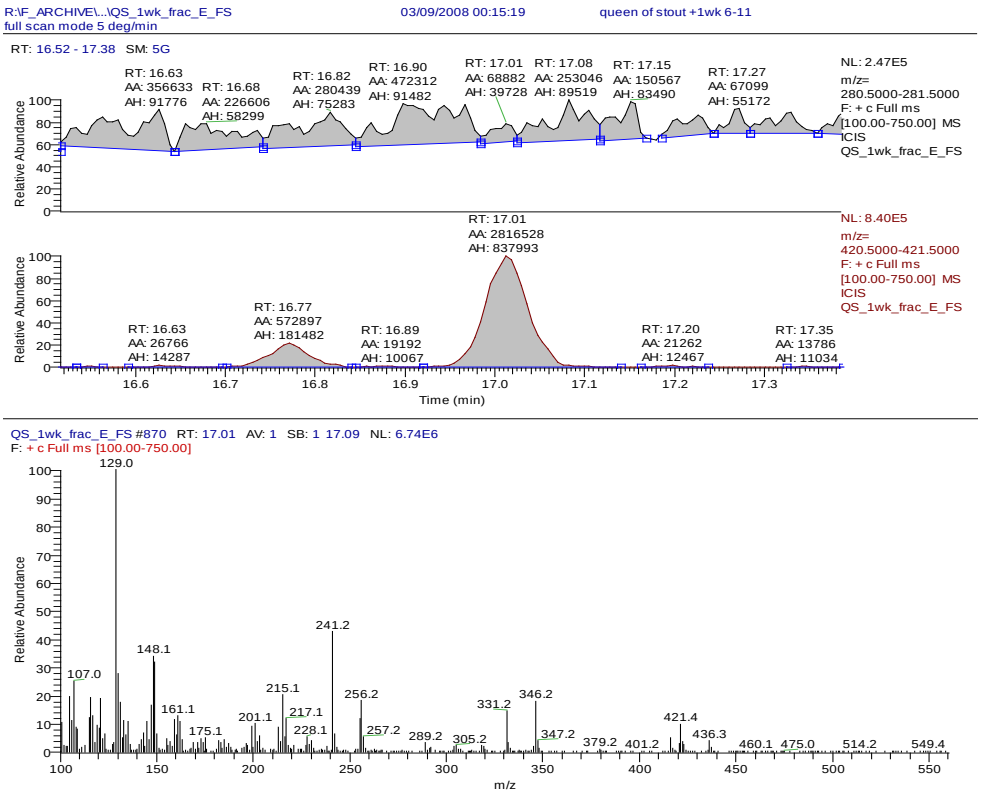


Figure 5.23: Ion chromatogram and mass spectrum for 5α-androstane-3β,17β-diol and ion chromatogram at m/z 281 for originally co-eluting compound.

Table 5.2 summarises the chromatographic separation obtained on the HPLC system for the main steroid analytes of interest. When derivatised and analysed by GCMS individually, these cleaner fractions provided further spectral information for some of the analytes of interest. Fraction 6 was analysed to try and detect the molecular ion of progesterone M1 and therefore support the interpretation that this compound was a pregnanetriol.

Table 5.2: Summary of HPLC fractions

LC Fraction	Analyte	MW	Ions	Comments
5 (4-5mins)	cortico-related	636		
	?Pregnane	550	117, 371, 460	* not in all bitches
	unknown		117	
6 (5-6mins)	?11B-OH epietio or related		168, 522	
	Major pregnanetriol	552	117, 372, 435, 537	*** breaking through into fraction 7**
	Pregnanetriol	552	117, 435,	
	?	550	407, 521	* 143 loss
7 (6-7mins)	*Major pregnanetriol*	*552*	*117, 372, 435, 537*	
	?unknown	638	231	
8 (7-8mins)	norepiandrosterone	420	420, 405, 315	
	aBB-Adiol	436	421, 346, 241	
	[$^2H_3$]-testosterone	435	435, 420	
	testosterone	432	432, 417	
9 (8-9mins)	**[$^2H_4$]-noretiocholanolone**	424	424, 409, 319	
	noretiocholanolone	420	420, 405, 315	
	epiandrosterone	434	434, 419, 329	
	epitestosterone	432	432, 417	
10 (9-10mins)	**[$^2H_4$]-norandrosterone**	424	424, 409, 319	
	aBa-Adiol	436	421, 346, 241	
	[$^2H_5$]-etiocholanolone	439	439, 424, 334	
	etiocholanolone	434	434, 419, 329	*** breaking through into fraction 11**
	[$^2H_3$]-androstanediol	439	439, 424, 349	
11 (11-12mins)	**[$^2H_4$]-androsterone**	438	438, 423, 333	
	etiocholanolone	*434*	*434, 419, 329*	
	[$^2H_5$]-BaB-Adiol	441	426, 351, 261	
	BaB-Adiol	436	421, 346, 241	
	17a-OH pregnanetriol	552	117, 255, 435, 537	
	17a-OH pregnanetriol	552	117, 255, 435, 537	

The analysis of the individual fraction from the HPLC clean-up procedure enabled the molecular ion to be determined for this compound, due to the overall reduction in background noise from the mass spectrum (figure 5.24). The increase in the detected signal of this metabolite, observed in the +3 week samples (after the onset of season) compared with the -6 week samples (prior to the onset of pro-oestrus), was on average 100 fold, for the 33 bitches analysed. This therefore reinforced the choice of this metabolite as the most significant urinary marker metabolite for monitoring progesterone changes throughout the oestrus cycle of the greyhound bitch.

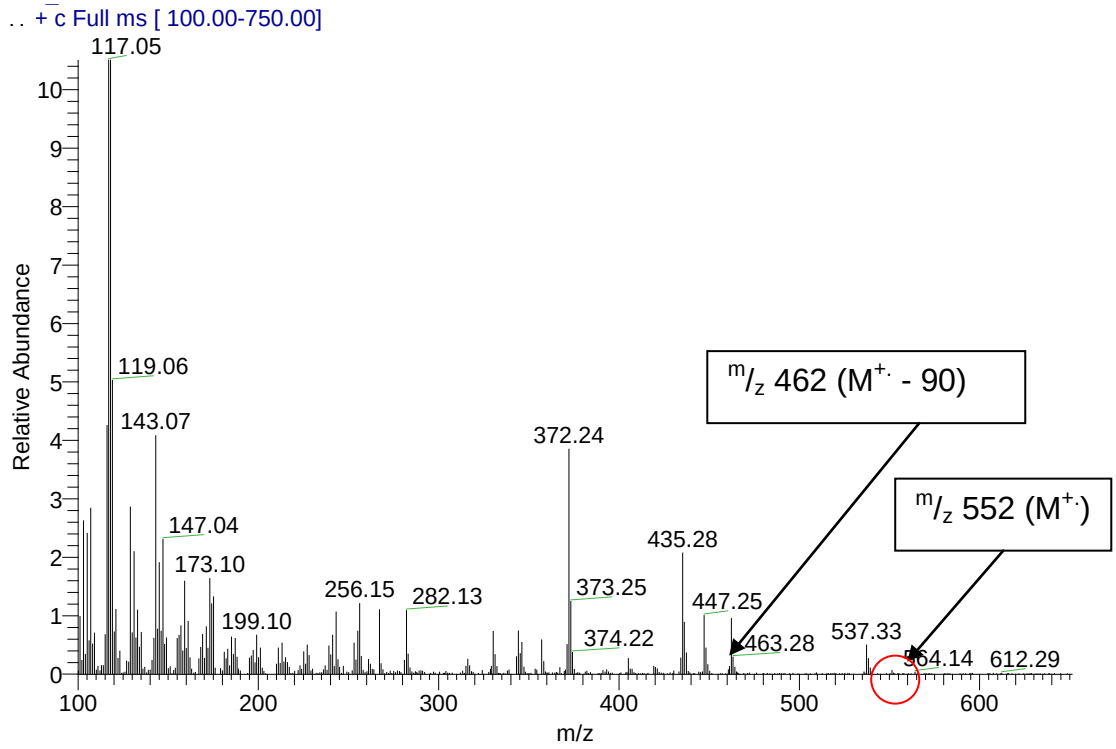


Figure 5.24: Zoomed in spectrum of proposed pregnanetriol metabolite (M1)

Figure 5.25 shows ion chromatograms for a mixed standard containing all the androgens and internal standards monitored on the quantitative analysis. Figure 5.26 shows ion chromatograms for a urine extract from Queen of Stout (+8days after the onset of her season), indicating the presence of the various androgens to be monitored along with the major pregnanetriol (progesterone metabolite M1). Both of these extracts were generated using the developed SPE/LC clean-up sample preparation procedure.

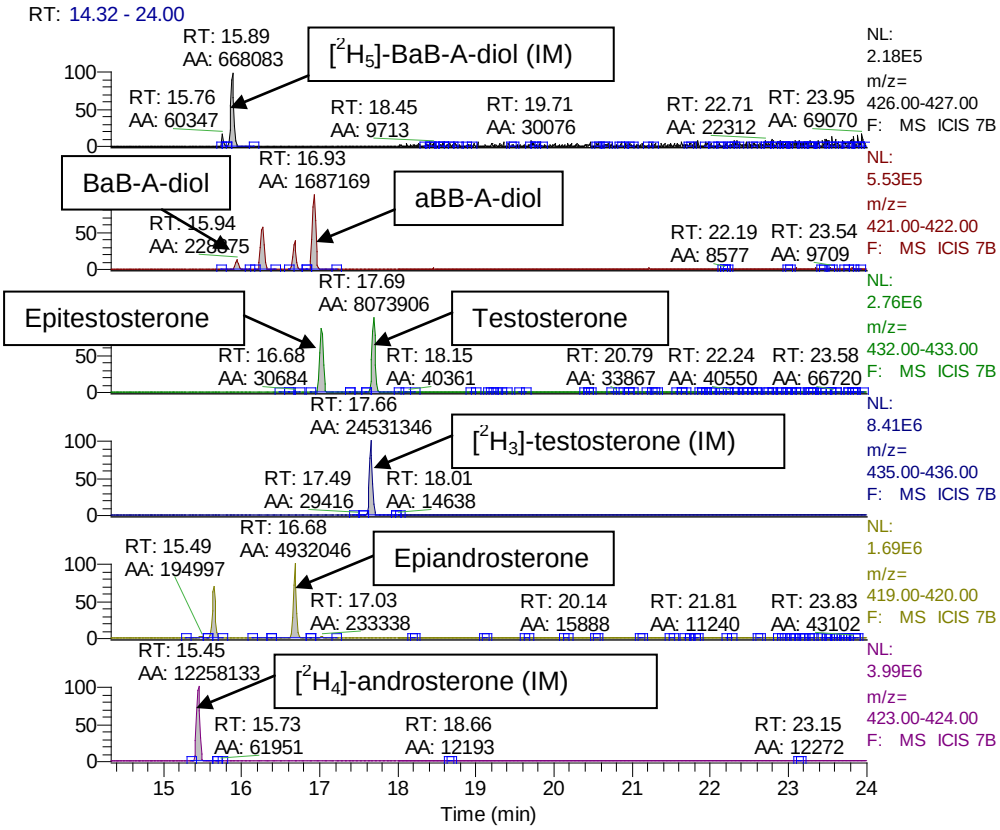


Figure 5.25: Extracted standard (7B): LC fractionation (5-11 incl.)

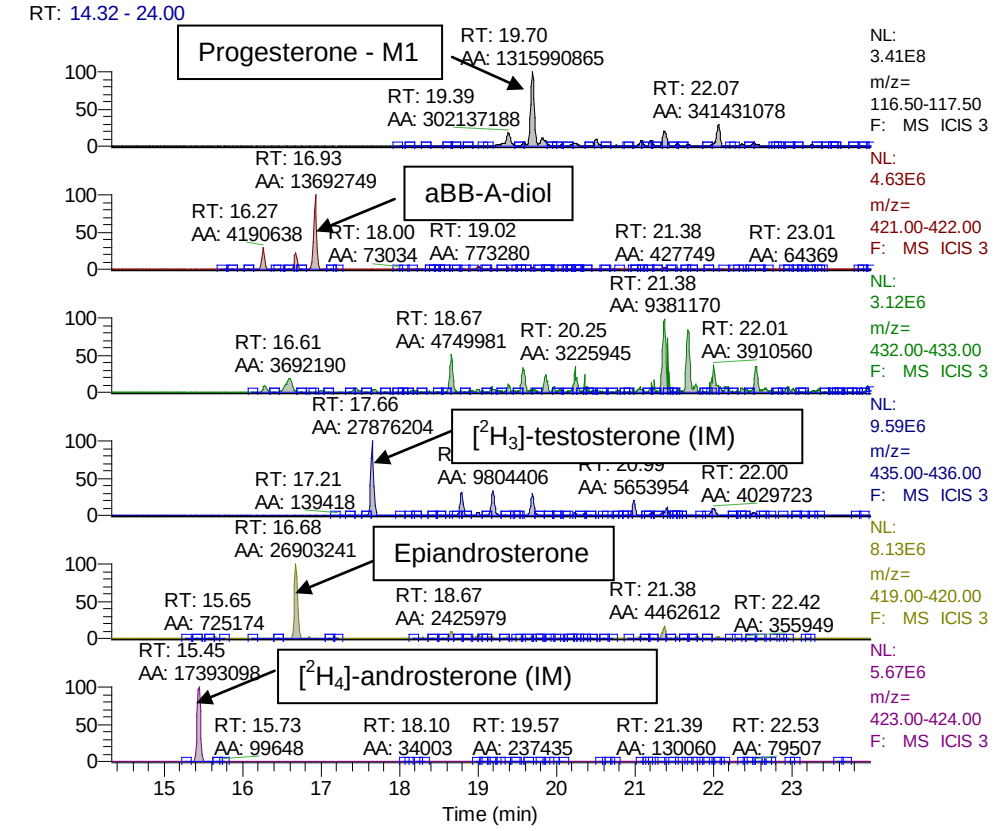


Figure 5.26: Queen of stout – 8 days post season: LC fractionation (5-11incl.)

5.3.5 Quantitative GC-MS analyses

Table 5.3 shows the final analyte retention times and relative retention times used for the quantitative steroid profiling.

Table 5.3: Summary of analyte retention times for the quantitative steroid analysis

Analyte	Retention Time (min)	Relative Retention Time
[$^2H_4$]-androsterone (IM)	15.45	1.000
etiocholanolone	15.65	1.013
epiandrosterone	16.68	1.080
[$^2H_5$]-BaB-androstanediol (IM)	15.89	1.000
BaB-androstanediol	15.94	1.003
aBB-androstanediol	16.93	1.065
[$^2H_3$]-testosterone (IM)	17.66	1.000
epitestosterone	17.02	0.964
testosterone	17.69	1.002
Progesterone metabolite Pregnanetriol (M1)*	19.70	1.116

* The analysis of pregnanetriol was semi-quantitative as no certified reference standard was available for calibration

Unless repeat analyses were required, all the samples from the complete longitudinal profile of a particular animal were analysed in a single run to reduce analytical variation.

The three major testosterone metabolites epiandrosterone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol along with testosterone, epitestosterone and etiocholanolone were quantified in all the urines collected from 33 greyhound bitches throughout their oestrus cycles. The major pregnanetriol (M1) was not available as a reference standard and the analysis was therefore only semi-quantitative for this compound.

The total number of urine samples analysed by this quantitative procedure involving SPE/Liquid-liquid extraction, HPLC fractionation, derivatisation and GC-MS analysis for all the samples collected for each bitch throughout her cycle was in excess of 1500.

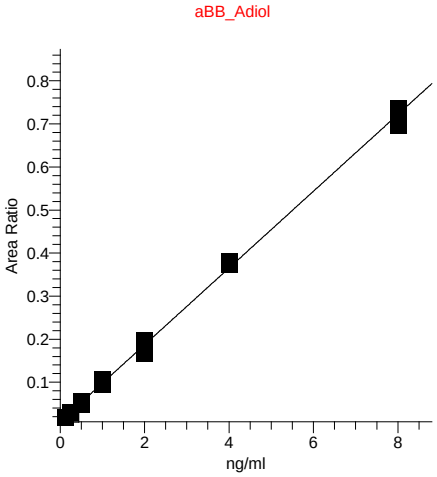
5.3.5.1 Batch acceptance

For each batch of samples extracted, a calibration line was generated and quality control samples were analysed alongside. Three out of four correct values were required for batch acceptance with a pass for at least one duplicate at each level. The

lowest level quality controls were required to fall within a ±20% error range and the other two within ±15%. The LLOQ of the assay was 125 $pgmL^{-1}$.

Percentage error, mean, coefficient of variation (CV) and relative error (RE) were calculated for each analyte at each calibration and quality control level for all batches analysed. Acceptance criteria were ±15% for all criteria. These tabulated values are detailed in Appendix III.

Example calibration data for 5α-androstane-3β,17β-diol and table showing calculated amounts from quality control samples



LLOQ = 125$pgml^{-1}$

Equation: Y = 0.00344154+0.048*X R^2 = 0.999

Sample	Specified Amount	Calculated Amount	% Difference
LOW A	0.375 $ngml^{-1}$	0.387 $ngml^{-1}$	3%
LOW B	0.375 $ngml^{-1}$	0.368 $ngml^{-1}$	2%
HIGH A	3.000 $ngml^{-1}$	3.084 $ngml^{-1}$	3%
HIGH B	3.000 $ngml^{-1}$	3.169 $ngml^{-1}$	6%

Details and values for the quantitative assay quality control and precision and accuracy data are shown in Appendices IV and V.

5.3.6 Oestrus Cycle Endogenous Steroid Profiles: Results

Urine samples from a total of 33 greyhounds were analysed throughout their oestrus cycle in order to determine the basal levels for each target analyte. The testosterone metabolites were quantified using calibration standards prepared from certified reference standards. However, due to the fact that there were no authentic reference standards available for the progesterone metabolites, a semi-quantitative analysis was performed and area ratios were calculated against the deuterated internal marker for the major progesterone metabolite (M1) detected in urine. This provided a trend for the relative amounts of progesterone metabolite that were excreted throughout the oestrus cycle for each animal. The raw data values for each analyte, in each individual bitch analysed, are shown in Appendix VII.

Figure 5.27 shows the results of the quantitative analysis of the daily sampling profile for Black Pansy. The daily sampling data enabled a clear picture of the steady rise in testosterone metabolites, in the lead up to the onset of oestrus, to be displayed. It is felt that the peak maximum level for testosterone may well be linked to the marked increase in secretion of LH prior to ovulation. However, it was not possible to establish a direct time link, as no commercial kit was available for the analysis of LH in canine urine. It has also been suggested that intact LH is not excreted into canine urine, which may explain the lack of available urinalysis LH kits available on the market[16]. However, data has been published relating to the detection of LH in both plasma and urine using a plasma RIA, with some apparent success, although no androgen data was obtained, precluding any direct link being made between the two analytes[17]. An oestradiol analysis in the cycle urines might provide some insight in the future, bearing in mind the link between the rapidly decreasing levels of oestradiol and the pre ovulatory LH surge. Figures 5.27 to 5.34 illustrate the quantitative excretion profiles of the major testosterone metabolites and semi-quantitative profiles of the major progesterone metabolite throughout the oestrus cycle of a selection of individual animals. The remaining profiles are located in appendix VI. N.B. The timeframe between urine sample collections (i.e. several days) means that the maximum concentration observed for the major androgen metabolites e.g. 5α-androstane-3α,17β-diol and epiandrosterone may well be less than the maximum concentration actually reached in urines that would have been voided on days just before or just after the urine sample with the highest concentration of metabolite in the profiles.

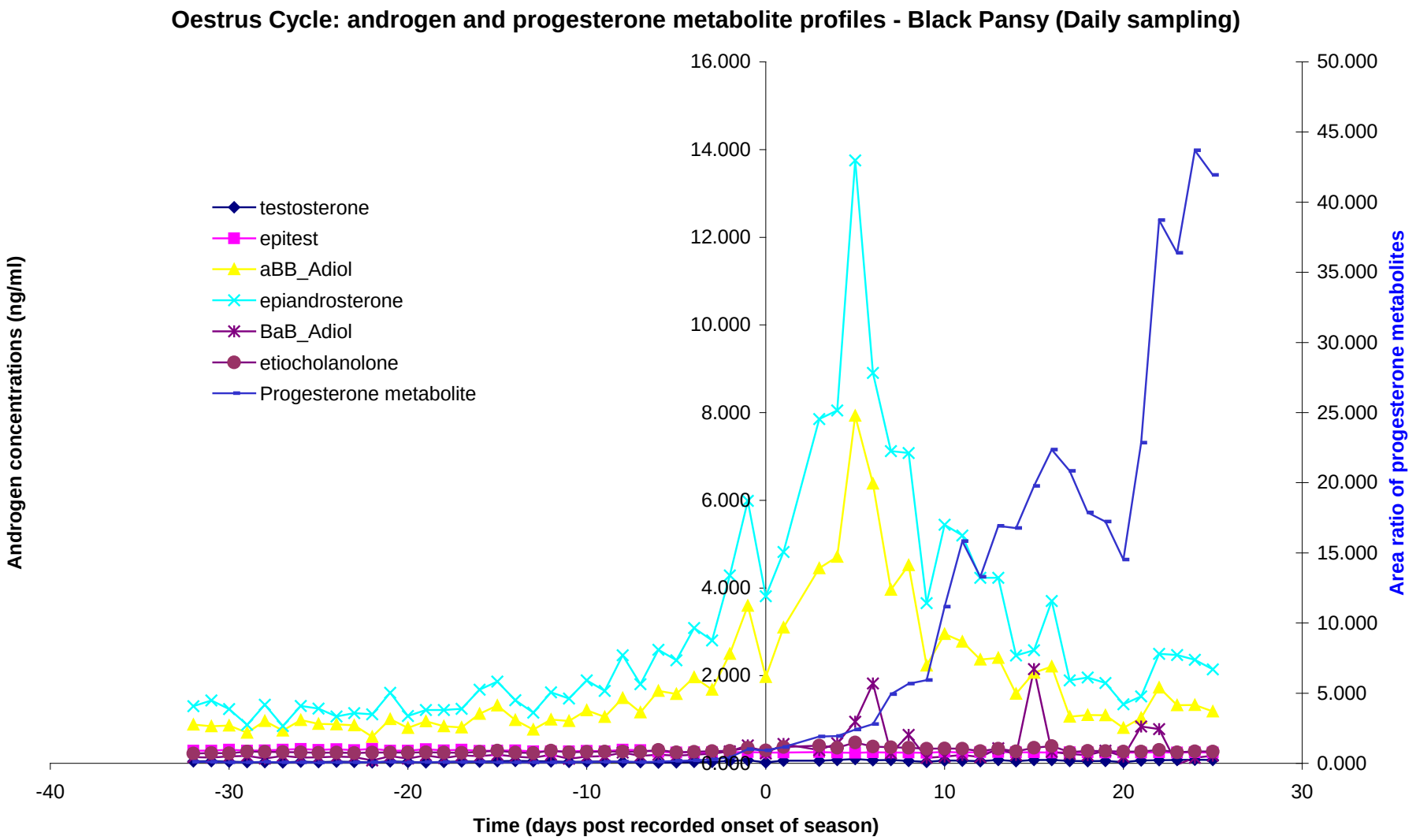

Oestrus Cycle: androgen and progesterone metabolite profiles - Black Pansy (Daily sampling)
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolites
Time (days post recorded onset of season)
testosterone
epitest
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
16.000
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
50.000
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
-40
-30
-20
-10
0
10
20
30

Figure 5.27

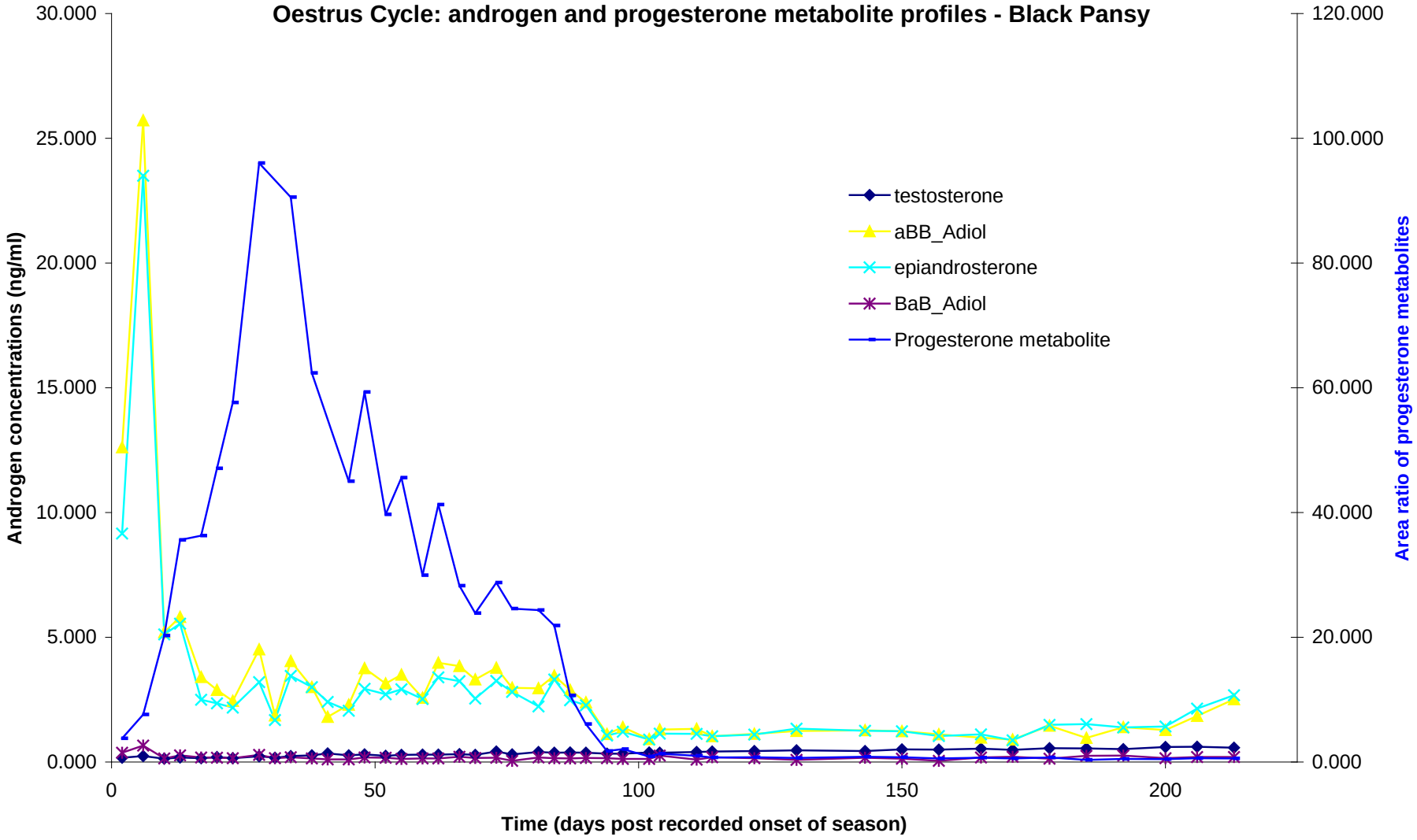

Oestrus Cycle: androgen and progesterone metabolite profiles - Black Pansy
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolites
Time (days post recorded onset of season)
30.000
25.000
20.000
15.000
10.000
5.000
0.000
120.000
100.000
80.000
60.000
40.000
20.000
0.000
0
50
100
150
200
testosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
Progesterone metabolite

Figure 5.28

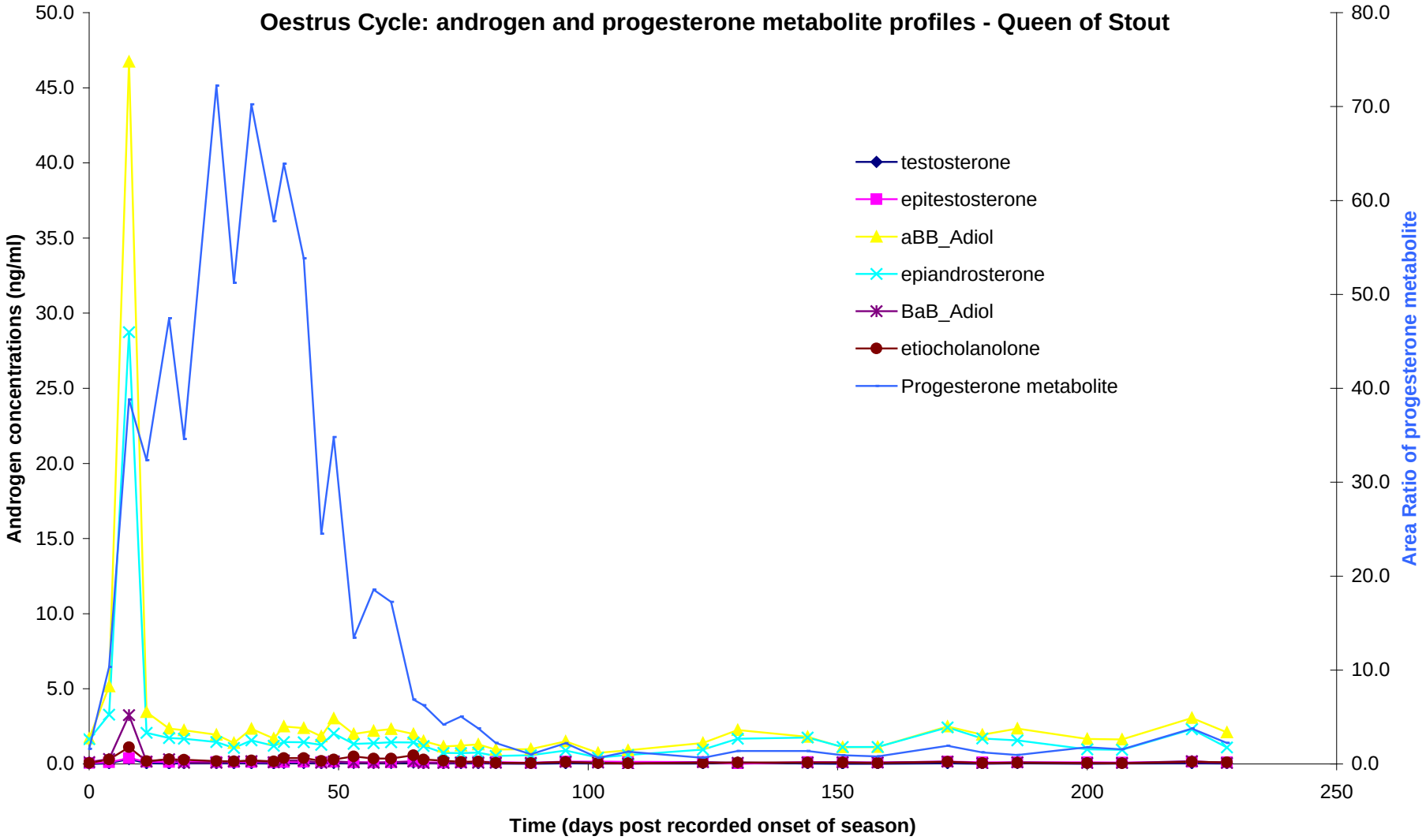

Oestrus Cycle: androgen and progesterone metabolite profiles - Queen of Stout
Androgen concentrations (ng/ml)
Area Ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
50
100
150
200
250

Figure 5.29

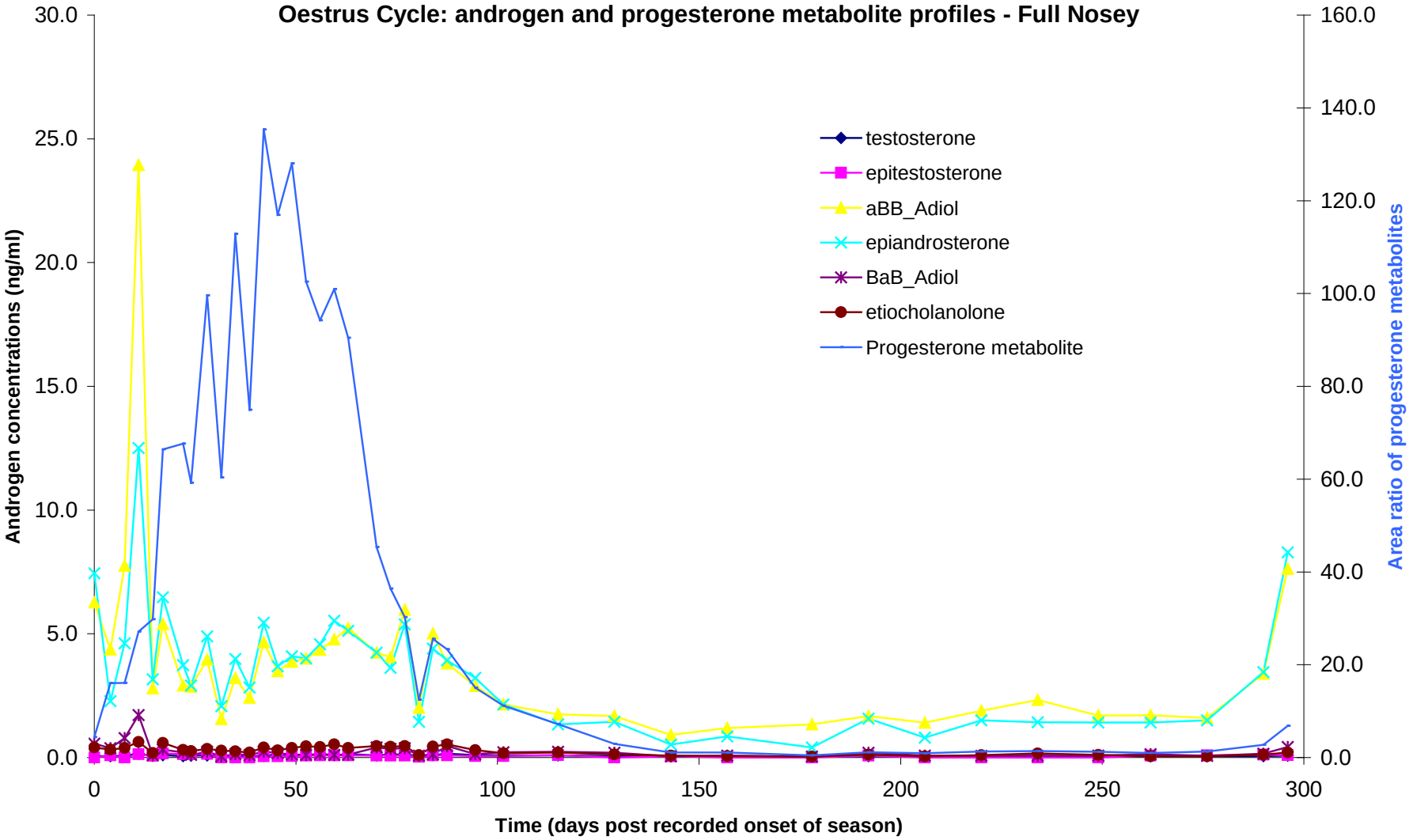

Oestrus Cycle: androgen and progesterone metabolite profiles - Full Nosey
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolites
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
30.0
25.0
20.0
15.0
10.0
5.0
0.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
50
100
150
200
250
300

Figure 5.30

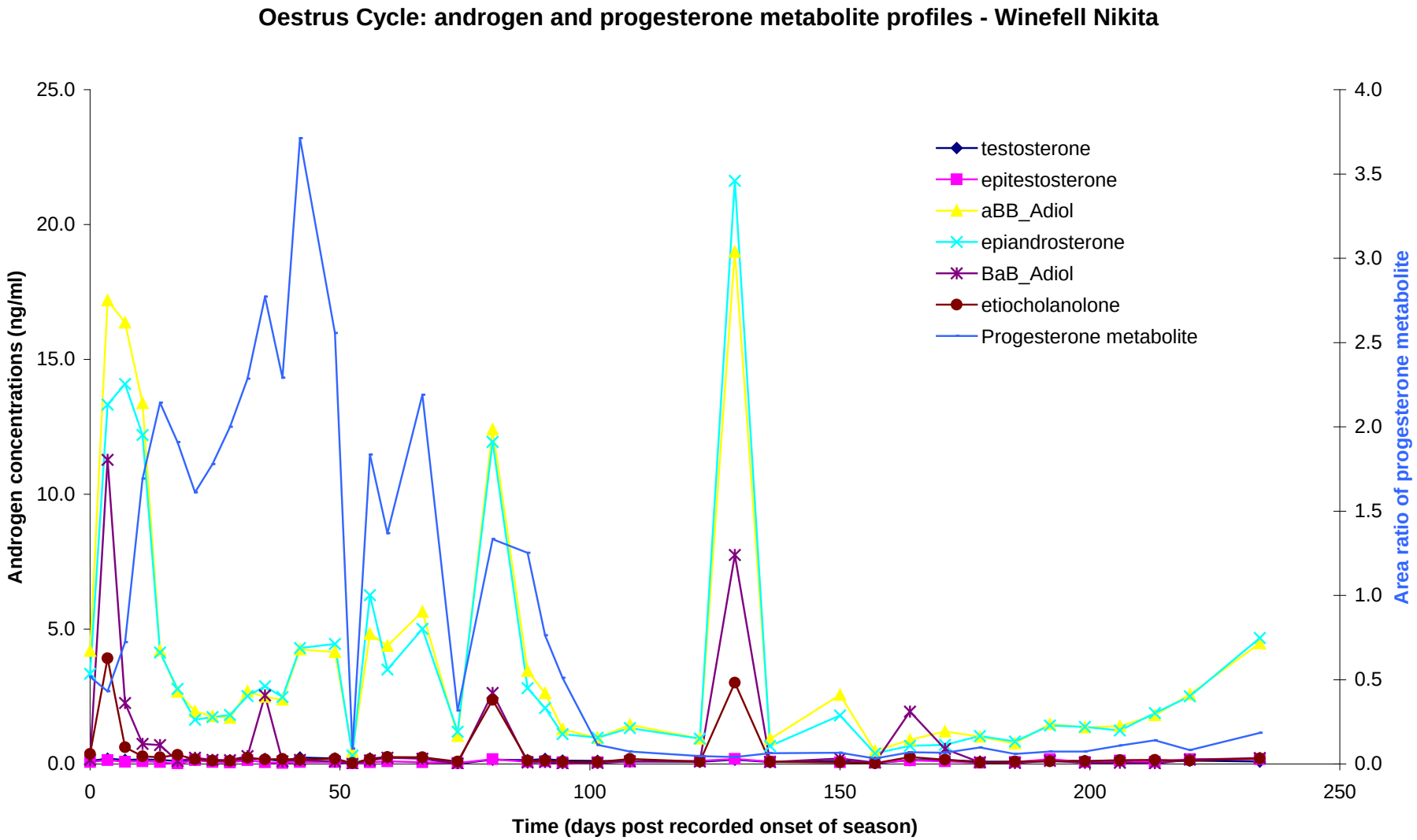

Oestrus Cycle: androgen and progesterone metabolite profiles - Winefell Nikita
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
25.0
20.0
15.0
10.0
5.0
0.0
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250

Figure 5.31

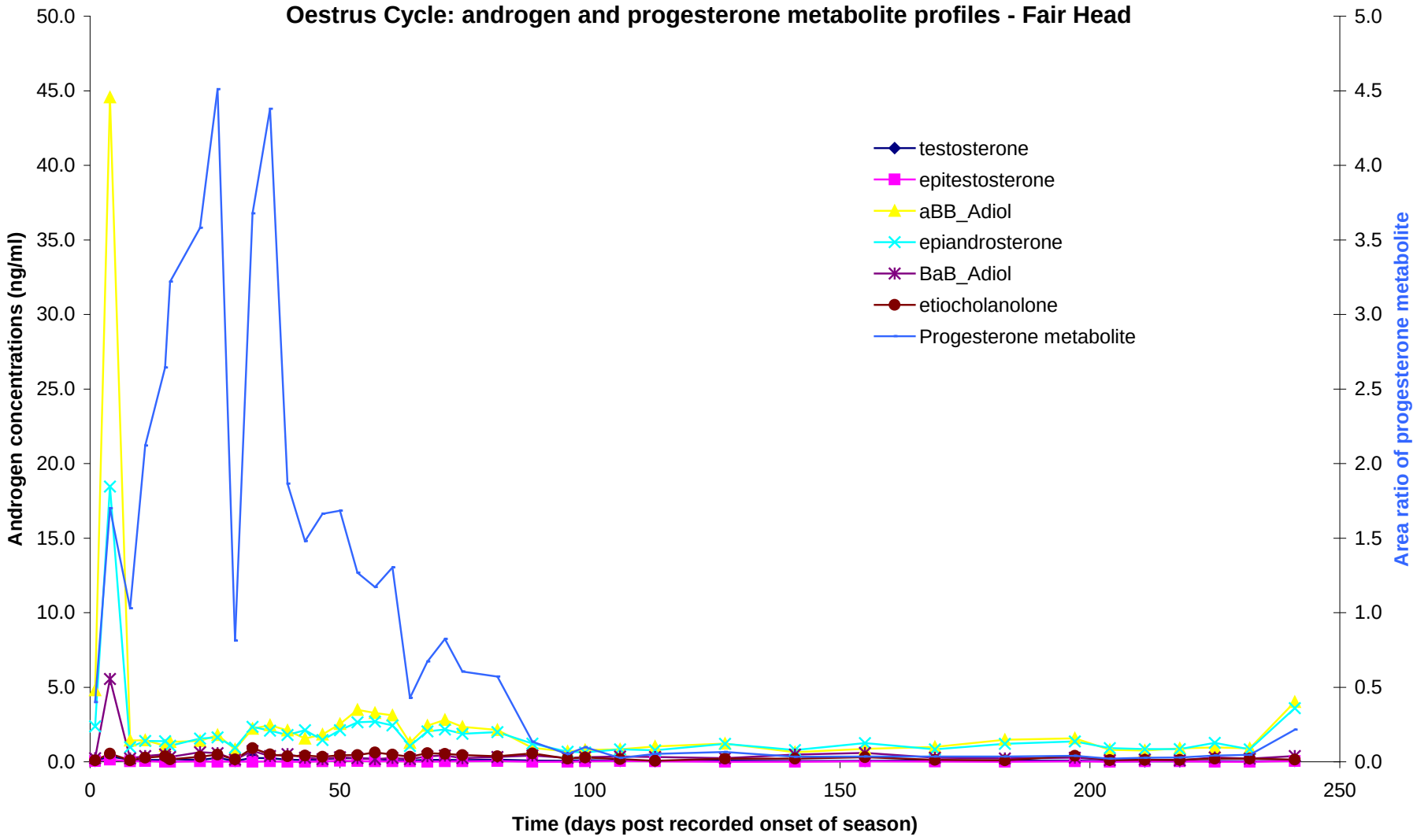

Oestrus Cycle: androgen and progesterone metabolite profiles - Fair Head
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250

Figure 5.32

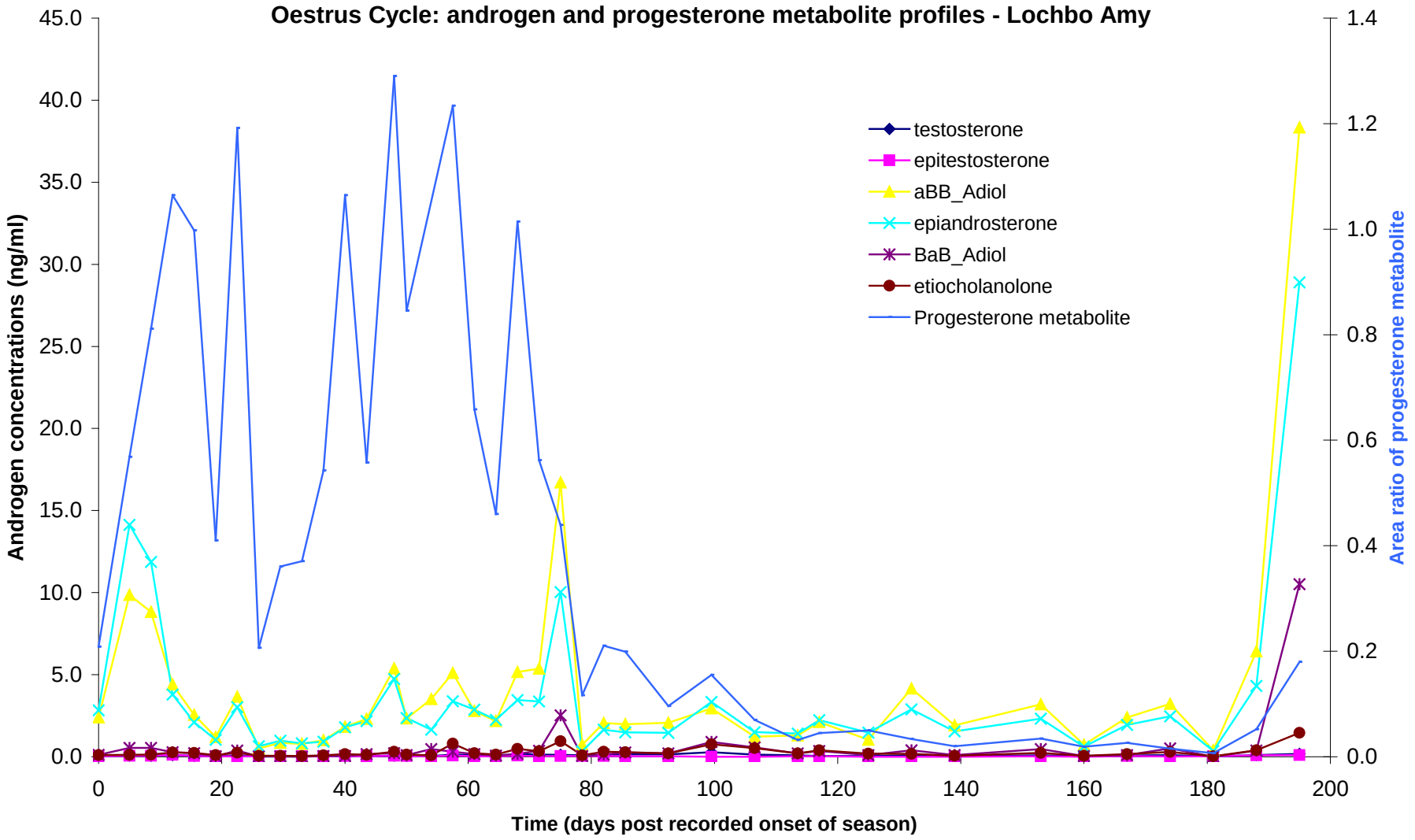

Oestrus Cycle: androgen and progesterone metabolite profiles - Lochbo Amy
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
180
200

Figure 5.33

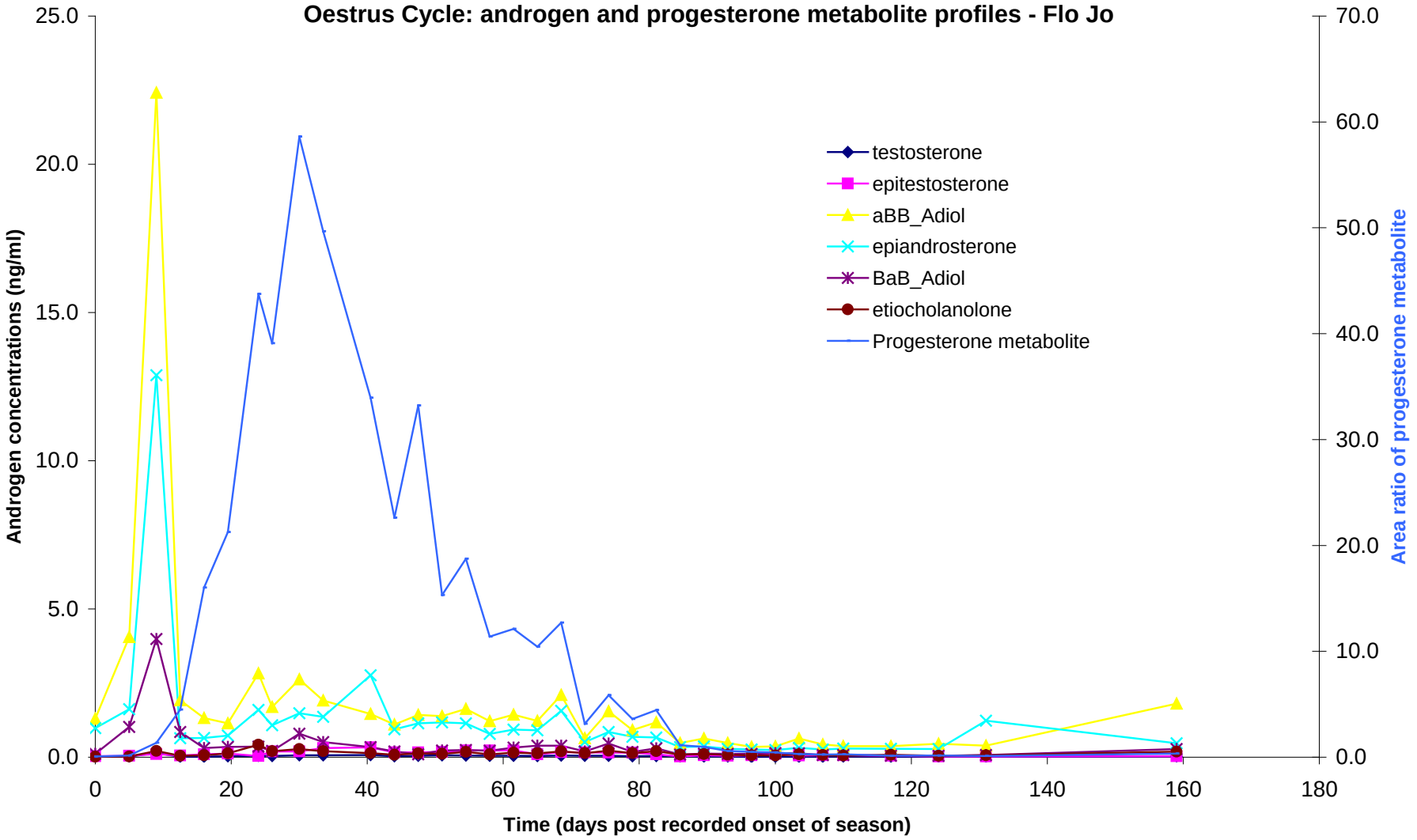

Oestrus Cycle: androgen and progesterone metabolite profiles - Flo Jo
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
25.0
20.0
15.0
10.0
5.0
0.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
20
40
60
80
100
120
140
160
180

Figure 5.34

5.3.7 Oestrus Cycle Endogenous Steroid Profiles: Interpretation

The data generated from the population of bitches analysed has produced some trends for a large percentage of the population, but has also served to show that there appears to be marked variation from one animal to another in relation to the hormone activity in a given oestrus cycle. This is not necessarily that unusual, given the general knowledge of the greyhound's oestrus cycle[1,8,9,10,11]. Examples of the oestrus cycle profiles are shown in figures 5.27 to 5.34. The remaining profiles are shown in appendix VI.

In most of the bitches the urinary androgen profiles were characterised by a marked increase in epiandrosterone and 5α-androstane-3α,17β-diol approximately 7 days into oestrus. It is reasonable to interpret that these metabolites are most likely derived from the surge in circulatory testosterone and androstenedione linked with the surge of LH prior to ovulation[3]. That being the case, it is therefore interesting to observe that in nearly all the bitches, the surge in epiandrosterone and 5α-androstane-3α,17β-diol is not accompanied by an equivalent surge in levels of 5β-androstane-3α,17β-diol. All three metabolites are excreted in similar quantities to one another, following the intramuscular administration of testosterone to the greyhound bitch (see chapter 4). This is likely to arise as a result of liver metabolism of the circulating levels of testosterone present following the drug administration. As such, it would be reasonable to assume that the liver would metabolise circulating testosterone, related to ovulation, in the same way, but this does not appear to be the case in the bitch during oestrus.

Following the surge in metabolites there follows a period of several months of slightly elevated excretion of epiandrosterone and 5α-androstane-3α-17β-diol (see figure 5.28). These levels return to basal during anoestrus, i.e. when progesterone levels have returned to basal, signalling the end of the luteal phase of the cycle. The levels therefore appear to be linked with the duration of excretion of progesterone in di-oestrus. In this case these metabolites are likely to be derived from metabolism of androstenedione which is thought to be prevalent throughout di-oestrus in the greyhound[3]. Once again there is very little 5β-androstane-3α,17β-diol excreted by comparison to the other two metabolites.

The difference in metabolism during the oestrus cycle compared with drug administration may be linked to the activity of 5α- and 5β- reductases and their relative tissue locations. No significant quantities or indeed changes in level of urinary

testosterone itself were observed throughout the cycle. The androgen profiles were considered 'normal' when similar to those discussed, e.g. profiles such as that obtained for Black Pansy (Figure 5.28), Queen of Stout (Figure 5.29), Full Nosey (Figure 5.30), Fair Head (Figure 5.32) and Flo Jo (Figure 5.34).

The lack of 5β reduced metabolites could be indicative of an alternative substrate to testosterone entering the circulation during the androgen surge to produce primarily 5α metabolic products. This could be androstenedione. Following the administration of testosterone to the bitch, 5α and 5β metabolites were produced in similar amounts and the ratio 5α:5β was similar throughout the excretion profile (see chapter 4 Figures 4.7 and 4.8). Thus, following administration of testosterone, it appears that the metabolic process, to induce alternative pathways, is not influenced by changes in circulating levels of testosterone. This would imply that the liver is not switching to 5β reductase activity due to saturation of 5α-reductase pathways, following an administration of testosterone. There is no 'lag phase' of induction of 5β activity in the drug administration, as 5β-androstane-3α,17β-diol is excreted in equal quantities to the 5α metabolites at 2 hours post dose.

Another explanation might be that androstenedione is indeed the majority androgen released from the ovary at the time of the surge and that it is then preferentially metabolised by 5α reductase in the canine liver. The structural specificity of this enzyme in the canine may be different than other species. The oral administration of androstenedione in humans, certainly gives rise to a large amount of 5β reduced metabolites excreted in the urine[18]. It seems unlikely that the liver would metabolise androstenedione in a different way when presented with it *via* the hepatic portal vein as opposed to the hepatic artery, but there are no known studies of intravenous androstenedione administration to the greyhound of which the author is aware that could provide the answer. It is also not known whether the androstenedione and/or testosterone that enter the circulation from the developing theca cells in the ovarian follicle are in the free form or bound to protein or sulphate, which would influence their ability to be metabolised. The data from this longitudinal cycle study certainly supports the observation that 5β-androstane-3α,17β-diol was undetectable in 80% of the samples analysed in the population study.

The progesterone metabolite profiles rose steadily following the first week of sample collection and remained elevated for up to three months in many of the bitches profiled. This was interpreted as a successful ovulation, as the developing corpora lutea for these

animals should continue to secrete progesterone for this length of time. Where levels returned to basal at approx 90-100 days the bitch was considered to have had a 'normal' cycle. Based upon these criteria, observations for the bitches profiled are listed in Table 5.4 and Table 5.5 provides a summary of interpretations and trainer observations (where applicable) for the oestrus cycle of each animal.

Table 5.4: Observations from the urinary profiles of the population of cycling bitches

Bitch name (listed in data profile report order)	"Normal" androgens	"Normal" progesterone metabolite (P-M1) levels	Max androgen conc. above postulated threshold	Progesterone: Ratio of max P-M1 level vs basal P-M1 level
Black Pansy	✓	✓	No	100:1
Queen of Stout	✓	✓	Yes	50:1
Suitable Choice	✓	✓	No	300:1
Full Nosey	✓	✓	(No)	100:1
Fair Head	✓	✓	Yes	100:1
Lochbo Amy	✓	✓	Yes	30:1
BPIMA	✓	✓	No	60:1
Saunders Pet	✓	✓	No	150:1
Whitefort Euro	✓	✓	Yes	100:1
Flo Jo	✓	✓	(No)	150:1
Tonightsthenight	✓	✓	No	50:1
Miss Honcho	✓	✓	Yes	75:1
Giveherthevote	✓	✓	Yes	75:1
Ringlestown Flos	✓	✓	Yes	35:1
Langies Posh Girl	✓	✓	No	50:1
Queen Boadicea	✓	✓	No	12:1
Queen of the Bar	✓	✓	(No)	100:1
Little Reaction	✓	✓	Yes	50:1
BPXYP	✓	✓	No	400:1
Cannon Clover	(✓)	(✓)	(No)	75:1
Wideopenspaces	(✓)	(✓)	No	30:1
Standard Whisper	✓	✓	No	150:1
Chop and Change	?	✓	Yes	70:1
Supreme Madonna	(✓)	✓	No	25:1
Distant Pearl	?	✓	(No)	50:1
Marshalls Promise	?	✓	No	150:1
Sparky Pacific	(✓)	(✓)	No	400:1
Cullane Chief	×	?	(No)	2:1
Drumpark Access	×	✓	No	20:1
Queen Sally	×	✓	No	50:1
Whinefell Nikita	×	✓	(No)	50:1
The Alarm's On	×	×	No	25:1
Bolly for Molly (P)	?	?	No	125:1

(No) = level below but close to the postulated threshold for αββ-A-diol in urine.

Table 5.5: Summary of interpretations observations including season dates for all bitches in the cycling programme (I)

Bitch	Season 1	Season 2	Cycle length	Comments
Black Pansy	31-Jul-05	12-Mar-06	224	Normal
Black Pansy(2)	12-Mar-06	22-Nov-06	255	Normal
BollyforMolly	15-Jan-07	12-Sep-07	240	Mated D12, had pups D75
Bonny BPIMA	04-Aug-05	30-May-06	299	very oscillating androgen and progesterone
Cannon Clover	09-May-07	19-Feb-08	286	?anovulation 2nd heat was silent as trainer did not report in season and very long subsequent luteal phase 100+ to 200+
Chop & Change	08-Mar-06	10-Nov-06	247	biphasic and anovulatory
Cullane Chief	10-Dec-05	02-Jun-06	174	very variable progesterone profile
distant pearl	24-Mar-07	05-Jul-08	469	very short luteal phase. Androgens biphasic - ?non ovulator
Drumpark access	24-Jul-07	22-Mar-08	242	very odd androgens during luteal phase / early completion
Fair Head	16-Jan-07	15-Sep-07	242	Normal (S.G.readings account for Progesterone M1 dip @D29)
FloJo	12-Jun-06	22-Nov-06	163	Normal
Fully Nosey (Daisy)	10-Nov-06	01-Sep-07	295	Normal
Giveherthevote	17-Mar-07	19-Dec-07	277	Normal
Honeywick Cleo	28-Sep-05	not known	224	
jess BPXYP	31-Jul-05	12-Mar-06	255	prolonged androgens and short luteal phase
Langies Posh Girl	12-Mar-06	22-Nov-06	240	activity before return season
Little Reaction	15-Jan-07	12-Sep-07	299	Normal
Lochbo Amy ALDLH	04-Aug-05	30-May-06	286	Activity before return season. Oscillating progesterone
marshall's promise	09-May-07	19-Feb-08	247	Normal although Progest. bit short? Split heat with 1st bit silent?
Miss Honcho EDH9	08-Mar-06	10-Nov-06	174	Normal
Posh Girl Honey	10-Dec-05	02-Jun-06	469	

Table 5.5: Summary of interpretations observations including season dates for all bitches in the cycling programme (II)

Bitch	Season 1	Season 2	Cycle length	Comments
Queen Boadicea	24-Mar-07	05-Jul-08	242	Ovulation before signs of season
Queen of Stout	04-Oct-05	21-May-06	229	Normal
Queen of the Bar	12-Dec-05	03-Jul-06	203	Normal
Queen Sally	07-Jan-06	no return for 2 years		No return – died about 2 years later. Silent heat – non-ovulatory at about 250 days.
Ringlestown Flos	09-Jul-05	28-Feb-06	234	Normal
Riverview Gypsy	21-Apr-08	13-Feb-09	298	
Riverview Slick	11-Feb-08	03-Aug-08	174	
Saunders Pet	25-Jul-05	31-Mar-06	249	Normal
Sparky Pacific	17-May-07	26-Dec-07	223	Normal
Standard Whisper	02-Mar-06	20-Sep-06	202	Normal
Suitable Choice	03-Aug-05	03-Mar-06	212	Normal
Supreme Madonna	23-Aug-07	28-Apr-08	249	Androgen activity in anoestrus – follicular activity ?silent split heat?
The alarms on	23-Sep-05	02-Jul-06	282	Silent heat about 100days after premature luteal regression
tonightsthe night	03-Sep-06	13-May-07	252	Normal
Uluru Baby	15-Jun-07	28-Apr-08	318	Not analysed
Whinfell Nikita	29-Jan-07	20-Sep-07	234	Follicular activity about day 130 ?Silent anovulatory heat (S.G. readings account for dips in progesterone)
Whitefort Euro	26-Nov-05	07-Sep-06	285	Normal
Wideopenspaces	20-Mar-07	11-Sep-07	175	Fair bit of androgen activity during luteal and anoestrus phases

(Average Cycle duration: 253 days)

The profiles for some of the bitches gave some markedly fluctuating values at certain time points during the oestrus cycle. Some of these could be explained in relation to the level of diuresis observed in some of the urine samples compared with other samples in the longitudinal profile which has clearly had an impact on the concentrations of steroid analytes detected during the analysis. Specific Gravity measurements were taken for the profiles of some of the bitches, where dramatic changes in analyte levels were observed and difficult to rationalise. As an example Figure 5.35 shows the specific gravity (S.G.) readings for each sample in the cycle of the greyhound Fair Head (profile in figure 5.32), plotted alongside her progesterone metabolite profile. The SG reading at day 29 shows the effect of the marked diuresis for that urine sample on the level of analyte recorded. This profile was therefore interpreted as 'normal' without the need to rationalise why there may have been a marked dip in progesterone at that stage of the bitch's cycle. The 'normalised' profile is shown in figure 5.36. Specific gravity values have shown good correlation with hormone levels in previous studies[19].

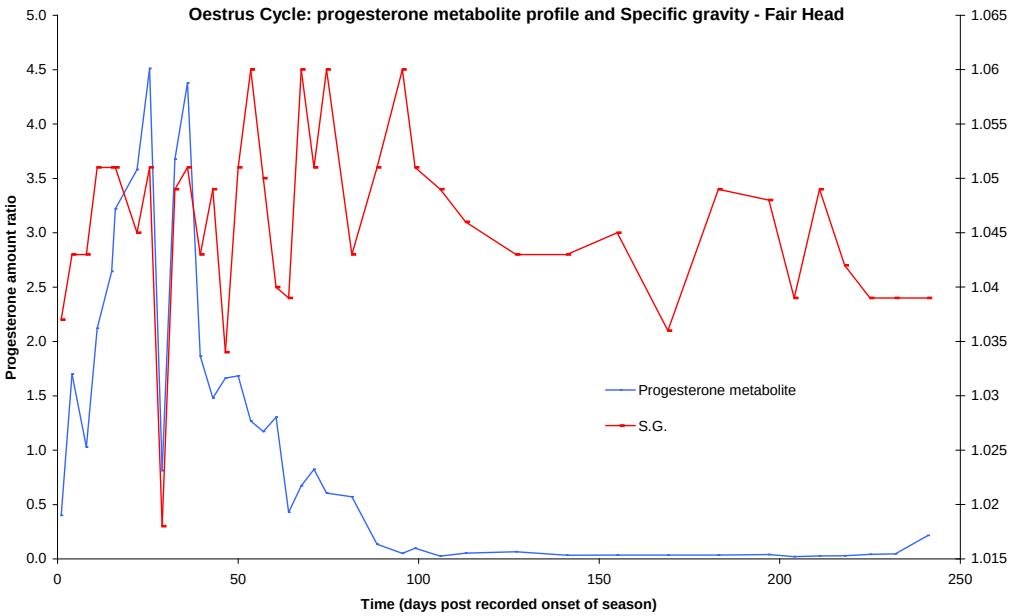


Figure 5.35: Progesterone metabolite (M1) profiled with Specific Gravity (S.G.) values

This marked diuresis was apparent for several other samples in a number of other greyhounds; Suitable Choice day 29 (Appendix VI); Saunders Pet day 46 (Appendix VI); Full Nosey day 81 (Figure 5.30); Chop and Change day 50 (Appendix VI); Langies Posh Girl day 40 (Appendix VI); Lochbo Amy day 195 (Figure 5.33) and Whinfell Nikita days 53 and 74 (Figure 5.31).

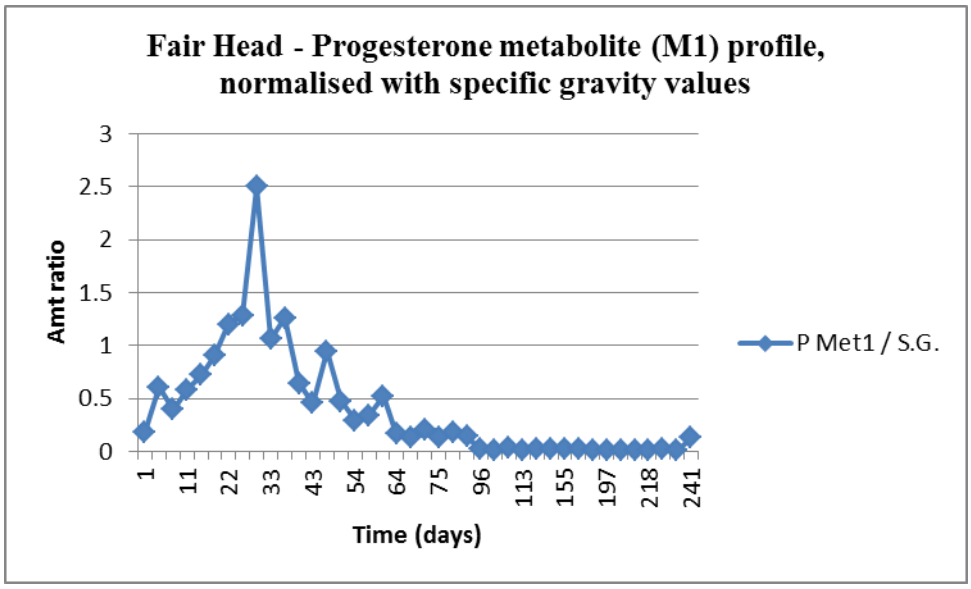


Figure 5.36: Progesterone metabolite (M1) profile normalised with Specific Gravity (S.G.) values for greyhound bitch Fair Head.

However, not all of the unusual observations could be explained by specific gravity, not least for the last named bitch, Whinfell Nikita. This was only one of only two bitches to excrete higher than average levels of 5β-androstane-3α,17β-diol at certain time points during her cycle. The first was at day 4. This sample gave an S.G reading off the scale on the refractometer, as it was extremely concentrated and above the top end of the normal clinical range for dogs of 1.065[19]. The second unusual sample was at day 129. This sample gave a normal S.G. reading in line with all the samples voided before and after. It was also re-analysed to check the concentrations of the androgens observed resulting in similar levels being quantified. This could represent a silent anovulatory heat, as no rise in progesterone was observed and no information was forthcoming from the trainer in relation to change in behaviour for this animal at the time of sampling. Despite this being unusual, the concentration of 5β-androstane-3α,17β-diol was no greater than10ng/ml at the time of sampling. This animal did appear to exhibit a greater propensity for excreting 5β-reduced metabolites overall. It could be that she has a particular condition resulting in this enzyme activity.

What did appear to be relatively 'normal' as it did occur in a number of bitches was the appearance of small quantities of 5β-androstane-3α,17β-diol at the point of the androgen surge. This was also observed for Lochbo Amy (Figure 5.33), but on day 195, which actually represented the first sample collected at the onset of her next season.

As previously discussed, the normal reproductive cycle of the bitch consists of a number of endocrinologically distinct parts, namely pro-oestrus, oestrus, di-oestrus and anoestrus. Of these, only the first two are detectable clinically, lasting on average 21 days and indicated by bleeding and attractiveness to male dogs. Di-oestrus, the progesterone producing phase, lasts about two to three months and the balance of the cycle is made up of anoestrus.

It is considered that if the bitch ovulates and therefore enters di-oestrus, the cycle length cannot be less than about four months. An interval less than this, is usually suspected as being either a split heat, a condition which is relatively common and presents as a bitch entering into a normal cycle which then stops and the bitch does not ovulate. This is then followed by a full, normal cycle, some 1-2 months later[20]. This was apparent for the bitch The Alarms On (Appendix VI). Clinically it is considered that bitches should cycle at least every 12 months and if it longer than this, the bitch has either been treated with hormones or may have some underlying physiological issue.

The results of the longitudinal sample collection and analysis throughout the oestrus cycle in this study have shown that urinary testosterone/androstenedione metabolites are elevated significantly during the early part of the cycle. Figures 5.37 to 5.40 show consolidated data plots (Box and Whisker plots) of the combined data for all 33 bitches in the longitudinal cycling study. These data show the considerable variation between animals that is apparent when measuring hormone concentrations. The elevated metabolite level trends at the early part of the cycle coincide with the current 21-day lay-off rule operated by the greyhound regulatory authority, where a bitch is not permitted to race for the first 3 weeks after the commencement of her oestrus cycle. The original population study to determine suitable threshold values for androgen metabolites (Chapter 4), contained some animals that were known to be in season at the time of their sampling. Therefore, as these samples represent animals that should not be on the racetrack, it was considered that they should be removed from the dataset prior to statistical analysis, such that the final dataset is more representative of the actual racing population available to be sampled at the racetrack.

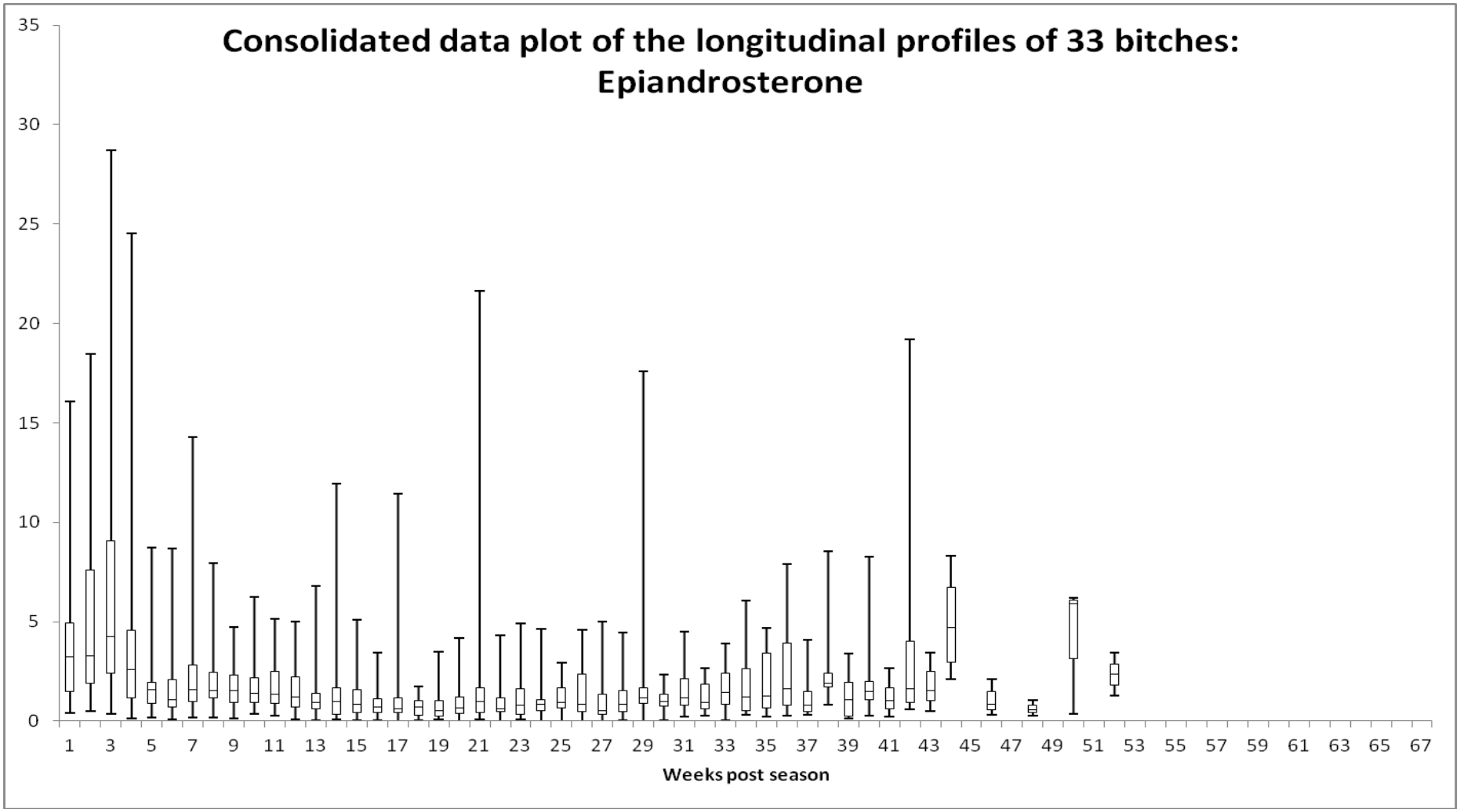


Figure 5.37: Box and whisker plot of epiandrosterone levels in 33 bitches throughout the entire oestrus cycle.

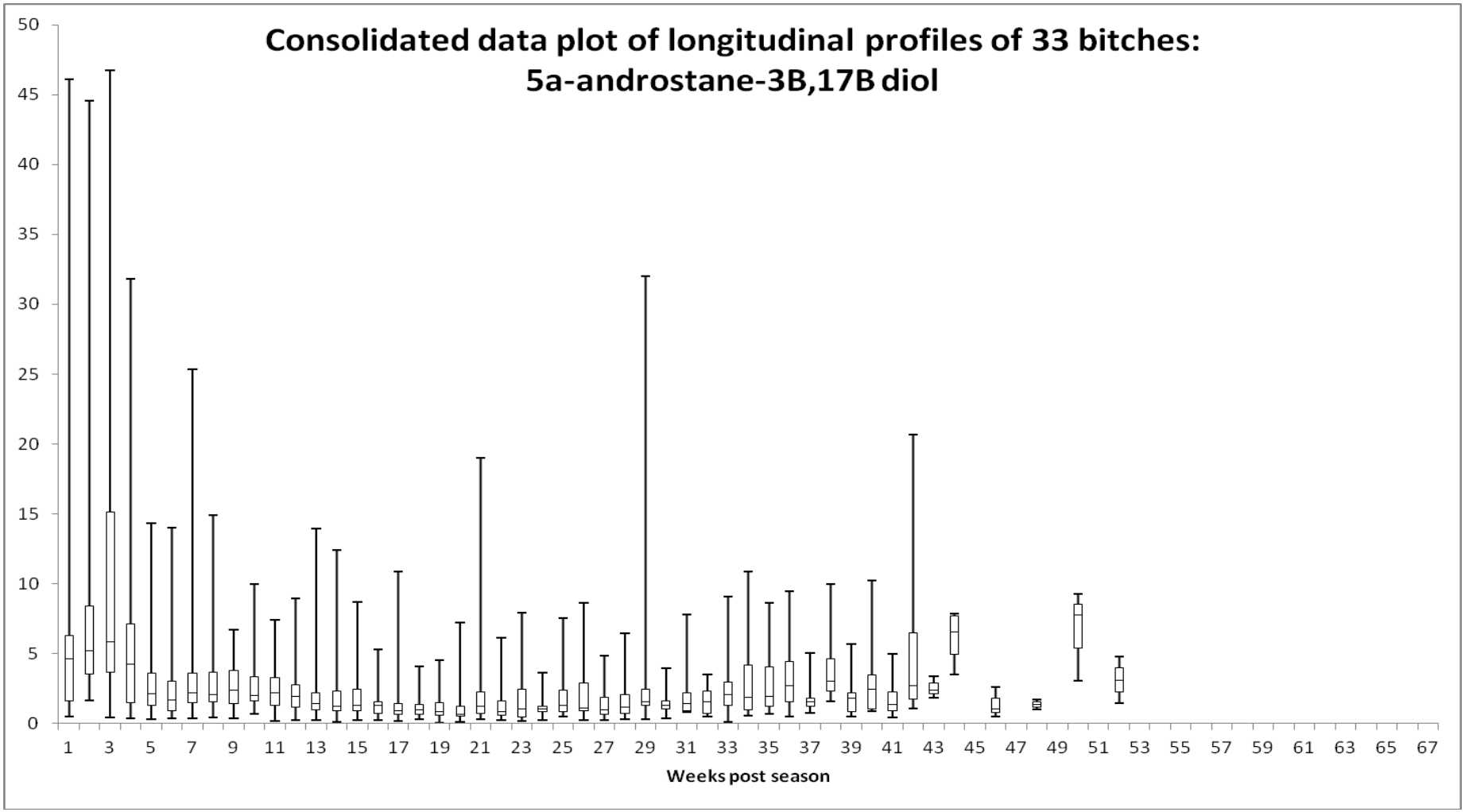


Figure 5.38: Box and whisker plot of 5a-androstane-3B,17B-diol levels in 33 bitches throughout the entire oestrus cycle.

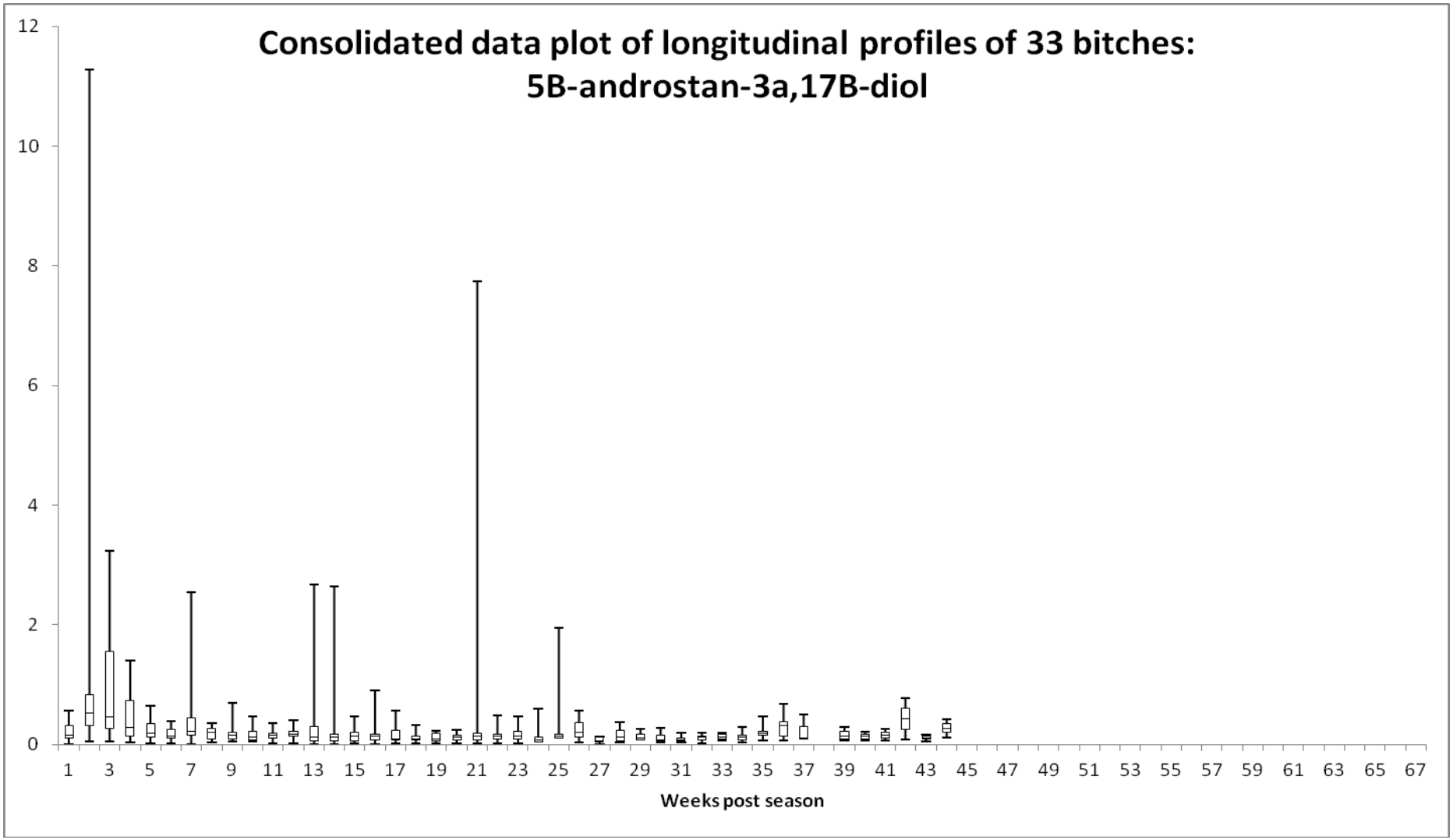


Figure 5.39: Box and whisker plot of 5B-androstane-3a,17B-diol levels in 33 btches throughout the entire oestrus cycle.

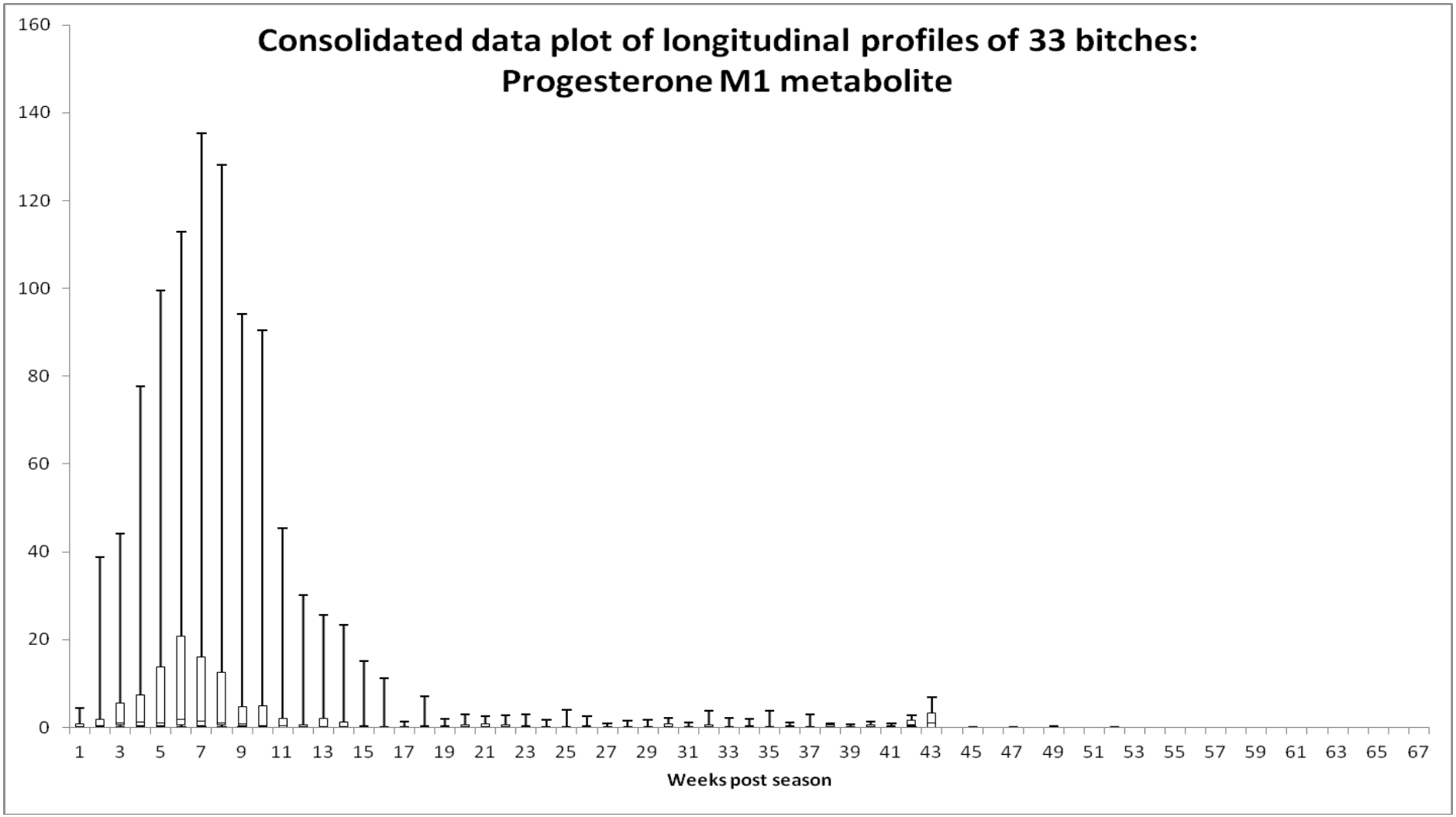


Figure 5.39: Box and whisker plot of progesterone M1 metabolite levels in 33 btches throughout the entire oestrus cycle.

The results from this cycling study, and those from an adjoining study, on short term suppression of oestrus in racing bitches (Chapter 1[24]), reinforce the fact that greyhound bitches can, and indeed do, have an incidence of both split heat and silent heat. These phenomena are clearly not uncommon considering the size of the populations involved in the oestrus cycle studies, where at least 10% of the animals appeared to have a 'non-standard' cycle (as defined by the urinary profiles of the majority of the animals), including two animals that clearly had silent heats, as they went through an entire 'hormonal cycle' unbeknown to their trainers.

Whilst there is no reason to suspect any of the trainers involved in the cycling study of wilfully administering any drugs to their animals at any stage, without declaration, it should be noted that in essence, the data has been acquired from a non-scientifically controlled environment. However, such a collection of data may well not have been possible at all, without the co-operation of such a large number of trainers.

5.4 CONCLUSIONS

The greyhound bitch has been studied throughout her oestrus cycle to determine reference concentrations of steroid hormones during this time.

When considering that the average cycle time for the bitch is considered to be 8-9 months, then a number of the bitches observed in this study appeared to conform to this. The study is the first of its kind to look at the urinary metabolites of testosterone and progesterone throughout the entire cycle of greyhound bitches. Knowing that the cycle is already a variable one from animal to animal and indeed within a particular animal then it is not straightforward to say what constitutes 'normal' for the urinary profile. However, there would appear to be a number of bitches that have shown profiles that could be proposed as normal/reference profiles in relation to existing knowledge.

Following on from the original testosterone metabolite population study (chapter 4) one of the main aims of this study was to establish whether or not there were particular time points in a bitch's cycle where urinary testosterone metabolite levels could be expected to be elevated. The answer to this question has been provided by this work. At the onset of the cycle there are marked increases of urinary testosterone metabolite levels within the first week in most of the bitches analysed. The metabolites that were elevated were primarily the 5α-androstane-3β,17β-diol and epiandrosterone, with a slight elevation in 5β-androstane-3α,17β-diol in one or two instances. The levels are elevated for a period of about 7-10 days. This is best shown in Black Pansy's profile (Figure 5.27) where daily sample collections were available for analysis. As samples were collected intermittently (twice a week) for the majority of the animals in the scheme the peak maximum levels observed at the time of the testosterone surge cannot be assumed to represent the maximum as this may have been reached on one of the days in between sample collections.

The elevated level of androgen metabolites is probably related to the LH surge that occurs prior to ovulation in the bitch as a result of the shut-down in production of oestrogens, resulting in a surplus of substrates that are no longer converted to oestrogen.

Under the current rules greyhound bitches should not be racing for 21 days after the onset of their seasons, which would mean that they should not be sampled at the track

during this time and therefore would not present a false positive result for elevated testosterone metabolites. This is based on the assumption that the animal has exhibited external signs of coming into season during pro-oestrus to the trainer and/or the racetrack veterinary steward.

Lochbo Amy (Figure 5.33), Tonightsthenight, Little Reaction, Wideopenspaces, Marshalls Promise, Cullane Chief, Drumpark Access, Queen Sally and The Alarms On (all figures in Appendix VI), were all bitches that either did not show an obvious surge in androgens or did, but it may have been at maximum the day before the first sample collection. This may be due to the bitch not showing visible signs of being in season or may simply be related to the sampling regime and the time that the trainer/handler was available to inspect the animals. Nevertheless some consideration needs to be given to the possibility that a bitch may undergo a ‘silent heat‘ that results in her being at the races and available for testing when her urine might contain levels of androgen metabolites that would be above a proposed threshold for a positive finding. Such a finding could be overcome by collecting a subsequent urine sample 2 to 3 weeks later and checking for the presence of elevated progesterone metabolites to indicate that the bitch had ovulated.

However, although informative for some animals this would not be a completely failsafe approach, as indicated by the progesterone metabolite profiles for some of the bitches in the scheme, where the levels of progesterone were variable at this time. This was most apparent for Cannon Clover (Appendix VI), who appeared to undergo a possible premature luteal regression as her progesterone metabolite levels returned to basal for two months after her initial testosterone surge, implying that she had either not ovulated or that she had ovulated but there was no subsequent development of a corpus luteum. A few of the bitches also showed variable testosterone metabolite levels at certain times during anoestrus (Distant Pearl, Marshalls Promise, Queen Sally, Whinfell Nikita). Of these Whinfell Nikita and Queen Sally displayed the most unusual elevations of metabolites. Repeat analyses of the urine samples showed these values to be correct. Whinfell Nikita’s profile (Figure 5.31) is probably of greatest interest in relation to the levels of 5β-androstane-3α,17β-diol (βαβ-diol), observed at 4 and 130 days in the cycle. These are the two highest values observed for any untreated animal in this study as well as those analysed during the population study of more than 200 urines. However, the values are still below the ‘action’ and threshold levels tentatively proposed from the testosterone population study. The levels of epiandrosterone

observed for some of the bitches at the point of their androgen surge are close to the proposed threshold level for epiandrosterone. Whether it is possible for this level to be breached during a silent heat is not known. It could be that silent or split heats are characterised by different levels of hormones, but should that be proved, it would almost certainly be variable from one bitch to another.

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CHAPTER

CONCLUSIONS

6. CONCLUSIONS

Gas chromatography-mass spectrometry and selected derivatisation techniques have been applied to the identification and structural elucidation of anabolic and progestagenic steroids in the greyhound. Coupled with a variety of sample extraction and preparation methods, the successful use and value of these techniques, for the study of steroid metabolism, has been demonstrated in this thesis.

The requirement to control the use of various steroidal preparations in racing greyhounds, and in particular greyhound bitches, from both a regulatory and a welfare perspective, has been discussed throughout, and this need has directed the vast majority of the work that has been carried out in this body of research.

Following the administration of proprietary steroid preparations to the greyhound, urine samples were collected and prepared for analysis using solid phase and liquid-liquid extraction techniques. After oral administration of methyltestosterone to the greyhound, several metabolites were identified, including reduced, mono-, di- and tri-hydroxylated steroids. The major metabolites observed were 17α-methyl-5β-androstan-3α-17β-diol, 17α-methyl-5β-androstan-3α,16α,17β-triol and a further metabolite tentatively identified as 17α-methyl-5z-androstan-6z,17β-triol. The most abundant of these was the 17α-methyl-5β-androstan-3α,16α,17β-triol. This metabolite was identified by comparison with a reference standard synthesised using a Grignard procedure and characterised using trimethylsilyl (TMS) and acetonide-TMS derivatisation techniques. The structures of the major metabolites 17α-methyl-5β-androstan-3α-17β-diol and 17α-methyl-5β-androstan-3α,16α,17β-triol have been fully elucidated. There did not appear to be any evidence for 16β-hydroxylation as a phase one metabolic transformation in the greyhound. However significant quantities of 16α-hydroxy metabolites were detected. Selective enzymatic hydrolysis procedures indicated that the major metabolites identified were excreted as glucuronic acid conjugates and their detection and identification in urinary extracts by mass spectrometric techniques will provide an effective approach to screening and confirmatory analysis for the control of methyltestosterone use in the greyhound.

Similar approaches were used to identify urinary metabolites of norethisterone, a progestagenic compound recently permitted for use as an oestrus suppressant by the GBGB. Following oral administration to the greyhound, several metabolites were

identified including reduced, mono-, di- and tri-hydroxylated steroids. The major metabolites observed were 17α-ethynyl-5β-estrane-3α,17β-diol, 17α-ethynyl-5α-estrane-3β,17β-diol, three 17α-ethynylestranetriol stereoisomers and two 17α-ethynylestranetetrol stereoisomers. The major metabolites were predominantly excreted as glucuronic acid conjugates, as observed for methyltestosterone, and detection of the administration of norethisterone was possible for up to 8 days post dose using the methods described. The nandrolone metabolites, 19-norepiandrosterone and 19-noretiocholanolone were also identified in the post administration urine samples collected up to 8 hours after dosing the treated animals. The detection of these metabolites resulted in an investigation into minor contaminants of the norethisterone tablet preparation itself, which included nandrolone and 19-norandrostenedione. Their presence was considered the most likely cause for the detection of 19-norepiandrotserone and 19-noretiocholanolone in the post administration urines.

The urinary metabolites identified in these two particular studies have provided an understanding of the *in vivo* phase I metabolism of methyltestosterone and norethisterone in the greyhound. The metabolic pathways include reduction of the 4-ene-3-one group of the A-ring, and oxidations at C16 and probably C6. The 5α3β and 5β3α stereochemistry of the fully A-ring reduced metabolites is comparable to the stereochemistry of the major metabolites observed in the metabolism of testosterone in the greyhound namely, epiandrosterone, 5β-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. The urinary metabolites identified in these studies have therefore further enhanced the general knowledge of steroid metabolism in the greyhound, providing information to support routine drug testing programmes.

To support the detection of the naturally occurring endogenous hormone testosterone, a quantitative GC-MS method was developed to determine the concentrations of the major metabolites of testosterone in a population of “normal” untreated animals. These data were compared with results from the analysis of post-administration urine samples, following the intramuscular administration of testosterone esters to the greyhound. Some approaches for statistical treatment of the population data have been proposed to establish threshold values for the major metabolites, with a view to providing a routine methodology for discriminating between ‘normal’ and ‘positive’ post administration samples, thereby detecting the abuse of testosterone in the racing greyhound bitch.

During the course of the analysis of the normal population samples it became apparent that the metabolites of testosterone were potentially elevated at certain stages of the oestrus cycle in the greyhound bitch. Therefore in order to provide a greater understanding of the profile of endogenous hormones in the bitch, the major metabolites of testosterone and progesterone were quantified throughout the entire oestrus cycle in a population of 33 greyhound bitches.

A preliminary study of the metabolism of progesterone resulted in the identification of a suitable pregnanetriol metabolite to monitor alongside the androgen metabolites, thereby providing information on the hormonal status of the bitch at the various stages of her cycle. A normal progesterone profile, i.e. the excretion of progesterone metabolites for two to three months, provided likely indications that ovulation had occurred.

The results of the study clearly indicate a surge in androgen metabolites during the first 7-10 days of the oestrus cycle, in particular epiandrosterone and 5α-androstane-3β,17β-diol, both of which are 5α reduced analytes. The stereo chemistries of the metabolites observed have indicated that the source of these metabolites may well be androstenedione as opposed to testosterone. This conclusion was drawn due to the fact that only minor amounts of 5β-reduced metabolites were observed in the majority of bitches during their normal oestrus cycles. However, the 5β-reduced metabolite, 5β-androstane-3α,17β-diol was observed in significant, and equal quantities to the 5α metabolites following the administration of testosterone to the greyhound bitch. It is not known whether the canine liver metabolises androstenedione in a different manner to testosterone and the possibility of other androgens being released from the ovary cannot be ruled out, although most serum based studies in this area of research suggest testosterone and androstenedione to be the major androgens.

The androgen surge takes place during the current 21 day layoff period enforced by the rules of the GBGB, once the bitch comes into season. In some animals the peak levels of 5α-androstane-3β,17β-diol and epiandrosterone, at this time were close to the proposed action level/threshold for the detection of testosterone administration.

Many of the bitches involved in the study showed similar trends in their longitudinal steroid profiles and the data from these animals has been considered to represent the profile of a bitch going through a ‘normal’ oestrus cycle of known average length. There are also several examples amongst the population of where the steroid profiles are variable, with some animals showing signs of having had either a silent heat or a

split heat. This is not unusual for greyhound bitches and the resulting variation in steroid profiles needs significant consideration in relation to future data interpretation as part of routine drug screening.

This thesis contains a unique set of data and is the first of its kind relating to the study of urinary steroid profiles in greyhound bitches. The data acquired is invaluable in relation to increasing the understanding of the hormonal status of racing bitches at various times throughout the racing calendar and it may even be possible with good record keeping to maintain a database of longitudinal values obtained from particular greyhounds of interest to the racing authorities. This may help to increase the ability of the authorities to direct their sampling strategy in relation to suspicious findings for particular animals or indeed trainers.

Due to the complexity and variability of the hormonal profile in certain animals observed in this study, urinary thresholds and/or action levels may prove challenging, though not insurmountable, in relation to providing a relevant and useful detection period following testosterone administration. The key analyte appears to be the 5β-androstane-3α,17β-diol (βαβ-diol). Further work with more sensitive methods may provide a more workable and useful threshold level for this metabolite, and this will definitely be suggested.

It may also be of interest to investigate the detection of intact testosterone esters in canine plasma as a potential associated means of detecting testosterone administration to the greyhound. This will also be of interest in relation to testosterone abuse in the male greyhound as well.

Another approach may be the use of carbon isotope ratio mass spectrometry to evaluate whether the androgen metabolites detected above an implemented action level to highlight suspicious samples, are actually from an exogenous source. This technique is powerful but by no means mainstream in the industry as yet.

Detecting the administration of endogenous hormones to animals is probably one of the greatest analytical challenges faced by testing laboratories. The greyhound bitch is no exception, but the data obtained from this study have provided a firm foundation on which to base a future testing strategy both in the UK and internationally.

7. APPENDICES

APPENDIX I: LIST OF PUBLICATIONS (2005-2013)

[1] S.Biddle, A.O'Donnell, J.Peterson, W.Hyde and E.Houghton. (2005) Monitoring the use of anabolic oestrus suppressants in the racing greyhound bitch: testosterone; 15th International Conference of Racing Analysts and Veterinarians (ICRAV), Dubai, UAE p.107

[2] J.Roberts, S.Scrimshaw, S.Mayoss, C.Elliot and S.Biddle. (2005) The application of a surface plasmon resonance biosensor to drug screening in equine samples; 15th International Conference of Racing Analysts and Veterinarians (ICRAV), Dubai, UAE p.178

[3] P.Teale, L.Grainger, A.Turrell and S.Biddle; Corticosteroids. (2005) Effect of the route of administration. Part1:Triamcinolone acetonide in the horse; 15th International Conference of Racing Analysts and Veterinarians (ICRAV), Dubai, UAE p.443

[4] P.Teale, P.Beck, C.M. Pearce, L.Hillyer and S.Biddle. (2006) Development of a method for the detection of cartrophen administration to the horse; Proc. 16th International Conference of Racing Analysts and Veterinarians (ICRAV), Tokyo, Japan p.526

[5] P.Teale, S.Biddle, P.Beck, M.Wieder, L.Hillyer and C.Pearce (2006) Blood as the matrix of choice: detection of sedatives using LCMS/MS; Proc. 16th International Conference of Racing Analysts and Veterinarians (ICRAV), Tokyo, Japan p.527

[6] S.Biddle, P.Teale, A.Robinson, J.Bowman, E.Houghton (2007) GCMS/MS analysis to determine natural and post administration levels of oestrogens in bovine serum and urine; Analytica Chimica Acta : special edition for 5th International Symposium on Hormone and Veterinary Drug Residue Analysis Vol. 586; p115

[7] C.Pearce, P.Beck, K.Woodward, S.Biddle and L.Hillyer (2009) The development of a multi-residue method for the detection of a range of anabolic steroids in equine plasma; Proc. 17th International Conference of Racing Analysts and Veterinarians (ICRAV), Antalya, Turkey p168

[8] J.Scarth, S.Maynard, A.Clarke, P.Taylor, S.Biddle, S.Hudson, P.Teale, B.Gray, C.Pearce and L.Hillyer. (2009) The use of in-vitro drug

metabolism studies to complement, reduce and refine in vivo administrations in medication and doping control; Proc. 17th International Conference of Racing Analysts and Veterinarians (ICRAV), Antalya, Turkey p213

[9] S.Hudson, S.Biddle and S.Maynard. (2009) Screening and confirmation of Stanozolol administration in the greyhound; Proc. 17th International Conference of Racing Analysts and Veterinarians (ICRAV), Antalya, Turkey p381

[10] S.Biddle, A, O'Donnell, E.Houghton and C.Creaser. (2009) Metabolism of methyltestosterone in the greyhound; Rapid Communications in Mass Spectrometry; 23, p713

[11] B. Gray, S.Biddle, C.Pearce and L.Hillyer. (2012) Detection of fluticasone propionate in horse plasma and urine following inhaled administration. *Drug Testing and Analysis*. Early view, first published online 18 April 2012. DOI: 10.1002/dta.1329.

[12] B.Gray, S.Biddle, C.Pearce and L.Hillyer. (2012) Approaches to the detection of fluticasone propionate in horse plasma and urine following inhaled administration at high and low doses. In Proceedings of the 19th International Conference of Racing Analysts and Veterinarians (In publication).

[13] S.Biddle, A, O'Donnell, E.Houghton and C.Creaser. (2013) Metabolism of norethisterone in the greyhound; Rapid Communications in Mass spectrometry 27, p2229

CONFERENCE PRESENTATIONS:

Presented: Monitoring the use of anabolic oestrus suppressants in the racing greyhound bitch: Testosterone: at the 15th International Conference of Racing Analysts and Veterinarians, (Dubai, United Arab Emirates) 2004

Presented**:** GCMS/MS analysis to determine natural and post administration levels of oestrogens in bovine serum and urine and Metabolism and detection of boldenone in bovine urine and plasma: at the 5th International Symposium on Hormone and Veterinary Drug Residue Analysis (Antwerp, Belgium) 2006.

APPENDIX II: PROJECT LICENCE AND ETHICAL REVIEW NOTIFICATION FOR RACING CHEMISTRY IOWA STATE UNIVERSITY.

	Review Date: 12/20/03	Committee ID: 6-2-5183-KE
	Approval Date: 12/20/03	Length of Approval: 1 yr
	Continuing Review Date: 12/04/04	FULL Committee Review Date: n/a
	Date OSPA Database:	Date in Compliance Database:
	Project Closure Date:	
	Key Personnel Training: X Complete / Date of Completion: ☐ Incomplete	

ISU RESEARCH ASSURANCES REVIEW FORM - USE OF ANIMALS IN RESEARCH

SECTION I: GENERAL INFORMATION

Principal Investigator (PI): Dr. Walter Hyde	Phone: 515-294-1950	Fax: 515-294-3182
Degrees: BS, MS, PhD	Correspondence Address: 1600 S. 16th Street, 1505 College of Vet. Med., Ames, IA 50011	
Department: VDPAM	Email Address: wghyde@iastate.edu	
Center/Institute:	College: CVM	
PI Level: ☒ Faculty ☐ Staff ☐ Postdoctoral ☐ Graduate Student ☐ Undergraduate Student		
Alternate Contact Person: Ms. Jackie Peterson	Email Address: jdpeterson@iastate.edu	
Correspondence Address: 1600 S. 16th Street, 1505 College of Vet. Med., Ames, IA 50011	Phone: 515-294-1950	
Title of Project: Drug Metabolism and Chemical Detection in the Racing Greyhound and Horse. Old COAC#6-2-5183-KE		
Start Date for involvement of Animals or Biohazards (*mm/dd/yy)*: 1/1/2004		

KEY PERSONNEL

List all members and relevant experience of the project personnel. This information is intended to inform the committee of the training and background related to the specific procedures that the each person will perform on the project.

NAME	DEGREE	SPECIFIC DUTIES ON PROJECT	TRAINING & EXPERIENCE RELATED TO PROCEDURES PERFORMED, DATE OF TRAINING
Walt Hyde	Ph.D, M.S., B.S.	Direct the project and assist in any and all duties in the Chem Lab	37 years in toxicology, Ph.D. in analytical and diagnoistic toxicology
Steve Hopkins	DVM,	Doses equine and collects samples	DVM
Jackie Peterson	B.A.	Manage the daily project logisitics, supervise and train the hourly and technical assistants, dose, collect specimens etc. as needed within the project	Trained by Drs. Hyde & Mullin (ISU-CVM-LAR).
Dan Gilloon	BS	Dose, collect and walk greyhounds under supervision.	Has been shown how to walk, dose and collect the greyhounds by current personel, Jackie Peterson.
Colin Novak	student	Dose, collect and walk greyhounds under supervision.	Has been shown how to walk, dose and collect the greyhounds by current personel, Jackie Peterson.
Melinda Thede	B.S	Dose, collect and walk greyhounds under supervision	Has been shown how to walk, dose and collect the greyhounds by current personel, Jackie Peterson.
Pat Simmons	Student.	Dose, collect and walk greyhounds under supervision..	Has been shown how to walk, dose and collect the greyhounds by current personel, Jackie Peterson.

FUNDING INFORMATION

Provide the funding source for the project below. Do not skip this question. If not applicable, please explain under "Other."

Internally funded, please provide account number: 202-05-29
Externally funded, please provide funding source and account number:
Funding is pending please provide OSPA Record ID on GoldSheet:
Title on GoldSheet if Different Than Above:
Other:

SCIENTIFIC REVIEW

☐ Yes ☒ No Has or will this project receive peer review?

If "yes", please indicate who did or will conduct the review:
If a review was conducted, please indicate the outcome of the review:

COLLECTION OR RECEIPT OF BIOLOGICAL SAMPLES

Will you be: (Please check all that apply.)

☐ Yes ☒ No Receiving samples from outside of ISU? See examples below.

☒ Yes ☐ No Sending samples outside of ISU? See examples below.

Examples include: genetically modified organisms, body fluids, tissue samples, blood samples, and pathogens.

If you will be receiving samples from or sending samples outside of ISU, please identify the name of the outside organization(s) and the identity of the samples you will be sending or receiving outside of ISU:

canine and equine urine and blood, as appropriate to the study component

SECTION II: APPLICATION FOR INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APPROVAL

☒ Yes ☐ No Does this project involves live vertebrate animals? If "no" is checked, you don't need to answer the questions in SECTION II.

SECTION III: APPLICATION FOR INSTITUTIONAL BIOSAFETY COMMITTEE (IBC) APPROVAL

☐ Yes ☒ No Does this project involve any of the following: Recombinant DNA (including transgenic animals); human, plant or animal pathogens; biological toxins; or administration of experimental biological products? If "no" is checked, you don't need to answer the questions in SECTION III.

SECTION IV: ENVIRONMENTAL HEALTH AND SAFETY INFORMATION (EH&S)

☐ Yes ☒ No Does this project involve laboratory chemicals, human cell lines or tissue culture (primary OR immortalized), or human blood components, body fluid or tissues? If "no" is checked, you don't need to answer the questions in SECTION IV.

ASSURANCE

- I certify that the information provided in this application is complete and accurate and consistent with any proposal(s) submitted to external funding agencies.
- I agree to provide proper surveillance of this project to ensure that the health and welfare of animal subjects are protected. I will report any problems to the appropriate compliance review committee(s).
- I certify that this project does not unnecessarily duplicate previous experiments involving animals.
- I agree that modifications to the originally approved project will not take place without prior review and approval by the appropriate committee(s), and that all activities will be performed in accordance with all applicable federal, state, local and Iowa State University policies.
- I will follow applicable biosafety level requirements, comply with all shipping requirements and required waste management practices.
- I will ensure that all personnel have appropriate training including but not limited to: biosafety principles and techniques, accidental spills, shipping regulations, proper handing of biohazardous materials and waste management, animal welfare regulations, and human subject regulations training.
- **CONFLICT OF INTEREST**
-
- ☐ Yes ☒ No Will you or any member of your research team have an actual or potential conflict of interest?
- ☐ Yes ☐ No If "yes", have the appropriate disclosure form(s) been completed?

Summary of ethical review considerations:

Ethical approval for animal drug administrations is required when dosing animals with non-licensed preparations for a given species. Approval is also required when licensed veterinary medications are administered above a normal therapeutic dose and if invasive sampling techniques are utilised (such as blood sample collection).
Northisterone is a human contraceptive that was not licensed for use in greyhounds at the time of the drug administration study described in this thesis. Therefore, ethical approval was sought, and obtained, by the drug administration facility at Racing Chemistry in IOWA State, USA, for the administration of this compound.
All the remaining drug administrations in this study involved the use of licensed veterinary preparatons (methyltestosterone was licensed for oestrus suppression in greyhounds in the USA at the time of the study), at or below therapeutic doses. Naturally voided urines were the only samples collected during the course of each drug administration. Therefore none of the remaining drug administration studies technically required ethical approval. However, each one was still discussed regarding its scientific merits, by the committee prior to commencement.

APPENDIX IIA: HEALTH AND SAFETY REQUIREMENTS FOR THE ANALYTICAL EXPERIMENTS CONDUCTED THROUGHOUT THIS THESIS

No special health and safety approvals were required prior to commencement of the work in this thesis. All health and safety requirements regarding the use of PPE (personal protective equipment) and appropriate handling of potentially hazardous chemicals, solvents and reagents (e.g. under fume extraction) in the laboratory environment, were adhered to throughout the course of this research. Where required/appropriate, the chemicals and reagents used during the laboratory experiments were disposed of using suitable commercial procedures. Particular attention was paid to the disposal of the enol-tms and MO-tms reagents used for derivatisation prior to GC-MS analysis, and the Grignard reagent used for the chemical synthesis of the methyltestosterone metabolite reference standard.

APPENDIX III: QUALITY CONTROL AND PRECISION AND ACCURACY DATA FROM QUANTITATIVE TESTOSTERONE METABOLITE ASSAY.

βαβ-Androstanediol:

βαβ-Androstanediol		**βαβ-Androstanediol**		**βαβ-Androstanediol**		**R^2**	**Batch**
QC Low 0.375ng/ml		*QC Med 3.000ng/ml*		*QC High 12.000ng/ml*			
Amount	%Error	Amount	%Error	Amount	%Error		
0.400	7%	3.268	9%	11.579	-4%	0.9952	1
0.390	4%	3.051	2%	12.230	2%		
0.445	19%	3.045	2%	11.643	-3%	0.9978	2
0.391	4%	3.182	6%	11.397	-5%		
0.306	-19%	2.878	-4%	11.493	-4%	0.9966	3
0.384	2%	2.631	-12%	11.683	-3%		
0.408	9%	2.956	-1%	11.790	-2%	0.9992	4
0.364	-3%	2.836	-5%	11.027	-8%		
0.386		2.981		11.605		**Mean**	
0.040		0.202		0.343		**Std Dev**	
10.312		6.787		2.954		**%CV**	
2.933		-0.638		-3.290		**%RE**	

Epiandrosterone:

Epiandrosterone:		**Epiandrosterone:**		**Epiandrosterone:**		**R^2**	**Batch**
QC Low 0.375ng/ml		*QC Med 3.000ng/ml*		*QC High 12.000ng/ml*			
Amount	%Error	Amount	%Error	Amount	%Error		
0.410	9%	3.155	5%	12.716	6%	0.9984	1
0.420	12%	3.188	6%	12.902	8%		
0.323	-14%	2.866	-4%	12.000	0%	0.9988	2
0.331	-12%	2.938	-2%	13.545	13%		
0.384	3%	3.011	0%	11.876	-1%	0.9992	3
0.372	-1%	2.892	-4%	12.193	2%		
0.314	-16%	3.146	5%	12.534	4%	0.9990	4
0.317	-15%	3.161	5%	13.397	12%		
0.359		3.045		12.645		**Mean**	
0.043		0.133		0.618		**Std Dev**	
12.001		4.376		4.885		**%CV**	
-4.300		1.488		5.378		**%RE**	

αββ--Androstanediol:

αββ--Androstanediol:		**αββ--Androstanediol:**		**αββ--Androstanediol:**		**R^2**	**Batch**
QC Low 0.375ng/ml		*QC Med 3.000ng/ml*		*QC High 12.000ng/ml*			
Amount	%Error	Amount	%Error	Amount	%Error		
0.384	2%	3.434	14%	13.527	13%	0.9970	1
0.432	15%	3.441	15%	**14.265***	**19%***		
0.400	7%	2.961	-1%	12.538	4%	0.9976	2
0.386	3%	3.227	8%	12.276	2%		
0.315	-16%	3.176	6%	12.850	7%	0.9922	3
0.401	7%	2.989	0%	12.854	7%		
0.374	0%	3.059	2%	12.411	3%	0.9987	4
0.376	0%	3.070	2%	12.943	8%		
0.384		3.170		12.772		**Mean**	
0.033		0.187		0.4170		**Std Dev**	
8.691		5.905		3.265		**%CV**	
2.267		5.654		6.427		**%RE**	

βαβ-Androstanediol:

Batch	**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**		**16.000ng/ml**	
1	0.311	0.292	0.580	0.545	1.025	0.982	1.828	1.905	4.159	3.609	8.605	7.754	16.743	15.162
2	0.245	0.266	0.556	0.542	1.126	1.080	1.958	2.064	3.880	4.041	8.239	7.246	16.418	15.840
3	0.268	0.273	0.501	0.521	0.948	0.913	2.036	1.942	4.156	3.859	8.857	7.293	16.104	15.828
4	0.199	0.179	0.415	0.451	1.007	0.989	2.013	1.910	4.019	4.258	7.888	8.341	15.734	16.098

Mean	0.254	0.514	1.009	1.957	3.998	8.029	15.991
Std dev	0.045	0.056	0.069	0.078	0.209	0.587	0.474
%CV	17.676	10.873	6.812	3.987	5.225	7.308	2.966
%RE	1.650	2.775	0.875	-2.150	-0.060	0.349	-0.057

Epiandrosterone

Batch	**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**		**16.000ng/ml**	
1	0.260	0.276	0.549	0.610	1.085	0.995	2.010	1.996	4.030	3.979	8.040	7.409	16.457	15.805
2	0.178	0.200	0.431	0.445	0.993	0.969	1.918	1.903	4.043	4.169	8.205	8.342	15.704	**14.722***
3	0.265	0.266	0.515	0.522	1.058	0.997	2.008	2.020	4.233	3.999	7.775	7.589	16.168	16.083
4	0.198	0.191	0.421	0.441	1.010	0.985	2.014	2.114	3.762	4.215	7.833	8.472	15.915	15.929

Mean	0.230	0.492	1.012	1.998	4.054	7.958	16.009
Std dev	0.041	0.068	0.039	0.065	0.154	0.371	0.252
%CV	17.819	13.787	3.901	3.270	3.796	4.668	1.574
%RE	-8.300	-1.650	1.150	-0.106	1.343	-0.523	0.054

αββ-Androstanediol:

Batch	**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**		**16.000ng/ml**	
1	0.254	0.259	0.531	0.525	1.033	0.938	1.770	1.967	4.194	3.667	8.284	**7.371***	16.170	**14.763***
2	0.277	0.266	0.437	0.458	1.129	1.037	1.863	2.029	3.800	3.914	8.738	7.948	15.893	15.831
3	0.207	0.267	0.492	0.407	1.013	1.078	2.046	2.270	4.362	4.161	9.301	7.199	15.647	15.446
4	0.231	0.257	0.501	0.485	1.023	1.084	2.077	1.973	3.710	4.199	7.840	8.364	15.982	15.843

Mean	0.252	0.480	1.042	1.999	4.001	8.239	15.830
Std dev	0.023	0.043	0.057	0.149	0.261	0.673	0.232
%CV	8.977	8.951	5.472	7.449	6.522	8.172	1.468
%RE	0.900	-4.100	4.188	-0.031	0.022	2.989	-1.061

APPENDIX IV: QUALITY CONTROL DATA - OESTRUS CYCLES

βαβ-Androstanediol:

QC Low 0.375ng/ml		*QC High 3.000ng/ml*		R^2	Batch
Amount	**%Error**	**Amount**	**%Error**		
0.373	0%	2.899	3%	0.9992	1
0.393	5%	3.010	0%		
0.411	10%	3.046	2%	0.9996	2
0.402	7%	3.058	2%		
0.345	8%	2.931	2%	0.9993	3
0.406	8%	3.039	1%		
0.346	8%	2.945	2%	0.9989	4
0.388	3%	3.036	1%		
0.353	6%	2.917	3%	0.999	5
0.384	2%	2.923	3%		
N/A	N/A	N/A	N/A	N/A	6
N/A	N/A	N/A	N/A		
0.397	6%	2.850	5%	0.9994	7
0.369	2%	2.910	3%		
0.371	1%	2.862	5%	0.9997	8
0.391	4%	2.690	10%		
0.421	12%	3.114	4%	0.9996	9
0.384	2%	3.114	4%		
0.355	5%	2.826	6%	0.9996	10
0.340	9%	2.707	10%		
0.427	14%	3.004	0%	0.9997	11
0.397	6%	2.761	8%		
N/A	N/A	N/A	N/A	N/A	12
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	13
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	14
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	15
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	16A
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	17
N/A	N/A	N/A	N/A		
N/A	N/A	N/A	N/A	N/A	18
N/A	N/A	N/A	N/A		
0.407	8%	2.895	3%	0.9961	19
0.317	16%	2.597	13%		
0.381		2.915		**Mean**	
0.03		0.14		**Std Dev**	
7.54		4.73		**%CV**	
-1.54		2.82		**%RE**	

Epiandrosterone:

QC Low 0.375ng/ml		*QC High 3.000ng/ml*		R^2	Batch
Amount	**%Error**	**Amount**	**%Error**		
0.348	7%	2.813	6%	0.9975	1
0.368	2%	3.4	13%		
0.375	0%	3.146	5%	0.9866	2
0.364	3%	3.075	3%		
0.373	0%	3.370	12%	0.9983	3
0.413	10%	3.428	14%		
0.366	3%	3.294	10%	0.9983	4
0.370	1%	3.374	12%		
0.389	4%	3.359	12%	0.9912	5
0.440	17%	3.270	9%		
0.306	18%	2.963	1%	0.9941	6
0.319	15%	2.775	7%		
0.362	4%	3.092	3%	0.9981	7
0.379	1%	**4.724***	57%		
0.402	7%	3.174	6%	0.9986	8
0.373	1%	2.805	7%		
0.377	0%	3.110	4%	0.9993	9
0.417	11%	3.110	4%		
0.375	0%	3.097	3%	0.9992	10
0.391	4%	**4.599***	53%		
0.390	4%	3.233	8%	0.9981	11
0.409	9%	3.152	5%		
0.382	2%	2.971	1%	0.9965	12
0.373	1%	2.955	2%		
0.360	4%	3.089	3%	0.9802	13
0.402	7%	3.285	9%		
0.386	3%	2.783	7%	0.9923	14
0.350	7%	2.830	6%		
0.373	0%	3.021	1%	0.9925	15
0.394	5%	3.001	0%		
0.375	0%	2.799	7%	0.9872	16B
0.416	11%	2.770	8%		
0.328	13%	2.663	11%	0.9885	17
0.385	3%	2.603	13%		
0.354	6%	2.723	9%	0.9857	18
0.307	18%	2.638	12%		
0.377	0%	2.874	4%	0.9992	19
0.386	3%	2.680	11%		
0.375		3.020		**Mean**	
0.03		0.24		**Std Dev**	
7.63		8.02		**%CV**	
-0.03		-0.67		**%RE**	

αββ--Androstanediol:

QC Low 0.375ng/ml		*QC High 3.000ng/ml*		R^2	Batch
Amount	**%Error**	**Amount**	**%Error**		
0.434	16%	2.688	10%	0.9977	1
0.435	16%	2.892	4%		
0.361	4%	2.908	3%	0.9936	2
0.349	7%	2.878	4%		
0.307	18%	2.920	3%	0.9915	3
0.330	12%	3.174	6%		
0.333	11%	3.242	8%	0.9977	4
0.355	5%	3.412	14%		
0.332	11%	3.001	0%	0.9969	5
0.340	9%	2.748	8%		
0.377	1%	2.695	10%	0.9943	6
0.337	10%	2.568	14%		
0.449	20%	2.912	3%	0.9997	7
0.384	2%	3.375	12%		
0.430	15%	2.908	3%	0.999	8
0.372	1%	3.287	10%		
0.378	1%	2.938	2%	0.9995	9
0.372	1%	2.938	2%		
0.387	3%	3.084	3%	0.9999	10
0.368	2%	3.169	6%		
0.432	15%	3.429	14%	0.9954	11
0.432	15%	**3.962***	32%		
0.384	2%	3.028	1%	0.9971	12
0.360	4%	2.868	4%		
0.302	20%	2.786	7%	0.9993	13
0.357	5%	3.139	5%		
0.382	2%	2.998	0%	0.9977	14
0.369	2%	2.966	1%		
0.383	2%	3.174	6%	0.9937	15
0.394	5%	2.905	3%		
0.384	2%	2.941	2%	0.9858	16
0.373	0%	2.880	4%		
0.371	1%	2.86	5%	0.9899	17
0.431	15%	2.669	11%		
0.335	11%	2.894	4%	0.996	18
0.311	17%	2.614	13%		
0.325	13%	2.953	2%	0.9795	19
0.370	1%	2.577	14%		
0.372		2.957		**Mean**	
0.04		0.22		**Std Dev**	
10.27		7.46		**%CV**	
0.87		1.43		**%RE**	

APPENDIX V: PRECISION AND ACCURACY DATA – OESTRUS CYCLES

βαβ-Androstanediol:

Batch	**0.125ng/ml**		**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**	
1	**0.157***	**0.162***	0.281	0.283	0.492	0.459	1.035	1.03	1.965	1.969	3.816	3.873	8.039	8.125
2	0.121	0.131	0.230	0.239	0.467	0.516	1.003	0.933	1.990	1.991	3.955	4.025	7.990	8.158
3	0.124	0.139	0.253	0.222	0.515	0.525	0.955	0.962	1.963	1.958	3.999	4.124	8.128	7.881
4	0.126	0.129	0.273	0.245	0.528	0.495	0.903	0.900	1.969	2.070	4.050	4.039	7.984	8.039
5	0.147	0.128	0.226	0.222	0.498	0.492	1.028	1.043	2.012	1.923	4.000	3.922	7.885	8.223
6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	0.142	0.121	0.234	0.219	0.541	0.502	1.023	0.976	2.013	1.990	3.931	4.101	7.934	8.025
8	0.118	0.129	0.230	0.265	0.499	0.528	0.994	1.012	1.981	1.982	3.924	4.054	7.981	8.054
9	0.116	0.132	0.261	0.243	0.507	0.508	1.043	0.990	1.900	1.980	3.941	4.014	8.104	8.010
10	0.108	0.131	0.275	0.247	0.517	0.499	1.008	1.010	1.949	1.948	3.934	4.048	8.062	8.014
11	0.132	0.123	0.237	0.245	0.508	0.506	1.020	1.004	2.001	1.971	4.042	3.871	8.114	7.974
12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
13	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
19	0.115	0.138	0.262	0.253	0.521	0.502	1.105	1.001	1.738	1.929	4.399	4.207	7.568	8.012
Mean	0.128		0.248		0.506		0.999		1.963		4.012		8.014	
Std dev	0.01		0.02		0.02		0.05		0.06		0.13		0.13	
%CV	7.59		7.95		3.74		4.73		3.12		3.12		1.63	
%RE	-2.00		1.00		-1.14		0.10		1.84		-0.31		-0.17	

Epiandrosterone:

Batch	**0.125ng/ml**		**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**	
1	**0.160***	**0.161***	0.266	0.26	0.476	0.452	0.951	0.956	1.914	1.849	4.062	4.085	**9.363***	9.187
2	0.124	0.132	0.236	0.278	0.454	0.434	0.889	0.905	1.889	2.075	4.406	4.035	9.196	8.680
3	**0.163***	0.122	0.284	0.238	0.506	0.505	0.907	1.085	1.748	2.042	4.043	4.088	7.791	8.195
4	**0.158***	0.124	0.258	0.245	0.520	0.473	0.907	1.059	1.769	2.053	3.992	4.290	7.878	8.030
5	**0.169***	0.149	0.226	0.253	0.477	0.462	0.864	1.131	1.815	2.130	4.054	4.227	8.640	7.198
6	**0.079***	0.128	0.228	0.269	0.521	0.458	0.972	0.931	1.933	1.940	4.163	4.445	7.994	8.286
7	0.119	0.118	0.240	0.247	0.493	0.503	1.086	1.053	2.170	1.990	3.827	4.007	8.198	7.699
8	0.131	0.113	0.232	0.257	0.510	0.495	1.070	1.033	1.908	1.905	4.131	4.097	7.666	8.202
9	0.113	0.113	0.241	0.247	0.496	0.474	1.005	1.019	2.130	1.999	3.889	3.917	7.952	8.155
10	0.125	0.122	0.239	0.253	0.492	0.488	1.042	1.060	2.153	2.020	3.972	3.872	8.039	7.875
11	0.128	0.117	0.242	0.245	0.514	0.490	0.998	1.035	1.991	1.821	3.975	4.017	7.690	8.488
12	0.136	0.112	0.258	0.258	0.493	0.476	0.990	0.963	2.073	2.046	3.927	3.938	8.139	8.120
13	0.104	0.148	0.281	0.244	0.477	0.459	0.853	0.879	1.911	1.969	4.296	4.094	8.827	9.160
14	0.160	0.104	0.257	0.283	0.507	0.504	0.983	1.002	2.032	2.046	3.911	3.730	7.956	7.757
15	0.126	0.137	0.233	0.214	0.486	0.488	1.002	0.983	2.175	2.139	4.149	4.022	8.450	7.293
16	0.123	0.111	0.290	0.272	0.477	**0.334***	1.045	1.103	2.034	2.109	3.670	4.107	7.222	6.903
17	0.112	0.126	**0.293***	0.252	0.465	**0.324***	1.050	1.108	2.045	2.106	3.772	4.068	7.135	7.173
18	0.127	0.122	0.218	0.280	0.458	0.559	1.092	1.016	2.094	1.842	3.469	3.643	9.190	7.868
19	0.121	0.104	0.279	0.305*	0.514	0.558	1.141	0.919	2.045	1.872	3.875	3.903	6.834	7.239

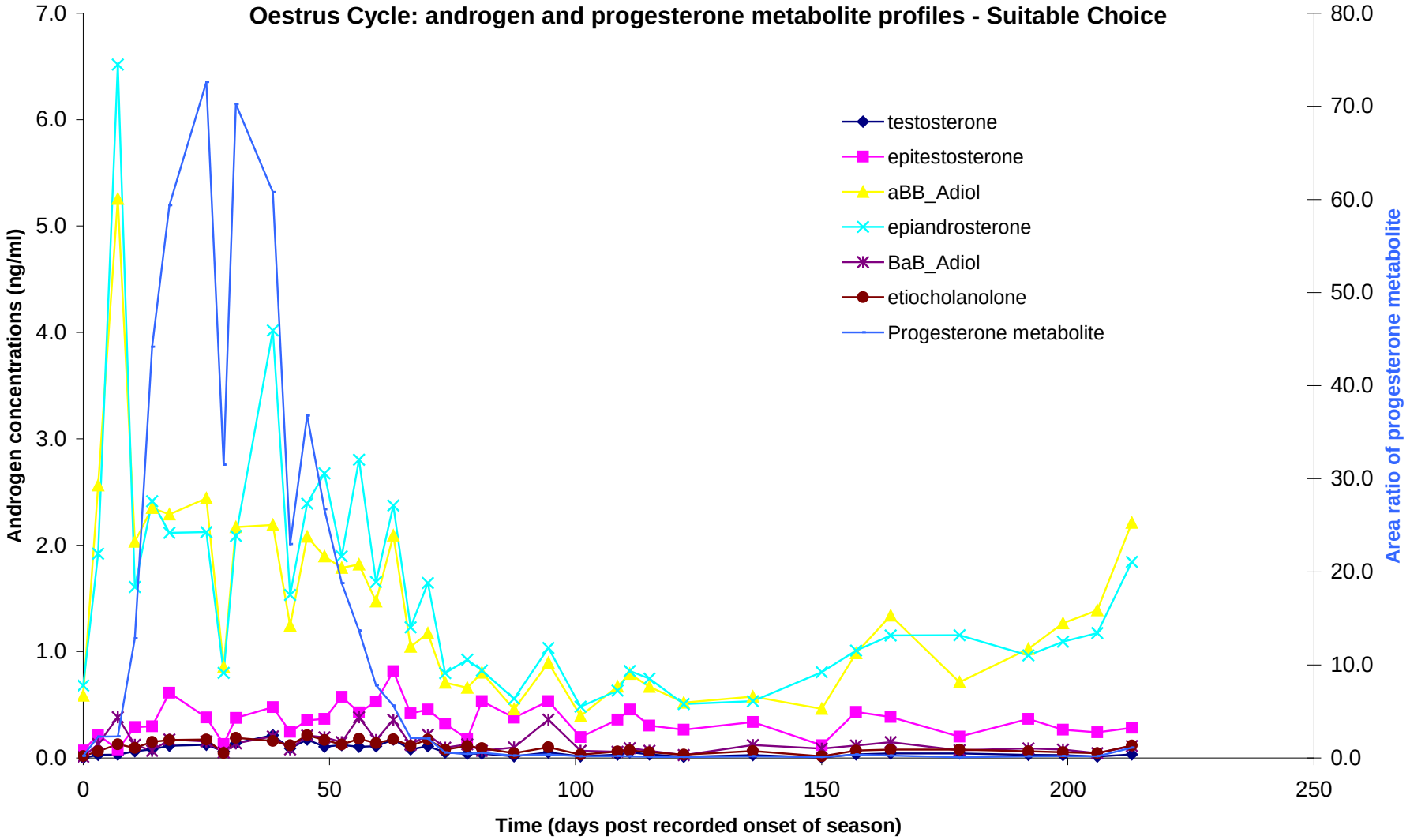
Mean	0.123	0.253	0.489	1.002	1.994	4.004	8.008
Std dev	0.01	0.02	0.03	0.08	0.12	0.20	0.64
%CV	10.40	7.72	5.54	7.66	5.80	4.97	7.96
%RE	1.22	-1.14	2.14	-0.23	0.29	-0.11	-0.10

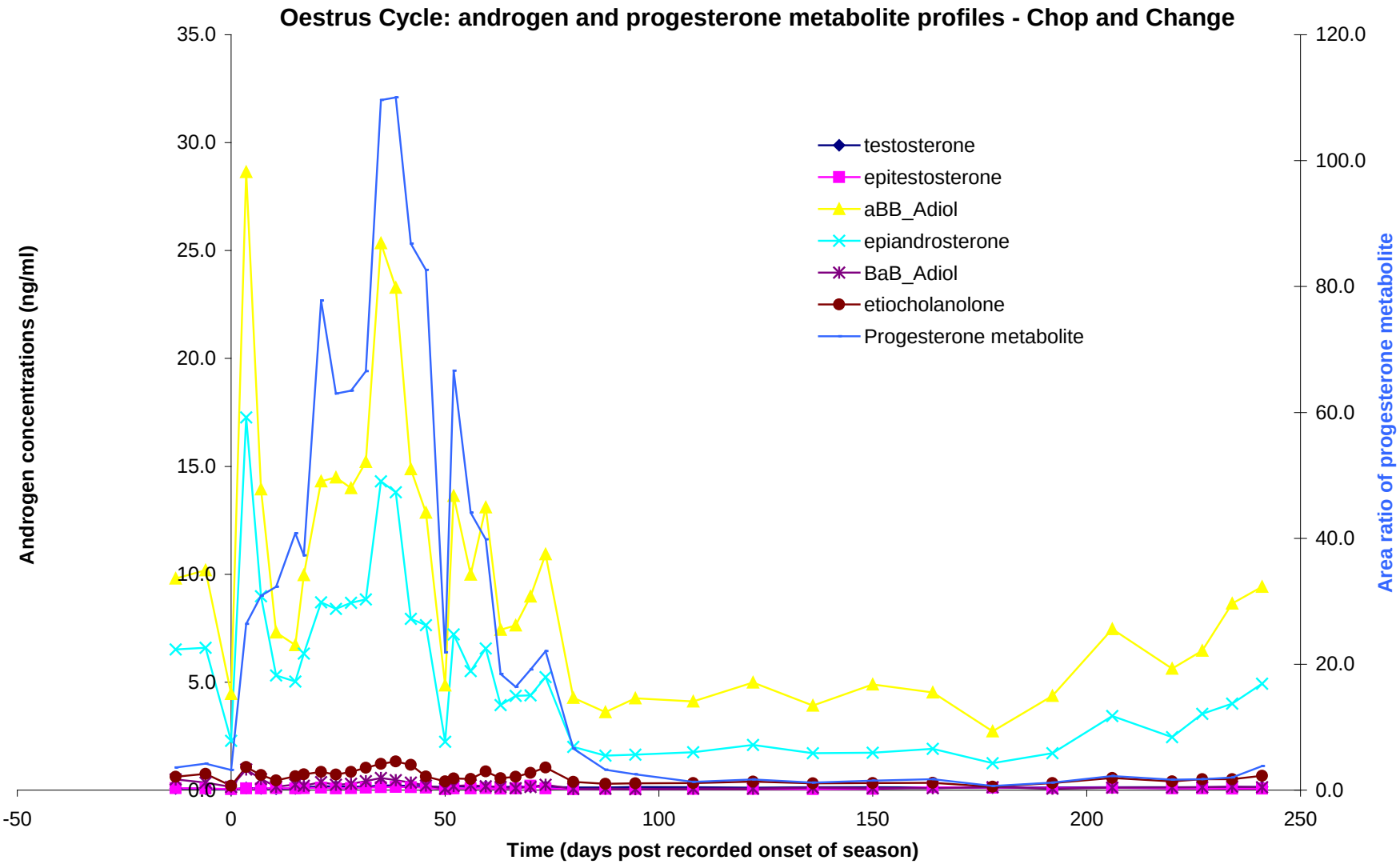
αββ-Androstanediol:

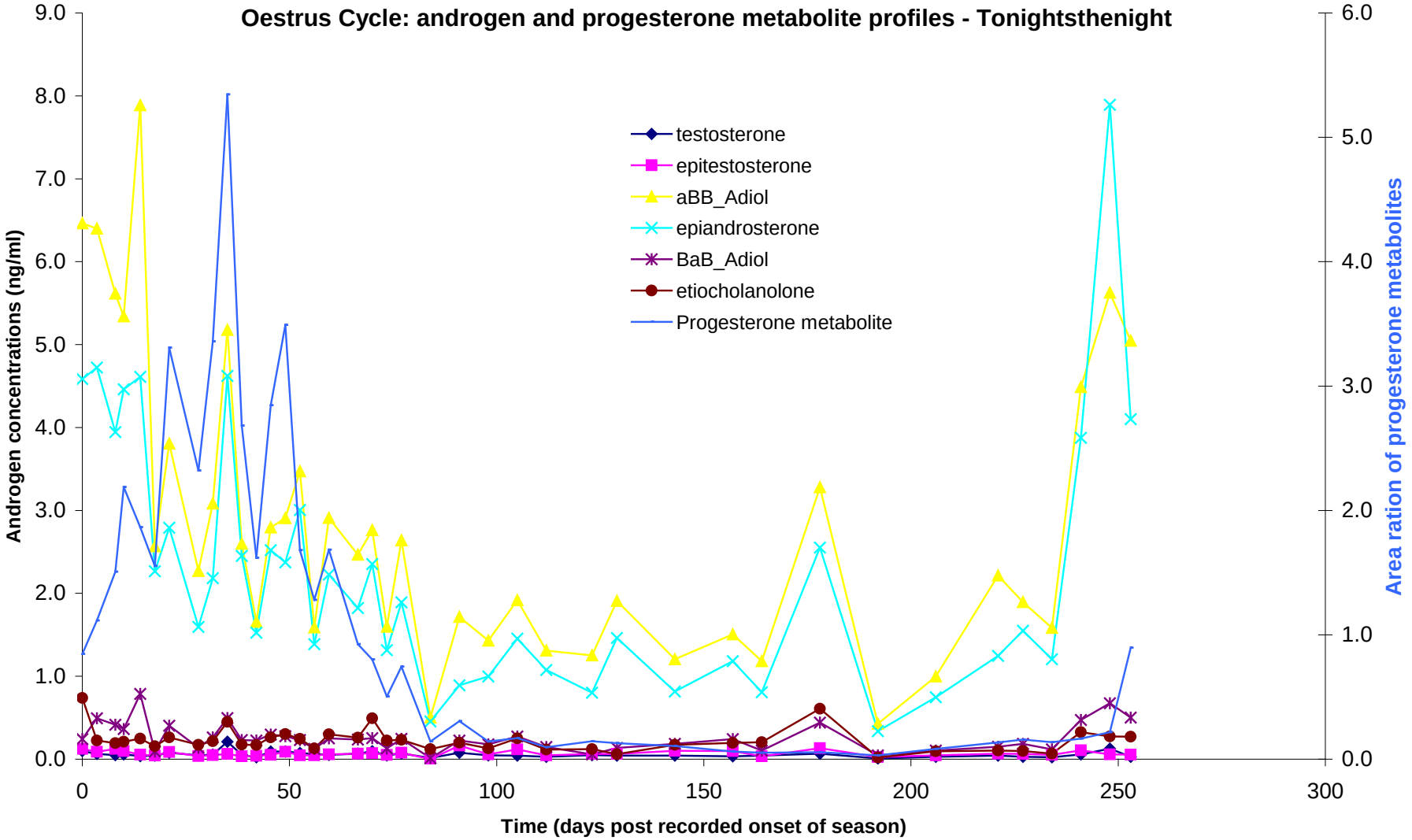
Batch	**0.125ng/ml**		**0.250ng/ml**		**0.500ng/ml**		**1.000ng/ml**		**2.000ng/ml**		**4.000ng/ml**		**8.000ng/ml**	
1	**0.399***	**0.393***	**0.358***	**0.360***	0.54	0.537	0.975	0.921	1.834	1.83	3.824	3.861	8.142	8.101
2	0.121	0.124	0.213	0.218	**0.416***	0.454	0.879	0.843	1.852	1.892	4.064	3.897	8.542	8.389
3	0.149	0.143	0.236	0.219	0.512	0.461	0.951	1.041	1.721	2.086	4.079	4.090	8.361	7.306
4	0.117	0.114	0.228	**0.192***	0.517	0.464	0.980	1.096	1.773	2.109	4.111	4.172	8.170	7.711
5	0.142	0.106	0.257	0.266	0.431	0.515	1.165	1.013	1.924	1.797	3.988	3.849	8.137	8.160
6	0.144	0.156	**0.698***	0.282	0.561	0.532	0.919	0.920	1.911	1.804	3.915	4.457	7.591	8.307
7	**0.171***	0.145	0.262	0.280	0.473	0.477	0.987	0.972	1.894	1.946	3.977	4.073	8.043	7.978
8	0.137	0.135	0.232	0.261	0.523	0.481	1.010	0.965	1.901	1.936	3.958	4.242	7.863	8.081
9	**0.153***	0.147	0.275	0.270	0.519	0.502	1.040	0.965	1.942	1.910	3.852	4.009	8.030	8.072
10	0.134	0.132	0.250	0.263	0.526	0.480	1.049	1.005	1.968	1.960	3.918	4.008	8.023	8.025
11	0.132	0.109	0.231	0.224	0.500	0.470	1.040	1.018	2.007	1.971	4.083	4.234	8.572	8.879
12	0.120	0.106	0.260	0.269	0.519	0.505	0.997	0.979	2.115	2.048	3.867	3.920	8.216	7.991
13	**0.085***	0.159	**0.315***	0.267	0.534	0.443	1.059	0.997	1.984	1.977	3.937	3.843	8.134	7.978
14	0.132	**0.085***	0.266	0.274	0.464	0.509	0.947	0.950	1.994	2.022	3.920	3.833	8.054	7.920
15	0.141	0.150	0.253	0.233	0.504	0.480	0.972	0.949	2.061	2.010	3.877	3.923	7.879	6.899
16	0.109	0.121	0.295	0.286	0.497	**0.305***	1.025	0.990	2.005	2.023	3.682	4.370	7.503	6.886
17	0.137	0.111	0.276	0.231	0.466	**0.267***	1.046	1.136	1.971	2.102	3.763	4.093	7.707	7.316
18	**0.205***	**0.149***	0.260	0.258	0.460	0.492	0.915	0.931	2.112	1.987	3.772	3.658	8.176	8.456
19	**0.213***	0.141	0.240	0.256	0.426	0.520	0.918	0.948	2.083	2.018	3.983	4.179	7.859	8.055

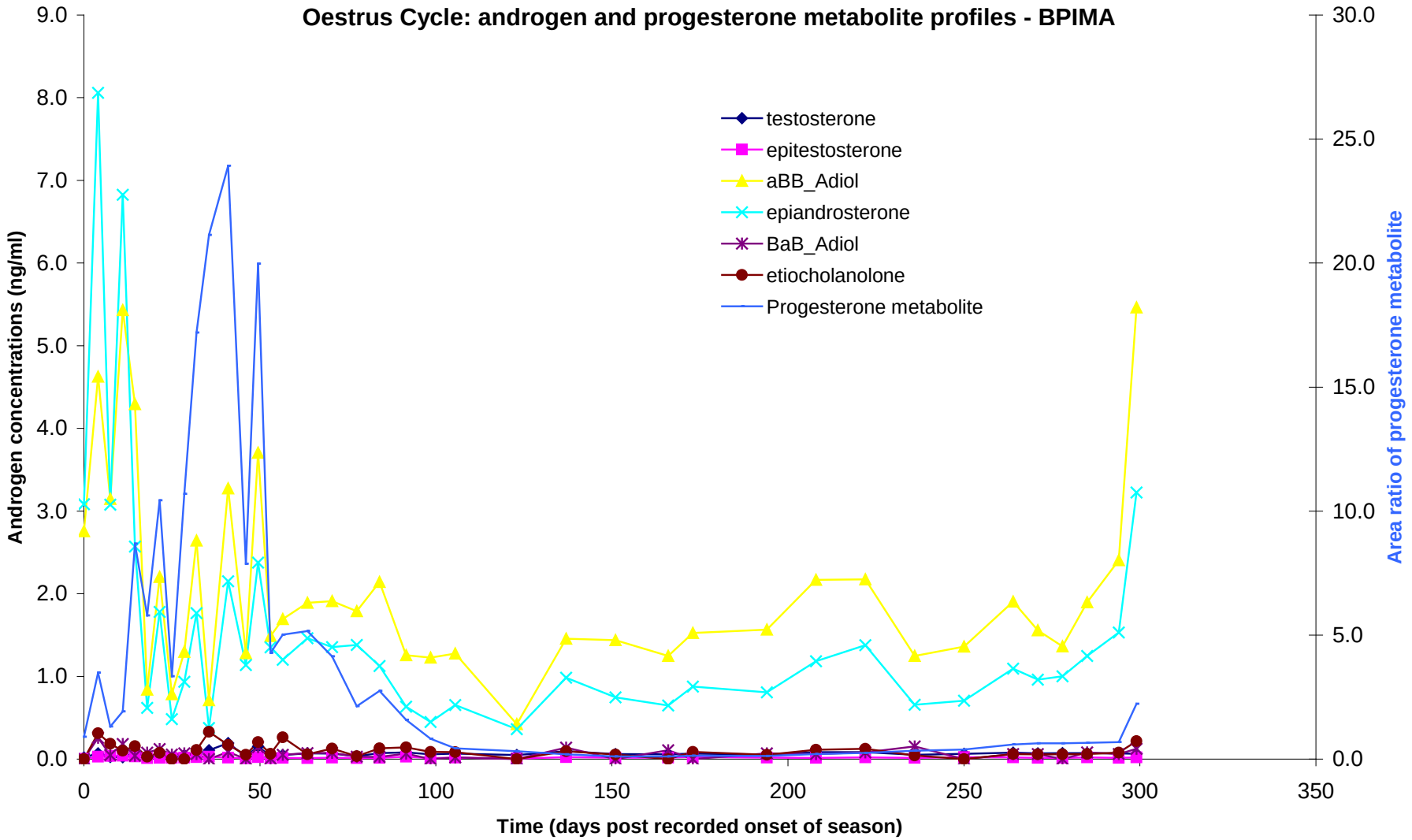
Mean	0.132	0.254	0.494	0.987	1.960	3.981	7.987
Std dev	0.02	0.02	0.03	0.07	0.10	0.17	0.41
%CV	11.80	8.52	6.64	6.64	5.08	4.36	5.14
%RE	-5.21	-1.71	1.18	1.28	2.00	0.47	0.16

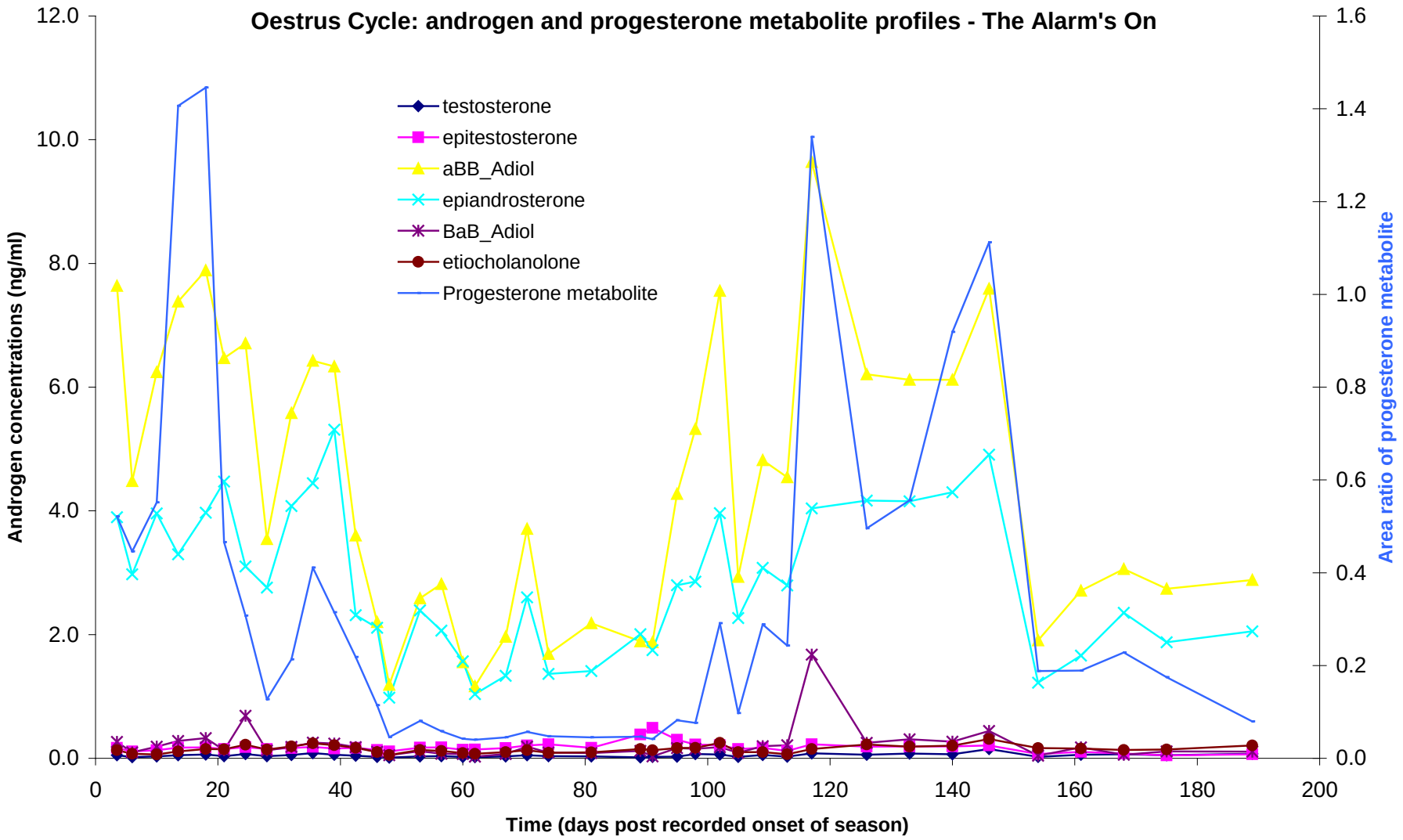
APPENDIX VI: OESTRUS CYCLE STEROID PROFILES

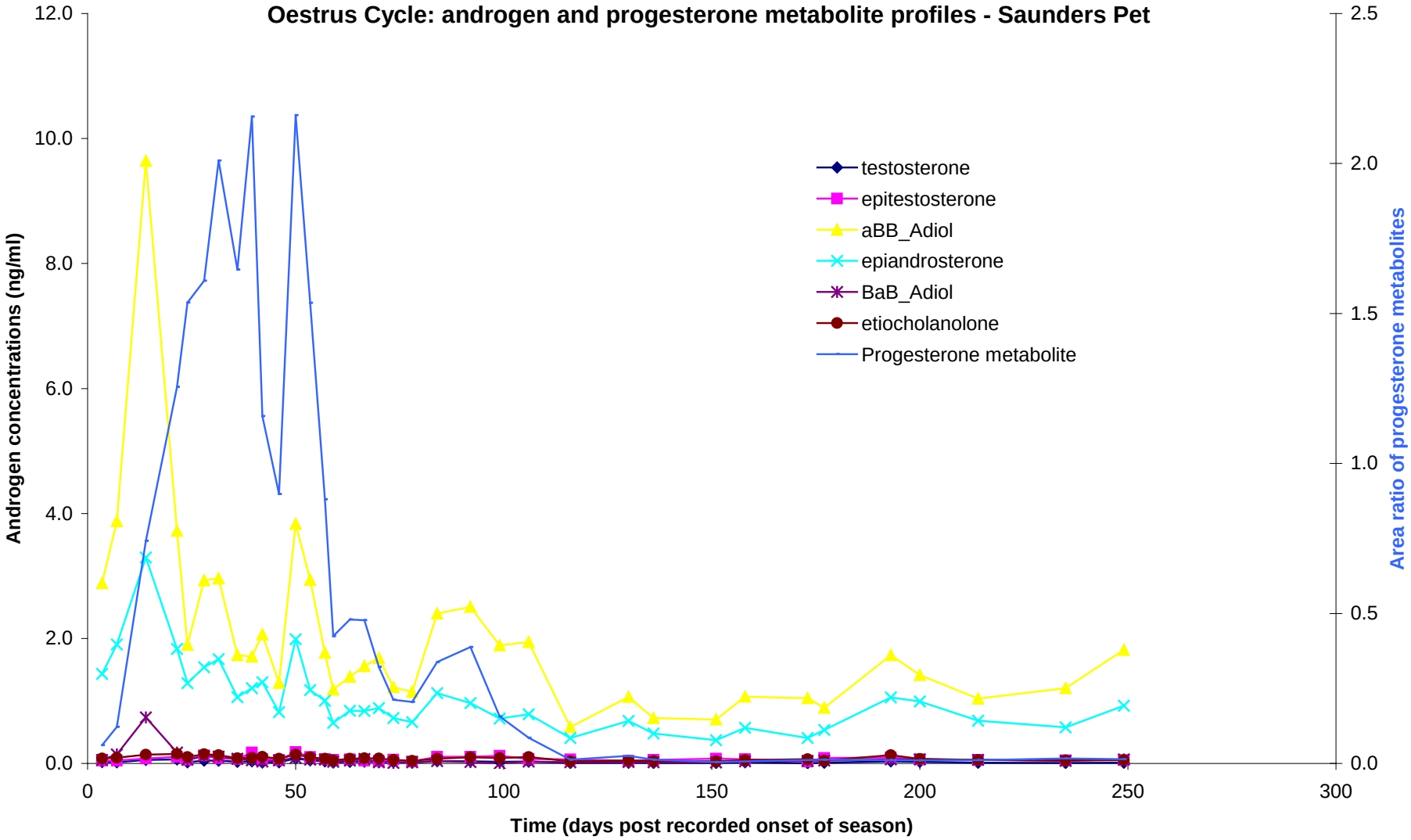

Oestrus Cycle: androgen and progesterone metabolite profiles - Suitable Choice
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
50
100
150
200
250

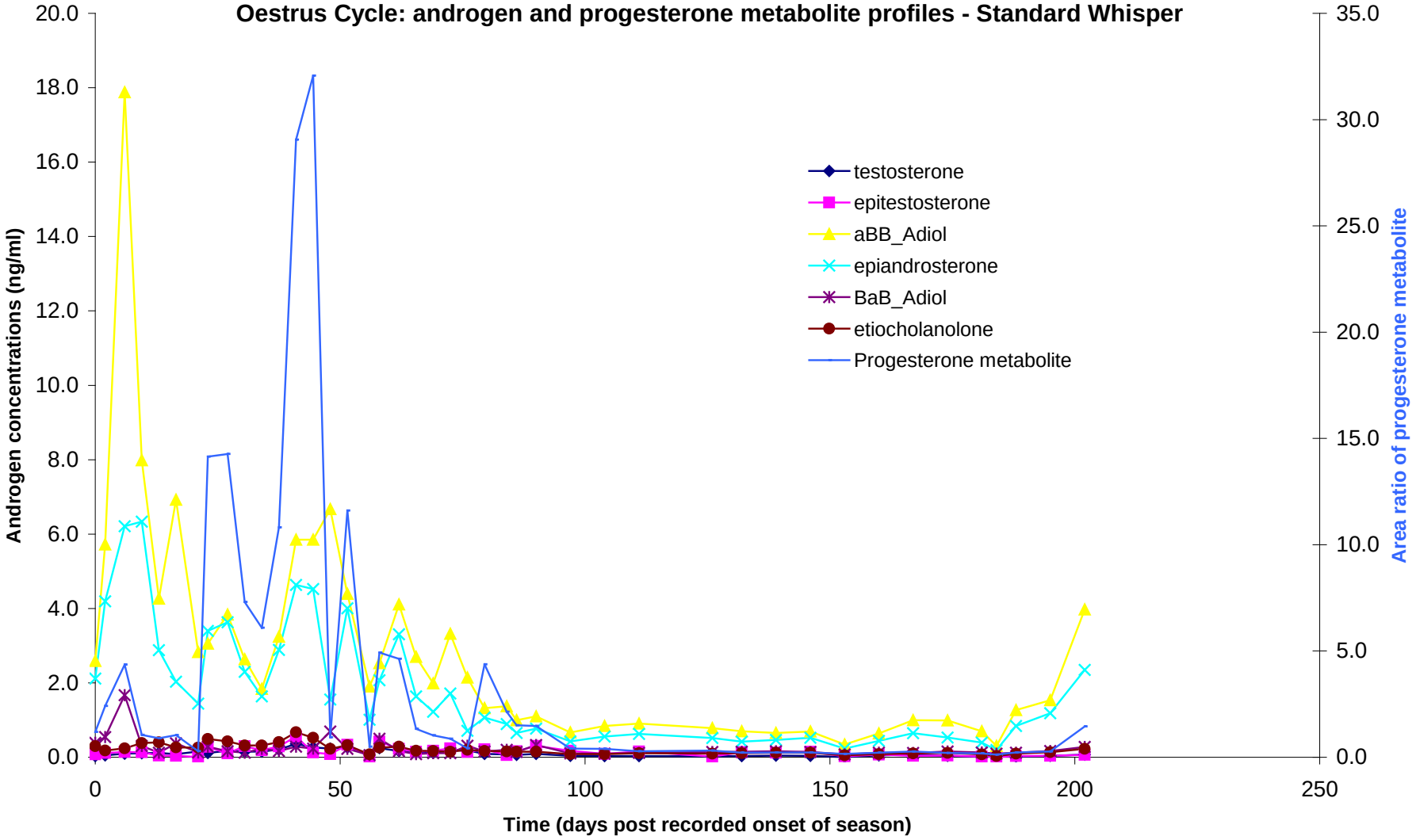

Oestrus Cycle: androgen and progesterone metabolite profiles - Chop and Change
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
-50
0
50
100
150
200
250

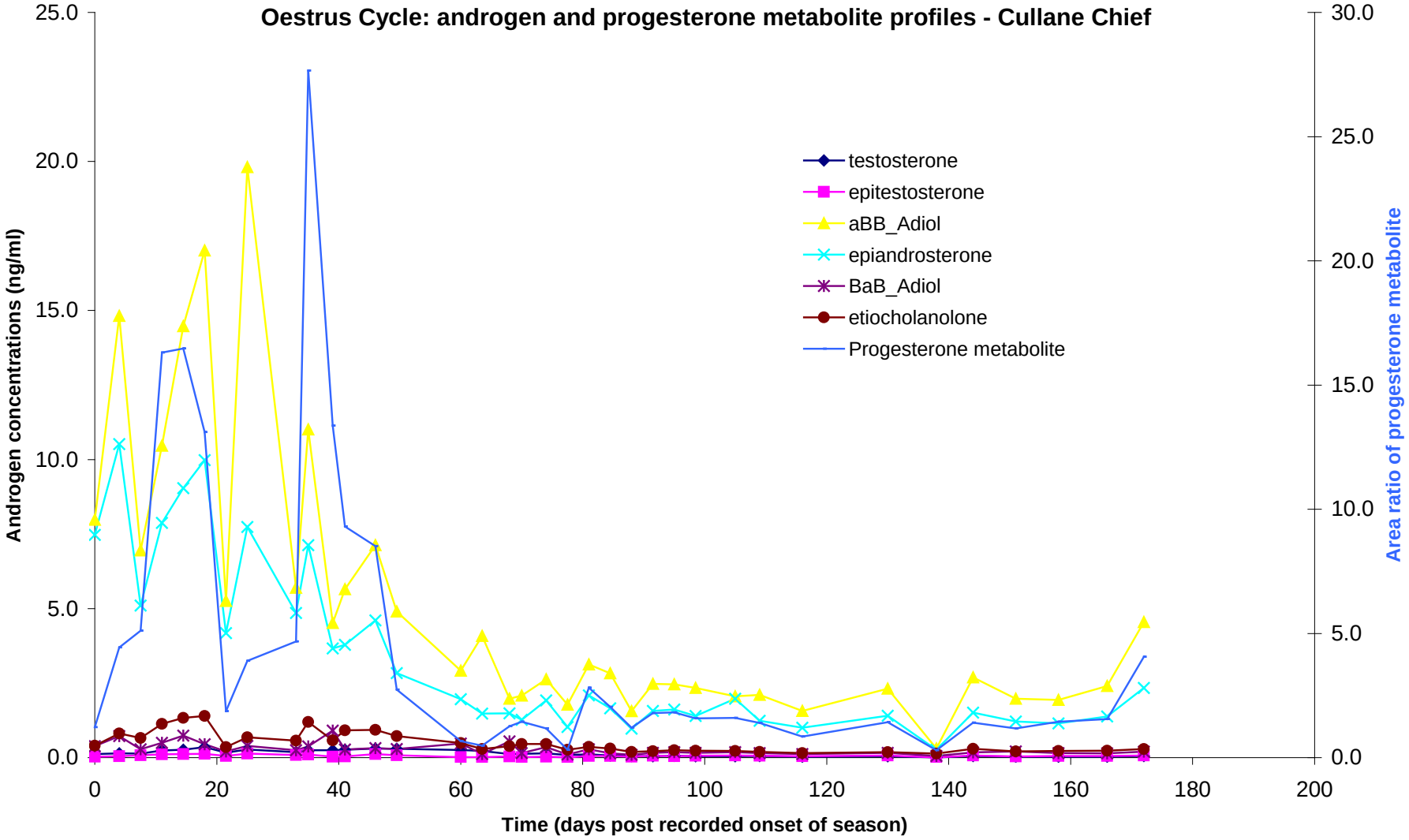

Oestrus Cycle: androgen and progesterone metabolite profiles - Tonightsthenight
Androgen concentrations (ng/ml)
Area ration of progesterone metabolites
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300

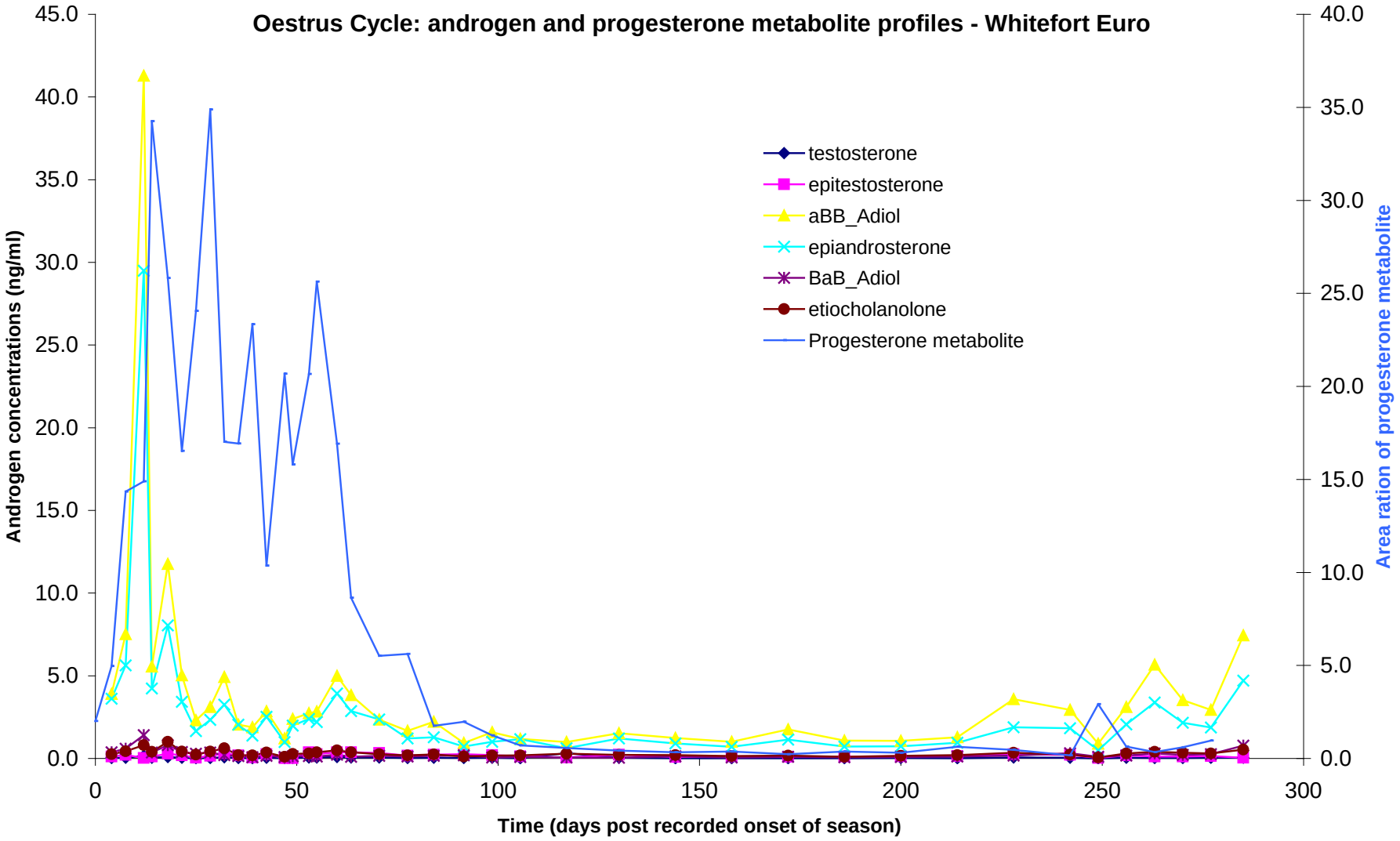

Oestrus Cycle: androgen and progesterone metabolite profiles - BPIMA
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250
300
350

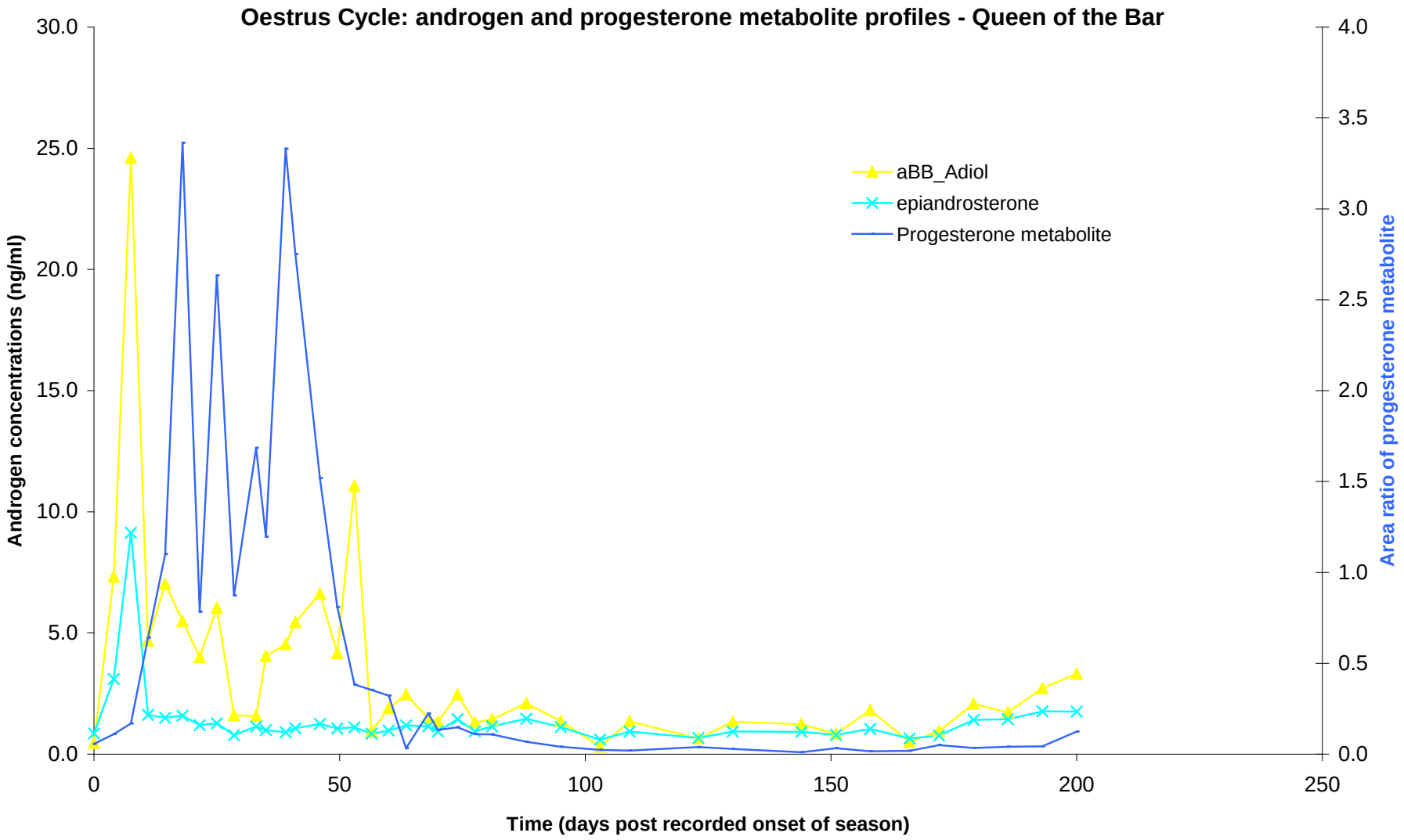

Oestrus Cycle: androgen and progesterone metabolite profiles - The Alarm's On
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
180
200

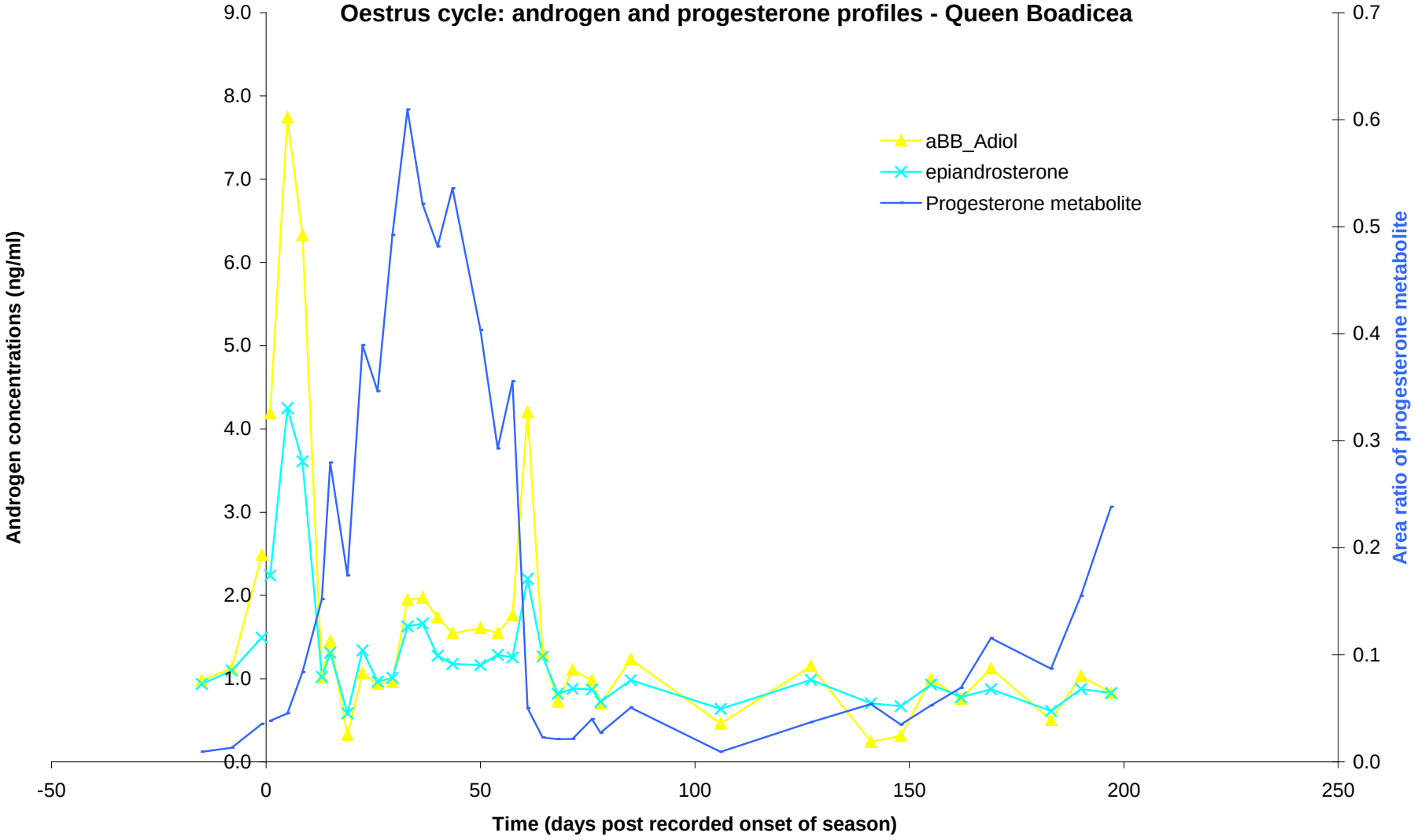

Oestrus Cycle: androgen and progesterone metabolite profiles - Saunders Pet
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolites
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
12.0
10.0
8.0
6.0
4.0
2.0
0.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300

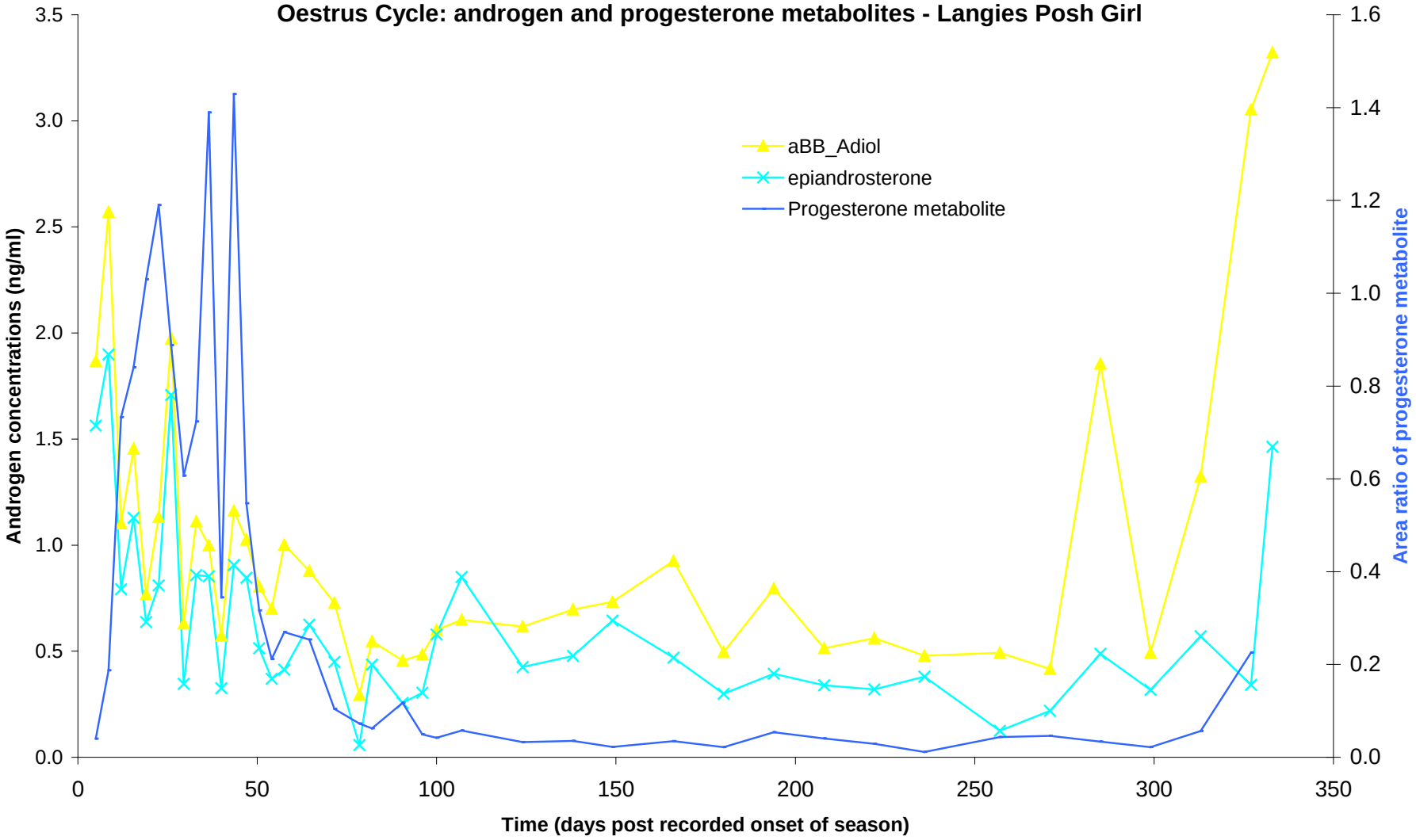

Oestrus Cycle: androgen and progesterone metabolite profiles - Standard Whisper
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250

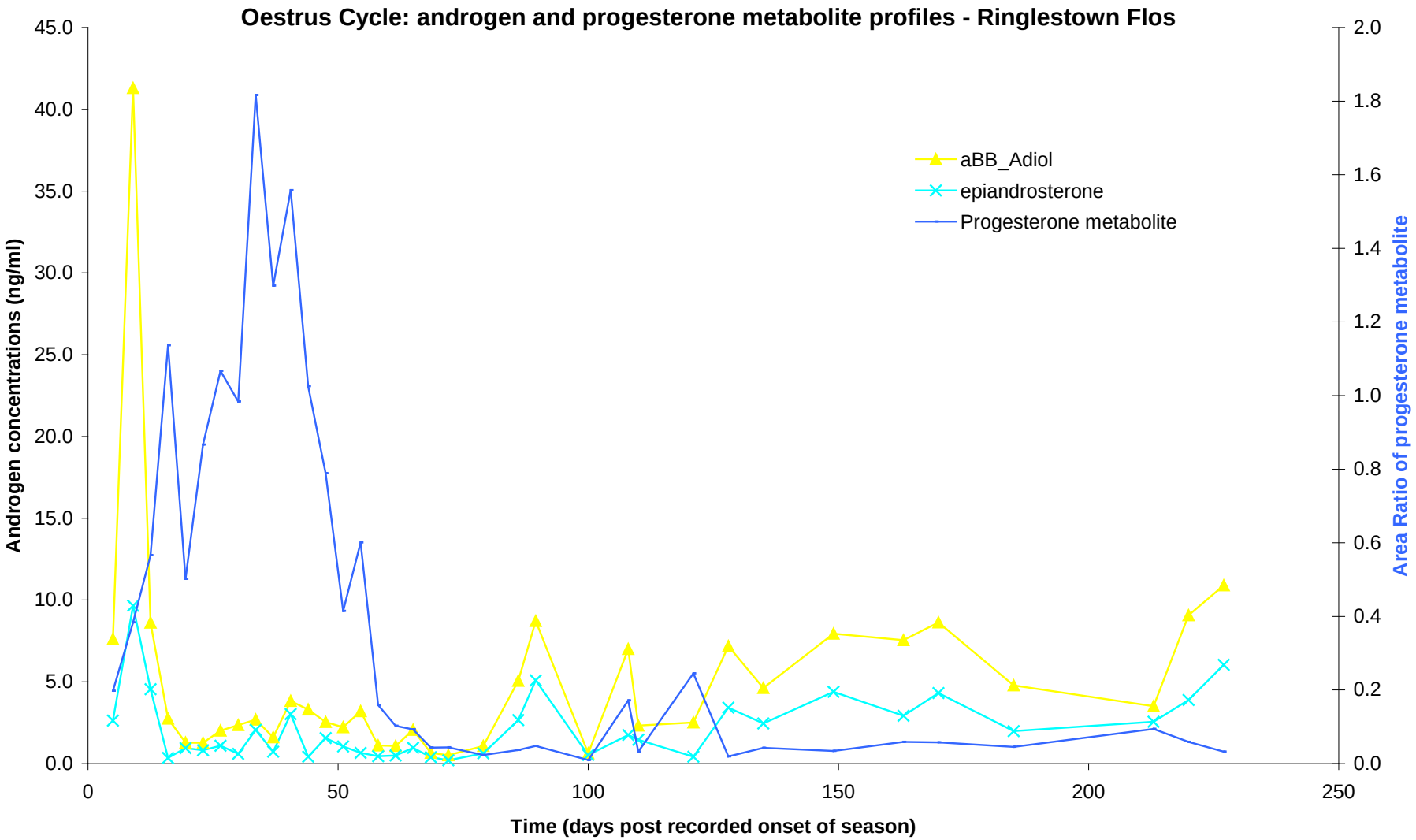

Oestrus Cycle: androgen and progesterone metabolite profiles - Cullane Chief
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
25.0
20.0
15.0
10.0
5.0
0.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
20
40
60
80
100
120
140
160
180
200

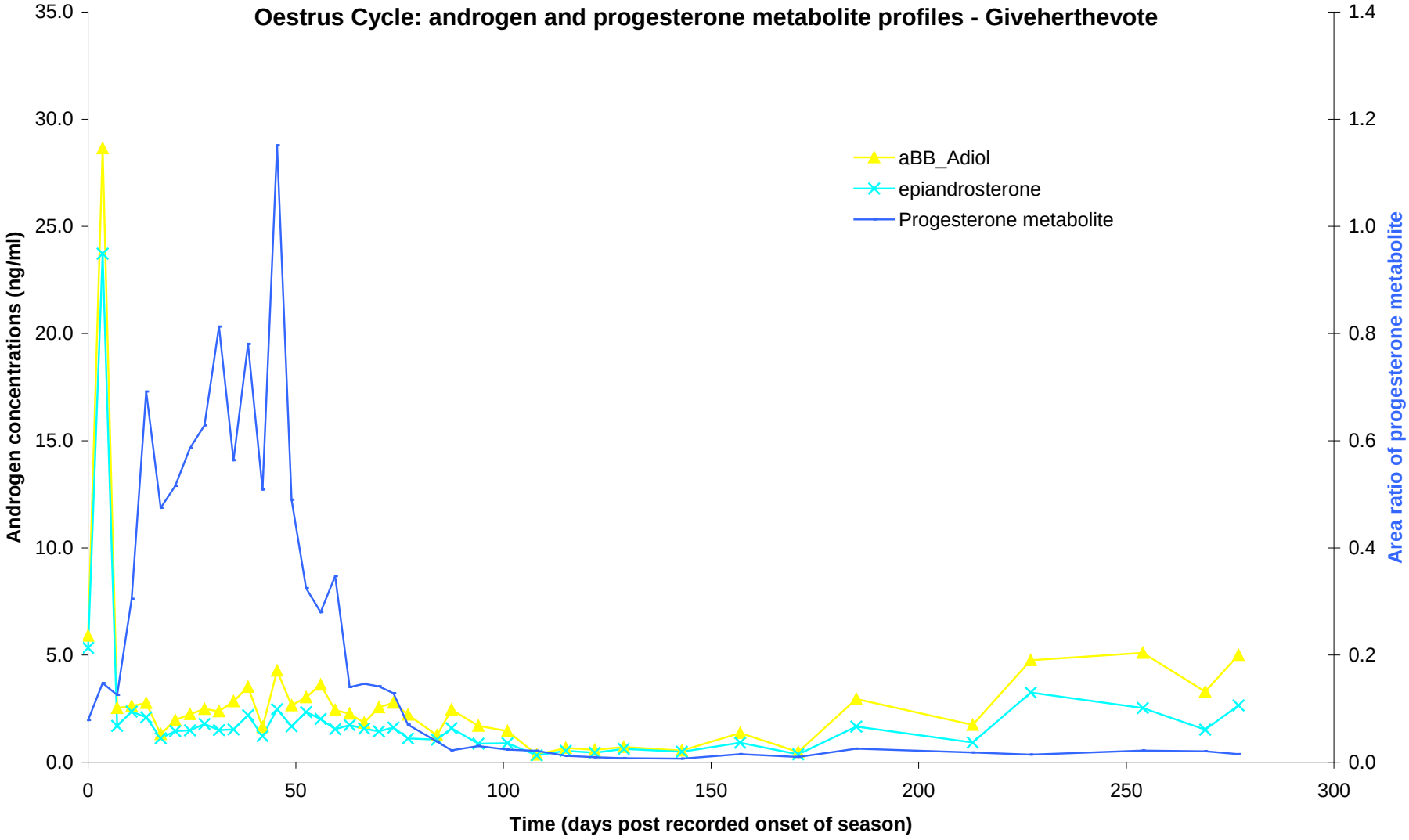

Oestrus Cycle: androgen and progesterone metabolite profiles - Whitefort Euro
Androgen concentrations (ng/ml)
Area ration of progesterone metabolite
Time (days post recorded onset of season)
testosterone
epitestosterone
aBB_Adiol
epiandrosterone
BaB_Adiol
etiocholanolone
Progesterone metabolite
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250
300

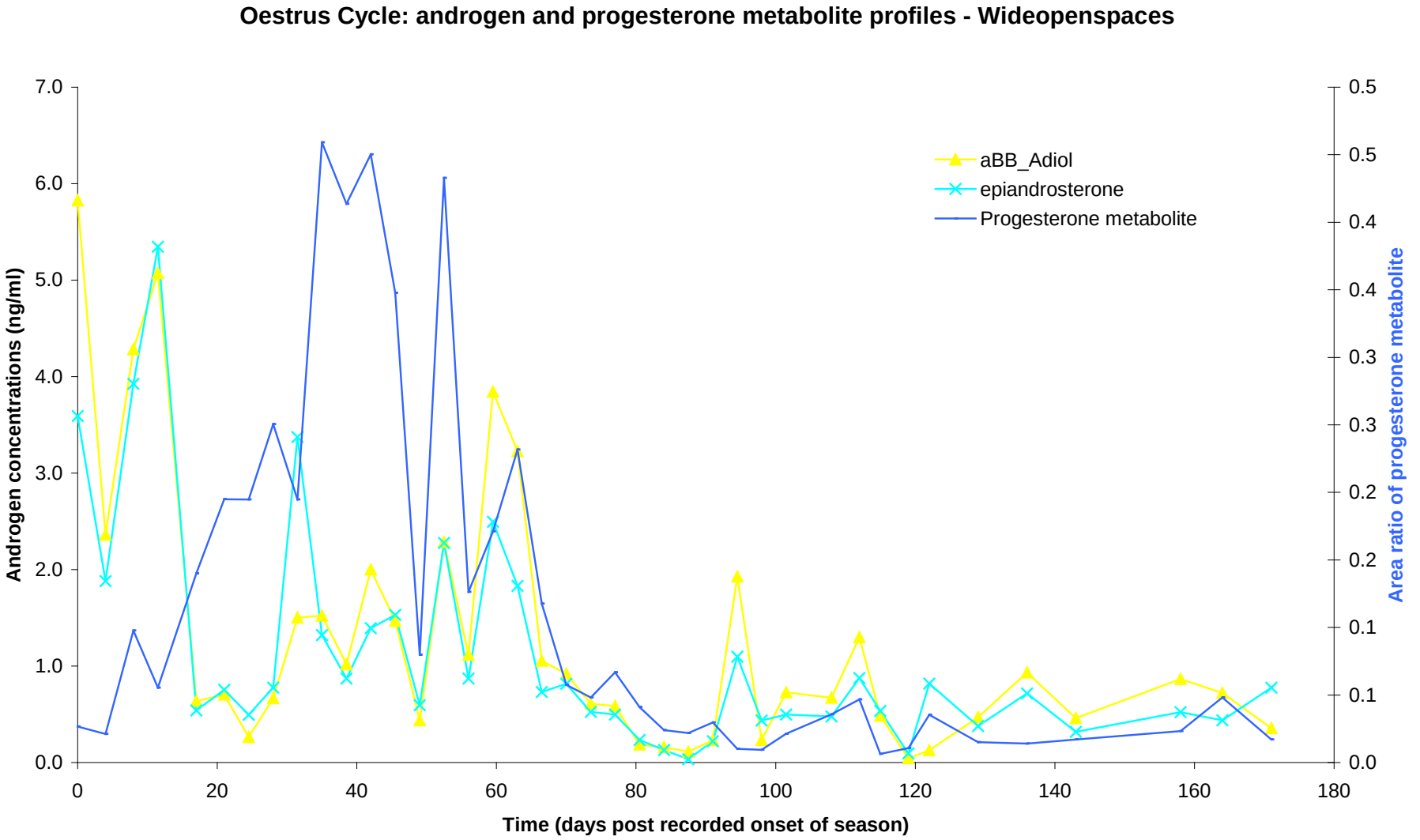

Oestrus Cycle: androgen and progesterone metabolite profiles - Queen of the Bar
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
30.0
25.0
20.0
15.0
10.0
5.0
0.0
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250

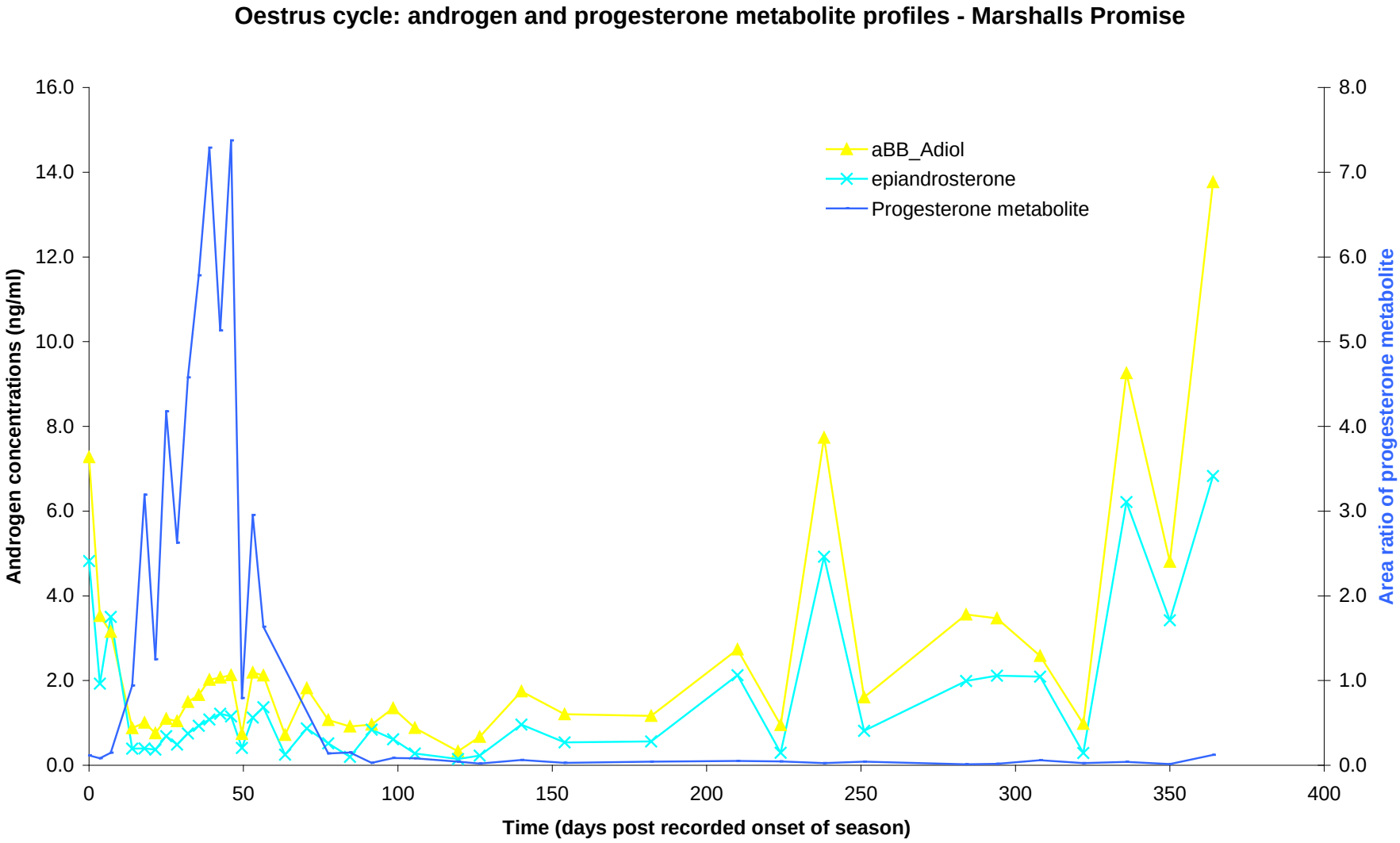

Oestrus cycle: androgen and progesterone profiles - Queen Boadicea
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-50
0
50
100
150
200
250


Oestrus Cycle: androgen and progesterone metabolites - Langies Posh Girl
aBB_Adiol
epiandrosterone
Progesterone metabolite
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
50
100
150
200
250
300
350

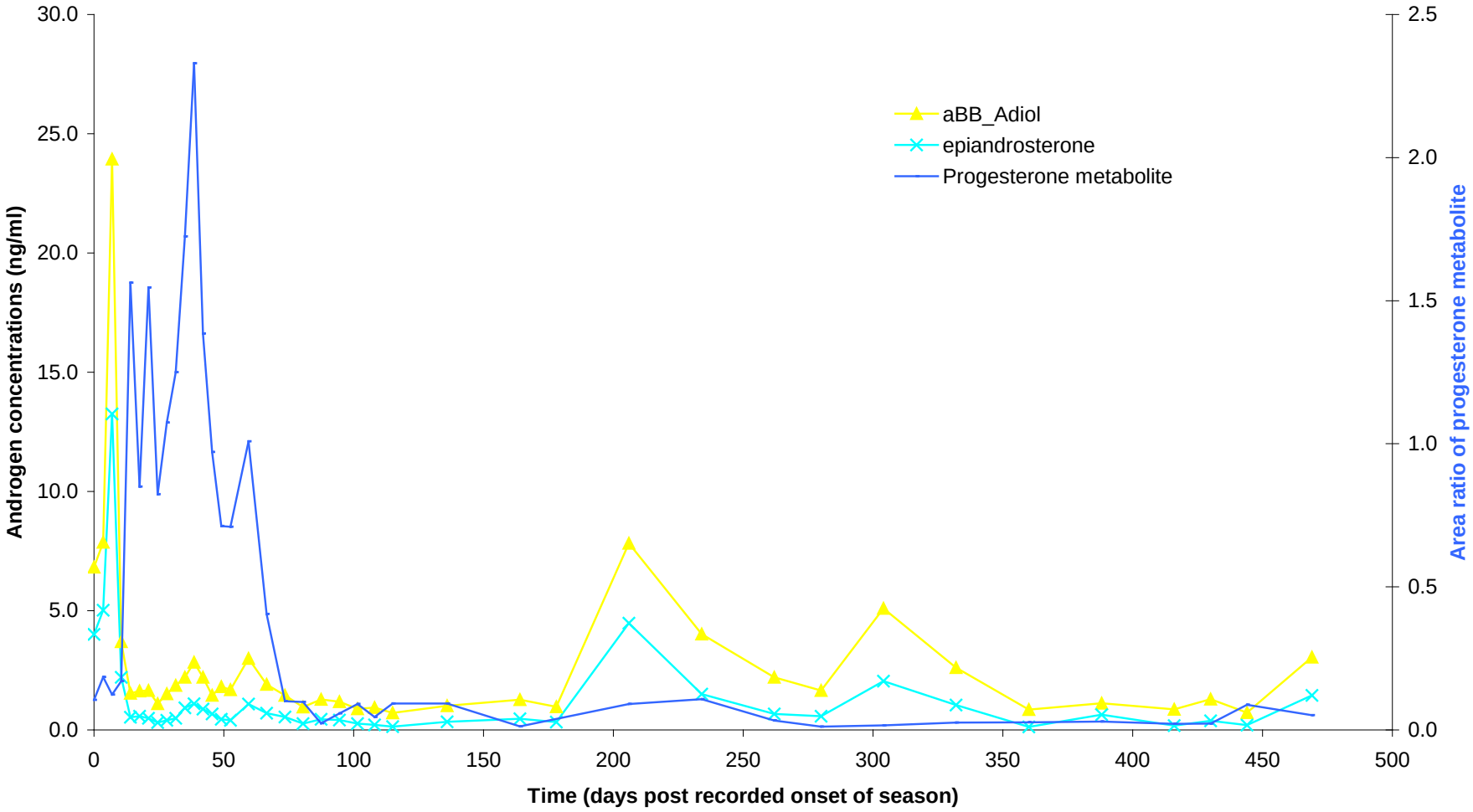

Oestrus Cycle: androgen and progesterone metabolite profiles - Ringlestown Flos
Androgen concentrations (ng/ml)
Area Ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250


Oestrus Cycle: androgen and progesterone metabolite profiles - Giveherthevote
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
300

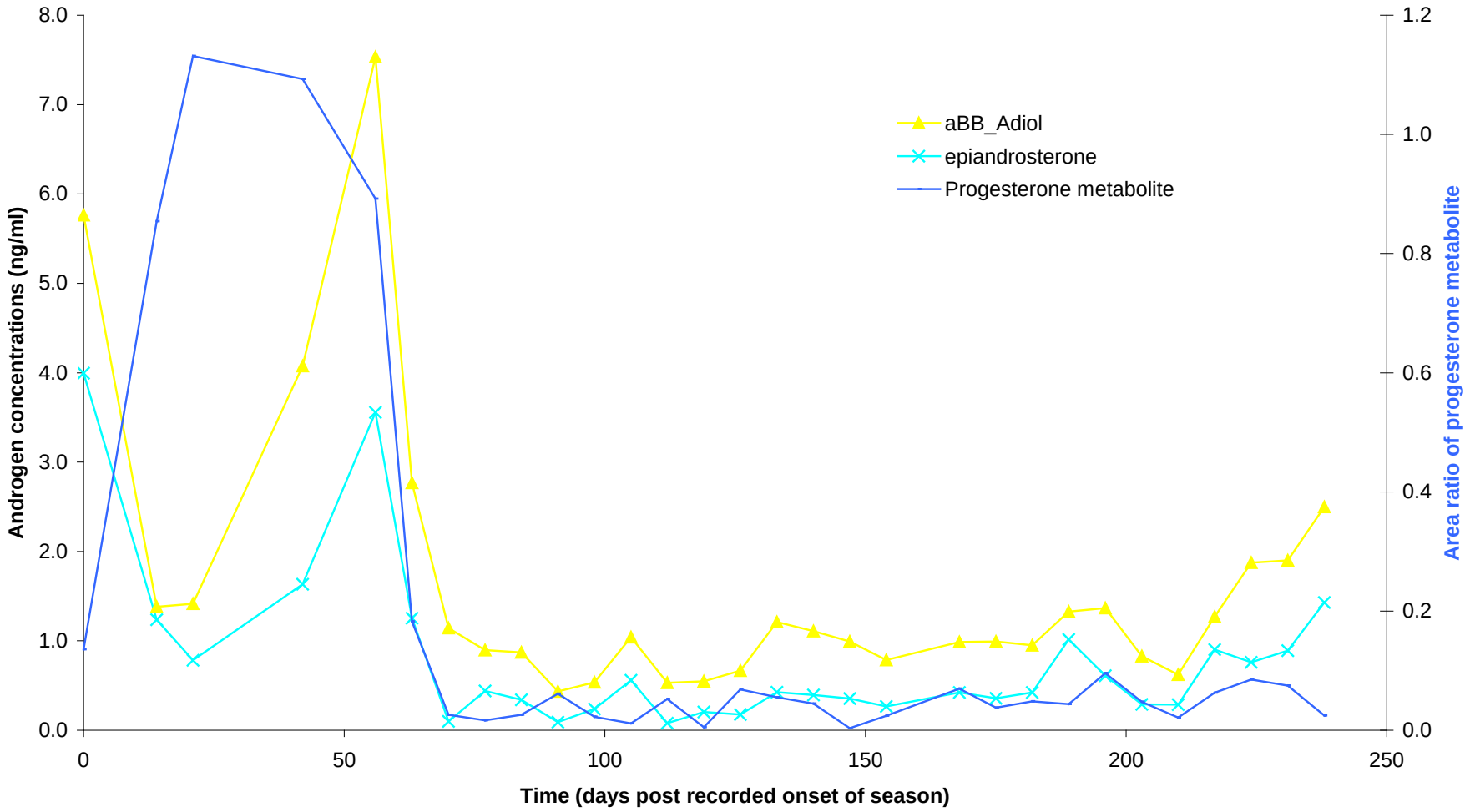

Oestrus Cycle: androgen and progesterone metabolite profiles - Wideopenspaces
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80
100
120
140
160
180

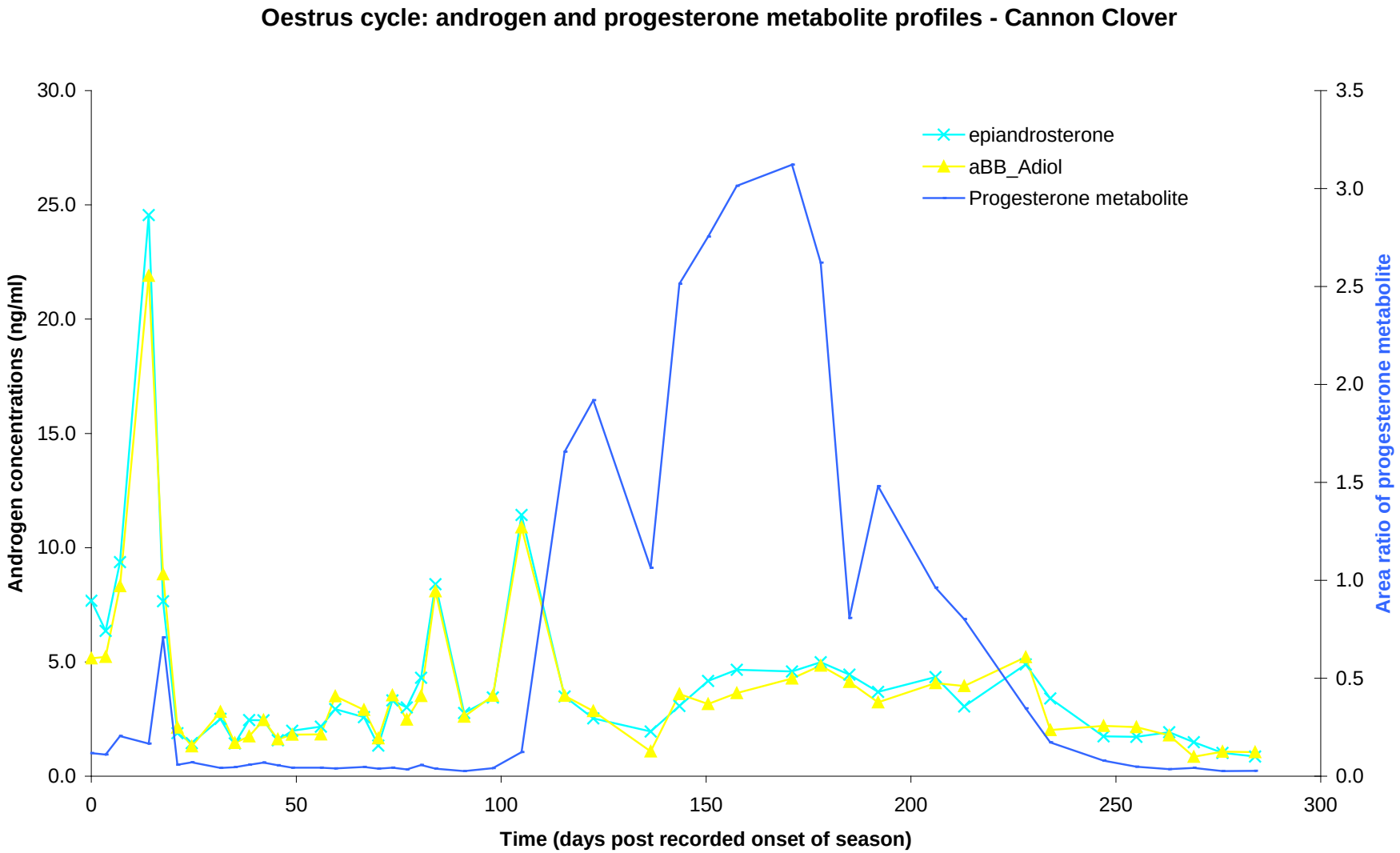

Oestrus cycle: androgen and progesterone metabolite profiles - Marshalls Promise
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
400

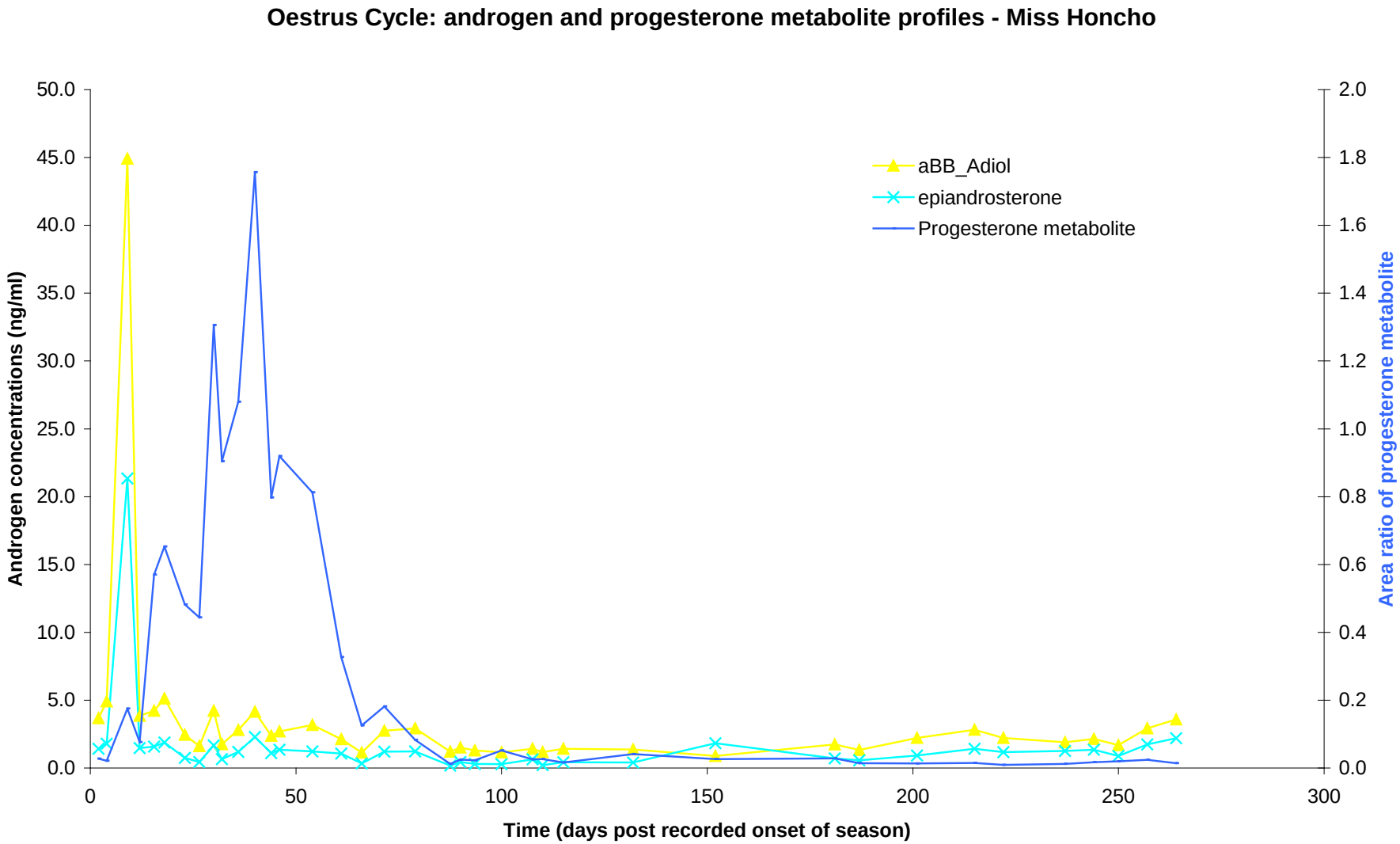
Oestrus Cycle: androgen and progesterone metabolite profiles - Distant Pearl

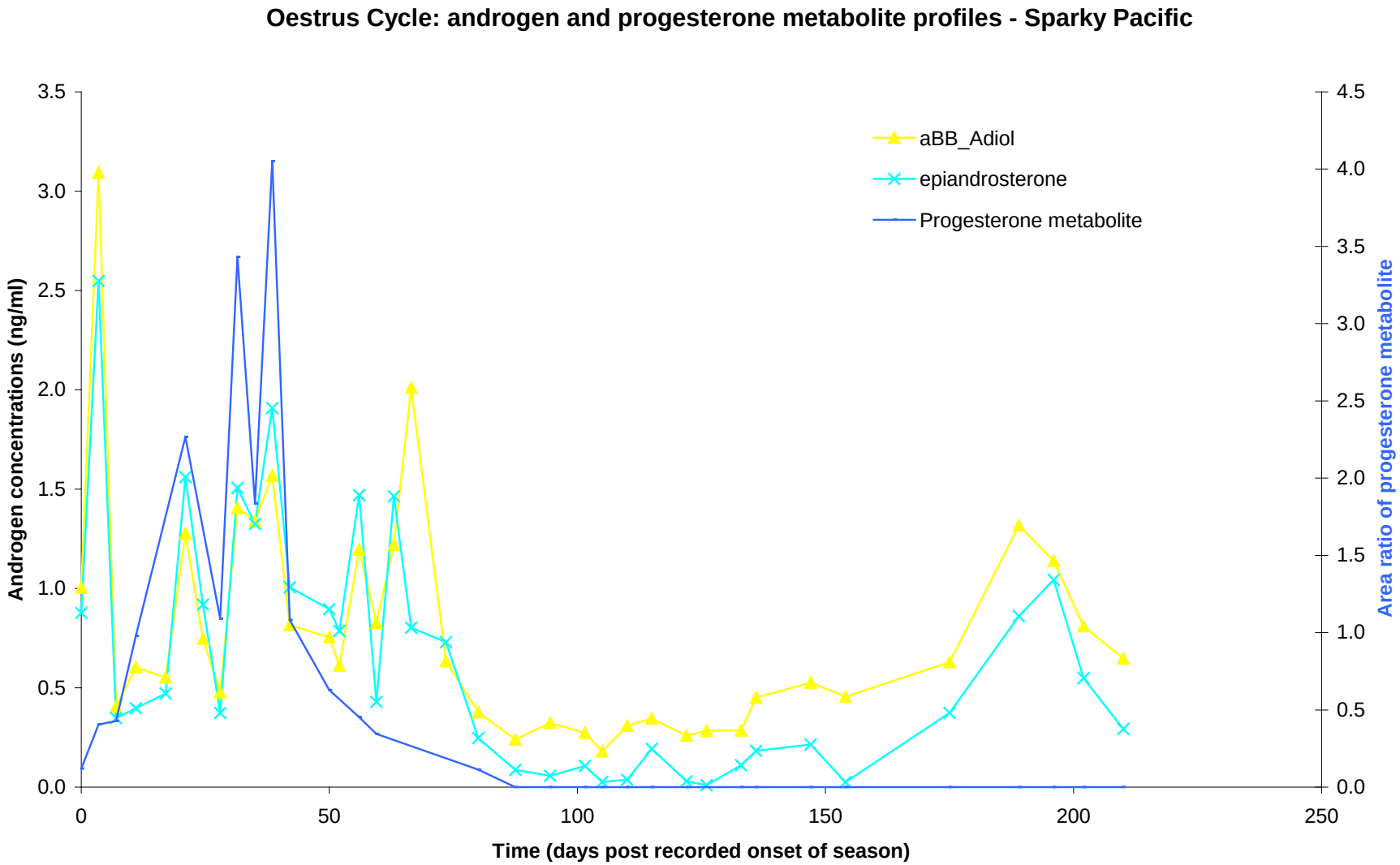


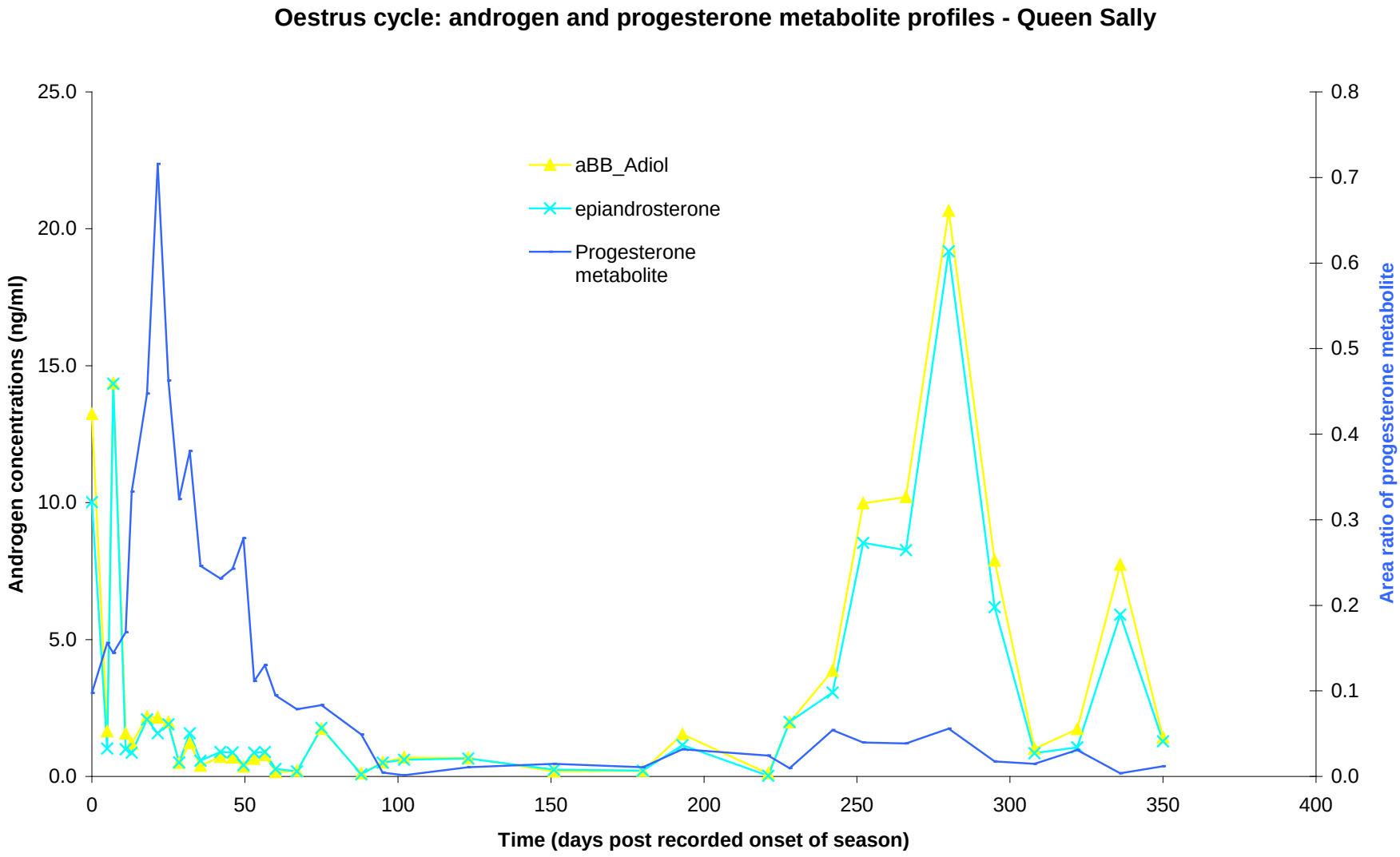
Oestrus Cycle: androgen and progesterone metabolite profiles - Drumpark Access

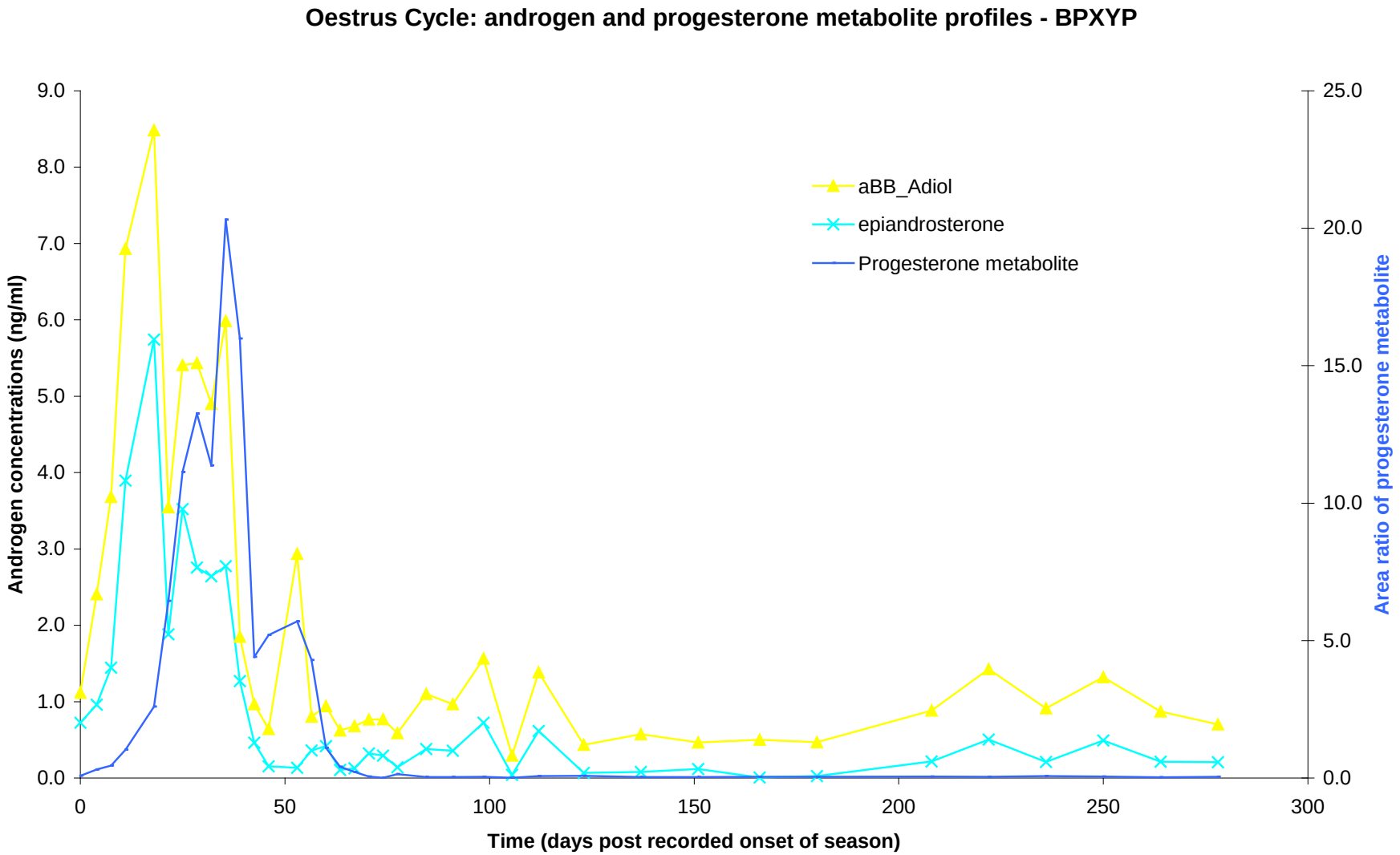



Oestrus cycle: androgen and progesterone metabolite profiles - Cannon Clover
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
epiandrosterone
aBB_Adiol
Progesterone metabolite
30.0
25.0
20.0
15.0
10.0
5.0
0.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300

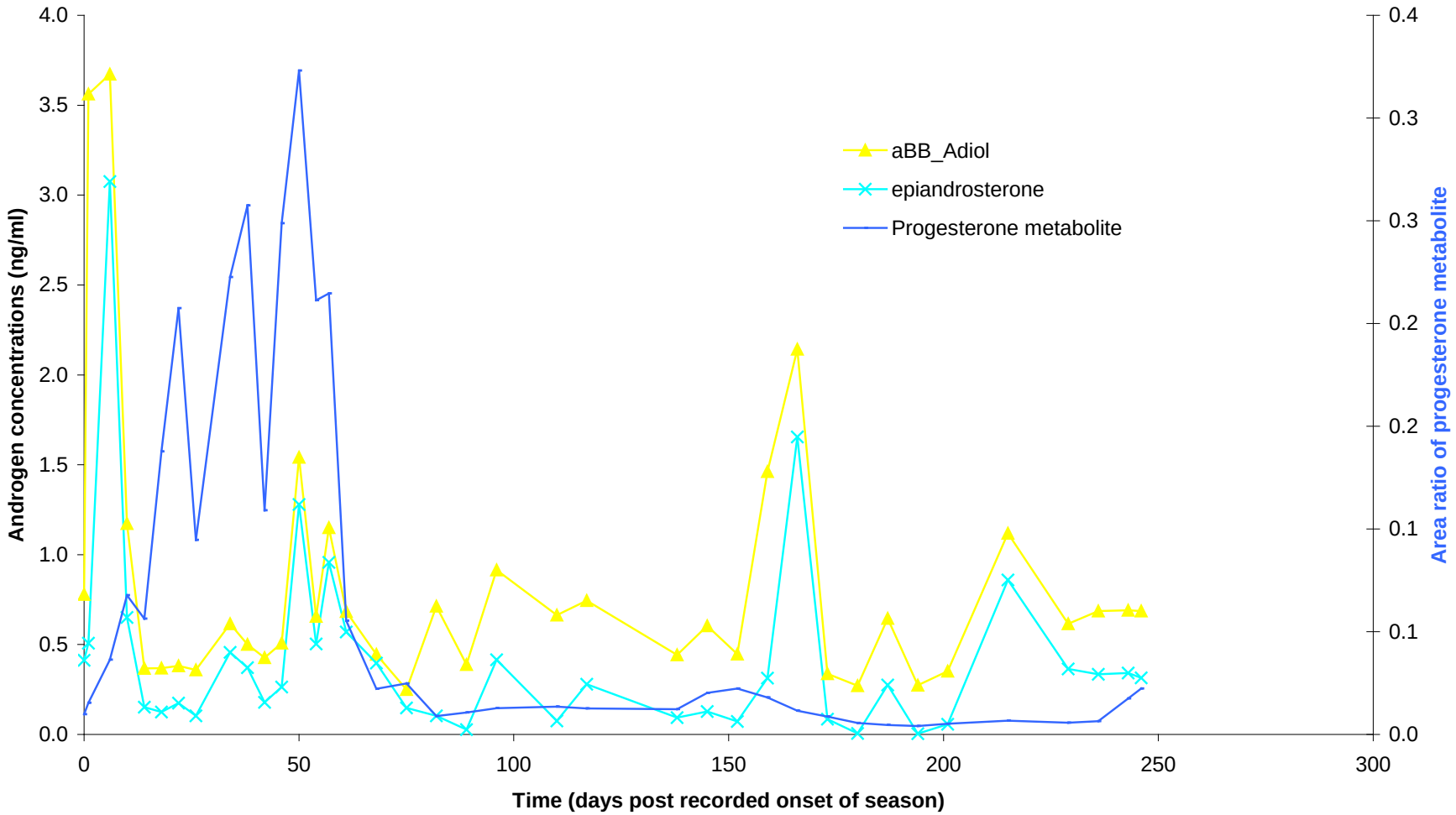

Oestrus Cycle: androgen and progesterone metabolite profiles - Miss Honcho
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
300

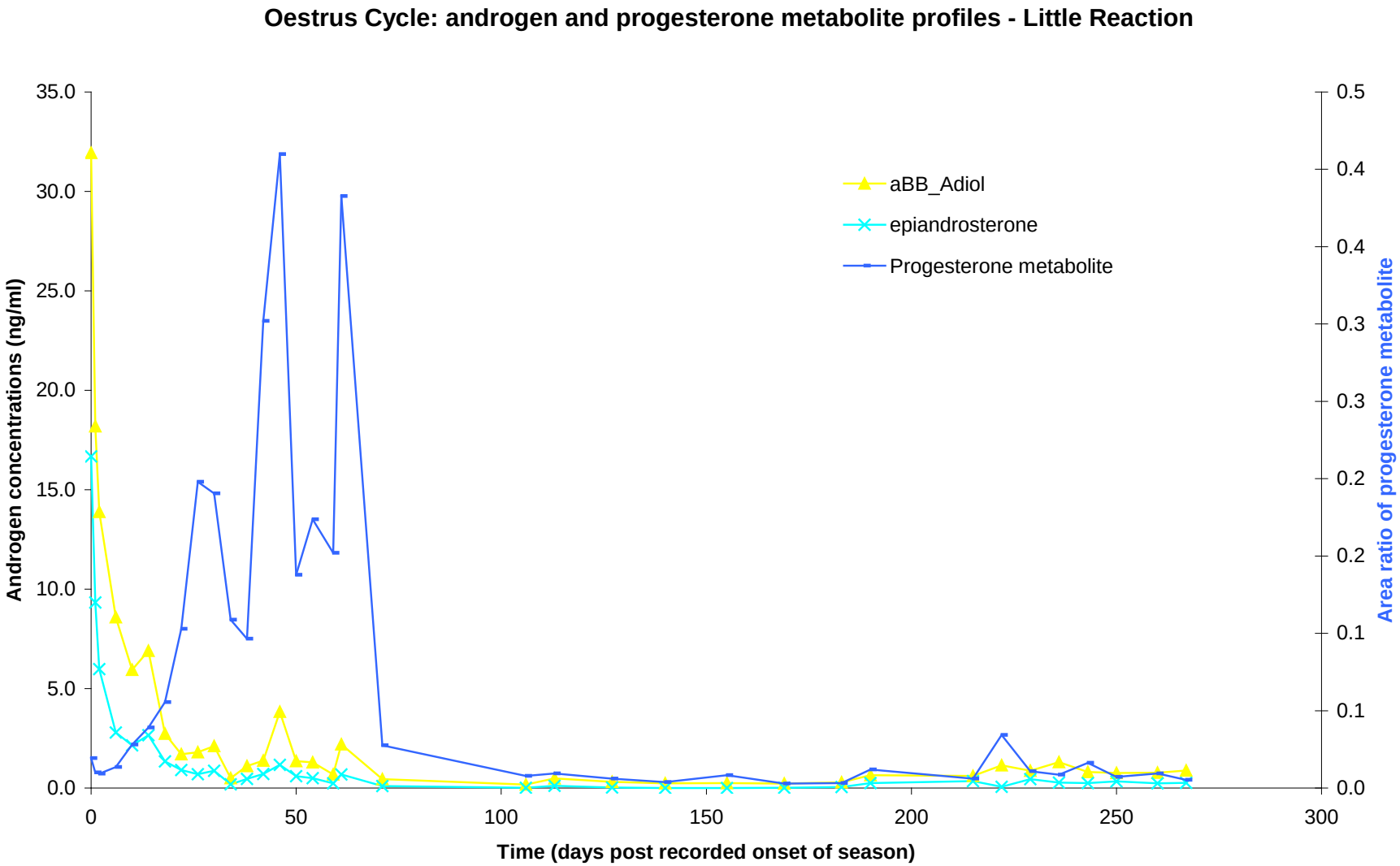

Oestrus Cycle: androgen and progesterone metabolite profiles - Sparky Pacific
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
50
100
150
200
250


Oestrus cycle: androgen and progesterone metabolite profiles - Queen Sally
aBB_Adiol
epiandrosterone
Progesterone metabolite
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
25.0
20.0
15.0
10.0
5.0
0.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
250
300
350
400

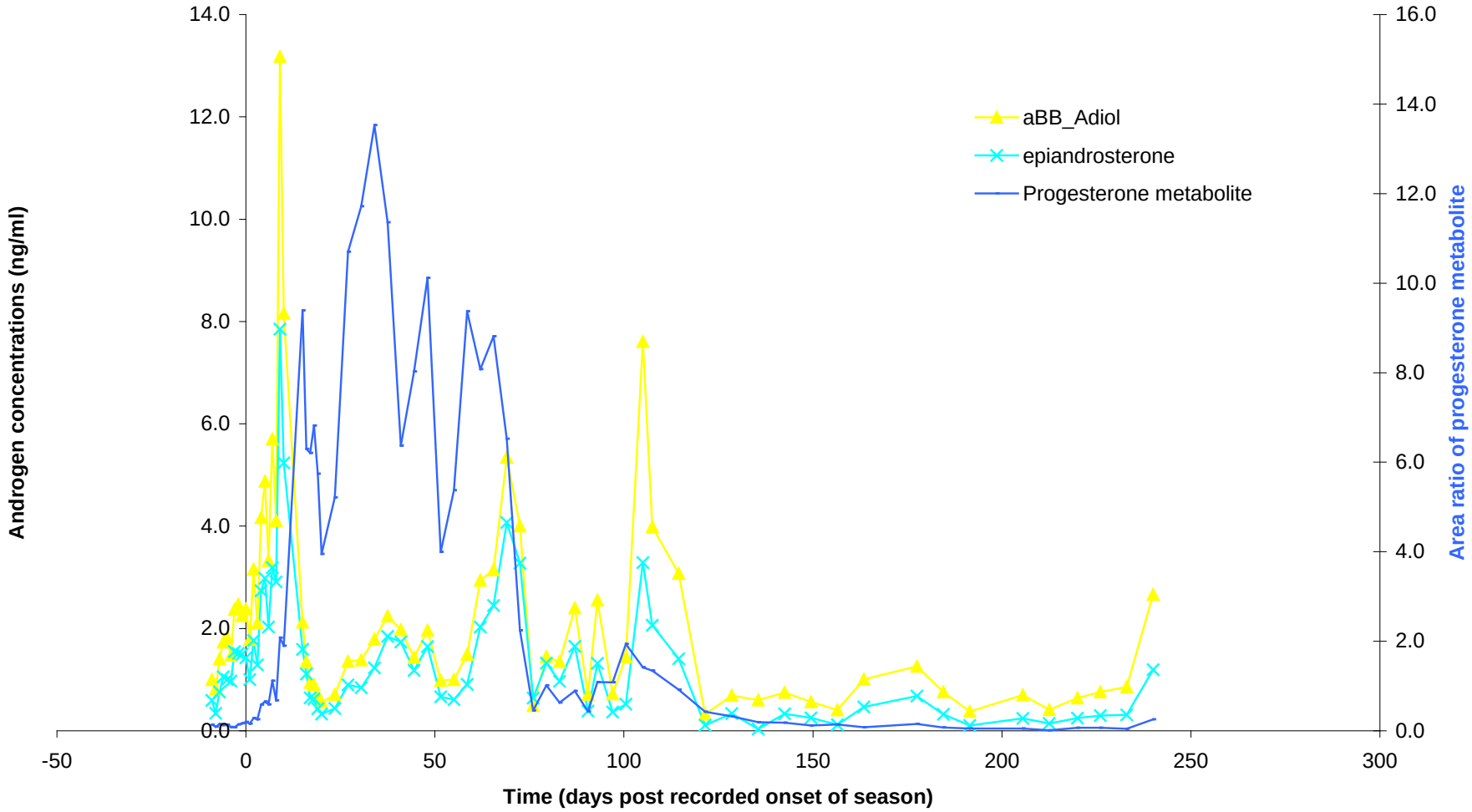

Oestrus Cycle: androgen and progesterone metabolite profiles - BPXYP
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
25.0
20.0
15.0
10.0
5.0
0.0
0
50
100
150
200
250
300

Oestrus Cycle: androgen and progesterone metabolite profiles - Supreme Madonna




Oestrus Cycle: androgen and progesterone metabolite profiles - Little Reaction
Androgen concentrations (ng/ml)
Area ratio of progesterone metabolite
Time (days post recorded onset of season)
aBB_Adiol
epiandrosterone
Progesterone metabolite
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.5
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0.0
0
50
100
150
200
250
300

Oestrus Cycle: androgen and progesterone metabolite profiles - Bolly for Molly - (Pregnant)



APPENDIX VII: RAW DATA FOR OESTRUS CYCLE STEROIDS

- The highlighted results are those which had a repeat analysis

Black Pansy	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
-32	0.045	0.291	0.882	1.301	0.142	0.225	0.103
-31	0.049	0.290	0.847	1.437	0.131	0.237	0.107
-30	0.037	0.310	0.860	1.240	0.160	0.231	0.071
-29	0.027	0.291	0.697	0.877	0.169	0.266	0.073
-28	0.029	0.294	0.963	1.334	0.107	0.264	0.095
-27	0.023	0.311	0.749	0.850	0.166	0.257	0.061
-26	0.038	0.328	0.986	1.308	0.129	0.251	0.071
-25	0.027	0.304	0.899	1.250	0.137	0.241	0.053
-24	0.037	0.319	0.889	1.070	0.153	0.245	0.063
-23	0.035	0.298	0.869	1.146	0.144	0.236	0.064
-22	0.020	0.332	0.607	1.117	0.065	0.245	0.064
-21	0.042	0.287	1.008	1.611	0.165	0.244	0.097
-20	0.032	0.288	0.806	1.081	0.109	0.245	0.065
-19	0.019	0.323	0.962	1.218	0.178	0.250	0.084
-18	0.035	0.295	0.843	1.217	0.118	0.243	0.089
-17	0.045	0.313	0.817	1.242	0.182	0.240	0.083
-16	0.039	0.286	1.131	1.679	0.162	0.264	0.100
-15	0.049	0.290	1.318	1.863	0.194	0.288	0.074
-14	0.054	0.294	0.988	1.440	0.152	0.245	0.067
-13	0.049	0.275	0.770	1.156	0.126	0.233	0.063
-12	0.043	0.275	0.998	1.620	0.196	0.290	0.075
-11	0.034	0.275	0.963	1.476	0.102	0.251	0.067
-10	0.038	0.283	1.213	1.892	0.154	0.263	0.079
-9	0.042	0.271	1.058	1.655	0.164	0.265	0.102
-8	0.033	0.311	1.490	2.467	0.244	0.269	0.122
-7	0.029	0.298	1.162	1.805	0.169	0.260	0.093
-6	0.018	0.262	1.651	2.592	0.195	0.307	0.116
-5	0.020	0.248	1.579	2.349	0.176	0.251	0.178
-4	0.030	0.251	1.966	3.086	0.194	0.263	0.205
-3	0.030	0.248	1.677	2.801	0.216	0.276	0.278
-2	0.052	0.253	2.497	4.285	0.275	0.286	0.427
-1	0.072	0.256	3.593	5.986	0.412	0.357	1.005
0	0.023	0.247	1.967	3.809	0.221	0.295	0.921
1	0.061	0.244	3.099	4.819	0.442	0.372	1.162
3	0.062	0.256	4.448	7.851	0.300	0.402	1.912
4	0.076	0.245	4.714	8.050	0.457	0.355	1.947
5	0.100	0.240	7.929	13.755	0.950	0.474	2.404
6	0.068	0.249	6.380	8.907	1.820	0.380	2.787
7	0.076	0.248	3.957	7.124	0.232	0.365	4.928
8	0.057	0.244	4.519	7.079	0.652	0.356	5.670
9	0.035	0.240	2.229	3.653	0.125	0.330	5.926
10	0.065	0.259	2.948	5.442	0.151	0.337	11.154
11	0.063	0.245	2.778	5.197	0.190	0.332	15.828
12	0.050	0.255	2.369	4.232	0.141	0.280	13.291
13	0.076	0.257	2.403	4.232	0.343	0.330	16.928

14	0.048	0.252	1.587	2.458	0.192	0.273	16.770
15	0.074	0.249	2.066	2.578	2.150	0.355	19.770
16	0.077	0.248	2.211	3.705	0.261	0.395	22.366
17	0.055	0.248	1.064	1.891	0.214	0.258	20.845
18	0.048	0.250	1.101	1.959	0.188	0.282	17.875
19	0.056	0.248	1.097	1.834	0.290	0.279	17.225
20	0.029	0.247	0.806	1.349	0.152	0.268	14.513
21	0.068	0.247	1.035	1.526	0.840	0.265	22.861
22	0.070	0.250	1.729	2.496	0.781	0.308	38.715
23	0.075	0.247	1.323	2.471	0.025	0.250	36.383
24	0.080	0.251	1.330	2.363	0.128	0.275	43.702
25	0.076	0.248	1.184	2.145	0.173	0.269	41.945

Black Pansy	testosterone	aBB_Adiol	epiandro	BaB_Adiol	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
2	0.176	12.606	9.156	0.377	3.778
6	0.239	25.717	23.500	0.664	7.592
10	0.136	5.200	5.111	0.144	20.274
13	0.184	5.813	5.549	0.269	35.613
17	0.156	3.407	2.491	0.183	36.288
20	0.203	2.892	2.348	0.168	47.067
23	0.156	2.443	2.183	0.157	57.624
28	0.229	4.508	3.205	0.286	96.004
31	0.175	1.860	1.681	0.152	N/A
34	0.242	4.048	3.450	0.193	90.536
38	0.271	3.000	3.000	0.141	62.369
41	0.345	1.803	2.407	0.102	N/A
45	0.268	2.292	2.053	0.107	44.997
48	0.307	3.755	2.939	0.185	59.307
52	0.248	3.146	2.716	0.176	39.683
55	0.284	3.495	2.914	0.122	45.578
59	0.296	2.570	2.527	0.143	29.935
62	0.298	3.974	3.398	0.148	41.280
66	0.315	3.845	3.242	0.207	28.279
69	0.287	3.304	2.539	0.164	23.851
73	0.425	3.780	3.254	0.168	28.770
76	0.309	2.974	2.813	0.049	24.573
81	0.404	2.955	2.232	0.186	24.366
84	0.373	3.463	3.310	0.149	21.878
87	0.387	2.884	2.472	0.146	10.654
90	0.369	2.392	2.273	0.161	6.086
94	0.338	1.119	1.062	0.156	1.755
97	0.331	1.396	1.212	0.126	2.068
102	0.380	0.919	0.897	0.124	0.887
104	0.370	1.304	1.135	0.260	1.354
111	0.398	1.326	1.129	0.092	0.995
114	0.423	1.035	1.029	0.191	0.710
122	0.440	1.129	1.099	0.155	0.748
130	0.465	1.242	1.342	0.082	0.656
143	0.438	1.272	1.252	0.175	0.840
150	0.507	1.234	1.232	0.136	0.750
157	0.496	1.104	1.040	0.044	0.581
165	0.534	0.973	1.112	0.178	0.687
171	0.490	0.897	0.873	0.209	0.576
178	0.556	1.454	1.493	0.134	0.723
185	0.545	0.967	1.522	0.251	0.337
192	0.517	1.392	1.386	0.260	0.492
200	0.605	1.282	1.422	0.154	0.444
206	0.613	1.845	2.138	0.196	0.606
213	0.575	2.513	2.677	0.202	0.587

Queen of Stout	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.005	0.066	1.692	1.645	0.119	0.062	1.598
4	0.045	0.104	5.156	3.284	0.333	0.297	10.319
8	0.334	0.392	46.743	28.729	3.244	1.107	38.799
12	0.055	0.146	3.447	2.068	0.155	0.193	32.346
16	0.029	0.123	2.349	1.723	0.307	0.321	47.467
19	0.055	0.143	2.252	1.672	0.072	0.288	34.606
26	0.052	0.131	1.941	1.456	0.083	0.195	72.221
29	0.051	0.122	1.403	1.074	0.180	0.173	51.223
33	0.055	0.123	2.340	1.563	0.189	0.228	70.238
37	0.037	0.107	1.690	1.215	0.111	0.173	57.801
39	0.063	0.125	2.498	1.459	0.238	0.395	63.904
43	0.069	0.137	2.378	1.445	0.219	0.392	53.847
47	0.047	0.120	1.832	1.272	0.076	0.210	24.507
49	0.067	0.138	3.029	2.030	0.170	0.302	34.806
53	0.067	0.116	1.996	1.335	0.116	0.506	13.445
57	0.055	0.106	2.199	1.386	0.084	0.368	18.551
61	0.064	0.116	2.326	1.437	0.122	0.363	17.247
65	0.087	0.169	2.001	1.434	0.160	0.580	6.859
67	0.049	0.111	1.530	1.199	0.070	0.296	6.260
71	0.030	0.092	1.156	0.730	0.069	0.219	4.190
75	0.042	0.118	1.227	0.720	0.099	0.148	5.042
78	0.041	0.160	1.308	0.756	0.082	0.155	3.802
82	0.037	0.119	0.960	0.529	0.080	0.076	2.242
89	0.022	0.085	0.988	0.586	0.065	0.070	1.035
96	0.052	0.164	1.508	0.898	0.148	0.131	2.204
102	0.030	0.162	0.741	0.418	0.095	0.056	0.655
108	0.036	0.146	0.909	0.597	0.023	0.054	1.300
123	0.033	0.125	1.401	0.951	0.119	0.076	0.662
130	0.099	0.020	2.262	1.675	0.073	0.089	1.392
144	0.034	0.123	1.804	1.765	0.092	0.096	1.385
151	0.022	0.103	1.117	1.126	0.106	0.098	0.933
158	0.019	0.091	1.130	1.131	0.096	0.060	0.793
172	0.045	0.153	2.489	2.426	0.163	0.137	1.941
179	0.022	0.103	1.945	1.687	0.050	0.070	1.229
186	0.046	0.114	2.348	1.569	0.117	0.075	0.966
200	0.023	0.116	1.663	0.981	0.068	0.069	1.777
207	0.025	0.090	1.638	0.945	0.068	0.055	1.550
221	0.040	0.137	3.065	2.314	0.192	0.142	3.759
228	0.024	0.104	2.110	1.104	0.063	0.112	2.242

Suitable Choice	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.001	0.071	0.586	0.680	0.012	0.019	0.225
3	0.030	0.218	2.563	1.921	0.130	0.064	2.305
7	0.032	0.079	5.255	6.518	0.384	0.129	2.325
11	0.064	0.291	2.032	1.606	0.123	0.095	12.850
14	0.081	0.298	2.351	2.414	0.069	0.152	44.166
18	0.115	0.614	2.289	2.117	0.173	0.169	59.366
25	0.123	0.381	2.441	2.124	0.156	0.174	72.613
29	0.063	0.131	0.857	0.801	0.051	0.047	31.493
31	0.139	0.376	2.171	2.088	0.137	0.190	70.239
39	0.215	0.477	2.191	4.019	0.204	0.160	60.793
42	0.091	0.246	1.244	1.532	0.081	0.119	22.975
46	0.174	0.355	2.080	2.392	0.212	0.215	36.793
49	0.106	0.368	1.896	2.675	0.194	0.169	26.700
53	0.122	0.575	1.788	1.896	0.148	0.126	18.777
56	0.105	0.429	1.818	2.803	0.383	0.179	13.669
60	0.110	0.530	1.471	1.653	0.168	0.139	7.808
63	0.173	0.816	2.091	2.374	0.359	0.175	5.612
67	0.082	0.419	1.045	1.228	0.125	0.116	2.152
70	0.111	0.454	1.173	1.645	0.219	0.174	2.083
74	0.053	0.320	0.707	0.798	0.097	0.079	0.592
78	0.040	0.180	0.661	0.923	0.129	0.113	0.450
81	0.042	0.534	0.802	0.823	0.067	0.091	0.579
88	0.016	0.379	0.457	0.556	0.099	0.045	0.249
95	0.053	0.535	0.896	1.034	0.360	0.102	0.391
101	0.017	0.196	0.393	0.482	0.067	0.032	0.177
109	0.030	0.362	0.671	0.634	0.059	0.061	0.198
111	0.053	0.456	0.791	0.817	0.093	0.072	0.174
115	0.033	0.305	0.670	0.746	0.067	0.054	0.128
122	0.012	0.265	0.520	0.507	0.027	0.031	0.110
136	0.026	0.337	0.577	0.535	0.122	0.066	0.177
150	0.005	0.120	0.461	0.807	0.088	0.015	0.100
157	0.033	0.434	0.985	1.009	0.118	0.070	0.362
164	0.043	0.386	1.338	1.152	0.150	0.079	0.255
178	0.043	0.201	0.712	1.154	0.073	0.079	0.081
192	0.028	0.368	1.026	0.964	0.089	0.064	0.169
199	0.028	0.266	1.267	1.094	0.079	0.054	0.196
206	0.014	0.241	1.387	1.175	0.045	0.044	0.187
213	0.034	0.283	2.210	1.842	0.113	0.118	1.117

Full Nosey (Daisy)	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.061	NA	6.266	7.448	0.559	0.403	4.382
4	0.063	0.087	4.365	2.283	0.380	0.319	16.019
8	0.080	NA	7.752	4.613	0.783	0.388	16.068
11	0.156	0.133	23.937	12.507	1.717	0.633	27.150
15	0.065	0.068	2.788	3.157	0.100	0.182	29.803
17	0.097	0.166	5.394	6.473	0.321	0.588	66.409
22	0.061	0.138	2.911	3.735	0.207	0.312	67.655
24	0.076	0.137	2.864	2.912	0.102	0.261	59.233
28	0.091	0.149	3.958	4.898	0.237	0.350	99.582
32	0.054	NA	1.553	2.079	0.050	0.283	60.353
35	0.059	NA	3.212	3.989	0.140	0.252	112.835
39	0.047	NA	2.416	2.827	0.097	0.207	74.931
42	0.068	0.047	4.658	5.448	0.244	0.409	135.354
46	0.121	0.049	3.487	3.685	0.175	0.295	116.912
49	0.096	0.056	3.873	4.096	0.121	0.385	128.044
53	0.100	0.076	3.997	4.001	0.108	0.452	102.564
56	0.127	0.086	4.355	4.578	0.145	0.422	94.222
60	0.102	0.087	4.774	5.526	0.103	0.531	100.932
63	0.136	0.091	5.229	5.120	0.116	0.384	90.446
70	0.084	0.081	4.242	4.239	0.412	0.470	45.356
74	0.126	0.073	4.052	3.626	0.352	0.435	36.426
77	0.113	0.079	5.975	5.383	0.380	0.465	30.256
81	0.069	0.031	2.003	1.443	0.081	0.093	12.463
84	0.092	0.091	5.010	4.399	0.130	0.442	25.551
88	0.158	0.083	3.797	3.931	0.466	0.529	23.341
95	0.096	0.055	2.892	3.220	0.140	0.304	15.033
102	0.089	0.055	2.141	2.146	0.216	0.178	11.184
115	0.085	0.096	1.749	1.341	0.232	0.206	7.199
129	0.061	NA	1.678	1.446	0.190	0.122	2.929
143	0.052	0.049	0.910	0.524	0.051	0.074	1.110
157	0.043	NA	1.190	0.849	0.081	0.058	1.093
178	0.038	NA	1.336	0.403	0.043	0.036	0.446
192	0.057	0.073	1.675	1.572	0.194	0.112	1.141
206	0.049	NA	1.405	0.806	0.081	0.046	0.908
220	0.057	NA	1.887	1.502	0.067	0.100	1.288
234	0.069	NA	2.327	1.424	0.067	0.165	1.384
249	0.043	NA	1.692	1.419	0.082	0.105	1.231
262	0.054	0.077	1.710	1.412	0.139	0.052	0.960
276	0.056	0.086	1.600	1.504	0.067	0.046	1.268
290	0.056	0.115	3.384	3.447	0.160	0.113	2.755
296	0.077	0.093	7.625	8.291	0.424	0.203	6.836

Chop & Change	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
-13	0.081	0.087	9.806	6.523	0.490	0.623	3.579
-6	0.067	0.076	10.183	6.595	0.344	0.755	4.201
0	0.037	0.048	4.458	2.286	0.149	0.206	3.196
4	0.098	0.074	28.640	17.269	0.954	1.068	26.395
7	0.080	0.084	13.939	8.988	0.516	0.694	30.862
11	0.069	0.088	7.307	5.309	0.157	0.456	32.308
15	0.076	0.084	6.720	5.037	0.261	0.638	40.783
17	0.138	0.092	9.957	6.326	0.200	0.729	37.297
21	0.182	0.111	14.305	8.704	0.369	0.849	77.762
25	0.169	0.081	14.492	8.395	0.251	0.725	62.985
28	0.162	0.095	13.992	8.684	0.288	0.840	63.467
32	0.162	0.116	15.209	8.832	0.417	1.036	66.537
35	0.215	0.132	25.344	14.303	0.558	1.216	109.602
39	0.215	0.145	23.277	13.804	0.458	1.326	110.028
42	0.195	0.132	14.874	7.939	0.352	1.176	86.804
46	0.183	0.108	12.856	7.647	0.208	0.629	82.629
50	0.133	0.049	4.851	2.244	0.062	0.405	21.872
52	0.178	0.083	13.628	7.221	0.191	0.541	66.602
56	0.149	0.083	9.984	5.512	0.220	0.517	44.083
60	0.174	0.090	13.106	6.562	0.182	0.867	39.821
63	0.141	0.072	7.428	3.938	0.147	0.547	18.464
67	0.158	0.079	7.635	4.369	0.093	0.620	16.390
70	0.159	0.206	8.973	4.385	0.148	0.795	19.148
74	0.156	0.084	10.937	5.238	0.253	1.051	22.083
80	0.127	0.050	4.283	2.007	0.054	0.384	6.585
88	0.120	0.059	3.612	1.596	0.068	0.288	3.243
95	0.142	0.054	4.257	1.645	0.044	0.319	2.521
108	0.133	0.054	4.105	1.758	0.071	0.321	1.302
122	0.115	0.060	4.986	2.089	0.069	0.395	1.696
136	0.140	0.050	3.918	1.713	0.117	0.312	1.189
150	0.137	0.057	4.902	1.737	0.091	0.330	1.500
164	0.126	0.134	4.527	1.917	0.095	0.340	1.753
178	0.117	0.117	2.727	1.245	0.140	0.154	0.649
192	0.125	0.099	4.363	1.710	0.071	0.325	1.191
206	0.135	0.116	7.468	3.436	0.114	0.559	2.229
220	0.130	0.077	5.627	2.460	0.125	0.403	1.648
227	0.139	0.082	6.457	3.535	0.140	0.503	1.729
234	0.145	0.070	8.642	4.011	0.161	0.504	2.005
241	0.152	0.066	9.428	4.927	0.137	0.670	3.837

Whinfell Nikita	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.116	0.074	4.192	3.340	0.149	0.368	0.517
4	0.191	0.138	17.190	13.326	11.279	3.917	0.431
7	0.152	0.081	16.362	14.086	2.264	0.623	0.722
11	0.165	0.095	13.375	12.202	0.744	0.286	1.692
14	0.153	0.067	4.203	4.130	0.696	0.244	2.143
18	0.107	NA	2.676	2.781	0.110	0.338	1.909
21	0.153	0.140	1.948	1.636	0.227	0.177	1.611
25	0.135	0.069	1.777	1.734	0.154	0.121	1.779
28	0.126	0.055	1.713	1.820	0.138	0.112	2.001
32	0.188	0.139	2.699	2.518	0.291	0.231	2.285
35	0.182	0.065	2.494	2.882	2.548	0.169	2.771
39	0.107	0.040	2.388	2.485	0.097	0.189	2.290
42	0.235	0.072	4.244	4.299	0.149	0.163	3.712
49	0.200	0.067	4.148	4.447	0.088	0.197	2.556
53	0.004	NA	0.462	0.313	0.004	0.035	0.090
56	0.204	0.060	4.827	6.255	0.129	0.173	1.836
60	0.250	0.097	4.380	3.504	0.228	0.260	1.368
67	0.209	0.065	5.649	5.008	0.196	0.245	2.189
74	0.011	0.038	1.033	1.196	0.005	0.088	0.318
81	0.156	0.173	12.399	11.940	2.635	2.372	1.333
88	0.141	0.078	3.450	2.813	0.057	0.123	1.253
91	0.187	0.100	2.626	2.073	0.070	0.136	0.763
95	0.124	0.060	1.288	1.110	0.008	0.078	0.510
102	0.117	0.060	0.968	0.972	0.036	0.072	0.114
108	0.096	0.091	1.450	1.326	0.099	0.183	0.073
122	0.078	0.117	0.944	0.938	0.092	0.088	0.046
129	0.156	0.188	18.989	21.619	7.744	3.012	0.041
136	0.074	0.090	0.923	0.660	0.072	0.082	0.064
150	0.111	0.066	2.564	1.799	0.203	0.061	0.066
157	0.029	0.044	0.476	0.393	0.057	0.026	0.032
164	0.142	0.135	0.893	0.673	1.945	0.261	0.070
171	0.147	0.096	1.205	0.709	0.559	0.162	0.067
178	0.085	0.042	1.007	1.036	0.059	0.050	0.098
185	0.076	0.068	0.763	0.836	0.040	0.093	0.060
192	0.136	0.180	1.480	1.428	0.116	0.094	0.074
199	0.103	0.074	1.354	1.370	0.040	0.104	0.073
206	0.095	0.120	1.399	1.240	0.040	0.138	0.110
213	0.076	0.080	1.806	1.889	0.021	0.150	0.141
220	0.124	0.179	2.578	2.513	0.163	0.123	0.083
234	0.091	0.177	4.473	4.674	0.226	0.207	0.184

tonightsthe night	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.098	0.125	6.463	4.585	0.242	0.740	0.847
4	0.063	0.088	6.399	4.724	0.493	0.224	1.116
8	0.049	0.125	5.614	3.946	0.414	0.194	1.507
10	0.057	0.099	5.341	4.462	0.362	0.210	2.188
14	0.038	0.055	7.886	4.612	0.787	0.249	1.865
18	0.042	0.042	2.568	2.267	0.092	0.156	1.553
21	0.079	0.087	3.806	2.792	0.403	0.262	3.308
28	0.047	0.038	2.270	1.595	0.138	0.172	2.320
32	0.056	0.050	3.082	2.183	0.261	0.215	3.359
35	0.210	0.063	5.177	4.622	0.496	0.449	5.344
39	0.052	0.034	2.600	2.450	0.231	0.176	2.681
42	0.024	0.040	1.656	1.527	0.225	0.170	1.618
46	0.093	0.051	2.795	2.522	0.298	0.262	2.846
49	0.092	0.089	2.908	2.374	0.277	0.303	3.492
53	0.076	0.046	3.473	3.006	0.229	0.244	1.681
56	0.062	0.046	1.590	1.388	0.123	0.130	1.281
60	0.053	0.058	2.910	2.226	0.252	0.301	1.684
67	0.066	0.067	2.466	1.822	0.236	0.259	0.925
70	0.096	0.071	2.762	2.353	0.254	0.492	0.801
74	0.048	0.053	1.599	1.316	0.130	0.224	0.504
77	0.069	0.077	2.640	1.890	0.244	0.235	0.745
84	0.015	NA	0.502	0.454	0.011	0.120	0.141
91	0.079	0.163	1.716	0.892	0.226	0.198	0.306
98	0.045	0.059	1.429	0.999	0.178	0.130	0.143
105	0.044	0.118	1.919	1.453	0.275	0.252	0.168
112	0.029	0.045	1.311	1.077	0.146	0.115	0.095
123	0.050	0.063	1.252	0.803	0.059	0.122	0.144
129	0.042	0.064	1.908	1.461	0.136	0.059	0.129
143	0.044	0.102	1.205	0.817	0.186	0.174	0.107
157	0.035	0.098	1.506	1.182	0.242	0.196	0.062
164	0.043	0.034	1.182	0.807	0.103	0.206	0.052
178	0.067	0.133	3.279	2.553	0.442	0.610	0.055
192	0.009	0.025	0.427	0.336	0.047	0.021	0.031
206	0.028	0.047	0.999	0.747	0.104	0.095	0.082
221	0.042	0.067	2.216	1.245	0.149	0.105	0.135
227	0.030	0.064	1.893	1.547	0.185	0.098	0.158
234	0.022	0.053	1.584	1.205	0.120	0.067	0.137
241	0.057	0.107	4.488	3.874	0.473	0.325	0.164
248	0.127	0.057	5.626	7.893	0.676	0.273	0.217
253	0.029	0.054	5.048	4.101	0.500	0.271	0.896

Fair Head	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
1	0.066	0.046	4.795	2.373	0.240	0.096	0.399
4	0.349	0.143	44.551	18.458	5.550	0.540	1.700
8	0.151	0.036	1.434	0.979	0.221	0.102	1.029
11	0.120	0.046	1.443	1.397	0.391	0.265	2.120
15	0.118	NA	1.075	1.385	0.505	0.364	2.644
16	0.174	NA	1.334	1.071	0.321	0.165	3.221
22	0.123	0.038	1.402	1.562	0.642	0.342	3.581
26	0.261	NA	1.797	1.640	0.580	0.486	4.511
29	0.058	0.037	0.832	0.939	0.145	0.165	0.812
33	0.252	NA	2.207	2.339	0.721	0.911	3.677
36	0.247	0.039	2.455	2.086	0.382	0.492	4.379
40	0.152	NA	2.099	1.797	0.520	0.371	1.865
43	0.107	NA	1.561	2.124	0.268	0.425	1.479
47	0.172	0.032	1.822	1.462	0.184	0.329	1.662
50	0.234	0.061	2.531	2.116	0.312	0.440	1.684
54	0.244	0.041	3.479	2.653	0.244	0.454	1.268
57	0.190	0.034	3.271	2.703	0.183	0.630	1.172
61	0.105	0.032	3.118	2.446	0.251	0.480	1.303
64	0.078	0.033	1.274	1.062	0.178	0.342	0.428
68	0.130	NA	2.424	2.036	0.365	0.574	0.672
71	0.126	0.036	2.801	2.170	0.414	0.538	0.824
75	0.106	0.035	2.343	1.872	0.244	0.455	0.604
82	0.138	0.046	2.133	1.998	0.319	0.375	0.572
89	0.079	NA	0.925	1.212	0.419	0.556	0.135
96	0.065	NA	0.697	0.676	0.257	0.207	0.052
99	0.107	0.037	0.782	0.632	0.309	0.293	0.098
106	0.063	0.041	0.839	0.830	0.353	0.172	0.026
113	0.056	0.029	1.029	0.774	0.319	0.063	0.052
127	0.078	NA	1.213	1.199	0.247	0.204	0.066
141	0.046	NA	0.642	0.793	0.482	0.211	0.034
155	0.063	0.026	0.867	1.249	0.594	0.309	0.036
169	0.079	0.024	0.996	0.835	0.276	0.121	0.036
183	0.077	NA	1.477	1.210	0.228	0.098	0.035
197	0.078	0.028	1.572	1.361	0.273	0.409	0.040
204	0.029	NA	0.801	0.915	0.111	0.110	0.020
211	0.037	0.026	0.747	0.847	0.146	0.134	0.028
218	0.029	0.031	0.906	0.844	0.094	0.127	0.029
225	0.048	NA	0.970	1.263	0.296	0.204	0.042
232	0.038	NA	0.893	0.842	0.210	0.216	0.047
241	0.072	0.046	4.018	3.595	0.387	0.144	0.214

Lochbo Amy	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.025	0.028	2.391	2.828	0.111	0.107	0.209
5	0.080	0.056	8.759	10.053	0.642	0.120	0.568
9	0.059	0.059	7.535	7.797	0.393	0.128	0.811
12	0.097	0.120	4.410	3.795	0.279	0.263	1.064
16	0.060	0.047	2.552	2.093	0.216	0.236	0.998
19	0.028	0.033	1.191	1.038	0.027	0.107	0.410
23	0.084	0.035	3.661	3.013	0.377	0.254	1.192
26	0.017	0.028	0.517	0.641	0.015	0.062	0.206
30	0.022	0.028	0.849	0.971	0.024	0.059	0.361
33	0.020	0.028	0.791	0.819	0.002	0.048	0.371
37	0.039	0.034	1.001	0.916	0.044	0.086	0.543
40	0.056	0.037	1.824	1.789	0.050	0.164	1.064
44	0.030	0.028	2.309	2.167	0.167	0.113	0.558
48	0.075	0.064	5.383	4.739	0.222	0.311	1.290
50	0.053	0.037	2.329	2.366	0.096	0.138	0.846
54	0.039	0.028	3.510	1.644	0.471	0.085	N/A
58	0.134	0.072	5.110	3.377	0.301	0.807	1.234
61	0.097	0.036	2.791	2.875	0.155	0.213	0.658
65	0.054	0.027	2.171	2.244	0.069	0.134	0.460
68	0.166	0.070	5.159	3.462	0.169	0.478	1.014
72	0.130	0.028	5.373	3.374	0.359	0.329	0.562
75	0.120	0.045	13.930	6.802	2.671	0.942	0.439
79	0.088	0.044	0.730	0.338	0.038	0.087	0.117
82	0.118	0.049	2.056	1.649	0.125	0.302	0.210
86	0.143	0.028	1.977	1.494	0.256	0.281	0.199
93	0.140	0.036	2.067	1.457	0.199	0.208	0.096
100	0.279	NA	2.946	3.334	0.899	0.754	0.155
107	0.130	NA	1.227	1.507	0.568	0.519	0.070
114	0.094	0.026	1.300	1.410	0.198	0.203	0.032
117	0.049	0.028	2.114	2.225	0.349	0.399	0.045
125	0.037	NA	1.041	1.471	0.097	0.191	0.050
132	0.094	NA	4.161	2.882	0.395	0.174	0.033
139	0.026	NA	1.903	1.541	0.095	0.065	0.020
153	0.093	0.023	3.189	2.322	0.464	0.227	0.034
160	0.024	NA	0.734	0.612	0.040	0.057	0.019
167	0.068	0.049	2.385	1.942	0.108	0.159	0.026
174	0.099	0.021	3.218	2.469	0.516	0.291	0.016
181	0.011	0.025	0.395	0.352	0.004	0.036	0.007
188	0.108	0.089	6.422	4.323	0.377	0.398	0.052
195	0.179	0.100	32.010	17.574	14.415	1.464	0.180

BPIMA	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.022	0.003	2.755	3.082	NA	NA	0.901
4	0.070	0.030	4.626	8.059	0.258	0.314	3.482
8	0.025	0.037	3.147	3.078	0.049	0.185	1.309
11	0.022	0.041	5.430	6.827	0.183	0.100	1.917
15	0.084	0.031	4.293	2.571	0.039	0.157	8.680
18	0.026	0.012	0.839	0.618	0.077	0.030	5.787
22	0.032	0.015	2.202	1.780	0.122	0.074	10.427
25	0.022	0.014	0.783	0.483	0.055	NA	3.336
29	0.046	0.017	1.298	0.935	0.068	NA	10.692
32	0.062	0.022	2.643	1.765	0.062	0.110	17.192
36	0.106	0.030	0.713	0.376	NA	0.331	21.133
41	0.193	0.017	3.274	2.150	0.088	0.171	23.920
46	0.032	0.009	1.279	1.136	NA	0.051	7.870
50	0.154	0.023	3.706	2.378	0.144	0.205	19.970
53	0.035	0.011	1.488	1.351	0.007	0.064	4.280
57	0.049	0.014	1.693	1.202	0.052	0.263	5.019
64	0.068	0.011	1.891	1.463	0.073	0.058	5.164
71	0.066	0.017	1.912	1.354	0.075	0.126	4.140
78	0.034	0.012	1.790	1.380	0.030	0.033	2.132
84	0.073	0.015	2.142	1.126	0.025	0.129	2.749
92	0.081	0.028	1.255	0.634	0.066	0.141	1.578
99	0.058	0.011	1.227	0.449	NA	0.086	0.824
106	0.068	0.016	1.277	0.653	0.019	0.084	0.437
123	0.048	0.007	0.423	0.363	NA	NA	0.315
137	0.095	0.022	1.457	0.984	0.139	0.096	0.198
151	0.060	0.017	1.438	0.747	NA	0.055	0.109
166	0.057	0.018	1.249	0.647	0.107	NA	0.105
173	0.067	0.026	1.525	0.878	NA	0.085	0.136
194	0.056	0.017	1.566	0.807	0.069	0.053	0.121
208	0.085	0.016	2.169	1.185	0.061	0.113	0.201
222	0.084	0.020	2.175	1.379	0.081	0.123	0.252
236	0.052	0.015	1.247	0.658	0.155	0.044	0.336
250	0.063	0.024	1.360	0.705	NA	NA	0.383
264	0.077	0.021	1.904	1.093	0.061	0.061	0.593
271	0.068	0.014	1.558	0.962	0.061	0.057	0.634
278	0.073	0.014	1.362	1.002	NA	0.057	0.642
285	0.073	0.019	1.895	1.248	0.082	0.060	0.658
294	0.070	0.015	2.404	1.530	0.060	0.079	0.686
299	0.063	0.022	5.462	3.226	0.124	0.219	2.229

The Alarm's On	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
4	0.050	0.170	7.637	3.899	0.264	0.129	0.521
6	0.021	0.113	4.482	2.975	0.096	0.072	0.446
10	0.030	0.132	6.242	3.961	0.190	0.061	0.551
14	0.051	0.173	7.383	3.297	0.279	0.110	1.406
18	0.060	0.175	7.888	3.972	0.328	0.149	1.446
21	0.034	0.150	6.467	4.473	0.129	0.144	0.466
25	0.070	0.182	6.709	3.103	0.689	0.219	0.308
28	0.036	0.151	3.544	2.761	0.128	0.142	0.127
32	0.054	0.167	5.580	4.077	0.186	0.193	0.213
36	0.084	0.184	6.428	4.446	0.253	0.243	0.411
39	0.057	0.159	6.334	5.314	0.239	0.212	0.315
43	0.043	0.168	3.601	2.314	0.179	0.169	0.218
46	0.023	0.136	2.201	2.108	0.103	0.098	0.114
48	0.011	0.113	1.189	0.980	0.050	0.055	0.046
53	0.030	0.174	2.585	2.389	0.115	0.134	0.081
57	0.034	0.179	2.817	2.064	0.073	0.117	0.058
60	0.023	0.138	1.552	1.567	0.069	0.083	0.042
62	0.017	0.144	1.166	1.038	0.028	0.073	0.040
67	0.033	0.165	1.965	1.333	0.070	0.098	0.045
71	0.049	0.208	3.708	2.601	0.187	0.132	0.057
74	0.034	0.227	1.687	1.363	0.092	0.088	0.048
81	0.031	0.168	2.184	1.412	0.085	0.094	0.045
89	0.015	0.385	1.893	2.008	0.120	0.150	0.047
91	0.024	0.491	1.876	1.747	0.030	0.132	0.042
95	0.027	0.300	4.273	2.797	0.165	0.167	0.082
98	0.068	0.225	5.324	2.857	0.153	0.171	0.077
102	0.061	0.212	7.558	3.961	0.181	0.249	0.291
105	0.023	0.151	2.931	2.268	0.087	0.099	0.098
109	0.053	0.168	4.820	3.078	0.195	0.101	0.288
113	0.027	0.118	4.543	2.795	0.213	0.065	0.243
117	0.081	0.227	9.640	4.038	1.675	0.149	1.339
126	0.059	0.189	6.208	4.165	0.252	0.225	0.496
133	0.075	0.190	6.120	4.154	0.306	0.188	0.556
140	0.065	0.191	6.119	4.301	0.269	0.205	0.919
146	0.151	0.206	7.590	4.908	0.444	0.313	1.112
154	0.025	0.069	1.906	1.223	0.041	0.166	0.188
161	0.059	0.104	2.709	1.662	0.176	0.159	0.189
168	0.065	0.079	3.060	2.352	0.056	0.133	0.228
175	0.051	0.047	2.741	1.875	0.112	0.141	0.175
189	0.074	0.067	2.883	2.053	0.108	0.209	0.079

Saunders Pet	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
4	0.021	0.045	2.883	1.434	0.056	0.079	0.061
7	0.019	0.045	3.876	1.903	0.144	0.092	0.122
14	0.055	0.080	9.643	3.296	0.738	0.141	0.742
22	0.063	0.105	3.723	1.833	0.174	0.153	1.255
24	0.018	0.058	1.895	1.285	0.062	0.101	1.536
28	0.044	0.128	2.931	1.538	0.112	0.147	1.609
32	0.046	0.085	2.962	1.667	0.109	0.133	2.010
36	0.027	0.068	1.732	1.061	0.078	0.085	1.647
40	0.034	0.175	1.711	1.205	0.048	0.089	2.156
42	0.016	0.067	2.062	1.302	0.030	0.107	1.158
46	0.020	0.052	1.288	0.822	0.051	0.075	0.898
50	0.080	0.181	3.834	1.986	0.089	0.143	2.161
54	0.050	0.103	2.937	1.176	0.061	0.101	1.535
57	0.033	0.070	1.773	1.005	0.062	0.077	0.881
59	0.015	0.057	1.183	0.649	0.026	0.050	0.424
63	0.025	0.063	1.388	0.845	0.044	0.076	0.480
67	0.030	0.045	1.560	0.839	0.071	0.084	0.477
70	0.024	0.038	1.687	0.887	0.020	0.078	0.322
74	0.024	0.062	1.220	0.727	0.008	0.059	0.212
78	0.012	0.029	1.146	0.663	0.018	0.043	0.205
84	0.037	0.108	2.399	1.124	0.036	0.075	0.338
92	0.035	0.108	2.503	0.967	0.024	0.100	0.388
99	0.028	0.120	1.890	0.717	0.001	0.087	0.157
106	0.030	0.072	1.942	0.787	0.030	0.104	0.085
116	0.008	0.064	0.583	0.407	0.017	0.033	0.012
130	0.011	0.043	1.064	0.681	0.024	0.045	0.025
136	0.009	0.057	0.725	0.480	0.019	0.038	0.013
151	0.007	0.077	0.705	0.372	0.014	0.035	0.005
158	0.021	0.068	1.068	0.572	0.033	0.053	N/A
173	0.004	0.030	1.045	0.408	0.040	0.067	N/A
177	0.011	0.088	0.889	0.535	0.047	0.045	0.013
193	0.034	0.094	1.733	1.056	0.064	0.132	0.011
200	0.026	0.065	1.416	0.991	0.063	0.074	0.010
214	0.010	0.056	1.036	0.684	0.056	0.057	0.011
235	0.008	0.048	1.206	0.578	0.042	0.049	0.016
249	0.012	0.053	1.818	0.924	0.063	0.053	0.014

Standard Whisper	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.032	0.078	2.588	2.116	0.388	0.295	1.183
2	0.055	0.116	5.723	4.195	0.545	0.184	2.415
6	0.098	0.141	17.877	10.247	1.670	0.240	4.354
10	0.108	0.134	7.982	6.334	0.303	0.385	1.051
13	0.115	0.048	4.265	2.879	0.124	0.400	0.891
17	0.082	0.039	6.925	2.032	0.378	0.268	1.040
21	0.153	0.005	2.826	1.443	0.130	0.257	0.278
23	0.118	0.279	3.052	3.394	0.286	0.489	14.140
27	0.174	0.106	3.835	3.632	0.163	0.430	14.267
31	0.150	0.297	2.636	2.298	0.123	0.317	7.303
34	0.157	0.187	1.837	1.635	0.226	0.318	6.096
38	0.206	0.288	3.244	2.886	0.171	0.406	10.813
41	0.365	0.556	5.847	4.630	0.286	0.676	29.057
45	0.266	0.128	5.849	4.524	0.235	0.523	32.064
48	0.234	0.081	6.674	1.554	0.690	0.224	0.936
52	0.268	0.337	4.391	4.006	0.225	0.326	11.612
56	0.059	0.015	1.900	1.008	0.060	0.078	0.498
58	0.246	0.418	2.532	2.069	0.501	0.259	4.925
62	0.168	0.229	4.106	3.307	0.166	0.285	4.633
66	0.121	0.148	2.697	1.634	0.087	0.173	1.333
69	0.123	0.177	1.984	1.222	0.108	0.167	1.035
73	0.180	0.236	3.322	1.716	0.108	0.150	0.873
76	0.188	0.143	2.143	0.709	0.311	0.201	0.422
80	0.088	0.215	1.323	1.069	0.152	0.168	4.367
84	0.079	0.057	1.372	0.890	0.201	0.157	2.137
86	0.062	0.161	0.999	0.650	0.139	0.148	1.501
90	0.089	0.303	1.102	0.772	0.331	0.151	1.473
97	0.040	0.175	0.665	0.428	0.101	0.086	0.406
104	0.034	0.093	0.844	0.555	0.098	0.087	0.393
111	0.031	0.158	0.905	0.628	0.119	0.111	0.282
126	0.027	0.019	0.780	0.516	0.143	0.096	0.312
132	0.029	0.138	0.700	0.421	0.146	0.109	0.219
139	0.047	0.121	0.655	0.464	0.163	0.123	0.232
146	0.040	0.151	0.692	0.524	0.141	0.124	0.228
153	0.009	0.027	0.346	0.231	0.075	0.053	0.164
160	0.053	0.068	0.643	0.438	0.126	0.085	0.212
167	0.071	0.040	0.997	0.647	0.117	0.101	0.277
174	0.041	0.042	0.987	0.529	0.155	0.114	0.198
181	0.011	0.018	0.696	0.393	0.128	0.078	0.179
184	0.024	0.009	0.304	0.196	0.097	0.031	0.129
188	0.020	0.030	1.266	0.843	0.122	0.095	0.230
195	0.037	0.040	1.535	1.184	0.165	0.133	0.274
202	0.074	0.064	3.975	2.351	0.276	0.227	1.448

Cullane Chief	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.120	0.033	7.981	7.477	0.387	0.405	1.228
4	0.143	0.046	20.313	10.517	0.721	0.814	4.438
8	0.137	0.079	6.952	5.101	0.284	0.661	5.109
11	0.240	0.113	12.544	7.874	0.501	1.130	16.311
15	0.269	0.124	18.260	9.040	0.741	1.331	16.471
18	0.357	0.132	16.624	9.978	0.451	1.395	13.102
22	0.169	0.059	5.258	4.180	0.212	0.355	1.875
25	0.274	0.134	10.355	7.741	0.392	0.682	3.892
33	0.184	0.092	5.696	4.857	0.250	0.568	4.673
35	0.251	0.106	13.418	7.129	0.378	1.201	27.659
39	0.247	0.031	4.517	3.668	0.906	0.580	13.361
41	0.269	0.043	5.648	3.785	0.273	0.919	9.306
46	0.302	0.121	7.126	4.606	0.323	0.932	8.514
50	0.300	0.079	4.906	2.838	0.290	0.724	2.739
60	0.260	0.016	2.920	1.957	0.470	0.490	0.663
64	0.228	0.017	4.079	1.481	0.117	0.286	0.478
68	0.122	0.040	1.977	1.484	0.548	0.385	1.259
70	0.128	0.016	2.078	1.272	0.153	0.458	1.444
74	0.145	0.036	2.627	1.920	0.354	0.457	1.175
78	0.088	0.008	1.779	1.028	0.106	0.261	0.318
81	0.111	0.059	3.127	2.087	0.280	0.362	2.815
85	0.069	0.055	2.831	1.654	0.146	0.309	2.045
88	0.054	0.035	1.559	0.976	0.094	0.197	1.207
92	0.046	0.062	2.473	1.564	0.158	0.220	1.798
95	0.065	0.051	2.460	1.619	0.186	0.251	1.818
99	0.045	0.066	2.336	1.402	0.161	0.230	1.582
105	0.049	0.070	2.059	1.978	0.183	0.229	1.599
109	0.060	0.061	2.109	1.235	0.152	0.195	1.400
116	0.033	0.044	1.569	1.002	0.122	0.157	0.850
130	0.051	0.074	2.314	1.407	0.160	0.184	1.427
138	0.009	0.008	0.307	0.268	0.060	0.140	0.297
144	0.059	0.071	2.695	1.510	0.179	0.295	1.406
151	0.036	0.040	1.976	1.212	0.210	0.211	1.181
158	0.036	0.060	1.939	1.148	0.150	0.222	1.445
166	0.034	0.054	2.405	1.385	0.142	0.230	1.557
172	0.053	0.069	4.546	2.342	0.202	0.289	4.064

Flo Jo	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
0	0.015	0.033	1.295	0.979	0.112	0.016	0.062
5	0.024	0.052	4.055	1.620	1.020	0.028	0.166
9	0.134	0.102	17.020	6.247	1.527	0.209	1.353
13	0.019	0.060	1.921	0.638	0.853	0.046	4.494
16	0.017	0.097	1.320	0.652	0.308	0.064	16.011
20	0.031	0.123	1.143	0.726	0.351	0.133	21.264
24	0.051	0.040	2.827	1.593	0.353	0.420	43.741
26	0.038	0.193	1.694	1.076	0.207	0.205	39.087
30	0.064	0.198	2.625	1.481	0.794	0.273	58.619
34	0.068	0.308	1.910	1.359	0.509	0.206	49.661
41	0.073	0.328	1.461	2.761	0.338	0.141	33.952
44	0.033	0.160	1.102	0.938	0.193	0.083	22.616
48	0.051	0.158	1.426	1.142	0.098	0.133	33.224
51	0.068	0.181	1.382	1.173	0.229	0.110	15.307
55	0.055	0.227	1.627	1.142	0.241	0.182	18.752
58	0.046	0.225	1.213	0.785	0.220	0.095	11.397
62	0.047	0.230	1.434	0.932	0.322	0.154	12.121
65	0.044	0.097	1.223	0.904	0.388	0.128	10.428
69	0.053	0.168	2.096	1.561	0.388	0.191	12.716
72	0.052	0.165	0.638	0.514	0.193	0.134	3.159
76	0.048	0.153	1.546	0.852	0.466	0.218	5.851
79	0.035	0.153	0.912	0.691	0.175	0.135	3.605
83	0.040	0.099	1.177	0.668	0.299	0.205	4.454
86	0.029	0.023	0.474	0.344	0.088	0.092	1.139
90	0.021	0.065	0.634	0.365	0.124	0.104	0.981
93	0.028	0.047	0.479	0.285	0.116	0.077	0.594
97	0.008	0.061	0.347	0.261	0.111	0.062	0.488
100	0.017	0.114	0.362	0.243	0.136	0.060	0.443
104	0.017	0.053	0.631	0.316	0.130	0.093	0.303
107	0.017	0.064	0.427	0.257	0.070	0.074	0.259
110	0.022	0.066	0.375	0.295	0.075	0.059	0.279
117	0.020	0.045	0.377	0.285	0.049	0.078	0.146
124	0.020	0.026	0.453	0.277	0.046	0.049	0.153
131	0.017	0.023	0.388	1.234	0.075	0.063	0.122
159	0.032	0.030	1.807	0.468	0.274	0.189	0.345

Whitefort Euro	testosterone	epitest	aBB_Adiol	epiandro	BaB_Adiol	etio	117 ion
Days post season	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	Area Ratio
4	0.028	0.122	3.897	3.614	0.376	0.260	2.008
8	0.044	0.219	7.515	5.627	0.596	0.429	4.971
12	0.152	0.028	31.846	15.939	1.409	0.801	14.349
14	0.045	0.117	5.560	4.233	0.364	0.403	14.889
18	0.091	0.288	7.349	3.365	0.801	1.013	34.256
22	0.043	0.192	5.023	3.429	0.382	0.410	25.828
25	0.017	0.049	2.300	1.649	0.285	0.232	16.536
29	0.032	0.163	3.109	2.329	0.366	0.412	24.059
32	0.079	0.323	4.918	3.252	0.202	0.622	34.879
36	0.026	0.190	2.048	2.040	0.139	0.198	17.021
39	0.021	0.059	1.882	1.389	0.069	0.193	16.932
43	0.050	0.252	2.847	2.514	0.152	0.366	23.343
47	0.016	0.015	1.212	0.995	0.083	0.105	10.359
49	0.029	0.018	2.393	1.991	0.135	0.275	20.680
53	0.057	0.381	2.739	2.395	0.137	0.309	15.808
55	0.062	0.266	2.839	2.200	0.112	0.380	20.662
60	0.089	0.370	4.990	3.929	0.226	0.485	25.632
64	0.056	0.377	3.833	2.870	0.108	0.374	16.921
71	0.059	0.332	2.352	2.361	0.154	0.256	8.640
78	0.024	0.171	1.667	1.204	0.114	0.190	5.521
84	0.046	0.237	2.214	1.274	0.155	0.244	5.620
92	0.032	0.255	0.937	0.726	0.186	0.119	1.753
99	0.048	0.205	1.582	1.002	0.057	0.172	1.977
106	0.021	0.131	1.175	1.172	0.088	0.181	1.238
117	0.320	0.053	1.000	0.614	0.055	0.288	0.701
130	0.040	0.232	1.525	1.213	0.037	0.214	0.561
144	0.021	0.089	1.231	0.903	0.133	0.198	0.420
158	0.019	0.132	1.010	0.701	0.058	0.141	0.339
172	0.018	0.096	1.759	1.137	0.105	0.166	0.369
186	0.018	0.109	1.078	0.721	0.068	0.104	0.231
200	0.027	0.126	1.068	0.731	0.084	0.156	0.369
214	0.017	0.140	1.288	0.950	0.117	0.197	0.309
228	0.059	0.259	3.585	1.880	0.171	0.350	0.628
242	0.050	0.215	2.931	1.826	0.319	0.226	0.455
249	0.001	0.033	0.893	0.493	0.100	0.058	0.167
256	0.043	0.183	3.109	2.052	0.169	0.301	2.917
263	0.031	0.109	5.673	3.383	0.298	0.403	0.638
270	0.036	0.118	3.521	2.157	0.210	0.345	0.341
277	0.043	0.145	2.939	1.874	0.258	0.309	0.598
285	0.050	0.044	7.438	4.711	0.781	0.531	0.957

Queen of the Bar	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	0.466	0.852	0.056
4	7.317	3.098	0.110
8	24.607	9.134	0.169
11	4.678	1.618	0.641
15	7.023	1.499	1.100
18	5.492	1.590	3.362
22	4.002	1.192	0.783
25	6.029	1.273	2.634
29	1.596	0.780	0.873
33	1.576	1.136	1.684
35	4.040	0.991	1.196
39	4.528	0.904	3.331
41	5.441	1.066	2.750
46	6.610	1.241	1.518
50	4.159	1.055	0.810
53	11.065	1.113	0.384
57	0.930	0.839	0.353
60	1.891	0.975	0.321
64	2.461	1.187	0.033
68	1.492	1.144	0.223
70	1.335	0.940	0.133
74	2.453	1.444	0.147
78	1.305	0.941	0.110
81	1.428	1.146	0.108
88	2.101	1.462	0.068
95	1.362	1.122	0.040
103	0.301	0.601	0.023
109	1.358	0.928	0.019
123	0.652	0.671	0.039
130	1.332	0.928	0.029
144	1.238	0.925	0.010
151	0.834	0.792	0.033
158	1.812	1.043	0.016
166	0.502	0.647	0.018
172	0.951	0.763	0.050
179	2.084	1.410	0.034
186	1.727	1.446	0.041
193	2.714	1.768	0.043
200	3.317	1.757	0.124

Queen Boadicea	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
-15	0.978	0.936	0.009
-8	1.122	1.098	0.013
-1	2.489	1.493	0.036
0	N/A	N/A	N/A
1	4.188	2.237	0.038
5	7.739	4.252	0.045
9	6.329	3.610	0.084
13	1.016	1.021	0.152
15	1.451	1.311	0.280
19	0.326	0.583	0.174
23	1.066	1.341	0.389
26	0.935	0.964	0.346
30	0.961	1.009	0.492
33	1.946	1.628	0.610
37	1.971	1.661	0.521
40	1.737	1.273	0.482
44	1.544	1.176	0.536
50	1.607	1.161	0.404
54	1.546	1.285	0.293
58	1.762	1.255	0.355
61	4.203	2.198	0.050
65	1.310	1.267	0.023
68	0.724	0.817	0.021
72	1.106	0.878	0.021
76	0.978	0.871	0.040
78	0.697	0.723	0.027
85	1.230	0.981	0.051
106	0.459	0.637	0.009
127	1.150	0.986	0.037
141	0.241	0.704	0.054
148	0.311	0.670	0.035
155	0.994	0.931	0.053
162	0.756	0.777	0.069
169	1.121	0.873	0.116
183	0.503	0.611	0.087
190	1.031	0.874	0.155
197	0.828	0.826	0.238

Langies Posh Girl	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
5	1.868	1.563	0.040
9	2.571	1.899	0.187
12	1.103	0.792	0.733
16	1.454	1.128	0.840
19	0.768	0.637	1.030
23	1.133	0.810	1.190
26	1.975	1.708	0.888
30	0.631	0.347	0.607
33	1.112	0.859	0.724
37	0.999	0.853	1.389
40	0.573	0.325	0.344
44	1.161	0.906	1.429
47	1.026	0.846	0.547
51	0.805	0.514	0.316
54	0.700	0.370	0.211
58	1.001	0.413	0.270
65	0.878	0.625	0.254
72	0.727	0.449	0.104
79	0.294	0.058	0.073
82	0.547	0.437	0.062
91	0.456	0.256	0.117
96	0.484	0.304	0.049
100	0.600	0.579	0.042
107	0.648	0.849	0.058
124	0.616	0.426	0.033
138	0.697	0.478	0.035
149	0.733	0.644	0.023
166	0.925	0.470	0.035
180	0.496	0.300	0.022
194	0.795	0.394	0.054
208	0.514	0.340	0.041
222	0.561	0.320	0.029
236	0.478	0.380	0.012
257	0.492	0.125	0.044
271	0.416	0.218	0.046
285	1.855	0.488	0.034
299	0.492	0.318	0.022
313	1.324	0.570	0.056
327	3.053	0.341	0.226
333	3.322	1.463	

Ringlestown Flos	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
5	7.601	2.627	0.198
9	41.294	9.649	0.383
13	8.618	4.553	0.566
16	2.756	0.347	1.137
20	1.282	0.953	0.502
23	1.270	0.825	0.867
27	2.013	1.097	1.067
30	2.362	0.605	0.984
34	2.687	2.070	1.817
37	1.614	0.733	1.298
41	3.833	3.025	1.558
44	3.308	0.421	1.025
48	2.552	1.567	0.789
51	2.218	1.058	0.415
55	3.206	0.654	0.601
58	1.117	0.449	0.159
62	1.081	0.493	0.103
65	2.062	0.966	0.093
69	0.662	0.393	0.044
72	0.506	0.208	0.044
79	1.077	0.636	0.024
86	5.064	2.664	0.037
90	8.715	5.096	0.048
100	0.622	0.524	0.009
108	7.014	1.756	0.172
110	2.333	1.465	0.033
121	2.518	0.420	0.245
128	7.190	3.427	0.019
135	4.623	2.462	0.043
149	7.942	4.389	0.034
163	7.548	2.923	0.059
170	8.627	4.318	0.058
185	4.788	1.993	0.046
213	3.512	2.553	0.095
220	9.071	3.894	0.059
227	10.896	6.040	0.033

GIVEHERTHEVOTE	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	5.904	5.332	0.079
4	18.937	16.769	0.148
7	2.528	1.693	0.125
11	2.618	2.366	0.305
14	2.759	2.092	0.692
18	1.313	1.126	0.475
21	1.964	1.450	0.515
25	2.245	1.482	0.586
28	2.482	1.791	0.629
32	2.376	1.494	0.813
35	2.842	1.526	0.563
39	3.520	2.195	0.780
42	1.643	1.221	0.509
46	4.268	2.478	1.151
49	2.649	1.671	0.490
53	3.024	2.343	0.325
56	3.618	2.018	0.280
60	2.427	1.543	0.348
63	2.261	1.736	0.140
67	1.840	1.564	0.146
70	2.557	1.447	0.141
74	2.768	1.617	0.129
77	2.218	1.109	0.070
84	1.256	1.052	0.039
88	2.450	1.585	0.022
94	1.692	0.871	0.030
101	1.449	0.883	0.024
108	0.350	0.320	0.021
115	0.666	0.537	0.012
122	0.580	0.440	0.009
129	0.708	0.619	0.008
143	0.543	0.487	0.007
157	1.369	0.910	0.015
171	0.475	0.364	0.010
185	2.951	1.667	0.025
213	1.743	0.919	0.018
227	4.760	3.252	0.014
254	5.105	2.529	0.022
269	3.296	1.519	0.020
277	4.998	2.647	0.015

Wideopenspaces	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	5.825	3.593	0.027
4	2.360	1.880	0.021
8	4.285	3.925	0.098
12	5.072	5.347	0.056
17	0.636	0.540	0.140
21	0.704	0.754	0.195
25	0.263	0.494	0.195
28	0.666	0.776	0.251
32	1.501	3.373	0.195
35	1.523	1.320	0.459
39	1.017	0.871	0.414
42	2.000	1.392	0.450
46	1.468	1.530	0.348
49	0.437	0.594	0.080
53	2.287	2.276	0.433
56	1.117	0.872	0.126
60	3.845	2.496	0.171
63	3.226	1.831	0.232
67	1.054	0.730	0.118
70	0.920	0.816	0.058
74	0.609	0.525	0.048
77	0.586	0.498	0.067
81	0.186	0.234	0.041
84	0.158	0.129	0.024
88	0.113	0.036	0.022
91	0.232	0.220	0.030
95	1.927	1.097	0.010
98	0.233	0.435	0.009
102	0.725	0.498	0.021
108	0.671	0.478	0.036
112	1.297	0.875	0.047
115	0.486	0.535	0.006
119	0.045	0.093	0.011
122	0.127	0.819	0.035
129	0.470	0.377	0.015
136	0.932	0.717	0.014
143	0.461	0.319	0.017
158	0.866	0.523	0.023
164	0.722	0.438	0.048
171	0.356	0.776	0.017

Marshal Promise	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	7.271	4.824	0.117
4	3.525	1.927	0.081
7	3.149	3.502	0.148
14	0.876	0.389	0.941
18	1.005	0.399	3.193
22	0.754	0.369	1.251
25	1.096	0.690	4.176
29	1.038	0.491	2.626
32	1.494	0.748	4.576
36	1.660	0.935	5.783
39	2.020	1.079	7.290
43	2.072	1.217	5.132
46	2.125	1.155	7.374
50	0.741	0.412	0.792
53	2.189	1.126	2.954
57	2.122	1.371	1.634
64	0.717	0.250	N/A
71	1.821	0.872	N/A
78	1.070	0.516	0.139
85	0.916	0.201	0.150
92	0.962	0.839	0.028
99	1.347	0.614	0.085
106	0.882	0.278	0.081
120	0.328	0.146	0.041
127	0.669	0.229	0.019
140	1.745	0.961	0.061
154	1.206	0.540	0.029
182	1.167	0.561	0.044
210	2.733	2.126	0.052
224	0.950	0.295	0.046
238	7.735	4.923	0.025
251	1.601	0.812	0.042
284	3.559	1.992	0.010
294	3.468	2.117	0.018
308	2.580	2.090	0.059
322	0.980	0.291	0.025
336	9.258	6.213	0.039
350	4.802	3.423	0.014
364	13.768	6.828	0.123

Distant Pearl	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	6.823	4.012	0.105
4	8.072	3.174	0.185
7	6.833	2.613	0.124
11	3.694	2.206	0.170
14	1.538	0.537	1.563
18	1.628	0.578	0.850
21	1.655	0.488	1.546
25	1.094	0.305	0.823
28	1.515	0.401	1.074
32	1.870	0.494	1.250
35	2.205	0.926	1.724
39	2.838	1.096	2.330
42	2.207	0.875	1.385
46	1.450	0.676	0.971
49	1.809	0.446	0.712
53	1.682	0.402	0.710
60	2.999	1.084	1.008
67	1.909	0.703	0.405
74	1.447	0.548	0.101
81	0.962	0.265	0.098
88	1.277	0.451	0.023
95	1.178	0.412	0.058
102	0.887	0.271	0.091
108	0.937	0.217	0.045
115	0.712	0.135	0.093
136	1.008	0.336	0.092
164	1.271	0.470	0.012
178	0.972	0.329	
206	7.823	4.482	0.091
234	4.018	1.500	0.108
262	2.210	0.668	0.033
280	1.654	0.574	0.011
304	5.091	2.043	0.016
332	2.615	1.045	0.026
360	0.849	0.130	0.027
388	1.117	0.635	0.030
416	0.860	0.180	0.021
430	1.293	0.389	0.023
444	0.724	0.208	0.088
469	3.054	1.449	0.052

Drumpark Access	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	5.762	3.997	0.136
14	1.381	1.236	0.854
21	1.415	0.783	1.132
42	4.076	1.633	1.093
56	7.531	3.557	0.892
63	2.768	1.255	0.182
70	1.142	0.100	0.026
77	0.897	0.439	0.016
84	0.872	0.339	0.026
91	0.434	0.090	0.061
98	0.537	0.238	0.022
105	1.040	0.560	0.012
112	0.530	0.080	0.053
119	0.548	0.204	0.005
126	0.667	0.175	0.068
133	1.212	0.424	0.055
140	1.108	0.393	0.045
147	0.992	0.354	0.003
154	0.785	0.268	0.024
168	0.987	0.424	0.070
175	0.992	0.356	0.038
182	0.951	0.423	0.049
189	1.327	1.016	0.044
196	1.366	0.609	0.096
203	0.827	0.288	0.047
210	0.620	0.286	0.021
217	1.270	0.902	0.063
224	1.875	0.761	0.085
231	1.900	0.890	0.075
238	2.498	1.428	0.025

Cannon Clover	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	5.160	7.679	0.118
4	5.222	6.356	0.111
7	5.711	4.412	0.205
14	21.899	24.551	0.166
18	8.834	7.653	0.708
21	2.122	1.885	0.058
25	1.306	1.441	0.070
32	2.813	2.505	0.043
35	1.453	1.438	0.046
39	1.748	2.460	0.058
42	2.461	2.451	0.070
46	1.617	1.576	0.055
49	1.819	1.985	0.044
56	1.833	2.169	0.043
60	3.498	2.937	0.040
67	2.898	2.589	0.048
70	1.662	1.338	0.038
74	3.537	3.323	0.044
77	2.476	3.009	0.035
81	3.507	4.303	0.058
84	8.088	8.399	0.038
91	2.604	2.766	0.026
98	3.525	3.450	0.041
105	10.895	11.436	0.123
116	3.520	3.490	1.656
123	2.855	2.530	1.920
137	1.087	1.958	1.063
144	3.597	3.072	2.513
151	3.163	4.167	2.754
158	3.632	4.653	3.013
171	4.271	4.581	3.122
178	4.844	4.985	2.621
185	4.126	4.444	0.808
192	3.234	3.691	1.480
206	4.076	4.342	0.963
213	3.948	3.052	0.801
228	5.215	4.890	0.346
234	2.024	3.403	0.173
247	2.197	1.739	0.080
255	2.150	1.723	0.049
263	1.786	1.925	0.036
269	0.854	1.491	0.042
276	1.077	1.018	0.026
284	1.058	0.862	0.028

Miss Honcho	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
2	3.677	1.416	0.028
4	4.914	1.789	0.021
9	46.540	20.450	0.175
12	3.846	1.464	0.076
16	4.226	1.585	0.570
18	5.129	1.878	0.653
23	2.446	0.741	0.482
27	1.605	0.451	0.444
30	4.226	1.671	1.306
32	1.762	0.658	0.905
36	2.803	1.181	1.080
40	4.155	2.288	1.757
44	2.372	1.099	0.798
46	2.697	1.350	0.919
54	3.171	1.220	0.812
61	2.134	1.065	0.327
66	1.138	0.341	0.126
72	2.751	1.198	0.181
79	2.920	1.217	0.083
88	1.198	0.175	0.012
90	1.512	0.436	0.024
94	1.292	0.292	0.023
100	1.151	0.281	0.051
108	1.418	0.646	0.026
110	1.177	0.220	0.026
115	1.412	0.426	0.016
132	1.359	0.410	0.041
152	0.882	1.831	0.026
181	1.745	0.712	0.028
187	1.335	0.574	0.014
201	2.216	0.917	0.014
215	2.820	1.409	0.015
222	2.221	1.165	0.009
237	1.898	1.255	0.012
244	2.141	1.346	0.017
250	1.685	0.878	0.020
257	2.931	1.729	0.024
264	3.575	2.198	0.014

Sparky Pacific	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	1.004	0.877	0.119
4	3.093	2.549	0.405
7	0.406	0.347	0.428
11	0.603	0.397	0.978
17	0.552	0.472	N/A
21	1.276	1.561	2.267
25	0.747	0.920	N/A
28	0.477	0.374	1.090
32	1.405	1.507	3.432
35	1.340	1.325	1.837
39	1.569	1.908	4.052
42	0.816	1.006	1.082
50	0.754	0.895	0.629
52	0.612	0.786	N/A
56	1.196	1.471	0.453
60	0.826	0.429	0.344
63	1.219	1.464	N/A
67	2.011	0.802	N/A
74	0.634	0.730	N/A
80	0.377	0.247	0.114
88	0.240	0.087	0.000
95	0.324	0.057	0.000
102	0.273	0.107	0.000
105	0.180	0.026	0.000
110	0.309	0.038	0.000
115	0.345	0.193	0.000
122	0.259	0.029	0.000
126	0.284	0.010	N/A
133	0.285	0.110	0.000
136	0.450	0.182	0.000
147	0.526	0.215	0.000
154	0.456	0.025	0.000
175	0.629	0.373	0.000
189	1.318	0.861	0.000
196	1.140	1.044	0.000
202	0.811	0.549	0.000
210	0.648	0.293	0.000

Queen Sally	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	13.238	10.015	0.098
5	1.650	1.018	0.156
7	3.597	0.688	0.144
11	1.565	0.988	0.168
13	1.194	0.872	0.333
18	2.178	2.085	0.447
22	2.157	1.576	0.716
25	1.985	1.909	0.463
29	0.493	0.513	0.324
32	1.215	1.576	0.380
36	0.388	0.580	0.246
42	0.715	0.894	0.231
46	0.689	0.873	0.243
50	0.360	0.419	0.278
53	0.650	0.871	0.111
57	0.783	0.892	0.130
60	0.157	0.275	0.095
67	0.207	0.187	0.079
75	1.732	1.768	0.083
88	0.105	0.076	0.049
95	0.510	0.502	0.005
102	0.691	0.613	0.001
123	0.671	0.654	0.011
151	0.187	0.247	0.015
180	0.206	0.213	0.011
193	1.546	1.142	0.032
221	0.111	0.024	0.025
228	1.974	1.996	0.010
242	3.863	3.064	0.054
252	9.979	8.527	0.040
266	10.206	8.266	0.039
280	20.658	19.178	0.056
295	7.886	6.183	0.017
308	1.008	0.855	0.015
322	1.726	1.066	0.031
336	7.737	5.912	0.004
350	1.426	1.286	0.012

BPXYP	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	1.116	0.723	0.083
4	2.404	0.959	0.308
8	3.681	1.445	0.455
11	6.931	3.894	1.030
18	8.485	5.742	2.601
22	3.544	1.884	6.447
25	5.407	3.523	11.127
29	5.434	2.755	13.255
32	4.900	2.643	11.373
36	5.985	2.777	20.308
39	1.851	1.270	15.989
43	0.966	0.461	4.408
46	0.642	0.152	5.198
53	2.937	0.134	5.694
57	0.804	0.362	4.287
60	0.941	0.418	1.095
64	0.623	0.107	0.410
67	0.679	0.129	0.225
71	0.764	0.321	0.053
74	0.769	0.294	0.010
78	0.589	0.140	0.141
85	1.100	0.379	0.040
91	0.968	0.359	0.039
99	1.562	0.725	0.043
106	0.295	0.042	0.011
112	1.384	0.617	0.067
123	0.433	0.069	0.083
137	0.573	0.081	0.039
151	0.467	0.118	0.034
166	0.501	0.007	0.042
180	0.468	0.027	0.043
208	0.884	0.216	0.056
222	1.426	0.504	0.048
236	0.912	0.210	0.071
250	1.320	0.490	0.058
264	0.871	0.213	0.023
278	0.702	0.208	0.042

Supreme Madonna	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	0.778	0.412	0.010
1	3.562	0.508	0.015
6	3.673	3.076	0.036
10	1.173	0.651	0.068
14	0.368	0.152	0.056
18	0.368	0.125	0.138
22	0.381	0.175	0.207
26	0.358	0.104	0.095
34	0.615	0.457	0.223
38	0.501	0.372	0.257
42	0.427	0.179	0.109
46	0.508	0.264	0.249
50	1.541	1.279	0.323
54	0.657	0.504	0.211
57	5.116	0.957	0.215
61	0.683	0.570	0.055
68	0.445	0.397	0.022
75	0.250	0.147	0.025
82	0.713	0.103	0.009
89	0.388	0.027	0.011
96	0.915	0.416	0.013
110	0.664	0.075	0.013
117	0.744	0.280	0.013
138	0.443	0.093	0.012
145	0.605	0.128	0.020
152	0.446	0.073	0.022
159	1.462	0.314	0.018
166	2.142	1.655	0.012
173	0.337	0.084	0.009
180	0.272	0.006	0.006
187	0.646	0.275	0.005
194	0.273	0.004	0.004
201	0.352	0.057	0.005
215	1.118	0.859	0.007
229	0.616	0.365	0.006
236	0.686	0.335	0.006
243	0.691	0.342	0.018
246	0.687	0.314	0.022

Little Reaction	aBB_Adiol	epiandro	117 ion
Days post season	(ng/ml)	(ng/ml)	Area Ratio
0	46.126	16.071	0.019
1	19.951	8.817	0.010
2	17.935	7.107	0.009
6	5.869	2.236	0.014
10	5.948	2.143	0.028
14	6.892	2.655	0.039
18	2.725	1.334	0.055
22	1.703	0.908	0.103
26	1.796	0.692	0.198
30	2.105	0.874	0.190
34	0.499	0.188	0.109
38	1.103	0.462	0.097
42	1.377	0.712	0.302
46	3.833	1.170	0.410
50	1.357	0.601	0.138
54	1.293	0.491	0.174
59	0.687	0.230	0.152
61	2.192	0.687	0.383
71	0.451	0.098	0.028
106	0.178	0.009	0.008
113	0.486	0.117	0.009
127	0.307	0.028	0.006
140	0.239	0.001	0.004
155	0.252	0.001	0.008
169	0.228	0.007	0.003
183	0.289	0.046	0.003
190	0.646	0.251	0.012
215	0.602	0.350	0.006
222	1.142	0.064	0.034
229	0.866	0.442	0.011
236	1.305	0.274	0.009
243	0.812	0.248	0.016
250	0.761	0.332	0.007
260	0.767	0.235	0.009
267	0.885	0.265	0.005

	Bolly for Molly	aBB_Adiol	epiandro	117 ion
	Days post season	(ng/ml)	(ng/ml)	Area Ratio
	-9	0.997	0.596	0.138
	-8	0.815	0.340	0.089
	-7	1.403	0.757	0.149
	-6	1.732	1.055	0.125
	-5	1.819	1.004	0.138
	-4	1.472	0.974	0.087
	-3	2.369	1.546	0.080
	-2	2.468	1.512	0.142
	-1	2.246	1.496	0.167
Day of Season	0	2.374	1.429	0.201
	1	1.783	0.999	0.157
	2	3.161	1.763	0.283
	3	2.102	1.290	0.255
	4	4.165	2.735	0.581
	5	4.877	2.978	0.654
	6	3.329	2.027	0.587
	7	5.703	3.188	1.115
	8	4.105	2.913	0.679
	9	13.176	7.850	2.081
Mated between Day 11 and 14	10	8.158	5.239	1.899
	15	2.121	1.592	9.393
	16	1.344	1.110	6.296
	17	0.940	0.647	6.208
	18	0.897	0.625	6.819
	19	0.686	0.430	5.744
	20	0.558	0.328	3.948
	24	0.710	0.436	5.213
	27	1.356	0.897	10.700
	31	1.385	0.843	11.712
	34	1.789	1.231	13.527
	38	2.241	1.846	11.359
	41	1.972	1.733	6.372
	45	1.434	1.182	8.027
	48	1.964	1.647	10.114
	52	0.984	0.662	3.993
	55	0.998	0.609	5.373
	59	1.492	0.906	9.368
	62	2.942	2.028	8.078
	66	3.146	2.451	8.813
	69	5.346	4.070	6.526
Had pups Days 71/72	73	4.001	3.279	2.246
	76	0.492	0.641	0.453
	80	1.449	1.322	1.016
	83	1.347	0.965	0.629
	87	2.402	1.651	0.891
	91	0.710	0.389	0.433

	93	2.552	1.319	1.087
	97	0.717	0.366	1.081
	101	1.444	0.521	1.939
In season Day 105	105	7.608	3.289	1.418
	108	3.985	2.062	1.342
	115	3.076	1.409	0.918
Stopped bleeding Day 123	122	0.325	0.109	0.426
	129	0.687	0.335	0.324
	136	0.599	0.035	0.194
	143	0.747	0.332	0.179
	150	0.566	0.251	0.117
	157	0.409	0.117	0.143
	164	1.008	0.468	0.082
	178	1.258	0.678	0.156
	185	0.761	0.323	0.072
	192	0.375	0.103	0.049
	206	0.697	0.242	0.050
	213	0.409	0.145	0.013
	220	0.641	0.248	0.066
	226	0.757	0.298	0.069
	233	0.856	0.310	0.043
Date of next season	240	2.662	1.197	0.255