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CICLO XX

USE OF FAECAL SAMPLES TO MONITOR THE OESTROUS CYCLE, REPRODUCTIVE STATUS AND ADRENAL GLAND ACTIVITY IN THE BOTTLENOSE DOLPHIN (*Tursiops truncatus*)

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DATA CONSEGNA TESI

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ABBREVIATIONS

AI artificial insemination

ABP androgen-binding protein

ACTH Adrenocorticotropic hormone

MMPA Marine Mammal Protection Act

ANOVA analysis of variance

BL body lenght

BW body weight

C cortisol

cAMP cyclic adenosine monophosphate

CG chorionic gonadotropin

CITES Convention of International Trade in Endangered Species of Wild Fauna and Flora

CL corpus luteum

E estrogen

E2 17β-oeastrdiol

EC estrogen conjugates

eCG Equine chorionic gonadotropin

EDTA ethylenediaminetetraacetic acid

EIA Enzyme immunoassay

F females

FSH follicle-stimulating hormone

FSHR FSH-receptor

G girth

GCs glucocorticoids

GnRH gonadotropin-releasing hormone

hCG human chorionic gonadotropin

HPA hypothalamic-pituitary-adrenal axis

HPLC high performance liquid chromatography

HPO hypothalamic-pituitary-ovary axis

IM intramuscular

LH luteinizing hormone

M males

MHz mega hertz

P4 progesterone

PKA cAMP-dependent protein kinase

PTP plasma transport protein

RIA radioimmunoassay

StAR Steroidogenic Acute Regulatory proteins

SD standard deviation

SE standard error

TST testosterone

VS versus

WBC white blood cells

Summary

The detection of hormones in faecal samples has been used during the last years on many different domestic and exotic species, including humans. The information gained through the to application of non-invasive techniques has become an essential tool to investigate endocrinological and physiological mechanisms of several wild animal species, for which traditional methods of study would be difficult to perform.

The scope of this thesis was to develop analytical methods to assess adrenal and reproductive hormones in bottlenose dolphin (*Tursiops truncatus*) faecal samples and determine the feasibility of its use with wild cetaceans. Due to the lack of information regarding the levels of hormones present in the faeces of bottlenose dolphins, I compare the results obtained from faecal samples with those obtained from serum and blood samples, vaginal cytology, ultrasounds and behaviour observations.

After a general introduction to the production and role of steroids hormones and the non invasive techniques applied to study reproduction and endocrinological aspects of wildlife terrestrial and marine animals, in the second chapter I present the methodology applied to collect the samples from bottlenose dolphins used in the present study. All the samples were collected from the ten dolphins by voluntary behaviour.

The first step was to set up a radio immuno assay (RIA) to determine progesterone, estrogens, testosterone and cortisol concentrations in faeces of bottlenose dolphins and each test was validated and parallelism test and recovery test were performed.

In the fourth chapter I evaluate the presence of progesterone and estradiolo in faecal samples of eight females of different age and under different physiological conditions (Pre puberty, puberty, cycling, resting and pregnant). The results highlight as a major difference that in pregnant animals, progesterone never drop below 10 pmol/g of faeces, while in resting females it was always below that value. The results showed as well a correspondence between faecal levels and results observed with ultrasound and vagynal cytology.

Two males were studied and results are reported in the fifth chapter. A baseline level for testosterone in juvenile animals has been established at 30pmol/g faeces.

The results obtained for reproductive hormones suggest that the RIA can be applied to investigate the reproductive physiology in bottlenose dolphins.

In chapter six I investigate the cortisol levels in faecal samples and comparing the results obtained with those values obtained from serum samples, the technique seems feasible to monitor chronic stress, while, due to the lag time between the moment the hormone is produced from the adrenal gland, until gets excreted in the faeces, it does not seems feasible to detect state of acute stress.

On the contrary to results reported in literature for other marine mammals, no differences were noticed in the cortisol levels in comparison with different physiological status in females, while observing the group when male and females were kept together, rise in faecal cortisol levels were observed during the warm season, when animals were showing more frequently sexual behaviours and were more busy with shows and interactive activities.

Important observation was done comparing the sexual hormonal levels in peri-pubertal females and the male sharing the pool with those: a rise of progesterone level was noticed to follow the rise of testosterone in the male. This suggest the possible “male effect” on the hormones production and cycle in females.

It was further concluded that the radio immuno assay is effective to detect hormonal levels in faeces of bottlenose dolphins, but further steps are required to investigate the faecal presence of hormonal metabolites through the application of high-performance liquid chromatography and mass spectrometry (HPLC-MS)

Riassunto

La possibilità di utilizzare campioni di materiale fecale come matrice biologica per analizzare la presenza di ormoni è una tecnica che è stata utilizzata ed applicata durante gli ultimi anni in varie specie di animali esotici e domestici, uomo incluso. Le informazioni ottenute attraverso l'utilizzo di tecniche non invasive sono essenziali per studiare i meccanismi fisiologici attuati nell'attività endocrinologica e riproduttiva di molte specie di animali selvatici, per i quali l'utilizzo di metodi tradizionali renderebbero questi studi difficili da effettuare.

Lo scopo di questa tesi era di sviluppare un metodo analitico per estrarre e valutare la presenza di ormoni steroidei nelle feci di delfino tursiope (*Tursiops truncatus*) e determinare la possibilità di applicare la tecnica a campioni di feci provenienti da cetacei in ambiente naturale. Data la mancanza di informazioni riguardo i livelli di ormoni presenti nelle feci di tursiope, I risultati ottenuti dai campioni fecali sono stati confrontati con quelli ottenuti da campioni ematici, campioni di citologia vaginale, esami ecografici delle gonadi ed osservazioni comportamentali.

Dopo aver descritto i meccanismi che regolano la produzione degli ormoni steroidei, il loro ruolo e le tecniche non invasive utilizzate per lo studio dell'attività riproduttiva ed endocrinologica di animali selvatici, sia terrestri che marini, nel secondo capitolo ho presentato le tecniche utilizzate per la raccolta dei campioni usati per la ricerca. Tutti i campioni sono stati raccolti da delfini addestrati per i comportamenti medici, e quindi tutti i campioni sono stati raccolti senza dover catturare gli animali, ma avendo la loro piena collaborazione volontaria.
Il primo passo è stato quello di mettere a punto una metodica RIA per determinare la presenza di progesterone, estrogeno, testosterone e cortisolo nelle feci di delfino tursiope. Ogni test è stato validato attraverso il test di parallelismo ed il test di recupero.

Dopo I primi capitoli di introduzione e spiegazione dei materiali e metodi utilizzati, nel quarto capitolo ho valutato la presenza di progesterone ed estradiolo nelle feci di otto femmine, con età differente e che si trovavano in situazioni fisiologiche differenti (prepuberi, puberi, cicliche, in anestro ed in gravidanza). I risultati ottenuti hanno

dimostrato una sostanziale differenza tra femmine gravide e non, evidenziando come il progesterone durante la gravidanza non scenda mail al di sotto di 10 pmol/g di feci, mentre nelle femmine in anestro il progesterone è sempre inferiore a tale valore. I risultati ottenuti hanno inoltre evidenziato una corrispondenza tra i livelli di ormoni fecali e sierici ed una corrispondenza anche con osservazioni ecografiche e strisci vaginali.
I risultati ottenuti riguardo i livelli di testosterone fecale osservati nei maschi sono riportati nel capitolo cinque ed hanno permesso di stabilire un valore basale di 30 pmol/g per gli animali prepuberi.
I risultati ottenuti dalle analisi su ormoni sessuali suggeriscono che il RIA utilizzato è adatto per studiare la fisiologia della riproduzione nel delfino tursiope.

Nel sesto capitolo ho analizzato I livelli di cortisolo fecali e li ho confrontati con i livelli di cortisolo sierico. Dai risultati ottenuti si evince che l'utilizzo di campioni fecali è una metodologia valida per monitorare stati di stress cronico, mentre, dato il tempo che intercorre dal momento in cui gli ormoni sono prodotti dalle surrenali e riversati nel circolo ematico, ed il momento in cui l'ormone viene escreto con le feci, le molecole ormonali vengono modificate dalla flora batterica presente nell'intestino, non rendendo comparabili i valori ormonali ottenuti dalle due matrici per quanto concerne stati di stress acuto.

A differenza di quanto riportato in letteratura, nel presente studio non sono state osservate variazioni significative nei valori di cortisolo fecale in relazione allo stato fisiologico delle femmine esaminate. Sono state invece osservate delle differenze nel gruppo in cui maschi e femmine erano tenuti insieme. In quest' ultimo casi si è evidenziato un aumento dei valori di cortisolo fecale durante la stagione estiva, quando gli animali manifestavano più frequentemente comportamenti sessuali e quando erano più impegnati con attività di spettacoli ed interazioni.

Un'importante osservazione è stata notata confrontando I valori di ormoni sessuali quando le femmine peri-puberi erano tenute in compagnia del maschio: nelle femmine si è osservato una aumento del progesterone fecale preceduto da un aumento del testosterone fecale nel maschio. Questo suggerisce la possibilità che "l'effetto maschio" si manifesti anche sulla produzione e sull'attività ciclica delle femmine di delfino tursiope.

E' possibile quindi concludere che il RIA è un metodo analitico adatto allo studio degli ormoni fecali nel delfino tursiope, ma sono necessari ulteriori studi per evidenziare la presenza di metaboliti degli ormoni nelle feci, attraverso l'applicazione di tecniche quali la cromatografia liquida in colonna.

CHAPTER 1: INTRODUCTION

1.1- Structure and aim of the thesis

The main aim of this thesis is to verify whether the use of faecal samples can be applied to bottlenose dolphins (*Tursiops truncatus*) to monitor their reproductive status and adrenal gland activity. Due to the lack of knowledge of basal values for faecal hormones in bottlenose dolphins, the results obtained from faecal measurement were compared to others parameters already used in the normal clinical practice: serum hormones concentrations, white blood cells differential count, vagynal cytology, ultrasound and ethogram. The long term aim of the thesis is to establish a longitudinal profile and baseline values for faecal hormones, that can be considered as a basis for further studies on endangered species of cetaceans, for which blood or urine collection would be difficult.

In the **first chapter** I will make a review about the steroids hormones and the application of non invasive technique to wildlife to monitor reproduction and adrenal gland activity; methodologies and techniques applied to terrestrial and marine mammals and the difficulties to monitor these two types of animals, according to their habitat and environment.

In the **second chapter** I present the methodology used for the collection of the different samples and their storage. In the **third chapter** I assess the procedures applied for the hormones extraction and analysis. In the **fourth chapter** I investigated the methodology to extract and evaluate the presence of Progesterone and Estradiolo in faecal samples of females of different age and under different physiological conditions (Pre puberty, puberty, cycling and pregnant). I compared the hormones concentrations of faecal samples with others parameters already used in the normal clinic practice like serum hormones concentrations, vagynal cytology, and ultrasound. In the **fifth chapter** I investigated the methodology to extract and evaluate the presence of testosterone in faecal samples of males of different age; results were compared with serum testosterone and ultrasound examination of the testis. In the **sixth chapter** I investigated the methodology to extract and evaluate the presence of cortisol in faecal samples of females and males and results were compared to serum cortisol level, white blood cells differential count and ethogram.

In the **seventh chapter** I examine the relationships between the adrenal gland activity and the reproductive status.

1.2- Steroid hormones

Hormones are responsible for the activation and regulation of physiological system of mammals. They are synthesized and expressed by different organs and are responsible for specific activities. Hormones undergo metabolism and catabolism at varying rates according to the different hormone. Hormones were divided into three classes: steroid, polypeptide/protein, and amino-acid related hormones. Due to the strong correlation between steroid hormones and mammalian reproduction only this class of hormones will be considered.

Steroids hormones are lypophilic compounds of low molecular weight and they derive from cholesterol. Due to their lypophilic nature, they are not very soluble in the blood and need to be transported in the blood by plasma transport protein (PTP) that are synthesized in the liver (Siiteri *et al.* 1982).

The concentration of the PTP is subject to physiological regulation. The concentration of unbound hormone available for biological activity is subject to the amount of PTP present in the plasma.

Cholesterol is the precursor of steroids hormones, and being part of the cells membrane, it is responsible for allowing hormones to enter the cells and have their action.

Cholesterol is the precursor for the six classes of steroid hormone (androgens, estrogens, progestins, glucocorticoids, mineralcorticoids and Vitamin D), and its biosynthetic 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. Dietary cholesterol is not the only source of cholesterol, and more than 60% of the body's

cholesterol comes from the liver and the intestines (Popják and Cornforth 1960).

Steroids are mainly synthesized by endocrine glands like gonads (ovaries and testis) and adrenal glands. During pregnancy, with different ratio according to the different species, they can be synthesized by the fetoplacental unit. From the glands they are released into the blood circulation and can act both on the “peripheral” target tissue or on the central nervous system (Steimer, 1993).

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 corpus luteum in the ovaries to produce progestins and on the testes to produce androgens; follicle stimulating hormone (FSH) acts on the ovarian follicles to produce oestrogens. In human the placental chorionic gonadotropin (hCG) acts on pregnant female ovaries (corpus luteum) to produce progestins. CG have been found in few other mammals like primates and horses. Murphy and Martinuk (1991) identified CG in the horse (eCG) and the donkey and reported that the CG may acts as a LH-like hormone to induce supplementary ovulation and/or luteinization of follicles in the mare and assure that there is sufficient secretion of the primary corpus luteum to maintain pregnancy until the placenta assumes its role for progesteron secretion. Daels *et al*. (1998) showed how eCG have a luteotropic role during pregnancy of mares. Hobson and Wide (1986) report of chorionic gonadotropin extracted from a bottlenose dolphin placenta. Whether delphinidae produce functional placental gonadotropins is currently being examined in more details by Japanese scientists and Watanabe et al. (2007) reported for the fist time the identification of placental expression of LH-like substances in a bottlenose dolphin.

Adrenocorticotropic hormone (ACTH) produced at the level of the hypothalamus, acts at the level of the adrenal gland inducing the production of glucocorticoids.

Steroid hormone synthesis begins with the conversion of cholesterol into pregnenolone by the mitochondrial cytocrome P450scc (20,22 desmolase or 20,22 lyase). Transport of cholesterol from intracellular source into the mitochondria is the real rate-limiting step in steroid biosynthesis (Black et *al.*, 1994). This process is regulated by hormones like LH in gonads and ACTH in adrenal glands. At the level of adrenocortical cells and testicular Leyding and ovarian cells, the ACTH and LH respectively, via G-protein-coupled receptors, can activate the adenylate cyclase and induce the formation of cAMP (cyclic adenosine monophosphate). Four molecules of cAMP bind to the regulatory $(R)^2$ subunits

of the cAMP-dependent protein kinase (PKA) holoenzyme to release the catalytic subunits that phosphorylate specific substrates/effectors, thus transducing and amplifying the signal transmitted by the hormone. This highly efficient process requires a minimum amount of cAMP to induce, within few minutes of exposure of the cells to hormones, an elevated rate of cholesterol transport and synthesis of steroid (Liu et *al.*, 2006).

At the cellular level, in response to the activation of the cAMP second messenger system, ACTH and LH stimulate the synthesis of mitochondrial proteins called StAR (Steroidogenic Acute Regulatory proteins). StAR are responsible for the transfer of cholesterol within the mitochondria (Liu et *al.*, 2006).

Once the cholesterol is transported into the mitochondria, the cholesterol side chain cleavage enzyme P450scc can convert cholesterol into pregnenolone. Pregnenolone itself is not a hormone, but it is the precursor for the synthesis of all steroids hormones (Audi Parera, 1998)

Cytochrome P450 enzymes are involved in steroid hormone synthesis (Simpson *et al.* 1994). They are involved in the hydroxylation reactions that make up the metabolic pathways of steroid hormones. There are limited number of P450 enzymes involved in steroid synthesis in the ovaries, testes and adrenal cortex. Enzymes and steps for the hormones synthesis are reported in fig. 1.1. (Steimer, 1993).

Once pregnenolone is produced it may follow one of the following pathways. It is either converted to progesterone or it undergoes 17-hydroxylation, leading to cortisol production in the adrenal glands. Progesterone is also an intermediate in the synthesis of aldosterone in the adrenal glands. The 17-hydroxylation of pregnenolone can also lead into the androgen/oestrogen pathways.

Other important enzymes involved in the synthesis process are: hydroxilases, present either in the mitochondrial or the microsomal fraction of cells; Hydroxysteroid dehydrogenases and Aromatase.

The biological activity of steroid hormones depend on their ability to interact with specific binding site on the corresponding receptors. Target tissue is the one that has stereospecific receptors permitting the accumulation of the steroid in the target tissue against a concentration gradient. 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 the blood is determined by different 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.

The main site of peripheral steroid inactivation and catabolism is the liver, but some catabolic activity also occurs in the kidneys. Inactive hormones are mainly eliminated as urinary and faecal metabolites. Usually, steroids are eliminated once they have been inactivated. This elimination (e.g. as a urinary excretion products) requires conversion to hydrophilic compounds in order to ensure their solubility in biological fluids at rather high concentrations

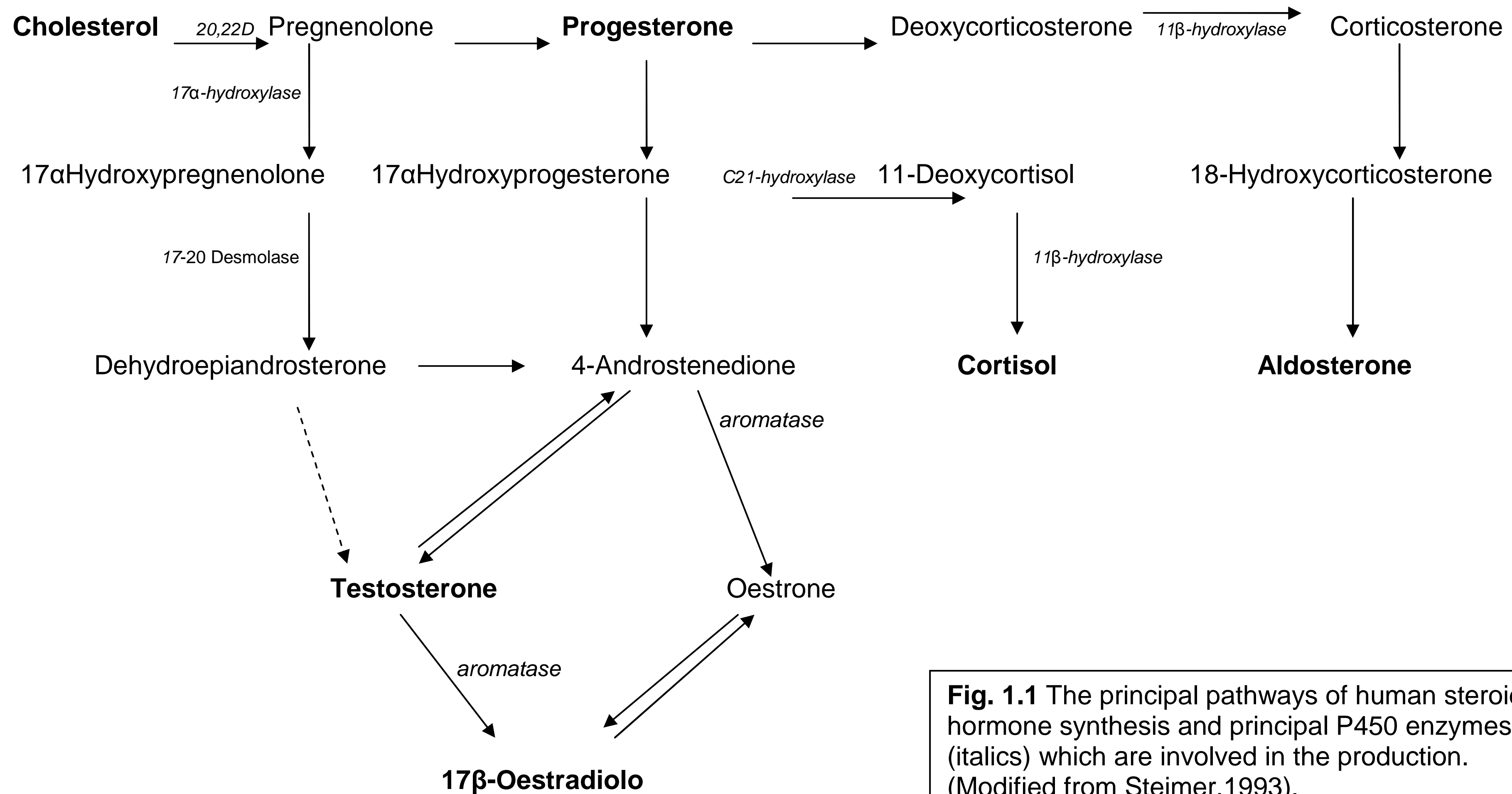


Fig. 1.1 The principal pathways of human steroid hormone synthesis and principal P450 enzymes (italics) which are involved in the production. (Modified from Steimer,1993).

1.3- Non invasive techniques applied to wildlife reproduction and endocrinology

Endocrinology is the branch of medicine that deal with endocrine glands and their secretions. From endocrine glands (i.e. adrenal glands and gonads) the hormones are secreted into the bloodstream to regulate the reproductive status, health and behaviour of humans and animals. The connection between endocrine and reproductive status has been studied over the past decades and it has been proved how abnormal activity of adrenal gland (i.e. elevated levels of glucocorticoids) resulting from chronic stress, can have detrimental effects on many physiological aspects, including inhibition of normal reproductive function (Dobson and Smith, 1995; Kirby, 1990; Ferin, 1999). Problems that many exotic and endangered species have to face are complex and often little understood: endocrinology, nutrition, behaviour and husbandry are all aspects of a bigger picture and the most valuable researches are still focusing on learning the basic biology of different species that are candidates for reproductive intervention.

Two are the major problems related to studies on endangered and threatened species: numbers of animals available for the studies is often limited and this makes difficult to get results on basic biological studies and difficult to make general statements or protocols useful for the entire species; the second major problem is the possibility to collect biological samples to monitor the hormonal status of these species.

The most traditional method to monitor endocrine activity is blood sampling, but especially when applied to wild animals (both free ranging or under human care) it results not very feasible due to its invasive nature: for blood collection most of the wild animals have to be caught and in same cases anesthetised. These processes cause stress that can alter the physiological levels of circulating hormones (Touma and Palme, 2005). For some other species, blood sampling on living animals results not possible (i.e. *Eubalaena glacialis*: Rolland *et al.,* 2005).
To overtake these problems, non-invasive sampling collection techniques have become more and more used during the last years and have been applied to different wild species.
The major advantage of non invasive techniques is that these offer the possibility to use faecal or urine samples to monitor steroids hormones and with repeated samples that can be collected from the same animal with more frequency without disturbing it from its

normal social activities or without modifying its environment. Furthermore, due to the cumulative secretion of steroid hormones in faeces and urine and their elimination over several hours (Palme *et al.*, 1996; Touma and Palme, 2005) these samples are less affected by the instant fluctuations of hormone secretion than the blood.
Reproductive technology results to be the key for maximizing genetic diversity and becomes essential when population of wild animals (both in wild and captivity) are fragmented and isolated, limiting this natural exchanging process.

The most high-tech reproductive methods (artificial insemination, in vitro fertilization, embryo transfer and genome resource banking) used nowadays to bring the race against extinction of different exotic animal's species rely on our knowledge of basic reproductive physiology of specific species.

Non invasive methods, as the faecal and urinary steroids analysis technique, have already been successfully used in many domestic and exotic species to asses the reproductive status, monitor the corpus luteum function and pregnancy, and to monitor the adrenal glands activity (Fieß *et al.*, 1999; Gomez et al., 1994; Schwarzenberger *et al.*, 1991; Schwarzenberger *et al.*, 1992;; Schwarzenberger *et al.*, 1996; Ziegler *et al*, 1989). During the last fifteen years even saliva has been use to measure hormones in human (Quissel, 1993), domestic animals (Beerda *et al.,* 1996) and exotic animals (Gomez *et al.,*2004).

Urine collection is not always simple, both from free ranging and captive not-trained animals, due to nature of the sample that can soak into ground before it can be collected and can be contaminated with water or wet. Nevertheless studies reported urine collection from bowl placed under the resting site of animals (Andelman *et al.*, 1985), snow (Kirkpatrick *et al.*,1990) and ground (Robbins and Czekala, 1997).

The identification and collection of faecal samples is easier. In 1976 Adlercreutz *et al.* reported the application of steroids extraction from human faeces to study the metabolism of estrogens in the intestine, and the first report on faecal steroid analysis to determine oestrogen presence in pregnant woman (Adlercreutz and Martin, 1976). During the following years this extraction has been applied to domestic and exotic animals (see Schwarzenberger *et al.,* 1996)

Although the nature of these samples can allow easy and repeated collection, it has to be considered that native steroid hormones that circulate in the blood stream undergo different metabolic processes before to be excreted in urine and faeces. These processes occur mainly in the liver, where the bioactive hormones (progesterone, oestradiol, testosterone and corticoids) are deactivated by conjugation with different molecules. This process is necessary to make these metabolites more hydrophilic thus to be possible the excretion into the urine through the kidneys or into the faeces through the bile and intestine (Steimer, 1993). Schwanzerberger *et al.* (1996) report that the excretion of steroids into the intestine is mainly through the bile and only a small proportion of circulating steroids is secreted through the mucosa of the large intestine. Before faecal excretion, hormones present in the bile are pour into the intestine where can be reabsorbed in the enterohepatic circulation, hydrolyzed by the intestinal flora and excreted as unconjugated metabolites (Palme *et al.*,1996; 2005).

There is a large difference among species regarding the route of excretion, the delay time between the circulation of hormones in the plasma and their appearance in urine, faeces or other biological liquids (lag-time) and the percentage of metabolites formed and excreted. The lag time of faecal steroids resulted to be of 12-24 hours in ruminants, and about 24 to over 48 hours in animals like primate, horse, pig and elephant that are hindgut fermenters (Schwarzenberger *et al.*, 1996). The percentage of hormones excreted in urine and faeces in several species were studied using radioactively labelled steroids. Known amount of ^{14}C steroids were infused intravenously over a period of hours and faeces were collected after defecation, while urine were collected either with permanent catheter or after spontaneous urination. It resulted that within the studied species, the Yellow baboon and the pig have the lowest excretion of P4 in faeces with respectively 40% (Wasser *et al.,* 1994) and 34% (Palme *et al.,* 1996). Heistermann *et al.*(1998) reported that the Sumatran rhino has the highest excretion of P4 with 99% in faeces and 1% of P4 excreted in the urine. Brown *et al.(*1994) reported that domestic cat excretes P4 mainly in faeces (97%) and in Cotton-top tamarind 95% of P4 is excreted in faeces and only 5% of the hormone is excreted in urine (Ziegler *et al.,* 1989). With the same technique the domestic cat and the Siberian polecat have been identified as the species with highest excretion of 17β-oeastrdiol (E2) in faeces with respectively 97% (Shille *et al.,* 1990) and 93% (Gross, 1992) of the radio-labelled hormone found in faeces, while the African elephant results to

have the lower faecal excretion of E2 (5%) (Wasser *et al.,* 1996). Within terrestrial mammals different studies managed to established the lag time between the secretions of hormones from the gonads and/or adrenal glands and the moment the hormone is found in the excretes, either faeces or urine (see tab 1.1).

Due to the large differences within animal species and metabolites excreted, it is of the utmost importance to establish which technique is the most applicable to each species and what kind of immunoassay is more reliable to monitor the reproductive status and adrenal activity. The type of steroids metabolites excreted in faeces of different terrestrial animal species have been identified by radioinfusion studies and with the use of chromatographic and immunoassay techniques (Kirkpatrick et al., 1991; Schwanzerberger *et al*., 1996; Schwanzerberger *et al.* 1992; Schwanzerberger *et al*., 1991; Shideler *et al*., 1993; Wasser *et al*., 1996; Wasser *et al*., 1994). These studies showed that progesterone undergoes several metabolic process before its faecal excretion and its faecal metabolites consist of several 5α- and 5β- pregnanes (pregnanediones and mono- and dyhidroxilated pregnanes). In contrast faecal estrogens, being estrogens endproducts of steroid metabolism, have similar compound in plasma and faeces and consist mainly of oestrone, estradiol-17α and estradiol-17β (Schwanzerberger *et al*., 1996). Different metabolites from P4, E, TST and glucocorticoids have been investigated in several different species (see Tab.1.2.)

Table 1.1. Hormone excretion and metabolism in domestic and exotic species - Part 1
(modified from Schwarzenberger *et al.*, 1996)

Species	Hormone	Faecal excretion %	Urinary excretion %	Faecal time lag (hrs)	% unconjugated in faeces	Reference
Sheep (*Ovis aries*)	E2	74	26	18	97	Adams *et al.*, 1994
	E1	87	13	10	100	Palme *et al.*, 1996
	P4	77	23	11	99	
	T	44	56	13	98	
	C	28	72	11	95	
Horse (*Equus caballus*)	E1	2	98	20	n.d	Palme *et al.*, 1996
	P4	75	25	24	97	
	T	28	72	22	82	
	C	41	59	26	77	
Pig (*Sus domesticus*)	E1	4	96	20-80	99	Palme *et al.*, 1996
	P4	34	66	57-113	96	
	T	14	86	24-49	98	
	C	7	93	21-100	94	

Table 1.1. Hormone excretion and metabolism in domestic and exotic species – Part 2
(modified from Schwarzenberger *et al.*, 1996)

Species	Hormone	Faecal excretion %	Urinary excretion %	Faecal time lag (hrs)	% unconjugated in faeces	Reference
Domestic cat (*Felis catus*)	E2	97	3	24-72	50	Shille *et al.*, 1990
	E2	97	3	11-21	55	Brown *et al.*, 1994
	P4	97	3	12-24	22	
	C	86	14	22	13	Graham and Brown, 1996
Siberian polecat (*Mustela eversmannii*)	E2	93	7	n.d.	61	Gross, 1992
	P4	93	7	n.d.	66	
	T	93	7	n.d.	70	
White rhino (*Cerathoterium simum simum*)	E2 and P4 a	59	41	24-48	92	Hindle and Hodges, 1990
Sumatran rhino (*Dicerorhinus sumatrensis*)	E2	33	67	n.d.	n.d.	Heistermann *et al.*, 1998
	P4	99	1	n.d.	n.d.	
African elephant (*Loxodonta africana*)	E2	5	95	48-50	80	Wasser *et al.*, 1996
	P4	55	45	48-50	80	
Slow lori (*Nycticebus coucang*)	E2	93	7	24-48	59	Perez *et al.*, 1988

Table 1.1. Hormone excretion and metabolism in domestic and exotic species – Part 3
(modified from Schwarzenberger *et al.*, 1996)

Species	**Hormone**	**Faecal excretion %**	**Urinary excretion %**	**Faecal time lag (hrs)**	**% unconjugated in faeces**	**Reference**
Ring-tailed lemur (*Lemur catta*)	E2	16	84	24-48	100	Perez *et al.*, 1988
Cotton-top tamarin (*Saguinus oedipus oedipus*)	E1	43	57	24-48	11	Ziegler *et al.*, 1989
	E2	13	87	24-48	15	
	P4	95	5	24-48	65	
Long-tailed macaque (*Macaca fascicularis*)	E2	45	55	24-56	100	Shideler *et al.*, 1993
	P4	58	42	32-56	100	
Yellow baboon (*Papio cynocephalus cynocephalus*)	E2	10	90	37	80	Wasser *et al.*, 1994
	P4	40	60	37	80	

Notes: E_2 = 17β-oestradiol; E_1 = oestraone; P_4 = progesterone; T = testosterone; C = cortisol.
n.d. = not determined.
[a] Combined application and determination of faecal E_2 and P_4 metabolites.

Tab 1.2. Faecal metabolites in different species (Part 1)

Author	Native hormone	Metabolites	Animal species
Billitti et al, 1998	TST	androsterone etiocholanone	Mouse (*Mus musculus*) Deer mouse (*Peromyscus maniculatus)*
Hunt et al, 2006	TST	A4, androstenedione T, testosterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone	North Atlantic right whale (*Eubalaena glacialis*)
Mohle et al., 2002	TST	testosterone, 5α-androstane-17α-ol-3-one (Epidehydrotestosterone) androsterone	common marmoset (Callithrix jacchus) long-tailed macaque (Macaca asciculari s) chimpanzee (Pan troglodytes)
Rolland et al., 2005	TST	Androgens metabolites	North Atlantic right whale (*Eubalaena glacialis*)
Foley et al., 2001	GC	Cortisol metabolites	African elephant
Hunt et al., 2006	GC	B-S, corticosterone sulfate; DHEA-S, dihydroepiandrosterone sulfate; F, cortisol; B, corticosterone	North Atlantic right whale (Eubalaena glacialis)

Tab 1.2. Faecal metabolites in different species (Part 2)

Author	Native hormone	Metabolites	Animal species
Wasser et al, 2000	GC	Cortisol Corticosterone Glucocorticoid metabolites	yellow baboons (Papio cynocephalus) long tailed macaque (Macaca fascicularis), African elephant (Loxodonta africana), Roosevelt elk (Cervus elaphus roosevelti), Alaskan sea otter (Enhydra lutris kenyoni). Malayan sun bear (Helarctos malayanus), gerenuk (Litocranius walleri), scimitarhorned oryx (Oryx dammah), black rhinoceros (Diceros bicornis), cheetah (Acinonyx jubatus), clouded leopard (Neofelis nebulosa), northern spotted owl (Strix occidentalis caurina).
Fieß et al, 1999	P4	5α- pregnane-3-ol-20-one (5 α-P-one) 5α-dihydroprogesterone (5-α-DNP)	African Elephant
Heistermann et al., 1998	P4	5β-pregnane-3α-ol-20-one 5β-pregnane-3α,20α-diol 5α-pregnane-3α-ol-20-one	Sumatran rhinoceros
O'Neill et al., 2004	P4	pregnanediol (PdG)	Japanese macaques (Macaca fuscata)

Tab 1.2. Faecal metabolites in different species (Part 3)

Author	Native hormone	Metabolites	Animal species
Ostner and Heistermann, 2003	P4	5α-pregnane-3α-ol-20-one (5- P-3OH) 20a-hydroxyprogesterone (20-OHP) 5α-dihydroprogesterone (5-DHP)	redfronted lemurs (Eulemur fulvus rufus)
Schwarzenberger et al, 1991	P4	20α-OH progestagens(20 α-G) 20β-OH progestagens (20 β- G)	Lipizzan, Trotter and Thoroughbred mares.
Schwarzenberger et al., 1992	P4 in Plasma	20 α-G 20 β- G	Mares and Przewalski mares
Schwarzenberger et al, 1996	P4	5α pregnane 5β pregnane	Wild-zoo animals
Heistermann et al., 1996	E	Estrn conjugates (E1C) Estriol (E3)	Bonobo (Pan paniscus)
Heistermann et al., 1998	E	oestradiol-17α (E2-17α) oestrone (E1) oestradiol-17β (E2-17β) Oestriol (E3)	Sumanrtan rhinoceros
Hunt et al., 2006	E	A4, androstenedione E1, estrone; E2, 17β-estradiol	North Atlantic right whale (Eubalaena glacialis)
Ostner et al., 2003	E	estriol (E3) estradiol-17β (E2-17β) estradiol-17α (E2-17α), estrone (E1)	Redfronted lemurs (Eulemur fulvus rufus)
Patzl et al., 1998	E	oestradiol-17β	Giant anteater (Myrmecophaga tridactyla)

1.4- Non invasive sample collection in marine mammals

Marine mammals, especially dolphins (members of the Order cetacea, Suborder Odontoceti, Family Delphinidae) have been kept under human care since the beginning of 1900 (Mead and Potter, 1990). The first world's ocenarium (Marine Studios) was open to the public in Florida (U.S.A.) in 1938 (www.marineland.net/aboutus.htm). Due to its capability to easily adapt to different situations the bottlenose dolphin (*Tursiops truncatus*) is the most common species of delphinidae kept in controlled environments, followed by the Killer whale (*Orcinus orca*).

The capture of wild cetaceans is nowadays strictly regulated by the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in 1972 the Unites States established the Marine Mammal Protection Act (MMPA) that imposed a moratorium in both capturing and importing marine mammals. Due to the increased demand for captive born dolphins during the last years, the possibility of monitoring the reproductive status and the effect of extraneous factors like increasing level of stress of these animals is of the utmost importance, both for animal management and to implement proper reproductive programs. Studies on wildlife conservation are nowadays dominated by researches into genetics, population growth, seasonal movements and reproductive rates, biometric studies and external factor that can affect the general health of population's survival. The key of most of these studies are the hormones that allowed us to better understand the reproductive physiology and physiological response to environmental changes.

Hormones concentrations is determined through invasive sample collection such as blood (serum or plasma), but during the last decades the application of non invasive techniques such as faecal, urine saliva or blowhole sampling has become more used with marine mammals as well to describe the reproductive cycle and/or the adrenal gland activity of Steller sea lions (Hunt *et al.*, 2004; Hunt *et al.*,2006; Petrauskas and Atkinson, 2006), North Atlantic Right Whale (Hunt *et al.*,2006; Rolland *et al.*, 2005), bottlenose dolphins (Pedernera-Romano *et al.*,2006), killer whale (Deffield *et al.*, 1995; Robeck *et al.*,1993), sea otter (Larson *et al.*, 2003).

In some species of marine mammal, like right whales, repetitive blood collection results to be very difficult if not impossible. For some species of dolphins, sirenians and pinnipeds,

blood samples can be collected from wild animals by capturing some specimens and release them after collection. But this method has the disadvantages that the animal got stressed by the all procedures of capturing, handling and venipuncture. Furthermore, blood samples give the hormone concentration of the instant of collection. While short period of stress might not induce an increase of sexual hormones, it can induce an increase of adrenal hormones making difficult to interpret specific situation that can be altered by the stress induced by capturing. St Aubin *et al*. (1996) reported that cortisol and aldosteron levels were both higher in 36 wild bottlenose dolphins captured with nets and sampled after a variable intervals of up to four hours (interval between initial encirclement and blood collection ranged between 23 and 260 minutes) than the levels of adrenal hormones obtained from 36 semidomesticated bottlenose dolphins conditioned to present their tail for blood collection.

The possibility to keep marine mammals in controlled environment allowed scientists to further investigate the anatomy and physiology of different species and apply the knowledge to better understand wildlife.

Due to the large difference between excretion route within different species and the difference of metabolites excreted into faeces, urine, saliva or blowhole, it is important to validate any new non invasive hormone measurement technique. The immunoassay has to be validated in order to demonstrate that the specific hormone that is searched, it is properly measured. Within marine mammals the differences between pinnipeds (sea lions and seals), sea otters and polar bears that come ashore to rest or to breed and cetaceans (dolphins and whales) and sirenians (Manatees and dugongs) that spend their entire life in the water, make the possibility to investigate the physiology and endocrinology of those animals even more challenging.

Validation needs to be done both from biological and laboratory aspects. Laboratory validation includes different parameters (sensitivity, specificity, accuracy and parallelism) of the test that need to meet specific criteria. Biologically, the test has to be validated comparing results obtained from different biological samples used to test the same hormone (i.e. Faeces VS blood; Urine VS faeces), or comparing ethogram and physiological events with hormones detected (i.e. end of pregnancy VS drop of faecal progesterone in faeces). The most effective method to biologically validate a non invasive

method is through the administration of exogenous long-acting hormones or with high dosages of hormones like LH, GnRH (gonadotropin releasing hormone) or ACTH. The animals considered in this study are registered in the Appendix 2 anex C of the CITES and following the European regulation CE 338/97 it was not authorized the injection of exogenous substances for research purposes. To my knowledge, few studies have been done on bottlenose dolphins where the administration of substances were used to study the renal function and water/electrolytes balance and hormonal regulation. For these purposes either fresh water or hypertonic NaCl solution were orally administrated (Fetcher and Fetcher, 1942; Ridgway, 1972). In 1983 Thomson and Geraci (1986) evaluated the adrenocortical and stress response of bottlenose dolphins to intramuscular injection of ACTH

Non-invasive methods that allow the determination of hormone concentrations need to be developed initially using captive populations to determine the concentration ranges and possible physiological baseline levels of hormones and then the validated techniques could be used to assess reproductive behaviour of wild cetaceans.

1.5- Bottlenose dolphin under human care

The bottlenose dolphin is the most common cetacean held in captivity, and reproductive success with this species has reached a point that parallels or exceeds that observed in wild populations (Duffield et al. 2000, Wells 2000).

Despite the large number of individuals present in oceanaria and dolphinaria, most animals are dispersed among numerous genetically isolated facilities. The movement of animals between facilities could enable genetic exchange, but many aquaria are unwilling to participate in this management practice (Robeck *et al*, 2005) for logistic and or economic reasons. In 1987 the costs for the transport of three false killer whale from United States to Japan and then to Holland, including medical equipment, trucks, aircraft charter and staff amounted at $ 166,237.11 (http://www.pbs.org/wgbh/pages/frontline/show/whales/seaworld/org). The cost of one-way shipping of a container with stored semen, including shipping company fees, lab fees and semen collection fees, varied from $ 300 to $600 (Homer-Drummond, 2006), resulting to

be cheaper than an international live-animal transport and involving zero risk for the welfare of the animals. Personnel costs for the staff in charged for monitoring and assisting females during parturition are the highest, but the same for both the procedures. Although there are some circumstances for which the transport of cetaceans have to be taken in consideration (i.e. necessity of social changes in the group, social incompatibility between animals, necessity to renew the structures of the pools) (R. Gojceta, curator Laguna Oltremare, personal communication, 2007), the applications of advanced reproductive techniques can solve the problems of high costs and long distances.

To achieve the best management of bottlenose dolphins from the breeding purpose point of view, the groups of animals kept under human care should mimic the natural social grouping. In the wild, bottlenose dolphins are divided in three main groups: 1-females: pregnant females, mothers and their youngest calves; 2- adult males, usually in small groups; 3- juvenile dolphins, of both sexes, that live temporary together (Wells *et al.*, 1999)

In order to establish a proper breeding program, several aspects have to be considered, especially if the final aim is to develop an assisted reproduction program with the application of the artificial insemination (AI). The reproductive endocrinology of the females has to be monitored on regular base in order to establish the right moment for the insemination. This include ultrasound monitoring of the ovarian follicles and hormonal levels.

In captive bottlenose dolphins, blood sampling is achieved nowadays mainly by trained behaviour. But due to its invasive nature, other excretes, such as urine or faeces, for which the collection is considered to be less invasive, can be use to monitor endocrine status of these animals. Due to the possibility for pinnipeds to spend several hours out of the water, there is not particular behaviour that needs to be trained with them for urine or faeces collection. Different is the situation with cetaceans that do not spend part of their life out of the water. To obviate this scarcity of possibility of collecting their urine from the environment, dolphins and killer whales can be trained for urine (Duffield *et al.,* 1995; Lenzi, 2000; Robeck *et al., 2005*) and faecal samples collection.

Although urine analysis have been successfully used to monitor oestrous cycle and predict the ovulation of bottlenose dolphins (Robeck *et al,* 2005; Van Elk *et al.,* 2005) not all the

facilities train their animals for urine collection and in the case of males, it is preferable to train only for semen collection to avoid the risk of contamination of the semen with urine.

Salivary samples have been used to monitor cortisol levels in trained bottlenose dolphins (Pedernera-Romano *et al.*,2006), while blowhole samples have been used to monitor the pregnancy (Accorsi *et al.,*2005 ; Tizzi *et al.,* 2004)

Animals in controlled environment have to cope with different stimuli that affect their health status by modulating their hypothalamic-pituitary-adrenal (HPA) axis' response. Inappropriate management or environment has been shown to have negative effect on the reproduction, physiology and behaviour of mammals (Estep and Dewsbury, 1996; Kirby, 1990). The activation of HPA induce release of ACTH that stimulate the release of glucocorticoids (GCs). Elevated levels of GCs can lead to chronic stress with negative effect on the immuno system, electrolytes balance, growth and reproduction (Kirby, 1990).

The possibility to train the animals for medical behaviours has been proved to result in less stressful situation for the animals and the voluntary collection can yield samples representative of resting ACTH hormones concentrations (St. Aubin *et al.,* 1996). Faecal material resulted to be the most easy to sample by voluntary behaviour in association with less stressful situation for the animals. It allowed us to collect frequent samples with the same methodology applicable to males and females.

CHAPTER 2: MATERIAL AND METODS

Sample collection

During the last fifteen years the necessity to improve our knowledge regarding reproductive hormones and adrenal activity of cetaceans has led researchers to broad the methodologies to investigate the physiology of these animals.
The first and still most known and used technique to asses physiological events in cetaceans is blood sampling (St. Aubin *et al*., 1996; St. Aubin *et al*., 2001; Suzuki *et al*.,1998). Within the last years different sampling methods were use to reach the best results, with less invasive and stressful techniques: urine sampling (Robeck *et al*., 1993), blubber sampling (Mansour *et al*., 2002), saliva sampling (Pedernera-Romano *et al*., 2006), blowhole sampling (Accorsi *et al*, 2004; Tizzi *et al*., 2004) and faecal sampling (Biancani *et al*., 2007a,b; Hunt *et al., 2006,* Rolland *et al.,* 2005). Radioimmunoassay (RIA) and/or Enzyme immunoassay (EIA) have been used to determine hormone concentrations.
The most applied procedures expect a comparison between concentrations of hormones in instantaneous samples (serum or plasma) with concentrations of hormones in pooled samples of faeces or urine. This comparison should be considered with caution due to the differences of secretion and excretion followed by the different hormones (Whitten *et al*., 1998). The lag-time between presence of hormones in blood stream and their appearance in exctreta differs between less than 5 hours in urine and up to over 48 hours for faeces according to different animals species, the length of the intestine and the passage rate time (Schwarzenberger *et al*., 1996). Furthermore, as explained in the first chapter (see Tab 1.1.) the percentage of hormones excreted into the urine or in the faeces varies among different species (Palme *et al*., 1996; Schwarzenberger *et al*., 1996).
Considering these variables and the absence of known lag time for hormones excretion in to the faeces of bottlenose dolphins, during the collection of samples used for my research, I tried to collect faecal samples and vagynal cytology samples on the same day of blood collection and as well as at least during the two following days.

2.1. Faecal sampling

The necessity to further investigate physiology and endocrinology aspects of wild animals has led during the last years to the more impelling need of developing non invasive techniques (Larson *et al.*, 2003; Palme *et al.*, 1996; Ostner and Heistermann, 2003; Wasser *et al.*, 1988; Ziegler *et al.*, 1989). Faecal samples are generally easy to collect and studies on different species showed how steroids hormones excreted in faeces reflect the reproductive status of the animals.

Glucocorticoids are usually used to evaluate the level of stress in animals (Reeder and Kramer, 2005; Romero, 2002; St. Aubin *et al.*, 1996). Due to the fact that hormones and their metabolites can be concentrated in faeces from hours to days before to be excreted, faecal hormones do not reflect the instant physiological situation of the adrenal gland activity, and for this reason faecal sample could be very useful for monitoring chronic stress or could be used to detect acute stress episodes by collecting them on daily base.

For the present study, faecal samples were collected at least once a week (from 1 to 4 times a week), with the aim of collecting sample of faeces on the same day of blood collection and at least the two following days.

All the dolphins used for the present study were trained for faecal sample collection as normal routine of the clinical examination of the animals. Faecal samples were collected by voluntary behaviour. The dolphin was asked for a “belly-up” position and the faeces were collected directly from the rectum (Fig.1) with “Levins tube” (Pennine healthcare, Derby- UK). The tube has a diameter of 4 mm and was inserted in the rectum (Fig. 2) for 10 to 20 cm. After collection, the faecal samples were placed in a plastic container and kept frozen at minus 23 ℃ until analysed for proge sterone (P_4), Oestradiol (E_2), Cortisol (C) and Testrtosterone (TST).

Fig.1: Voluntary faecal sampling (Picture by B. Biancani)

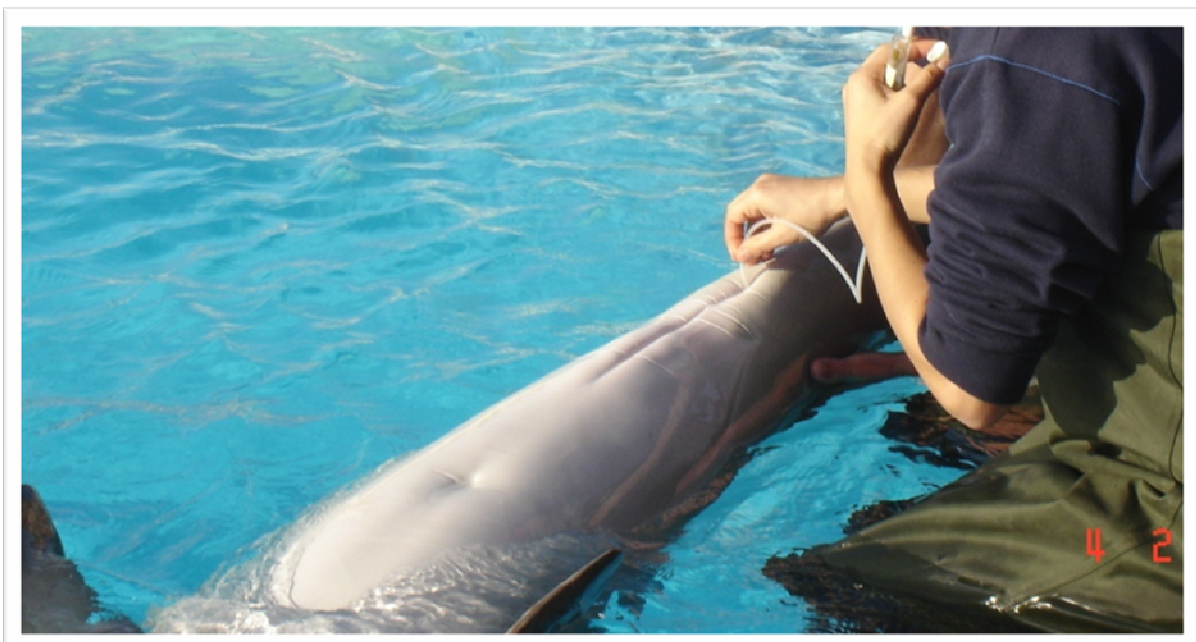


Fig. 2: Voluntary faecal sampling (Picture by B. Biancani)

2.2. Blood samples

Due to its invasive nature, most of the facilities that keep marine mammals under human care, prefer not to collect blood sample on a daily base if the animals do not show signs of sickness. For the present study blood was collected by voluntary behaviour from the tail fluke either (Fig.3) from the superficial vein or from the artery or the periarterial venous *rete* (Geraci and Lounsbury, 2005) at least once a month, or according to the medical protocol adopted at the facility. When possible, blood and faeces were collected on the same day. Blood was collected with 21 gauge butterfly needles and spun in plain red top tube (without anticoagulant). After collection the blood was centrifuged (2,500 rpm at 4℃) to separate the serum. Serum was kept in eppendorf and frozen at -23 ℃ until analysed.

Fig. 3: Blood sampling from the ventral side of the tail. (Picture by B. Biancani)

While sampling blood for serum, another blood sample was collected in a tube with anticoagulant (EDTA). Blood with EDTA was used to prepare count the white blood cells (WBC) with the Unopette Reservoirs (BD, Franklin Lakes, NJ,USA) and to prepare a slide

to check the differential of the WBC. The count was done up to 100 WBC differentiated in Neutrophils, Lymphocytes, eosinophils, monocytes, and basophiles (Fig.4).

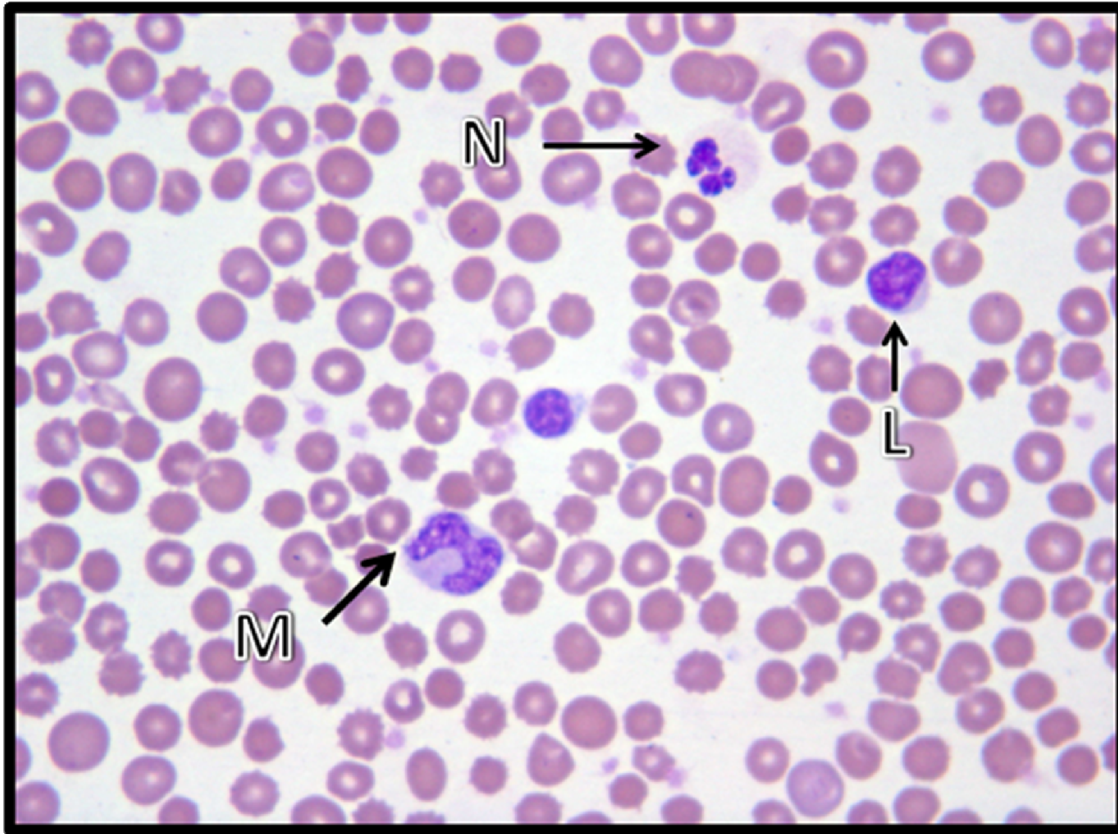


Figure 4: Blood smear with monocites (M), lymphocytes (L) and neutrophil (N)
(Picture by B. Biancani)

4.3. Vaginal cytology samples

Vaginal cytology has been used in different domestic and exotic animal species to asses the stage of the reproductive cycle and the onset of ovulation (Durrant *et al.*, 2002; Mills *et al.*, 1979; Muraco *et al.*, Williams *et al.,* 1992). The turnover of hormones during the oestrous cycle, particularly the estrogens, stimulate the changes of the mucosa of the vaginal tract. The increase of estrogens induce a progressive keratinisation of the vaginal epithelial layers. Muraco et al. (2004) reported that 80-100% of keratinized cells correspond to the onset of the ovulation and optimal time for breeding.

Details of animals and periods for vaginal cytology samples collection will be referred in chapter four.

For the present study vaginal cytology samples were collected by voluntary behaviour. The dolphins were trained for a ventral lay-out presentation. The genital area was dried with gaze, and the sample was collected (Fig.5) using a sterile culture swab Sterilin® (Stone, Staffs- UK) that was inserted in the vaginal opening for about 8 cm and turned for 360 degrees before removing. The swab was then rolled in a tube containing 1 ml of Hartmann solution. The liquid containing the cells was then placed on the slide in order to get dry. Once the smears were dry, they were fixed and stained with the rapid blood-dye ® (Diffquick), with the modification reported by Muraco *et al.* (2004):

- Dry slide was first dipped 1 second for 10 times into the Dip Quick stain fixative solution (Fast green in methanol – pale green colour);
- Once the slide was dry, it was dipped 15 times in the stain n°1 (Eosin G in phosphate buffer- red colour);
- then was dipped 15 times in the stain n°2 (Thiazi ne dye in phosphate buffer – blue colour);
- The slide was then rinsed with distillate water and once dryed up, it was ready to be examined under the microscope.

The slides were examined by light microscope under 10x and 40x. One-hundred cells were counted at 40x to determine the status of the cycle.

Cells were differentiated in:

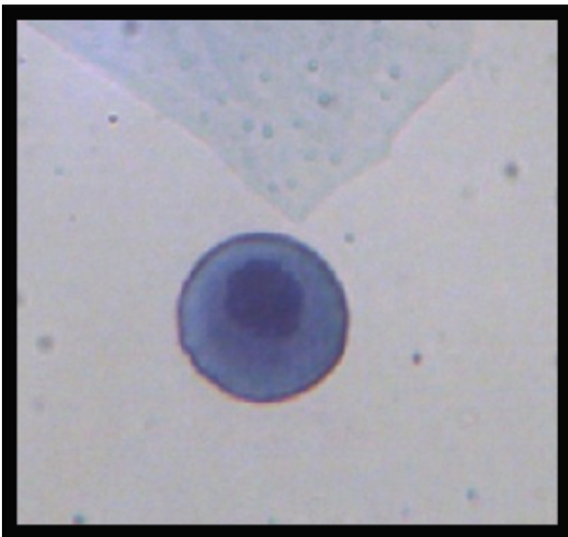
Basal cells: smallest cells with rounded shape, rounded nucleus and ratio nucleus: cytoplasm in favour of the nucleus (Fig.6).

Parabasal cells: rounded/oval shape with ratio nucleus: cytoplasm in favour of the nucleus, and margins of cells still very well delineated (Fig.7).

Intermediate cells: larger than parbasal cells, oval to polygonal in shape with a small nucleus to cytoplasmic ratio (Fig.8).

Superficial or keratinized cells: the largest cells, with polygonal shape, flat or even “rolled up” in appearance, nuclei are absent (anucleated) or very small and dark (Fig.9).

Fig.5: Vaginal cytology sampling: the animal has been trained for the position.
(Picture by B.Biancani)



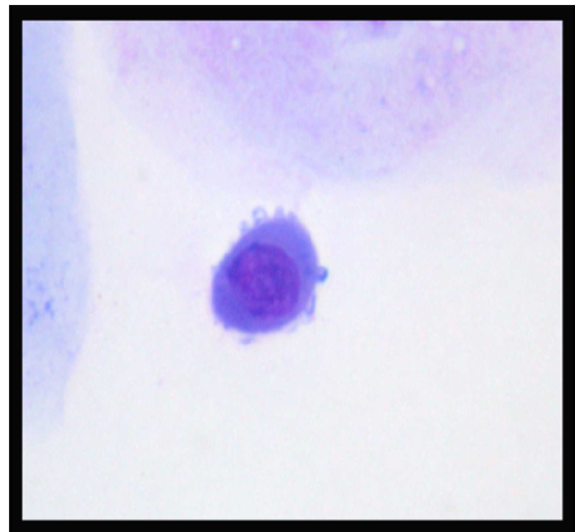
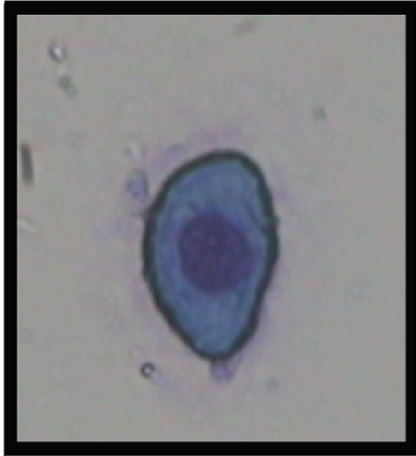


Fig.6: Basal cell 40x and 63x (Picture by B.Biancani)



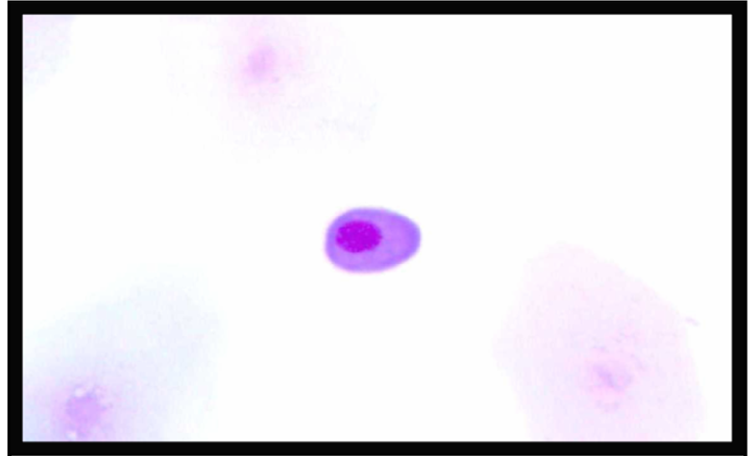


Fig.7: parabasal cell 40x and 20x (Picture by B.Biancani)

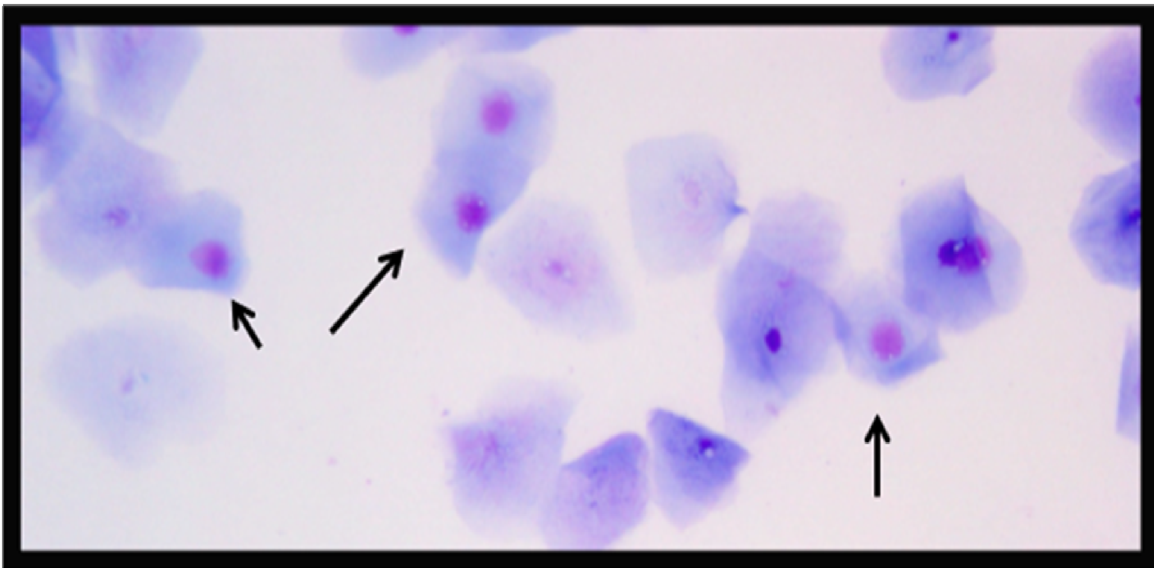


Fig. 8: intermediate cells 20x (arrows) (Picture by B.Biancani)

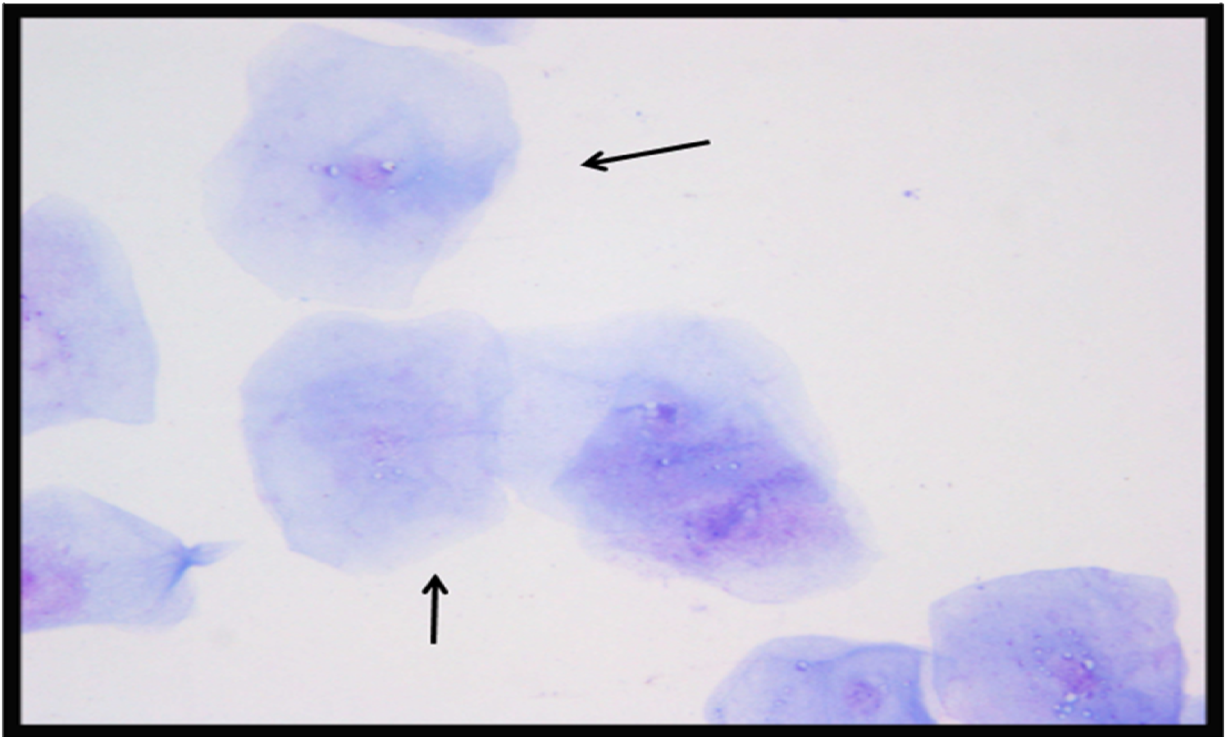


Fig.9: Superficial cells (arrows) (Picture by B.Biancani)

2.4. Ultrasound

Ultrasound examination was performed on different schedule according to the facility where the animal were kept. All ultrasound examinations were gathered through voluntary behaviour of the animals. The animal was asked for a lateral position and supported on the surface of the water with the help of one or two trainers that would have help the animal to breath without changing its position. The animal was trained to station in lateral position, adjacent to the edge of the pool for the necessary time to scan the ovary or the testis (time varies from 8 to 12 minutes). Both sides of the animal were examined.

Ultrasound were performed according to Brook (2001; 2000,b and 1999) to asses the stage of oestrous cycle in the females and the testis development in the males.

To examine the ovary the procedure reported by Brook (2001) was followed. The transducer was placed in the transverse (Fig. 10) plane midway along the genital slit and moved dorsally along the line where the junction between the triangular, lateral border of

the rectus abdominus muscle and the roundedvisceral surface of the hypaxialis lumborum muscle can be seen (Fig. 11).

Fig.10: transversal position of the probe (Photo by B. Biancani)

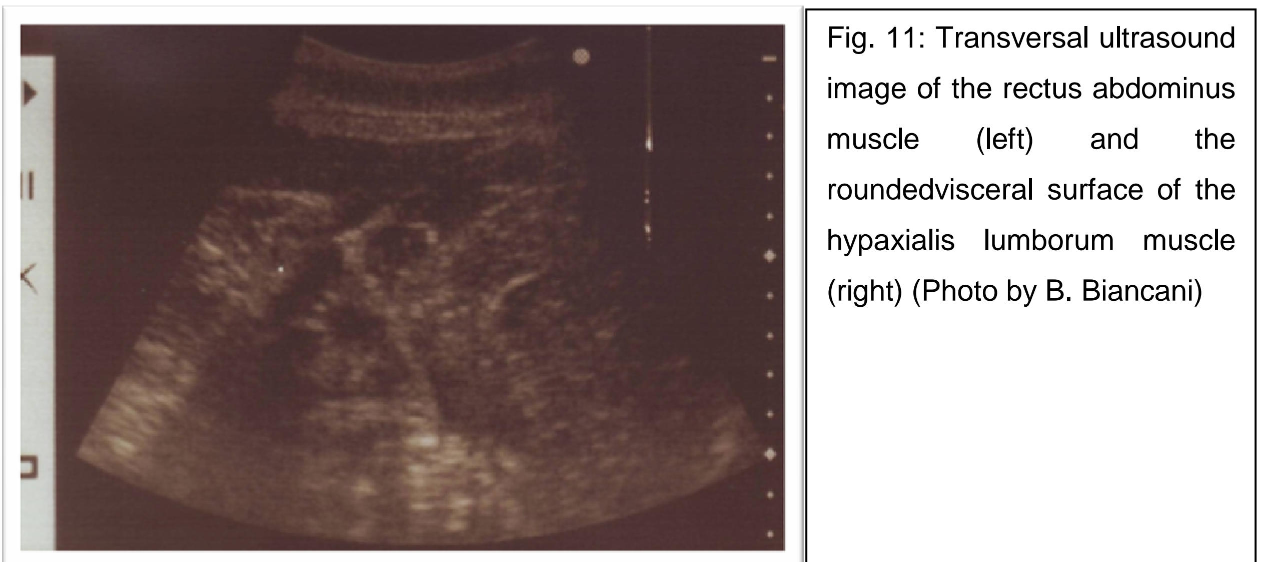


Fig. 11: Transversal ultrasound image of the rectus abdominus muscle (left) and the roundedvisceral surface of the hypaxialis lumborum muscle (right) (Photo by B. Biancani)

The ovaries usually lie between these, against the wall of the abdominal cavity, at a variable distance from the genital slit. There is a palpable depression in the flank where these muscles meet, which can also provide a guide for placement of the transducer (Fig. 12). The transducer was moved cranially until the transverse axis of the ovary was identified. The transducer could then be rotated by 90° to visualize the long axis of the ovary. The procedure was repeated for the contralateral ovary.

Fig.12: longitudinal position of the transducer for ultrasound examination of the ovary and testis (Photo by B.Biancani)

The ovary were located trans-abdominally and follicular size was determined by measuring its largest diameter (Fig. 13)

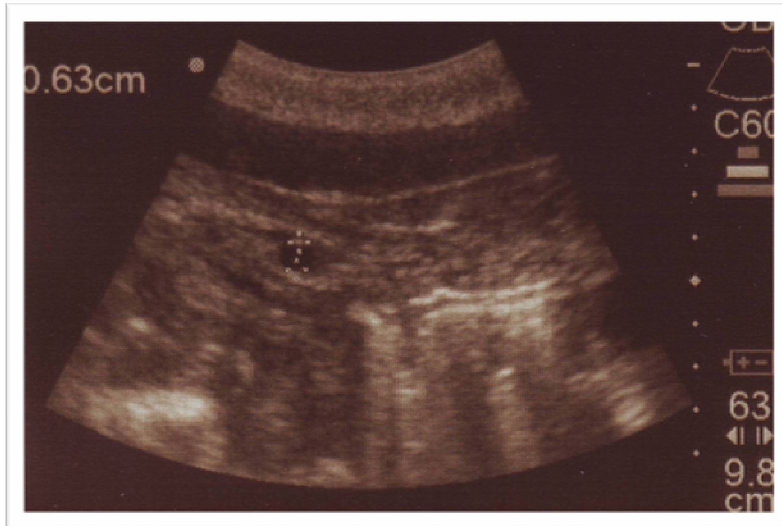


Fig. 13: Measurement of follicle in the left ovary.
(Picture by B.Biancani)

Pregnant females were monitored to asses the wellbeing of the foetus and to better establish the delivery date (Lacave *et al.,* 2002)

The testis can be visualized lying longitudinally, immediately caudoventral to the kidneys

In transverse section, immediately adjacent or close to the testis there are the hypaxialis lumborum muscle and the rectus abdominus muscle.

Of the gonads of the males both the cross section and the length were measured (Brook *et al*, 2000). The cross-sectional measurements of the testes were taken directly from the image display, using the in-built electronic caliper functions. However, the maximum width of the ultrasonographic field of view available was not always enough and it was not possible to take longitudinal measurements of longer testes directly from the monitor image. To double check the correct length of the gonads, each end of the testis was located ultrasonografically and noting these points on the surface of the skin, it was possible to make indirect measurements of testicular length. The transducer was placed perpendicular to the flank, at the level of the genital slit and moved dorsally or cranially until the testis was located. The proximal and caudal ends of the testis were identified, both points were noted on the skin surface and the distance between them measured (Figure 14) (Brook *et al*, 2000).

Fig. 14: external measurement of testis (Picture B.Biancani)

A SonoSite 180 Plus (Sonosite, Inc.-U.S.A.) ultrasound scanner with using a convex sectorial 3.5 MHz probe was used to monitor the ovarian activity in females 001, 002 and 003 and the testis of males 001 and 002.

The pregnancies of females 007 and 008 were monitored with an Aloka SSD-900 ultrasound scanner using a convex sectorial 3.5 MHz probe to measure the development of the foetus by measuring the head biparietal and thoracic diameters.

The probe was waterproofed by covering it with a plastic sleeve containing acoustic gel, which displaced any air and provided good acoustic contact. Plastic taping secured the covering sleeve and extended up the transducer cable, to ensure this was also waterproofed.

2.5. Ethogram

For the five animals hosted at Mediterraneo Marine Park (Malta), 3 females and 2 males, the appetite and attitude were evacuate on daily base. Trainers were giving a scores to each animals according to their attitude towards the trainers and other dolphins of the group and their appetite. Scores ranged from 1=low/bad to 5=very good (see Tab 2.1.). Specific notes regarding the clinical situation of the animals were recorded as well to compare with the cortisol levels.

2.6. Body measurement and body weight

The females (F-001, F-002, F-003) and males (M-001 and M-002) were measured mainly twice a month during the study period. Dolphin measurements were body length (BL) and girth (G) and were taken with a non-rigid rule. In order to measure BL, the trainer asked the dolphin for a voluntary ventral layout position and measurement was done from the tip of the rostrum to the navel (Fig. 15) and from the navel to the notch of the tail fluke. For G measurement (Fig.16), the trainer asked the dolphin for a voluntary dorsal layout position and data was recorded 5 cm in front of the dorsal fin right just after the animal breath out.
Body Weight (BW) was calculated using Morphometric Calc pre-release version 1.4 (Outernet Technologies International 1999) accessible at http://www.outernet-tech.com/research/download/ (Messinger and Weissensel, 1999).
Females 006,007 and 008 were weighted with the use of an electronic weight scale. The animals were trained to slide on the scale for the necessary time for the weight to be read.

Fig. 14: body length measurement (Picture by B.Biancani)

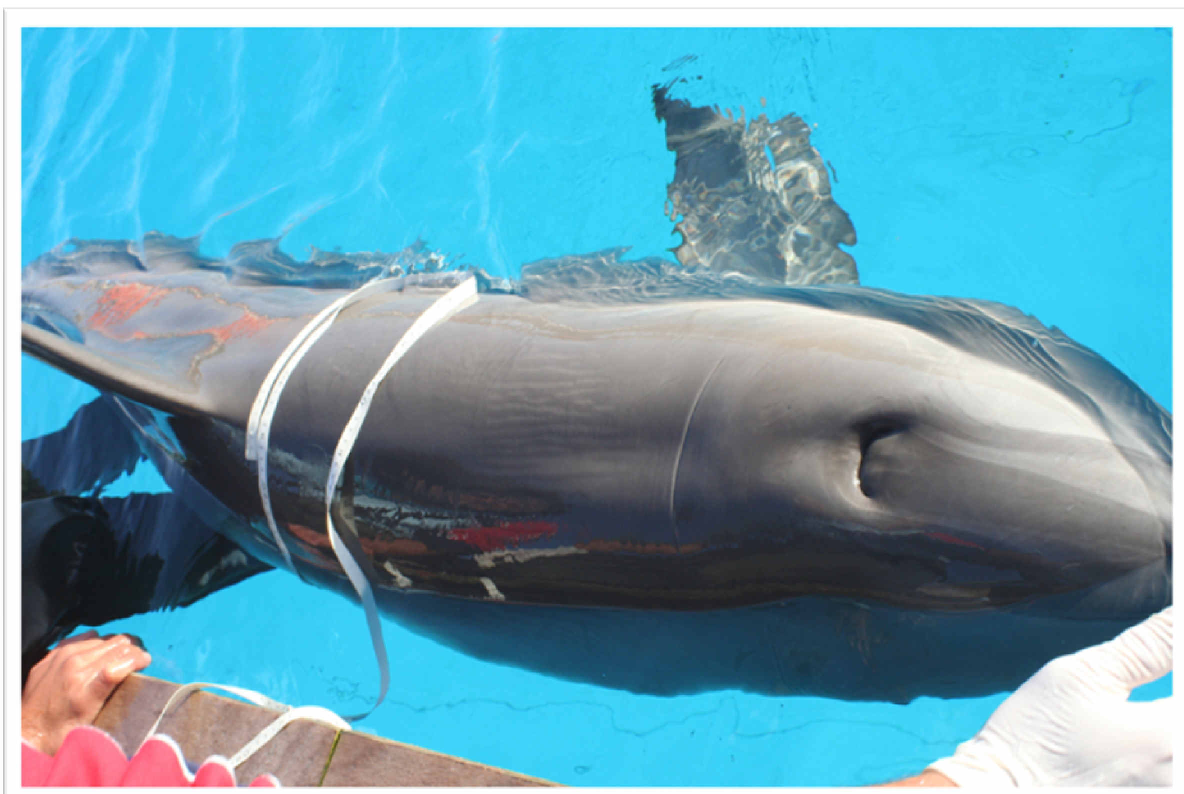


Fig. 15: measurement of the girth. Tape placed 4-5 cm in front of the dorsal fin (Picture by B.Biancani)

Tab 2.1.: Monthly ethogram

NAME :XXX							MONTH:				SPECIES: Tursiops			SEX : M/F			
DATE	**ATTITUDE**					**APETITE**					**FISH**						**K CAL**
	5	4	3	2	1	5	4	3	2	1	HR	CA	MK	WT	SP	SQ	**TOTAL**	
1	X					X					1	2		3			6	7590
2	X					X					3	3					6	10740
3	X					X					5						5	11200
4	X					X					5						5	11200
5		X					X				5	1,5					6,5	13210
6	X					X					2	2		2			6	8940
7	X					X					6						6	13440
8	X					X					2	2		2,5			6,5	9385
9	X					X					2	4					6	9840
10	X					X					5						5	11200
11					X					X	1,6						1,6	3584
12	X					X						5					5	6700
13	X					X					3,3	3					6,3	11412
14	X					X					4,5						4,5	10080
15			X					X			0,8	0,5		0,5			1,8	2907
16	X					X					2,5	2					4,5	8280
17	X					X					5						5	11200
18	X					X					5						5	11200
19	X					X						5					5	6700
20	X					X					2,5	3,5					6	10290
21		X					X				5						5	11200
22	X					X					2,2	3		2,5			7,7	11173
23	X					X					4,5	2,5					7	13430
24	X					X					5						5	11200
25			X					X			4	0,8					4,8	10032
26	X					X					1,75	5					6,75	10620
27	X					X					3	3,5					6,5	11410
28	X					X					6						6	13440

CHAPTER 3: HORMONES ANALYSIS

HORMONES ANALYSIS

The immunoassay used for the present study is a radioimmunoassay (RIA). RIA consists in a binding assay, where the quantification of the searched substance is measured depending on the progressive saturation of a specific antibody by that substance, followed by the determination of the free and bound phases (Chard, 1990). The differentiation between the bound and free phases is made by labelling the antigen with a radioactive isotope.

Due to the different steroids hormones and their metabolites present in the faeces, the extraction process has to be considered properly in order to maximize the concentration of the substances of interest and, at the same time, to exclude possible interfering compounds. As explained in the first chapter, the presence of different metabolites vary among species due to their specific metabolic process and the specific intestinal microbial flora. As the exact identification of faecal steroid metabolites (i.e. the percentage of conjugated and unconjugated substances, prevalence of specific metabolite) is not known for the *Tursiops truncatus,* for the present study petroleum and ethylic ether were used, keeping in consideration the possibility that these apolar solvents can dissolve many other lipids that can interfere with the analysis (Makin, 1975).

3.1. Hormone extraction

3.1.1. Faecal extraction

Prior to extraction samples of faeces were thawed and then one hundred mg were placed in a glass tube with 8 ml of petroleum ether or ethylic ether according to the different hormones and mixed vigorously for 10 minutes at room temperature. Petroleum ether was used to extract faecal P4 in the females while ethylic ether was used to extract TST and

E2. Cortisol was extracted from faecal with ethylic ether in males and petroleum ether and ethylic ether from female faeces.

The tubes were centrifuged (2500 rpm, 4℃) for 5 min and the aqueous phase was frozen at -20℃ for 1 hour. The organic phase was transferred to a fresh tube and evaporated under nitrogen current. The dry extracts of faeces were respectively carefully dissolved in 0.5 ml of radio immuno assay (RIA) buffer (61 mM di-sodium hydrogen orthophosphate, 40 mM sodium di-hydrogen orthophosphate, 154 mM sodium chloride, 0.1% BSA, pH 7.2). Extracts were opportunely diluted before the assay was used.

3.1.2. Serum extraction

Prior to extraction samples of serum were thawed and 200 µl serum were placed in a glass tube with 8 ml of petroleum ether or ethylic ether according to the different hormones and mixed vigorously for 10 minutes at room temperature. Petroleum ether was used to extract serum P4 in the females while ethylic ether was used to extract TST, Cortisol and E2 in serum. The tubes were centrifuged (2500 rpm, 4℃) for 5 min and the aqueous phase was frozen at -20℃ for 1 hour. The organic phase was transferred to a fresh tube and evaporated under nitrogen current. The dry extracts of serum were respectively carefully dissolved in 0.2 ml of radio immuno assay (RIA) buffer (61 mM di-sodium hydrogen orthophosphate, 40 mM sodium di-hydrogen orthophosphate, 154 mM sodium chloride, 0.1% BSA, pH 7.2) for P4, TST and E2 while Cortisol dissolved in 1 ml of radio immuno assay (RIA) buffer. Extracts were opportunely diluted before the assay was used.

3.2. RIA

3.2.1.RIA analysis

Extracts were analyzed by a solid-phase RIA (Progesterone: Battocchio *et al.* 1999; Estrogen: Gabai *et al.*, 2004; Cortisol: Gabai *et al.,*2006; Testosterone: Simontacchi *et., al*, 2004). Briefly, 96-well microtitre plates (Optiplate, Perkin-Elmer life sciences) were coated with an anti-rabbit γ-globulin serum raised in a goat. The antiserum was diluted 1:1000 in

Tab. 3.1. Specific characteristic for the RIA methods of different hormones from faecal samples.

Hormone	antibody	dilution	Standard curve (pg/well)	tracer	Specific activity (Ci/mmol)
P4	anti-progesterone-carboxymethyloxime-BSA	1:8,000	2.5 - 320	[1,2,6,7-^{3}H] Progesterone	97
E	Anti E2-6-CMO	1: 15,000	1,5 - 200	[2,4,6,7-^{3}H] Estradiol	72
TST	Anti-TST-3-CMO-BSA	1:32,000	3,15 -200	[1,2,6,7-^{3}H] Testosterone	78.5
C	Anti CORT-3-CMO-BSA	1: 20,000	3,15 - 200	[1,2,6,7-^{3}H] Hydrocortisone	70

Tab: 3.2.: Cross reactions measured in the antisera used in the RIA methods for sex steroids.

Antibody anti-TST-3-CMO-BSA	**%**	**Antibody anti- E**	**%**	**Antibody anti-P4-7-CMO-BSA**	**%**
Testosterone	100	17β Estradiolo (E_2)	100	Progesterone	100
5α- dihydrotestssterone	38	E_1	2.5	11-α- idrossipro	77
5α- androstan- 3α, 17β-DIOL	13.7	E_3	1.2	11-β- idrossipro	65
5α- androstan- 3β, 17β-DIOL	13.6	DHEA	0.007	17-α- idrossipro	2.9
Androstenedione	1.7	17α- E_2	0.004	20-α- idrossipro	0.01
5- androsten- 3β, 17β-DIOL	1.2	P4	0.004	20-β- idrossipro	<0.001
Dehydrppiandrosterone (DHEA)	0.01	TST	0.004		
Cholesterol	$<1x10^{-3}$	AND	0.004		
Cortisol	$<1x10^{-3}$				

Tab. 3.3. : Cross reactions of the antiserum used for Cortisol (Anti-CORT-3-CMO- BSA) RIA.

Antibody anti-CORT-3-CMO-BSA	**%**
Cortisol	100
Prednisolone	44.3
11- Deoxycortisol	13.9
Cortisone	4.9
Corticosterone	3.5
Prednisone	2.7
17-hydroxyprogesterone	1
11-Deoxyprogesterone	0.3
Dexametasone	0.1
Progesterone	<0.01
17- Hydroxypregnenolone	<0.01
Danazol	<0.01
Dhea sulphate	<0.01
Androsterone sulphate	<0.01
Pregnenolone	<0.01

Tab 3.4. Detection limit, intra-assay and inter-assay for different faecal hormones extraction

Hormone	Detection limit (pg/well)	intra-assay (%)	inter-assay (%)
P4	2.5	4.1	5.5
E	1.5	5.3	11.3
TST	3.15	4.5	14.6 (low values) 15.7 (medium values) 9.03 (high values)
C	3.15	5.4 (Dietyl ether) 8.7(Petroleum ether)	4.1(Dietyl ether) 4.1 (Petroleum ether)

0.15 mM sodium acetate buffer pH 9 and incubated overnight at 4℃. The plates were then carefully washed with RIA buffer, added with 200 µL of specific anti-body serum raise in the rabbit diluted with different dilution according to the hormones in RIA buffer, and incubated overnight at 4℃. Afterwards, the anti-se rum solution was decanted and plates were washed with RIA buffer. Standards, quality control, and unknown extracts (100 µl) were added in duplicate. The standard curve was made by serially diluting in RIA buffer a hormone preparation (Sigma, Milan, Italy). The tracer (Perkin-Elmer Life Sciences, Boston; with specific activity for different hormones (see Tab 3.1) was used at 30 pg/well/10 µl. The total volume of the reaction mixture was adjusted to 200 µl with RIA buffer and was incubated overnight at 4℃. Finally the plate was c arefully washed four times. Bound radioactivity was β-counted (Top-Count, Perkin-Elmer life sciences), after the addition of 200 µL/well of the scintillation cocktail (Microscint 20, Perkin-Elmer Life Sciences, Boston). Specific antibodies, dilutions, serial dilution for the standard curve, the tracer and its specific activity are reported for each hormones in table 3.1. Specific cross reactions for antibody anti sexual hormones are reported in table 3.2; Cross reaction for antibody anti cortisol are reported in table 3.3.

The detection limit of the assay was calculated by the software Riasmart (Perkin-Elmer life sciences). The results of the intra- and inter-assay precision test for faecal extraction, expressed as coefficients of variation (CV), are reported in table 3.4.

3.2.2.RIA validations

Validation procedures included parallelism and recovery tests for the four hormones assays (Progesterone, Estrogens, testosterone and cortisol) in bottlenose dolphin faeces and serum. Results are reported in pmol/gr faeces and nmol/L .

The parallelism test assesses the interferences from compounds, mainly of unknown origin, which affect the antibody-antigen binding reaction. If the parallelism is not achieved the assay is considered to be not valid and it means that either the sample or the standard contains substances not present in the other fraction with affects the binding reaction (Simontacchi *et al.,* 1999). Parallelism was assessed for each hormone calculating the regression curve between the observed hormone concentrations and the reciprocal of the dilution factors (1:2 to 1:32) faecal and serum extracts.

Parallelism curve: $$[H_{obs}]=a*1/df + b$$

Hobs : observed hormone

a : angular coefficient

b: intercept

df: dilution factor

Recovery was expressed as the regression curve obtained between observed and expected hormone concentrations measured in faecal and in charcoal-treated steroid free serum samples added with known amounts of hormone.

Recovery curve: $$[Hobs] = a [Hexp] + b$$

Hobs: observed hormone

Hexp: expected hormone

a: angular coefficient

b: intercept

Progesterone validation in faeces

Parallelism was expressed by the parallelism curve (Fig 3.1).

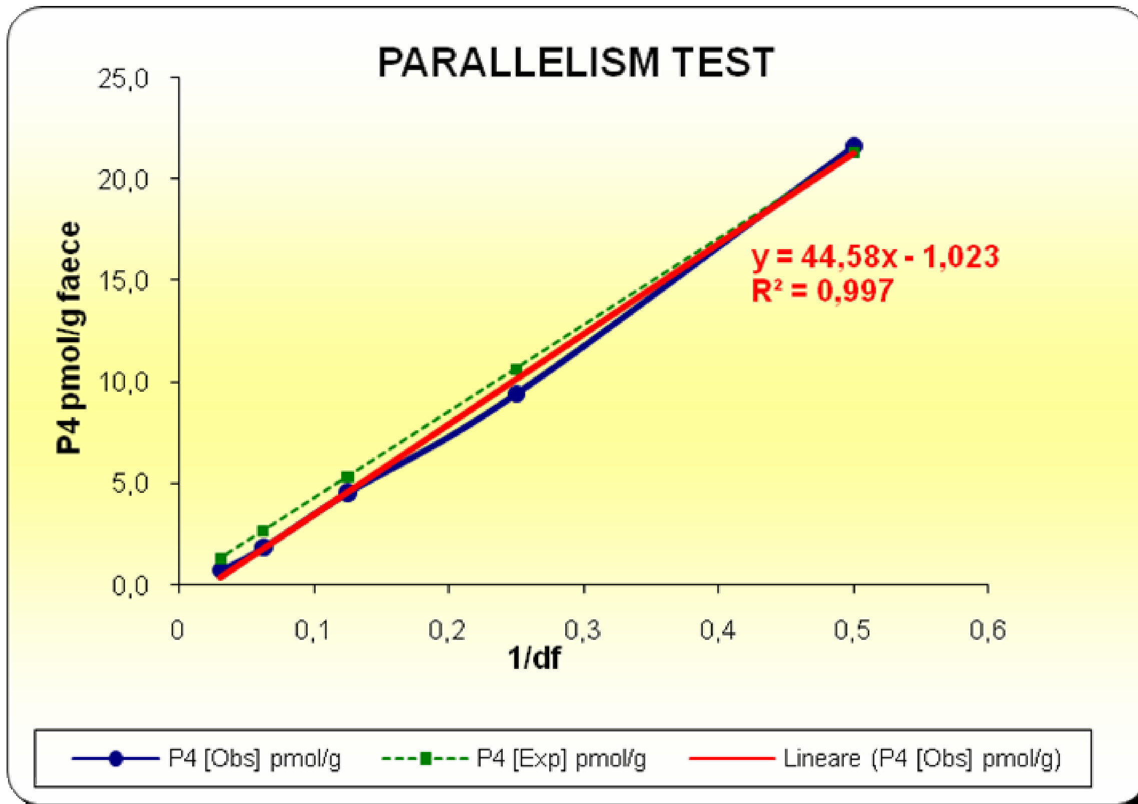


Fig. 3.1.: Parallelism test of the faecal progesterone.

The assay showed a good degree of parallelism, but there was a slight underestimation in low range, demonstrated by the values of the intercept (b). The coefficient of regression showed good result being grater than 0.98 and the underestimation was due to the lost of hormones during the extraction process with the solvent.

The underestimation was confirmed by the recovery test (Fig. 3.2.).

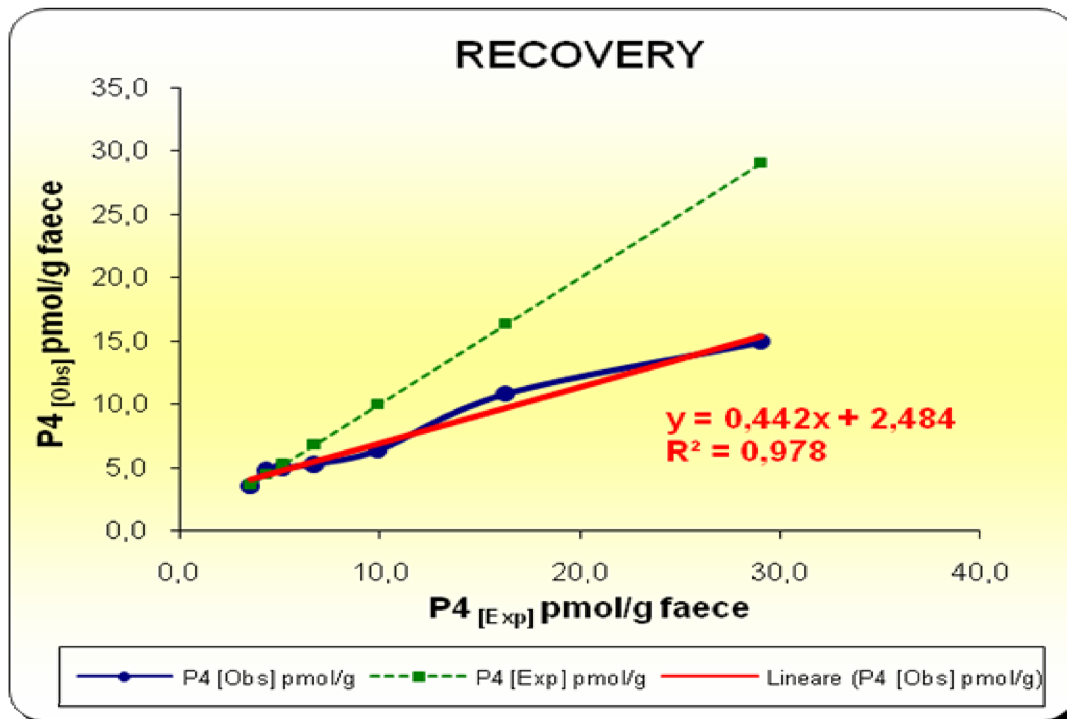


Fig 3.2.: Recovery test of the faecal progesterone

Both the intra-assay and the inter-assay (see table 3.4) showed a good level of the reproducibility of the method applied to P4 extraction from faecal samples.

Estrogens validation in faeces

Parallelism test was performed by analysing faeces with high (18.4 pmol/g faeces) hormone concentration, serially diluted with RIA Buffer. Parallelism was expressed by the parallelism curve (Fig. 3.3.)

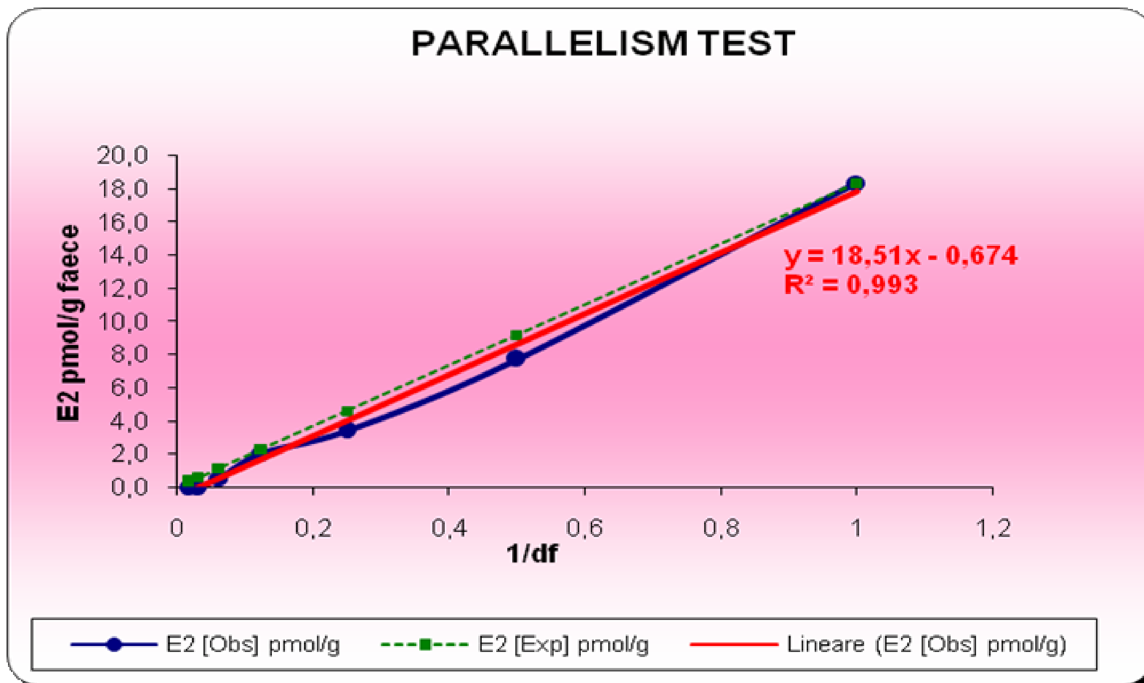


Fig. 3.3: Parallelism test of the faecal estrogens

The assay showed a good degree of parallelism, with a slight underestimation in low range, demonstrated by the values of the intercept (b).

For the recovery test the known amount of hormone ranged between 0.5 and 15.2 pmol/g in faeces and the recovery curve is shown in figure 3.4.

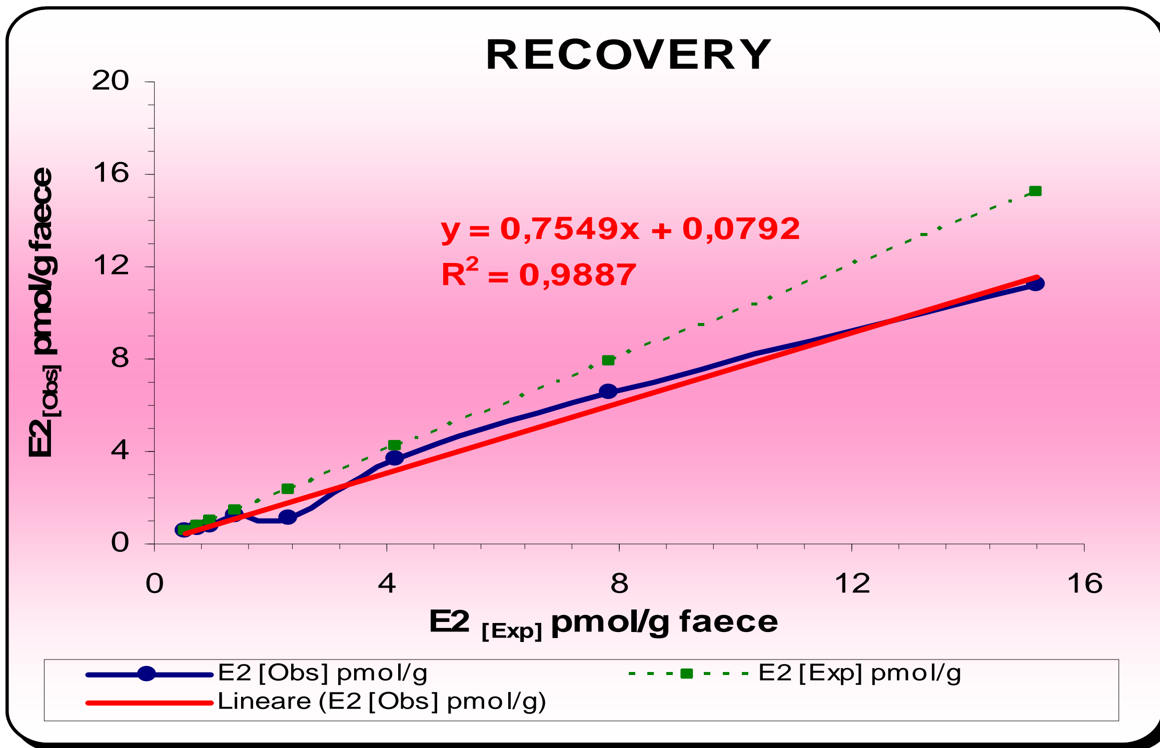


Fig. 3.4. Recovery test for the faecal estrogens

Both the intra-assay and the inter-assay (see table 3.4) showed a good level of the reproducibility of the method applied to E2 extraction from faecal samples.
(intra assay : 5.37; inter assay: CV% = 8)

Testosterone validation in faeces

Parallelism and recovery test were both performed.

Parallelism test is shown in figure 3.5

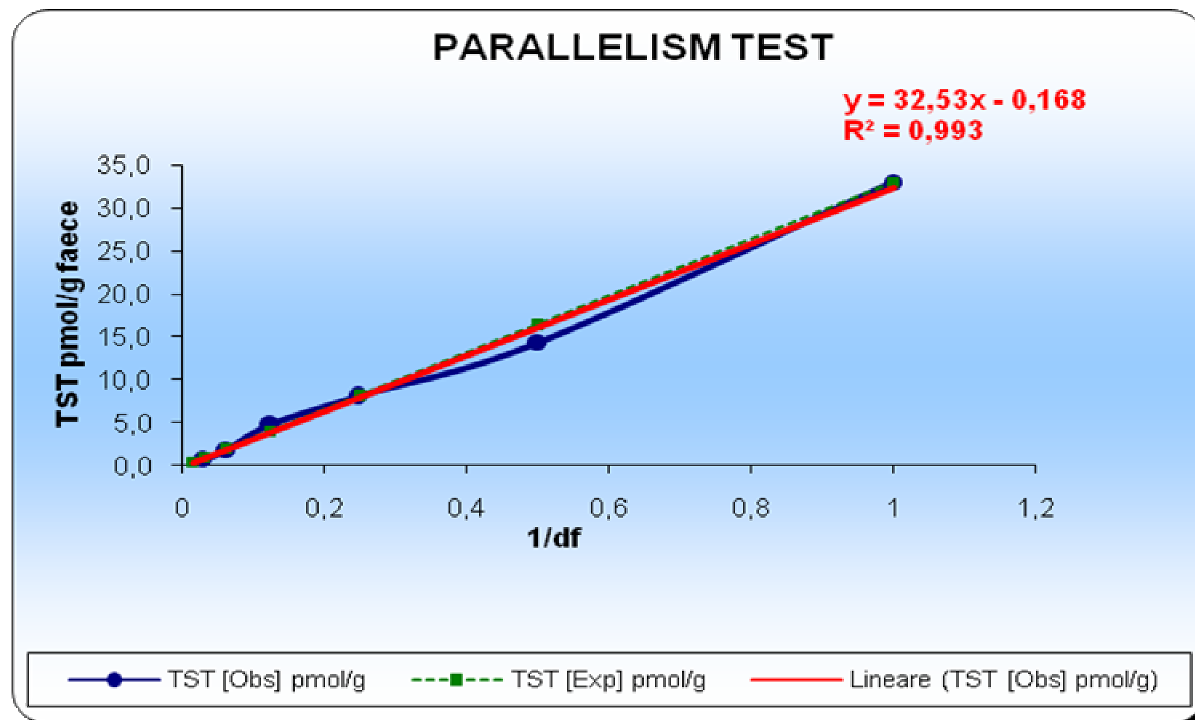


Fig. 3.5. Parallelism test of the faecal testosterone

Intra-assay precision was tested by analysing faecal samples with two different levels of hormones as reported above.

The assay showed a good degree of parallelism demonstrated by the values of the intercept (b) that is not significantly different from 0.

The recovery test is reported in figure 3.6.

Both the intra-assay and the inter-assay (see table 3.4) showed a good level of the reproducibility of the method applied to TST extraction from faecal samples of bottlenose dolphins.

The recovery test shows that there is an overestimation of samples with hormone concentrations higher than 10 pmol/g faeces

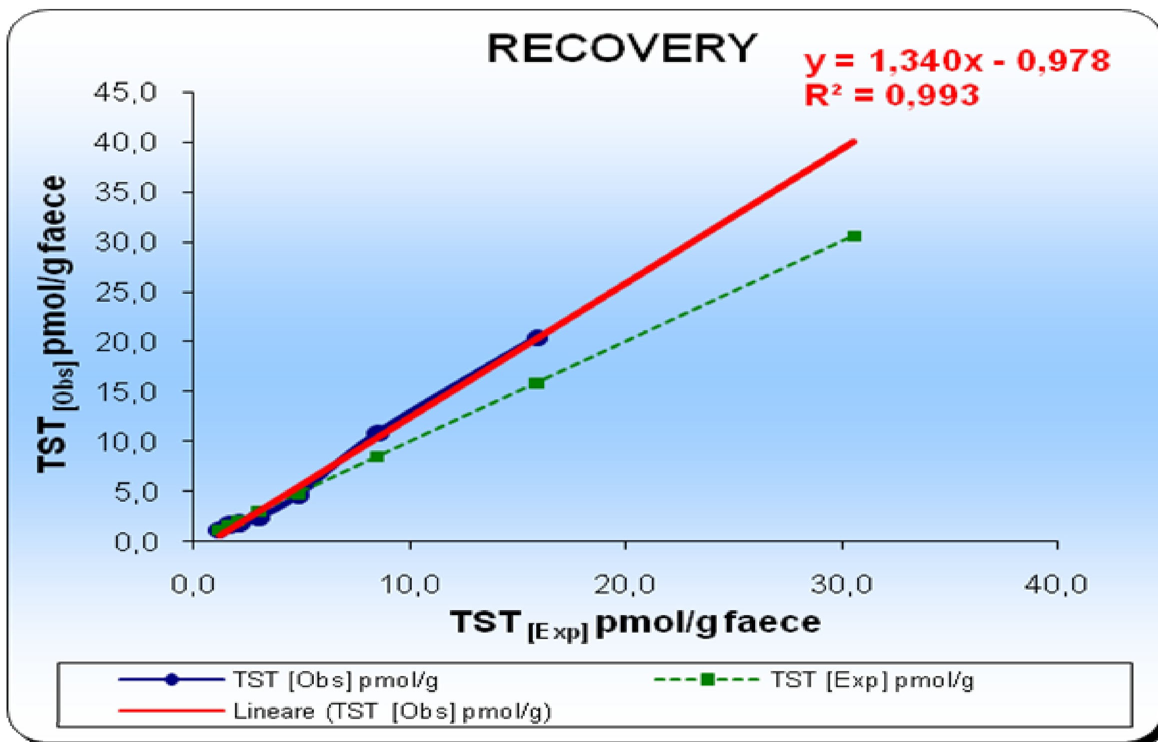


Fig. 3.6.: Recovery test of the faecal testosterone

Cortisol validation in faeces

The validation for faecal cortisol extraction was done both for the samples extracted with petroleum ether and with ethylic ether.

The first experiment was done considering females and males separately and validation for cortisol assay was done considering faecal samples from females (F) extracted with petroleum ether and faecal samples from males (M) extracted with ethylic ether. Serum samples were extracted with ethylic ether.

Parallelism for samples extracted with petroleum ether and ethylic ether are reported in figure 3.7.

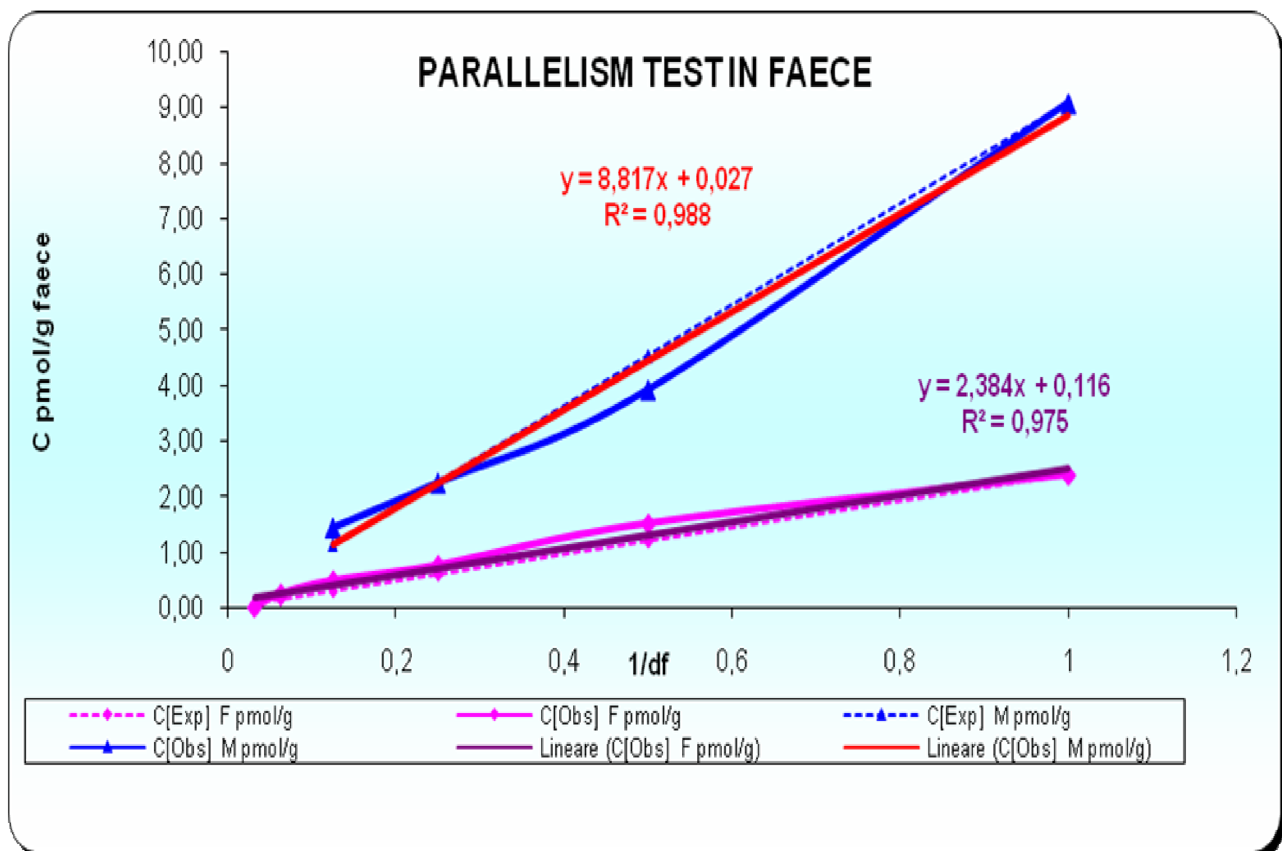


Fig. 3.7.: Prallelism test for faecal cortisol extracted with petroleum ether (purple curve) and with ethylic ether (blue curve)

The assay shows a good degree of parallelism, as demonstrated by the values of the intercept (b) that is not significantly different from 0 (P>0.05).

In the samples extracted with petroleum ether, the known amount of hormone ranger between 0,43 and 13,8 pmol/g of faeces.

In the samples extracted with ethylic ether, the known amount of hormone ranger between 0,7 and 5,55 pmol/g of faeces.

The recovery test for cortisol extracted with petroleum ether is shown in figure 3.8 and recovery test for cortisol extracted with ethylic ether is shown in figure 3.9.

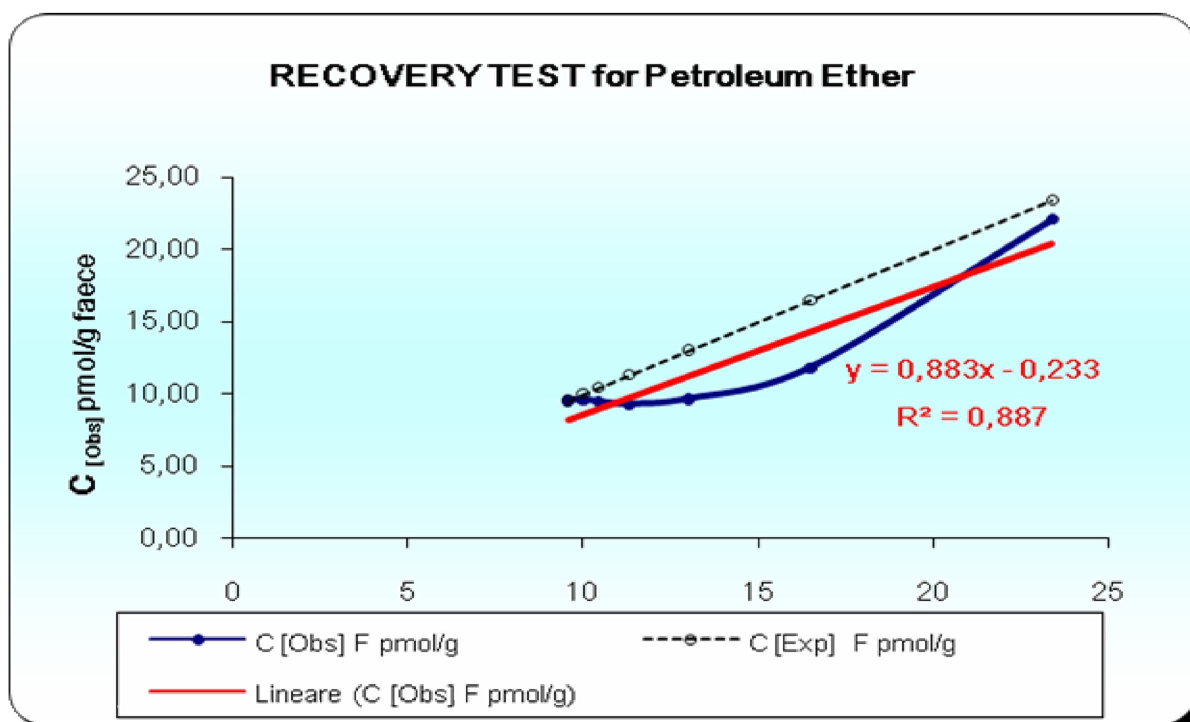


Fig 3.8. Recovery test for faecal cortisol extracted with petroleum ether

The graphic shows that within the samples extracted with petroleum ether there is a great underestimation of the lower values (R^2 = 0,88). The coefficients of variation were respectively 8,7% for the intra assay and 4,1% for the inter assay.

The recovery of extraction with ethylic ether shows that this organic solvent is more suitable than petroleum ether for the extraction of cortisol.

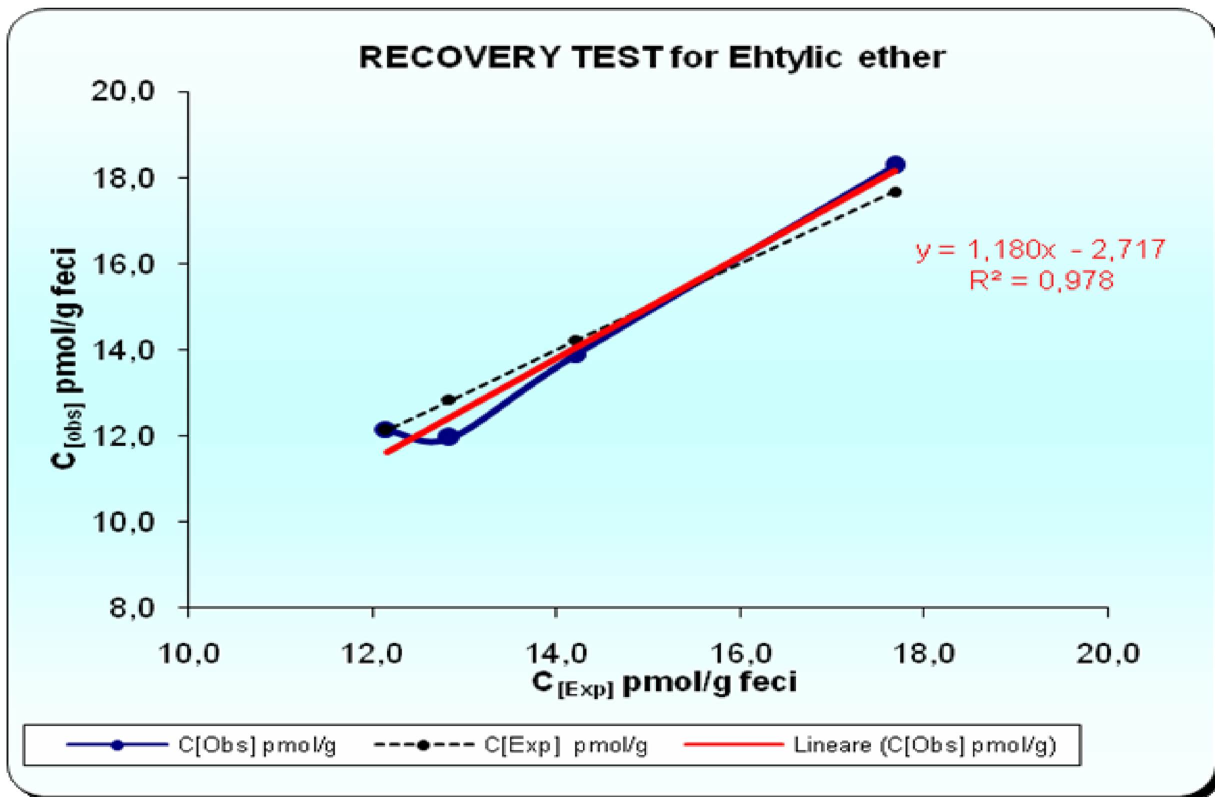


Fig 3.8. Recovery test for faecal cortisol extracted with ethylic ether

The coefficients of variation were respectively for the intra assay 5,4% and the inter assay 4,1% for the extraction with ethylic ether, while for the samples extracted with petroleum ether the coefficients of variation were respectively for the intra assay 8,7% and the inter assay 4,1%

CHAPTER 4: FEMALE REPRODUCTION

4.1. Anatomy and physiology of female reproductive tract

Reproductive tract of female bottlenose dolphins does not differ very much from those of terrestrial mammals. The vagina opens cranially to the anus and passing through the cervix, leads to the uterus. The uterus consists of the uterine body that lies dorsally on the urinary bladder, and two cornua that extend from the uterine body to the lateral sides of abdominal cavity (Figure 4.1.). Distally the cornua reduce drastically their diameter and extend as uterine tubes (fallopian tubes) to paired ovaries (Rommel and Lowenstine, 2001).

The uterus and the ovaries are connected to the abdominal wall through the broad ligaments.

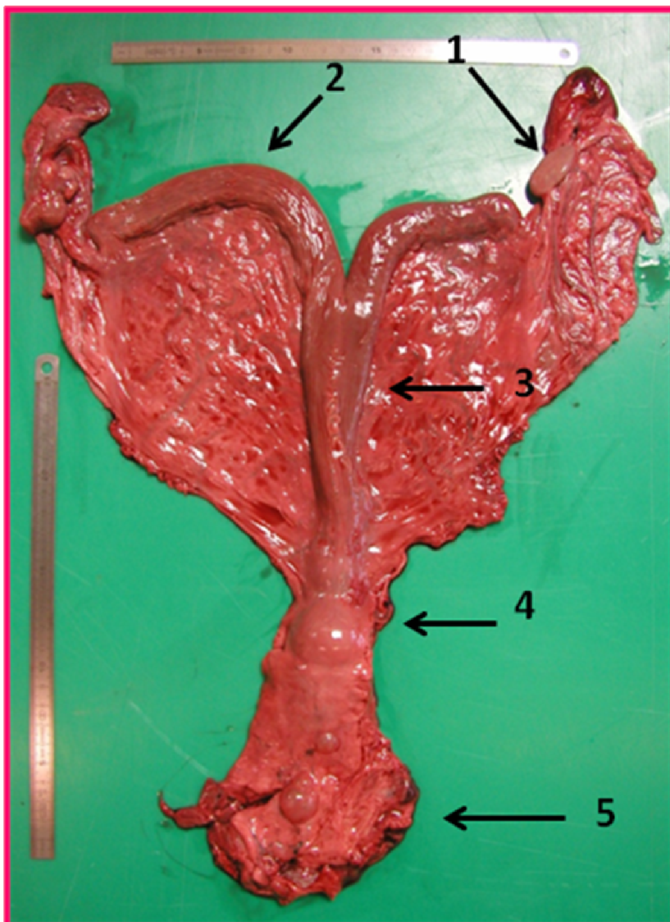


Fig: 4.1. Genital tract of female dolphin. Notice the presence of follicles in the left ovary.
1: ovary
2: uterine horn
3: uterine body
4: cervix
5: vagina

(Picture by B.Biancani)

The ovaries of sexually mature females are ovoid in outline and rounded in cross-section. Ovarian lengths in all sexually mature females showed some fluctuation over time; ovaries are slightly longer during periods of ovarian activity, and decreased in size during prolonged periods of anoestrus (Brook, 2001). Brook (2001) reported the length of ten sexual mature females monitored with ultrasound for 10 years, and these varied from 4.4 to 6.4 cm. In all animals, the left ovary was the same size, or slightly longer than the right ovary. A significant correlation between mean ovarian length and body length was reported.

Females of bottlenose dolphin can reach sexual maturity around 10 years old. Although the youngest captive bottlenose dolphin to give birth is reported to be 4 years of age (Robeck *et al.*, 2001), the majority of bottlenose dolphins first give birth between 7 and 10 years. Bottlenose dolphin is considered to have a seasonally polyestrus cycle and cycles can occur one or more time during the year, with the majority of ovarian activity observed from spring to fall. The gestation length of bottlenose dolphin is estimated of 12 months, and lactation, during which usually the oestrus is suppressed, can last up to 2 years (Robeck *et al.*, 2001). Bottlenose dolphins have a diffuse epitheliochorial placentae (Rommel and Lowenstine, 2001; Watanabe *et al.,* 2007).

Female reproductive endocrinology is based on the hypothalamic-pituitary-ovary (HPO) axis. GnRH (gonadotropin-releasing hormone) is episodically released from the hypothalamus (a gland situated in the third ventricle of the brain), stimulates pulsatile releases of FSH (Follicular-stimulating hormone) and LH (luteinizing hormone or lutropin) from the anterior lobe of the pituitary gland. Follicular-stimulating hormone (FSH) is primarily responsible for stimulation of follicular development and the triggering of the reproductive estradiol (E2) cascade in the female. FSH is slightly elevated during the early follicular phase of the oestrous cycle. FSH targets the FSH receptor (FSHR) that is expressed on the granulosal cells of the immature follicles, stimulating follicular development, increase in LH receptor concentrations in the follicles (Robeck *et al*, 2001) and estradiol synthesis (Senger, 2003). Increase of estrogens has also a positive correlation with numbers of LH receptors in the preovulatory follicle. As the follicles continue to grow, estrogen (E) is produced through paracrine interaction between granulosa and theca cells that line the follicle. Increased E production initially inhibits the

secretion from the pituitary gland of FSH and LH. When the follicle reaches the preovulatory stage, the Es reach the maximal production (the preovulatory estrogen surge) inducing a positive effect on frequency and amplitude of GnRH secretion. This leads to the preovulatory LH surge and the production of a glycoprotein, the inhibin, from the follicle. The inhibin induce suppression of FHS production and increase the sensitivity of the theca cells of the preovulatory follicle to the LH. The increased number of LH receptors and the sensitivity to LH act together to assure an adequate response to the LH surge and the following ovulation (Robeck *et al*, 2001). The LH acts at the level of the theca interna and luteal cells to induce ovulation and the formation of corpus luteum (CL). Due to its luteinic activity, the pre-ovulatory 'LH surge' is responsible for starting the ovulation process in female mammals, and is therefore used to direct prediction of ovulation (Robeck, 1993; 2005). The prediction of the time interval in which ovulation occurs is crucial for planning artificial insemination and managing breeding in the female. One of the difficulties in defining this window lies in the fact that LH has a short half-life. Robeck *et al.* (2005) reported that for bottlenose dolphins the LH surge duration is 20.3 ± 5.1 h (range 12– 25 h) and the time from the onset of LH surge to peak LH is 9.4 ±3.1 h (range 5.5–12.5 h). They determined by ultrasonography that the time of ovulation occurred 32.1± 8.9 h and 24.3 ± 7.0 h after the onset of the LH surge and LH peak, respectively, concluding that the period from peak LH to ovulation in the bottlenose dolphin is of 24 h.

Especially when the artificial insemination is planned, end even more when the artificial insemination is going to be done with sexed semen, due to the economical costs and time required for the sexing procedure and cryopreserve the semen, it is important to be able to detect the exact moment of the LH surge and peak. To achieve this, it is common practice (Muraco, *et al.,* 2006; Biancani, personal observation) to use a semi-quantitative LH assay (see fig. 4.2.) that can be used to check urine LH by collecting the samples 3 times a day.

Fig. 4.2: Semi-Quantitative Lutenizing Hormone (LH) Assay:
This assay can be used with urine or serum, gives test results in few minutes and identifies the LH surge.

Two important reproductive steroids that participate in the control of oestrous cycling are estradiols (E2) and progesterone (P4).

Estradiol-17β is the primary estrogen in the female reproductive cycle. Ovarian estradiol is increasingly secreted during diestrus, proestrus and oestrus, and towards the end of the oestrus assists the increase of LH production leading to the ovulation and the following increase of progesterone. After ovulation the ovary presents a CL and its persistency varies according to the establishment of a pregnancy or not. If the pregnancy is established, the CL persists all over the gestation and it is responsible for consistently maintaining a high blood serum level of P4. Progesterone plays a role in maintaining pregnancy by preventing a return to oestrous cycling and the specificity of CG in dolphins and their functionality is currently under study (Watanabe *et al.,* 2007).

To characterize the oestrous cycle of bottlenose dolphins, Robeck *et al.*(2005) monitored the urinary estrogen conjugates (EC), urinary luteinizing hormone (LH), and urinary progestins of twelve females during the peri-ovulatory interval. Three females had natural cycles and nine had post altrenogest (a synthetic progestagen) cycle. Based on the three natural cycles, the interval between successive peak EC and LH was 35.5 days and 36.0 days.

Using the results obtained from the two different situations mean oestrous cycle phase durations were characterized and the length of the follicular phase resulted of 8.1 ± 3.0 days (n = 10, range 4–14 days) and the luteal phase of 19.3 ± 2.8 days (n = 6, range 16–23 days). The preovulatory EC rise was 2.4 ±1.8 days (n =10, range 0–5 days). The interval between peak EC and peak LH was 7.5 ± 10.6 h (n = 12, range 0.3 to 24 h). The time from the onset of LH surge to peak LH was 9.4 ± 3.1 h (n = 6, range 5.5–12.5 h). The LH surge duration was 20.3 ± 5.1 h (n =6, range 12– 25 h).

4.2 Experimental design (ultrasound and vaginal cytology)

The females of bottlenose dolphins (*Tursiops truncatus*) examined during the study were housed in three different European facilities (A, B, C).
The pools of each facility had a different shape. Facility A (one mail pool and 4 auxiliary pools) had 9 millions litres of water. Facility B had two pools: pool "L" of 4 millions of litres of water and pool "D" (one mail pool and two holding pools) with 5 millions of litres of water respectively. The pool of facility C consisted of one main pool and four auxiliary pools and the total volume was of 2.1 millions of litres of water.
All the eight females used for the study were maintained in public display in out-door pools; 5 of them were used for shows, 2 for swim programs and one animal was used both for shows and swim programs (table 4.1.).
Animal management was slightly different between the facilities; however, all the animals were fed with frozen fish and the diet consisted mainly of herring (*Clupea harengus*), capelin (*Mallutus villosus*), sprat (*Sprattus Sprattus*), blue whiting (*Micromesistius poutassou*), mackerel (*Scomber scombrus*) and squid (*Loligo Opalescens*). The quantitative composition of the diets differed according to the facilities and the requirements of the animals related to different factors such as sex, age, and physiological status (Worthy, 2001). Diets were supplemented with vitamin and mineral.
Body length and body weight were monitored for six dolphins during the period of the study and initial and final measurements are reported in table 4.1.
Females 001, 002 and 002 were monitored from October 2005 until June 2007 and faecal samples were collected at least twice a week.
The female 005 was monitored through faecal samples randomly for 6 months during the anoestrus post partum, while still lactating her calf.
Female 006 was sexually mature and her faeces were collected ten months during which she was placed under Regumate® (Regumate is a man made progestane that has strong progesterone properties and can be placed in feed) to prevent her oestrus.
Faecal samples from the pregnant females (004, 007, 008) were collected once a week after the pregnancy had been confirmed by serum progesterone and ultrasound monitoring.

Table 4.1.: Females used for the study

Animal	Facility	Estimated year of birth	Sex stage	activity	Body weight (Kg)		Body length (cm)	
					Initial	Final	Initial	Final
001	B	1996-97	prepubertal	Swim program	117	141	218	230
002	B	1997-98	peripubertal	Swim program	123	144	228	237
003	B	2000	peripubertal	Swim and show	109	123	210	225
004	A	1994	Pregnant	Show	n.a	n.a	n.a	n.a
005	A	1976	Lactating	Show	n.a	n.a	n.a	n.a
006	C	1984	Sexually mature	Show	180	169		
007	C	1968	Pregnant	Show	230	204 (*) 210(**)		
008	C	1980	Pregnant	Show	210	190 (*)		

(*)body weight taken approximately one month after parturition
(**) body weight at the end of the period during which faecal samples were collected from the animal
(n.a) not available

4.3 Faecal progesterone and estradiol: material and methods

Faecal samples collection began in June 2005 and it was carried on till June 2007 for different periods and frequency according to the possibilities of the facilities involved in the study. Period of faeces collection is reported in table 4.2. The methodology used for samples collection of faeces, vaginal cytology, blood and ultrasound examination is reported in chapter 2. The method used for hormone extraction and RIA applied to evaluate the levels of P4 and E in faeces and blood is reported in chapter 3.

Table 4.2: Faecal sample period collection

N animal	Faecal sample
001	Oct 05- June 07
002	Oct 05- June 07
003	Oct 05- June 07
004	Jun – Dec 05
005	Jun 05- Dec 05
006	Jun 06- Apr 07
007	Jun 06 - Apr 07
008	Jun 06- Apr 07

4.4 RESULTS AND DISCUSSION

Peri-pubertal females

At the beginning of the study, in October 2005, **female 001** was considered to be in a prepubertal status. First ultrasound examination occurred in December 2005 and the ovaries were measured and they had a mean length of 3,1 cm and width of 0,77 cm (the mean was calculated considering the dimension of both the ovaries). No presence of follicles was observed. The samples of vaginal cytology (see table 4.4) collected during the first five months showed constantly the presence of keratinized grater than 70%. The progesterone values obtained from faecal samples ranged between 0,83 pmol/g and 8,67 pmol/g faeces (mean 2 ± 1,56 pmol/g). Serum progesterone measured varied from 0,25 nmol/L to 0,97 nmol/L (mean 0,58 ± 0,22 nmol/L), comparable with results reported in table 4.5. by Sawyer-Steffan *et al.,* (1983) .

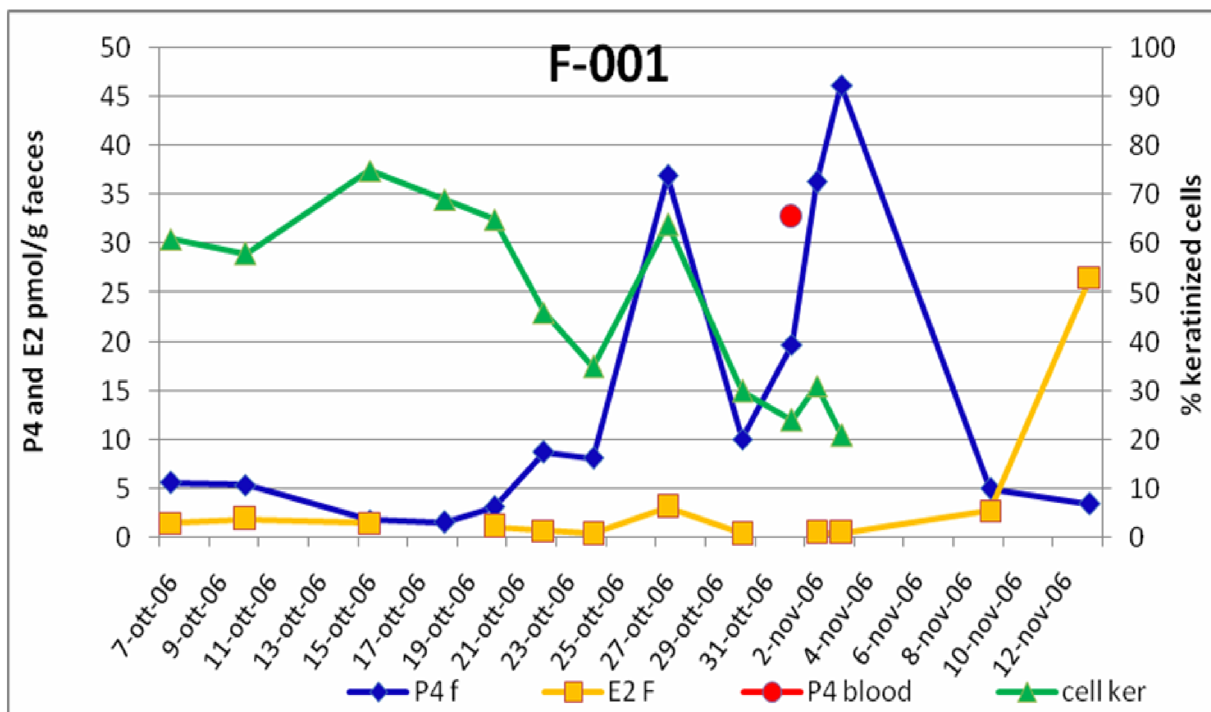
In February 2006 analysis showed an increase of faecal and serum progesterone. During the following months, from February 2006 until February 2007, the faecal P4 varied between 0,43 pmol/g and 78,35 pmol/g and serum P4 varied between 0,16 nmol/L and 32,78 nmol/L. From June until December 2006 the ovary were checked at least on monthly with ultrasound and their size were of 5.07 ± 0,32 cm length and 1,91 ± 0,45 cm width for the left ovary, and 5,34 ± 0,26cm length and 2,23 ± 0,48 cm width the right ovary.

During the summer months of 2006 trainers observed an increase of sexual behaviour (attempts of copulation, approaching with rostrum of flipper to the genital area) between the female and male 002. Due to the very busy weeks that animals and trainers had with shows and swimming programs, it was not possible to perform ultrasound examination on weekly base.
By the end of October 2006 the animal displayed changes in behaviour, showing reduced interest to food and trainers during the normal training session, spending time floating on the surface and sinking. On the 9th October ultrasound examination was performed and a follicle of 0,59 cm was observed (figure 4.3.). Twenty days later serum progesterone reached 32,78 nnoml/L, faecal cortisol was 19,71 nnom/g and basal and parabals cells were at 7%, indicating a possible effect of progesterone on the mucosa of the uterus. In the graphic 4.1. the variation of vaginal cytology and faecal hormones are showed from

the 7th October to the 13th November 2006. Although when the follicle was measure it did not reached the size of a preovulatory follicle (17-23 mm as reported by Brook, 2000) it can be feasible to asses that the follicular phase lasted until the 24th October when ovulation might have occurred. Comparing the serum progesterone of the female with the ranges reported for bottlenose dolphins by Sawyer-Steffan *et al.,* 1983 (see table 4.5.), although no ultrasound were performed to follow the growth of the follicle, but a rise of serum progerterone was noticed on the 1st of November suggesting a possible luteal phase. Faecal progesterone also increased from the 26th October to the 6th November was of 29,85 ± 14,63 pmol/g. According to this parameters it is feasible to believe that this was the luteal phase of the cycle and that lasted 11 days.

Complete results of hormonal and cytology results of female 001 are reported in table 4.3.

Graphic 4.1.: Vaginal cytology and hormonal levels observed in female 001 from 7th October to 13th November 2006



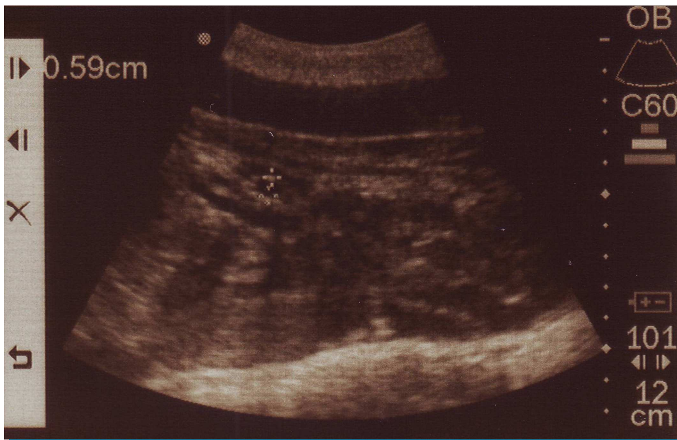


Fig. 4.3.: Ovarian follicle developing in the right ovary of female 001. Ultrasound performed on the 9th of October 2006. Faecal sample was not collected on the same day, but the mean of faecal P4 between the 7th and 10th October was 5,49 ± 0,15 nmol/g

From march 2007 until June, when sample collection was terminated, no follicles were observed in the ovaries. Faecal P4 ranged between 0,52 pmol/g and 4,30 pmol/g (n=46; mean 1,86 ± 0,89 pmol/g). During the same period four samples of blood were collected and P4 varied between 0,32 nmol/L and 0,64 nmol/L (0,46 ± 0,14 nmol/L), suggesting that no ovarian activity was present. Vaginal cytology was checked daily from 26th March to the 29th April and the percentage of superficial cells ranged between 37% and 71% (mean 58,57% ± 9,29), confirming not strong flotation in hormones concentration.

Table 4.3: clinical evaluation of the physiological status of peripubertal female 001during the experimental period

Time interval	Ultrasonography	Vaginal cytology (*)				Faecal P4 pmol/g ± s.d (a)	Faecal E pmol/g ± s.d (b)	diagnosis
		Basal	Parabasal	Intermediate	keratinized			
Oct 05- Jan 06		0	1-3	3-19	80-97	1,95 ± 1,61	n.a	prepubertal
28 June- 6 July 06		1-4	3-5	53-76	18-43	30,57 ± 11,70	0,85 ± 0,25	luteal phase
9 July – 24 Aug 06	Small follicles < 0,5 cm		n.a.	n.a.	n.a.	5,84 ± 5,02	0,85 ± 0,07	follicular phase
26 Aug- 3 Sept 06	n.a	n.a.	n.a.	n.a.	n.a.	53,30 ± 35,43	n.a	luteal phase
9-24 Oct 06	0,59 cm follicle	0-3	0-7	25-64	35-75	4,80± 3,11	1,12 ± 0,61	follicular phase
26oct- 6 Nov 06	n.a	0-1	3-6	36-79	21-64	29,85± 14,63	1,10 ±1,33	luteal phase
Jan –June 07		0-3	0-6	63-26	37-71	2,57 ± 4,35	n.a.	anoestrus

(*) ranges of % of cells are reported for vaginal cytology
(a,b) Values for faecal hormones are reported as mean ± standard deviation.
n.a = not available

Table 4.4.: Frequency of Vaginal cytology sample collection of female 001-002-003

Animal	Every other day	3 times/week	daily
001	6 Oct 05- 28 Oct 05		
	3 Dec- 11 Dec 05		
		1 Feb- 13 march 06	
			26 march-30 April 06
			16-22 may 06
			1 June- 7 July 06
			1 Oct – 6 Nov 06
			26 march – 29 April 07
002	6 Oct 05- 28 Oct 05		
	3 Dec- 11 Dec 05		
		31 jan-13 march 06	
			26 march-30 Apr 06
			16-23 may 06
			1 June- 7 July 06
			1 Oct – 6 Nov 06
			26 march – 29 April 07
003	6 Oct 05- 28 Oct 05		
	3 Dec- 11 Dec 05		
		1 Feb- 9 march 06	
			26 march-30 Apr 06
			17-22 may 06
			1 June- 7 July 06
			2- 11 October 06
			26 march – 19 April 07

At the beginning of the study, in October 2005, **female 002** was considered to be in a prepubertal status. Ultrasound examination occurred in December 2005 and the ovaries were measured and they had a mean length of 2,95 cm and width of 0,75 cm (the mean was calculated considering the dimension of both the ovaries) and there was not presence of follicles. In June 2006 the ovaries had reached the dimension of 4,5 cm length and 1,35 cm width, but there was not evidence of follicles. Within the same months, from October 2005 until July 2006, faecal progesterone varied between 0,51pmol/g and 5,70pmol/g (mean 1,82 ± 1,12pmol/g of faeces) and serum P4 varied between 0,16 nmol/L and 1,28nmol/L (mean of 0,5 ± 0,31 nmol/L). During the same period, vaginal cytology samples were collected as reported in table 4.4. The superficial cells varied between 29% and 98% (mean 64,50 ± 22,04%). All parameters considered indicated that the animal was still in a pre-pubertal status.

Between the end of July and end of September 2006 follicles were seen with ultrasound (average of 0,3 cm). On the 22nd of August a follicle of 0,3 cm was seen on the left ovary. During the month, faecal P4 varied between 0,55 pmol/g and 9,82 pmol/g (n=7; mean 3,33 ± 3,03 pmol/g) and faecal estrogen varied from 0,26 pmol/g to 9,16 pmol/g (n=7; mean 2,4 ± 3,07pmol/g), reaching the higher value six days after the follicle was seen. On the 4th of September 2006 ultrasound was performed and the follicle had grown to 0,65 cm. On the same day faecal P4 was at 5,92 pmol/g and serum P4 was at 2,58 nmol/L, while faecal E2 was at 6,83 pmol/g and serum E was at 140,6 pmol/L, suggesting that the follicle was still growing. Faecal samples collected from the 10th to the 24th of September 2006 increased from 42,83 pmol/g to 50,94 pmol/g suggesting that possible her first ovulation had occurred (see table 4.6.). Due to the lack of possibility to examine the animal with ultrasound, not CL was detected, but if we consider that the values of faecal P4 are indicating a luteal phase, comparing with the length of follicular phase detected in female 001 being of 16 days and considering the range of 4 to 14 days referred by Robeck *et al*, (2005), the follicular phase in female 002 could have occurred from 28th August when faecal estrogens were higher to the 8th September, lasting eleven days. During the following months small follicles were seen in march and April 2007 (respectively 0,56cm and 0,96 cm), but not high levels of faecal P4 were detected (min 0,30 pmol/g - max 3,83 pmol/g; n= 64, mean 1,42 ± 0,79 pmol/g).

Vaginal cytology collected in October 2006 and April 2007 showed a percentage of superficial cells that varied between 31% and 77%, and never reached values greater than 80%. Muraco *et al* (2004) refer that percentage of superficial/keratinized cells of cycling females reaches values greater than 80% during the oestrous and 100% is considered to be the optimal time for artificial insemination. Following this reference it is possible to say that during the months of October and April, when the vaginal cytology of female 002 was checked, she did not have complete ovarian activity.

Furthermore it is possible that the female was cycling without ovulating, being in the physiological phase of “adolescent sterility” (Whitehead and Mann, 2000)

Table 4.6.: Clinical evaluation of the physiological status of female 002 during the experimental period.

Time interval	Ultrasonography	Vaginal cytology (*)				Faecal P4 pmol/g ± s.d [(a)]	Faecal E pmol/g ± s.d [(b)]	diagnosis
		Basal	Parabasal	Intermediate	keratinized			
Oct 05- Jul 06	2,95 cm length 0,75 cm width (*A)	0-2	0-9	2-70	30-98	1,82 ± 1,12	n.a	prepubertal
Aug 06	0,3-0,42 cm follicle	n.a	n.a	n.a	n.a	3,33 ± 3,03	2,4 ± 3,07	prepubertal
4 Sept 06	0,65 cm follicle	n.a	n.a	n.a	n.a	5,92	6,83	possible follicular phase
10-20 Sept 06	n.a	n.a	n.a	n.a	n.a	48,83 ± 50,94	4 ± 4,86	luteal phase
October 06		0-1	0- 4	25- 60	41-75	1,56 ± 0,64	1,02 ± 0,84	anoestrous
November 06- Feb 07	No follicles seen	n.a	n.a	n.a	n.a	1,19 ± 0,75	n.a	anoestrous
27 March 07	0,56 cm follicle	0	1	27	72	0.97	n.a	anoestrous
8 April 07	0,96cm follicle	0	0	25	75	n.a	n.a	anoestrous
27 April 07	0,81 cm follicle	0	1	28	71	n.a	n.a	anoestrous
27 march- 21 June 07	5,08 cm length 1,73 cm width (*B)	0-2	0-5	22-55	41-77	1,19 ± 0,83	n.a	anoestrous

(*) ranges of % of cells are reported for vaginal cytology; (a,b) Values for faecal hormones are reported as mean ± standard deviation.
n.a = not available; (*A)= measurement detected in December 2005; (*B) = measurement obtained as mean of the measurements taken during the period.

Female 003 was the youngest female examined during the present study. At the beginning of the study she was examined with ultrasound without being able to detect the ovary, probably due to the small size. From November 2006, it was possible to observe the ovary ultrasonographically and the dimension of the ovaries (considering the mean of both ovaries) during the following months was of 3,43 ± 0,25cm length and 1,13 ± 0,17cm width. During the months of the study no follicles were seen in the ovaries. Vaginal cytology samples (see tab. 4.4) were collected following approximately the same periods than female 001 and 002. The average of superficial cells was of 62,83 ± 17,11%. The faecal progesterone during all the months of study varied between 0,30 pmol/g and 9,78 pmol/g (mean 1,58 ± 1,71 pmol/g) and the serum P4 showed always low values varying between 0,04 nmol/L and 1,00 nmol/L (mean 0,32 ± 0,23 nmol/L). Few samples were checked also for estrogen. Faecal estrogen varied between 0,27 pmol/g and 32,78 pmol/g (n=5; mean = 7,38 ± 14,04) and serum estrogen in two samples was of 107,65 ± 3,24 pmol/L. Complete profile of the female 003, with clinical observations, during the period of the study are reported in table 4.7. The results obtained for serum estrogens seems to be slightly higher than those reported in table 4.5. Sawyer-Steffan *et al.,* (1983) and Atkinson *et al.,* (1999,b) suggesting that much of the sexual hormones (P4 and E) found in the serum of non pregnant females could be of adrenal origin. This result could be the reason of the differences. Since the difference is not high, it could be due also to the differences of the method applied for the analysis.

Under these hypothesis, faecal estrogens of 7,38 ± 14,04 pmol/g could be considered for anovulatory levels, but it is necessary to analyze the faecal hormone through HPLC-MS in order to identify the possible metabolites.

Table 4.7.: Clinical evaluation of the physiological status of female 003 during the experimental period

Time interval	Ultrasonography	Vaginal cytology (*)				Faecal P4 pmol/g ± s.d (a)	Faecal E pmol/g ± s.d (b)	diagnosis
		Basal	Parabasal	Intermediate	keratinized			
Oct 05- Oct 06	n.d.	0-1	0-9	4-63	36-96	1,84 ± 1,98	0,33 (**)	prepubertal
Nov 06- June 07	3,43 ± 0,25cm length 1,13 ± 0,17cm width	0-1	0-3	30-63	36-74	1,07 ± 0,77	9,70 ± 15,47	prepubertal

(*) ranges of % of cells are reported for vaginal cytology
(**) only one value was present
(a,b) Values for faecal hormones are reported as mean ± standard deviation.
n.d.= not detectable
n.a = not available

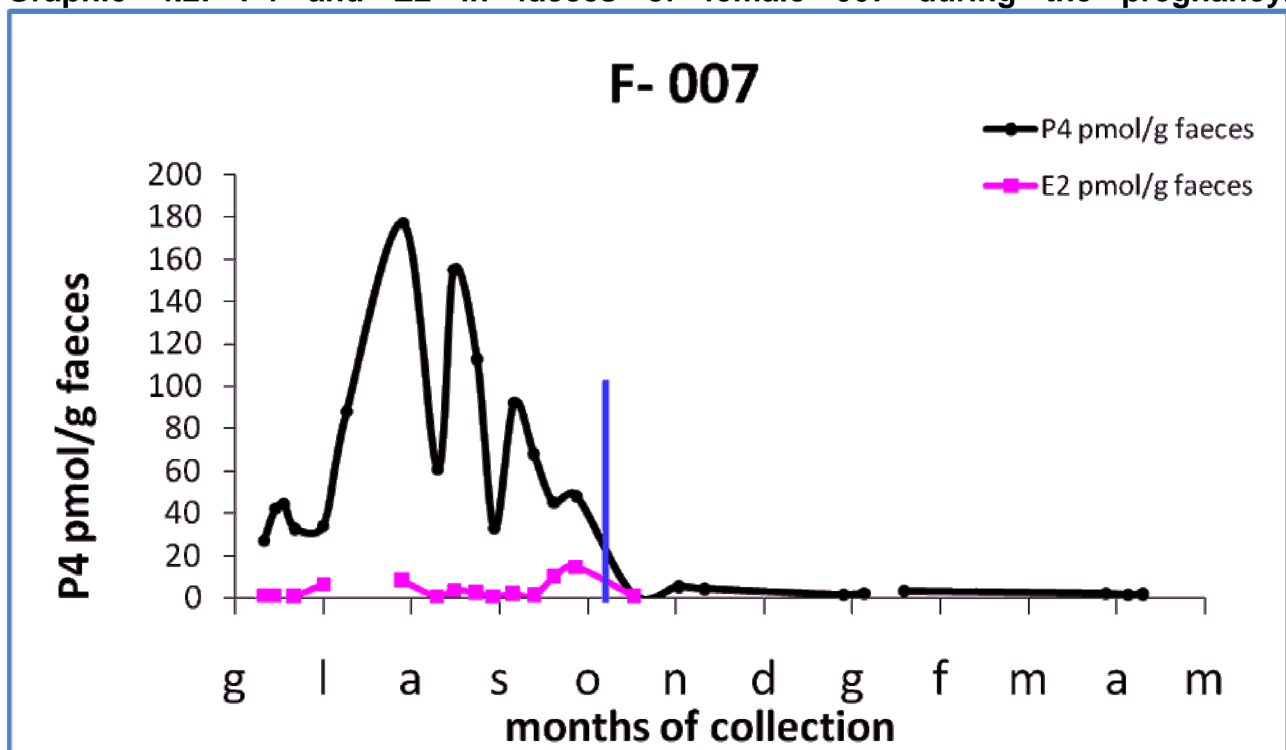
Pregnant females

Three females were monitored during the pregnancy for different period.

From **female 004** only few faecal samples were collected during the first seven months of pregnancy and the P4 ranged between 15,29pmol/g and 42,58 pmol/g of faeces (21,32 ± 10,54 pmol/g). Estrogen was monitored as well and it varied between 0,93 pmol/g and 2,08 pmol/g faeces (mean 1,46 ± 0,53 pmol/g faeces)

Female 007 was monitored during the last five months of pregnancy for P4 and E2 and after parturition the progesterone was monitored for the following six months. During the pregnancy the P4 varied between 26,99 pmol/g and 176,92 pmol/g faeces (mean 67,65± 46,36 pmol/g) and the estrogens varied from 0,5pmol/g to 14,8pmol/g (mean 4± 4,43 pmol/g). Eight days after parturition the sample collection started again and her progesterone was 1,74 pmol/g and estrogen was 0,45 pmol/g. For the following six month, while the female was lactating, the progesterone varied between 1,57pmol/g and 5,43pmol/g (mean 2,7± 1,35 pmol/g faeces). The profile of faecal hormones of female 008 during the pregnancy are shown in the graphic 4.2.

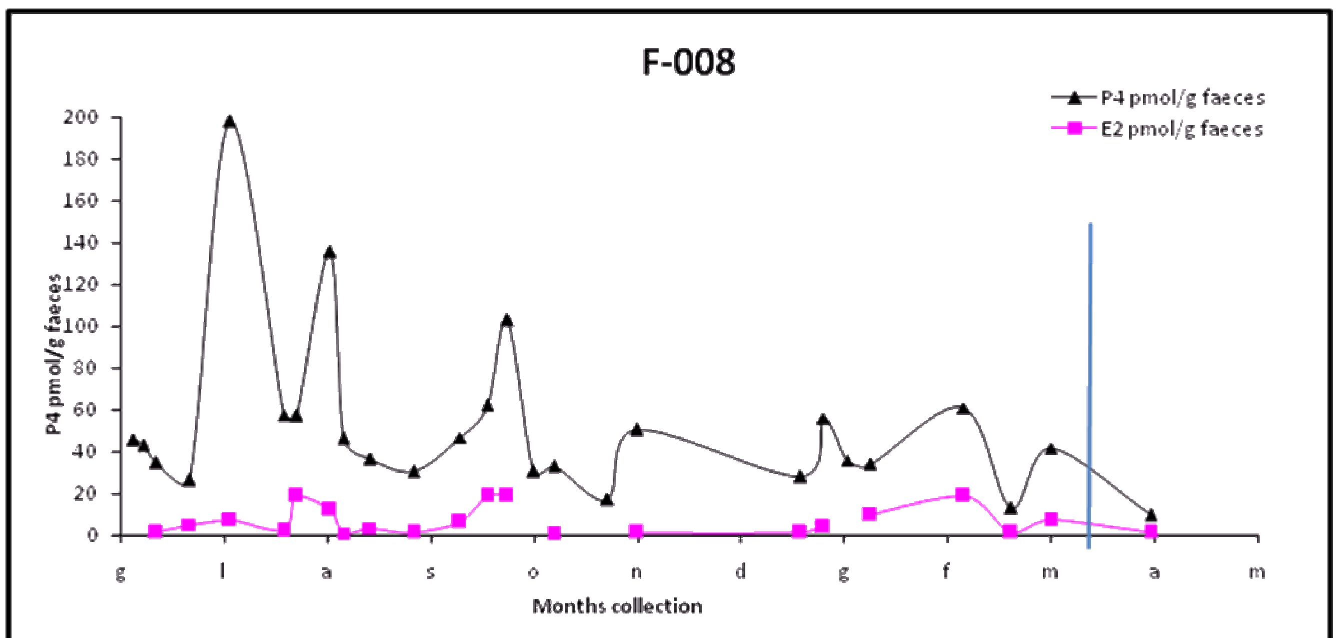
Graphic 4.2: P4 and E2 in faeces of female 007 during the pregnancy.



The blue line refers to parturition.

Female 008 was monitored during the last ten months of pregnancy. The progesterone varied between 12,56 pmol/g and 197,69 pmol/g faeces (mean 52,68± 39,68 pmol/g faeces) and estrogens ranged from 0,54pmol/g to 18,36pmol/g faeces (mean 6,74 ± 6,7 pmol/g faeces). Twenty days after parturition faecal progesterone was 9,40 pmol/g faeces. The profile of faecal hormones of female 008 during the pregnancy are shown in the graphic 4.3.

Graphic 4.3.: P4 and E2 in faeces of female 008 during the pregnancy



. The blue line refers to parturition.

Following the faecal progesterone during the pregnancy, it seems to follow the same patter reported for the killer whales by Walker *et al.,* (1988) and Katsumata *et al,* (2006). They reported that during the pregnancy the progesterone increase during the first part, stabilizing itself to lower value during the mid and late period of pregnancy as reported in graphic 4.4. and table 4.8.

Rolland et al., (2005) reported the mean faecal progestins in pregnant females of right Whales (201,240 ± 27,025 ng/g dry faecal weight) that were several orders of magnitude higher than those of all other female sub-groups (juvenile, resting and lactating). In our study the faecal progesterone in pregnant females was of 59,42 ± 42,46 pmol/g wet faeces

(mean ± s.d) and it never dropped below 10 pmol/g which can be considered as baseline level to detect a pregnancy in faecal sample of bottlenose dolphin.

Graphic 4.4.: Serum progesterone concentration (pmol/L) during pregnancy of two killer whales (modified from Walker *et al.*, 1988).

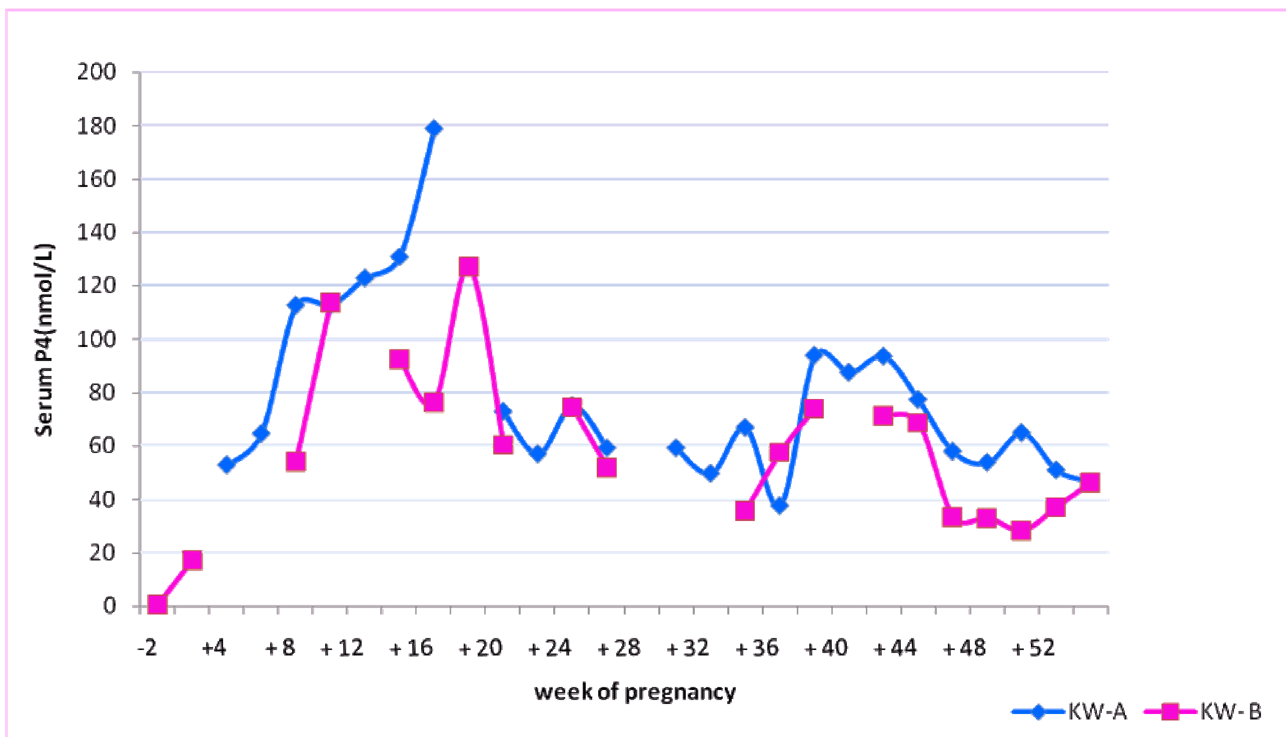


Table 4.5.: Serum progesterone and total estrogen values (pmol/L) in adult females of bottlenose dolphins (modified from Sawyer-Steffan *et al.,* 1983)

Physiological phase	Serum P4 (pmol/L)	Serum Estrogen (pmol/L)	References
Anovulatory level	0,91± 0,08	91,78 ± 3,67	**Sawyer-Steffan *et al.,* 1983**
Assumed ovulation without pregnancy	Pick values (range) 18,57- 70,38	Pick values (range) 33,04- 132,16	
Pregnancy level throughout gestation	Pick values (range) 18,32 – 137,26	Pick values (range) 40,38 – 234,95	
Ovariectomized females (10years)	2,14 ± 0,41	73,42 ± 8,36	

Table 4.8.: Serum progesterone values (pmol/L) during reproductive phases in killer whales (modified from Katsumata et al, 2006 and Walker et al., 1988)

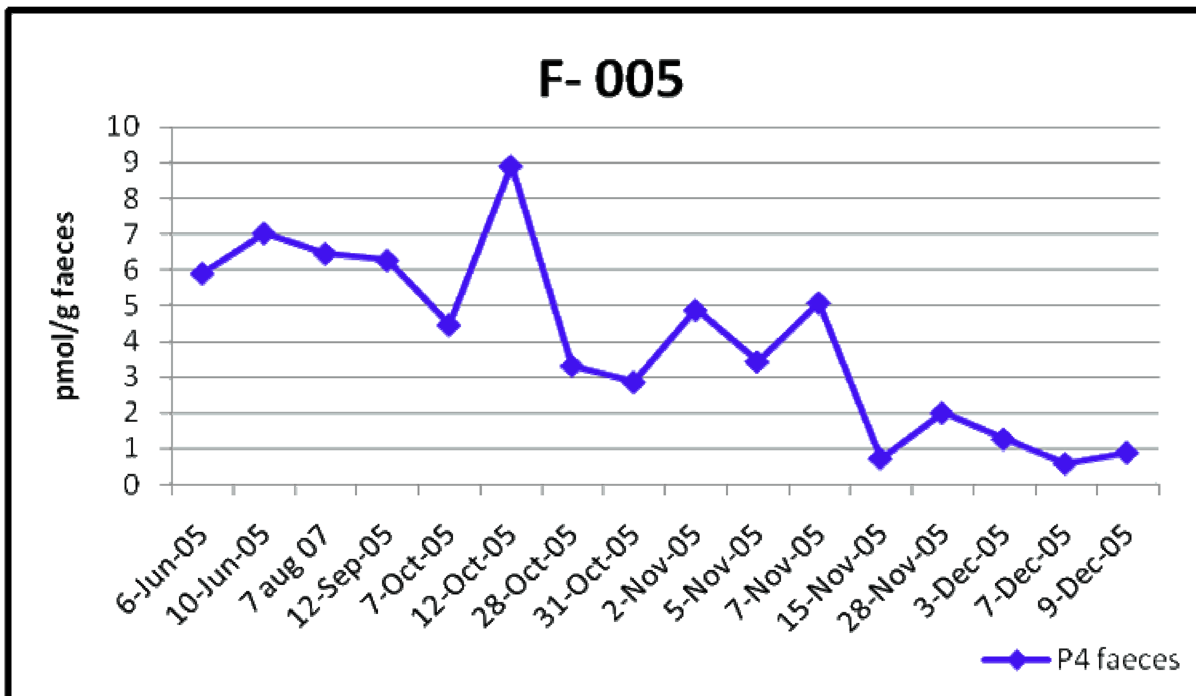
Physiological phase	Serum P4 (pmol/L)		References
Anestrum	0,06- 0,38		Katsumata *et al.,* 2006
Luteal phase	7,25 – 10,79		
First part pregnancy	11,76-70,27[g1] 6,71- 75,99	19,08- 74,72[g2] 16,85 – 101,75	
Mid- late pregnancy	31,80 – 95,39		Walker *et al., 1988*
Not pregnant	0,32- 31,80		

Anoestrus females

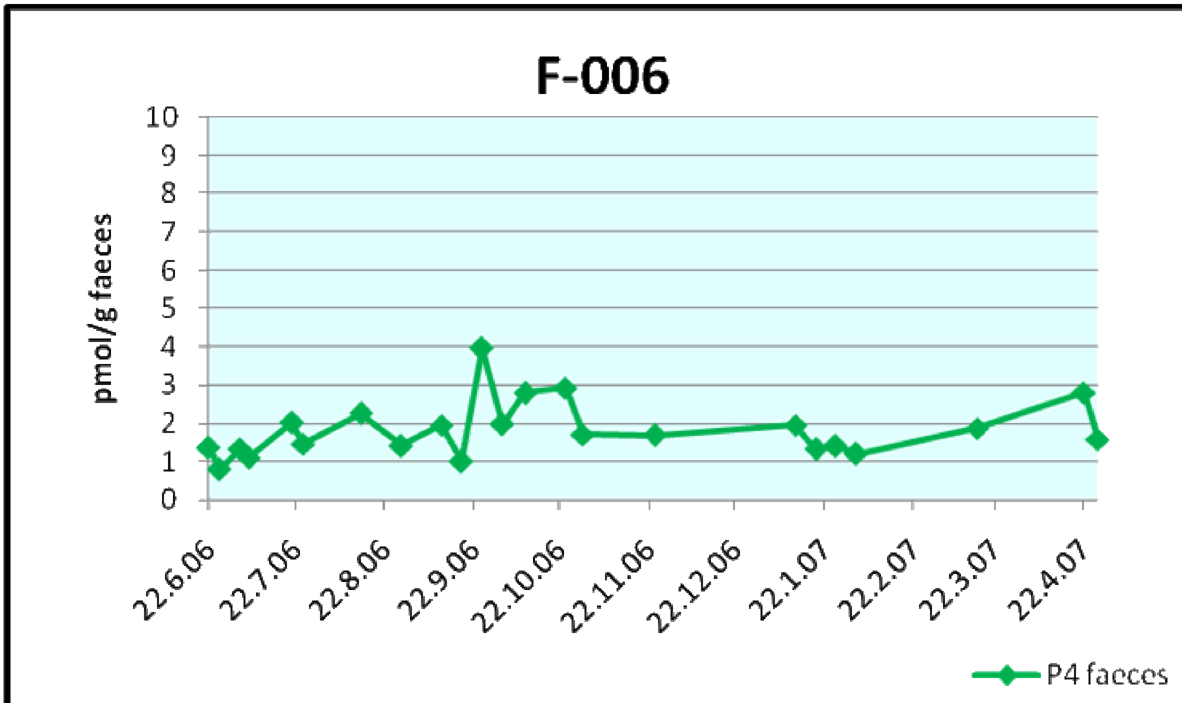
Females 005 and **006** are considered in the same group due to the fact that the first one was still in anoestrus post partum and lactating, while the second one was placed under oral treatment with altrenogest (Regumate ®) to prevent the animal to cycle. Altrenogest mimics the action of endogenous progesterone to induce a hormonal state resembling pregnancy. During treatment, the cyclical release of gonadotrophins is suppressed, blocking follicle development and oestrus. When treatment is stopped, pituitary inhibition rapidly declines, allowing a rapid rise in follicle development and subsequent ovulation.

The progesterone in the lactating female , as presented in graphic 4.5., ranged between 0,58pmol/g and 8,90 pmol/g faeces (mean 3,41 ± 2,43pmol/g), while in the female under Regumate progesterone varied between a minimum of 0,78 pmol/g and a maximum of 3,95 pmol/g of faeces (mean of 1,82 ± 0,73 pmol/g faeces) (see graphic 4.6.)

Graphic 4.5.: Faecal progesterone levels observed in Female 005 during six months she was lactating.



Graphic 4.6.: Faecal progesterone levels observed in Female 006 during the months she was treated with Regu-Mate®.



The results obtained from female 005 and 006 showed that lactating female had progesterone slightly higher than the resting female and this is comparable with results obtained by Rolland et al. (2005) for the right whales.

Robeck *et al.* (2001) reported that animals treated with altrenogest for 31 days, showed a delay on ovarian activity after the hormone withdrawal, but they went back to oestrous within 17.6 days. On the other hand Sanchez *et al.* (2004) reported that females treated with altrenogest for one year showed sexual activity (with pregnancy confirmed soon after) within 4-5 days after the Regu-mate was removed. By the end of the present study the animal 006 was still receiving the altrenogest and it made not possible to detect the moment the ovarian activity would have started again.

Conclusion

The aim of this study was to evaluate the possibility to use faecal sample to monitor the reproductive status of captive females bottlenose dolphins.

Results show good possibilities to use the RIA to detect progesterone and estrogens in the faeces.

Prepubertal values for faecal progesterone were detected as 1,87 ±1,57 pmol/g (mean ± s.d.) , while faecal estrogens were 9,70 ±15,47 pmol/g (mean ± s.d.). Whitehead and Mann (2000) reported of cetaceans females that may begin to cycle without ovulating (non ovulatory cycles), and describe this period as the physiological phase of "adolescent sterility". The serum P4 values obtained in the present study for prepubertal females do not differ much from those reported by Sawyer-Steffan *et al.* (1983) for anovulatory cycles confirming that faecal values can be used as baseline levels for prepubertal or females in anestrum.

Two possible follicular phase have been identified through faecal hormones and they lasted 11 and 16 days respectively, potentially in agreement with Robeck *et al.* (2005) that report follicular phases of 4-14 days, with estrogens varying from 0,51 pmol/ ang 6,83 pmol/g during the follicular phase P4 was 4,80 ± 3,11 pmol/g.

Two luteal phases were detected in the femal 001 lasting respectively 8 and 10 days, shorted than the range of 16-23 days reported by Robeck *etal.,* (2005). This is possible due to the fact that the animal is still young and not having a full developed cycle or to the fact that some metabolites became more present during the last days of the luteal phase and they were not detected throught the methodology applied. Atkinsons *et al.,* (1999,b) reported that suppressed progesterone serum concentrations in false killer whales may have reflected increased progesterone-binding proteins rendering less free progesterone to be assayed. Similar situation could occur to the progesterone and its metabolites in the faeces.

This results suggest the necessity to perform the HPLC-MS analysis to better characterized the faecal metabolites of P4.

Faecal progesterone observed during the pregnancy showed a similar pattern to those observed with the serum of killer whales reported by Walker *et al.* (1988), indicating that

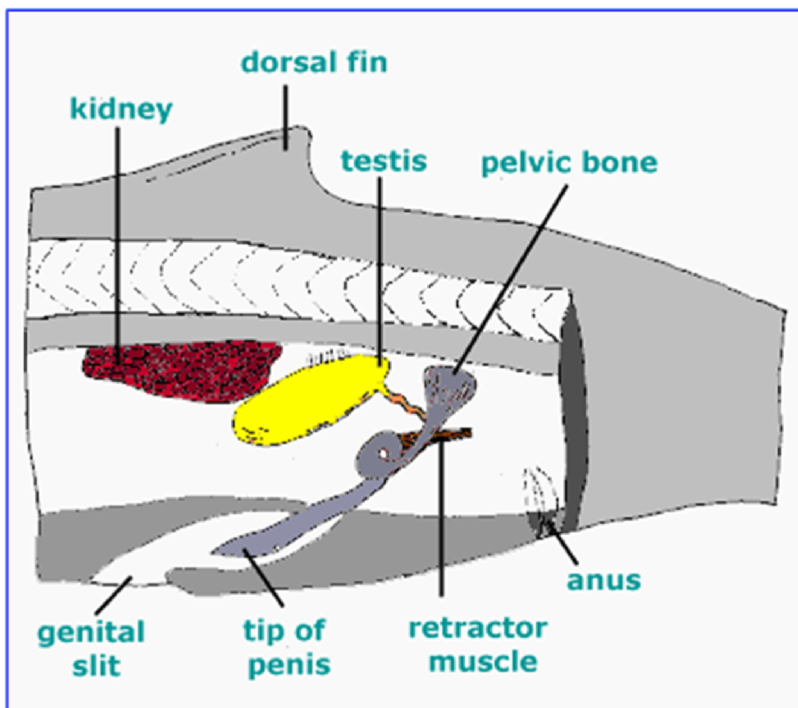
faeces can be used to follow the pregnancy and in the present study P4 during pregnancy never dropped below 10 pmol/g.

Observing the vaginal cytology samples the percentage of keratinized cells noticed during the pre pubertal phase of the females examined resulted to be opposite to the trend reported for females of other species in anoestrous (Feldman and Nelson, 1998). Wide ranges of intermediate and keratinized cells were noticed in the prepubertal females, although the faecal hormones did not show any significant variation. Concerning bottlenose dolphins I am not aware of data published on vagynal cytology in pre pubertal females.

CHAPTER 5: MALE REPRODUCTION

5.1. Anatomy and physiology of male reproductive tract

The male external reproductive tract consists of a genital opening placed anterior to the anal slit. Internal to the genital opening there is the fibro-elastic penis similar to that of ruminants, which originates at the median surface of vestigial pelvic bones (Atkinson, 2002; Robeck *et al.*2001, Schroeder, 1990a). Medially, the penis presents a sigmoid flexure that is lost during erection. The testes are intra-abdominal (Schroeder, 1990a) and are placed ventrally to the caudal end of the kidneys (Picture 5.1).



Picture 5.1.: Internal anatomy disposition of the genital apparatus in male bottlenose dolphin.

The testes vary in morphology, size and function with age and season (Atkinson, 2002). The epididymes are placed on the dorsolateral surface of the testis and merge into the ductus deferens, which are cranial medial to the epidiymes and incorporated into the spermatic cord leading to the urethra. The epididymis is responsible for storage and transport of spermatozoa. The prostate, the only accessory gland, is tubuloalveolar and is constituted by cuboidal to low-columnar to pseudostratified lining cells (Rommel and Lowenstine, 2001). The testes are suspended by two ligaments, and are highly structured.

The testis capsule, the tunica albuginea, is tough, fibrous and vascularised. From the internal surface of the tunica albuginea, septa extend to the interior of the organ, and join in the mediastinum testis, which is a network of connective tissue containing the rete testis. The testis are divided into lobules by septa; the lobules containing the seminiferous tubules that are continuous with the rete testis tubules. The rete tubules provide a portal for the spermatozoa, which are produced in the seminiferous tubules, and carry them to the ducts.

Based on post mortem examination of testis weight, diameter of seminiferous tubules, presence of seminal fluid in the epididimus and spermatozoa in the seminiferous tubules males of bottlenose dolphin are considered to reach sexual maturity at approximately 9-13 years of age (Cockcroft and Ross , 1990; Robeck *et al*. 2001), with gonads having wide range of sizes. Brook (1997), referred that testes cross-section size and weight can be used as general indicators of males fertility and recently Yuen *et al. (*2007) reported males of *Tursiops truncatus aduncus* that resulted to be reproductively effective soon after the onset of spermatogenesis, which occurred at the age of 6.5-7.5 years.

Testosterone concentrations in plasma is generally used to classify dolphins as immature, pubescent or sexually mature (Kirby 1990; Schroeder 1990a). Sexually mature males are considered to have testosterone concentrations of 5 to 54 ng/ml, whereas in pubescent animals testosterone concentrations range from 3 to 5 ng/ml. In immature animals plasma testosterone concentrations is less than 3 ng/ml (Schroeder, 1990a; Brook ,1999).

Schroeder (1990a) specify that it is possible that during the breeding season the serum testosterone of a mature male can drop below 5 ng/ml, but it was never observed that an immature male showed TST > 5ng/ml.

Schroeder and Keller (1989) demonstrated significant differences in serum testosterone levels and sperm density in *T. truncatus* over a 28 month period. Peak sperm densities were demonstrated during September and October, while serum testosterone levels peaked in June decreasing during July and August. Robeck *et al.* (2001) report that peak sperm density occurred from late august through October, while peak plasma testosterone levels occurred from April throughout July and August to a low in September.

Schroeder (1990b) reported of clear seasonality in male bottlenose dolphin reproductive activity. He referred of seasonal fluctuations in serum TST and sperm production, with peak TST levels preceding peak sperm production by 2-6 weeks.

Byers *et al.* (1983) and Matsubayashi *et al.* (1991) as reported by Robeck *et al.* (2001) referred of serum TST peak preceding peak sperm production as characteristic of seasonal reproduction of stallion and Japanese monkeys respectively. This mechanism is most probably due to the inhibitory effect of high TST levels on spermatogenesis (Matsumoto,1990 as referred by Robeck *et al*, 2001).
Cunningham and Huckins (1979, reported by Robeck *et al*, 2001) observed that normal spermatogenesis occurs when intratesticular concentration of TST is at lower levels, suggesting that lower TST concentration can be necessary to optimize sperm recruitment. Robeck *et al.* (2001) reported that the mechanism of spermatocyte recruitment occurring during the peak of TST and the following sperm maturation taking place while TST is at lower concentrations can apply also to dolphins, with the consequence of having peak of sperm production during the breeding season. Correspondence between peaks of male reproductive behaviour and pick of sperm density was observed also by Schroeder and Keller (1989).

Male reproductive endocrinology is based on the hypothalamic-pituitary-testicular (HPT) axis. Pulsatile release of GnRH (gonadotropin-releasing hormone) from the hypothalamus stimulates pulsatile releases of FSH (Follicular-stimulating hormone) and LH (luteinizing hormone) from the anterior pituitary. LH positively regulates the Leydig cells of the testicle to synthesize testosterone (TST) which in turn stimulates the Sertoli cells of the seminiferous tubules to generate androgen-binding protein (ABP). The AB proteins have their effect in the lumen of the seminiferous tubules where, concentrating the TST, help the maturation of the spermatozoa (Kirby, 1990). High level of TST from the Leydig cells negatively feed back the hypothalamus to down-regulate GnRH that down-regulate anterior pituitary gland to produce LH.
TST and FSH maintain the gametogenic function of the testis, but FSH and LH in males are not cyclically produced (as it happens in females); the FSH production is inhibits by inhibin (kirby, 1990).

The reproductive endocrinology of many male cetaceans is seasonally regulated. Seasonal effects are seen in behaviour, seminal production and quality, reproductive hormones and reproductive steroids (Robeck et al., 1993; Atkinson, 2002).

The factors responsible for these seasonal effects on cetacean's reproduction is not well understood. Water temperature, type of preys and their availability, female receptivity, environmental changes due to seasonal migration, and seasonal changes in threats to health may all play a role. What does seem to be clear is that complex regulatory mechanisms affect the output of key gonadotropins and steroids by season. Read (1989) reports in 244 females of harbour porpoises monitored for a period of over three year, suggesting that possibly female seasonality regulates the reproductive seasonality of males.

5.2. Experimental design

Two males of bottlenose dolphin (*Tursiops truncatus),* considered to be prepubertal at the beginning of the study, and located at the facility B were used for the present endocrinological study. The animals were kept in an outdoor pool, with sea water. From the beginning of April 2006 until the end of November 2006 and from the beginning of April 2007 until the end of the study the two males were separated in two different pools. The characteristics of the pools are reported in chapter 4. Moreover, male 001 was kept in a pool with a young female (F-003), while male 002 was kept with two cycling females (F-001 and F-002). During the other months the five animals were kept together in pool D. The animals were fed with frozen fish and the diet consisted mainly of herring (*Clupea harengus*), capelin (*Mallutus villosus*), sprat (*Sprattus Sprattus*), blue whiting (*Micromesistius poutassou*), mackerel (*Scomber scombrus*) and squid (*Loligo Opalescens*). The quantitative composition of the diets differed according to the season and the requirements of the animals related to different factors such as sex, age, and physiological status (Worthy, 2001).

5.3. Faecal testosterone: material and methods

At the beginning of the study the animals were estimated to be of 5 and 6 years of age. The samples were collected from October 2005 till June 2007, one to three times a week with the methodology explained in chapter 2. Serum samples were collected at least once a month, following the preventive medicine protocol used at the facility where the animals were kept. The methodology used for serum collection is explained in chapter 2. From the male 001(M-001) 133 samples of faeces and 31 sample of serum were collected. From male 002 (M-002) 146 samples of faeces and 26 samples of serum were collected. Both the animals were also examined with ultrasound to monitor the development of testis. The methodology used for ultrasound examination is explained in chapter 2. The frequency of ultrasound examination varied during the period of the study, becoming more frequent (up to at least be-monthly) from September 2006.

All the samples were analysed with the RIA methods explained in chapter 3 for the determination of testosterone levels.

Each animal was measured at the beginning of the study and body weight was calculated as explained in chapter 2. Measuring and recording of body length and weight is a routine part of the medical care protocol at the facility. Body weights and body length recorded at the beginning and at the end of the study are reported in Table 5.1.

Table 5.1.: Males considered for the study

Animal	Estimated age in years (*)	Initial body weight (Kg)	Final body weight (Kg)	Initial body length (cm)	Final body length (cm)
M-001	8	126	152	225	240
M-002	7	99	126	197	220

(*)estimated age at the end of the study

5.4. Results and discussion

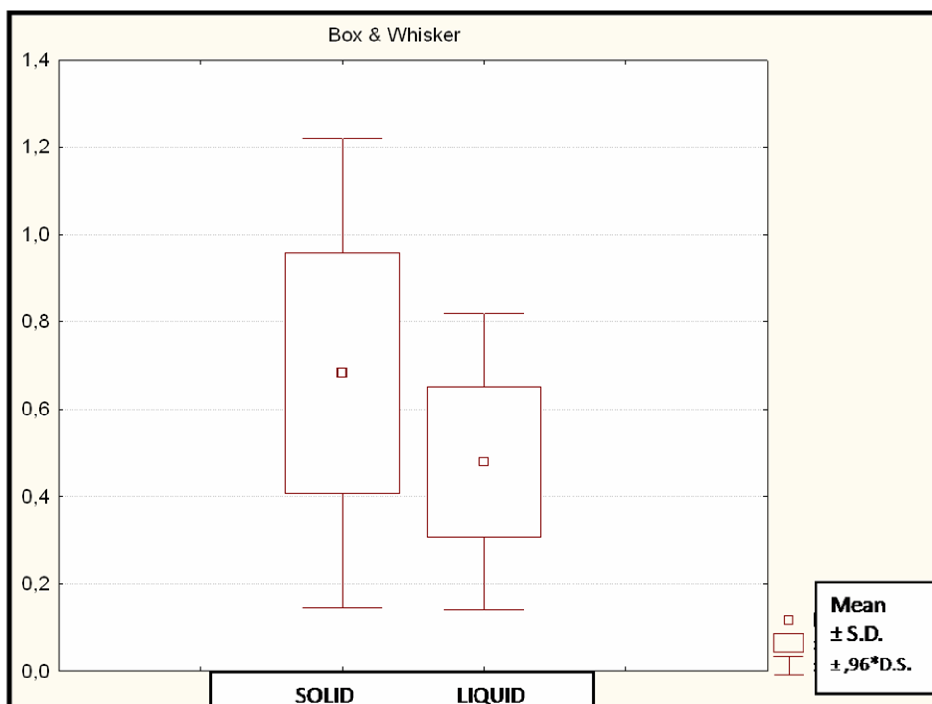
The RIA applied to measure the faecal testosterone was validated and the results are presented in chapter 3 (Figure 3.5 and 3.6).

Faecal TST varied between 0,39 pmol/g and 119,95 pmol/g in male 001 and between 0,38 pmol/g and 167,79 pmol/ g of faeces in male 002. Serum testosterone varied in M-001between 0,31 and 46,49 nmol/L and between 0,21 nmol/L and 62,38 nmol/L in male 002 (see Graphic 5.1a and 5.1b).

The results reported for faecal TST are referred to the sample analysed *in toto,* considering both the liquid and the solid part. To evaluate if the composition of the sample could have affected the results, eight samples selected *at random* were first centrifuged to separate the liquid part from the solid one. The two phases were then analysed with the method explained in chapter 2 and the results, expressed as mean ± standard deviation, showed that the difference of testosterone within the two matrixes was of 0,8 ± 0,7 pmol/g of faeces.

As shown in graphic 5.1. the hormone concentrations in the two matrixes are not significantly different. However a different distribution of the hormone concentrations have been observed.

Graphic 5.1.: Testosterone concentration in the two faecal matrixes



Complete profile of faecal and serum testosterone level of the two males considered in the study are shown in graphic 5.2a. and 5.2b.
Two picks of serum TST were observed in both males at the beginning of the study (see graphics 5.2a and 5.2b), reaching values that would be considered physiological for an adult male. At the ultrasound examinations performed at the beginning of the study, the animals could not be considered as sexually mature. Atkinson *et al.,* (1999) reported that increased of ovarian hormones could some times be due to the adrenal gland production. This could explain at least in part the high level of TST found in the serum of prepubertal males in October and November 2005. Most probably the increment was due to an instant fluctuation produced by the adrenal gland which, like for cortisol, do not find correspondence in the faeces examined in the present study.
During the following year, until the end of 2006, serum TST (N=40; mean ± s.d.) was of 1,23 ± 0,78 nmol/L (below than 3 ng/ml reported by Schroeder, (1990) as reference for immature males). Faecal TST (N= 160) was of 4,05 ± 5,93 pmol/g. Within the 2006 some rising of faecal TST were noticed, but possibly due to adrenal gland activity. During the study period the size of the testes were monitored with ultrasound as reported in chapter 2 and the length varied from a minimum of 6.82 cm in male 002 to a maximum of 12,6 cm in male 001. Right testis were usually longer than the left one as reported by Brook *et al.* (2000). The length and width of the testis at the beginning and at the end of the study are reported in table 5.4. During the ultrasound examination the parenchyma of the testis was examined. At the beginning of the study the parenchyma of the testis was hypoechoic in relation to the hypaxialis lumborum muscle, poorly differentiated and the testicular mediastinum was not visualized. These observation, confronted with faecal TST value obtained could be used to detect faecal baseline values as references for prepubertal males.

Studies on different animal species reported a seasonality in testosterone production (Noonan et al, 1991; Robeck and Monfort, 2006; Strier *et al.,* 1999). Robeck *et al.* (2001) reported that evidence of seasonal variation of blood testosterone levels in the bottlenose dolphins. In the present study the seasonal variation of TST in faeces and serum was considered and results are reported in table 5.2. and 5.3. The seasons were considered following the calendar (i.e. Spring from 21st of March till the 20th of June). The highest levels of faecal TST were found between February and May, partially in agreement with

the period reported by Harrison and Ridgway (1971) who found higher levels of TST in the blood in April and May.

Within the two males considered in the present study, higher increase of TST level in faecal samples was observed in male 002. This animal was the younger, and with smaller testis size, but it was kept in a pool with two cycling females. Ruiz de Elvira *et al.* (1982) reported that in rhesus monkeys the exposure of males to sexually active females further stimulated testosterone elevations. It is possible that a similar mechanism is present in the bottlenose dolphins, and this may explain TST production in male 002.

From January to June 2007 the TST of male 002 varied significantly from the values obtained during the previous months. Serum TST (mean ± d.s.) 9,92 ± 12 nmol/L and faecal TST was 16,37 ± 31,31 pmol/g.

Due to the schedule followed for sampling collection, only in two occasion it was possible to observe serum TST > 10,40 nmol/L (=3 ng/ml) on the same day when high values of faecal TST were observed (30,19 and 167,79 pmol/g respectively). Although further study are necessary to better identify the faecal TST metabolites and the possibility of those to interfere with the exact value result, it is possible to hypothesize that value of faecal inferior to 30pmol/g can identify prepubertal males and that values superior can be referred to pubertal males. This hypothesis can be supported by the ultrasound examination done during the first months of 2007, during which the testicular mediastinum was visualized as an hyperchoic linear structure on of the longitudinal length of the testis. Brook et al (2000) reported three different level of echogenicity of the testis parenchyma to identify whether a male is juvenile, sub-adult or adult. To evaluate the echogenicity of the testis, the hypaxialis lumborum muscle is used as reference, since its echogenicity is found to be similar in all the animals, with no distinction of age and size.

During the examination of the parenchyma, in the animals used for the present study there was not evidence of parenchymal lobolation, which is considered by Brook et al (2000) to be a sign of reproductively mature testis. The echogenicity was homogeneous but less echogenic than the muscle. For these observations the males considered in this study can be defined as sub-adult or pubertal at the end of the study. Although it was not possible to check the quality of the semen, the observation reported above and the lack of pregnancies in the facility where those animals were kept sustains the hypothesis that the animals were prepubertal at the beginning of the study and maturing at the end.

Table. 5.2. Faecal and serum seasonal TST in Male 001

M-001	Faeces	pmol/g		serum	nmol/L	
Season	N. sample	mean	s.d.	N. sample	mean	s.d.
Autumn 05	16	5,56	8,3	5	16,18	22
Winter 05	21	1,19	0,9	5	0,96	0,5
Spring 06	21	3,19	6,9	6	0,89	0,2
Summer 06	11	4,29	3,3	5	1,20	0,6
Autumn 06	14	7,05	7,7	3	2,45	1,4
Winter 06	21	3,22	6,3	4	0,56	0,1
Spring 07	29	11,61	30	3	2,20	1,8

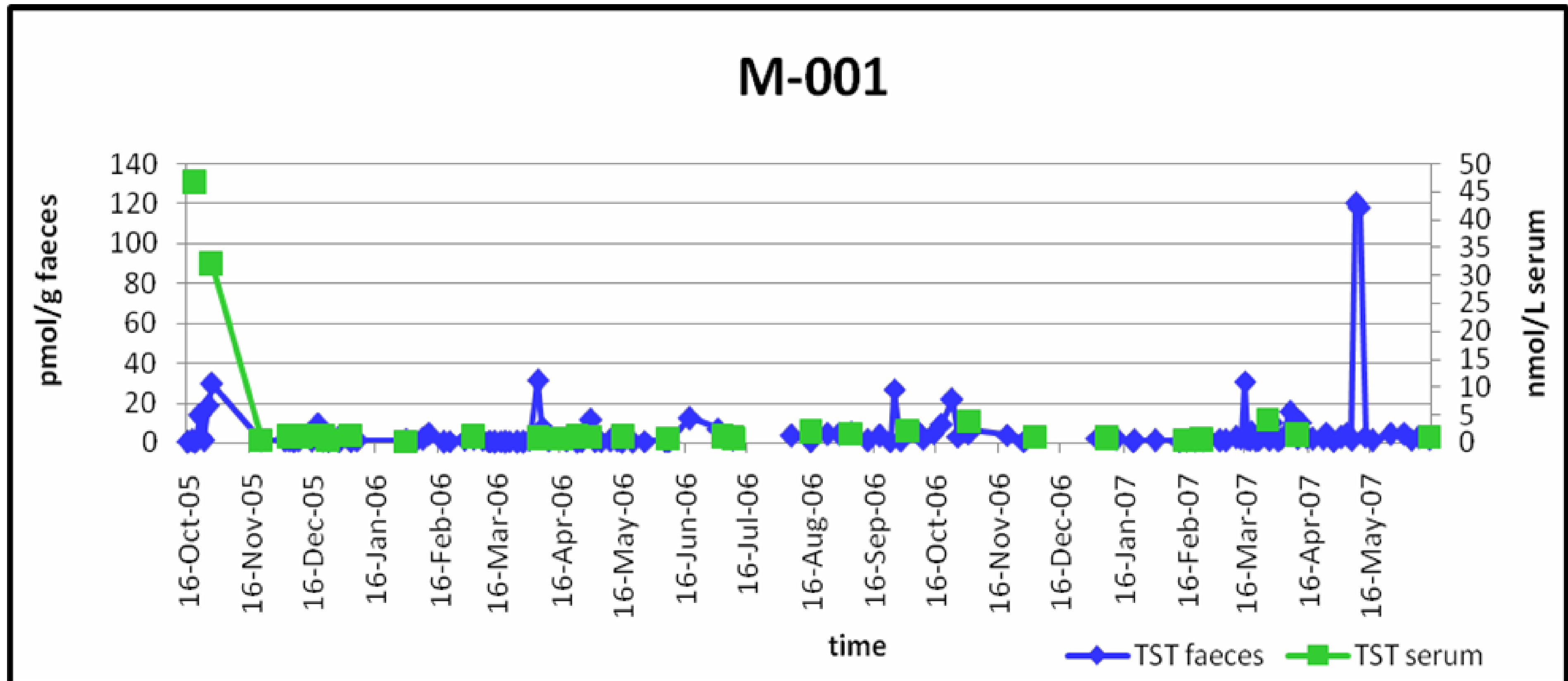
Tab 5.3 Faecal and serum seasonal TST variation in Male 002

M-002	Faeces	pmol/g		Serum	nmol/L	
Season	N. sample	mean	s.d.	N. sample	mean	s.d.
Autumn 05	17	4,98	7,98	5	18,43	27,10
Winter 05	18	2,58	3,31	2	0,48	0,08
Spring 06	25	3,72	5,00	6	1,49	1,03
Summer 06	13	4,20	6,70	4	1,50	0,41
Autumn 06	19	5,94	6,96	3	1,57	1,13
Winter 06	17	16,55	30,67	2	0,25	0,05
Spring 07	35	16,80	32,36	4	0,40	0,18
Summer 07	2	1,01	0,22			

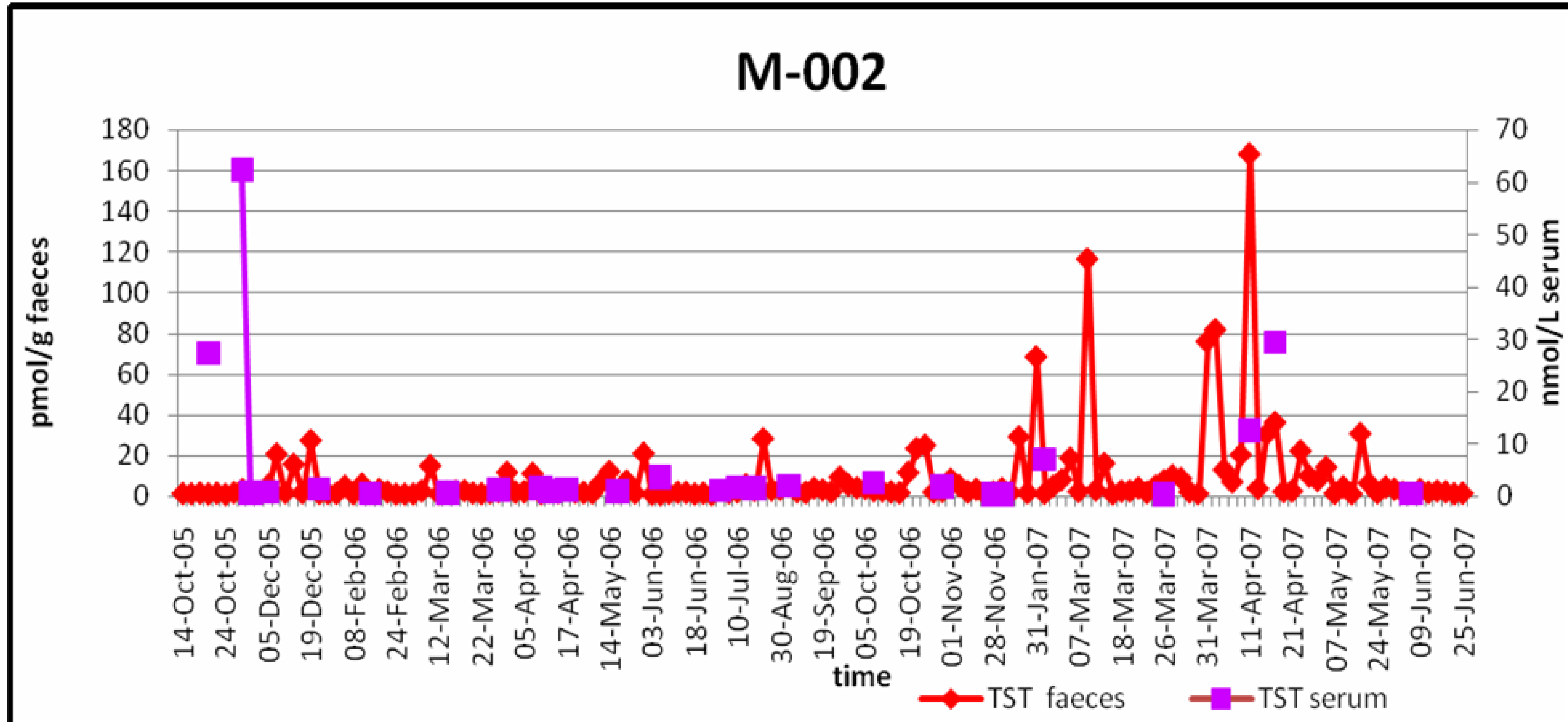
Table 5.4: Testicular size of male 001 and 002 at the beginning and at the end of the study

Animal	Right testis length (cm)	Right testis width (cm)	Left testis length (cm)	Left testis width (cm)
M-001(beginning study)	9,1	1,5	8,95	1,7
M-001(end study)	12,6	2,27	10,3	2,23
M-002(beginning study)	7,3	1,8	7,3	1,9
M-002(end study)	8,2	2,22	7,7	1,95

Graphic 5.2a.: Complete profile of TST level of Male 001

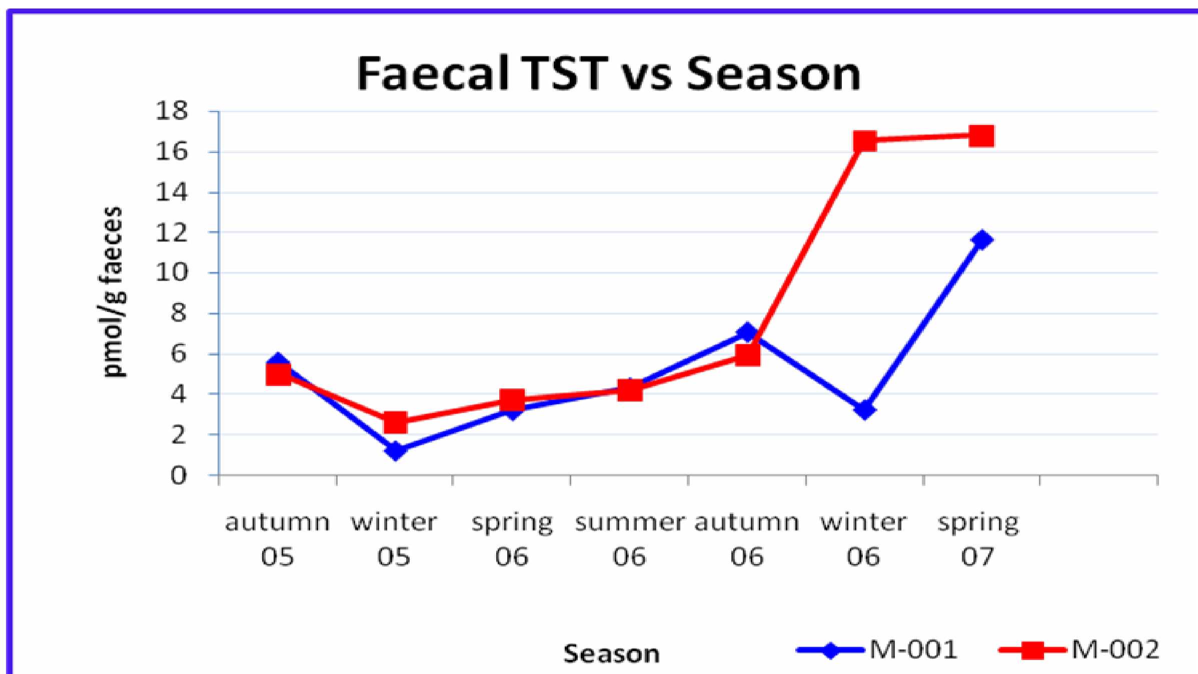


Graphic 5.2b.: Complete profile of TST level of Male 002



When the effect of the season on the TST was considered, a specific patter was not found (graphic 5.3.). Male 001 showed lower values of faecal TST in winter for two consecutive years. On the contrary it was noticed that male 002 in winter 2005-2006 had lower faecal TST values, while the following winter showed higher values of faecal TST. The rise of faecal TST in male 002 took place in correspondence of the period when both the females kept usually with him had shown both to have an ovarian activity.

Graphic 5.3.: Correlation between faecal testosterone and season



Several authors (Robeck *et al*, 2001;Boyd *et al.*, 1999; Schroeder, 1990b; Schroeder, and Keller, 1989) reported of a seasonality of serum TST and sperm production. The lack of specific patter of faecal TST in relationship with the season observed in the present study it is most probably due to the young age of the animals considered and the prepubertal stage they were during the first year of the study.

5.5. Conclusion

Considering the serum TST values obtained and the ultrasonography observation and comparing with the reference values given by Schroeder (1990) and Brook (1999), the animals used for the present study can be considered as being prepubertal at the beginning of the study and peri pubertal or at the onset of the puberty at the end of the study.

Reproducible RIA method has been validated for testosterone in faecal samples of bottlenose dolphins and both the intra-assay and inter-assay resulted to be acceptable for RIA analysis. The method validated in the present study appear to be reliable to monitor TST in faeces of bottlenose dolphins. The measurement of hormone concentrations in captive dolphins using an easily trainable technique provides an outstanding opportunity to further understand the physiology of marine mammals. Faecal samples allow for multiple sampling, and demonstrate no observable stress to the animals, allowing for diurnal and seasonal data measurements. Due to the similarity of the behaviours that could be necessary to train, in order to avoid the possible contamination of semen with urine, males are generally not trained for urine collection. The use of faecal samples can provide information about the endocrinological situation of the animals, without the risk of contamination of valuable samples as semen.

Nevertheless the technique need to be further studied: faecal metabolites need to be individualized with HPLC- MS and the possibility that rise of sexual hormones in the serum possibly related to adrenal gland activity needs to be verified.

In the present study it was not possible to detect statistically a correlation between TST in faeces and serum. This could be due to the small number of faecal and serum samples collected on the same days, especially when animals started to show higher hormonal values, but it could be also due to the lipophilic nature of the steroidal hormones. Atkinson (1999) reported that steroids extraction from faeces was attempted in hawaiian monk seals. She noticed that the diet and faeces of these seals are extremely fatty. Since steroids are lipophilic, it was difficult to get good extractions of steroids without having interference problems. Atkinson suggested to keep this hypothesis in account for the relationships between the serum concentrations and the faecal concentrations not being linear. Due to the diet based on fatty fish that the dolphins used for the present study were

receiving, it is feasible to believe that this could have partially affected the present results and the correspondence between faeces and serum.
Seasonal patter of faecal testosterone was not identified, due probably to the young age and the prepubertal phase of the males during the first months of this study. To better identify the seasonal patter of faecal testosterone further studies should be done and addressed to older animals.

To my knowledge, faecal sampling has not been tested previously to monitor testosterone in bottlenose dolphins. A comprehensive and longitudinal study of this species using the method reported will provide an helpful tool to better understand the fluctuations in testosterone concentrations in bottlenose dolphins and a further step would be to examine the correlation between faecal testosterone levels with spermatic production.

CHAPTER 6. ADRENAL GLAND AND ITS HORMONES

6.1. Review of adrenal gland endocrinology in marine mammals

The adrenal glands are situated at the cranial pole of the kidneys and are represented by two endocrine portions that secret different hormone: the cortex that surrounds the medulla. However, in cetaceans mammal, due to the presence of adrenal medullary and connective tissue septae that extend into the cortex, the adrenal glands appear to be pseudolobulated (Rommel and Lowenstine, 2001).

The medulla produces catacholamines: epinephrine, norepinephrine and dopamine. The cortex secretes glucocorticoids (cortisol and corticosterone), mineralcorticoids (aldosterone) and steroids (androgens, progestin and estrogens). It can represent the 90% of the gland in adult animals (Kirby, 1990). The hormones secreted by the cortex are primary under the control of the adrenocorticotropin (ACTH) secreted by the anterior part of the pituitary gland.

Histologically and endocrinologically the cortex can be divided in three zones: the zona glomerulosa secretes aldosterone, while the zona fasciculata and the zona reticularis secrete glucocorticoids (Cortisol, Corticosterone and dehydroepiandrosterone) and the majority of adrenal androgens and estrogens. All the zona of the cortex produce Deoxycorticosterone (DOC) (Kirby, 1990).

The amount of Cortisol and corticosterone produced differs among the different species of vertebrates. In dogs Cortisol and corticosterone are secreted at a ratio of 1:1 (Kirby, 1990).

Thomson and Geraci (1986) reported that in bottlenose dolphins cortisol predominates over corticosterone in a ratio of 5:1 and they observed that this ratio varied from 5:1 to 10:1 in long term captive animals (Kirby, 1990). The same cortisol:corticosterone ratio with the mean value of 4.7 is reported by Ortiz and Worthy (2000)

Thomson and Geraci (1986) have established that in cetaceans the cortisol prevails over corticosterone as the principal glucocorticoid, and the presence of aldosterone as the mineralcorticoid hormone.

The functionality of the adrenal glands is correlated with the pituitary gland and St. Aubin (2001) and Ortiz and Worthy (2000) reported how the systemic consequences of increasing of cortisol concentrations, including hyperglycemia, and stress leukograms (leukocitosis, lymphopenia and eosinopenia) work for the most part in accordance with the hypothalamic-pituitary-adrenal axis (HPA) relationship established for other mammals. However an important difference was noticed regarding the low circulating level of cortisol in cetaceans and the modest rising of the hormone following "stressful" observed situation. This suggests that even small changes can be of clinical importance. Studies of St. Aubin and Geraci (unpublished data as referred by St. Aubin, 2001) showed that in bottlenose dolphins 50% or less of the total cortisol circulating correspond to the bound fraction and this might be the reason for the small changes observed for cortisol levels.

Glucocorticoids have different functions. Some of them are: increase in hepatic glycogenesis, increase gluconeogenesi, increase protein catabolism, have anti-insulin action on peripheral tissues (brain and heart excluded). Other functions affected by the glucocorticoids are reproduction, immune response, electrolytes and water balance and growth.

Glucocorticoids are measured in serum and urine with radio immuno assay (RIA) [in dolphins] and in faeces in different animal species. There is usually some cross reactivity with antisera for cortisol and corticosterone even if physiological levels are different (Kirby, 1990). To avoid this possibility it is necessary to evaluate properly the method and the antiserum used.

Free cortisol, to monitor adrenal gland activity, has been measured as well in plasma and saliva (Pedernera-Romano *et al.*, 2006; Theodorou and Atkinson, 1998).

6.2. Experimental design

For the present study, 10 bottlenose dolphins were used: eight females and two males. The age, reproductive status and physical characteristics (body weight, body length) of the animals are reported in the tables presented in chapter 4 and 5. Pools characteristics and diets used to feed the animals are reported as well in chapter 4 and 5.

6.3. Faecal cortisol: material and methods

The techniques used to collect the faecal sample and blood are explained in chapter 2. The sampling period and frequencies used for monitoring the faecal cortisol were similar to those applied for the collection of other hormones samples for females and males as explained in chapter 4 and chapter 5 respectively . Methodology applied for extraction of cortisol from sample of faeces and serum and the validation of method used to evaluate the cortisol concentration in faeces are explained in chapter 3.

6.4.Results and discussion

As explained in chapter 3, the cortisol in females was extracted both with petroleum ether and ethylic ether. Cortisol extracted with Ethylic ether varied within the females from 0,60 pmol/g to 26,31pmol/g (N= 215; 6,85 ± 4,51pmol/g), while cortisol extracted with Petroleum ether varied from 0,19 pmol/g to 15,22pmol/g (N=309; 0,79 ± 0,98). The results obtained with the two extraction showed that ethylic ether is more effective to extract cortisol from faecal sample, although a positive correlation (P< 0,01) was observed between extraction with ethylic ether and petroleum ether. Serum cortisol varied between 1,74 nmol/L and 53,29 nmol/L (N= 101; mean 14,36 ± 9,19 nmol/L).

Faecal cortisol in males varied between 0,28 pmol/g and 21,29 pmol/g (N= 281; mean 5,43 ± 3,94 pmol/g). Serum cortisol varied between 2,87 nmol/L and 29,40 nmol/L (N= 57; mean 12,33 ± 6,72 nmol/L). Thomson and Geraci (1986) applied the RIA to determine the serum cortisol in bottlenose dolphins. The method they used was modified to include 30 nmol/L standard and the sensitivity of the assay used was 10nmol/L. Through the results obtained, they considered serum cortisol concentration of 30-40 nmol/L as resting/basal value.

The correlation between faecal and serum cortisol was not significant, but tendencial (R= -0,246; P= 0,096; N=47) and this result is supported by the fact that serum cortisol is affected by the instant fluctuations. Instant fluctuation does not affect the level of cortisol in the faeces, because before to be excreted in the faeces the hormone undergo to different physiological metabolic processes (Palme *et al.*, 2005;1996). After being secreted into the blood stream, steroids are usually inactivated within minutes by metabolic processes mainly in the liver. The liver acts as a filter by deactivating highly bioactive steroids that could be toxic, by conjugating them to glucuronide or sulphate molecules. Conjugation can be complex, such as 17ßoestradiol- 3-glucuronide-17- sulphate, or simple as oestrone-glucuronide and oestradiol-sulphate. Conjugation causes increases in molecular polarity of steroids to improve their water-solubility (Ziegler and Wittwer, 2005). The inactivated metabolites are then eliminated from the body, either by excretion into the urine via the kidney or via the bile and the gut into the faeces. Before faecal excretion steroids may be reabsorbed from the bile into the bloodstream (enterohepatic circulation) prolonging their permanence in the body and the intestinal microbial flora can hydrolyze steroids and break apart some of the conjugates (Ziegler and Wittwer, 2005). Therefore in the majority of species hormones excreted into the faeces are predominantly unconjugated. Several metabolites for each hormone can be found in the excreta, and steroid metabolism pathways are different among species and even sexes, resulting in a diverse array of metabolites.
Furthermore, Atkinson (1999) reports of studies done in Hawaiian monk seals and that due to their diets and faces extremely fatty, it was difficult to get good extractions of steroids from these lipids without having interference problems. This would account for the relationships between the serum concentrations and the faeces concentrations not being linear.
Considering the pathway steroids hormones follow before to be excreted into the faeces and the results obtained in the present study, faecal material seems to be a suitable sample to monitor situations of chronic stress.

Significant relationship was found between faecal sexual hormones and faecal cortisol concentration ($P<0,05$).

The T-Student test revealed differences between sex and social grouping as reported in table 6.1.

Table 6.1.: T-student test between sex and social grouping.

	GROUP		SEX	
	5 animals together	Two groups (3 and 2 animals)	Females	Males
C faeces	5,2 ± 0,7	6,7 ± 0,6	7,0 ± 0,5	5,1 ± 0,7 *
C serum	17,6 ± 1,2	13,1 ± 1,0 *	15, 9 ± 1,1	13,8 ± 1,0
Neutrophils	66,4 ± 1,4	59,9 ±1,4 *	64,4 ± 1,4	60,3 ± 1,4 *
Lymphocytes	21,3 ± 1,3	26,1 ± 1,3 *	22,6 ± 1,3	26,0 ± 1,1 *
Eosinoplhils	9,4 ± 0,6	11,7 ± 0,9 *	10,4 ± 0,7	11,0 ± 0,9

*: P< 0,05

The difference observed in faecal cortisol is possibly due to a different metabolism between sex, as excretion in females can occur earlier than in males. Hunt *et al. (2004)* studied the faecal glucocorticoids excretion in Steller sea lion (*Eumetopias jubatus*) by injecting ACTH and collecting faecal samples for 2 days before and 4 days after injection. They reported that while females have peak glucocorticoid excretion at 5 and 28 hours post injection, males had peak excretion at 71 and 98 hours post injection. Considering that in our study blood was usually collected at 9am, while faeces were collected at 4-5pm, it is possible that the different time excretion between sex explains the differences between faecal and serum results.

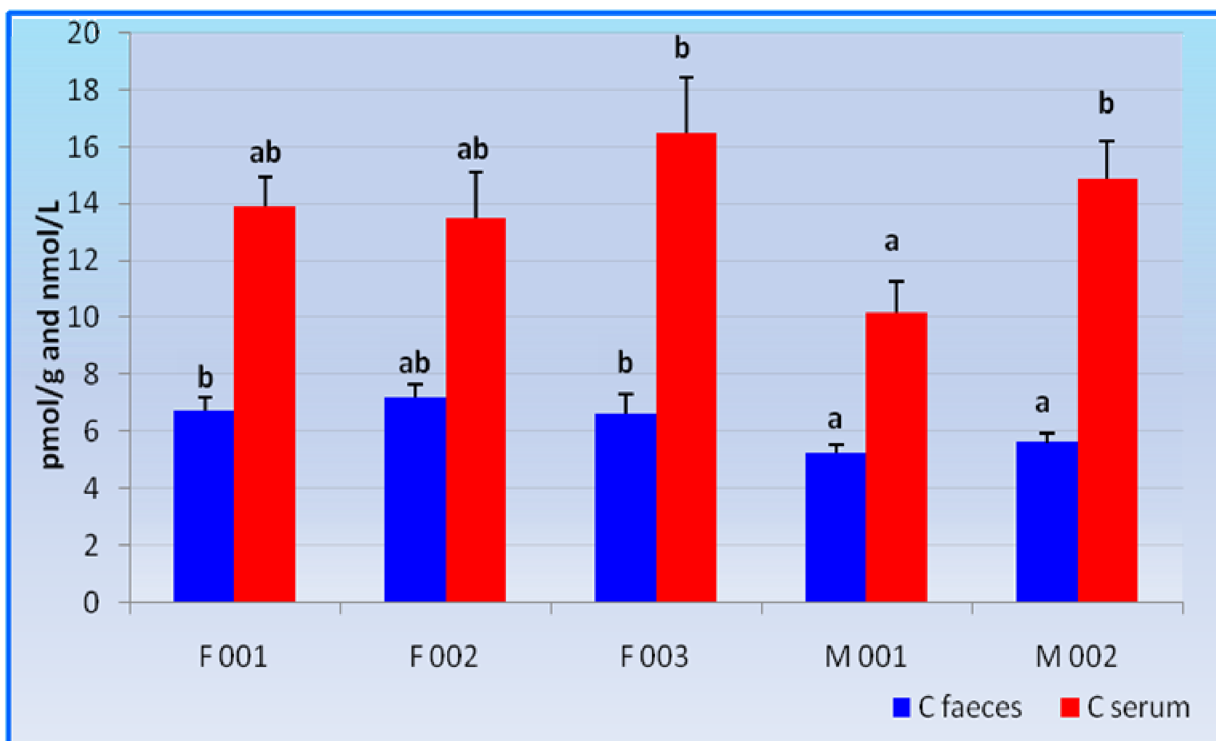
Faecal cortisol was examined considering two social situation: first when the five animals were separated in two groups (male 001 and female 003 were in a pool and male 002 was in another pool with female 001 and 002). Secondly faecal cortisol was considered when the five animals were kept together in the same pool. Results showed that when the animals were all together the faecal cortisol levels did not vary (no significant correlation), but serum cortisol was higher (P< 0,001) than when they were kept separated in two groups.

This can be due to the fact that in both groups there were female and male and the difference in faecal cortisol is not evident because of the homogeneity of the groups.

Although the environmental temperature was not considered in the present study, it was noticed that faecal cortisol was higher when the animals were all together and it usually happened during the winter season. The increase of faecal cortisol during those months could have been because of the lower temperature (Suzuki *et al.*, 2003) or because males were fighting for dominancy on the females.

The ranges and mean faecal cortisol levels of the three females and two males kept together are shown in graphic 6.1.

Graphic 6.1.: Faecal and serum cortisol when the five animals of the facility B were kept together.



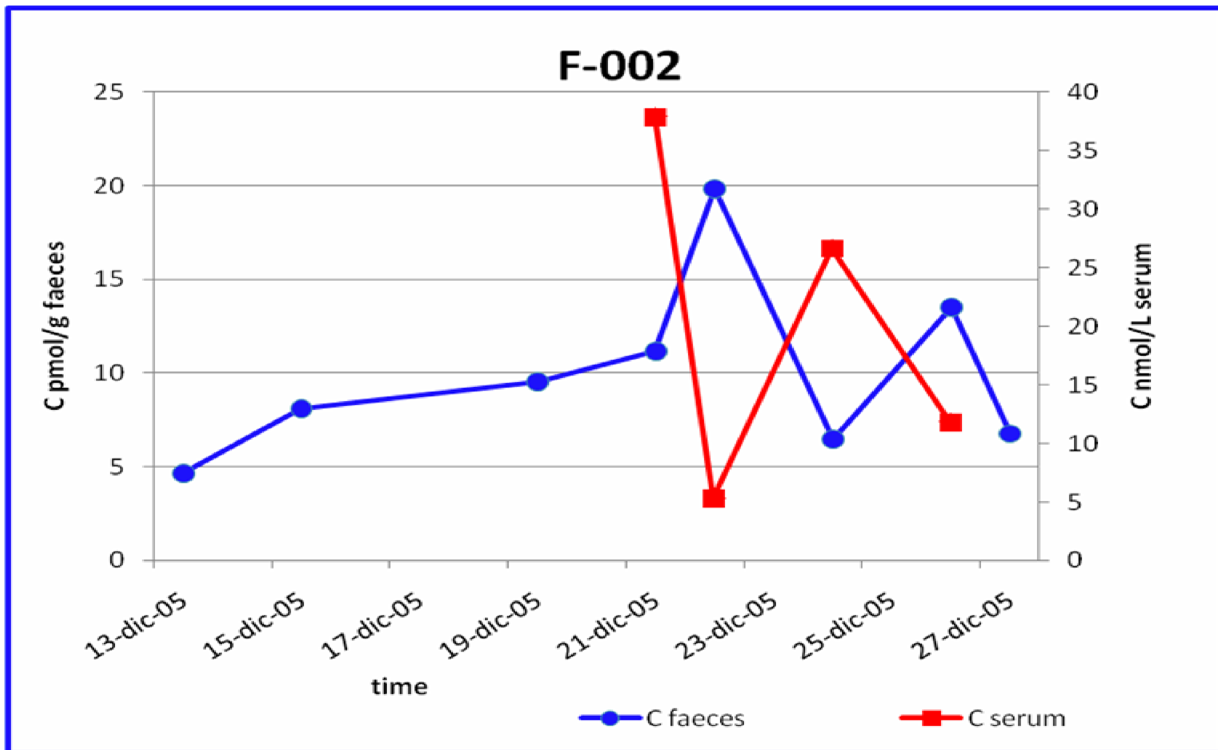
Female 001 and 002 showed similar level of serum cortisol, female 003 and male 002 were similar and male 001, the older and the dominant between the five, showed the lower serum cortisol level. In the social structure of the group female 003 and male 002 were the younger and under the dominancy of male 001 and female 002. Thus the hierarchic positions could explain the differences in serum cortisol levels.

The faecal cortisol, shows that female 002 has hormonal levels partially similar to both the group of males and females, due probably to her dominant position on females and subdominant with males.
The differences can be due to specific metabolites present in the faeces, or to the higher number of faeces collected during the study period and further studies are necessary to better understand the differences.
Significant correlations were found between season vs behaviour (N= 133; P= 0,01; Rho= -0,29), season vs appetite (N= 133; P<0,001; R= -0,29) and behaviour vs appetite (N= 134; P< 0,001; Rho= 0,82). Significant correlation was found also between season and serum cortisol (N= 133; P= 0,018; R= -0,205).
A significant negative correlation, in agreement with Thomson and Geraci (1986), was found between serum cortisol and eosinophils (Rho= -0,317; P=0; N=131). Not significant, but negative correlation was found between faecal cortisol and eosinophils showing that eosinophils follow the same trend of the cortisol. The lack of significant correlation can be possibly referred to the delay that intercourse between cortisol excretion in the blood and cortisol excretion in faeces after the metabolic processes it has to undertake. In this study no significant correlation was found between serum and faecal cortisol and lymphocytes.

To validate indirectly the measurements of faecal cortisol, clinical observations were compared with the results obtained in the laboratory. Female 002 on the 20th December 2005 started to refuse food and during the day she did not eat all the fish she was supposed to. On the following day she refused to eat, but she was still collaborative and blood was collected by behaviour: serum cortisol was of 37,89 nmol/L and faecal cortisol was of 11,17 pmol/g. All the chemical and haematological parameters were within the normal ranges. During the following two days the animal was refusing food and was not collaborative. It was decided to catch the animal twice a day for hydration, collection of cytology sample (gastric and faeces) and blood sampling. The ethogram reported for attitude and appetite marks of 1 for those days. After two days of not eating completely, the animal started to eat cut fish in small amount and within 3 days her attitude and appetite was back to normality. During the days the animals had to be caught for medical procedure, the elevation in serum cortisol were modest and her serum cortisol level did not exceeded the 40 nmol/L reported by Thomson and Geraci (1986) as normal levels. Serum cortisol increased compared to the baseline values presented from the animal in normal

clinical situation, but never overtake the levels referred by Thomson and Geraci (1986) as baseline levels. Of interest it was to observe that the faecal cortisol was rising and decreasing following the trend of serum cortisol within one day of difference.

Graphic 6.2.: Comparison between cortisol in faeces and serum.



In October 2005, female 001 and 002 had to receive a 15 days intra muscular (IM) antibiotic treatment. Every day, the animals were put in a medical pool, lifted up to the surface and hold in position for the necessary time for the injection. The procedure was lasting around 5-10 minutes. After then the animals was left back into the medical pool and observed by veterinarian and trainers for one hour, before it was allowed to go back to the main pool with other dolphins. Faecal cortisol and serum cortisol were check before, during and after the treatment (see table 6.3.) and serum cortisol never exceeded the 40 nmol/L, showing that the animals, accustomed to human contact, did not exhibit a glucocorticoid response to the situation that could have been considered as a stressful one. Nevertheless it was noticed that during the IM treatment serum cortisol increased of the 50% compared to the values obtained before and after the treatment.

Tab. 6.3: Cortisol levels presented by two females before, during and after IM antibiotic treatment

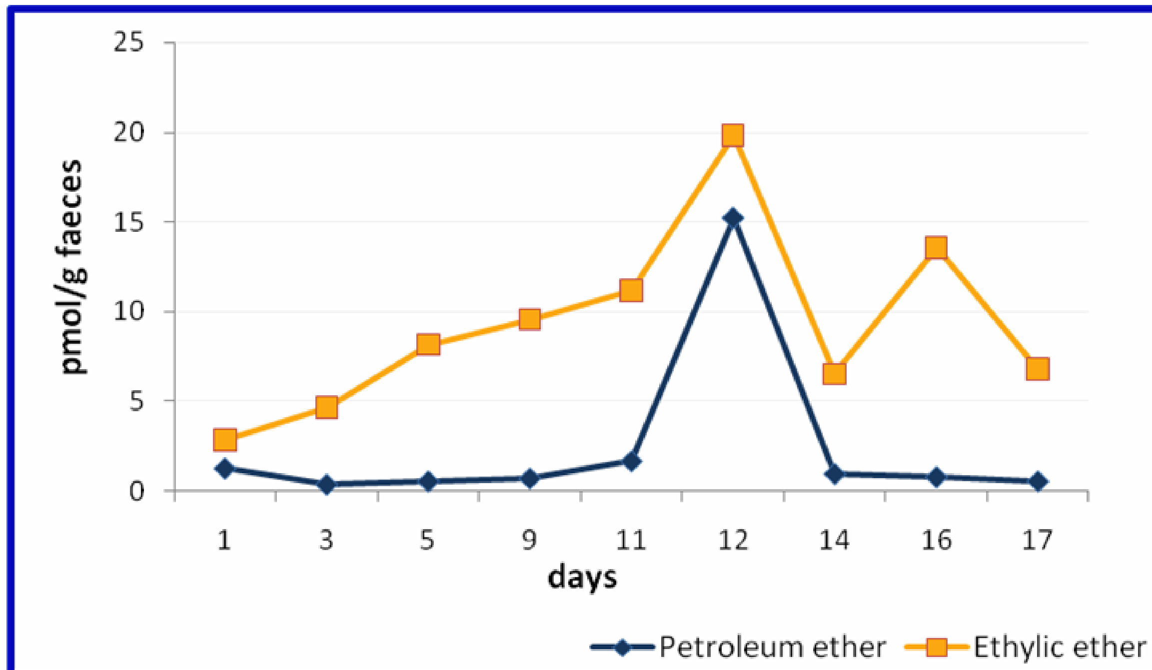
		Faeces- Ethylic ether (b)		Serum	
		C pmol/g		C nmol/L	
	N°faecal sample	Mean	s.d.	Mean	s.d.
Before IM treatment	3	12,58	2,97	14,93	2,46
During IM treatment	7	8,30	3,78	28,68	8,37
After IM treatment	5	8,58	2,56	18,69	0,59

Results reported are the mean of the values obtained from the two animals treated.

Tataranni et al., (1996), as referred by Ortiz end Worthy (2000), report that in response to stress circulating glucose concentrations increases due primarily to elevated glucocorticoids. During the IM treatment, as part of the normal medical protocol, glucose was checked and the mean value was of 141 ml/dl, within the ranges referred for bottlenose dolphins by Bossart *et al.* (2001), who consider the normal values between 90-170 ml/dl. These results are in agreement with Ortiz and worthy, (2000) who referred that relatively short capture and restraint times did not induce a significant neuroendocrine stress response.

Although from the present study resulted that ethylic ether is more appropriate to extract cortisol from faeces, petroleum ether (as shown in graphic 6.3.) seems to be more appropriate to extract specific metabolites that can be more representative of the adrenal gland activity and the enteric metabolism that cortisol has to undertake in the intestine.

Graphic 6.3.: Faecal samples extracted with petroleum ether and ethylic ether



Female 006, considered as resting female because of the progestin treatment (Regumate), was monitored for 10 months. Faecal samples varied from 2,15 pmol/g to 11,25 pmol/g (mean ± s.d.: 5,48 4,09 pmol/g)

Hunt *et al.* (2006) reported the values obtained from faecal samples of lactating females and resting females of North Atlantic Right Whales (*Eubalena glacialis*). Their results showed that lactating females have higher faecal glucocorticoids metabolites than resting females (39,33±5,82 ng/g versus 23,11±4,25 ng/g of dry faeces, corresponding to 108,65 ± 16,08 pmol/g versus 63,84 ± 11,74 pmol/g). Our results show no particular differences between lactating and resting females and this is possible due to the fact that the bottlenose dolphins examined during the study were kept under human care and not exposed to environmental factors that usually marine mammals can be exposed in the wild. The difference can be also due to the different species we examined.

Faecal cortisol in pregnant females varied between 3,02 pmol/g and 34,67 pmol/g (mean 11,12± 10,20 pmol/g).

Hunt *et al.* (2006) reported that pregnant North Atlantic right whales have much higher glucocorticoids concentration (238,14 ±74,37 ng/g) compared to lactating, resting and

immature females, while in our study pregnant females did not show great differences comparing with females in other physiological status.

6.5. Conclusion

The present study shows that the RIA is suitable to monitor faecal cortisol in bottlenose dolphins. As first attempt petroleum ether was used to extract the cortisol with the intent to use the same technique to evaluate both cortisol and progesterone. The aim was to use the same method to evaluate the relationship between gonad and adrenal gland activity.

The different extraction procedures tested in this experiment resulted in an important discrepancy between the faecal cortisol extracted with petroleum ether and samples extracted with ethylic ether. The results obtained from the extractions done with petroleum ether showed that this solvent resulted to be less efficient to extract cortisol from faeces and should not be recommended as the best faecal extraction method for cortisol.

Furthermore, given that faecal steroid metabolites are a mixture of several chemical molecules with different polarities, selection of an appropriate extraction procedure can represent a critical issue when working this sample and further studies should be address to the identification of specific metabolites present in the faeces of bottlenose dolphins. Consideration has to be given also to the physiological situation that needs to be characterized to evaluate the best method of extraction.

Although a significant correlation between faecal and serum cortisol was not found, it was possible to detect a correspondence between the hormone extracted from the two matrixes within situations potentially stressful. Empirically, it seems that the delay time between cortisol secretion in the blood stream and its excretion in the faeces could be of 24 hours, comparable with the lag-time reported for carnivorous animals like dog and cats (Schatz and Palme, 2001; Graham and Brown, 1996).

Because the different steroid metabolism pathways between species result in a diverse array of metabolites in the faeces, and the excretion lag time appear to be specie-specific,

further studies would be required to isolate the metabolite of interest and the specific enteric hormones excretion in the bottlenose dolphin.

CHAPTER 7: GENERAL DISCUSSION

ENDOCRINOLOGY AND REPRODUCTION

The fertility is of valuable importance and priority for animals and it is one of the most important and basic drivers for all the species. Reproduction allowed the survive of the specie and the genetics differences, without which the entire group would be in danger.

For these reasons the animal would make physiological sacrifice to ensure reproductive success and only the most severe threats to its well-being will prevent the animal from reproducing (Moberg, 1985) .

During the last decades, many studies have been done with the intent of studying the correlation between stress and reproduction (Maeda and Tsukamura, 2006; Moore and Jessop, 2003; Tilbrook et al, 2000; Dobson and Smith, 1995; Moberg, 1985), and successful reproduction is nowadays considered a good parameter to monitor the well-being of animals and a useful indicator of stressful condition.

St.Aubin and Dierauf (2001) report as definition of stress like a “ three stage syndrome of (1) alarm and adaptation, (2) hormonal events, and (3) resistance, exhaustion, and death”, with the updated definition of Moberg (1985) of stress as (1) recognition of the stressful stimulus, (2) the body’s actual response to the stimulus and (3) the resulting consequence to the body.

Nevertheless, not all the stressful stimulus have to be considered as negative, and not all the stimuli have the same results on different individuals or species: periodic activation of stress response can have a beneficial effect on health, and only when a prolonged, uncontrolled and excessive response to stress is present, it can result in a state of distress (St. Aubin and Dierauf, 2001).

There are several factors that can be considered as potential stressor for animals in wild and/or in captivity, like environment condition (i.e. chemical and noises pollution, temperature), predators, social changes, nutrition, systemic diseases. McBain (2001) said that “ a dolphin that does not have a positive social environment is likely to become a sick dolphin”. Considering the connection and overlap between physiology, immunology, endocrinology and neurology, all the potential stressor can have an impact on the physiological homeostasis and by that on reproduction. The capability of animals to

maintain homeostasis under stressful condition has a cost and it depends on how long the animal has to use its energy to maintain it while under stress. According to Moberg (1985) short period of stress should have minimal effect on reproduction, unless the stress occurs during a particular sensitive period of the oestrous cycle or pregnancy (Gabai *et al,* 2006).

Although many information are still missing on the hypothalamic-pituitary-adrenal-gonad axis pathway in marine mammals, it is plausible to believe that it works as for terrestrial mammals. Rivier and Rivest (1991) as referred in St. Aubin and Dierauf (2001) reported that elevation of glucocorticoids induced by stress may affect the reproductive system by inhibiting hypothalamic secretion of gonadotropin-relising hormone, blocking the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH), and altering the gonadal response to LH and FSH secretion from the anterior pituitary.
Studies done on several species indicate that glucocorticoids can affect reproduction primarily by inhibiting LH secretion at different sites: at the hypothalamic level by interacting with gonadal steroids and suppressing GnRH secretion (Tilbrook et al., 2000; Dobson et al., 2003); at the pituitary level by reducing gonadotroph sensitivity to GnRH (Breen and Kersch, 2004), and/or at the ovarian level by altering androgen synthesis (Spicer and Chamberlain, 1998).

7.1. Cortisol – female reproduction (Progesterone – Estradiolo)

Being under the direct control of the endocrine and central nervous system, the oestrus cycle presents different events that can be vulnerable to the effect of stress and cortisol.
In order to achieve a successful reproduction, it is important that all the phases of the oestrous cycle take place at the right moment with a specific temporal relationship between endocrinological events and behaviour. When oestrogen increase during the follicular phase, it synchronizes the fundamental events by stimulating the preovulatory release of LH, inducing the oestrous behaviour and modifying the uterus that changes to be ready for an eventual pregnancy. Gabai *et al.* (2006) refer as prolonged or intense adrenal gland activity can block the expression of oestrous behaviour or delay the preovulatory release of LH, resulting in a not successful reproduction event.
Different studies done on species that have social behaviour and in which the social structure and dominance are an important aspects, tend to show that high-ranking females exhibit better reproductive performances. Keverne (1979, as referred by Molberg,1985),

Holekamp *et al*. (1996) and Alvarez *et al.* (2007) showed that dominant females of talapoin monkeys, spotted hyaena and Australian cashmere goats have better reproductive results in term of LH secretion, ovulation, expression of oestrus and successful pregnancy. Moberg (1985) concluded that the basic reproductive physiology of a female can be modified by the stress she experiences when receipting aggression from the dominant members of the group.

On the contrary Creel *et al.* (1997) showed that in African wild dog (*Lycaon pictus*) dominant females (with better reproductive performances) had higher corticosterone levels than subdominant females, indicating that chronic glucocorticoids elevation does not suppress reproductive activity.

Cortisol levels, oestrous cycle manifestations and behaviour of females 001 and 002 used for the present study were monitored for 21 months. Although no particular differences regarding the cortisol levels were observed among the two animals, it was noticed that during the second reproductive season (during they year 2007), when both the females had already started to cycle, female 002 showed better ovarian activity (more follicles were present and the primary follicle reached bigger size more often), while female 001 (that was the older and the one who first started to show ovarian activity) did not present ovarian activity. At the same time, female 002 was receiving the attention of the male, while female 001 was left out of the attention of the other animals. Considering the group situation, the fact that female 001 resulted to be under the dominance of female 002, the lack of ovarian follicles detected by ultrasound in female 002 and that this female did not show faecal progesterone variation indicating ovulations, it is possible to indicate that her reproductive activity was suppressed by the dominance of female 001, in agreement with the hypothesis of “dominance as reproduction suppression” as reported by Whitehead and Mann (2000).

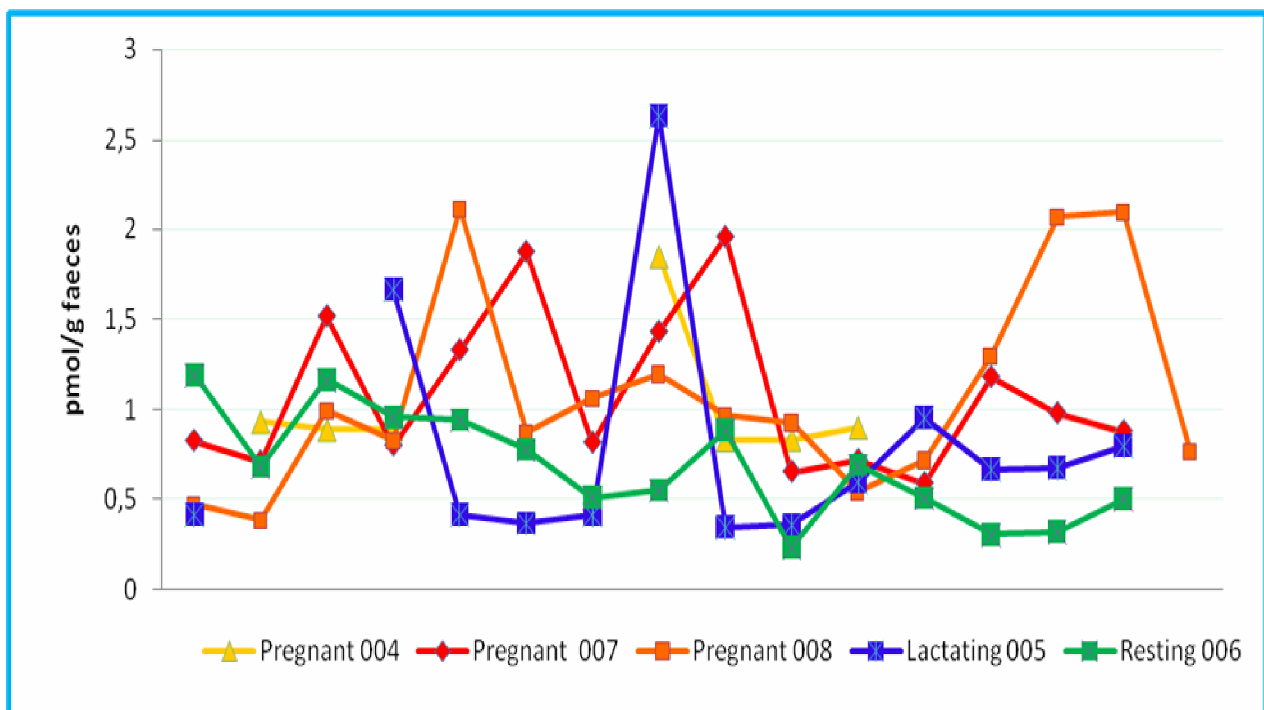
Pregnancy is another moment during the reproductive life of an animal that is considered to be potentially at risk of stressors influence. Nutritional deficiencies, increased physical demand, altered metabolism, physical injuries and psychological factors are considered as potentially stressful conditions. Implantation is a phase during which the neuroendocrine support is essential for a successful pregnancy. If the first part of the pregnancy is crucial, the same attention has to be given to the last part of it: Moberg (1985) report that even thought the maintenance of pregnancy, once implantation has

taken place, seems to be buffered from stress, attention has to be given to possible traumas that can occur near the term and that could induce premature parturition.

Although bottlenose dolphins kept under human care can become quite tolerant to transportation in a stretcher (St.Aubin and Dierauf, 2001), some facilities that host pregnant bottlenose dolphins and might experience the necessity to move one animal during pregnancy, prefer not to move the animal during the first and the last quadrimester to avoid any possible stressful situation (R. Gojceta, personal communication).

Within the pregnancies monitored during this study, none of the animals had to be moved from their original pool, but faecal cortisol was constantly monitored. None of the pregnant females showed values of cortisol levels significantly different from lactating females or resting females (Graphic 7.1.) .

Graphic 7.1.:Faecal cortisol in females during different physiological situations



Results showed in the graphic 7.1. are presented only for cortisol extracted with petroleum ether.

7.2. Cortisol- male reproduction (testosterone)

Contrary to females, reproduction in males is not determined by several and different steps that characterise the oestrous cycle. Once the male reach the puberty, its fertility is ensured by a continuous spermatogenesis and male became receptive to oestrous females. Moeberg (1985) report that although elevated environment temperature (which can be considered as a stressor factor) for prolonged period could affect the quantity and quality of semen (motility and capability to fecundate) it would not be due to an alteration of the endocrine system that control spermatogenesis, but only a physical effect and that the testis response to the effect of the adrenal axis and glucocorticoids during stress has no significant effect on fertility of the male. (Moberg, 1991)

On the other hand Wildt et al. (1993) and Munson (1993) showed how reproductive dysfunction in captive cheetahs (*Acinoynx jubatus*) was due to hypercortisolemia from chronic stress and that corticoids were acting as hormone disruptors inhibiting gonad function. This conclusion was supported by the observations that the anatomy of the genital was normal and responsiveness to exogenous hormones, there was an adrenal cortical hyperplasia, a suboptimal spermatogenesis and the level of faecal corticoids were four times higher than those found in wild cheetahs.

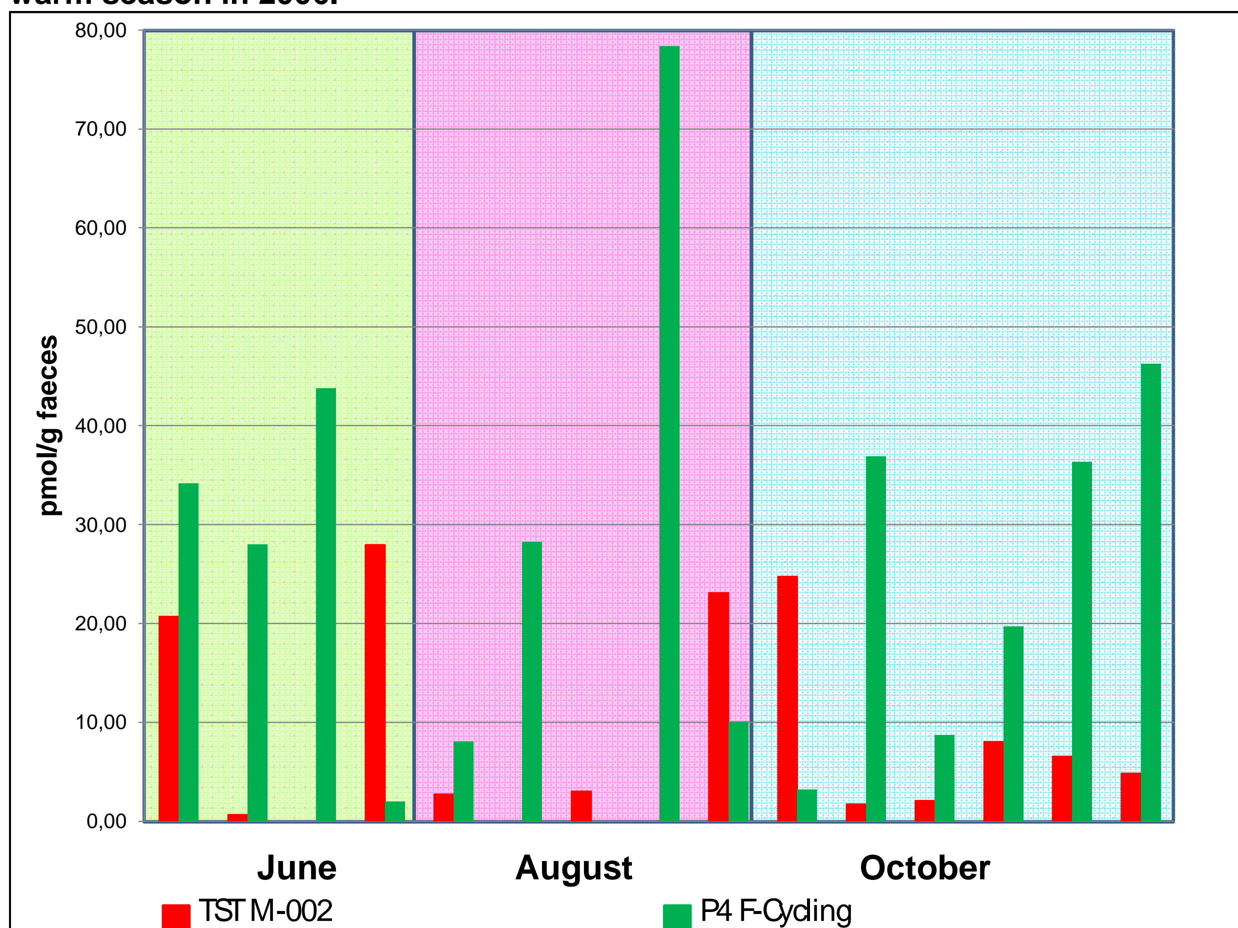
In the males used for the present study, the correlation between cortisol and testosterone in faecal samples showed that there is a significant correlation between the hormones (R=0,481; P= 0,01; N=47). These results are in agreement with Creel *et al.* (1997) who observed that in subordinate wild African dogs TST decrease during the mating season and that dominant had higher cortisol levels than subdominant.

Suzuki et al (2003) reported that in killer whale and bottlenose dolphin cortisol was higher during the cold season, when the energy demand was higher due to cold environment. In our study faecal cortisol was higher in spring and summer, when energy demand for activities (show and swims program) was higher and there is an overlap between TST and C: TST start to increase in February in the male housed with the cycling females and females started cycling in march. Although at that point no other male was present in the pool, it is possible that cortisol increased in relationship to the dominant position of the male on the females. This hypothesis is sustained by the positive correlation between cortisol and testosterone (R=0,481; P=0,001) obtained through the analysis of faecal samples.

During 2006 it was noticed that female 001, that started to show ovarian activity in march, showed constant ovarian activity during the warm season, with rising of progesterone and presence of follicles monitored with ultrasound. Examining the TST level of the male that was kept with her, we noticed that the increase of faecal P4 in the female was usually preceded by rises of TST (graphic 7.2.), suggesting that even on bottlenose dolphins there might be a “male effect” on female’s reproductive activity.

During the same months, sexual behaviours were noticed between the animals. Although dolphins start to have sexual behaviour when very young and the erected penis is not always an indication of sexual activity (Boyd *et al.,*1999), trainers referred that more copulative attempts were taking place from the male’ side and that the attitude of the female was more reflecting the oestrous behaviour (as reported in chapter 4).

Graphic 7.2.:Faecal TST in male versus Faecal P4 in females observed during the warm season in 2006.



No pregnancies were noticed during the following months, and the lack of semen samples did not make possible to establish precisely the stage of sexual maturity in males.

In 1964 Marsden, and Bronson established the qualities of the pheromones in male urine of laboratory mice and how those synchronize the cycle of females. Vandenbergh (1969) noticed that sexual maturation in female mice was accelerated by male pheromones and later on Drickamer (1977) reported that sexual maturation in females was retarded by female pheromones. The effect of pheromones to induce oestrous in females has been studied in different species of mammals as pigs (Kirkwood *et al.,*1981), prairie voles (Carter *et al.*1980) and opossum (Harder and Jackson, 2003).
The nervous systems of bottlenose dolphins has rudimental or absent peripheral olfactory components (Dehnhardt, 2002; Ridgway,1990), however the presence of a gustatory nucleus of the thalamus and the elaboration of the seventh and ninth cranial nerves suggested to neuroanatomists that the gustatory sense is well developed (Kruger, 1959 as referred by Ridgway, 1990). Although some study have been done to test the chemical sense of dolphins (Kuznetsov, 1979, as referred by Tyack., 2000), little is know about how cetaceans may sense the waterborns chemicals (Tyack*.,* 2000). Norris and Dohol (1980) suggested the possibility that also cetaceans can use pheromones. Caldwell and Caldwell (1972, 1977- as reported by Mann *et al.*, 2000) suggested that bottlenose dolphins can release pheromones from pores present in the anal glands. Norris (1991- reported by Tyack., 2000) hypothesized that the male spinner dolphins (*Stenella longirostris*) can use chemical sign to feel the reproductive state of females. Many information regarding the chemical sense of cetaceans and their capability of detecting pheromones are still missing. If they have limited use to chemical communication, due in part to the limited range of diffusion in water compared to their motility (Tyack 2000), the possibility to study these animals while kept under human care, gives us an extremely important chance to learn more about their physiology.
Never the less it has to be considered that if from one side the limited environment can help us to detect different factor more precisely, on the other side the presence in the pools' water of substances like chlorine, or systems like UV, used to disinfect the water of the pools can alter the presence of hormones and pheromones in the water.

Further studies would be necessary to better investigate the possible influence of pheromones on bottlenose dolphins reproductive activity.

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7.3. General Conclusion

Faecal samples can be easily collected from bottlenose dolphins kept under human care, and in the present study the RIA technique, already used for many different animal species was developed and validated to be used for the detection of steroid hormones in faecal samples of these cetaceans as well.

This research demonstrates that the analysis to evaluate the levels of steroid hormones in faecal samples of bottlenose dolphins can be used to asses their reproductive status. It also demonstrate that the methodology can be applied to the study of adrenal gland activity to detect situation of chronic stress.

When larger number of samples collected from more animals of different age and physiological status become available, further studies should be done to better define the hormonal ranges for each age class and reproductive status class and thus this technique could be applied to study wild population of bottlenose dolphins.

Due to the methodology used for the collection of faecal samples and that samples were immediately frozen after collection, the degradation of the hormones during the time was not considered in the present study.

In this study, levels of faecal progesterone discriminated accurately between pregnant females and resting females. Not significant differences were observed within progesterone levels in lactating females, resting females and pre pubertal females. Faecal estrogens were not evaluated for lactating and resting females thus it was not possible to detect the differences with pregnant females.

The possibility to collect repeated samples from the same individual during several months gave the possibility to detect the moment the pre pubertal females started to show ovarian activity and to detect individual differences.

Although for the present study it was not possible to monitor males of certain sexual maturity, the results obtained from faecal samples of the two juvenile males and their comparison with serum testosterone made possible to detect the moment the hormones started to rise, identifying the moment the animal were becoming peri-pubertal.

Longitudinal studies using faeces can provide baseline values for both male and female captive dolphins.

Furthermore the possibility to study those animal over a longitudinal period of months, gave the possibility to establish whether the cortisol level was changing during the months

of more intense activity and no chronic rise of hormone was detected. This make the possible conclusion that dolphins trained for shows or exposed to constant contact with human do not show signs of chronic distress.

Although this study showed that it is possible to use faeces as biological material to detect hormonal levels and follow the reproductive and adrenal gland activity, further studies should be done to better characterized any single physiological status and HPLC-MS analysis should be done to identify the dominant metabolites in faeces for gonadic hormones and glucocorticoids.

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