CHAPTER 1

Introduction

On such a silent night a silvery jet was seen far in advance of the white bubbles at the bow. Lit up by the moon, it looked celestial; seemed some plumed and glittering god uprising from the sea. –Moby Dick

1.1 Introduction

Wildlife conservation research is currently dominated by studies into population size and growth, genetics, seasonal movements and reproductive rates. Hormones are key factors in reproductive rates and general health and so are crucial to a population’s survival. Hormones control many basic functions of the body including reproductive systems in addition to the physiological responses to changing environmental parameters. It has been reported that measured hormonal concentrations can offer a better understanding of factors that impair a species’ demographic vitality or identify early warning signals for changes in reproductive rates (Kirby and Ridgway 1984; Schoech and Lipar 1998). In order to successfully manage any population and understand the factors that may be influencing it, basic biological knowledge is required on internal physiology, reproductive cycling, and effects of extraneous factors (such as increasing levels of stress) on both individual animals and populations. The use of non-invasive techniques to monitor hormone metabolites in wildlife is growing (Creel et al. 1991; Lasley and Kirkpatrick 1991; Wasser 1995; Robbins and Czekala 1997; Rolland et al. 2005). These techniques can be used to determine reproductive fitness and further our understanding of the relationship between behaviour, physiology and the environment (Wildt and Wemmer 1999).
With advances in technology, animal reproductive parameters in wildlife populations, such as hormone concentrations and reproductive cycling, have been described for a number of terrestrial mammal species including: bandicoots (Gemmell et al. 1985); Asian elephants (Brown et al. 1991); black rhinoceroses (Berkeley et al. 1997); gorillas (Czekala and Robbins 2001); feral horses (Lasley and Kirkpatrick 1991); Siberian tigers (Seal et al. 1985); one-humped camels (Marie and Anouassi 1987); and muriquis (Ziegler et al. 1997).

Although not as prominent as their terrestrial counterparts, there has been some description of reproductive cycling in marine mammal species such as killer whales (Duffield et al. 1995); polar bears (Ramsay and Stirling 1988); harbour seals (Raeside and Ronald 1981); and captive bottlenose and common dolphins (Kirby and Ridgway 1984). There are a number of advantages in studying terrestrial mammals over marine mammals as they are easier to locate, can be anaesthetised, tracked, and tagged with relative ease. In contrast, marine mammals provide researchers with a number of complex problems when assessing their basic biology and internal physiology. When comparing marine mammals, pinnipeds (seals and sea lions) and polar bears and sea otters can be accessed when they come ashore to breed or rest, but cetaceans (whales and dolphins) and sirenians (dugongs and manatees) do not come ashore at all and novel methods must be developed.

Hormone concentrations are determined after collection of a sample through invasive techniques such as blood (plasma or serum) sampling or through non-invasive techniques, such as faecal or urine sampling. Obtaining samples for hormone analysis from cetaceans can be difficult because of their aquatic locations. Anaesthesia of cetaceans, although possible, has only been accomplished by a few individuals (Haulena and Heath 2001) and therefore is not common practice,
particularly with wild animals. Blood sampling has been achieved in captive cetaceans (Duffield et al. 1995; Suzuki et al. 1998) and in some smaller wild cetaceans (St. Aubin et al. 1996) but unfortunately with fatal results in great whales (Kjeld et al. 1992; Kjeld 2001; Suzuki et al. 2001; Kjeld et al. 2004). Mansour et al. (2002) recently developed a protocol to assess progesterone concentrations in the blubber of minke whales using whales from the Greenland hunt. They suggest that blubber from biopsy sampling may be another way to assess hormone concentrations in great whales. Captive cetaceans have been trained for urine sampling (Robeck et al. 1993; 1994; Duffield et al. 1995) although some species can be particularly difficult to train (G. Bedford, personal communication). Faecal sampling has been undertaken in the North Atlantic right whale (Rolland et al. 2005) but can be difficult as great whales do not defecate at regular intervals and tend to defecate only when in their feeding grounds. Faecal sampling has been also used to determine stress in some seal species (Gulland et al. 1999; Hunt et al. 2004; Mashburn and Atkinson 2004).

Most great whales migrate annually from their breeding grounds, usually in warmer tropical waters at low latitudes, to their feeding grounds in cooler Artic or Antarctic waters at high latitudes (Dawbin 1966). As there is little to no feeding on the breeding grounds or along the migratory route, faecal samples can only be systematically collected in the feeding grounds. In the Northern Hemisphere, this is in the Arctic or Sub-Arctic areas which are readily accessible and for the coastal great whales, such as humpback whales and right whales, close to shore. In the Southern Hemisphere, faecal sampling is more difficult as the feeding areas are in the Southern Ocean near Antarctica and systematic samples cannot be easily obtained.
Assessment of the internal physiology (such as reproductive hormone concentrations) of great whales and other large cetaceans is problematic without restraint, which can endanger the animal and the lives of those that attempt it. Currently the only reproductive hormone data of great whales comes from commercial whaling on minke whales and fin whales (Kjeld 2001; Suzuki et al. 2001; Mansour et al. 2002). Subsistence whaling in the Arctic on bowhead whales also allows for further opportunity to assess hormone concentrations in some great whale species (Reeves et al. 2001). The challenge remains to assess hormone concentrations of large free-swimming whales without harm and to sample the population multiple times over a given period to gain a better understanding of their basic biology.

Reproduction and population growth are fundamental to whale conservation. In this chapter I will present our current understanding of mammalian reproductive hormones; review our current knowledge of cetacean reproductive hormones; discuss the suitability of using existing non-invasive techniques to determine reproductive hormones in cetaceans; and propose a new non-invasive method to determine reproductive hormones in free-swimming whales.
1.2. **Whales and whaling**

There are two suborders in the order Cetacea, the Odontoceti (toothed whales) and the Mysticeti (baleen whales). The great whales are all those species found in the Mysticeti, including humpback, right and fin whales; and one species of Odontoceti, sperm whales.

Great whale populations have been hunted from the time of the early Basque whalers in the sixteenth century to the present day. Commercial whaling catch limits were set to zero by the International Whaling Commission in 1982, coming into effect in 1986 (IWC Convention III-11). Scientific and subsistence whaling continues today.

With the decline of whales in the Atlantic in the 1800s, many European and American whaling ships moved to the Indian, Pacific and Southern Oceans. Until the late 1890s whales were hunted from shore-based stations and so whalers were limited in their catches by the number they could tow back to shore. The factory ship and the exploding harpoon were invented in the early 1900s (Harmer 1931; Day 1992). This meant that more whales could be caught and processed at sea, producing a sharp decline in great whale stocks in Antarctic waters.

Humpback whales in Antarctic waters were divided into six areas designated by their respective feeding areas (fig. 1.1). The areas were originally designated according to whaling areas in the Antarctic and are now used to describe the different feeding and breeding populations. Dawbin (1966) describes how those animals found in particular feeding areas in the Southern Ocean tend to be found in specific breeding areas as well. The whales off the west coast of Australia are known as the Area IV (70°E-130°E) population and, off the east coast as the Area V (130°E-170°W) population (Dawbin 1966). Area V humpback whales are thought to migrate up the east coast of
Figure 1.1: World map depicting the six areas of the Antarctic as designated by the International Whaling Commission (IWC).
Australia as well as New Zealand and Tonga. Prior to whaling in Australian waters it was estimated that there were approximately 17,000 humpback whales in Area IV (Bannister 1994) and 10,000 humpback whales off the east coast, a portion of the Area V population (Paterson and Paterson 1984). The portion of the Area V humpback whale stock that migrates along the east Australian coast is estimated to have numbered 1900 ± 250 in 1992 compared with as few as 100 at the conclusion of commercial whaling in 1962. The average annual rate of increase from 1984 to 1992 was estimated at 11.7% with a 95% confidence interval of 9.6-13.8% (Paterson et al. 1994). Brown et al. (2003) estimated the annual rate of increase to be 8.54% (s.e. = 0.05) from 1991 to 2000. This level of recovery is at a higher limit for a large mammal. This suggests that there have been no detrimental environmental impacts upon this particular stock post whaling (Paterson et al. 1994).

In 1946, the International Whaling Commission (IWC) was established to monitor catch rates and quotas for the whaling industry. As the number of whales decreased the IWC became responsible for the protection of the great whales. The Commission consists of 52 member nations and focuses on the conservation of whales to allow for an orderly development of the whaling industry. Part of the conservation program is the comprehensive assessment of whale stocks by the Scientific Committee. In 2001, a workshop into the causes of reproductive failure in the critically endangered North Atlantic right whale was convened. Unlike humpback and southern right whale populations, this northern right whale population is declining and will be extinct within 200 years (Caswell et al. 1999). This decline has been attributed to a number of different causes such as shipstrikes, entanglement and reproductive failure (IWC 2000). The workshop into reproductive failure highlighted the need to assess the internal physiology (hormonal concentrations, disease, basal health values) of great
Chapter 1 – Introduction

whales. It recommended to the IWC the development of non-lethal techniques to achieve this (Reeves et al. 2001). This study is based on these recommendations.

1.3. Hormones

Hormones are the messengers that initiate and regulate the physiological systems of higher organisms and are the means of cell-cell communication. The study of hormones has been limited in the past by the technology of the age. From the beginning of the twentieth century until around the 1960s, hormones were studied predominantly using physiological methods. These studies resulted in the discovery of approximately 25 hormones (Norman and Litwack 1997). Improved methodology for hormone assessment paralleled advances in biotechnology. The knowledge of hormones in the twentieth and twenty-first centuries is due primarily to rapid advances in technology, namely the coupling of labelled radio-isotopes with chemical means such as chromatography, mass spectrometry and x-ray crystallography.

In mammals, hormones are responsible for specific activities, are generally synthesised and expressed by different organs, and undergo metabolism (and catabolism) at varying rates. Initially, hormones were divided into three classes: polypeptide/protein, steroid and amino-acid related hormones. This review will consider only steroid hormones. For many animal populations an important factor in conserving a species is a greater understanding of population growth and reproduction (Wildt and Wemmer 1999). Steroid hormones are the class of hormones that are directly associated with mammalian reproduction.
1.3.1. Steroid Hormones

In 1929 oestrone was the first steroid hormone to be isolated in the urine of pregnant women (Butenandt and Hanisch 1935; Freeman et al. 2001). Currently there are over 225 naturally occurring steroids that have been isolated (Norman and Litwack 1997). In mammals there are six families of steroids that can be classified on both a structural and biological basis, they are the oestrogens (female sex steroids), the androgens (male sex steroids), the progestins, the mineralocorticoids, the glucocorticoids and vitamin-D (fig. 1.2). All of these steroids are biologically derived from cholesterol. For the purposes of this review, only mammalian oestrogens, androgens and progestins will be discussed.

Most steroid hormones have limited solubility in plasma, due to the presence of the ‘steroid’ rings which give them an intrinsically hydrophobic nature. Steroid hormones are transported in the blood by plasma transport proteins (PTP) from their sites of biosynthesis and secretion to the target tissues (Siiteri et al. 1982). All five of the PTPs are synthesised in the liver (Siiteri et al. 1982). The concentration of the PTP is subject to physiological regulation, where it can be increased or decreased. In theory, the amount of ‘free’ (unbound) hormone available for biological activity is subject to the amount of PTP present in the plasma.

1.3.1.1. Cholesterol

Cholesterol is the most commonly occurring steroid (fig. 1.2) and is found in practically all living organisms, including blue-green algae and bacteria (Norman and Litwack 1997). Animal products are a rich source of cholesterol, with above average concentrations in the skin, sperm cells, and egg yolk. Virtually all cell membranes in higher animals include cholesterol as an integral component and most organisms also have the capacity to biosynthesise cholesterol (Popják and Cornforth 1960).
Figure 1.2: Five ring cholesterol structure and the derivatives of these into the six classes of steroids: estrogens, androgens, progestins, mineralcorticoids, glucocorticoids and vitamin-D.
ANDROGENS

PROGESTINS

ESTROGENS

CHOLESTEROL (C-27)

VITAMIN D

ANDROGENS

Oestradiol

PROGESTINS

Oestrogen

CHOLESTEROL (C-27)

GLUCOCORTICOIDS

MINERALCORTICOIDS

Progesterone

Testosterone

Cortisol

Aldosterone
Since cholesterol is the precursor for the six classes of steroid hormone, it is important to consider its biosynthetic pathway. This pathway can be divided into four steps: 1) formation of mevalonic acid from acetate; 2) conversion of mevalonic acid into the hydrocarbon squalene (30-carbon structure); 3) oxidation of squalene into lanosterol; and 4) the processing of lanosterol to produce cholesterol. The early precursors of cholesterol biosynthesis are water soluble, but after the production of squalene the cholesterol precursors become hydrophobic. The concentration of total body cholesterol in mammals is a dynamic interplay of biosynthetic cholesterol, dietary cholesterol and excretion of cholesterol. It is important to note that dietary cholesterol is not the only source of cholesterol as more than 60% of the body's cholesterol comes from the liver and the intestines (Popják and Cornforth 1960).

1.3.1.2. Steroid synthesis

The principal tissues that synthesise the five classic steroid hormones are the ovaries (oestrogens, progestins), testes (androgens) and the adrenal gland (glucocorticoids, mineralcorticoids). During pregnancy, the foetal-placental unit can be a source of oestrogen and other hormones. The production of each steroid hormone is dependent on the stimulation of cells of origin by a specific stimulatory peptide hormone. Luteinising hormone (LH) acts on the testes to produce androgens and on the ovaries (corpus luteum) to produce progestins; follicle stimulating hormone (FSH) acts on the ovarian follicles to produce oestrogens; and placental chorionic gonadotropin (hCG) acts on pregnant female ovaries (corpus luteum) to produce progestins (fig. 1.3).
Figure 1.3: Relationship between secreted peptide hormones and the production of steroid hormones by different endocrine glands.
Peptide Hormone

- Luteinising Hormone (LH)
- Follicle Stimulating Hormone (FSH)
- Placental Chorionic Gonadotropin (hCG)
- Adrenocorticotropic Hormone (ACTH)

Endocrine Gland

- Testes (interstitial cells)
- Ovaries (follicles)
- Ovaries (corpus luteum)
- Adrenal cortex (zona fasciculata)

Steroid Hormone

- Androgens (e.g. testosterone)
- Estrogens (e.g. oestradiol)
- Progestins (e.g. progesterone)
- Glucocorticoids (e.g. cortisol)
1.3.1.3. Enzymes in steroid synthesis

Cytochrome P450 enzymes are involved in classical steroid hormone synthesis (Nerbet et al. 1987; Simpson et al. 1994). They are involved in the hydroxylation reactions that make up the metabolic pathways of steroid hormones. There are a total of six P450 enzymes involved in steroid synthesis in the ovaries, testes and adrenal cortex (fig. 1.4) (Norman and Litwack 1997). The first step of classical steroid hormone synthesis is the conversion of cholesterol into pregnenolone by the cholesterol side chain cleavage enzyme P450scc (fig. 1.4). The rate-limiting step of adrenal and gonadal steroid hormones synthesis, however, is not this enzyme but rather the side chain cleavage reaction. Specifically the delivery of cholesterol from the cytoplasm across the outer mitochondrial membrane to the inner mitochondrial membrane is actually the rate-limiting step (Black et al. 1994).

Once pregnenolone is produced it may undergo one of two conversions. It is either converted to progesterone (fig. 1.5a) or it undergoes 17-hydroxylation, leading to cortisol production in the adrenals. Importantly, progesterone is also an intermediate in the synthesis of aldosterone in the adrenals. The 17-hydroxylation of pregnenolone can also lead into the androgen/oestrogen pathways (fig. 1.5b).

1.3.1.4. Transport

The hormonally active form of steroids are free molecules, while the readily available form of most steroids are those bound to PTP, which are transported to the target tissues from the endocrine gland. A target tissue is defined as one that has stereospecific receptors permitting the accumulation of the steroid in the target tissue against a concentration gradient. This in turn permits the generation of the appropriate biological response in that target tissue for the specific steroid in
Figure 1.4: The principal pathways of human steroid hormone synthesis, P450 enzymes which are involved in the production (italics), and tissues where the production occurs: adrenal cortex (A), ovaries (O), placenta (P), testes (T) and skin (S). Modified from (Norman and Litwack 1997).
Cholesterol → Vitamin D3

O,P,T,A  Cholesterol scc

Pregnenolone → Progesterone

O,P,T,A  17α-hydroxylase/C-17-C-20 lyase

17OH-Pregnenolone → 17OH-Progesterone

O,P,T,A  17α-hydroxylase/C-17-C-20 lyase

Dehydroepiandrosterone (DHEA) → Androstenedione

O,P,T,A  O,T

Testosterone → Oestradiol

O aromatase
Figure 1.5: 

a) 21-Carbon pathway for steroid metabolism; C-21 ring is derived from the side-chain cleavage of cholesterol.  
b) Androgen and oestrogen metabolism that is a lead from the C-21 metabolic pathway; adapted from DBGET (Kanehisa 1997; Fujibuchi et al. 1998)
question. A key factor in determining the ability of a target tissue to bind the hormone is the actual blood hormone concentration. The concentration of the steroid hormone in plasma is determined by three factors: the rate at which the hormone is biosynthesised and enters the blood; the rate at which the hormone is biologically inactivated by catabolism or metabolism (in the case of steroid hormones) and the ‘tightness’ of the hormone binding to its PTP.

1.3.2. Androgens

Androgens (testosterone, dehydroepiandrosterone, androstenedione and 5α-dihydrotestosterone), commonly known as the male hormones, are the hormones that are responsible for the development and maintenance of male characteristics and are produced by the gonads (Norman and Litwack 1997; Squires 2003).

The two most important steroid hormones of the adult male are testosterone and 5α-dihydrotestosterone (DHT) (Parkinson 2001). Both are produced by the testes and circulate in the plasma. The primary site of testosterone production is in the Leydig cells of the testes (Braunstein 1994) and the manner in which it is produced is analogous with other steroid hormones. These cells are under the influence of LH which is secreted by the anterior pituitary gland (Norman and Litwack 1997). As with other steroidogenesis, side-chain cleavage of cholesterol into pregnenolone is the rate-limiting step in androgen production. There is significant target tissue metabolism of testosterone into DHT.

Testosterone plays an important role in the differentiation, growth and maintenance of the sexual reproductive tissue. In addition, testosterone, along with other hormones, plays an important role in the maintenance of the secondary sexual characteristics as well as skeletal and muscle growth (Parkinson 2001).
biological responses of the androgens may be divided into four categories: 1) growth-promoting or androgenic effects on the male reproductive system; 2) development of the male secondary sexual characteristics; 3) actions on the central nervous system and brain; and 4) a stimulatory or anabolic effect on body weight and nitrogen balance (Handelsman 1995; Parkinson 2001). Although it has been shown that testosterone and DHT are the predominant androgens, it has not been determined which is the primary initiator of the biological responses in certain tissues.

The onset of puberty is believed to occur as a result of a change in the pre-pubescent pituitary-gonadal system. Changes in the androstenedione/testosterone ratios with age and increased P450 enzyme activity in the testes of animals are indicative of sexual maturity (Pineda 2003). The rate of testosterone biosynthesis is positively correlated to the LH concentrations in the plasma (Parkinson 2001). The secretion of LH is reduced by increasing concentrations of sex steroid in the blood. These bind to steroid receptors in the hypothalamus and the pituitary and create a ‘suppressive negative feedback’ system (fig. 1.6).

Testosterone concentrations are highly variable among different individuals of a species. In seasonal breeders testosterone concentrations tend to be more variable as they vary between individuals and across seasons. Hormone concentrations are dependent on the metabolic clearance rate, secretion and release of the hormone and also on the circadian rhythm, season and age of the animal (Pineda 2003).
Figure 1.6: Negative feedback system of the hypothalamic-pituitary-gonadal axis in males (testes) and females (ovaries). Dashed lines indicate negative feedback influences and solid lines indicate positive influences.
1.3.3. Progestins and oestrogens

Progestins and oestrogens are associated with foetal development, birth, growth and female reproduction (Odell 1979). There are two distinct types of hormones involved in the female phenotype. The first of these are the peptide gonadotropin hormones, including LH and FSH, and both are produced by the anterior pituitary gland. The second type is the steroid hormones which are produced by the gonads in response to the gonadotropins. These are primarily the progestins and the oestrogens.

The ovary is the primary site for the biosynthesis of the female steroid hormones (Norman and Litwack 1997). The two most important steroid hormones in the adult female are progesterone and oestradiol (Squires 2003). Oestrone, oestriol and dehydroepiandrosterone play important roles in pregnancy (Norman and Litwack 1997). Naturally occurring progesterone has a 21-carbon (21-C) ring with an oxo-functionality on both the C-3 and C-20 (fig. 1.7a). The naturally occurring oestrogens are 18-carbon (18-C) steroids that have an aromatic A ring with a phenolic hydroxyl (fig. 1.7b).

The female reproductive system is governed by the secretion of FSH and LH from the anterior pituitary gland (Hall and Crowley 1995). The secretion of both these peptide hormones is dictated by gonadotropin-releasing hormone (GnRH). GnRH is released in a pulsatile fashion from the hypothalamus and is a primary stimulator of the release of FSH and LH. Once FSH and LH have been released they affect the ovaries and the female reproductive tract. Progesterone and oestradiol function in a negative feedback system at both the hypothalamic and the pituitary level to affect GnRH production and release (fig. 1.6) (Hall and Crowley 1995; Squires 2003).
Figure 1.7: a) The naturally occurring progestins have a 21-carbon ring with an oxo-functionality on both the C-3 and C-20. b) The estrogens have a 18-carbon ring with an aromatic ring and phenolic hydroxyl.
With the onset of puberty there is an increased output of both LH and FSH (Noakes 2001). As the ovaries become more sensitive to these hormones there is a gradual increase in the production of oestrogens and androgens. These help stimulate the development of the vagina, uterus, external genitalia and mammary glands. The onset of puberty is species dependent and is also influenced by external factors such as nutrition, season and proximity of a male (Noakes 2001). In non-seasonal polycyclic animals (such as in cows and pigs) the cyclic activity is only interrupted by pregnancy, lactation and disease (Noakes 2001; Squires 2003). In species which are seasonally polycyclic (such as horses, deer and dolphins) there are periods of anoestrous (Squires 2003). The oestrous cycle and related hormonal concentrations in animals is described in depth in Chapter 5.

1.4. Cetacean reproduction

As the analysis of hormone concentrations in cetaceans can be technically difficult much of the current knowledge of cetacean reproductive endocrinology comes from captive species (Sawyer-Steffan et al. 1983; Walker et al. 1988; Robeck et al. 1994; Duffield et al. 1995; West et al. 2000).

The reproductive system works on a negative feedback system where the increase in hormones from the gonads causes a decrease in the release of hormones from the hypothalamus and pituitary glands (fig. 1.6). There are detailed anatomical descriptions of both male and female reproductive anatomy in great whales (Matthews 1937; Chittleborough 1954; b; 1955ba). However, there is little literature on hormonal concentrations or related reproductive cycling for many great whale species. The reproductive cycles of cetaceans were determined during the whaling days (Chittleborough 1958) before assay techniques for the determination of
hormone concentrations had been developed. Chittleborough (1958) described the cycle by histological examination of the ovaries and the presence or absence of the corpus albicans.

In more recent years there has been an increased interest in assisted reproductive technologies (ART) in captive cetaceans, such as bottlenose dolphins and false killer whales (Robeck et al. 1994; Brook 1999). Concurrent with these technological advancements is a greater understanding of the endocrinology of cetaceans. However, only a limited number of cetacean species (those in captivity) have been studied and inaccuracies can occur when using one species as a model for others (Robeck et al. 2001).

1.4.1. Females

In the dolphins (Delphinidae), females are believed to have seasonal reproduction, yet this is from studies of a few species, the bottlenose dolphins, harbor porpoises, killer whales and false killer whales (Kirby and Ridgway 1984; Duffield et al. 1995; Read and Hohn 1995; Atkinson et al. 1999). Through histologic examination of the corpus albicans, it has been shown that bottlenose dolphins reach sexual maturity between 9.5 to 11 years of age (Sergeant et al. 1973; Perrin and Reilly 1984; Cockcroft and Ross 1990). With the availability of newer chemical analytical methods, baseline serum progesterone concentrations have been measured at 0.29 ng/ml for captive bottlenose dolphins during the non-cycling phase and oestrogen concentrations at 25 pg/ml (Sawyer-Steffan et al. 1983). When serum progesterone concentrations are higher than 3 ng/ml and then returned to less than 1 ng/ml during one month, it is considered that the animal has ovulated but pregnancy has not ensued (Sawyer-Steffan et al. 1983). Immature animals have progesterone concentrations less than 1 ng/ml (Kirby 1990).
In killer whales, serum progesterone concentrations range from 0.10 – 0.42 ng/ml during non-cycling to 1 - 13.7 ng/ml during non-conceptive ovulatory cycles (Duffield et al. 1995). During pregnancy, progesterone concentrations range from 1.5 - 68 ng/ml (Duffield et al. 1995). Oestrogen conjugate concentrations in killer whale urine ranged from 0.5 - 55 ng/mg Creatinine (Cr) in nonpregnant animals and 0.5 - 25 ng/mg Cr in pregnant females (Walker et al. 1988). In pregnant females pregnanediol-3α-glucuronide (PdG) range from 50 - 100 ng/mg Cr while in nonpregnant females PdG concentrations were below the level of detectability (Walker et al. 1988).

A small study of three captive false killer whales showed that plasma progesterone concentrations were below 1 ng/ml in an immature female while plasma progesterone concentrations ranged from 0.8 - 30 ng/ml in two adult females (Atkinson et al. 1999). There were seasonal changes in plasma progesterone concentrations. They were highest in summer and lowest in winter. Salivary progesterone was also measured in this study. There were no seasonal trends seen in salivary progesterone (Atkinson et al. 1999).

Using whaling carcasses, hormone concentrations have been determined for minke whales through blubber and plasma sampling and fin whales through serum sampling (Kjeld et al. 1992; Kjeld 2001; Suzuki et al. 2001; Mansour et al. 2002; Kjeld et al. 2004). In minke whales a mean blubber progesterone concentration of 132.96 ng/g was an indication of pregnancy. Non-pregnant females had a blubber progesterone concentration of 1.95 ng/g where males had a concentration of 1.86 ng/g (Mansour et al. 2002). Mean plasma progesterone concentrations were less than 0.05 ng/ml in immature minke whales, 7.08 ng/ml in mature minke whales and 6.71 – 13.84 ng/ml in pregnant minke whales (Suzuki et al. 2001; Kjeld et al. 2004).
In minke whales plasma $17\beta$-oestradiol concentrations were 0.63 pg/ml, 0.79 pg/ml and 0.77 pg/ml in immature, mature and pregnant animals respectively (Suzuki et al. 2001). In fin whales plasma progesterone concentrations ranged from 0.03 to greater than 3.14 ng/ml. Concentrations higher than 3.14 ng/ml indicated pregnancy (Kjeld et al. 1992). Serum oestradiol concentrations varied widely in the fin whale with a mean of 15.8 pg/ml (Kjeld et al. 1992).

### 1.4.2. Males

Compared to female cetaceans male hormonal concentrations have only been described for a few species. It has been shown that male wild bottlenose dolphins reach sexual maturity between 10 and 13 years (Sergeant et al. 1973; Perrin and Reilly 1984; Cockcroft and Ross 1990). There is seasonal variation in testosterone concentrations in bottlenose dolphins with concentrations being the highest during the breeding season (Schroeder 1990a). Testosterone concentrations in plasma can be utilised to classify dolphins as immature, pubescent or sexually mature (Kirby 1990; Schroeder 1990a). Sexually mature males are said to have testosterone concentrations of 2 - 5 ng/ml, rising as high as 10 ng/ml in the breeding season, whereas in pubescent animals testosterone concentrations change from 1 - 10 ng/ml. Immature animals have plasma testosterone concentrations less than 1 ng/ml (Kirby 1990; Brook 1999). In killer whales, testosterone concentrations fluctuated seasonally, ranging from 1.4 - 2.2 ng/ml (Röbeck et al. 2001).

Testosterone concentrations have been determined for minke and fin whales through sampling whale carcasses (Kjeld et al. 1992; Suzuki et al. 2001; Kjeld et al. 2004). Plasma testosterone concentrations in minke whales were 0.14 - 0.81 ng/ml in immature animals and 0.51 - 2.1 ng/ml in mature animals (Suzuki et al. 2001; Kjeld et
al. 2004). In fin whales serum testosterone concentrations ranged from 0.11 - 0.5 ng/ml depending on the season when the animal was captured (Kjeld et al. 1992).

Plasma concentrations of different reproductive hormones are useful for captive cetacean management. However plasma sampling in wild populations can be problematic. Non-invasive methods that allow for the determination of reproductive hormone concentrations of wild populations need to be developed initially using captive populations to determine the likely concentration range. These validated techniques can then be used to assess reproductive behaviour of wild cetaceans.

1.5. **Current hormonal sampling techniques**

The techniques that are currently used to assess reproductive hormones in cetaceans are blood sampling (St. Aubin et al. 1996; Suzuki et al. 1998; St. Aubin 2001), urine sampling (Robeck et al. 1993; Robeck et al. 2004) and recently blubber sampling (Mansour et al. 2002) and faecal sampling (Rolland et al. 2005). These techniques employ either radioimmunoassay (RIA) or enzyme immunoassay (EIA) to determine hormone concentrations. These methods are discussed in detail in Chapter 2.

Correlations between concentrations of hormones in pooled samples (urine or faeces) with hormone concentrations in instantaneous samples (plasma or serum) are common place. These comparative results should be treated with caution as there are different passage rates for hormones in urine and faeces (Whitten et al. 1998). Urinary steroids tend to be excreted within 4 to 8 hours with complete excretion by 24 hours (Ziegler et al. 1989; Crockett et al. 1993; Wasser et al. 1994;
Whitten et al. 1998) whereas faecal steroids can be excreted from 0.3 to 3 days after they have been secreted (Ziegler et al. 1989; Heistermann et al. 1993; Wasser et al. 1994; Whitten et al. 1998). This depends on the length of the intestinal tract and the passage rate time. Making correlations between instantaneous and pooled samples can be further complicated by other factors, such as diet and lean body mass, which may cause changes in the respective hormone concentrations.

Considering these variables, arguments arise as to whether correlations should be made between plasma and other forms of sampling, or should each sampling method describe an independent hormonal concentration range for diurnal and seasonal variations as was done for plasma? Once the trends and variations have been established for the new sampling methods, such as urine or faeces, the trends then can be compared to verify if the new method is a reliable alternative and parallels the pattern of hormone fluctuations.

1.5.1. Blood

Blood has been used in a wide range of mammalian species to ascertain hormone concentrations: from the reproductive cycle in females to glucocorticoid production during acute stress, to diurnal and seasonal changes in reproductive hormones (Gemmell et al. 1985; Pietraszek and Atkinson 1994; Romero 2002; Brown et al. 2004). Although this is a practical method for assessing hormonal fluctuations in captive management situations it is problematic when working with wild populations. Due to its invasive nature, blood samples cannot be collected on a daily basis.
1.5.2. Faecal

Faecal sampling is a non-invasive technique that has become more prominent in recent years and particularly with wild animal studies (Wasser et al. 1988; Ziegler et al. 1989; Berkeley et al. 1997; Goymann et al. 1999; Wasser et al. 2000; Lynch et al. 2003). Faeces are easy to collect and the steroid hormone concentrations reflect the reproductive cycle of the animal. But as faecal hormone concentrations are pooled over time they do not indicate instantaneous hormone concentrations. Faecal hormones are concentrated for 0.3 to 3 days prior to defecation (Whitten et al. 1998). This is particularly important if assessing whether glucocorticoids are affected by a specific event. Glucocorticoids are commonly used to determine stress in animals (Romero 2002). In order to detect if an event was “stressful” to an animal faecal samples 1 to 3 days after the event should be collected. But would be useful in situations of chronic stress.

It is also questionable whether faecal sampling is suitable for assessment of hormone concentrations in seasonal feeders such as baleen whales. Since these animals are feeding only for a portion of the year, it is not likely that faecal samples can be collected outside of their feeding areas. These samples cannot be easily collected during migration or on their breeding grounds. Baleen whales have a 10 - 12 month gestation (Matthews 1937; Jonsgård 1951; Chittleborough 1958; Laws 1961). Assessing hormone concentrations at the time of ovulation and fertilisation is important. As baleen whales are migratory, and do not feed in their breeding grounds, faecal samples may not be suitable for assessment of reproductive cycling.

1.5.3. Urine

Urine sampling has been used widely to assess hormone concentrations in mammals. In humans the most common use for hormone assays of urine is to
assess pregnancy. Urine samples have been used to determine hormone concentrations in feral horses (Kirkpatrick et al. 1988), timber wolves (McLeod et al. 1996), gorillas (Czekala et al. 1988), and killer whales (Walker et al. 1988; Robeck et al. 2004). Similar to faecal samples, urine is pooled in the bladder over a number of hours before urination occurs. It is relatively easy to collect from captive animals, for example by training in killer whales (Walker et al. 1988), or by placing trays under cages in order to catch the voided urine, such as with primates (Ziegler et al. 1989). In wild species, however, urine collection can be difficult. It is absorbed in soft ground before it can be collected (Kirkpatrick et al. 1988). In species that live in snow covered regions it is possible to obtain a urine sample from the ice or snow (McLeod et al. 1996; Constable 2001; Parslow 2002). For wild cetaceans urine collection is not possible.

1.5.4. Saliva

A variety of hormones and peptides have been assessed in the saliva of humans (Riad-Fahmy et al. 1982; Vining and McGinley 1987; Lu et al. 1999; Granger et al. 1999a; Anfossi et al. 2002) and animals (Pietraszek and Atkinson 1994; Bettinger et al. 1998; Theodorou and Atkinson 1998; Atkinson et al. 1999). Routinely RIA or EIA is used to determine hormone concentrations but recently liquid chromatography-mass spectrometry (LC-MS, described in Chapter 2) has been used to determine peptide concentrations in humans (Vickers et al. 2001). Saliva is favoured over blood sampling in many studies, particularly with children (Granger et al. 1999a). It is less stressful and allows for daily or multiple sampling. In wildlife, saliva has been used to determine progesterone concentrations in captive monk seals (Pietraszek and Atkinson 1994) and Californian sea lions (Iwata et al. 2003). In addition it has been used to determine stress in captive white-tailed deer (Millspaugh et al. 2002) and captive gorillas (Bettinger et al. 1998).
1.5.5 Other infrequently used hormone sampling methods

Milk has been used as a non-invasive alternative to plasma sampling in determining progesterone concentrations in dairy cows (Lamming and Darwash 1998) and captive bottlenose dolphins (West et al. 2000). This method is only possible with female animals and needs the animal to be lactating in order to monitor changes in the reproductive status.

Alternatively, Mansour et al. (2002) showed the feasibility of using blubber samples from whales to determine progesterone concentrations. Although the samples in this study were taken from dead whales it was reported that blubber samples collected during biopsy operations could be used as a viable option to determining pregnancy in minke whales by measuring progesterone concentrations (Mansour et al. 2002). Although the biochemical composition of fin (Lockyer et al. 1984), sei (Lockyer et al. 1985) and minke whale (Olsen and Grahl-Nielsen 2003) blubber has been described very little other work has been conducted on blubber. It is unknown if steroid hormones are found uniformly throughout blubber or if there is some form of stratification. It is difficult to use blubber biopsies as a successful measure of hormonal concentrations until hormone distribution through the blubber is described. That is, is the blubber biopsy an indication of the most recent hormone concentration or is it an indication of months past?
1.6. **Aims of this study**

Unlike terrestrial mammals, cetaceans breathe less frequently but tend to use a greater portion of their lungs with each breath (Pabst *et al.* 1999). The blow (exhalation) comes from deep inside the lungs and theoretically should carry with it small amounts of mucus from the lungs. If sufficient mucus is collected then the possibility to determine hormone concentrations in cetaceans is feasible. The hypothesis of the work presented in this thesis is that blow sampling can be used to determine reproductive hormone concentrations in cetaceans.

As no chemical assays exist for the analysis of blow samples, the primary focus of this study was to develop validated analytical techniques to determine reproductive hormones in blow samples. This two-part program consisted of method development and validation; and a field program for sample collection. Due to the nature and novel methodology employed for this study, validation and method development for reproductive hormones in cetaceans used saliva and blow samples from captive bottlenose dolphins.

The specific objectives of this research were to:

- develop a method for the determination of testosterone using liquid chromatography-mass spectrometry (LC-MS) (Chapter 3);
- describe testosterone concentrations in captive male bottlenose dolphin saliva and blow (Chapter 3);
- determine parameters of testosterone stability in saliva and blow particularly storage conditions and enzyme inhibition (Chapter 4);
- develop a method for the determination of female reproductive hormones (progesterone, oestradiol and oestrone) using LC-MS (Chapter 5);
• describe female hormone concentrations in captive female bottlenose dolphin saliva and blow (Chapter 5);
• determine parameters of progesterone stability in saliva and blow particularly storage conditions and enzyme inhibition (Chapter 6);
• develop a method to collect blow samples from free-swimming great whales (Chapter 7); and
• determine the feasibility of using blow samples to assess reproductive hormones in humpback whales using LC-MS (Chapter 7).

Blow sampling could prove to be a powerful tool to improve our understanding of reproductive cycling in great whales as it can be used in both the breeding and feeding areas. This is particularly important for critically endangered species, such as the North Atlantic right whale, as understanding reproductive cycling can lead to a better comprehension of causes of reproductive dysfunction.
CHAPTER 2

General Methodology

There is no validated analytical method to determine reproductive hormone concentrations in the blow of cetaceans. Accordingly the primary focus of this study was to determine a method that could use the small sample volumes that are obtained from blow sampling. This method needed to have a high degree of selectivity and sensitivity. Immunoassays are used routinely to assess reproductive hormone concentrations for a number of wildlife species (Schneyer et al. 1985; Czekala et al. 1986; Schroeder and Keller 1989; Robeck et al. 1993; Whitten et al. 1998; Hagey and Czekala 2003; Deschner et al. 2004). Recently an alternative method, mass spectrometry, has been used in pharmacokinetic and physiological studies (Gelpi 1995; Tiller et al. 1997; Vickers et al. 2001; Wren et al. 2003) and to assess reproductive and adrenal hormones in human plasma (Wu et al. 2002; Nelson et al. 2004), urine (Taylor et al. 2002; Rockwood et al. 2003) and environmental water samples (Croley et al. 2000).

2.1. Immunoassays

The introduction of immunoassay (Ekins 1960; Yalow and Berson 1960) was the single most important advance in biological measurement in the past 50 years (Chard 1990). Several different immunoassay methods have been developed but radioimmunoassay (RIA) and enzyme immunoassay (EIA) have been the most frequently used types to assay reproductive hormones. Both are binding assays, with the quantification of the substance being measured depending on the progressive saturation of a specific antibody by that substance and the subsequent determination of the bound and free
phases (Chard 1990). The differentiation between the bound and free phases is made by labelling the antibody with either a radioactive isotope (RIA) or an enzyme (EIA). There are advantages to each method when comparing RIA with EIA but there is very little difference between the two with respect to final quantitative results (Chard 1990).

2.2. Mass Spectrometry

A mass spectrometer produces gas-phase ions of a molecular compound. These ions are separated according to their mass-to-charge ratio ($m/z$) and the proportion to which they are detected. Using mass spectrometry (MS) coupled with gas chromatography (GC) or liquid chromatography (LC) provides a highly selective and sensitive method of chemical analysis.

Although there is a variety of different methods that can be employed when using a mass spectrometer, such as different ionisation techniques and the type of mass spectrometer used, there are common fundamental analytical requirements of the various types of MS. These requirements include: a device to introduce the compound to be analysed; an ionising source; one or more analysers to separate the ion; a detector to quantitate the ions and a data processing system (a computer).

2.2.1. History

Mass spectrometry is now used routinely in many biochemical and pharmacological laboratories. Surprisingly, the earliest recorded use of a mass spectrometer is from 1912 with the discovery of mass spectra for oxygen ($O_2$), nitrogen ($N_2$), carbon dioxide ($CO_2$), carbon monoxide (CO) and carbonyl chloride.
Chapter 2 – General Methodology

(COCl₂) (Thomson 1913). Although there were developments in mass spectrometers, it was not until 1958 that it was coupled with GC. In 1968, with the beginning of the computer era, a data processing unit was added to the GC-MS system.

The MS used for this program consisted of a quadropole analyser and an electrospray ionisation method. The quadropole analyser was invented in 1958 but it was not until the development of the electrospray in 1988 (Yamashita and Fenn 1984) that compounds over 20,000 Da could be analysed. Although MS had been around for nearly a hundred years it has been only in the past fifteen years that technological advances have allowed mass spectral analysis of large compounds. It is anticipated that mass spectral analysis will become a more commonly used method in the twenty-first century (De Hoffman et al. 1996).

2.2.2. Chromatography-Mass Spectrometry

There have been a number of technical issues associated with coupling MS with LC. LC requires chemical separation compared with GC and is used with compounds that are not volatile (i.e. not suitable for GC). In addition polar compounds for LC do not need to be derivatized as with GC. GC samples are usually derivatized to render polar substances more volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular rearrangement (Scott 1998). Samples are derivatized by either esterification or acetylation. The problem with LC is that, unlike GC, the compounds leave the column in a liquid state and it is more difficult to eliminate the solvent in the MS. Different ionisation methods have been used including thermospray, ionspray or chemical ionisation but electrospray ionisation is the preferred method for the majority of applications (De Hoffman et al. 1996).
2.2.3. Mass spectrometric analysis

Electrospray ionisation (ESI) is produced by applying a strong electric field under atmospheric pressure to a liquid passing through a capillary tube and is charged by a few kilovolts. The liquid emerges as a mist of very fine charged droplets (De Hoffman et al. 1996; Chapman 1998). A flow of drying gas (usually nitrogen to prevent chemical interaction with the analytes) assists the solvent in evaporating, causing the droplets to decrease in size. As the droplets get smaller they become unstable and explode to form smaller droplets. A cascade of smaller and smaller droplets are created until the electric field on the droplet surface is large enough to produce the desorption of ions (De Hoffman et al. 1996).

ESI is a very mild process with almost no thermal input so there is no fragmentation of the ions and only the molecular weight information is available for the mass spectrum (Chapman 1998). The most important feature of ESI is that the ions are multiple charged as MS separates ions according to their $m/z$. Multiple charged ions are also advantageous as they allow for greater sensitivity as the scanning times are reduced over the selected $m/z$ range and allow for the analysis of very large molecules using a low mass analyser.

There are three modes for MS: namely scan mode; selected-ion monitoring mode (SIM); and selected-reaction monitoring mode (SRM). Scan mode measures the complete spectra between two extreme masses, for example 50-900 Da. Although scanning allows for detection of all $m/z$ within the scan range, the sensitivity for substances is decreased due to the detection of multiple ions (fig. 2.1). Using SIM increases the sensitivity by looking for pre-determined specific masses (fig. 2.2). In SIM mode the MS acts as a sensitive and highly selective detector that can discriminate between interference from background noise or
Figure 2.1: Chromatogram of dolphin blow in scan mode (top), the mass spectrum (bottom) shows the different m/zs that can be found in the sample where the line is marked.
other materials, so quantitative analysis can be achieved with a high degree of confidence (Chapman 1998). If further sensitivity and selectivity is required then SRM can be used with a tandem mass spectrometer (MS-MS).

The data recorder and processor show the mass spectra in an interpretive format for analysis (fig. 2.2). The data are given as a function of time with the area under the curve being directly proportional to the number of ions detected. A concentration curve can be developed by analysing a number of known concentration samples. This concentration curve shows the relationship between the area under the mass spectral curve and the concentration of a particular substance by using a least-squares regression analysis (Bressolle et al. 1996).

The sensitivity of the liquid chromatography – mass spectrometry (LC-MS) assay is dependent on the choice of analyser, ion source and chromatographic conditions (Chapman 1998). It is important to develop a replicable, simple method for analysis, particularly if measuring multiple analytes.

### 2.3. Immunoassays vs. LC-MS

There are a number of advantages of LC-MS to immunoassays. Although MS may not be as sensitive compared to some immunoassays, when coupled with LC it provides a highly selective environment which has no cross-reactivity and which can analyse very small sample volumes. LC-MS can analyse sample volumes as small as 50 µl while for many immunoassays sample volumes of at least 200 µl and sometimes higher are needed. A further advantage is that not all of the sample is used during the LC-MS injections so it can be stored and re-used for further analyses which is not possible with immunoassays. Collecting samples
Figure 2.2: Chromatogram and mass spectrum of dolphin blow looking for a specific \( m/z \) ratio of 289.20.
from wild animals is difficult and expensive so reusing samples is a significant advantage. In addition, when using the scan mode in LC-MS, other biological substances can be preliminarily identified which may be of interest at a later date. Sample information is stored on CD and can be analysed or archived for future researchers to use. The unknown $m/z$ chromatographic peaks can be collected after LC column elution using a splitter directing a portion of the unknown analyte into the MS for $m/z$ identity and purity. The other portion is collected at the same time into a vial for amino acid sequencing providing complete information on previously unknown analytes.

A disadvantage of LC-MS in comparison to immunoassay is the initial capital setup cost of the equipment. Consumables are comparative in price to RIA and EIA kits. For some analytes RIA sensitivity is higher than that achieved with LC-MS but further work with tandem mass spectrometers may increase the sensitivity of current LC-MS methods.

Recent hormone concentration studies have shown that there are considerable variations between immunoassay results from different laboratories but there is sufficient reproducibility within a laboratory to be able to compare hormone concentrations of individuals (Gail et al. 1996; McShane et al. 1996). Comparative studies have been conducted to highlight the differences between immunoassays and mass spectrometry. Further to this Dorgan et al. (2002) showed that although the absolute concentrations between immunoassay and mass spectrometry may differ for some hormones, the two methods showed similar estimates of between-subject differences in serum concentrations of most sex steroid hormones. In summary there is a range of established hormone analysis techniques to choose from as an analytical method.
2.4. General Method Development

Due to the small sample volumes collected in this study immunoassay was not a viable technique. The preferred method for this study was high performance liquid chromatography (HPLC) coupled with a mass spectrometer due to sample volume constraints. As blow samples yielded volumes between 50 and 100 μl LC-MS would allow for a higher degree of accuracy and specificity to determine reproductive hormone concentrations.

The LC-MS was located at the research laboratories of the Department of Anaesthesia and Pain Management, University of Sydney at Royal North Shore Hospital, Sydney. The system compromised of a Shimadzu LC-10AD liquid chromatograph (Shimadzu, Japan) and Shimadzu SIL-10Axl autoinjector, attached to a Shimadzu LCMS-2010. The system was controlled by Shimadzu LC-MS solution 1.0 software. An Alltech Macrosphere 300Å, C8, 5µm, 150 mm x 2.1 mm HPLC column (Alltech Associates Ltd., Sydney, Australia) was used. The larger pore columns were found to be more economical than the traditional columns (100Å) as they tended to use less solvent, 0.2 ml/min (300Å) as opposed to 1 ml/min (100Å). Different LC mobile phases were used for testosterone and the female hormones due to conflicting m/zs.

LC-MS analysis was performed on a Shimadzu LC directly connected to a quadrupole Shimadzu LCMS-2010 equipped with an electrospray interface. The solvent delivery system consisted of LC10AD dual pumps. The system was conducted in positive SIM mode using the m/z determined for each of the following hormones: testosterone, progesterone, oestrone and oestradiol and in negative SIM mode for the internal standard (Fmoc-L-Gln(Trt)-OH).
The MS instrumental parameters for all hormones were: detector gain, 2kV; capillary voltage, 4.5kV; drying gas was nitrogen at 4.5 L/min; drying gas temperature, 250°C; nebulizer pressure, 6.89 MPa; ionization source at 200°C.

At the commencement of this study it was anticipated that a single LC-MS method could be developed to determine reproductive hormones in cetaceans. However, during the preliminary developmental stage, it was found that testosterone had the same m/z as formic acid (fig. 2.3) which was being used in the separation of hormones through liquid chromatography. Formic acid is added to the mobile phases to allow for the protonation of the hormones in the mass spectrometer. Acetic acid was used as an alternative to formic acid to overcome the difficulty posed by common m/z values of formic acid and testosterone but this caused analytical problems with oestradiol (fig. 2.4). Hence, separate methods were developed using acetic acid in the mobile phase for testosterone (Chapter 3) and formic acid for the remaining female reproductive hormones (Chapter 4).

In order to validate the liquid chromatographic method the following criteria were met (Bressolle et al. 1996):

i. Long-term and short-term stability of the compound in the biological matrix (Stability);

ii. Identification and inclusion of a suitable internal standard (Internal Standard);

iii. Limit of detection of the compound;

iv. Limit of quantification of the compound;

v. Concentration curve;

vi. Ruggedness of the method; and

vii. Intra- and inter-assay variability (Accuracy and Precision).
Figure 2.3: Formic acid has the same $m/z$ as testosterone.
Figure 2.4: Acetic acid has the same m/z as oestradiol.
2.4.1 Stability

Early experimental data of testosterone analysis showed the stability of testosterone in saliva and blow was markedly variable. Recovery of testosterone in saliva during the extraction process was 203 ± 55%, and in blow was 582 ± 66% (n = 10). During the extraction process samples were at room temperature (21°C) for approximately eight hours. Due to the dramatic increase in testosterone concentration, a comprehensive study on the stability of the hormones at 21°C, -20°C and -80°C was conducted. The majority of the literature which discusses stability of reproductive hormones has been conducted using human matrices of plasma and serum. To determine wildlife reproductive hormone concentrations a number of biological matrices are used, such as plasma, faeces and urine. However there is a paucity of information concerning reproductive hormone stability over both short-term and long-term storage. As stability of reproductive hormones in cetacean samples has not been validated a stability program was investigated (Chapter 4 & 6).

2.4.2 Internal Standard

GC-MS or LC-MS requires inclusion of an internal standard to control for variability in the extraction process, during chromatographic injection and from ionisation of the compound. Ideally the internal standard should have similar physical chemical properties to the substance under investigation, good resolution, and be close to the elution time of the substance under investigation (Kawakami and Montone 2002). In steroid hormone analysis internal standards are chosen because they are substances that are stable isotopes or close derivatives of the substance under investigation, such as trideuterated testosterone for testosterone (Wang et al., 2004) or 5α-cholestan for testosterone (Kawakami and Montone 2002).
However as the exact metabolic pathways for steroid hormones in dolphin saliva and dolphin and whale blow were unknown, it was inappropriate to choose an internal standard that was similar to these substances. For example, an internal standard that was labelled testosterone or a testosterone analogue would likely alter the testosterone concentration (or other hormone concentrations) in the highly complex androgen metabolic pathway (fig. 1.5). An internal standard was needed which would not interfere in any part of this pathway. As the research aim was to yield the maximum information possible from free-swimming whale blow samples a distinct neutral internal standard was investigated that would not alter the steroid hormones of interest.

A number of different compounds that were eluted in a similar manner to reproductive hormones were considered as internal standards (table 2.1). The retention times given in table 2.1 are for a 55% mobile phase B isocratic gradient (mobile phase A = 0.5% acetic acid; mobile phase B = 0.5% acetic acid, 90% acetonitrile). Initially a number of local anaesthetics and anti-depressants were tested. As synthetic compounds they were unlikely to occur in dolphin saliva and blow. The local anaesthetic, lidocaine, eluted off the liquid chromatography column close to the steroid hormones but the peak shape grossly changed when added to dolphin saliva (fig. 2.5).

A number of anti-depressants, such as trimipramine and carbamazepine, were tested but were found to bind to proteins in the saliva (Ulrich and Martens 1997) and were eluted off during solid phase extraction, e.g. trimipramine recovery was 3.5 % (n = 5).
Table 2.1: Substances tested as internal standards, molecular weight (mw), mass-to-charge ratio (m/z) and retention time on the column (RT) when using a 55% isocratic mobile phase B method (mobile phase A = 0.5% acetic acid, mobile phase B = 0.5% acetic acid, 90% acetonitrile).

<table>
<thead>
<tr>
<th>Substance</th>
<th>mw</th>
<th>m/z</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>336.46</td>
<td>337.25</td>
<td>8.8 min</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>234.30</td>
<td>235.15</td>
<td>6.5 min</td>
</tr>
<tr>
<td>Caffeine</td>
<td>194.20</td>
<td>195.05</td>
<td>3.0 min</td>
</tr>
<tr>
<td>Theophylline</td>
<td>180.20</td>
<td>181.25</td>
<td>2.4 min</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>236.26</td>
<td>237.00</td>
<td>2.8 min</td>
</tr>
<tr>
<td>Trimipramine</td>
<td>294.42</td>
<td>295.10</td>
<td>14.0 min</td>
</tr>
<tr>
<td>Sufentany</td>
<td>386.5</td>
<td>387.2</td>
<td>11.2 min</td>
</tr>
<tr>
<td>Cortisone</td>
<td>360.4</td>
<td>302.85</td>
<td>2.8 min</td>
</tr>
<tr>
<td>Penta-Substance P</td>
<td>631.0</td>
<td>613.25</td>
<td>3.4 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>635.15</td>
<td></td>
</tr>
<tr>
<td>Fmoc-L-Gln(Trt)-OH</td>
<td>610.7</td>
<td>609.15*</td>
<td>9.2 min</td>
</tr>
</tbody>
</table>

* negative ion SIM
Figure 2.5: a) Chromatogram of lidocaine standard, using 55% mobile phase B isocratic method (mobile phase A = 0.5% acetic acid, mobile phase B = 0.5% acetic acid, 90% acetonitrile), retention time = 6.5 min, m/z = 235.15.  

b) Chromatogram of lidocaine, testosterone standard and mixed dolphin saliva, using 55% mobile phase B.
Fmoc-Glutamine (Fmoc-L-Gln(Trt)-OH, Auspep, Melbourne, Australia) is a synthetically produced substance that is used in the synthesis of peptides. It was found to be the most appropriate internal standard. It fulfilled all the requirements of an internal standard for this program, as it was not endogenous in dolphin saliva or blow, it did not bind to proteins and had approximately 80% recovery in both saliva and blow (n = 20). In addition it demonstrated ideal retention times of 9.2 minutes using a 55% mobile phase B isocratic method and 11.5 minutes using a 50% mobile phase B isocratic method.

2.4.3. Other validation criteria

The remaining validation criteria: limit of detection of the compound (LOD); limit of quantification of the compound (LOQ); concentration curve; ruggedness of the method; and intra- and inter-assay variability (accuracy and precision) (Bressolle et al. 1996) are addressed in Chapter 3 (testosterone) and Chapter 5 (female reproductive hormones).
CHAPTER 3

Testosterone

3.1. Introduction

Testosterone is an androgenic steroid hormone produced by the gonads (fig. 1.1). It plays a key role in the growth, differentiation and maintenance of sexual tissues, and the development of secondary sexual characteristics (Norman and Litwack 1997; Squires 2003). In mammalian males, testosterone is one of the principal androgens and is secreted by the testes. In females, testosterone is produced in limited quantities by the ovarian follicles (Norman and Litwack 1997).

Testosterone is required for the production of sperm in the testes and for sperm maturation in the epididymis (Parkinson 2001). In addition, an animal’s libido, which is responsible for mating and aggressive behaviour, is primarily dependent on the androgenic hormones such as testosterone and 5α-dihydrotestosterone (Parkinson 2001). Although the correlation between testosterone concentration and libido has been debated, a positive correlation has been shown in some domestic species (Boyd and Corah 1988; Chenoweth 1997).

Human testosterone research has been well documented but there is less known about testosterone concentrations in wildlife. With the present focus on captive breeding programs there is a priority to understand wildlife reproductive hormonal cycles to improve management programs of potentially threatened species.
Historically, problems of declining populations or those which may be exhibiting reproductive dysfunction were assessed by studying female reproductive cycles. Little attention has been paid to male infertility as it was assumed that permanently sterile males would leave no offspring, and so would be a generational and evolutionary redundant component of the species survival (Malo et al. 2005). However, males exhibit varying degrees of fertility as a result of their genetic makeup, or may be temporarily infertile due to stress, poor nutrition or disease. In many mammalian species, male fertility is determined by semen traits or sperm motility (Malo et al. 2005). Yet in those species where semen samples cannot be collected easily, such as cetaceans, alternatives are needed to measure male fertility. Determining testosterone concentrations in relation to semen traits in captive cetaceans would allow for a clearer understanding of male fertility in wild populations.

The extensive and enduring presence of endocrine disrupting chemicals (EDCs) in the environment has been of concern for the last decade (Colburn et al. 1993). These chemicals are known to affect the endocrine system and so reproductive capacity is at risk (Baillie et al. 2003). Although studies into humans and laboratory rodents show that EDCs have an effect on male fertility, there is little knowledge of the effects on wild mammals. Testosterone concentrations are rarely reported in reproductive biology. However, concerns about the decline in male mammalian fertility highlight the need to determine baseline fluctuations in testosterone concentrations in different species (Baillie et al. 2003). Novel non-invasive methods are required to describe baseline testosterone concentrations in wild mammals. Without this background knowledge it will be difficult to determine if reproductive dysfunction in a population is due to female and/or male infertility.
Chapter 3 – Testosterone

This chapter describes a novel non-invasive sampling technique to determine testosterone concentrations in captive bottlenose dolphin saliva and blow samples. The aim of this chapter was to:

- develop a non-invasive method to determine testosterone concentrations in saliva and blow samples using LC-MS; and to
- determine a preliminary biological range of salivary and blow testosterone in captive bottlenose dolphins of different ages.

This chapter has been published in the Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences in January 2005 (Appendix 1).

3.2. Materials and Methods

3.2.1. Chemicals and solutions

All chemical reagents were of HPLC grade: acetonitrile (BioLab Scientific, Melbourne, VIC, Australia), acetic acid 17.5M (BDH Laboratory Supplies, Poole, England), water was purified by a Milli-Q system (Millipore®, Sydney, NSW, Australia) and nitrogen gas (5.0, BOC, Sydney, NSW, Australia) was of ultra-high purity.

Crystalline testosterone (Sigma-Aldrich, Sydney, NSW, Australia) was >99% purity as tested by thin layer chromatography by the manufacturer. Stock solutions were made fortnightly by dissolving testosterone in 60% acetonitrile (1 mg/ml) and stored at 4°C away from light. From the stock solution, working concentrations of testosterone at 100, 10, 1 µg/ml and 100 ng/ml, were prepared volumetrically by serial dilution with 60% acetonitrile. Stock solutions of the internal standard (Fmoc-L-Gln(Trt)-OH) were prepared in 60% acetonitrile at a concentration of 10 µg/ml. To maintain consistency with testosterone analysis in samples, 100 mM manganese...
Chapter 3 – Testosterone

chloride ($\text{MnCl}_2$; Clyde Industries, NSW, Australia), stabilising agent for testosterone, was also added to each working concentration (Chapter 4). Thus, each working concentration was 60% acetonitrile in 100mM MnCl$_2$.

3.2.2. LC analysis

The LC elution conditions for testosterone analysis were as follows: mobile phase A, 0.5% acetic acid and mobile phase B, 90% acetonitrile, 0.5% acetic acid, at isocratic conditions of 55% B and a flow rate of 0.2 ml/min. The HPLC column was equilibrated for 30 mins prior to a series of runs and maintained its performance for over 500 injections.

3.2.3. LC-MS methods

The LC-MS and settings used for the analysis are described in Chapter 2. Data acquisition was set for 11 mins using a m/z 289.20 $[\text{M} + \text{H}]^+$ (fig. 3.1a) for testosterone with zero variability of detector range. A second m/z 330.25 $[\text{M} + \text{H} + \text{CH}_2\text{CN}]^+$ was the acetylated adduct of testosterone and was used to further confirm the retention time RT (Bressolle et al. 1996). The acetylated adduct of 330.25 was present in both biological samples and testosterone standard with a ratio of approximately 30% (fig. 3.1a) compared to the m/z 289.2 peak. The Fmoc-Glutamine m/z was 609.15 $[\text{M} - \text{H}]^+$.

The data acquisition time was lengthened to 27 mins when analysing biological samples due to an interfering peak at 16 - 24 mins RT (fig. 3.2). Isocratic conditions were used as gradient conditions and equilibration of the column were found to have a longer run time.
Figure 3.1: Determination of testosterone $m/z$ by direct infusion into the MS (5 µl injection of 100 µg/ml in 60% acetonitrile), testosterone ($m/z$ 289.20, 100% relative intensity), acetylated adduct ($m/z$ 330.25, approximately 30% relative intensity).
Figure 3.2: MS chromatogram (m/z 289.20) of saliva sample from an adult male dolphin (Sirius) showing the native testosterone peak at 3.6 mins and peak at 16 - 24 mins requiring duration of isocratic conditions to have a 27 mins assay time.
3.2.4. **Sample collection and preparation**

Saliva and blow samples were collected just prior to the breeding season from four male captive bottlenose dolphins, two adults: Sirius (23 years) and Delbert (36 years); and two sub-adults: Zac, (10 years) and Buck (4.5 years). The dolphins were trained to open their mouths on cue and the saliva was collected from the oral cavity by wiping fresh cotton gauze (Smith & Nephew, Sydney, Australia; 7.5 x 7.5 cm x 4 ply) along the roof of the mouth. Saliva was then expressed from the gauze by placing it inside a 6 ml syringe and applying pressure to the plunger. Blow samples were collected using 50 ml polypropylene tubes (Sigma-Aldrich, Sydney, Australia). The tube was held inverted over the animal’s blowhole at a distance of 2 - 3 cm and the dolphins were trained to exhale on cue. Two exhalations were made per tube. There was no apparent stress to the dolphins observed by the dolphin trainers from either collection method.

To prevent degradation of testosterone in the saliva samples, 200 µl of 100mM MnCl₂ was added to each sample. For blow samples 500 µl of MnCl₂ was added and the tube was shaken vigorously to ensure that all blow mucus was collected in the solution.

Saliva samples were stored in 1.8 ml round bottom cryo-storage tubes (Sigma-Aldrich, Sydney, NSW, Australia) and blow samples were stored in 50 ml polypropylene tubes. Samples were stored at -20°C for two days before transporting on dry ice and further storage for one week at -80°C.

Testosterone was extracted from unspiked dolphin saliva and blow using a ‘Envi-Chrom P’ solid phase extraction (SPE) cartridge (Sigma-Aldrich, Sydney, NSW,
Chapter 3 – Testosterone

Australia) and was adapted from the method described by Vickers et al. (2001) for SPE of human saliva. The SPE procedure was as follows: (1) 5 ml Milli-Q water added to 200 µl of sample (saliva or blow) in a 10 ml polypropylene centrifuge tubes (Crown Scientific, Sydney, NSW, Australia), vortexed for 30 secs and then centrifuged for 10 mins at 3000 rpm to separate particulate matter before being loaded onto the SPE cartridge; (2) SPE cartridges were preconditioned using 20 ml acetonitrile and then 5 ml Milli-Q water (higher than the manufacturer’s recommendations, to completely remove interfering extraneous material) (Vickers et al. 2001); (3) samples were loaded onto the cartridges at 5 ml/min; (4) cartridges were then washed with 7.5 ml of Milli-Q water to remove salts and the eluant discarded; (5) elution of testosterone was carried out with 5 ml acetonitrile and eluant dried under nitrogen; (6) samples were reconstituted in 50 µl 60% acetonitrile (to prevent testosterone precipitation) for LC-MS analysis.

3.2.5. Validation

The method was validated for specificity, linearity, accuracy, precision, limits of quantification and detection, and stability. A calibration curve was calculated for testosterone in 60% acetonitrile for: 50, 20, 10, 5, 1 and 0.5 ng/ml, four curves were run on four separate non-consecutive days. The calibration curves were calculated with unweighted least-squares regression method (Bressolle et al. 1996). Three different runs of six repeats on separate non-consecutive days were performed to evaluate intra- and inter-batch accuracy and precision respectively using 1, 20 and 50 ng/ml spiked saliva and blow. Precision was determined by calculating the relative standard deviation (RSD, %) and accuracy by calculating the difference between the nominal and spiked values for the spiked saliva and blow samples (RE, %) (Starcevic et al. 2003). Ruggedness was ascertained by assaying stock solutions at two different concentrations: 50 ng/ml and 5 ng/ml, using the same method but with two
different 300Å C8 columns (Lot #: 02110886-1 and 02110885-1). Recovery of the SPE extraction was determined by spiking samples with high (50 ng/ml) and low (1 ng/ml) concentrations of testosterone. Samples were separated into 2 x 200 µl aliquots, one 200 µl aliquot was used for the pre-extraction assay (n = 6). The other was extracted and reconstituted in 200 µl 60% acetonitrile, and used for the post-extraction assay (n = 6).

Cotton-based sampling materials have been known to affect immunoassay results for some salivary steroid hormones (Dabbs 1991; Shirtcliff et al. 2001). To observe if this was also the case with LC-MS, a series of direct infusions with the gauze were conducted to identify possible interfering m/z values. Direct infusions were conducted in scanning mode, scan range 100 – 500 m/z.

Biological samples of saliva and blow were spiked with 50 ng/ml and 5 ng/ml testosterone standard and stored at 21°C, -20°C and -80°C. Stability experiments and results are presented in Chapter 5.

3.2.6. Statistical Analysis

Pearsons r correlation and residual analysis were used to assess linearity of the calibration curves.
3.3. Results

3.3.1 Validation

Retention time (RT) for testosterone was 3.6 min and the internal standard (IS) was 9.2 min (fig. 3.3). RTs for column A (Lot # 02110886-1) and B (Lot # 02110885-1) were 3.62 min and 3.71 min, respectively. The RSD between the results of the two columns was 8.4% (n = 12) at 5 ng/ml and 10.1% (n = 12) at 50 ng/ml. The equation for the calibration curve for testosterone (0.5 - 50 ng/ml) was $y = 0.01x + 0.0045$ and was linear ($r^2 = 0.959$, $r = 0.979$, $p < 0.001$). The residuals were normally distributed and centred around zero. The LOD (S/N 3:1, ± 5% RT) was 0.2 ng/ml, and the LOQ (S/N 5:1, ± 5% RT) was 0.5 ng/ml. Table 3.1 shows intra-batch and inter-batch precision and accuracy data.

Recovery of the SPE for saliva at 50 ng/ml testosterone was 93 ± 7.9% and at 1 ng/ml was 91.5 ± 3.72%. Recovery of 50 ng/ml of testosterone in blow was 83.3 ± 6.8% and for 1 ng/ml was 85.8 ± 4.6%. Recovery of the internal standard in saliva was 73.0 ± 14.2% and in blow was 78.63 ± 4.29%.

There were no m/z ratios in the cotton gauze that interfered with testosterone identification (fig 3.4).
Figure 3.3: Retention times of testosterone 3.59 mins (20 ng) and the IS Fmoc-Gln 9.22 mins (20 ng). HPLC conditions: 55% isocratic B, mobile phase A 0.5% acetic acid, mobile phase B 0.5% acetic acid 90% acetonitrile, 0.2 ml/min.
Figure 3.4: Direct infusion of cotton gauze into the MS. No m/z coincides with testosterone (m/z = 289.20).
**Table 3.1:** Intra-batch and inter-batch precision and accuracy for testosterone in dolphin saliva and blow.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Concentration (ng/ml)</th>
<th>n</th>
<th>Mean (ng/ml)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (RE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>1</td>
<td>6</td>
<td>0.9</td>
<td>1.6%</td>
<td>4.4%</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6</td>
<td>20.0</td>
<td>5.3%</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6</td>
<td>48.7</td>
<td>14.1%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Blow</td>
<td>1</td>
<td>6</td>
<td>1.0</td>
<td>2.5%</td>
<td>2.6%</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6</td>
<td>20.0</td>
<td>5.3%</td>
<td>-0.1%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6</td>
<td>49.4</td>
<td>10.9%</td>
<td>-1.2%</td>
</tr>
<tr>
<td>Inter-batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
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<td>18</td>
<td>1.0</td>
<td>4.5%</td>
<td>0.2%</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>18</td>
<td>20.2</td>
<td>5.5%</td>
<td>1.2%</td>
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<tr>
<td></td>
<td>50</td>
<td>18</td>
<td>49.5</td>
<td>12.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Blow</td>
<td>1</td>
<td>18</td>
<td>1.0</td>
<td>4.9%</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>18</td>
<td>20.2</td>
<td>7.3%</td>
<td>1.1%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>18</td>
<td>49.7</td>
<td>9.8%</td>
<td>0.7%</td>
</tr>
</tbody>
</table>
3.3.2. *Dolphin samples (endogenous testosterone concentrations)*

Testosterone could be measured by LC-MS in all male saliva samples (fig. 3.5a) ranging from 9.7 - 23 ng/ml (n = 10) (table 3.2). The two adult dolphins had a mean testosterone concentration of 15.2 ± 5.7 ng/ml (n = 5) and the two sub-adult dolphins had a mean testosterone concentration of 13.5 ± 3.5 ng/ml (n = 5). Testosterone in blow samples (fig. 3.5b) ranged from 14.7 - 86.2 ng/ml (n = 11) (table 3.2). The two adult dolphins had a mean testosterone concentration of 42.5 ± 21.8 ng/ml (n = 6) and the two sub-adult dolphins had a mean testosterone concentration of 59.9 ± 23.9 ng/ml (n = 5) in the blow.
Figure 3.5: a) MS chromatogram of endogenous testosterone (8.9 ng/ml) in an adult male dolphin (Sirius) saliva, (m/z 289.20, RT = 3.6 mins). (b) MS chromatogram of endogenous testosterone (4.9 ng/ml) in an adult male dolphin (Sirius) blow.
Table 3.2: Endogenous testosterone concentrations (ng/ml) in saliva and blow of male adult and sub-adult bottlenose dolphins. Three different samples were collected over three consecutive days.

<table>
<thead>
<tr>
<th></th>
<th>Saliva testosterone (ng/ml)</th>
<th>Blow testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adults</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sirius</td>
<td>19.0</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
<td>59.9</td>
</tr>
<tr>
<td></td>
<td>Not obtained</td>
<td>30.5</td>
</tr>
<tr>
<td>Delbert</td>
<td>9.9</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>14.4</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>9.9</td>
<td>28.6</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td><strong>15.2 ± 5.7</strong></td>
<td><strong>42.5 ± 21.8</strong></td>
</tr>
<tr>
<td><strong>Sub-adults</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buck</td>
<td>17.2</td>
<td>86.2</td>
</tr>
<tr>
<td></td>
<td>16.2</td>
<td>Not obtained</td>
</tr>
<tr>
<td></td>
<td>14.4</td>
<td>84.7</td>
</tr>
<tr>
<td>Zac</td>
<td>9.9</td>
<td>48.1</td>
</tr>
<tr>
<td></td>
<td>Not obtained</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>46.7</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td><strong>13.5 ± 3.5</strong></td>
<td><strong>59.9 ± 23.9</strong></td>
</tr>
</tbody>
</table>
3.4. Discussion

3.4.1 Validation

A reproducible, accurate LC-MS method has been validated for testosterone in saliva and blow in bottlenose dolphins. The intra- and inter-batch precision results were less than 15% which was acceptable for chromatographic analyses (Dadgar et al. 1995).

High recoveries for both 50 ng/ml and 1 ng/ml spiked testosterone in both saliva and blow samples indicated that the SPE is suitable for both matrices. SPE was chosen over liquid-liquid extraction as the global objective of this program was to develop a universal method that could be used to determine reproductive hormones (male and female) in great whale blow. The primary reason that SPE was chosen over the liquid-liquid extraction is that SPE allows retention and elution of virtually all analytes in the saliva and blow matrices. This method optimises the data available from these matrices particularly concerning detection of steroid hormones and in the future protein hormones (such as LH and FSH). Although liquid-liquid extraction is more rapid than SPE, it only allows retention and elution of the steroid hormones in the non-polar liquid (e.g. ether). As the composition of blow is unknown, it was considered that liquid-liquid extraction would result in loss of valuable compounds.

Biological samples collected with cotton gauze or cotton salivettes have artificially elevated concentrations of reproductive hormones when using immunoassays (Dabbs 1991; Shirtcliff et al. 2001). 'Cotton-based' sampling materials, such as gauze, can alter the results of hormonal RIAs (Dabbs 1991; Shirtcliff et al. 2001), but do not appear to affect ‘male’ hormonal results with LC-MS as demonstrated in this study.
3.4.2. Dolphin samples (endogenous testosterone concentrations)

It is possible to determine endogenous testosterone concentrations in bottlenose dolphin saliva and blow. Although sample sizes for this study were small, expected trends were observed in saliva where the two adult male dolphins had higher concentrations of testosterone than the two sub-adult males. The variability of both adult and sub-adult samples may be explained due to age. One of the adult males (Delbert) had lower concentrations of testosterone in both saliva and blow samples. Delbert at 36 years old may have fluctuating testosterone concentrations, as male and female bottlenose dolphins are known to have slowing reproductive rates from the mid-thirties onwards (Duffield et al. 1999). One of the sub-adult males (Zac) had lower testosterone concentrations due to the presence of a contraceptive implant.

Concentrations of testosterone in blow samples were higher than those in saliva. As blow sampling has not been trialled previously a comprehensive and longitudinal study of this species using the method described will provide a greater understanding of the fluctuations in testosterone concentrations in bottlenose dolphins. The measurement of hormone concentrations in captive dolphins using a non-invasive and easily trainable technique provides an outstanding opportunity to further understand the physiology of wild marine mammals. Saliva and blow samples allow for multiple daily sampling, and demonstrate no observable stress to the animals, allowing for diurnal and seasonal data measurements.
3.5. Conclusion

An accurate, reproducible and rapid LC-MS method has been developed and validated to determine testosterone concentrations in biological matrices (saliva and blow) obtained from bottlenose dolphins. Future longitudinal studies will determine diurnal and seasonal fluctuations in testosterone concentrations in saliva and blow. Used in conjunction with semen sampling, a greater understanding of the relationship between testosterone concentration and male fertility in captive dolphins could be established. This may provide guidelines to understanding male fertility in great whales.
CHAPTER 4

Testosterone Stability

4.1. Introduction

Saliva has been used for a number of years to assess steroid hormone concentrations in humans (Riad-Fahmy et al. 1982; Luisi and Franchi 1984; Vining and McGinley 1987; Dabbs 1991; Lu et al. 1999; Granger et al. 1999a) but it is only more recently salivary hormones have been measured in animals such as the monk seal (Pietraszek and Atkinson 1994; Theodorou and Atkinson 1998); western lowland gorilla (Grassi 2003); false killer whale (Atkinson et al. 1999) and bottlenose dolphin (Hogg et al. 2005).

As early as the 1980s, assessing the stability of steroid hormones in saliva became paramount (Luisi and Franchi 1984; Banerjee et al. 1985; Vining and McGinley 1987; Lipson and Ellison 1989). These studies showed that leaving saliva samples at room temperature for a number of days did not seem to have any adverse effects on the stability of different reproductive hormones (Luisi and Franchi 1984; Dabbs 1991). There are, however, differing results in reference to the stability of testosterone in saliva. Vining and McGinley (1987) found that testosterone in male salivary samples did not change over time but that testosterone in female salivary samples increased over time for no apparent reason. Other studies (Luisi and Franchi 1984; Banerjee et al. 1985; Granger et al. 1999b) have assessed the stability of testosterone in saliva at lower temperatures (−20°C and −80°C) and found that samples could be stored for six to twelve months at these temperatures with no adverse change in hormone concentrations. Studies have tended not to include independent hormone stability experiments. Granger et al. (2004), however, have recently
shown a 330.8% increase in testosterone concentrations in human saliva after four weeks storage at 4°C but found very little change in testosterone concentration at -20°C or -80°C. It is becoming a requirement for researchers to assess steroid hormone stability under different storage conditions in order to optimise sampling regimes (Khan et al. 2002; Washburn and Millspaugh 2002; Lynch et al. 2003; Granger et al. 2004).

Analytical chemists determine the stability of compounds differently to biologists and a number of articles have been published that discuss how to assess stability of compounds in biological matrices (Dadgar et al. 1995; Bressolle et al. 1996). The fundamental difference is that chemically-based studies spike biological samples with a known concentration of the compound being measured (Dadgar and Burnett 1995; Bressolle et al. 1996). This is done to ensure that observed changes in hormone concentration are at the true rate of change. Measurement of endogenous hormone concentrations, without comparable spiked standards, in the matrix to be investigated is inadvisable as the state of degradation is unknown.

In order to determine the stability of a compound in a biological matrix the following should be assessed: long-term stability of the compound in the biological matrix, stability of the reference standard, short-term stability of the biological matrix stored at different temperatures, in-process stability during the extraction process, stability of the processed sample over the maximum time from collection to analysis and the freeze-thaw stability of compounds (Dadgar and Burnett 1995). If these factors are not determined then changes that occur due to storage can mask the biological significance of a study’s results (Lynch et al. 2003).
Chapter 4 – Testosterone Stability

The structures of steroid hormones are generally consistent across mammals but there are other influencing factors in each biological matrix, whether it is plasma, saliva, urine or faeces. These affect hormones in different species in various ways. Each of these matrices contains different enzymes, bacteria and proteins which may have diverse effects on hormones. So the stability of steroid hormones in each biological matrix of a species should be assessed prior to that matrix being used to measure different life history parameters or behavioural patterns. The aim of this chapter was:

- to determine the short-term stability of testosterone in saliva and blow; and
- to determine long-term stability of testosterone in saliva and blow of captive bottlenose dolphins.

4.2. Materials and Methods

4.2.1. Sample preparation

Saliva and blow collection from four male bottlenose dolphins was described in Chapter 3. Samples were stored at –20°C for two days; shipped on dry ice; and stored at –80°C for two months prior to analysis. As this was not a kinetic study, the saliva samples from different individuals were pooled. The same was done for blow. A series of experiments was set up to determine stability of testosterone in both saliva and blow.

4.2.2. LC-MS assay

LC-MS was used to determine testosterone concentrations as described in Chapter 3.
4.2.3. Inhibitors

Preliminary analysis of spiked saliva and blow at room temperature (21°C) showed changes in testosterone concentrations. At the commencement of this program a cocktail of inhibitors consisting of leupeptin, pepstatin A (Auspep, Melbourne, Australia) and a mini-tab (Roche Diagnostics, Sydney, Australia) was used to prevent the degradation of the hormones from oral bacteria (Dadgar and Burnett 1995; Vickers et al. 2001). However, during the pilot study it was noted that when spiking samples for stability analysis at 21°C, hormone concentrations were still changing even with the cocktail mix. Two alternative inhibitors were trialled to combat any degradation that may be occurring, manganese chloride (MnCl$_2$, Clyde Industries, NSW, Australia) and amoxycillin/potassium clavulanate (Augmentin®, SmithKline Beecham, Sydney, Australia).

In order to determine the most suitable concentration of MnCl$_2$ to be used, a series of test experiments were run using 400 mM, 100 mM and 1 mM MnCl$_2$ with a 5 ng/ml spike of testosterone in saliva. Testosterone concentration was measured at time 0, 1, 2, 3 and 6 hours.

4.2.4. Freeze-thaw experiments

Pooled saliva and pooled blow were spiked with either 50 ng/ml, 20 ng/ml or 1 ng/ml of testosterone (n = 5). Due to the difficulties associated with weighing small sample volumes of crystalline testosterone the following method was used: stock standard solutions (1 mg/ml) were made in 60% acetonitrile. Serial dilution was used to obtain the desired concentrations of hormone required for spiking and the acetonitrile was dried off under air. Dry standards were then reconstituted in the equivalent volume of pooled saliva or blow. Samples were spiked and stored at -80°C between sample runs. Each spiked sample was measured after
Chapter 4 – Testosterone Stability

initial spiking and then after each thaw cycle, three freeze-thaw cycles were measured in total. Samples were not extracted but were injected directly onto the column.

4.2.5. **Short-term storage**

Samples were spiked with 50 ng/ml or 5 ng/ml of testosterone standard in order to determine which inhibitor, MnCl₂ or Augmentin®, would be more suitable to prevent degradation of testosterone in saliva and blow. Measurements at 21°C were made at time 0, 6, 9, 12, 15, and 18 hours after initial spiking. In addition to testosterone and the internal standard, either 100 mM MnCl₂ or 100 µg/ml Augmentin® was added to each sample.

4.2.6. **Long-term storage**

From this room stability experiment, MnCl₂ was deemed to be the superior inhibitor for testosterone and was used as the inhibitor for the -20°C and -80°C experiments. After initial spiking, samples were stored at -20°C and analysed at weeks 1, 2, 3 and 4; and at weeks 4, 6, 8, 10 and 12 when stored at -80°C.

All spiked samples were analysed according to the time schedules given until they exhibited a significant change to the initial spiking concentration.

4.2.7 **Statistical analysis**

Analysis of variance (ANOVA) was used to determine any significant differences in the freeze-thaw and stability studies. If the data did not meet the assumptions of ANOVA, Mann-Whitney U or Kruskal-Wallis tests were used where appropriate.
4.3. Results

4.3.1. Inhibitors

During the initial validation of the methods for this study, saliva and blow spiked with 50 ng/ml or 5 ng/ml of testosterone showed a marked increase in testosterone concentration over an eight hour period. The cocktail inhibitor mix, consisting of leupeptin, pepstatin A and a mini-tab, did not prevent the changes in testosterone concentration at both high and low spiking levels (fig. 4.1). Testosterone concentrations increased in saliva by 203 ± 55% (n = 5), and in blow by 582 ± 66% (n = 5) after eight hours.

The concentration of 100 mM MnCl$_2$ was used for all stability experiments as it had the best stabilising effect on testosterone concentrations (fig. 4.2). Comparative analysis of testosterone stability at 21°C using 100 mM MnCl$_2$ or 100 µg/ml Augmentin® as inhibitors showed MnCl$_2$ to be the superior inhibitor for testosterone in both saliva and blow (fig. 4.3).
Figure 4.1: a) Changes in 50 ng/ml spiked testosterone in bottlenose dolphin saliva and blow after eight hours at 21°C with first cocktail inhibitor used (leupeptin, pepstatin A and mini-tab). b) Changes in 5 ng/ml spiked testosterone in bottlenose dolphin saliva and blow after eight hours at 21°C with first cocktail inhibitor used (leupeptin, pepstatin A and mini-tab). Mean ± SE.
Chapter 4 – Testosterone Stability

a)

b)
Figure 4.2: Differences in testosterone concentration in saliva over six hours, following a 5 ng/ml spike of testosterone using different MnCl$_2$ concentrations (400 mM, 100 mM and 1 mM). Mean ± SE. (Data points are slightly offset in graph due to the software used).
**Figure 4.3:** (a) Stability of saliva spiked with testosterone (5 ng/ml) at 21°C with amoxycillin/potassium clavulanate (Augmentin®) and MnCl₂ over 18 hours. Mean ± one SE. (b) Stability of blow spiked with testosterone (50 ng/ml) at 21°C with amoxycillin/potassium clavulanate (Augmentin®) and MnCl₂ over 18 hours. Mean ± SE. Data are not continuous; lines linking points are shown to allow for easy interpretation of the observed trends. (Data points are slightly offset in graph due to the software used).
Chapter 4 – Testosterone Stability

(a) 

Testosterone concentration (ng/ml) vs. Hours

- MnCl\(_2\)
- Augmentin\(^{®}\)

(b) 

Testosterone concentration (ng/ml) vs. Hours

- MnCl\(_2\)
- Augmentin\(^{®}\)
4.3.2. Freeze-thaw experiments

When saliva was spiked with 50 ng/ml testosterone there was no significant change in testosterone concentration over the three freeze-thaw cycles (fig 4.4a). Yet when blow was spiked with 50 ng/ml testosterone, concentrations increased significantly (ANOVA: $F = 221.6$, df = 3, $p < 0.001$) after each cycle of freeze-thawing (fig. 4.4a). When saliva was spiked with 5 ng/ml testosterone, concentrations decreased significantly (ANOVA: $F = 13.4$, df = 3, $p < 0.001$) with each freeze-thaw cycle (fig. 4.4b). Yet when blow was spiked with 5 ng/ml testosterone, concentrations increased significantly after the first thawing and then decreased after subsequent thawing (ANOVA: $F = 7.56$, df = 3, $p = 0.002$; fig 4.4b).

4.3.3. Short-term storage

4.3.3.1. Saliva at 21°C

With no inhibitors added, testosterone concentrations in saliva samples at 21°C changed significantly within one hour if concentrations were low and within three hours if concentrations were high (table 4.1). The presence of MnCl$_2$ or Augmentin® slowed the rate of change (fig. 4.3). MnCl$_2$ proved to be the superior inhibitor as significant changes did not occur until 15 hours for 5 ng/ml spiked testosterone and at 18 hours for 50 ng/ml spiked testosterone (table 4.1). Although Augmentin® improved the stability of testosterone at 21°C, significant changes were observed at 18 hours for 50 ng/ml spiked testosterone and at 12 hours for 5 ng/ml spike testosterone (table 4.1, fig. 4.3).
Figure 4.4: a) Three cycles of freezing and thawing of saliva and blow with 50 ng/ml testosterone. b) Three cycles of freezing and thawing of saliva and blow with 5 ng/ml testosterone. Mean ± SE.
**Table 4.1:** Changes in spiked testosterone concentration in bottlenose dolphin saliva and blow at 21°C with no inhibitors or inhibitors (MnCl₂ or Augmentin®) added. Storage time is the time when changes were observed.

<table>
<thead>
<tr>
<th>Hormone concentration (ng/ml)</th>
<th>Inhibitor</th>
<th>Storage time</th>
<th>Statistical test, statistic</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ANOVA F = 2.7</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>No inhibitor</td>
<td>2 hours</td>
<td>ANOVA F = 16.6</td>
<td>3</td>
<td>&lt;0.001</td>
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<tr>
<td>5</td>
<td>No inhibitor</td>
<td>1 hour</td>
<td>Mann-Whitney U: Z = 2.3</td>
<td>4</td>
<td>0.021</td>
</tr>
<tr>
<td>50</td>
<td>MnCl₂</td>
<td>15 hours</td>
<td>ANOVA F = 2.2</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>50</td>
<td>MnCl₂</td>
<td>18 hours</td>
<td>ANOVA F = 7.8</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>MnCl₂</td>
<td>15 hours</td>
<td>K-W: chi-square = 2.6</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>Augmentin®</td>
<td>15 hours</td>
<td>ANOVA F = 1.2</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>Augmentin®</td>
<td>18 hours</td>
<td>ANOVA F = 6.3</td>
<td>5</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>Augmentin®</td>
<td>6 hours</td>
<td>ANOVA F = 0.2</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>Augmentin®</td>
<td>12 hours</td>
<td>ANOVA F = 7.6</td>
<td>3</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hormone concentration (ng/ml)</th>
<th>Inhibitor</th>
<th>Storage time</th>
<th>Statistical test, statistic</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ANOVA F = -2.6</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>No inhibitor</td>
<td>1 hour</td>
<td>Mann-Whitney U: Z = -2.6</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>No inhibitor</td>
<td>3 hours</td>
<td>ANOVA F = 0.9</td>
<td>4</td>
<td>0.003</td>
</tr>
<tr>
<td>5</td>
<td>No inhibitor</td>
<td>4 hours</td>
<td>ANOVA F = 5.5</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>MnCl₂</td>
<td>15 hours</td>
<td>ANOVA F = 2.2</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>MnCl₂</td>
<td>18 hours</td>
<td>ANOVA F = 4.2</td>
<td>5</td>
<td>0.007</td>
</tr>
<tr>
<td>5</td>
<td>MnCl₂</td>
<td>9 hours</td>
<td>ANOVA F = 2.7</td>
<td>3</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td>MnCl₂</td>
<td>13 hours</td>
<td>ANOVA F = 13.5</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>50</td>
<td>Augmentin®</td>
<td>15 hours</td>
<td>ANOVA F = 1.2</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>Augmentin®</td>
<td>18 hours</td>
<td>ANOVA F = 6.3</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>Augmentin®</td>
<td>9 hours</td>
<td>K-W: chi-square = 4.0</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>Augmentin®</td>
<td>13 hours</td>
<td>ANOVA F = 86.8</td>
<td>4</td>
<td>&lt;0.001</td>
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</tbody>
</table>
4.3.3.2. **Blow at 21°C**

Blow spiked with testosterone without inhibitors exhibited significant changes in testosterone concentrations at 21°C. Changes occurred within one hour for 50 ng/ml testosterone and within 4 hours for 5 ng/ml testosterone (table 4.1). There was no difference between MnCl₂ and Augmentin® in blow samples. Both inhibitors stabilised spiked testosterone concentrations for 18 hours at 50 ng/ml and for 13 hours at 5 ng/ml (table 4.1). However, the absolute increase in testosterone concentration was higher for samples with Augmentin® than those with MnCl₂:

**Augmentin®**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time (0) hrs</th>
<th>Time (18) hrs</th>
<th>Time (13) hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ng/ml</td>
<td>51.51 ng/ml</td>
<td>100.55 ng/ml</td>
<td></td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>5.07 ng/ml</td>
<td>11.09 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

**MnCl₂**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time (0) hrs</th>
<th>Time (18) hrs</th>
<th>Time (13) hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ng/ml</td>
<td>51.51 ng/ml</td>
<td>91.10 ng/ml</td>
<td></td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>5.10 ng/ml</td>
<td>8.00 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, at 21°C testosterone concentrations in both saliva and blow increased over a period of 12 to 18 hours before they began to decline. This was observed with both 50 and 5 ng/ml testosterone concentrations.

4.3.4. **Long-term storage**

4.3.4.1. **Saliva at -20°C**

Saliva samples spiked with 50 ng/ml testosterone showed a significant increase (ANOVA: F = 34.7, df = 4, p < 0.001) after four weeks at -20°C. Those samples spiked with 5 ng/ml testosterone showed a highly significant decrease in concentration after four weeks at -20°C (ANOVA: F = 36.0, df = 4, p < 0.001).
4.3.4.2. Blow at -20°C

Blow samples spiked with 50 ng/ml or 5 ng/ml of testosterone showed no significant changes in concentration after four weeks at -20°C.

4.3.4.3. Saliva at -80°C

When spiked with 50 ng/ml testosterone and stored at -80°C saliva samples showed a significant increase in testosterone concentration after ten weeks (ANOVA: F = 3.8, df = 4, p = 0.02). Saliva samples spiked with 5 ng/ml testosterone showed no significant change in testosterone concentration after twelve weeks at -80°C.

4.3.4.4. Blow at -80°C

Blow samples stored at -80°C showed no significant change in testosterone concentration when spiked with 50 ng/ml for 12 weeks. Blow samples spiked with 5 ng/ml testosterone significantly decreased after six weeks at -80°C storage (ANOVA: F = 5.5, df = 3, p = 0.007). Testosterone concentrations in blow spiked with 5 ng/ml were as follows: week 0 = 5.13 ± 1.29ng/ml, week 4 = 4.78 ± 0.78 ng/ml; week 6 = 3.07 ± 0.87 ng/ml; and week 8 = 4.07 ± 0.53 ng/ml (n = 5).
4.4. Discussion

Testosterone was not stable in bottlenose dolphin saliva and blow samples. Changes were observed in testosterone concentrations at a number of different stages of sample storage. Average concentrations for both saliva and blow over an eight hour period at room temperature (21°C) were two to five-fold greater than the original concentration. Although testosterone is thought to be stable at room temperature (Chattoraj and Watts 1987; Vining and McGinley 1987; Lipson and Ellison 1989), Granger et al. (2004) showed a 330.8% increase in testosterone concentrations in human saliva over 4 weeks at 4°C. This current study showed similar results for testosterone in dolphin saliva at 21°C (room temperature). For blow samples kept at 21°C with no inhibitors added, testosterone concentrations increased over time regardless of the initial spiking concentration.

All steroid hormones are derived from cholesterol (Norman and Litwack 1997) and are involved in a complicated metabolic pathway (fig. 1.5). The exact metabolic pathway of steroid hormones in bottlenose dolphins is unknown. Yet as certain steroid hormones metabolise, their structure is subsequently modified to other related hormones before further metabolism or catabolism occurs. The increases in hormone concentration seen at room temperature in the present study can be attributed to this complex system. It is believed that compounds higher up in the metabolic pathway are degrading through testosterone and so initially showing increases in concentration before decreasing. If there is no biosynthesis of hormones in saliva or blow, once compounds higher up in the pathway have degraded, no further increases in concentration should occur.
When inhibitors were added the rate of change slowed but did not cease. Some wildlife studies that use faecal samples have tested ethanol (Wasser et al. 1988; Khan et al. 2002) and sodium chloride (Wasser et al. 1988) as inhibitors. Sodium chloride was noted to alter extraction recovery (Wasser et al. 1988). Glacial acetic acid and boric acid have been recommended as inhibitors for urine, although no stability studies have been reported for them (Young and Bermes 1987). Sodium azide has been shown to stabilise testosterone in human saliva (Vining and McGinley 1987; Lipson and Ellison 1989) but was not tested as an inhibitor for this program due to its toxicity, particularly when it comes into contact with water. This study investigated marine animals and samples were transported by commercial airline so sodium azide was deemed unsuitable.

Manganese chloride was examined as an inhibitor for testosterone in this study as it is known to affect testosterone steroidogenesis in rat Leydig cells (Cheng et al. 2003). Cheng et al. (2003) showed that different concentrations of MnCl₂ had differing affects on testosterone steroidogenesis. The present study showed that a 100 mM concentration was required to stabilise testosterone in dolphin saliva and blow for a suitable length of time (more than six hours). The long-term focus of this program was to determine the feasibility of assaying reproductive hormone concentrations in whale blow. A suitable time for testosterone stability was deemed to be longer than ten hours. This takes into account samples being collected in the morning in the field, and not being put into -20°C until the end of the research day. Augmentin® was also tested as an inhibitor as it was thought that bacteria in the mouth and lungs may have an influence on testosterone concentrations. Bacterial and enzyme activity in urine and faeces are known to have an influence on steroid hormones (Chattoraj and Watts 1987; Wasser et al. 1988; Millspaugh et al. 2002). In order to maximise the stabilising power of
Augmentin® 100 µg/ml concentrations were used as an inhibitor. It was felt that anything less than this would prove ineffective.

Saliva and blow samples spiked with 50 ng/ml testosterone and blow samples spiked with 5 ng/ml testosterone showed no differences between the stabilising affects of MnCl$_2$ compared with Augmentin®. MnCl$_2$ was the superior inhibitor when saliva samples were spiked with 5 ng/ml testosterone. In addition, changes in the absolute testosterone concentrations in blow samples were higher with Augmentin® than with MnCl$_2$. Therefore, MnCl$_2$ was deemed the superior inhibitor and used for all subsequent long-term storage experiments.

Interestingly, at 21°C testosterone concentrations in both saliva and blow increased over a period of 12 to 18 hours before they began to decline. Yet at -20°C, the concentration of testosterone in saliva increased over time after spiking with 50 ng/ml testosterone whilst it decreased after spiking with 5 ng/ml testosterone. After six weeks at -80°C the blow testosterone concentration was 3.07 ± 0.87 ng/ml (a 40.16% decrease from the original concentration). Yet the concentration of the same sample after eight weeks at -80°C was 4.07 ± 0.53 ng/ml. The causes of this decrease and then increase of concentration are unknown. Further analysis of testosterone and its precursors and metabolites is required. This will determine if the increases and decreases in concentration are due to some form of metabolism.

Other studies have found no change in testosterone concentrations when samples are stored at -20°C and -80°C but those studies did not spike the samples with a known testosterone concentration, so it is unknown if there was hormone degradation when the initial sample was assayed (Luisi and Franchi
1984; Banerjee et al. 1985; Granger et al. 1999b; Granger et al. 2004). In addition, those studies assayed samples stored at lower temperatures for six to twelve months. As seen at the lower temperature analyses here, testosterone concentrations fluctuate over very short periods of time. Hormone concentrations decreased and then increased within the initial six month period. We assayed the samples every week at -20°C and initially at four weeks and every two weeks thereafter when stored at -80°C.

Further, small fluctuations in hormone concentration when samples are spiked with high concentrations of testosterone give results which may be explained by simple assay variation. The same fluctuations in hormone concentrations in samples that were spiked with low concentrations of testosterone showed significant changes. Vining and McGinley (1987) found that salivary testosterone concentrations in men reflected free hormone concentrations in plasma. However in women salivary testosterone concentrations varied widely and were often considerably higher than free plasma concentrations. Vining and McGinley (1987) did not report whether the fluctuations seen in male salivary testosterone and female salivary testosterone were similar. Small changes in concentration in low concentration samples can affect the apparent stability of a sample. This may explain why saliva and blow samples with low testosterone concentrations appear to be less stable than those samples with high concentrations.

The freeze-thaw experiments showed changes in hormone concentrations with each freeze-thawing cycle. Repetitive thawing is known to cause error with steroid hormones (Chattoraj and Watts 1987). For steroid hormones that are bound to proteins in a matrix, thawing may break these bonds causing an increase in “free” hormone concentrations. If the assay detects “free” hormone concentrations there will be an increase with each freeze-thaw cycle.
Alternatively, if the assay is designed to measure bound hormones, then breaking the hormone-protein bond would be reflected in a decrease in concentration with each freeze-thaw cycle. Caution should be taken to ensure that freeze-thawing is kept to a minimum with non-invasively and invasively collected samples prior to thawing for extraction and assay.

This study not only assessed short-term testosterone stability but long-term stability at -20°C and -80°C. This is because many wildlife studies that use non-invasive samples (Kirkpatrick et al. 1988; Wasser et al. 1988; Schoech and Lipar 1998; Goymann et al. 1999; Millspaugh et al. 2002; Terio et al. 2002; Monfort 2003) store samples at -20°C or -80°C prior to assay. Unfortunately the length of storage is not commonly mentioned in the literature. In addition, there is very little comment on the use of inhibitors to slow or cease changes in testosterone concentrations.
4.5. Conclusion

Few studies (Khan et al. 2002; Lynch et al. 2003; Granger et al. 2004) have looked at the changes of steroid hormone concentrations at varying temperatures for varying lengths of time, regardless of the biological matrix. It is now apparent that each hormone in every matrix from different species has the potential to change. Comprehensive stability studies, which include spiking the biological matrix with the hormone to be analysed, should be conducted at the different storage temperatures and lengths of storage. The complexity of the metabolic pathway of steroid hormones can complicate the manner in which hormone concentrations change over time. Concentrations can increase before they decrease and have the potential to increase again. Simply measuring the endogenous hormone concentrations for stability studies may not be indicative of hormone stability as it is unknown what stage of degradation is being measured. As non-invasive hormone analysis is becoming more widely used in wildlife studies (Monfort 2003) it is imperative that any changes in the hormone concentrations (due to: collection substrate, storage time and temperature, freezing and thawing, and extraction) are understood for the species studied and the matrix used. Unless comprehensive stability studies have been conducted caution should be used when formulating biological conclusions from a program that is based on non-invasive sampling.
CHAPTER 5

Female Reproductive Hormones

5.1. Introduction

Progesterone and oestradiol are the two most important female reproductive hormones. Progesterone is produced by the ovary (corpus luteum) and the placenta and function to prepare and maintain the body for pregnancy (Norman and Litwack 1997; Squires 2003). The major oestrogens are oestradiol, oestrone and oestriol. Oestrogens are produced by the female’s ovaries, the placenta and the foetus during pregnancy (Norman and Litwack 1997; Squires 2003). Under some circumstances the testes of males can produce significant amounts of oestradiol. For purposes of this study, progesterone, oestradiol and oestrone are discussed due to their major involvement in the oestrous cycle and pregnancy.

5.1.1. Oestrous cycle

The oestrous cycle can be divided into a number of phases: follicular, luteal and anoestrous (fig. 5.1) (Noakes 2001). The primary events of the follicular phase are the growth and development of the follicle and the maturation of the uterine endometrium. The primary events of the luteal phase are the growth and development of the corpus luteum (CL), and in the absence of pregnancy, regression of the CL (Squires 2003). Anoestrus is the prolonged period of sexual rest where there is minimal follicular growth and the corpora lutea, although identifiable is present, are non-functional. Anoestrus is generally confined to seasonal breeders (Squires 2003), although it also occurs post-partum and during lactation in many species.
During the follicular phase the dominant follicle(s) produce(s) oestradiol. This acts by positive feedback on the pituitary to increase luteinising hormone (LH) production (fig. 1.6). The ovarian follicle consists of a large round oocyte that is surrounded by follicular cells and when mature, follicular fluid that increases in volume prior to ovulation. The surge of LH causes the mature follicle to rupture releasing the oocyte (ovulation) (Norman and Litwack 1997; Squires 2003). After ovulation the granulosa cells of the follicle give rise to lutein cells which are responsible for the formation of the CL (Noakes 2001). The CL remains during most of the cycle. The lutein cells produce progesterone which inhibits gonadotropin releasing hormone (GnRH) by the hypothalamus which in turn decreases the release of LH by the pituitary (fig. 1.6) (Squires 2003).

During the luteal phase of the cycle, the CL grows quickly and produces progesterone, which increases rapidly at the beginning of the cycle (Norman and Litwack 1997). Progesterone concentrations remain high until luteolysis, which occurs 10 - 20 days after ovulation depending on the species and in the absence of pregnancy or pseudopregnancy. If fertilisation does occur then the embryo implants and generally produces proteins which prevent the regression of the CL (Squires 2003). Progesterone is required for the maintenance of pregnancy and is produced by the ovary, the placenta and the adrenal gland (Norman and Litwack 1997). The source of progesterone and the length of gestation is species-dependent.

As the CL regresses it produces a small white ovarian scar known as the corpus albicans. If the released ovum is fertilised the corpus luteum continues to grow for the first three months of pregnancy (Norman and Litwack 1997). At three months, in humans, the corpus luteum regresses and leaves a corpus albicans.
Figure 5.1: Outline of the oestrous cycle, this cycle is interrupted by periods of pregnancy and lactation and anoestrus in the case of seasonal breeders (modified from Squires 2003).
In other species, such as goats and horses, the CL is present throughout pregnancy (Squires 2003). So it is difficult to determine if the scars on an ovary are due to an ovulation resulting in pregnancy or an ovulation with no fertilisation. This is particularly important if histological examination is being used to determine the number of ovulations per cycle for a species.

In many species the onset of oestrus is determined by the acceptance of a male. As the onset and end of oestrus tend to be the only measurable points of the cycle it is used for determining cycle length (Noakes 2001). Cycle length and frequency vary among species, in domestic animals the cycle lasts for 21 days in cattle, pigs and goats (Squires 2003); 19 to 25 days in horses (Squires 2003) and 16 to 17 days in sheep (Squires 2003). In wildlife the cycle length is not as intensively studied but tends to be longer than domestic animals. Cycle length in chimpanzees is 25 to 84 days (Tutin and McGinnis 1981); in river buffalo 21 days (Ahmad 2001); in Asian elephants 52 to 64 days (Brown et al. 1991); in Nile hippopotami 29 to 40 days (Graham et al. 2002); and in killer whales 41 days (Robeck et al. 1993).

Seasonal differences in the anoestrus of many species are dependent on environmental conditions (Squires 2003). During the anoestrous period, low concentrations of oestradiol inhibit the secretion of LH by the pituitary. In some species follicular development continues during anoestrus but LH concentrations are insufficient for maturation of the follicle. In other species follicular development ceases entirely during anoestrus (Squires 2003).

Female steroid hormone production is more complex than male testosterone production. Female hormones are released to optimise reproductive capacity and
a variety of hormones, including progestins and oestrogens, are involved. Changes in progesterone concentration during human reproductive cycles have been well documented and concentrations have been determined using a number of biological matrices, such as plasma (Meulenberg and Hofman 1989; Brown et al. 1991; Wren et al. 2003), urine (Czekala et al. 1988; Li et al. 2001; O'Connor et al. 2003), and saliva (Pietraszek and Atkinson 1994; Lu et al. 1997; Laine and Ojanotko 1999; Lu et al. 1999; Groschl et al. 2001; Graham et al. 2002; Ishikawa et al. 2002).

More recently, the oestrous cycle in a number of wildlife species has been described. Changes in progesterone concentrations have been determined by RIA in plasma (Kjeld et al. 1992; Pietraszek and Atkinson 1994), urine (Czekala et al. 1988; Walker et al. 1988; Lasley and Kirkpatrick 1991), faeces (Kirkpatrick et al. 1988; Graham et al. 2002) and saliva (Pietraszek and Atkinson 1994; Atkinson et al. 1999). As progesterone plays a vital role during ovulation and throughout pregnancy it is used by conservation management agencies and captive institutions to monitor pregnancy and oestrous cycles. Knowledge of baseline progesterone concentrations and the naturally occurring changes that make up the oestrous cycle are important as unusual events or fluctuations can lead to a better understanding of reproductive dysfunction. This is particularly important in captive animal and endangered species management.

The reproductive cycle of a bottlenose dolphin can be described as polyoestrous to seasonally polyoestrous (Kirby and Ridgway 1984; Schroeder 1990a). Periods of anoestrus have been documented in this species (Brook 1997). Most cycling, however, appears to occur from spring until autumn, although calves are born all year round (Robeck et al. 2001). Detailed analysis of the bottlenose dolphin’s
cycle through blood collection has not been achieved due to ethical restrictions associated with multiple daily blood sampling (Robeck et al. 1994). Weekly samples collected over a 3-year period showed that bottlenose dolphins are spontaneous ovulators and have a 21 to 42 day oestrous cycle (Kirby and Ridgway 1984; Schroeder 1990a). Pregnancy is indicated by oestrogen concentrations remaining elevated and progesterone concentrations higher than 3 ng/ml for a period of longer than six weeks (Schroeder 1990a).

The aims of this chapter were to:

- determine progesterone, oestradiol and oestrone concentrations in female bottlenose dolphin saliva and blow samples using LC-MS; and
- describe differences in hormone concentrations for females at different stages of the oestrous cycle.

5.2. Materials and Methods

5.2.1. Chemicals and solutions

All chemical reagents were of HPLC grade: acetonitrile (BioLab Scientific, Melbourne, VIC, Australia), formic acid, 90%, 23.0M (Ajax Chemicals, Clyde Industries, Australia), water was purified by a Milli-Q system (Millipore®, Sydney, NSW, Australia) and nitrogen gas (5.0, BOC, Sydney, NSW, Australia) was of ultra-high purity.

Crystalline progesterone (Fluka, Switzerland) was >97% purity as tested by HPLC by the manufacturer. Crystalline oestrone (Sigma-Aldrich, Sydney, Australia) was of ultra high purity (99%), tested by HPLC by the manufacturer. Crystalline β-oestradiol (Sigma-Aldrich, Sydney, Australia) was of ultra high purity
(98%), tested by the manufacturer. Stock solutions were made fortnightly by dissolving crystalline compounds in 60% acetonitrile (1 mg/ml) and stored at 4°C away from light. From the stock solutions, working concentrations of progesterone at 10 and 1 µg/ml and 100 ng/ml, oestradiol at 100 and 10 µg/ml and oestrone at 100, 10 and 1 µg/ml, were prepared volumetrically by serial dilution with 60% acetonitrile. Stock solutions of the internal standard (Fmoc-L-Gln(Trt)-OH) were prepared in 60% acetonitrile at a concentration of 10 µg/ml.

5.2.2. LC Analysis

The LC elution conditions for the female hormone analysis were as follows: mobile phase A, 0.5% formic acid and mobile phase B, 90% acetonitrile, 0.5% formic acid, at isocratic conditions of 50% B and a flow rate of 0.2 ml/min. The HPLC column was equilibrated for 30 mins prior to a series of runs and maintained its performance for over 500 injections.

5.2.3 LC-MS methods

LC-MS analysis and instrumental parameters used for MS analysis have been described in Chapter 2. Direct infusions of 5 µl of 100 µg/ml of standard were used to determine the m/z of progesterone, oestradiol and oestrone. Calibration curves were determined using serial dilutions of stock standard solutions. Data acquisition for all hormones was 12 mins.

5.2.4 Sample collection and preparation

Saliva and blow samples were collected from eight female bottlenose dolphins, during the peak of the austral breeding season (February in Australia). There were seven adults and one sub-adult (Nila). Of the seven adults, two were lactating (Moki and Stormy); one was pregnant (Ava); one was possibly
reproductively senescent at 36 years old (Splash) and the remaining three were believed to be cycling (Gemma, Scooter and Squeak).

Saliva and blow collection for the female dolphins was the same as for the males. Nylon stocking was used in place of cotton gauze as it was more inert (Chapter 7). The stocking was pre-cleaned by sonicating in 100% acetonitrile for 15 mins and then sonicating in Milli-Q water for 15 mins, changing the water at 5 min intervals.

200 µl of inhibitor mix (100 mM MnCl$_2$ + 100 µg/ml amoxicillin/potassium clavulanate) was added to each sample to prevent degradation of progesterone in saliva samples (Chapter 6). For blow samples 500 µl of inhibitor mix was added and the tube shaken vigorously to ensure that all blow mucus was collected in the solution. Saliva samples were stored in cryo-storage tubes and blow samples in 50 ml polypropylene tubes. Samples collected at the dolphin’s location were stored at -20°C overnight, transported on dry ice and stored for two weeks at -80°C prior to analysis.

Samples were extracted using the method previously developed to determine testosterone concentrations in male dolphin saliva and blow (Chapter 3).

5.2.5 Validation

The method was validated for specificity, linearity, accuracy, precision, limits of quantification and detection, and stability. Calibration curves were calculated for:

1. progesterone in 60% acetonitrile at 50, 20, 10, 5, 1, 0.5 and 0.2 ng/ml;
2. oestradiol in 60% acetonitrile at 200, 100, 50 and 20 ng/ml; and
3. oestrone in 60% acetonitrile at 100, 50, 20, 10 and 5 ng/ml.
Four curves were run on four separate non-consecutive days. The calibration curves were calculated using an unweighted least-squares regression method (Nelson et al. 2004).

For progesterone, three different runs of six repeats on separate non-consecutive days were performed to evaluate intra- and inter-batch accuracy and precision, using 0.5, 5 and 50 ng/ml spiked saliva and blow. Ruggedness of the method was ascertained by assaying stock solutions at two different concentrations (50 ng/ml and 0.5 ng/ml) using the same method but with two different 300Å C8 columns (Lot #: 02110886-1 and 02110885-1). Recovery of the SPE was determined by spiking samples with high (50 ng/ml) and low (0.5 ng/ml) concentrations of progesterone. Samples were separated into 2 x 100 µl aliquots. A 100 µl aliquot was used for the pre-extraction assay (n = 6) and the other was extracted and reconstituted in 100 µl 60% acetonitrile, and used for the post-extraction assay (n = 6).

Saliva and blow samples were spiked with 50ng/ml or 0.5 ng/ml progesterone and assessed for stability at 21°C (room temperature), -20°C and -80°C. Stability experiments and results are presented in Chapter 6.

### 5.2.6 Statistical Analysis

Pearson's $r$ correlation was used to assess linearity of the calibration curves.
5.3. Results

5.3.1 Progesterone validation

The $m/z$ for progesterone was $315.20$ [$M + H]^+$ (fig. 5.2) and a second $m/z$ $356.25$ [$M + H + CH_2CN]^+$ was the acetylated adduct of progesterone and was used to further confirm the retention time (RT) (Bressolle et al. 1996). The acetylated adduct of 356.25 was present in both biological samples and progesterone standard with a ratio of approximately 70% (fig. 5.2) compared to the $m/z$ 315.20 peak. The Fmoc-Glutamine $m/z$ was $609.15$ [$M - H]^+$.

The calibration curve equation for progesterone (0.2 - 50 ng/ml) was $y = 0.076x - 0.0123$ and was linear ($r^2 = 0.995; r = 0.997; p < 0.001$). The residuals were normally distributed and centred around zero. The LOD (S/N 3:1, ± 5% RT) was 0.1 ng/ml, and the LOQ (S/N 5:1, ± 5% RT) was 0.2 ng/ml. RT for progesterone was 5.0 mins and the internal standard was 8.3 mins (fig. 5.3). RTs for column A (Lot # 02110886-1) and B (Lot # 02110885-1) were 4.9 mins and 5.0 mins, respectively. The RSD between the results of the two columns was 13.2% ($n = 12$) at 0.5 ng/ml and 8.3% ($n = 12$) at 50 ng/ml. Intra and inter-batch accuracy and precision of the method are given in Table 5.1.

SPE recovery of 0.5 ng/ml progesterone in saliva was 96.9 ± 0.1% and for 50 ng/ml was 89.2 ± 3.8%. Recovery of 0.5 ng/ml progesterone in blow was 98.7 ± 0.2 % and for 50 ng /ml, 87.7 ± 5.2%. Recovery of the internal standard in saliva was 74.0 ± 3.2% and in blow was 76.3 ± 3.4%.
Figure 5.2: Determination of progesterone m/z by direct infusion into the mass spectrometer (5 µl injection of 100 µg/ml in 60% acetonitrile), progesterone (m/z 315.20, 100% relative intensity), acetylated adduct (m/z 356.25, approximately 70% relative intensity).
Figure 5.3: RT of progesterone 5.0 mins (5 ng) and the internal standard (IS) Fmoc-Gln 8.3 mins (5 ng). HPLC conditions: 50% isocratic B, mobile phase A 0.5% formic acid, mobile phase B 0.5% formic acid 90% acetonitrile, 0.2 ml/min.
**Table 5.1**: Intra- and inter-batch precision and accuracy for progesterone in dolphin saliva and blow.

<table>
<thead>
<tr>
<th>Intra-batch</th>
<th>Sample type</th>
<th>Concentration (ng/ml)</th>
<th>n</th>
<th>Mean (ng/ml)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (RE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saliva</td>
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<td>6</td>
<td>0.5</td>
<td>3.8%</td>
<td>10.4%</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td>4.9</td>
<td>11.4%</td>
<td>-0.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>6</td>
<td>49.3</td>
<td>1.4%</td>
<td>-1.5%</td>
</tr>
<tr>
<td></td>
<td>Blow</td>
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<td>0.5</td>
<td>7.0%</td>
<td>-6.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td>5.8</td>
<td>0.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>6</td>
<td>53.3</td>
<td>1.2%</td>
<td>6.6%</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-batch</th>
<th>Sample type</th>
<th>Concentration (ng/ml)</th>
<th>n</th>
<th>Mean (ng/ml)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (RE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saliva</td>
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<td>10.8%</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>18</td>
<td>4.8</td>
<td>9.5%</td>
<td>-4.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>18</td>
<td>50.7</td>
<td>5.7%</td>
<td>1.4%</td>
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<tr>
<td></td>
<td>Blow</td>
<td>0.5</td>
<td>18</td>
<td>0.5</td>
<td>9.1%</td>
<td>-3.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>18</td>
<td>4.9</td>
<td>0.2%</td>
<td>-0.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>18</td>
<td>53.1</td>
<td>3.9%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>
5.3.2. Oestradiol validation

The \( m/z \) for oestradiol was 255.20 \([M - OH]^+\), MW = 272.39 (fig. 5.4a). The calibration curve for oestradiol (20 – 200 ng/ml) was \( y = 3524x - 15688 \) and was linear \((r^2 = 0.997; r = 0.998; p < 0.001)\). The residuals were normally distributed and centred around zero. For oestradiol the LOD was (S/N 3:1, ± 5% RT) was 10 ng/ml, and the LOQ (S/N 5:1, ± 5% RT) was 20 ng/ml. RT for oestradiol was 3.60 mins (fig. 5.5a).

5.3.3. Oestrone validation

The \( m/z \) for oestrone was 271.25 \([M + H]^+\), MW = 270.37, (fig. 5.4b). The calibration curve equation for oestrone (5 - 100 ng/ml) was \( y = 8157x - 20616 \) and was linear \((r^2 = 0.996; r = 0.998; p < 0.001)\). The residuals were centred around zero and normally distributed. The LOD for oestrone (S/N 3:1, ± 5% RT) was 2 ng/ml, and the LOQ (S/N 5:1, ± 5% RT) was 5 ng/ml. The RT for oestrone was 3.95 mins (fig. 5.5b).
Figure 5.4: a) Determination of oestradiol m/z by direct infusion into the MS (5 µl injection of 100 µg/ml in 60% acetonitrile), oestradiol (m/z 255.20, 100% relative intensity). b) Determination of oestrone m/z by direct infusion into the MS (5 µl injection of 100 µg/ml in 60% acetonitrile), oestrone (m/z 271.25, 100% relative intensity).
Figure 5.5: RTs of a) oestradiol (3.60 mins) and b) oestrone (3.95 mins), using a 50% isocratic B, mobile phase A 0.5% formic acid, mobile phase B 0.5% formic acid 90% acetonitrile, 0.2 ml/min.
5.3.4 *Dolphin samples (endogenous progesterone concentrations)*

Progesterone was quantified in the pregnant female dolphin (Ava) in both saliva and blow samples (fig. 5.6). Salivary progesterone concentration was $14.9 \pm 0.7$ ng/ml ($n = 3$) and in blow was $30.9 \pm 16.2$ ng/ml ($n = 3$).

Progesterone peaks were qualified in five of the eight remaining dolphins. Four of these animals were adults and one was the sub-adult (Nila). The progesterone peak height in the four adults was greater than the sub-adult female, but none were quantifiable (i.e. S/N ratio < 5:1, fig 5.7). Three of the adult animals were the females that were cycling, Gemma, Scooter and Squeak. The last adult was a lactating female (Stormy). No progesterone peaks were observed in the oldest adult female, Splash, or in the other lactating female, Moki.
Figure 5.6: a) MS chromatogram of endogenous progesterone (15.4 ng/ml) in pregnant dolphin (Ava) saliva (arrowed), (m/z 315.30, RT = 5.0 mins). b) MS chromatogram of endogenous progesterone (19.5 ng/ml) in pregnant dolphin (Ava) blow (arrowed).
Figure 5.7: a) MS chromatogram of endogenous progesterone in a sub-adult female dolphin (Nila) saliva (arrowed), \((m/z\ 315.30, \text{RT} = 5.0 \text{ mins})\), peak too small to quantify. b) MS chromatogram of endogenous progesterone in a sub-adult female dolphin (Nila) blow (arrowed) peak too small to quantify.
5.4. Discussion

The LOD for oestradiol and oestrone with this LC-MS method was 10 ng/ml and 2 ng/ml, respectively. As the LOD was relatively high it was felt that the sensitivity would not be low enough to determine these hormones in dolphin saliva and blow. This is because plasma oestrogen concentrations in non-cycling bottlenose dolphins are 25 pg/ml (Sawyer-Steffan et al. 1983). The LC-MS method was only fully validated for determining progesterone concentrations in saliva and blow.

The progesterone method developed here can be used to determine pregnancy in bottlenose dolphins using saliva or blow samples. Currently pregnancy is determined in captive dolphins when plasma progesterone concentrations are greater than 3 ng/ml (Schroeder 1990a) or through ultrasonography (Williamson et al. 1990). In most wild studies it is impossible to determine pregnancy and so reproductive rates are determined by the presence of a calf (Whitehead and Mann 2000). As seen with testosterone in male dolphins (Chapter 3), the concentration of progesterone in the blow sample was greater than in saliva. The reasons for this are currently unknown and further analysis of the composition of blow is warranted.

Unfortunately, only the presence or absence of progesterone can be determined in samples from cycling females as the developed method did not achieve the required sensitivity for determining precise concentrations. The presence of progesterone in saliva and blow samples shows promise for this method. Further analysis using tandem mass spectrometry should lower the limit of detection for progesterone improving the sensitivity of the method.
The progesterone peak in adult cycling females was higher than that observed in the sub-adult. The sub-adult female (Nila) was eight years old and had only recently reached puberty. Bottlenose dolphins are sexually mature at five to seven years of age (Schroeder 1990b) and reach reproductive maturity at 9.5 to 11 years (Cockcroft and Ross 1990). It is important to distinguish between sexual maturity (puberty) and reproductive maturity. Reproductive maturity is when a female is cycling regularly and has reached full adult size (Schroeder 1990b). Further, it has been theorised that bottlenose dolphins may exhibit adolescent sterility (Whitehead and Mann 2000). That is, females will begin cycling without ovulating or ovulate without conceiving. Evidence of female adolescent sterility has been observed in other dolphin species, such as killer whales and common dolphins. In many mammals the first oestrous cycle tends to be short and ‘silent’ i.e. the animal does not exhibit open signs of oestrous, but it does have increasing concentrations of progesterone (Noakes 2001). Lower detection limits of the method and longitudinal sample collection will indicate whether Nila is cycling regularly.

The complete lack of progesterone in the old female (Splash) was to be expected as she was over 36 years old. Duffield et al. (1999) showed that female bottlenose dolphins have slowing reproductive rates from mid-thirties onwards. Bottlenose dolphins are believed to live as long as 45 years (Whitehead and Mann 2000). Studies on short-finned pilot whales (Marsh and Kasuya 1984) and killer whales (Olesiuk et al. 1990) show clear evidence of menopause but data of reproductive senescence in bottlenose dolphins are inconclusive (Marsh and Kasuya 1984).
Interestingly, one of the lactating females (Stormy) showed the presence of progesterone and the other female (Moki) did not. Suckling stimuli generally suppresses the secretion of LH in lactating animals, which prevents normal follicular growth and ovulation (McNeilly 1988). However this is not the case with bottlenose dolphins. Calves are generally weaned during the first ten months of their mother’s next pregnancy (Mann et al. 2000) indicating that females begin cycling while lactating. As the calving interval for females is between three and six years (Connor et al. 1996) the difference between the two lactating females in the present study was likely due to individual variability. The calves of both females were the same age and so theoretically they should have exhibited similar concentrations of reproductive hormones.

High variability was observed in the concentrations of female reproductive hormones in blow samples and this could be due to dilution from seawater. Every effort was made to ensure that seawater did not contaminate saliva and blow samples. However, when a dolphin surfaces to breathe, seawater sometimes collects on top of the blowhole. Problems with dilution can be reduced by using an endogenous standard to standardise sample concentrations. One possible standard is creatinine which is used with urinary hormonal assays (Berman et al. 1980; Lasley and Kirkpatrick 1991). Creatinine is a by-product of endogenous muscle metabolism and is excreted in urine in a constant fashion (Ruckebusch et al. 1991). At this time, it is not known whether creatinine is present in the blow samples of cetaceans. If creatinine proves to be an impractical internal standard, then the concentration of specific trace minerals found in seawater but not in blow could be used to estimate the degree of dilution. Further work is required to determine a suitable comparative dilution compound for dolphin saliva and blow samples to ensure that seawater dilution is not an issue.
5.5. Conclusion

A replicable and accurate method for determining pregnancy in bottlenose dolphins has been developed using non-invasively collected saliva and blow samples. It is currently unsuitable for daily monitoring of oestrous cycles or for determining sexual maturity in dolphins. This is due to an inadequate limit of detection for progestins and oestrogens in the acquired samples from a single quadropole LC-MS. Recently, a comparative study between GC-MS and LC-MS found that, due to the complexity of progestins and oestrogens, LC-MS-MS is the method of choice for analysis of these compounds (Diaz-Cruz et al. 2003). Unfortunately, there was no access to a LC-MS-MS for the present program. Further development of the methods described here using LC-MS-MS may improve the assay sensitivity so that dolphin oestrous cycles may be monitored using saliva and blow samples.
CHAPTER 6

Progesterone Stability

6.1. Introduction

Methods of determining human salivary progesterone concentrations used to describe the female reproductive cycle were developed over 25 years ago (Walker et al. 1979; Connor et al. 1982; Metcalf et al. 1984). Since that time salivary progesterone has been assayed frequently in humans (Wang and Knyba 1985; Ellison et al. 1986; Meulenberg and Hofman 1989; Ellison 1993; Lu et al. 1999; Lu et al. 1999; Gröschl et al. 2001; Ishikawa et al. 2002; Stikkelbroeck et al. 2003; Schultheiss et al. 2004). Salivary progesterone concentration has been determined also in animals such as false killer whales (Atkinson et al. 1999); monk seals (Pietraszek and Atkinson 1994); Californian sea lions (Iwata et al. 2003); and capuchin monkeys (DiGiano et al. 1992).

Although salivary progesterone is used commonly there seems to be a lack of consistency in the literature in reference to the stability of progesterone in saliva samples. Early literature gives varying results for progesterone stability. It has been considered stable in human saliva samples at room temperature for 48 hours (Riad-Fahmy et al. 1982), for 72 hours (Banerjee et al. 1985), for up to six weeks (Ellison et al. 1986), and for up to six months (Lipson and Ellison 1989). Progesterone concentrations in saliva samples have been also reported to decrease over a three week period at room temperature (Gröschl et al. 2001).
Some of this variability in stability of salivary progesterone concentration comes from the use of different inhibitors to prevent degradation, such as sodium azide (Lipson and Ellison 1989; Gröschl et al. 2001) or trifluoro-acetic acid (TFA) (Gröschl et al. 2001). Different inhibitors stabilise progesterone for varying lengths of time. Other variability may come from the variety of assay techniques used in different laboratories. Hagen et al. (2003) showed that there was a large amount of variability between different laboratories using the same immunoassays. Inconsistent progesterone results were obtained from the same saliva samples when sent to different laboratories (Hagen et al. 2003). In addition, Banerjee et al. (1985) showed that storing saliva samples in polyethylene, polypropylene or glass vials could result in differing progesterone concentrations. Polyethylene vials appear to absorb approximately 30% of progesterone within three days of being stored at room temperature (Banerjee et al. 1985).

In light of these inconsistencies in the literature about the stability of human salivary hormones, a comprehensive study was developed on progesterone stability in bottlenose dolphin saliva and blow. In addition, the testosterone stability experiments that were conducted using dolphin saliva and blow (Chapter 4) highlight the need to determine hormone stability with each new hormone in different matrices.

The aim of the study presented in this chapter was to:

- determine the stability of progesterone in dolphin saliva and blow; and
- determine optimum storage conditions (including temperature and inhibitors) for dolphin saliva and blow samples.
6.2. **Materials and Methods**

6.2.1. *Sample Preparation*

Saliva and blow were collected from six female bottlenose dolphins at Sea World, the Gold Coast, Queensland, Australia (Chapter 5). The saliva samples from different individuals were pooled. The same was done for blow. Progesterone stability experiments follow those described for testosterone (Chapter 4).

6.2.2. *LC-MS assay*

LC-MS was used to determine progesterone concentrations as described in Chapter 5.

6.2.3. *Inhibitors*

Progesterone stability experiments were set up to determine which was the superior inhibitor, either 100 µg/ml amoxycillin/potassium clavulanate (Augmentin®) or 100 mM MnCl₂. Experiments with no inhibitors were used as controls.

6.2.4. *Freeze-thaw experiments*

Pooled saliva from different individuals and pooled blow from different individuals were spiked with 50 ng/ml or 0.5 ng/ml progesterone (n = 5). The method of drying off stock standard solutions to obtain these low concentrations of progesterone has been described (Chapter 4). Samples were spiked and stored at -80°C between sample runs. Each spiked sample was analysed after initial spiking and then after each thaw cycle. A total of three freeze-thaw cycles were conducted. Samples were not extracted but were injected directly onto the column.
6.2.5. Short-term storage

In order to assess stability of progesterone at 21°C, saliva and blow samples were spiked with 50 ng/ml or 0.5 ng/ml progesterone. Three different treatments were set up: no inhibitors (NI), addition of 100 μg/ml Augmentin®, or addition of 100 mM MnCl₂. Measurements at 21°C were made at time 0, 3, 6, 9, 12, 15 and 18 hours after initial spiking.

6.2.6. Long-term storage

The stability of all three treatments (no inhibitors, MnCl₂, and Augmentin®) was assessed at -20°C and -80°C. After initial spiking, samples were stored at -20°C and analysed at weeks 0, 1, 2, 3 and 4; and at weeks 0, 4, 6, 8, 10 and 12 when stored at -80°C. All spiked samples were analysed according to the time schedules given until they exhibited a significant change relative to the initial spiking concentration.

6.2.7. Statistical analysis

Significant differences in progesterone concentration in the freeze-thaw and stability studies were determined by ANOVA. Mann-Whitney U or Kruskal-Wallis tests were used if the data did not meet the assumptions of ANOVA.
6.3. Results

6.3.1. Freeze-thaw experiments

When samples were spiked with 50 ng/ml progesterone, the progesterone concentration in the saliva (ANOVA: $F = 10.61$, $df = 3$, $p < 0.001$) and in the blow (ANOVA: $F = 47.39$, $df = 3$, $p < 0.001$) increased significantly after each cycle of freeze-thawing (fig. 6.1a). Similarly, when samples were spiked with 0.5 ng/ml progesterone, the progesterone concentration in the saliva (ANOVA: $F = 35.90$, $df = 3$, $p < 0.001$) increased significantly after each freeze-thaw cycle. Interestingly, blow samples spiked with 0.5 ng/ml progesterone increased slightly over two freeze-thaw cycles and significantly decreased (ANOVA: $F = 3.41$, $df = 3$, $p = 0.047$) after the third freeze-thaw cycle (fig. 6.1b).

6.3.2. Short-term storage

6.3.2.1. Saliva at 21°C

At 21°C with no inhibitors added, progesterone concentrations in saliva significantly decreased (table 6.1) within three hours when spiked with 50 ng/ml progesterone. Yet when spiked with 0.5 ng/ml progesterone, concentrations of progesterone increased significantly within one hour of initial spiking (table 6.1).

When MnCl$_2$ was added to the saliva significant increases in progesterone concentration were observed after 12 hours with 50 ng/ml and after nine hours with 0.5 ng/ml spiking (table 6.1). Progesterone concentrations increased by 98.1% for 50 ng/ml spiking over 18 hours ($t_0 = 49.04 \pm 4.74$ ng/ml vs. $t_{18} = 97.13 \pm 26.95$ ng/ml) then started to decrease by 21 hours ($t_{21} = 77.38 \pm 12.67$ ng/ml).
Figure 6.1: a) Three cycles of freezing and thawing of saliva and blow with 50 ng/ml progesterone. b) Three cycles of freezing and thawing of saliva and blow with 0.5 ng/ml progesterone. Mean ± SE.
Table 6.1: Changes in spiked progesterone concentration in bottlenose dolphin saliva and blow at 21°C with no inhibitors or inhibitors (Augmentin® or MnCl₂) added. Storage time indicates the time when changes were first observed.

**SALIVA**

<table>
<thead>
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<th>Hormone concentration (ng/ml)</th>
<th>Inhibitor</th>
<th>Storage time</th>
<th>Statistical test statistic</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NS</td>
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**BLOW**

<table>
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<th>Statistical test statistic</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
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<td>NS</td>
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<td>No inhibitor</td>
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<td>ANOVA: F = 2.7</td>
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<td>NS</td>
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<td>9 hours</td>
<td>ANOVA: F = 5.8</td>
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<td>18 hours</td>
<td>ANOVA: F = 1.23</td>
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<tr>
<td>0.5</td>
<td>Augmentin®</td>
<td>18 hours</td>
<td>ANOVA: F = 1.3</td>
<td>6</td>
<td>NS</td>
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</table>
In saliva spiked with 0.5 ng/ml progesterone concentrations increased by 104.0% over 18 hours ($t_0 = 0.54 \pm 0.04$ ng/ml vs. $t_{18} = 1.10 \pm 0.24$ ng/ml) and then began to decrease by 21 hours ($t_{21} = 0.78 \pm 0.14$ ng/ml).

Similarly, when Augmentin® was added to saliva significant increases in progesterone concentrations occurred after nine hours for both 50 ng/ml and 0.5 ng/ml spiking (table 6.1). Progesterone concentrations increased 79% over 18 hours for 50 ng/ml ($t_0 = 49.22 \pm 3.31$ ng/ml vs. $t_{18} = 87.87 \pm 16.69$ ng/ml) but after 21 hours concentrations in the the 50 ng/ml spiked samples began to decrease ($t_{21} = 77.38 \pm 5.66$ ng/ml). For those samples spiked with 0.5 ng/ml, progesterone concentrations increased by 73% over 18 hours ($t_0 = 0.53 \pm 0.02$ ng/ml vs. $t_{18} = 0.91 \pm 0.17$ ng/ml). As with the higher spiking concentration, after 21 hours the progesterone concentrations began to decrease for 0.5 ng/ml spiked samples ($t_{21} = 0.80 \pm 0.06$ ng/ml).

6.3.2.2. Blow at 21°C

When blow samples were spiked with 50 ng/ml progesterone and no inhibitors were added, significant changes in progesterone concentration occurred after four hours at room temperature (table 6.1). Significant increases were observed after two hours at room temperature when blow was spiked with 0.5 ng/ml progesterone (table 6.1).

The addition of inhibitors greatly improved the stability of progesterone in blow samples. When MnCl$_2$ was added to blow samples spiked with 50 ng/ml progesterone, no significant changes in concentration were observed over 18 hours. However when blow was spiked with 0.5 ng/ml progesterone a significant increase was observed after 12 hours (table 6.1). When Augmentin® was added
to blow samples that were spiked with either 50 ng/ml or 0.5 ng/ml progesterone, there were no significant changes in progesterone concentration over 18 hours.

6.3.3. Long-term storage

6.3.3.1. Saliva at -20°C

When saliva was stored at -20°C, significant changes were observed in spiked progesterone concentrations (table 6.2). However, the time it took for changes to be observed differed depending on whether or not an inhibitor had been added to the sample. Progesterone concentrations decreased significantly after one week for both 50 ng/ml and 0.5 ng/ml spiking (table 6.2) if no inhibitors were added. When MnCl$_2$ was added, significant decreases occurred in both 50 ng/ml and 0.5 ng/ml samples after one week (table 6.1). The addition of Augmentin® improved the stability of progesterone at -20°C to more than three weeks for 50 ng/ml and more than four weeks for 0.5 ng/ml progesterone (table 6.2).

6.3.3.2. Blow at -20°C

Progesterone concentrations decreased significantly in blow samples spiked with 50 ng/ml or 0.5 ng/ml progesterone without any inhibitor after one week at -20°C (table 6.2). The addition of MnCl$_2$ did not improve the stability of progesterone in blow. Significant decreases in progesterone concentration were seen after one week for both 50 ng/ml and 0.5 ng/ml spiked samples (table 6.2). The addition of Augmentin® improved progesterone stability in blow at -20°C. There were no significant changes in progesterone concentration for both 50 ng/ml and 0.5 ng/ml spiked samples after four weeks at -20°C (table 6.2).
Table 6.2: Changes in spiked progesterone concentration in bottlenose dolphin saliva and blow at -20°C with no inhibitors or inhibitors (Augmentin® or MnCl₂) added. Storage time indicates the time when changes were first observed.

<table>
<thead>
<tr>
<th>Hormone concentration (ng/ml)</th>
<th>Inhibitor</th>
<th>Storage time</th>
<th>Statistical test statistic</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>ANOVA: F = 143.5</td>
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<td>Mann-Whitney: Z = 2.9</td>
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<td>NS</td>
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<th>df</th>
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<tr>
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<td>4 weeks</td>
<td>ANOVA: F = 1.4</td>
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6.3.3.3. Saliva at -80°C

When saliva was stored at -80°C, significant changes in progesterone concentration were observed (table 6.3). With no inhibitors added, progesterone concentrations decreased after 4 weeks for both 50 ng/ml and 0.5 ng/ml spiked samples. The addition of MnCl₂ to the spiked saliva samples (50 ng/ml or 0.5 ng/ml) did not improve progesterone stability (table 6.3). However, the addition of Augmentin® improved salivary progesterone stability at -80°C. Significant increases in progesterone concentration were seen in 50 ng/ml spiked samples after 12 weeks. There was no significant change in 0.5 ng/ml spiked samples with Augmentin® after 12 weeks at -80°C (table 6.3).

6.3.3.4. Blow at -80°C

When no inhibitors were added the concentration of progesterone significantly decreased in both 50 ng/ml and 0.5 ng/ml spiked samples after four weeks (table 6.3). The addition of MnCl₂ to spiked blow samples did not improve the stability of progesterone. Significant decreases in progesterone concentration were seen after four weeks for both 50 ng/ml and 0.5 ng/ml spiked samples (table 6.3). When Augmentin® was used as an inhibitor, progesterone stability in blow samples improved. Significant changes in progesterone concentration were observed after eight weeks when spiked with 50 ng/ml and after six weeks when spiked with 0.5 ng/ml progesterone (table 6.3).
Table 6.3: Changes in spiked progesterone concentration in bottlenose dolphin saliva and blow at -80°C with no inhibitors or inhibitors (Augmentin® or MnCl₂) added. Storage time indicates the time when changes were first observed.

### SALIVA

<table>
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<tr>
<th>Hormone concentration (ng/ml)</th>
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### BLOW

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<td>6 weeks</td>
<td>K-W: Chi-Square = 6.9</td>
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</table>
6.4. Discussion

Progesterone was unstable in bottlenose dolphin saliva and blow samples. The degree of stability varied depending on storage temperature and whether inhibitors were used.

The freeze-thaw experiments showed increases in progesterone concentrations with each freeze-thaw cycle except for 0.5ng/ml progesterone in blow which decreased at the third freeze-thaw cycle. As discussed with testosterone stability, repetitive thawing is known to cause error with steroid hormones (Chattoraj and Watts 1987). The increase and/or decrease in concentration could be due to the breaking of chemical bonds during thawing. Every effort should be made to ensure that freezing and thawing is kept to a minimum when storing and shipping biological samples for hormone analysis.

When inhibitors were not added, progesterone concentrations changed rapidly in both saliva and blow samples. As with testosterone the addition of either MnCl$_2$ or Augmentin$^\text{®}$ improved the stability of progesterone in saliva and blow at room temperature. Although MnCl$_2$ was a slightly better inhibitor for saliva at room temperature, Augmentin$^\text{®}$ proved to be the superior inhibitor overall. At lower temperatures, MnCl$_2$ did not stabilise progesterone as there was no difference between samples with or without MnCl$_2$. This indicates that bacteria may have influenced progesterone stability at lower temperatures, as stability improved with the addition of a broad spectrum antibiotic.

Interestingly, salivary progesterone concentrations at room temperature increased over an 18 hour period. This trend was seen in saliva samples regardless of the presence or absence of the inhibitor. Yet when the same
samples were assayed at 21 hours after spiking the progesterone concentrations had begun to decrease. There is some progesterone metabolism in human saliva (Laine and Ojanotko 1999) but this did not affect salivary progesterone concentrations significantly. Progesterone metabolism in human saliva was attributed to the cellular content of saliva. Metabolic activity was greater in whole saliva than in centrifuged saliva (Laine and Ojanotko 1999). As steroid metabolism has not been determined in dolphin saliva, the rate of metabolic activity may be higher than in humans. In addition the stability experiments conducted in this study used whole saliva.

There were no significant changes in progesterone concentration when Augmentin® was used with blow samples. This indicates that changes in blow progesterone concentrations are more than likely due to bacterial changes rather than metabolic ones. Cetaceans are known to have a number of infectious and non-infectious diseases in their respiratory system (Dunn et al. 2001; Gulland et al. 2001). Microbiological studies of cetacean blow will allow for a greater understanding of the different types of bacteria found in blow.

MnCl₂ was tested as an inhibitor in this program as it was an effective inhibitor for testosterone. However, it does not appear to have an affect on progesterone stability at low temperatures. There may be more bacterial activity occurring at lower temperatures and MnCl₂ has no affect on bacteria. Alternatively, there may be metabolic activity occurring at lower temperatures. Although MnCl₂ is known to influence testosterone steroidogenesis (Cheng et al. 2003) it may have no effect on progesterone metabolism because the androgen/oestrogen metabolic pathway is separate to the progesterone metabolic pathway (fig. 1.5). Progesterone stability studies in dolphin saliva and blow that assess both
progesterone and its metabolites will clarify this issue. Alternatively, it may be possible to use a progesterone steroidogenesis inhibitor.
6.5 Conclusion

From this study it has been determined that dolphin saliva can be stored for six hours at 21°C, four weeks at -20°C and eight weeks at -80°C with the addition of 100 µg/ml Augmentin® before changes in progesterone concentrations occur. Dolphin blow can be stored with 100 µg/ml Augmentin® added for 18 hours at 21°C, four weeks at -20°C and four weeks at -80°C before changes in progesterone concentrations occur. In order to minimise any changes in endogenous hormone concentration samples should be extracted as soon as possible. It is also advised that samples are shipped on dry ice or in liquid nitrogen to prevent any freeze-thawing during transit.

Many biological programs are conducted under the premise that samples are stable at -20°C for a number of months or years without inhibitors added. Stability studies of progesterone in non-invasively collected samples, such as faeces (Schlenker et al. 1999; Lynch et al. 2003), urine (Kesner et al. 1995) and saliva (Lipson and Ellison 1989; Gröschl et al. 2001), have revealed changes in progesterone concentration over varying lengths of time and storage temperature. Similar results were found in this study. If stability is not addressed then observed changes in hormone concentrations may be an artefact of storage time and temperature, rather than an indication of an animal’s biological activity.
CHAPTER 7

Whale Blow Collection: Is It Feasible?

“They [whales] are blowing on every point of the compass, and frequently taint the whole atmosphere with the utmost disagreeable effluvia that can be conceived.” Charles Clerke, Lieutenant of the Resolution in his journal, 30 Dec 1774, off Tierra del Fuego.

7.1. Introduction

Anatomical and physiological studies of whales were carried out on whaling ships or in whaling stations (Matthews 1937; Chittleborough 1955; Chittleborough 1965). Since commercial whaling ceased in the 1980s, reproductive studies on large whale species have been limited post whaling.

It is problematic to determine the reproductive hormone cycles of great whales (Chapter 1). So our current understanding of their breeding behaviour is from histological analysis from the whaling days and more recent photo-identification studies. Behavioural studies have led to a greater understanding of breeding biology in some species, such as humpback whales, but many questions remain unanswered. Most whale populations around the world appear to be recovering, while others, such as the blue whales in the Gulf of St. Lawrence and the North Atlantic right whale, have low reproductive rates (Caswell et al. 1999). Why the reproductive rates of some populations are so low is unknown. It is impossible to determine if these low reproductive rates are a result of reproductive dysfunction or due to other external factors.
7.1.1. Humpback whale reproduction

Humpbacks (Cetacea: Balaenopteridae) whales migrate annually from their polar and temperate feeding grounds to their tropical breeding areas (Matthews 1937; Dawbin 1966).

Age is determined in this species by the number of growth layers that are present in the animal’s ear wax plugs (Chittleborough 1959; Lockyer 1984). Chittleborough (1959) believed that there were four growth layers per year and so age of sexual maturity was between five and six years. However, Lockyer (1984) estimated that there were only two growth layers per year, indicating that the age of sexual maturity was from ten to twelve years. Ongoing photo-identification studies of different humpback whale populations have indicated that the age of sexual maturity is between five and seven years with some animals reaching maturity as late as ten years (Clapham and Mayo 1987; Clapham et al. 1993). These estimates were obtained from the presence or absence of calves with known aged females. Using genetic analysis it is possible to know which males are siring calves (Clapham and Palsbøll 1997) and so age of sexual maturity may also be estimated for known males.

Males have a seasonal cycle indicated by the reduced testes weight in male humpback whales in the feeding grounds compared with testes weight in the breeding areas (Matthews 1937; Chittleborough 1955; Chittleborough 1955a). Due to the high variability in body length of male humpback whales during puberty it is difficult to determine sexual maturity on body length alone (Chittleborough 1955a). Testes weight increases rapidly before puberty and continues to rise sharply for some time after sexual maturity has been reached (Chittleborough 1955a). Without a method to determine testosterone
concentrations in humpback whales it is difficult to determine the proportion of mature whales that have the capacity to sire calves. Reproductive decline in a population can be a result of reproductive dysfunction in males or females or a combination of the two.

In female humpback whales puberty (first ovulation) and sexual maturity (when first pregnancy occurs) are not reached at the same time. Typically pregnancy does not occur in the first but rather in the female’s second ovulatory season (Chittleborough 1955b). From histological examination of ovaries and distribution of near-term foetuses, the gestation of humpback whales is estimated to be 12 months (Chittleborough 1958). Females are thought to be impregnated in the calving areas or along the migration to and from these areas (Chittleborough 1958; Dawbin 1966). When females return to their breeding sites the following season they give birth to a single calf (Clapham and Mayo 1990). Ongoing photo-identification studies of known individuals from different populations indicate that females are on a two to three year breeding cycle. Some animals calve annually while others have longer calving intervals (Chittleborough 1955b; Chittleborough 1958; Chittleborough 1965; Glockner-Ferrari and Venus 1983; Clapham and Mayo 1987; Glockner-Ferrari and Ferrari 1990; Hogg 1998).

Pod composition varies throughout the migration but generally consists of two to three whales (Paterson and Paterson 1984). Larger aggregations are found in the breeding areas (Herman and Antinoja 1977; Tyack and Whitehead 1983) and feeding grounds (Baker et al. 1987; Clapham and Mayo 1987). Males in the breeding areas are competing for mates and so aggressive behaviours are often observed (Tyack and Whitehead 1983). Competition pods are seen frequently and are associated with courtship behaviours. These pods
consist of a number of males jostling for the primary escort position behind a female, although some pods have been observed without a female present (Herman and Antinoja 1977; Tyack and Whitehead 1983). It is believed that when a female comes into oestrus she will mate with the male closest to her (Tyack and Whitehead 1983).

Females with last year’s calf are generally the first to arrive in the breeding areas, followed by juveniles, adults and pregnant females (Dawbin 1966). It is thought that males associate with sexually mature females at the beginning of the breeding season. As these females depart the breeding areas males then associate with females and calves (Tyack and Whitehead 1983). Catch data of females with calves showed that post-partum ovulation occurs and pregnancy may result (Matthews 1937; van Lennep and van Utrecht 1953; Chittleborough 1958). So males may escort females with calves waiting for the opportunity for mating to arise (Glockner-Ferrari and Ferrari 1984). This lends itself to a number of interesting hormonal questions.

− Is there a group of males that are more successful breeders in any population and do they have higher testosterone concentrations?
− Are these animals dominating the primary escort position in competition pods?
− When do females come into oestrus and how long is their cycle?
− Do subordinate males escort female-calf pods on the migration as this leads to a greater opportunity to breed?
− Do all females have post-partum ovulation?
− How can the concentrations of reproductive hormones in free-swimming humpback whales be determined?
By using captive bottlenose dolphins a method for determining the presence of reproductive hormones in cetacean blow was developed and validated (Chapter 3 and 5). The aim of the study presented in this chapter was to ascertain whether this method could be used to determine the concentration of reproductive hormones in free-swimming great whales by:

- determining the feasibility of collecting blow from free-swimming humpback whales, and
- determining if testosterone or progesterone could be found and then quantified in samples of humpback whale blow using LC-MS.

7.2. Materials and Methods

7.2.1. Sample collection materials

Three different sample collection materials were trialled. The first was cotton gauze (Smith-Neilsen, Beecham, Australia), the second was Millipore filtration net (Millipore, Sydney, Australia), which is inert and has a small micropore mesh, and the third was Koltex nylon knee high stockings (Koltex, Australia).

Experiments were conducted to determine which collection material was the most inert for whale blow collection. The collection material (either gauze, net or stocking) was placed in the collection tube with 5 ml of inhibitor mix (100 mM MnCl₂ and 100 µg/ml amoxicillin/potassium clavulanate) and frozen at -80°C overnight. The materials were then extracted as per the extraction method described in Chapter 3. Positive and negative scans of all extracts were conducted on a 0-100% mobile phase B gradient over 50 mins. Both mobile phases were used for the scans: scan 1 - mobile phase A = 0.5% acetic acid,
mobile phase B = 0.5% acetic acid, 90% acetonitrile; and scan 2 - mobile phase A = 0.5% formic acid, mobile phase B = 0.5% formic acid, 90% acetonitrile.

To ensure that nylon stocking was inert, two different methods of cleaning were examined. In the first the nylon stocking was sonicated for 15 mins with 0.5% methanol (MeOH) and then sonicated for a further 15 mins with Milli-Q water, changing the water every 5 mins. In the second the stocking was sonicated for 15 mins in 100% acetonitrile and then for a further 15 mins with Milli-Q water, changing the water every 5 mins.

7.2.2. Blow sample collection

Blow samples were collected from southward migrating humpback whales at Peregian Beach, Queensland, Australia (fig. 7.1) in October 2003 and 2004. The samples were collected using a 13-metre carbon fibre pole (Composite Engineering, Concord, Mass., USA) that was bracket mounted to the bow of a 5-metre aluminium vessel (fig. 7.2) (Moore et al. 2001). The blow collection device was a 5-inch bamboo double ring with a collection material secured over it (fig. 7.3). The blow collection device was mounted on the top of the pole (fig. 7.4) and the blow was collected as the whale surfaced.
Figure 7.1: Study site at Perregian Beach, Queensland, Australia
Figure 7.2: Thirteen metre carbon fibre pole used to collect blow samples from whales. The pole was bracket-mounted to the vessel (a) and steered by a crew member (b).
Chapter 7 – Whale Blow Collection

a) 

b)
Figure 7.3: The blow collection device was a 5-inch bamboo double ring with nylon stocking that had been sonicated with 100% acetonitrile stretched across it.
Figure 7.4: a) The blow collection device was mounted to the top of the pole and samples were collected when approaching the whale. b) This picture documents the collection of a blow that was graded as a medium quality sample.
Blow samples were graded based on the amount of blow that was seen to pass over the blow collection device during sampling. Samples were rated as 3 if the end of the pole was within 5 metres of the whale’s blowholes when it surfaced. However the sample was rated as 2 if there were high winds during collection or if the sample was collected when the pole approached within 5 - 10 metres of the whale. Any sample collection that was further than 10 metres from the whale was rated as the poorest quality, 1.

Once the blow had been collected, the collection material was removed from the ring and placed in a 50 ml polypropylene collection tube with 5 ml inhibitor mix. Samples were kept on ice in a shady area of the boat until arrival on-shore where they were stored at -20°C for two to three weeks. Samples were shipped on dry ice and stored in -80°C for three weeks until extraction.

Seawater samples were collected after each blow collection and extracted and assayed using the same method for blow.

7.2.3. Blow sample analysis
Whale blow samples were extracted as per Chapter 3 and the dry extracts from the SPE were stored at -80°C for one to four weeks prior to analysis. To minimise any changes to hormone concentrations in the samples, only four blow samples were extracted at a time to ensure that extraction time remained less than nine hours.

Extracted blow samples were reconstituted in 60 µl 60% acetonitrile and 5 µl injections used for all analyses. Samples were run using both the 55% isocratic testosterone method (Chapter 3), referred to as the “testosterone method” here.
and the 50% isocratic female hormone method (Chapter 5), referred to as the “female method”. Samples were scanned using both positive and negative scan mode on a 0 - 100% mobile phase B gradient over 50 mins. Two scan runs were done, one with mobile Phase A = 0.5% acetic acid and mobile Phase B = 0.5% acetic acid, 90% acetonitrile; and the other with mobile Phase A = 0.5% formic acid and mobile phase B = 0.5% formic acid, 90% acetonitrile. The scanning protocol was: 0 - 100% phase B for 30 mins; 100% Phase B for 5 mins; 100 - 0% phase B for 5 minutes; and 0% phase B for 10 mins (to allow for equilibration of the column). Blow samples from an individual whale were analysed with all methods (testosterone, female and scanning gradients) on the same day. Different individual whales were analysed on different days.

Seawater samples were analysed from multiple locations in the Peregian Beach region using the testosterone and female methods and the scanning gradients. Seawater results were compared to whale blow. To ensure that results obtained were from the whale and not from any other factors, the inhibitor mix was frozen at -80°C with collection material and then extracted. This extract was analysed with the same methods as the samples and results compared with whale blow.

During the 2003 season, nine blow samples were collected from nine individual humpback whales. All animals were adults and the sex was assumed for three whales, due to the presence of a calf in the case of females (n = 1) or due to the animal singing in the case of males (n = 2) (Table 7.1a). Of the nine samples, three were graded as high quality (3), two were graded as medium quality (2) and four were rated as poor quality (1).
During the 2004 season, a total of 26 blow samples were collected (Table 7.1b). Twenty were females with calves and one was an adult male (an escort to a female-calf pod). The sex was unknown for three samples as the identity of the individual (female or escort) was unable to be determined when the blow was collected. Two samples were collected from the same individual. Of the 26 samples collected, eight samples were graded as high quality (3), 15 were graded as medium quality (2) and three were graded as poor quality (1).

7.2.4. 0-100% gradient analysis

Analysis of individual $m/z$ of the scanning gradients was achieved by dividing the chromatograms into 1 minute time periods. For 2004 whale blow samples, seawater samples and cleaned stocking extracts, $m/z$s with a greater than 50% relative intensity were recorded in any given time period. The $m/z$s that were due to seawater and stocking/inhibitors were then excluded from the whale blow samples. Any $m/z$s which may be attributed to precursors or metabolites of progesterone and testosterone were noted. The $m/z$ for these substances was determined by adding or subtracting one to the molecular weight (mw) of the compound:

\[ \text{i.e. } [M+H]^+ \text{ or } [M+H]^\text{-} \]

where: \[ M = \text{the molecular mass} \]
\[ H = \text{the addition or subtraction of a proton during mass spectrometric analysis (Chapter 2).} \]
Table 7.1: a) Humpback whale blow samples collected from Peregian Beach in 2003.

### 2003

<table>
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<th>Time</th>
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Table 7.1: b) Humpback whale blow samples collected from Peregian Beach in 2004.

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</table>
Chapter 7 – Whale Blow Collection

The precursor of testosterone was androst-4-ene-3,17-dione (mw = 286.7) and the metabolites were 5α-dihydrotestosterone (mw = 290.44) and 19-hydroxytestosterone (mw = 304.42). The precursor for progesterone was pregnenolone (mw = 316.49) and the metabolites were 11-deoxycorticosterone (mw = 330.47) and hydroxyprogesterone (mw = 330.46).

7.3. Results

7.3.1. Sample collection materials

Scans showed nylon stocking and Millipore net were more inert than cotton gauze (fig. 7.5). Millipore net did not absorb liquid in the same manner as nylon stocking. Untreated nylon stocking showed some interfering peaks, i.e. not completely inert (fig. 7.5c). Scans of 0.5% methanol stocking (fig. 7.5d) showed improvements but there were still interfering peaks. The 100% acetonitrile cleaning method yielded optimal results (fig. 7.5e) and was the method used for whale blow sample collection.

7.3.2. Blow sample analysis

Of the nine 2003 whales, two were found to have testosterone present (fig. 7.6), Mn03-07 and Mn03-09. The blow samples from both Mn03-07 and Mn03-09 were graded as high quality samples. Mn03-09 was a female-calf-escort pod and the sample was collected from the escort. Mn03-07 was a single adult whale of unknown sex. Progesterone was not detected in any of the samples.

In 2004, 24 whale blow samples were collected. Two blow samples were found to have testosterone present, Mn04-13 and Mn04-16. The single sample from the adult escort (Mn04-20) did not have testosterone present. Seven blow samples
from the 2004 whales had progesterone present. Six of these samples had quantifiable peaks, i.e. signal-to-noise ratio greater than 5 to 1 (fig. 7.7). Fourteen blow samples from known females did not have any progesterone present.

7.33. 0-100% gradient analysis

There were 341 different m/zs found in whale blow (Appendix 2). Each of the scanning gradients using acetic acid phase and formic acid phase were compared against the control chromatograms for stocking and the inhibitors (fig. 7.5e) as well as the sea water samples.

The positive and negative m/zs for the precursors and metabolites of testosterone and progesterone are given in Table 7.2. An m/z representative of dihydrotestosterone was observed in two whales. The m/z representative of hydroxy-testosterone was observed in five individuals. Six whales had the m/zs representative of pregnenolone.
**Figure 7.5:** MS chromatograms of scans of different collection materials. All scans were carried out using both formic acid and acetic acid in the mobile phases. However, only those chromatograms using mobile phase A: 0.5% formic acid and mobile phase B: 0.5% formic acid, 90% acetonitrile, are shown here.

a) 0-100% mobile phase B gradient over 50 mins of cotton gauze;
b) 0-100% mobile phase B gradient over 50 mins of Millipore net;
c) 0-100% mobile phase B gradient over 50 mins of stocking;
d) 0-100% mobile phase B gradient over 50 mins of stocking washed in 0.5% MeOH;
e) 0-100% mobile phase B gradient over 50 mins of stocking washed in 100% acetonitrile.
a) 0-100% mobile phase B gradient over 50 mins of cotton gauze

b) 0-100% mobile phase B gradient over 50 mins of Millipore net

c) 0-100% mobile phase B gradient over 50 mins of stocking
d) 0-100% mobile phase B gradient over 50 mins of stocking washed in 0.5% MeOH

![Graph showing the gradient of MeOH with time and intensity.]  

![Graph showing the gradient of MeOH with time and intensity.]  

e) 0-100% mobile phase B gradient over 50 mins of stocking washed in 100% acetonitrile

![Graph showing the gradient of acetonitrile with time and intensity.]  

![Graph showing the gradient of acetonitrile with time and intensity.]
Figure 7.6: MS chromatogram showing presence of testosterone in humpback whale (Mn03-07) blow collected in 2003 season. 55% isocratic mobile phase B method using mobile phase A: 0.5% acetic acid, mobile phase B: 0.5% acetic acid, 90% acetonitrile over 15 mins.
Figure 7.7: MS chromatogram showing presence of progesterone in humpback whale (Mn04-07) blow collected in 2004 season. 50% isocratic mobile phase B method using mobile phase A: 0.5% formic acid, mobile phase B: 0.5% formic acid, 90% acetonitrile over 12 mins.
Table 7.2: Positive ([M+H]^+) and negative ([M+H]^-) m/z for some of the precursors and metabolites of testosterone and progesterone (mw = molecular weight, M = molecular mass and H = proton).

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</tbody>
</table>
Chapter 7 – Whale Blow Collection

7.4. **Discussion**

7.4.1 **Sample collection materials**

Analysis of different collection materials showed that cotton gauze was unsuitable for whale blow collection. The same brand, but a smaller size, did not have interfering results (Chapter 3). Due to this inconsistency, and that cotton gauze is known to effect immunoassay results (Shirtcliff *et al.* 2001), the use of cotton gauze for steroid hormone analysis is inadvisable. Although inert, Millipore net was unsuitable as it did not absorb enough liquid. There were concerns that if the pole bounced whilst at sea that any blow on the net would be dislodged. Nylon stocking absorbed liquid easily but interfering compounds were noted if the stocking was not cleaned. Sonication with 100% acetonitrile proved an affective cleaning method. These results support the need for analysis of different collection materials prior to sample collection. Different materials may cause interference with hormonal results depending on the chemical analysis used.

7.4.2 **Blow sample analysis**

Three of the blow samples with testosterone present were collected from whales in female-calf-escort pods. In humpback whales it is believed that escorts of female-calf pods are typically males waiting for the opportunity to mate (Glockner-Ferrari and Ferrari 1984). The presence of testosterone in these samples supports this hypothesis. No testosterone was found in any of the high quality samples from known females. The fourth sample with testosterone present was collected from an adult of unknown sex. There are no distinguishing features that can be used from the surface to determine sex in humpback whales unless the genital area is observed (Glockner-Ferrari and Ferrari 1990). However, this animal may have been male due to the presence of testosterone in the sample. Future collection of blow samples from individuals of known sex or in conjunction...
with genetic sampling will determine the practicality of sex determination from blow sampling. High testosterone concentrations in blow samples is likely to indicate sexually mature males due to the marked difference in testes size and weight between pre-pubescent and pubescent whales (Chittleborough 1955a).

All 2004 female blow samples were collected from known lactating females (due to the presence of a calf). Humpback whales nurse their young until they return to the breeding area the following season (Chittleborough 1958). The presence of progesterone in seven samples, and not in the other fourteen, may be attributed to a number of different factors. Chittleborough (1958) showed that humpback whales experience post-partum ovulation. If this is the case, progesterone in some samples and not in others may be due to the different stages of the oestrous cycle or that ovulation has resulted in pregnancy. Although the resight histories of East Australian female humpback whales show calves in consecutive years (Hogg 1998) it is not commonplace. If there is post-partum ovulation it does not always result in pregnancy. The lack of progesterone in some samples may be also due to the whales lactating. Suckling stimuli suppresses gonadotropin release (particularly luteinising hormone) preventing normal follicular maturation and growth (McNeilly 1988). Or alternatively the lack of progesterone in the samples may be due to the quality of the blow samples themselves.

Progesterone could not be quantified in the blow of cycling female bottlenose dolphins (Chapter 5). Using the same LC-MS method progesterone could be quantified in 30% (6 of 20) of the humpback whales sampled. In humpback whales, where successful pregnancy from post-partum ovulation is not common, perhaps progesterone concentrations in the blow of cycling females is higher than in bottlenose dolphins. Recently, Rolland et al. (2005) reported the need to
dilute North Atlantic right whale faecal samples when assaying for reproductive hormones due to higher than normal concentrations (for mammals) of reproductive hormones in the samples. As progesterone concentrations in cycling and pregnant female humpback whales are unknown, it is difficult to ascertain why progesterone was determined in some blow samples and not in others.

7.4.3. 0-100% gradient analysis

Humpback whale blow contains a plethora of different compounds. The gradient analyses showed a number of different substances, some of small molecular weight and others which make up larger compounds, such as proteins. At this stage it is unclear whether these compounds are specific fluid from the lung mucosa, are part of the oil found in whale blow or whether they have passed across the capillary wall of the lungs from the bloodstream.

Steroid hormones can be excreted in conjugated or unconjugated forms. In addition they can be metabolised in certain body fluids such as saliva (Whitten et al. 1998; Laine and Ojanotko 1999). The form in which hormones are secreted is species dependent (Whitten et al. 1998). Preliminary assessment of the m/z values indicates that the precursors and metabolites of testosterone and progesterone may be present in the samples of whale blow. Whether these compounds are from the bloodstream or whether they have been produced through metabolism/catabolism in the lungs is undetermined. By using more sensitive technologies like MS-MS it may be possible to better understand which form of the reproductive hormones are present in whale blow. However the gradient analyses showed that a variety of different compounds were present. With further analytical development and enhanced collection techniques, whale blow has the potential to provide answers to a range of physiological questions.
7.5. **Conclusion**

It is feasible to collect blow from a free-swimming whale. Testosterone and progesterone can be quantified in samples of whale blow using the LC-MS methods developed here. The collection of blow samples from humpback whales in conjunction with life history and genetic data will further our understanding of their breeding cycle. Development of more sensitive analysis and collection techniques will enable blow sampling to be used to determine reproductive function in great whales in both their breeding areas and feeding grounds. Moreover these techniques make possible the assessment of reproductive dysfunction in declining or threatened populations.
CHAPTER 8

General Discussion

Reproductive physiology plays a vital role in population growth and vitality. Understanding how an animal reproduces both physiologically and behaviourally is important in population conservation. The aim of this study was to develop a non-invasive technique that could be used to determine reproductive hormones in cetaceans. Although a number of non-invasive methods exist for captive cetaceans none of these were feasible to use with great whales. The new method developed and validated in this thesis proposed the use of blow samples to determine (and eventually monitor) reproductive hormones in cetaceans.

8.1. Analytical chemistry

Traditional methods of hormonal analysis, such as immunoassays, were not practical for this program as they required approximately 200 µl of sample. The blow samples were typically 50 µl in volume so an alternative method was needed. LC-MS was found to offer great promise. Reproducible and accurate LC-MS methods were developed for the determination of testosterone in male dolphins (Chapter 3) and progesterone in pregnant female dolphins (Chapter 5). Interestingly the concentration of hormones was greater in blow samples than in saliva samples. At this time the reasons for this are unknown. Future comparative analysis of blow and saliva samples with plasma samples may provide further insight.

Although promising, there are still limitations to the LC-MS methods at this stage. The limit of detection of the oestradiol and oestrone methods was not low enough
for their concentrations to be assessed in cycling dolphins. The progesterone method allowed for the detection of progesterone but was not sensitive enough to quantify concentrations in cycling female dolphins. Due to the complexity of progestins and oestrogens, LC-MS-MS may be required to analyse these compounds (Diaz-Cruz et al. 2003). The use of MS-MS or time-of-flight mass spectrometry (TOF) may permit further development of this method as this technology decreases the limits of detection for reproductive hormones. In addition these methods will allow for the determination of the molecular breakdown of the compounds found in both the saliva and blow of cetaceans.

Due to the aquatic nature of cetaceans it is difficult to ensure that there is no seawater contamination of the samples. To combat problems with seawater dilution a comparative dilution factor is required. Creatinine is an endogenous by-product of muscle metabolism and is excreted in urine in a constant fashion (Ruckebusch et al. 1991). It is used widely as a dilution factor to determine hormone concentrations in urine. Creatinine is a small molecule (mw = 149.59) and so could easily pass into saliva and lung mucosa. However, it is unknown at this time if creatinine is present in cetacean saliva and blow. Alternatively other substances which are excreted at a constant rate could be used to determine dilution. The next step for this program would be to find a compound to identify dilution rates so concentrations can be compared across animals.

8.2. Stability

One major research area that was investigated in this study was the stability of testosterone (Chapter 4) and progesterone (Chapter 6). There is a lack of consistency in the literature in reference to testosterone and progesterone
stability. A number of recent studies have shown that steroid hormones are not stable and that each compound reacts differently depending on the species and type of biological sample (Gröschl et al. 2001; Khan et al. 2002; Millspaugh et al. 2002; Lynch et al. 2003). No uniform method has been used to determine stability in biological samples. Some studies analyse samples frequently (daily or weekly) at room temperature and in the refrigerator (4°C) (Granger et al. 2004) but only analyse samples at six-monthly intervals when they have been stored at lower temperatures (-20°C and -80°C). But as was shown in this study, hormone concentrations can change over hours at room temperature and over weeks, rather than months, at lower temperatures (-20°C and -80°C).

The addition of inhibitors improved the stability of testosterone and progesterone in saliva and blow which has been shown with other biological samples (Gröschl et al. 2001; Hunt and Wasser 2003; Lynch et al. 2003). Sodium azide is commonly used as an inhibitor (Lipson and Ellison 1989; Gröschl et al. 2001) but due to its toxicity it was not considered for this study. MnCl₂ proved to be the best inhibitor for testosterone as it stops testosterone steroidogenesis (Cheng et al. 2003). As testosterone and progesterone belong to different metabolic pathways (fig. 1.5), it was not surprising that MnCl₂ was not effective as an inhibitor for progesterone. However, amoxycillin/potassium clavulanate (Augmentin®) proved to be an effective inhibitor for progesterone. The different stabilising effects of MnCl₂ and Augmentin® with testosterone and progesterone indicated that there are a number of different factors affecting stability in saliva and blow. Further analysis of the precursors and metabolites of testosterone and progesterone will indicate if metabolism is occurring in the samples. The use of a broad spectrum antibiotic is useful as bacteria are present in both saliva and blow.
Samples used in the stability experiments were spiked with a known concentration of either testosterone or progesterone. Using endogenous concentrations of hormones to assess stability can be problematic. This is because the endogenous concentration of these hormones may have already undergone changes prior to the commencement of the stability experiment. By spiking samples with known concentrations of hormone this problem is resolved. It is important also to spike samples with high and low hormone concentrations as small fluctuations in hormone concentrations were seen in these experiments. Fluctuations did not affect the stability of the high concentration samples but gave significant changes when lower concentrations were present.

Why testosterone and progesterone concentrations increase and then decrease over time in saliva and blow samples of bottlenose dolphins is unknown. There is very little published information on the composition of cetacean saliva and blow. Lipid micelles in faeces breakdown over time releasing steroid hormones that are bound to them (Yalkowsky 1999) and so increasing hormone concentrations (Hunt and Wasser 2003). Cetaceans have large quantities of fat in their bodies. Blow is oily, and perhaps lipids are binding to the steroid hormones which are released as the lipids degrade, so increasing hormone concentrations in the blow samples.

Alternatively, the instability of testosterone and progesterone in saliva and blow may be due to the presence of cells in the samples. Saliva samples contain epithelial cells, as the samples originate from the roof of the animal’s mouth. Progesterone metabolism is known to occur in human saliva due to the presence of cells (Laine and Ojanotko 1999). Cetacean blow exits the blowholes at enormous speeds. It is possible that epithelial cells that line the trachea are
expelled with the blow. Cytological examination of both saliva and blow samples would determine if this is the case.

Non-invasively collected samples are becoming more popular in the study of hormone fluctuations of wildlife (Monfort 2003). The different sample matrices that are used by wildlife biologists would contain different bacteria, proteins, and enzymes which may be influencing hormone concentrations. It is imperative that stability studies of each steroid hormone are conducted when using different matrices for different species. The stability of the steroid hormone studied needs to be addressed prior to using non-invasively collected samples as part of long-term biological studies. This will ensure that changes seen in hormone concentration are due to biological activity rather than an artefact of storage.

8.3. Whale blow collection

Australian humpback whales are known to be most reproductively receptive between June and September when they are off the coasts of Australia (Chittleborough 1954). Unlike dolphins these animals are not polyoestrous. Histological examination of the ovaries has shown that humpback whales ovulate only once during their ovulatory period, with a few ovulating twice (Chittleborough 1954). A method has been developed to determine reproductive hormones in faecal samples of northern right whales (Rolland et al. 2005) but, as there is no feeding off the Australian coast, faecal sampling is not a possibility when determining hormone concentrations of humpback whales. An alternative to faecal sampling is required to determine hormone concentrations in breeding areas.
A method was developed to determine the concentration of reproductive hormones present in the blow of free-swimming humpback whales (Chapter 7). Although a number of analytical variables remain, such as dilution and stability, this method could lead to a greater understanding of great whale reproduction. Further development of the analytical method using MS-MS should allow for the determination of concentrations of progesterone as well as oestradiol. A technique for sexing adult whales from blow samples becomes possible by determining an oestradiol/testosterone ratio (Gross et al. 1995). Sexing animals by their blow will take some time as it will need to be done in conjunction with studies that identify the sex of animals either by genetic sampling or photo-identification.

To determine ranges for reproductive hormones humpback whales could be sampled on their northward and southward migration over a number of years. Although there is the possibility of sampling the same individuals within a season, it is more likely that the same individuals would be sampled across seasons. Biological ranges for a variety of reproductive hormones can be determined for different sexes and ages by using blow samples in conjunction with ongoing photo-identification studies and life history information.

It is anticipated that baseline information on reproductive hormones of great whale populations will lead to a better understanding of how their populations are interacting:

- Is it only a proportion of females that are breeding and, if so, is it because the other females are not cycling?
- Do primary male escorts have higher testosterone concentrations than other males?
Chapter 8 – General Discussion

- Do subordinate males find alternative mating strategies, such as escorting female-calf pods?

These are the types of questions that can be answered with further development of blow sample collection.

8.4. Conclusion

This study developed analytical methods to assess reproductive hormones in cetacean blow samples with the view to develop methods which could be useful to study free-swimming great whales. Reproducible and validated LC-MS methods now exist for determining testosterone and progesterone in saliva and blow. This study highlighted the need to assess steroid hormone stability in the biological matrices being used. Further development using the latest mass spectrometric technology will enhance the sensitivity and accuracy of these methods. Longitudinal studies using saliva and blow will provide baseline values for both male and female captive dolphins. Non-invasive sampling of cetaceans can be used on a daily basis with minimal stress to the animal. In addition, further sampling of humpback whales will provide reference ranges for reproductive hormone concentrations on a vulnerable species in an area where alternative non-invasive sampling is not possible.

Any signs of population decline or issues with reproductive efficiency may be due to changes in internal physiology and/or external environmental factors. Sampling and analytical methods which incorporate multiple hormone analysis from one sample would be useful due to the difficulty and expense of collecting samples from free-swimming whales. Developing a technique that could determine steroid
hormones associated with reproduction and stress would be beneficial as there is not just one factor that affects an animal or population.

Of all vertebrate species in the world, reproductive behaviour has been described for only 200 (Wildt and Wemmer 1999). Part of conservation management is a comprehensive understanding of population growth. Baseline data on reproductive physiology and a comprehensive knowledge of breeding biology is essential. The analytical methods and techniques developed in this study provide a solid foundation for future researchers and conservation managers in relation to the reproductive physiology of cetaceans.
REFERENCES


References


References


References


182


References


References


References


References


Thomson, J. J. (1913). Rays of positive electricity and their application to chemical analysis. Longmans Green, London


Appendix 1

Peer-reviewed journal publication from Chapter 3.
Appendix 2

All mass-to-charge ratios (m/z) found in humpback whale blow. Those m/z’s that were only found in one animal were excluded. Data is presented in the one minute time period in which the m/z is found dependent on which phase was used, either formic acid or acetic acid.
<table>
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