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DEVELOPMENT OF NON-INVASIVE TECHNIQUES TO MEASURE TESTOSTERONE IN THE NORTHERN FUR SEAL (*CALLORHINUS URSINUS*)

（キタオットセイにおけるテストステロンの非侵襲的な測定手法の開発）

by

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Abstract

The reproductive physiology of wildlife can provide useful insights into population dynamics. Information about the physiological state of animals is generally collected through blood sampling, which involves capture and anaesthesia of animals. In the past few decades, however, non-invasive techniques of endocrine monitoring have been developed to study the reproductive status of animals using indicators such as faeces and hair. The least invasive method examines faeces, since faecal collection does not require physical contact with animals. As a result, faecal hormones are now commonly studied in captive animals. An advantage of hair sampling is that hair can be biopsied in the wild. In the present study, I used faeces and hair in place of blood to study the reproductive status of the northern fur seal (*Callorhinus ursinus*) males.

The sexually dimorphic northern fur seal congregates in rookeries during the breeding season in summer, and occurs in offshore waters without hauling out during the non-breeding season in winter. Males become sexually mature at age 4, but usually become capable of establishing breeding territories (*i.e.*, socially mature) at age 8. Socially mature males return to their rookeries to establish territories before the breeding season, while juveniles remain offshore during the breeding season. Spatial segregation of males occurs during the non-breeding season off southern Hokkaido in the Sea of Japan where they have been increasingly plundering fish from fishing gear. Since demographic information about males can help evaluate the impact of this plundering, development of a maturity-assessment method for free-ranging seals using non-invasive techniques could enhance our understanding of males in this community. The objective of this study was to measure testosterone in male northern fur seals using non-invasive techniques and to determine the sexual maturity of free-ranging seals during the non-breeding season.

In Chapter 2, I examined the relationships between serum testosterone and faecal testosterone metabolites in a captive seal to develop non-invasive monitoring of faecal testosterone metabolite levels. I found a significant relationship between the faecal testosterone metabolites and serum testosterone levels when the faeces were collected approximately one day after blood withdrawal. In addition, the seasonal changes in faecal testosterone metabolites showed similar trends with serum testosterone levels, showing higher testosterone levels during the breeding season. These results indicate that serum testosterone levels can be estimated using faecal testosterone metabolites. This method will be a useful tool to non-invasively monitor the reproductive cycles of male northern fur seals.

In Chapter 3, I compared the concentrations of faecal testosterone metabolites with age, sampling months and testis weight of free-ranging northern fur seals to evaluate their sexual maturity. Forty-five males were collected in 2011–2017 in waters off Hokkaido. The testis weight and age of the fur seals showed a significant positive relationship although no monthly differences were obtained between mature and socially mature seals. Significant differences in monthly faecal testosterone metabolites between mature and socially mature seals were obtained only in April. Furthermore, the individual differences in the faecal testosterone metabolite levels in mature and socially mature animals were large, possibly because environmental cues such as prey availability and energetic demands can differ among individuals and differently affect endocrine systems. My results also indicate that faeces are a more useful tool in captivity since factors in the captive environment such as photoperiod and prey are often controlled so that faecal steroid metabolites likely are comparable in captive conditions.

Hair is another endocrine matrix that can assess the reproductive status of animals non-invasively. However, it is unknown if hair samples can assess pinniped reproductive status. In Chapter 4, I examined the sexual maturity of males during the non-breeding season off Hokkaido using hair testosterone levels. A total of 57 hair samples were collected from males during the non-breeding seasons of 2011–2018. The testosterone levels of juveniles were significantly lower than those of mature seals. Elongated spermatids, which occur in the final phase of spermatogenesis, were present in seals collected between April and June. Seals collected in May, during the spermatogenesis progresses, showed the highest testosterone levels. My results demonstrate that juvenile males can be distinguished from mature males using hair testosterone levels in May preceding the breeding season.

In conclusion, non-invasive techniques to assess the reproductive status of northern fur seal males were developed. In captivity, faecal testosterone metabolites were a useful tool to evaluate the status during the breeding season, whereas hair was an invaluable tool to determine the maturity of free-ranging seals. An advantage of these methodologies is that they do not disturb the animals before sampling. Furthermore, data on male reproductive status can enhance our understanding of fur seal ecology, such as the distribution of the seals off Hokkaido during the non-breeding season, and clarify the connection between non-breeding and breeding seasons. This method can be applied in various pinnipeds and will be especially useful for species that spend time offshore, since hair can be biopsied in the wild.

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Abbreviations	**Definition**
AFDW	Ash-free dry weight
AIC	Akaike's information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
CV	Coefficient of variability
DEL-FIA	Dissociation-enhanced lanthanide fluorescence immunoassay
DW	Dry weight
EIA	Enzyme immunoassay
GC-FID	Gas chromatography flame ionization detector
GC-MS	Gas chromatography mass spectrometry
GLM	Generalized linear model
J	Juvenile
M	Mature
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
SC	Spermatocytes
SG	Spermatogonium
SM	Socially mature
SP	Round and elongated spermatids and/or spermatozoan
T	Testosterone
TMSI-H	Hexamethyldisilazone: trimethylchlorosilane: pyridine
TR-FIA	Time resolved fluoroimmunoassay
WT	Weight

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Chapter One: Introduction

1.1. Non-invasive techniques of endocrine physiology

The reproductive physiology of wildlife provides useful insights into population dynamics. Wildlife studies often involve animal captures. The capture and recapture of animals, however, can adversely affect the animal body condition (Cattet *et al.*, 2008), and anaesthesia can cause them unexpected deaths (Arnemo *et al.*, 2006). Although animal capture can provide morphometric data, and allow age determination (Darimont *et al.*, 2008) through methods such as analysis of teeth obtained from a live animal, indirect non-invasive techniques can also be used to gain ecological (Putman, 1984), evolutionary (Kohn and Wayne, 1997), and physiological information (Wasser *et al.*, 1993) of animals without disturbing them. Non-invasive techniques, thus, are widely spreading in the field of animal science.

Endocrine studies require blood sampling, which can be impractical in free-ranging animals (Amaral, 2010). Non-invasive techniques of quantifying endocrine physiology have been developed in different taxa including amphibians (*e.g.,* Narayan, 2013; Szymanski *et al.*, 2006), birds (*e.g.,* Cockrem and Rounce, 1994; Goymann, 2005), reptiles (*e.g.,* Atkins *et al.*, 2002; Scheun *et al.*, 2018), and mammals (*e.g.,* Touma and Palme, 2005a). Non-invasive techniques of endocrine monitoring used as a replacement of blood sampling are urines (*e.g.,* Amaral *et al.*, 2009; Constable *et al.*, 2006), faeces (*e.g.,* Cockrem and Rounce, 1994; Graham and Brown, 1997), hair (*e.g.,* Davenport *et al.*, 2006; Koren *et al.*, 2002), saliva (*e.g.,* Amaral *et al.*, 2015), and blubber (*e.g.,* Kellar *et al.*, 2006, 2009; Mansour *et al.*, 2002). I choose faeces and hair to study the endocrine physiology of northern fur seals (*Callorhinus ursinus*). The reasons for the choices are as follows. Faeces can be obtained without any physical contact with animals, so it is the least invasive technique of all. Sampling of urines and saliva involve physical contacts with animals or require training. I also used hair analysis as a non-invasive technique, since hair can be remotely biopsied in the field using a crossbow or airgun.

1.1.1. Faecal steroid metabolites

Faecal steroid metabolites are increasingly studied in captive and free-ranging animals. Faecal collections do not disturb animals when collected after defaecation. Even though animals often have to be enclosed alone, it has an advantage that animal training is not required. However, there are several concerns when developing faecal steroid monitoring in a new species. Since steroid hormones are metabolized in the liver and excreted via bile, hormones found in faeces are already metabolized (Möstl and Palme, 2002; Palme *et al.*, 1996; Schwarzenberger *et al.*, 1996; Figure 1.1), which makes the interpretation of results convoluted. Another concern is that the time lag between serum and faecal steroid metabolites hormones is species-specific and a function of gut passage time (Palme *et al.*, 1996; Schwarzenberger *et al.*, 1996). In addition, differences in prey species can influence animal metabolism that can contribute changes in faecal steroid

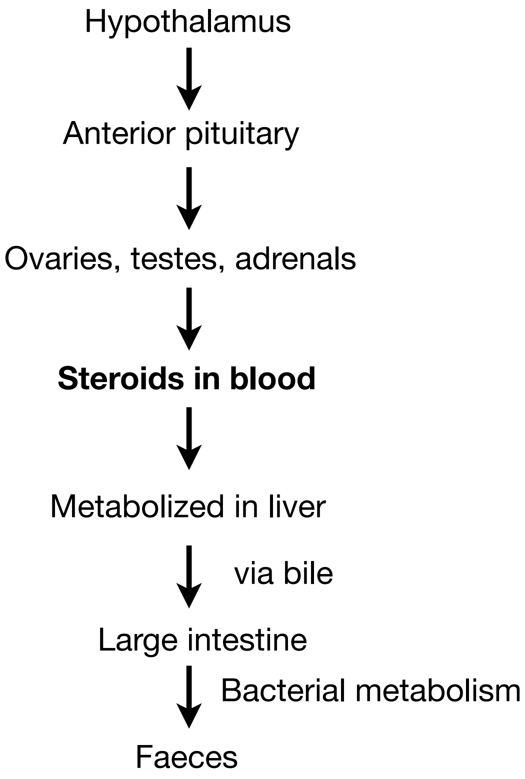

Figure 1.1. Diagram of the secretion and metabolism of steroid hormones.

(Modified from Möstl and Palme, 2002)

hormone levels (Dantzer *et al.*, 2011; Goymann, 2012). Therefore, it is important to examine the timing of sampling and effect on prey species in faecal steroid metabolites for a new species.

1.1.2. Steroid hormones in hair

Hair was first used as a steroid hormone matrix in the late 1990s. Since hair samples can be indirectly obtained by biopsy sampling, this method has been applied in the wild and captivity. The mechanism of steroid hormone incorporation into hair is still unknown (Stalder and Kirschbaum, 2012). The commonly accepted hypothesis is that the steroid hormones are incorporated into hair via passive diffusion from blood (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012). Steroid hormones in hair reflect the integrated free hormone fraction rather than the total concentration in serum (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012), reflecting endocrine activity integrated over several months (Bryan *et al.*, 2013b). The steroid hormones are likely incorporated into hair between moults, and thus the interpretation of hormone levels in hair will depend if hair is sampled before or after moulting (Ventrella *et al.*, 2018).

1.2. Steroid hormones

Steroid hormones are synthesized from cholesterol-based precursors. Steroid hormones are often classified into glucocorticoids and reproductive hormones. Glucocorticoids such as cortisol increase in response to stressful environment (Möstl and Palme, 2002). Reproductive hormones include androgens, progestogens, and estrogens, all of which are essential for reproduction of mammals. The majority of reproductive steroids are released from different targeting gonad organs. The hypothalamic–pituitary–gonadal axis regulates such as reproductive system of animals. This axis is activated by environmental factors such as photoperiod, temperature, and prey availability (Geyer, 2017).

1.2.1. Testosterone

Testosterone, a steroid hormone, is important for male sexual maturity and reproduction. This hormone is involved in spermatogenesis (Roberts and Zirkin, 1991), development of secondary sexual characteristics (Witschi, 1961), and expression of aggressive behaviours including songs to advertise and defend territories (Wingfield *et al.*, 1987). Animals with seasonal breeding cycles show an increase in testosterone levels prior to the onset of the breeding season (Mauget and Boissin, 1987). Testosterone concentrations often increase one month prior to the breeding seasons in pinnipeds (Griffiths, 1984; Noonan *et al.*, 1991). The onset of sexual maturity for males has been investigated with various morphological and physiological parameters. These include spermatogenesis stage, seminiferous tubule diameter, testis weight, spermatozoa abundance and testosterone levels (Plön and Ric, 2007). These parameters are all interconnected. Spermatogenesis is enhanced during the breeding season, and gonads are enlarged by an increase in testosterone levels (Blottner *et al.*, 1996; Holekamp and Talamantes, 1991). Therefore, these parameters are used to assess the sexual maturity of males.

1.3. Northern fur seals

1.3.1. Annual life cycle of males

The northern fur seal is distributed in the North Pacific Ocean (Bigg, 1990; Kenyon and Wilke, 1953). This species is sexually dimorphic, and the body size of males rapidly increases after sexual maturity (Trites and Bigg, 1996). Males at around the age of 4 years or above become sexually mature and capable of reproduction (Gentry, 1998). However, since northern fur seals are polygynous, socially mature males older than 8 years old form harems and participate in the reproduction (Vladimirov, 1987). They show a typical annual life cycle of pinnipeds. Their annual life cycle has two stages: breeding and non-breeding (Gentry, 1998). Socially mature males appear in rookeries about one month earlier than females and establish territories for reproduction, which peaks in July (Gentry, 1998; Vladimirov, 1987). After mating, the northern fur seals annually moult their pelage, which is mostly completed in fall (Scheffer and Johnson, 1963). Northern fur seals migrate to open waters during the non-breeding season in winter and remain in offshore waters without hauling out on land (Bigg, 1990; Kenyon and Wilke, 1953).

1.3.2. Sexual segregation during the non-breeding season

Sexual segregation in the distribution of animals is often seen in sexually dimorphic animals (Wearmouth and Sims, 2008). Sexual segregation of northern fur seals has been observed during the non-breeding season (Kenyon and Wilke, 1953; Wilke, 1951). Recently, more males than females have been observed off the southern coast of Hokkaido in the Sea of Japan (Horimoto *et al.*, 2017). Since mature males are larger in body size, they can dive deeper (Gentry, 1998), which likely contributes to the sexual segregation of this species. The energy gained during the non-breeding season will likely predict the success of following breeding (Beck *et al.*, 2003) since territorial males fast during the breeding season. Since 30% of mature males are excluded from reproduction for their entire lives (Vladimirov, 1987), and the survival rate to the age of breeding is only 3 % (Lander, 1981-82), juveniles and mature seals need to increase their body size to survive and reproduce. Knowledge of the distribution of males during the non-breeding season, therefore, can strengthen the understanding of the connections with breeding season of this species. Long-term monitoring of demography of northern fur seals can reveal the connection between the non-breeding and breeding season.

1.3.3. Fisheries competition and animal welfare

The northern fur seal has been observed near the coast of southern Hokkaido in the Sea of Japan since 2010 (Horimoto *et al.*, 2012). As a result of their occurrence nearshore waters, depredation of fisheries catches by fur seals has been of concern (Horimoto *et al.*, 2012). Northern fur seals were heavily exploited for pelts in the 18th centuries (Scheffer *et al.*, 1984), but exploitation of the fur seal is now prohibited in Japan except for scientific research. In order to study the ecology of this species, the capture of northern fur seals in the coast of southern Hokkaido in the Sea of Japan is conducted under the "*Noxious Organisms Fishery Damage Prevention Program*" by the Fisheries Agency of Japan.

The fisheries in Japan are often operated in small local communities whose economies can be impacted by depredation damage. It is, therefore, important to know the demography of this species in this water during the non-breeding foraging season for mitigation if necessary. On the other hand, for the northern fur seal perspective, it is important to know the demography to minimize bycatch and to maximize animal welfare. Bycatches of northern fur seals in fishing gear are observed around Hokkaido waters (Horimoto *et al.*, 2017). Entanglement with fishing gear is increasing in pinnipeds and cause serious damages to the animals (Butterworth and Sayer, 2017).

1.4. Research goals

Although non-invasive techniques of steroid hormone monitoring have been developed in various taxa, relatively little work has been done to use the techniques to study the demography of animals. The overall goal of this research is to build non-invasive techniques of testosterone measures in the northern fur seal in captivity and the wild and to relate the sexual maturity of free-ranging northern fur seals in the non-breeding season. The objective of Chapter 2 was to develop faecal testosterone metabolite monitoring in the northern fur seal. To accomplish this, I compared patterns in serum and faecal testosterone levels of a captive male northern fur seal. I examined the effect of prey species on faecal compositions, and compared the representations of testosterone metabolites in faecal DW and AFDW. The objective of Chapter 3 was to evaluate the sexual maturity of free-ranging northern fur seals using the concentrations of faecal testosterone metabolites. I compared the representation of faecal testosterone metabolite levels expressed between DW and AFDW. In Chapter 4, I used hair testosterone levels of free-ranging northern fur seals to develop non-invasive monitoring of hair testosterone levels. I compared the levels with testicular morphometries, sampling months, and age classes. Throughout my research, I have a goal to apply these non-invasive techniques to animals in captivity and the wild.

1.5. Thesis structure

My thesis contains five chapters: a general introduction (Chapter 1), comparison of serum testosterone levels and faecal testosterone metabolites in a captive northern fur seal (Chapter 2), analysis of faecal testosterone metabolite levels in free-ranging northern fur seals (Chapter 3), maturity of northern fur seals using hair testosterone levels (Chapter 4), and a general conclusion (Chapter 5). The data chapters (Chapters 2, 3, and 4) are written as manuscripts to submit for publication in peer-reviewed journals.

Chapter Two: Non-invasive monitoring of faecal testosterone metabolites in a captive northern fur seal

2.1. Abstract

The relationship between serum testosterone and faecal testosterone metabolites in a captive northern fur seal was examined using time-resolved fluoroimmunoassay to develop non-invasive testosterone monitoring technique. We found a significant relationship between the faecal testosterone metabolites and serum testosterone levels when the faeces were collected approximately one day after blood withdrawal. In addition, faecal testosterone metabolites showed seasonal fluctuations that were similar to those found in serum testosterone levels, with higher testosterone levels occurring during the breeding season. These results indicate that serum testosterone levels can be estimated using faecal testosterone metabolites. This method will be a useful tool to non-invasively monitor the reproductive cycles of male northern fur seals.

2.2. Introduction

Non-invasive monitoring of steroid hormone metabolites has been developed in various taxonomic groups, including mammals (*e.g.,* Kersey and Dehnhard, 2014), birds (*e.g.,* Ketterson *et al.*, 1992), and reptiles and amphibians (*e.g.,* Moore and Jessop, 2003; Narayan, 2013) to study environmental stress and reproductive cycles. Matrices such as faeces, saliva, hair, and urine are used to detect steroid hormones (*e.g.,* Kersey and Dehnhard, 2014; Moore and Jessop, 2003). One of the main advantages of these matrices is that they can be non-invasively obtained. Faeces are often the easiest to collect, as it is unnecessary to train animals (Kersey and Dehnhard, 2014), and are useful for monitoring the stress levels and reproductive status of captive animals. The time lag between serum steroid levels and faecal steroid hormone metabolite levels, however, is species-specific (Schwarzenberger *et al.*, 1996). It is thus important to determine the time lag of faecal metabolite levels with serum steroid levels when analyzing a new species (Touma and Palme, 2005b).

Steroid hormones are composed of organic matter. The hormone metabolites in faeces, however, are generally expressed as the amount of hormone per unit total weight (*i.e.,* dry weight (DW)) of faeces (Palme *et al.*, 2013). Depending on the ratio of organic to inorganic component in faeces, the mass of hormone metabolites in faeces is possibly misrepresented. To eliminate this discrepancy, the glucocorticoid metabolites in faeces of two aardwolves (*Proeles cristata*), which ingest soil during feeding, were expressed as per unit ash-free dry weight (AFDW) of faeces (Ganswindt *et al.*, 2012). Although the qualitative interpretations of the hormone metabolites do not change, diet can influence the concentrations of steroid hormones in faeces (Goymann, 2012). The presentation of the faecal hormone metabolites per unit AFDW, therefore, could allow for the detection of biologically significant differences in endocrine parameters (Ganswindt *et al.*, 2012). However, a feeding experiment was not conducted on aardwolves to determine how diet contributed to the organic component of faeces.

The composition of faeces depends mostly on diet. The organic portion of faeces in humans is made up of about 25 – 54% bacteria, and the rest includes undigested carbohydrates and proteins (Rose *et al.*, 2015). The inorganic part of the faeces comes from undigested dietary elements such as calcium phosphate and iron phosphate (Rose *et al.*, 2015). Since the amount and kinds of bacteria can depend on factors such as prey species (Ley *et al.*, 2008; Nelson *et al.*, 2013a,b), social behaviour (Nelson *et al.*, 2013b), and habitat (Nelson *et al.*, 2013a), these factors can also influence the composition of faeces. The composition of faeces in wildlife can be inconsistent within the same species when they hunt different prey species, whereas that of faeces in captivity is likely consistent between individuals, since captive animals are often fed the same diet.

Northern fur seals switch their prey consumption depending on the abundance and distribution of various prey species within their habitat (Yonezaki *et al.*, 2003). This implies that the prey species of wild northern fur seals can influence their faecal composition. Northern fur seals often feed on Pacific mackerel (*Scomber japonicus*), Japanese sardine (*Sardinops melanostictus*), myctophids, squid, arabesque greenling (*Pleurogrammus azonus*) and walleye pollock (*Theragra chalcogramma*) (Horimoto, 2015; Yonezaki *et al.*, 2008). The diet of these seals differs widely among individuals (Mori *et al.*, 2001; Yonezaki *et al.*, 2003), likely resulting in differences in the composition of faeces. Teleost species have less organic matter than cephalopods (Steimle and Terranova, 1985; Van Pelt *et al.*, 1997). The organic content in faeces is, therefore, expected to be greater when northern fur seals feed cephalopods as compared to teleost.

Northern fur seals breed between June and August (Gentry, 1998). Reproductively intact northern fur seals show seasonality in their testosterone levels (Kohyama *et al.*, 1999; Tsubota *et al.*, 2001). The size of seminiferous epithelia also increases during breeding season (Tsubota *et al.*, 2001). Monitoring faecal testosterone metabolites of northern fur seals in captivity, as an alternative to measuring serum steroid levels, will eliminate the stress experienced by animals during capture and blood withdrawal. In addition, captive monitoring will increase our understanding of testosterone levels in wild northern fur seals. Faecal testosterone metabolites can provide an effective tool to monitor the physiology of northern fur seals; here, we provide the first study examining testosterone metabolites in this species. We developed faecal testosterone metabolite monitoring expressed in DW and AFDW in a captive male northern fur seal by determining the time lag between faecal testosterone metabolites and serum testosterone levels and by examining the seasonal relationship between faecal testosterone metabolites and serum testosterone levels to test whether faecal monitoring provides a reliable replacement for information collected by sampling blood.

2.3. Materials and Methods

2.3.1. Sample collection

Samples were obtained from a reproductively intact 8-year-old male northern fur seal weighting on an average of 86 kg at the Izu Mito Sea Paradise (Numadzu, Japan). About 5 ml of blood was drawn from the hind flipper vein of the animal in the morning twice a month from April 2017 to January 2018, except that blood was taken only once in November 2017. The blood samples were centrifuged for 15 min at 1,550 ×*g* (H-26F, Kokusan, Japan), and the serum was frozen at –40 °C. The animal was enclosed alone from 1530 hr to 0830 hr consecutively for three days after the blood withdrawal. Faeces were collected at 0830 hr during the three mornings after the blood sampling.

As a prey experiment, Japanese common squid (*Todarodes pacificus),* Arabesque greenling, and walleye pollock were fed for four days in October, November, and December 2017, respectively. Japanese common squid was fed again for four days in January 2018. Otherwise, the daily diet of the seal was Pacific mackerel. He was fasted the day before the prey change. Faeces for this prey experiment were collected beginning on the second day of the prey change, as the first day of feeding provided a buffer for the change in prey species. The blood of the animal was withdrawn on the second day of each four-day prey experiment. When mackerel was fed outside of the four-day prey experiments, the faeces were collected on the three mornings following the blood withdrawal. All the faeces were also stored frozen at –40 °C in the aquarium until the faeces and serum were shipped to our laboratory in Hakodate.

2.3.2. AFDW of faeces

Faecal samples were dried at 60 °C (DKM600, Yamato Scientific Co., Ltd., Japan) until the weight of the faeces remain unchanged. The dried faeces were pulverized with a mortar and pestle and were kept frozen at –20 °C until analysis. Dried faecal sample (500 mg) of the seal for each of the prey experiment diets was weighted and burned in an electrical muffle furnace (Motoyama Co., Ltd., Japan) based on the method in JIS:M:8812:2006. The temperature of the furnace was increased to 500 °C for 1 h from 25 °C, and increased to 815 °C for 30 min, and kept at 815 °C for another 1 h. The mass of inorganic contents (*i.e.,* ash) in the dried faeces was measured. The weight of the ash was measured again after additional heating for 30 min at 815 °C and to make sure that each weight was constant. Inorganic weight of faeces was subtracted from the dry weight of faeces to find out the ash-free (*i.e.,* organic) weight of faeces.

2.3.3. Steroid extraction

Extraction methods of steroid hormones from serum and faeces were conducted as follows. The serum was centrifuged again at 21,600 ×*g* for 10 min (himac, CF15R, HITACHI, Japan). Diethyl ether (1 mL) was added to 100 μL of serum, and it was vortexed for 30 sec. The serum was frozen in a cold methanol bath for 3 min, and the supernatant was decanted. This procedure was repeated three times. The supernatant was evaporated in a water bath at 45 °C. Tris-HCl-based assay buffer with the same volume of the serum was added to reconstitute the sample for TR–FIA. Steroids were extracted from 10 mg of each dried faecal sample using 1 mLof 80 % methanol. The samples were shaken for 15 min, and centrifuged at 21,600 ×g for 5 min. The supernatant was evaporated using a centrifugal concentrator (CC-181, Tomy Seiko Co., Ltd, Japan). The extracts were reconstituted in the same volume of assay buffer.

2.3.4. Gas chromatography flame ionization detector (GC-FID)

We used GC-FID to qualitatively analyse the presence of testosterone in serum. Serum was extracted as previously described. The extracts were dried with N_2 and reconstituted with liquid chromatography mass spectrometry (LC-MS) grade methanol. Reconstituted samples (0.8 μL) and the samples with 0.5-mL standard samples (androsterone, dihydrotestosterone, Δ4-androsterone, testosterone) were analyzed by GC-FID (GC 390B, GL Sciences, Japan) using a 30-m long column, Zebron ZB-5HT Inferno (Phenomenex Inc., USA) with an internal diameter 0.25 mm and 0.25 μm film thickness. Helium was used as carrier gas with 2 mL min^{-1} constant flow compensation. The injection temperature was at 250 °C, and the detector temperature was at 350 °C. The oven temperature was set at

120 °C for 5 min, increased at the rate of 7 °C min^{-1} to 260 °C for 10 min, and 7 °C min^{-1} to 350 °C for 15 min.

2.3.5. Time-resolved fluoroimmunoassay (TR-FIA)

TR-FIA (DEL-FIA®, PerkinElmer, Waltham, MA) was used to assay the samples (Yamada *et al.*, 1997). The antigen, testosterone 3-(*O*-carboxymethyl)oxime: BSA (Sigma-Aldrich Japan, Tokyo, Japan) was coated in microplates (Greiner Bio-One Co., Ltd., Tokyo, Japan). The antibody to testosterone, testosterone 3-(*O*-carboxymethyl)oxime-BSA IgG was obtained from Sigma-Andrich Japan (Tokyo, Japan). The fluorescence of europium, Eu-N1 Goat anti-Rabbit IgG (PerkinElmer Japan Co., Ltd., Kangawa, Japan), was measured in the Wallac 1420 ARVOsx (PerkinElmer Japan Co., Ltd., Kangawa, Japan). The software WorkOut 2.5 (Dazdaq Ltd., UK) was used to calculate the concentrations. Manufacturer cross-reactivity data are as follows: testosterone (100%), dihydrotestosterone (7.0%), androstedione (2.0%), androsterone (0.2%), 3β – androstanediol (0.15%), 3α – androstanediol (0.1%), 5β – androstane – 3α, 17 β – diol (0.09%), cortisol (0.02%) and corticosterone (0.01%). Intra-assay and inter-assay variabilities of control samples were 11.9 % (n = 5) and 20.2 % (n = 4), respectively. Serial dilutions of serum (1:2, 1:4, 1:8, 1:16) and faecal samples (1:3, 1:6, 1:12, 1:24) were performed to obtain parallelism of the assay. Serum and faecal samples were spiked with known concentrations of standard solutions to determine the extraction efficiency. Known concentrations of standard solutions were added to these samples and extracted as previously described.

2.3.6. Statistical analysis

Parallelism of serum and faecal samples with standard solutions were compared using an ANCOVA test. When the interactions between binding and standards, and between binding and samples either serum or faeces were insignificant, the parallelism of the sample and standard solutions was verified. The testosterone metabolites per unit AFDW of faeces were computed by dividing testosterone metabolites by AFDW of faeces. Faecal testosterone metabolites per unit DW and AFDW including the samples collected during the prey experiments, was fitted with serum testosterone levels using a GLM. The models were fitted using gamma error distributions with a log link function. Significance of the models were tested with a null model using analysis of deviance (Bradshaw *et al.*, 2004). The testosterone metabolites per unit dry weight from faecal samples taken the following morning (Day 1), two days (Day 2), and three days (Day 3) after the blood withdrawal (Day 0) were compared with the serum testosterone. A one-sample t-test was conducted to determine if the AFDW percentages of faeces when the seal was fed teleost species was significantly different from mean percentage of AFDW of faeces when the seal was fed squid. The statistically significant level was set to $\alpha = 0.05$. The software used for analysis was R 3.5.1 (R Core Team, 2018).

2.4. Results

2.4.1. Organic contents of faeces

Prey experiment demonstrated that the organic weight percentages of faeces during teleost feeding were significantly less than the mean percentages of those during squid feeding (t (11) = –17.361, $p < 0.001$). The faeces during the squid feeding were only found on Day 1 in October and the Day 3 in January as faeces were absent in the pool, possibly because the seal did not excrete or faeces were drained. The faeces of lower and higher AFDW percentage when squid was fed was collected on Day 1 and Day 3, respectively. Similarly, those during greenling feeding were only collected on Day 1 and Day 3. We collected faecal samples from pollock feeding all three days. The difference in AFDW percentage of faeces between the teleost and cephalopod diets was large (Figure 2.1).

2.4.2. GC-FID and parallelism

The retention time of the testosterone standard was 27.30 min while the sample with testosterone spike also showed at 27.25 min with GC-FID (Figure 2.2). The results of the parallelism between the serially diluted serum of the fur seal and testosterone standards were similar ($F = 0.029$, $p = 0.872$, Figure 2.3a). In addition, the results of the parallelism between the serially diluted faecal steroid extracts of the fur seal and the testosterone standards were similar ($F = 0.297$, $p = 0.614$, Figure 2.3b). Extraction efficiencies of serum ($n = 5$) and faecal metabolites ($n = 4$) were

104.6 ± 7.4% and 106.8 ± 8.5%, respectively. These results can be used as a validation of assays. Furthermore,

2.4.3. Time lag between serum testosterone and faecal testosterone metabolite levels

The serum testosterone levels of Day 1, a time lag of between 6 and 22 h after blood withdrawal, were significant predictors of faecal testosterone metabolites per unit DW ($p = 0.0048$, Figure 2.4). The equation of the relationship is following:

$$\mathit{ln\ (Faecal\ T\ metabolites\ in\ DW)} = -0.7623 + 0.414 \times \mathit{ln\ (Serum\ T\ levels)}$$

An analysis of deviance showed strong support for the correlation between serum testosterone and faecal testosterone metabolites in DW ($Deviance = 1.15$, $F_{1,13} = 10.02$, $p = 0.0074$, Figure 2.4). The serum testosterone levels of Days 2 and 3 were insignificant predictors of faecal testosterone metabolites expressed per unit DW ($Estimate = 0.1733$, $SE = 0.1880$, $p = 0.3749$ for Day 2, $Estimate = 0.4153$, $SE = 0.2187$, $p = 0.0841$ for Day 3, Figure 2.4).

Similarly, the serum testosterone levels of Day 1 were also significant predictors of faecal testosterone metabolites per unit AFDW ($p = 0.0087$, Figure 2.4). The equation of the relationship is following:

$$\mathit{ln\ (Faecal\ T\ metabolites\ in\ AFDW)} = 0.102 + 0.385 \times \mathit{ln\ (Serum\ T\ levels)}$$

An analysis of deviance showed also strong support for the correlation between serum testosterone and faecal testosterone metabolites expressed in AFDW (*Deviance* = 0.98, $F_{1,13}$ = 8.20, p = 0.0133, Figure 2.4). The serum testosterone levels of Days 2 and 3 were insignificant predictors of faecal testosterone metabolites expressed per unit AFDW (*Estimate* = 0.1729, *SE* = 0.1878, p = 0.3754 for Day 2, *Estimate* = 0.5075, *SE* = 0.2314, p = 0.0507 for Day 3, Figure 2.4).

2.4.4. Seasonal changes in faecal testosterone metabolites

Seasonal changes in serum testosterone levels and faecal testosterone metabolites of a captive fur seal are examined when faeces were collected on Day 1 (Figure 2.5). The serum testosterone levels in this seal during May–August ranged from 1.18–6.31 ng $\mathrm{mL^{-1}}$. Although the greatest testosterone level measured in our study (6.31 ng $\mathrm{mL^{-1}}$) occurred in May, relatively high testosterone levels were observed throughout the breeding months of June–August.

Faecal testosterone metabolite levels expressed in DW in the fur seal in this study ranged from 0.02–1.63 μg $\mathrm{g^{-1}}$ whereas those in AFDW ranged from 0.05–3.68 μg $\mathrm{g^{-1}}$. Higher peaks in testosterone metabolite levels (0.21–1.63 μg $\mathrm{g^{-1}}$ for DW and 0.48–3.68 μg $\mathrm{g^{-1}}$ for AFDW) occurred around the breeding season of June–August.

Faecal testosterone expressed in AFDW and DW showed peak in July. The differences in faecal metabolite levels are greater in AFDW than DW.

2.5. Discussion

Prey experiment demonstrated that the organic weight percentages of faeces during teleost feeding were significantly less than the mean percentages of those during squid feeding. The faeces during the squid feeding were only found on Day 1 in October and the Day 3 in January as faeces were absent in the pool, possibly due to no excretion or faeces drained. Similarly, those during greenling feeding were only collected on Day 1 and Day 3. We collected faecal samples from pollock feeding all three days. One of the reasons for the greater organic content of the faeces is that the initial organic matter in squid is greater than that in fish. The mean ash weight percentage of mackerel is 12% (Steimle and Terranova, 1985; Van Pelt *et al.*, 1997), leaving 88% organic content. The organic percentages of greenling and pollock are also approximately 83% (Van Pelt *et al.*, 1997). On the other hand, the organic content of squid such as Gonatidae and *Illex sp.* are about 92% (Steimle and Terranova, 1985; Van Pelt *et al.*, 1997). Furthermore, squid are digested more quickly than fish in northern fur seals (Bigg and Fawcett, 1985). The organic content in faeces when the seal was fed squid was thus greater. In addition to the organic content of the prey species itself, switching prey species resulted in changes in the bacterial composition of the faeces. Since the amount and kind of faecal microbiota in animals are influenced by prey choice (Banks *et al.*, 2014; Ley *et al.*, 2008; Nelson *et al.*, 2013a), social behaviour (Nelson *et al.*, 2013b), and a wild or captive environment (Delport *et al.*, 2016; Nelson *et al.*, 2013b), this seal's microbiota may have changed, resulting in the different organic percentages between teleost and cephalopod. The large differences in the faecal composition within the squid diet probably came from the changes in the composition of microbiota due to prey changes from teleost to cephalopod.

The testosterone extracted from serum of northern fur seal was validated by a retention time of the GC-FID analysis. The testosterone metabolites and serum testosterone were validated also using parallelism tests. The species-specific time lag of serum and faecal steroid metabolites is observed. The gut passage time of female northern fur seal is around 6–22 h (Bigg and Fawcett, 1985), which is the same as the time between serum testosterone levels and faecal testosterone metabolites levels obtained from a male in this study. Although the gut passage time was determined in only female fur seals (Bigg and Fawcett, 1985), a comparable study of Steller sea lions (*Eumetopias jubatus*) injected with adrenocorticotropic hormones showed the peaks in serum and faecal corticosterone metabolites after 1 h and 32 h of its administration, respectively (Mashburn and Atkinson, 2004). Cheetah (*Acinonyx jubatus*) injected with testosterone reached peak in faeces after one day (Pribbenow *et al.*, 2016). Moreover, oestradiol, progesterone, testosterone, and cortisol that were injected in mammalian species appeared in faeces about 12–48 h after their administration (Graham and Brown, 1997; Schwarzenberger *et al.*, 1996). These studies agree with our finding that serum testosterone and faecal testosterone metabolites of the northern fur seal were most highly related at about a 1-day time lag. Faecal testosterone metabolites expressed in both DW and AFDW on Day 1 showed the significant relationship with serum testosterone levels. No relationship between serum testosterone and faecal metabolites per unit DW nor AFDW on both Days 2 and 3 indicate that Days 2 and 3 are unlikely to represent the same conditions as when the blood was drawn. The serum testosterone levels of northern fur seals thus are correlated to levels found in faeces deposited approximately 6–22 h after blood withdrawal.

Both serum testosterone levels and faecal testosterone metabolites are greater around the breeding season during June–August than the non-breeding season. In other marine mammal species that are seasonal breeders, faecal testosterone metabolites tend to be highest in males during the breeding season. For instance, two captive Australian fur seals (*Arctocephalus pusillus doriferus*) showed an increase in testosterone levels around the breeding season (Tripovich *et al.*, 2009). Similarly, free-ranging dugongs (*Dugong dugon*) showed a seasonal increase in faecal testosterone metabolites (Burgess *et al.*, 2012). These results coincide with the seasonal increase in testosterone metabolites levels we found in the northern fur seal. The testosterone metabolites per unit DW and AFDW showed the same tendency. This is because the initial faecal weight is constant and the organic contents of faeces when the seal is fed teleost species are the same. As a result, the qualitative explanation can be the same (Ganswindt *et al.*, 2012). Since the representation of faecal testosterone metabolites levels in DW and AFDW did not differ, it can be simplified by expressing in DW. Since this study was performed on a single male, further testing of this relationship in other individuals could provide more evidence supporting the use of this methodology as a replacement for blood sampling.

In conclusion, the present study has demonstrated the following results: firstly, a protocol for the measurement of faecal testosterone metabolites in the male northern fur seal was established. Secondly, testosterone metabolites in the faeces of a captive northern fur seal were found to reflect the serum testosterone levels if faeces were collected after a 1-day time lag from the time of blood sampling. Lastly, the faecal testosterone metabolite levels, as well as serum testosterone levels, were greater during the breeding season. Although further testing on prey experiment is required, faecal steroid metabolites, therefore, will be useful as a non-invasive method of studying the reproductive cycles of male northern fur seals.

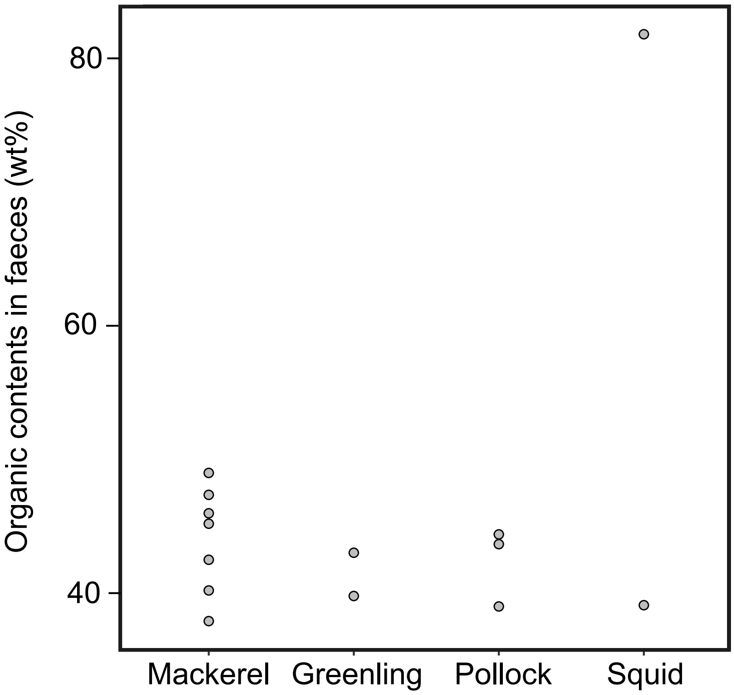

Figure 2.1. Percentages of organic contents in faeces of a captive northern fur seal when the seal was fed four species of prey. Greenling = Arabesque greenling, Mackerel = Pacific mackerel, Pollock = Walleye pollock, and squid = Japanese common squid.

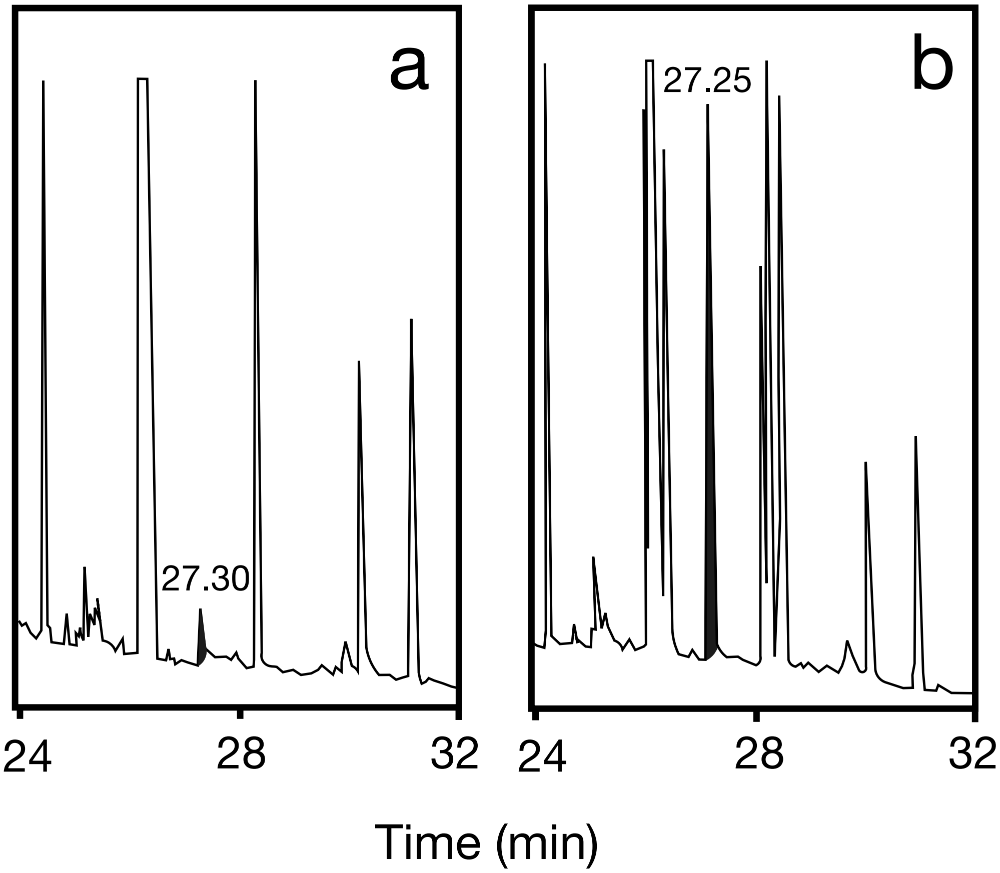

Figure 2.2. Chromatograms of (a) serum sample and (b) serum with four standard solutions (androsterone, dihydrotestosterone, Δ4-androsterone, testosterone). The retention time of testosterone is indicated in the figures.

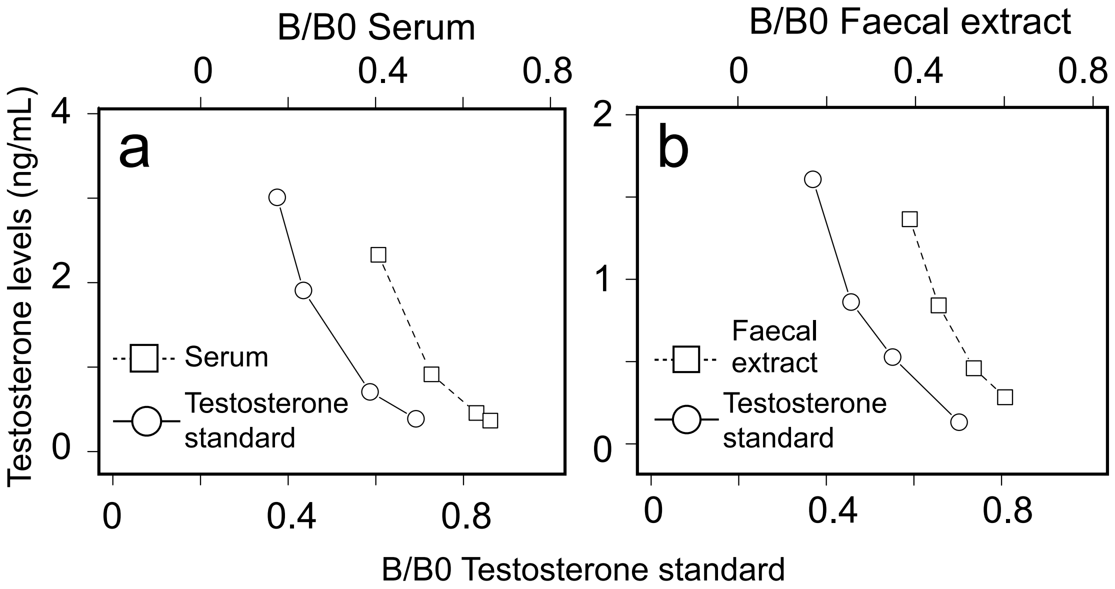

Figure 2.3. Parallelism for testosterone standards tested with serially diluted (a) serum and (b) faecal extracts. B/B0 shows the ratio of bound.

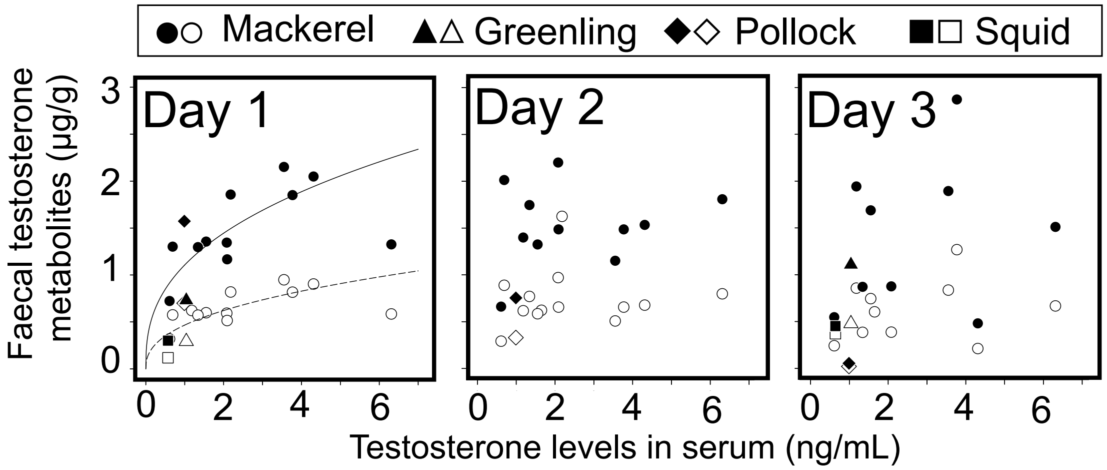

Figure 2.4. Testosterone levels in faeces expressed in dry weight and serum of a male northern fur seal. The lines on Day 1 is a fitted line from generalized linear models. The broken line indicates the fit of open symbols while the solid line shows the fit of closed symbols. Faeces were collected on Days 1, 2, and 3 after the blood withdrawal on Day 0. Open symbols indicate the faecal metabolites expressed in DW while closed symbols indicate those expressed in AFDW.

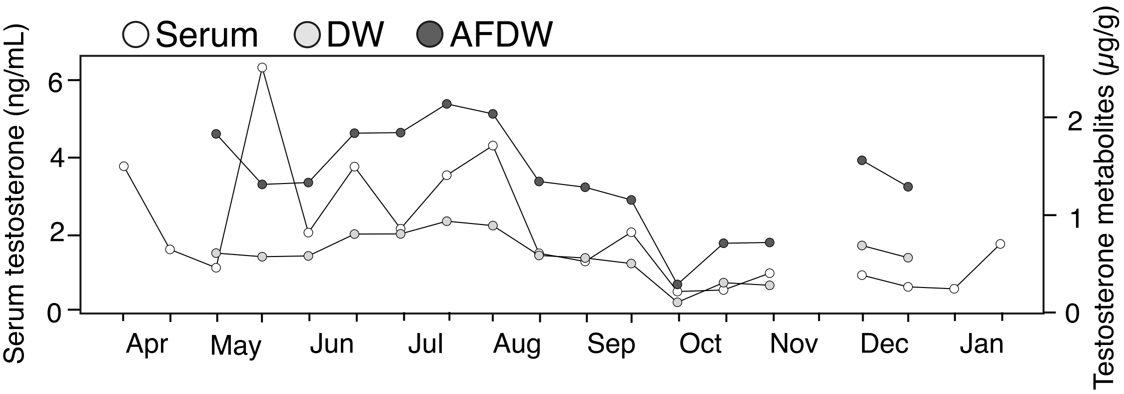

Figure 2.5. Seasonal changes in serum testosterone and faecal testosterone metabolite levels of a captive northern fur seal. Faecal testosterone metabolites are expressed in either DW or AFDW.

Chapter Three: Faecal testosterone metabolites of free-ranging northern fur seals during the non-breeding season

3.1. Abstract

Faecal steroid hormones have been used to monitor reproductive status of captive animals. Although faeces are composed of bacteria, undigested organic matters and inorganic elements, faecal steroid hormones are often represented as per unit DW. Since free-ranging animals feed on variable diets, it is controversial whether expressing faecal steroid hormones in per unit DW or weight of AFDW provides a more precise circulation of endocrine systems. Free-ranging northern fur seals feed on a variety of prey species that the faecal composition of the seals can differ among individuals. Here, we compare the sexual maturity of 45 free-ranging male northern fur seals using faecal testosterone metabolites expressed in per unit DW and AFDW. The faeces of the seals were combusted to determine the amount of organic contents in faeces. TR–FIA was used to measure the faecal testosterone metabolites of the seals. The testosterone metabolites in per unit DW and AFDW were compared with testis weight, age class, and month. Our results showed that the variances of differences in faecal testosterone metabolite levels between DW and AFDW become greater as the organic contents of faeces become lower, suggesting that conversion is preferable. However, the faecal testosterone metabolites in DW and AFDW show similar results, indicating the conversion is unnecessary. Furthermore, the individual differences in the faecal testosterone

metabolite levels in both DW and AFDW of adult seals were large, possibly because environmental cues such as prey availability and energetic demands can differ among individuals and differently affect endocrine systems.

3.2. Introduction

Faecal steroid metabolites are used as a non-invasive alternative to serum steroid hormones. It is a practical marker to monitor the reproductive status of free-ranging marine mammals as it is highly invasive and impractical to perform blood sampling in the field (Amaral, 2010). This method, thus, applies to both captive and free-ranging animals (*e.g.,* Mashburn and Atkinson, 2004; Schwarzenberger *et al.,* 1996).

Various factors such as metabolic rates and diet can affect the faecal steroid metabolite levels (Goymann, 2012). Steroid hormones are metabolized in a liver and gut, and excreted in faeces (Möstl and Palme, 2002). The faecal steroid metabolites are often expressed in per unit DW of faeces (Palme *et al.,* 2013). Dried faeces include both organic and inorganic matter as the faecal composition depends on diet (Klasing, 2005; Rose *et al.,* 2015). Because of differences in diet, the faecal steroid metabolites expressed in AFDW likely reduce the discrepancy. It has been a controversy whether DW or AFDW is a more precise representation of faecal steroid metabolite levels of circulating hormones. For example, Wasser *et al.* (1993) demonstrated that faecal steroid metabolites in per unit DW are sufficient to monitor longitudinal data. On the other hand, Ganswindt *et al.* (2012) argued that expressing faecal steroid metabolites in AFDW could represent the better circulating hormone levels. As a result, it is important to determine whether the conversion using organic content is necessary to present faecal steroid hormones.

The sexual maturity of males has been investigated using testis weight and faecal testosterone metabolites in various species. Faecal testosterone metabolite levels of sheep increase toward the breeding season (Pelletier *et al.,* 2003). Adult males produce greater concentrations of faecal testosterone metabolites than juvenile males (Burgess *et al.*, 2013; Graham and Brown, 1997; Rolland *et al.,* 2005). Faecal testosterone metabolites, therefore, can be used to distinguish the sexual maturity and reproductive status of mammals. Furthermore, although adult males secrete greater levels of testosterone, the range of serum testosterone levels are highly variable among them (Kita *et al.,* 1999). The variances in faecal testosterone metabolites in adults are also greater than those in juveniles (Burgess *et al.,* 2013; Pribbenow *et al.,* 2016; Rolland *et al.,* 2005). Once animals mature, individual variations and environmental factors surrounding the animals (Goymann, 2012) matter as testis weight plateaus as they age (Diamond *et al.,* 1975; Grocock, 1979; Lincoln, 1998).

Although faecal steroid analyses have been conducted in various species, the colour of dried faeces of northern fur seals was variable among individuals (Figure A.1). This indicates the possible differences in the composition of their faeces among them, which was not found when mackerel was exclusively fed to the captive northern fur seal in Chapter 2. Free-ranging northern fur seals feed on wide varieties of teleost and cephalopod species (Kajimura, 1984) since they depend on the changes in abundance of prey due to oceanographic regimes (Kiyota and Yonezaki, 2017). The amount of inorganic contents in faeces, therefore, possibly influence the representation of overall hormonal levels. Here, we have examined the maturities of male northern fur seals during the non-breeding season using faecal testosterone metabolites. We predicted that the testosterone metabolite levels expressing in AFDW would have more statistical power than those representing in DW. If the conversion to AFDW applies to faecal testosterone metabolites in the northern fur seal, we predicted the maturity of male northern fur seals would be classified using faecal testosterone metabolites.

3.3. Materials and Methods

3.3.1. Study animals

Forty-five bycaught and lethally taken male northern fur seals were collected from 2011 to 2017 around Hokkaido, Japan (Figure 3.1; Table 3.1). Lethal takes of northern fur seals are operated off southern Hokkaido in the Sea of Japan. The age of the seals was determined by the number of growth layers in tooth sections (Horimoto *et al.*, 2017). The age of the seals those without teeth were determined from the body length based on Trites and Bigg (1996). The body length from the tip of nose to tail of the animals was measured. The age classes of the seals were assigned as juvenile (< 4 years old), mature (4 ≤ age < 7 years old), and socially mature (≥ 8 years old) seals based on Gentry (1998) and Horimoto *et al.* (2017).

Right testes of the seals were preserved with 10% formalin and weighted (Hirakawa *et al., in prep*). The testis data are based on the results of Hirakawa *et al.* (*in prep*).

3.3.2. Sample preparation

Large intestines of the northern fur seals were kept frozen at –20 °C until sample preparation. These intestines were longitudinally cut and the contents (*i.e.,* faeces) were dried at 60 °C (DKM600, Yamato Scientific Co., Ltd., Japan) until the weight of the faeces remain unchanged. The dried faeces were pulverized with a mortar and pestle. The dried faeces were kept frozen at –20 °C until analysis.

3.3.3. Faecal composition using a muffle furnace and elemental analysis

The mass of inorganic contents (*i.e.,* ash) contained in the dried faeces were measured with a muffle furnace and a CHN analyzer. The sampling preparation for the two analyses is the same as in Chapter 2 (Otsuki *et al., under review*). In addition, dried faeces (2 mg) of the same five animals were combusted using a CHN analyzer at 900 °C to measure the residue of the faeces (Micro Corder JM10, J Science Lab Co., Ltd. Japan) and compare with the results from the muffle furnace. The ash of faeces was weighted. The AFDW of dried faeces were calculated by subtracting of ash weight from the total weight of dried faeces. Since a significant correlation of weight percentages of organic contents in faeces analyzed between the muffle furnace and CHN analyzer and was obtained ($r = 0.997$, $n = 5$, $p < 0.0001$, Figure 3.2), we used the elemental analyzer to determine the AFDW of the rest of the faecal samples.

3.3.4. Sample extraction and assay for testosterone metabolites

Testosterone metabolites were extracted from 10.0 ± 0.2 mg of dried faeces using 1-mL 80% methanol. This extraction method and TR-FIA were performed as in Otsuki *et al.* (*under review*). Intra-assay and inter-assay variabilities of control samples were 8.71% ($n = 4$) and 16.99 % ($n = 4$), respectively. The parallelism of the assay and the extraction efficiency of the faecal samples were obtained in Otsuki *et al.* (*under review*).

3.3.5. Analysis of faeces by GC-FID and gas chromatography mass spectrometry (GC-MS)

We used GC-FID and GC-MS to qualitatively analyse the presence of testosterone metabolites. The advantage of GC-FID over GC-MS is sensitivity though GC-MS libraries can identify the presence of particular substances. First, we used GC-FID to determine the presence of testosterone metabolites were present in faecal samples. Dried faeces (100 mg) were extracted as previously described. The extracts were dried with N_2 and reconstituted with LC-MS grade methanol. Reconstituted samples (1.5 μL) were analyzed by GC-FID (GC 390B, GL Sciences, Japan) using a 30-m long column, Ultra ALLOY-5 (MS/HT) with an internal diameter 0.25 mm and 0.25 μm film thickness. Helium was used as carrier gas with 2 mL min^{-1} constant flow compensation. The injection temperature was at 250 °C, and the detector temperature was at 350 °C. The oven temperature was set at 120 °C for 5 min, increased at the rate of 7 °C min^{-1} to 260 °C for 10 min, and 7 °C min^{-1} to 350 °C for 15 min. Next, we used GC-MS to identify what testosterone metabolites were present in faecal samples. Faecal samples (100 mg) was extracted as previously mentioned. Samples were derivatized with regents, MSTFA and TMSI-H (hexamethyldisilazane: trimethylchlorosilane: pyridine = 2:1:10), both of which were purchased from GL Science (Tokyo, Japan). The samples with the regents (MSTFA: TMSI-H = 50:1, *v*/*v*) were vortexed for 6 min and heated at 60°C for 30 min. 0.1μL of the derivatized mixture were analyzed by GC-MS (6890, Agilent, USA) using a 30-m long column, DB-5ms with an internal diameter 0.25 mm and 0.25 μm film thickness. Helium was again used as carrier gas with 2 mL min^{-1} constant flow compensation. The injection and detector temperatures and the rates and temperature of the oven were same as used in GC-FID.

3.3.6. Statistical analysis

Two-sample t-test was conducted to compare the means of differences in the testosterone metabolites between DW and AFDW. The two ranges of organic weight percentages (less than 60% and 60% or above) were created based on the mean of faecal organic weight percentages when a seal fed squid (Otsuki *et al.*, *under review*). A two-way ANOVA was performed to determine the effect of the months between April and June and three age classes on the testosterone metabolite levels of 42 seals after normality of data distribution was tested using a Shapiro–Wilk test. The ANOVA was conducted for testosterone metabolites expressed in DW and AFDW. Multiple comparisons using Holm were also performed to determine the monthly differences in faecal testosterone metabolites by age class when the interaction of the ANOVA was significant. In addition, multiple comparisons of the monthly changes in testis weight and age class were performed using a Tukey's HSD test. Simple linear regression models were performed to determine the effects of monthly testis weight on the faecal testosterone metabolite levels at age class. Since small samples were obtained in March and November, monthly analyses with testosterone metabolite levels were conducted for the seals collected between April and June. The statistically significant level was set to $\alpha = 0.05$. The software used for analysis was R 3.5.1 (R Core Team, 2018).

3.4. Results

3.4.1. Detection of testosterone metabolites with GC-FID and GC-MS

The retention time of the testosterone standard was 26.09 min while the sample with testosterone spike also showed at 26.15 min with GC-FID (Figure 3.3).

The analysis with GC-MS showed that the metabolites were a base skeletal structure of testosterone (Androst-4-en-3-one, 17[(trimethylsilyl)oxy]-,(17a)-) and androsterone (Androstan-17-one, 3[(trimethylsilyl)oxy]-,(3b,5b)).

3.4.2. Faecal testosterone metabolite levels expressed in DW and AFDW

The differences in faecal testosterone metabolites expressed as per unit DW and AFDW were determined. A significant difference in mean of faecal testosterone metabolite levels per unit DW and AFDW was obtained between above and below 60 % of faecal organic weight (Two sample t-test, $t = 6.1829$, $p < 0.0001$, Figure 3.4).

3.4.3. Faecal testosterone metabolites between month and age class

A two-way ANOVA of the faecal testosterone metabolites expressed in per unit DW and the effect of month and age classes did not show any significance (F (2, 35) = 4.429, $p = 0.184$, Figure 3.5a). As for the AFDW, a significant interaction between the effects of month and age class on faecal testosterone metabolites per unit AFDW was obtained (F (2, 35) = 5.341, $p = 0.0461$, Figure 3.5b). Multiple comparisons of Holm among these testosterone metabolites of age class in April showed that the metabolites of socially mature seals were significantly greater than those of mature seals ($p = 0.035$, Figure 3.5b).

3.4.4. Testis weight and age

The multiple comparisons of testis weight at age class was shown in Figure 3.6. The testis weight of mature seals in April was significantly greater than that of juveniles in May ($p = 0.0026$), and was significantly lower than that of socially mature seals in May ($p = 0.0114$) and June ($p = 0.0009$). The testis weight of socially mature seals in April was significantly greater than that in juvenile seals in May ($p < 0.0001$). The testis weight of juvenile seals in May were significantly greater than that of mature seals in May ($p < 0.0001$) and socially mature males in May ($p < 0.0001$) and June ($p < 0.0001$). The testis weight of mature seals in May was significantly greater than that of socially mature seals in May ($p = 0.0162$) and June ($p = 0.0005$).

3.4.5. Faecal testosterone metabolites and testis weight

Simple linear regression models were carried out to investigate the relationships between testis weight and faecal testosterone metabolite levels. The testosterone metabolite levels expressed in neither DW nor AFDW in April for socially mature animals were significant (DW: F (1,1) = 17.16, $p = 0.1508$, $R^2 = 0.9449$, Figure 3.7a; AFDW: F (1,1) = 19.15, $p = 0.143$, $R^2 = 0.9504$, Figure 3.7d). The testosterone metabolites expressed in DW in May for juveniles was significant (F (1,1) = 18860, $p = 0.0046$, $R^2 = 0.9999$, Figure 3.7b) whereas those in AFDW was insignificant (F (1,1) = 26.85, $p = 0.1214$, $R^2 = 0.9641$, Figure 3.7e). For mature animals in May, the metabolite levels expressed in neither DW nor AFDW were significant

(DW: F (1,5) = 0.0916, p = 0.7744, R^2 = 0.0180, Figure 3.7b; AFDW: F (1,5) = 1.438, p = 0.2842, R^2 = 0.2233, Figure 3.7e). Even though faecal testosterone metabolites expressed in neither DW nor AFDW of socially mature seals in May were significant (DW: F(1,8) = 2.369, p = 0.1623, R^2 = 0.2285, Figure 3.7b; AFDW: (F(1,8) = 2.319, p = 0.1663, R^2 = 0.2247, Figure 3.7e), testosterone levels increased as the testis weight became large. Lastly, as for the socially mature seals collected in June, those expressed in neither DW nor AFDW were significant (DW: F(1,4) = 2.258, p = 0.2074, R^2 = 0.3608, Figure 3.7c; AFDW: F(1,4) = 4.146, p = 0.1114, R^2 = 0.509, Figure 3.7f). Faecal testosterone metabolites of the seals in June decreased although the testis weight increased.

3.5. Discussion

Our GC-FID results showed that the retention time of the testosterone standard and the faecal samples with testosterone spike was nearly the same. Some peaks around testosterone in the control sample possibly indicate the presence of testosterone metabolites with a similar skeletal structure of testosterone. Little studies have been identified faecal steroid metabolites; however, the metabolites of testosterone in urine are such as androsterone, etiocholanolone, and epietiocholanolone (Krone *et al.,* 2010; Samuels *et al.,* 1998). Faecal testosterone metabolites of northern fur seals were composed with a main skeletal structure of androsterone and testosterone detected by GC-MS, indicating that TR-FIA likely measured these testosterone metabolites with the skeletal structures of testosterone and androsterone. Although almost all steroid hormones are metabolized before excretion, and no original hormones are present in faeces (Goymann, 2012), it might be possible that some of closely similar chemical skeletons to testosterone remain in faeces.

In non-invasive measurements of faecal hormonal analysis, diet is an important factor since free-ranging animals feed on a wide variety of diets (Goymann, 2012), resulting in altering the composition of faeces (Klasing, 2005; Rose *et al.,* 2015). In this study, the significantly lower mean of testosterone metabolite differences in DW and AFDW was observed when the faecal content is lower than 60%. This possibly suggests that the conversion of faecal metabolites is preferable. Since lower organic contents are found in teleost than cephalopod species (Van Pelt *et al.,* 1997), the small organic weight percentages of the seal faeces indicate that seals are possibly feeding on teleost prey. The stomach contents of seven fur seals in 2014 showed that seals with more than 85% of faecal organic contents fed only squid though those with less than 84% fed only fish or both fish and squid (Horimoto *per comm.*). Although these results are stomach contents not intestinal contents, the results suggest that diet might be the cause of different organic weight percentages among seals.

Although our hypothesis was expressing faecal testosterone metabolites in per unit AFDW likely reduce the differences in amount of inorganic contents in faeces, the faecal testosterone metabolite levels of northern fur seals in the present study show only a small significant difference between DW and AFDW, suggesting that presenting the faecal metabolite levels in DW will be sufficient. This agrees with previous studies in Wasser *et al.* (1993) and Goncalves *et al.* (2016). The only significant results of multiple comparisons of faecal testosterone metabolites expressed in AFDW was between mature and socially mature males in April. The AFDW conversion may not be statistically robust enough to see the significant differences when $\alpha = 0.05$.

Some of our results in the present study were in line with previous studies. The faecal testosterone metabolites of mature males increased from April to May. This result coincides with the increase in serum testosterone levels of a captive male northern fur seal from April to May (Kohyama *et al.,* 1999). The increase in faecal testosterone metabolites before the breeding season is also observed in other species such as lemurs (Ostner *et al.,* 2002). Another result that faecal testosterone metabolites of socially mature seals was greater than those of mature seals in April was also similar with other studies. For example, adult monkeys tend to have greater faecal testosterone metabolite levels than subadults (Teichroeb and Sicotte, 2008). Testosterone metabolite levels in cheetahs and right whales (*Eubalaena glacialis*) also were significantly higher in adults than juveniles (Pribbenow *et al.,* 2016; Rolland et al., 2005). The variances in the faecal testosterone metabolite levels in adults, however, were both greater than those in juveniles (Pribbenow *et al.,* 2016; Rolland *et al.,* 2005; Teichroeb and Sicotte, 2008), indicating individual differences should be considered. The previous research demonstrated that the faecal testosterone metabolites are an effective non-invasive technique for wild animals. However, faecal testosterone levels northern fur seals may not be used to differentiate the maturity of seals because of highly variable concentrations among individuals.

The male northern fur seals increased their testis weight as they get older (Hirakawa *et al., in prep*). Similar to the faecal testosterone metabolites, the variances of the testis weight become larger as they age. These results agree with the studies on other species such as rats (*Rattus norvegicus*) (Diamond *et al.,* 1975) and mouflon ram (*Ovis musimon*) (Lincoln, 1998), which demonstrated that the testis weight plateaus as they age. Since this tendency is the same in the testis weight of the northern fur seals, individual differences also matter once the animals mature. Although an increase in the testis weight toward the breeding season is demonstrated (Lincoln, 1998), no changes in the testis weight of northern fur seals between April and June were observed in mature and socially mature animals. The reason for this constant testis weight of these seals during April–June is that the testes of those seals were already large enough for the breeding season as the serum testosterone levels of intact northern fur seals peak in June (Kohyama *et al.,* 1999; Tsubota *et al.,* 2001). In addition, the seminiferous tubule diameter of a captive northern fur seal is larger in June than the non-breeding season (Tsubota *et al.,* 2001). This increase in seminiferous tubule diameters during the breeding season is also observed in elephant seals (Griffiths, 1984). Testis weight and sperm density were higher in the pre-breeding and breeding seasons (Griffiths, 1984; Müller *et al.,* 2014).

The relationship between faecal testosterone metabolites and testis weight is another evidence for individual differences of mature and socially mature animals. The comparison of faecal testosterone metabolite levels between DW and AFDW on testis weight showed that no significant differences were obtained for both, which further suggest that no evidence on the conversion of DW to AFDW. The testosterone metabolite levels of socially mature seals in May increased with the testis weight while those in June decreased. Since male northern fur seals mate only 1.5 seasons for their entire lives (Vladimirov, 1987), it is possible that some of the males are not participating breeding in the following months. Although the serum testosterone and faecal testosterone metabolite levels of a captive male northern fur seal are correlated (Otsuki *et al., under review*), it did not exactly apply for free-ranging seals. The reasons for this disagreement between captive and wild seals are possibly environmental factors. The hypothalamic-pituitary-gonadal axis is activated to produce testosterone by changes in such as prey availability (Geyer, 2017). Prey species are completely different between free-ranging and captive environment. Free-ranging northern fur seals migrate to rookeries in spring and summer, their metabolism likely higher than captive ones. These various factors thus have likely influenced the internal hormonal levels.

In conclusion, we show that the patterns in faecal testosterone metabolites in free-ranging northern fur seals during the pre-breeding season. We found no strong evidence to support the conversion of the AFDW when representing faecal testosterone metabolites in the northern fur seals. Expressing the faecal testosterone metabolites in DW would help to save time for sample preparation. The interpretation of faecal testosterone metabolite levels of free-ranging northern fur seals can be challenging as the individuality likely matters once the seals mature.

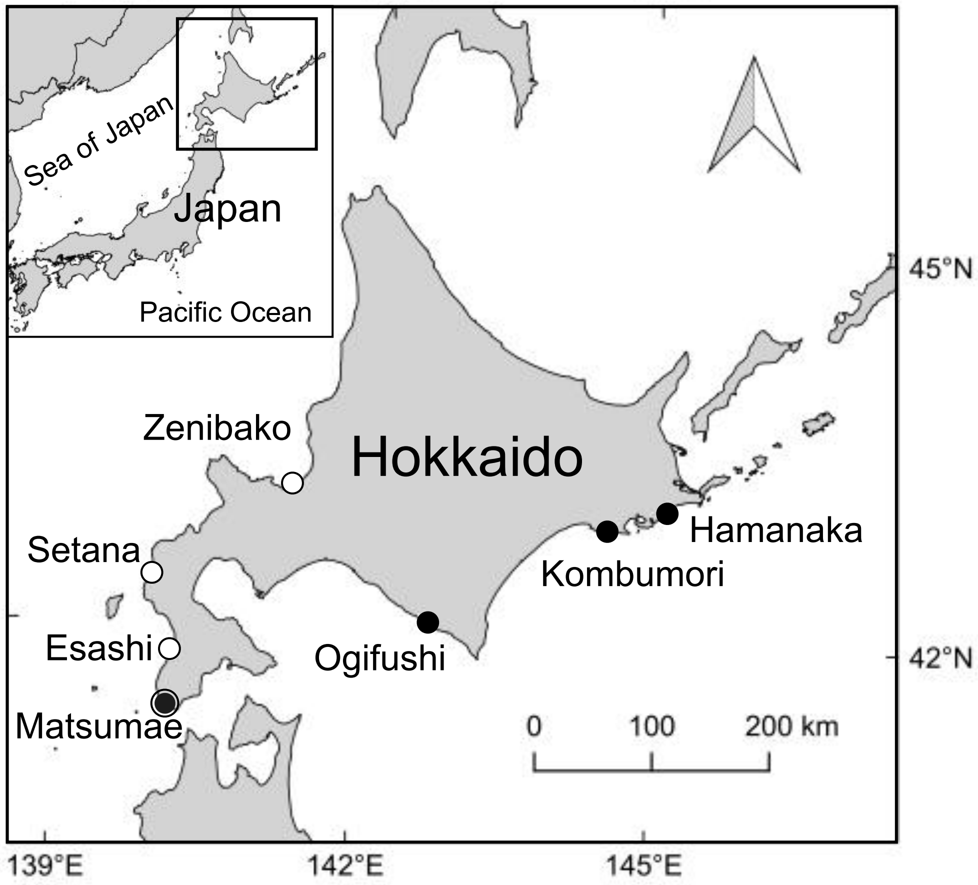

Figure 3.1. Locations of northern fur seals collected around Hokkaido, Japan. The fur seals analyzed in Chapter 3 were collected off Matsumae and at the locations showing the black circles. Those analyzed in Chapter 4 were collected in Matsumae and at the locations showing the white circles.

Table 3.1. The description of northern fur seals used in this study. Month represents the sampling month.

Month	Age	Location	Type	Number	Subtotal
March	7	Matsumae	Caught	1	
	9	Matsumae	Caught	1	2
April	4	Matsumae	Caught	2	
	5	Matsumae	Caught	1	
	6	Matsumae	Caught	1	
	9	Matsumae	Caught	1	
	10	Matsumae	Caught	1	
	13	Matsumae	Caught	1	7
May	1	Matsumae	Caught	1	
	2	Kombumori	Bycaught	1	
	2	Matsumae	Caught	2	
	4	Matsumae	Caught	4	
	5	Matsumae	Caught	1	
	6	Matsumae	Caught	3	
	7	Ogifushi	Bycaught	1	
	7	Matsumae	Caught	1	
	8	Matsumae	Caught	4	
	9	Matsumae	Caught	2	
	9	Matsumae	Bycaught	1	
	10	Matsumae	Caught	4	
	11	Matsumae	Caught	1	26
June	2	Kombumori	Bycaught	1	
	3	Kombumori	Bycaught	1	
	8	Kombumori	Bycaught	1	
	8	Matsumae	Caught	1	
	9	Matsumae	Caught	2	
	10	Matsumae	Caught	2	
	11	Matsumae	Caught	1	9
November	2	Hamanaka	Bycaught	1	1
		Total number of samples			45

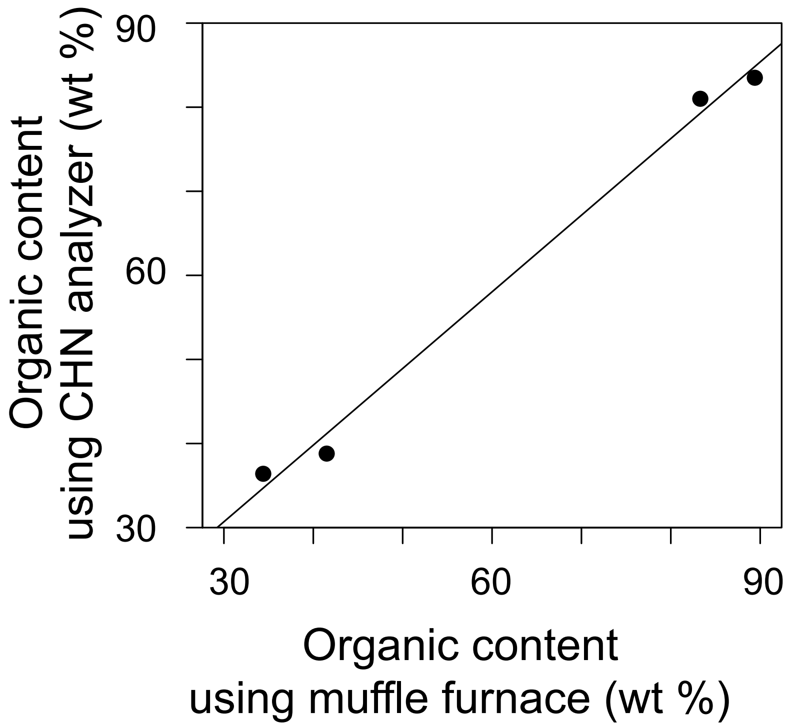

Figure 3.2. Correlation of organic weight percentages of five male northern fur seal faeces analyzed between a CHN analyzer and a muffle furnace.

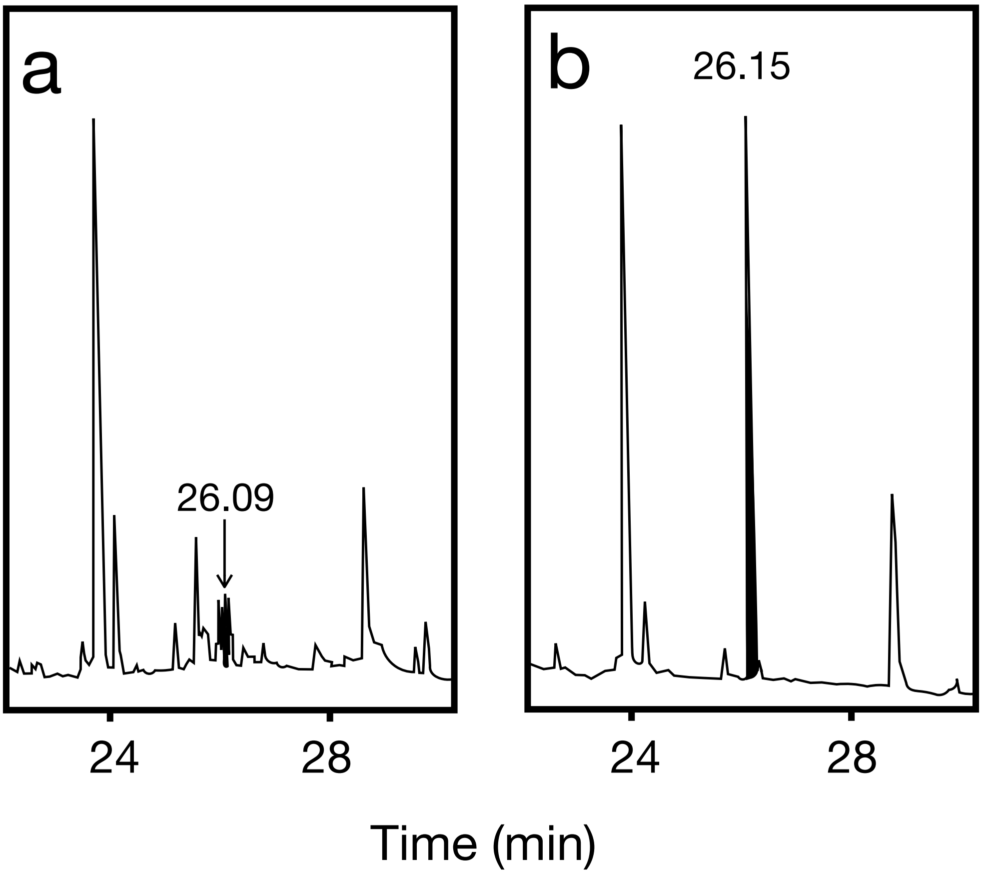

Figure 3.3. Chromatograms of (a) faecal samples and (b) faecal samples with testosterone. Retention time of testosterone is indicated in the figures.

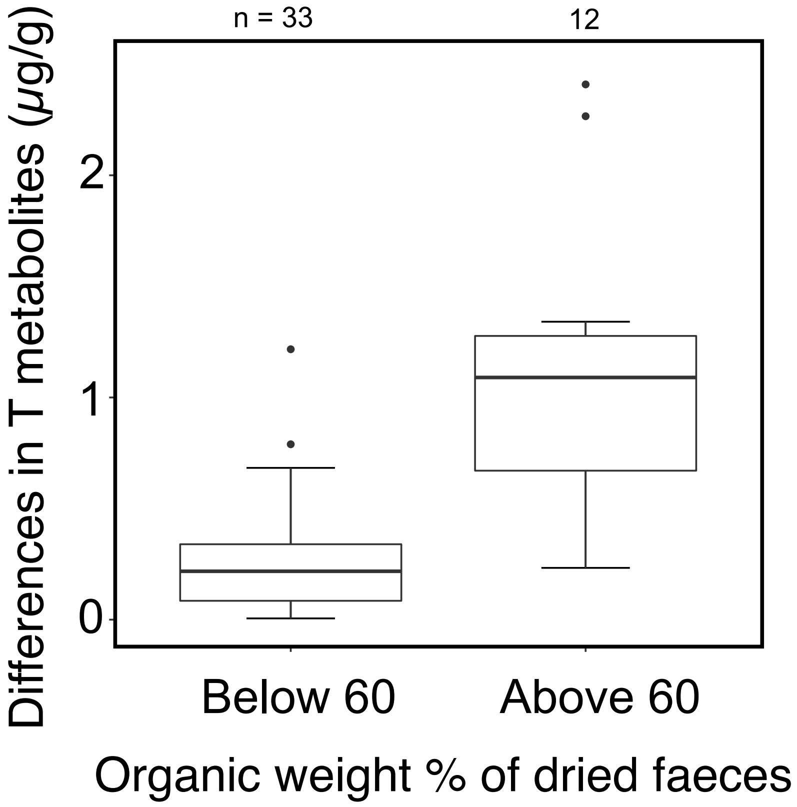

Figure 3.4. Differences in the faecal testosterone metabolite levels expressed as per unit AFDW and dry weight based on the organic weight percentages of dried faeces. The number on the top of the figure shows the sample size. The horizontal line through the box is the median. The lower and upper hinges represent to the first and third quartiles. The lower and upper whiskers correspond to 1.5 × inter quartile ranges from the lower and upper hinges, respectively. Data beyond the end of the whiskers are shown individually.

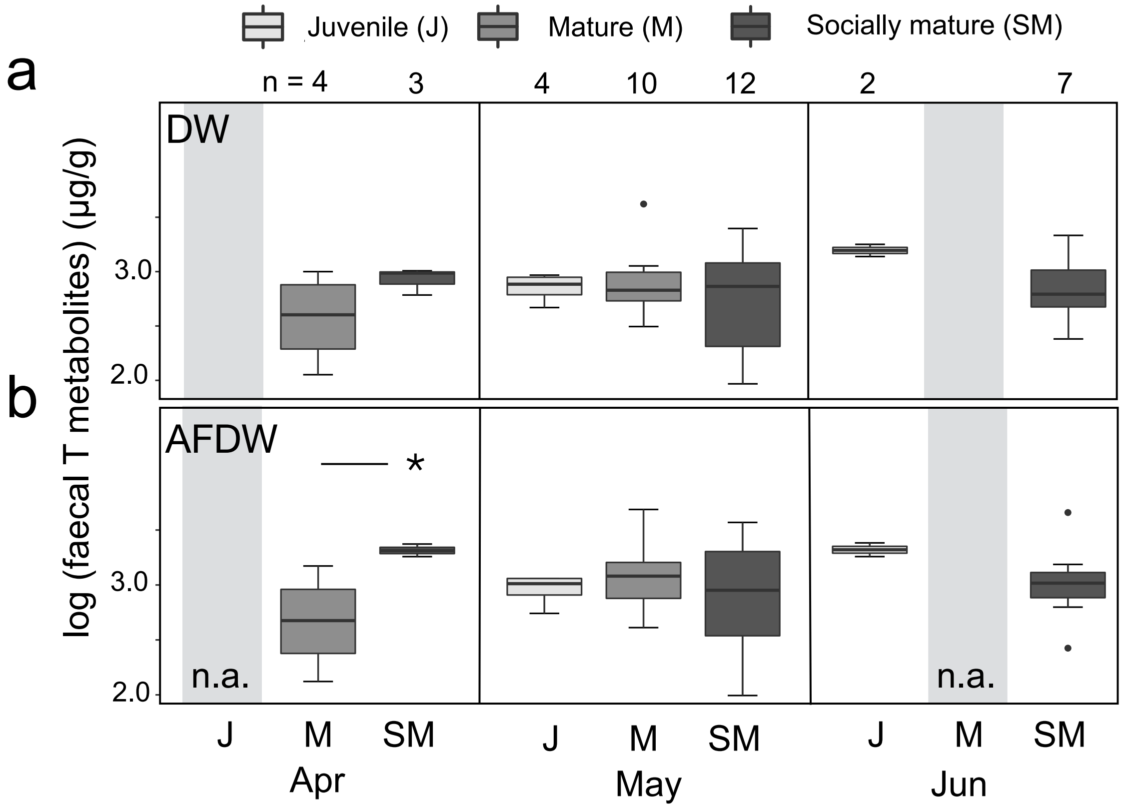

Figure 3.5. Monthly differences in faecal testosterone metabolites among three age classes of northern fur seals. (a) Upper three figures show the faecal testosterone metabolites expressed in DW while (b) the lower three show those in AFDW. The grey shaded areas have no data. The asterisk shows the significant of the testosterone levels ($p < 0.05$).

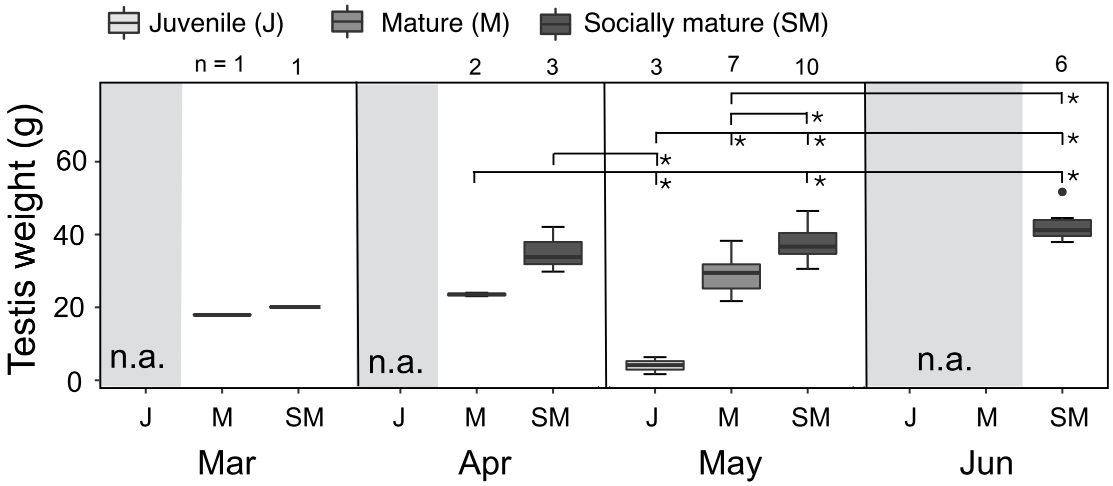

Figure 3.6. Monthly testis weight of northern fur seals among age class. The grey shaded areas show no samples. The asterisks show the significance of the testis weight ($p < 0.05$).

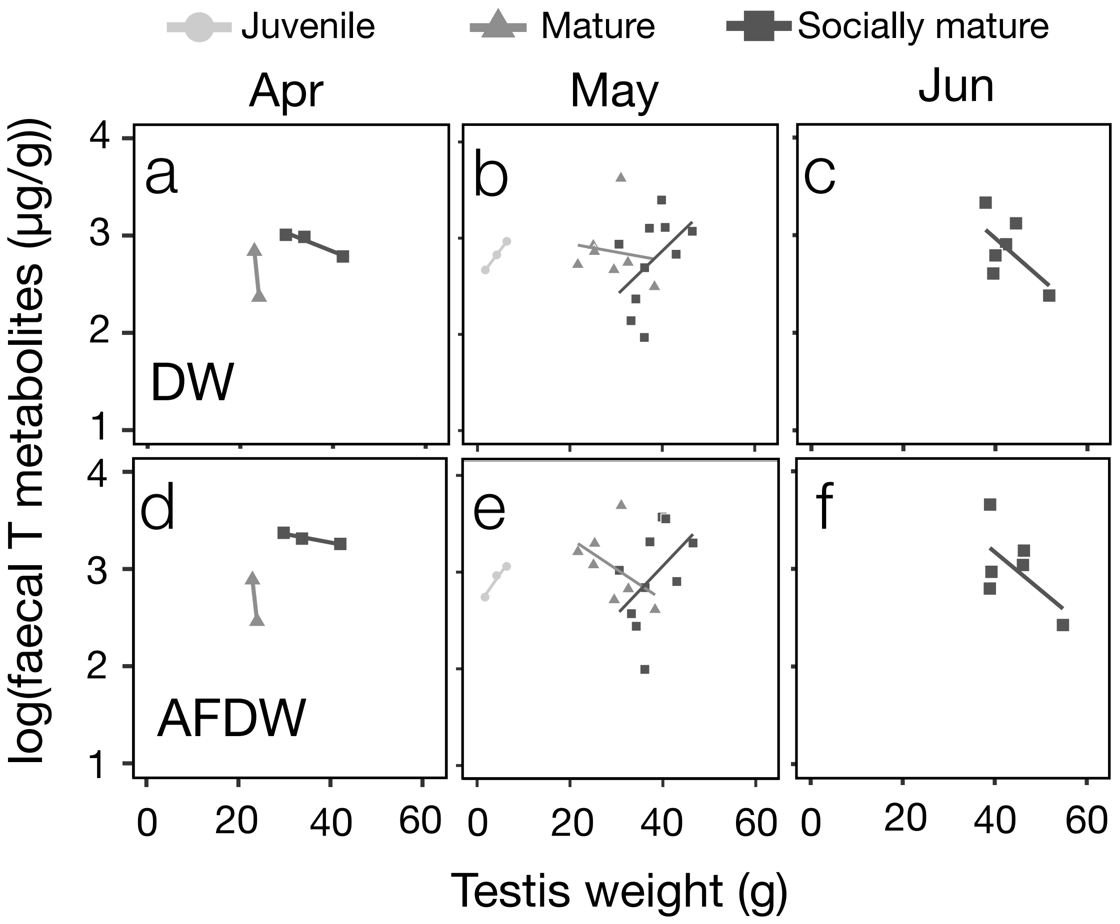

Figure 3.7. Monthly faecal testosterone levels and testis weight of northern fur seals categorized into three age classes. Upper three figures (a–c) show the faecal testosterone metabolites expressed in DW while the lower three (d–f) show those in AFDW.

Chapter Four: Hair testosterone levels of free-ranging northern fur seals correlate with month, age, and testicular morphometry

4.1. Abstract

Hair as an endocrine marker has been used to assess the reproductive status of animals. However, it is unknown whether pinniped reproductive status can be assessed from hair samples. Male northern fur seals are found off southern Hokkaido in the Sea of Japan during the non-breeding season in winter and spring. Knowledge about the reproductive status of the free-ranging seals provides important data to inform population dynamics analyses. Here, we show that sexual maturity of northern fur seal males during the non-breeding season off Hokkaido can be distinguished using hair testosterone levels. We analyzed for testosterone using GC-FID, and measured the testosterone levels of 57 male fur seals from hair collected during the non-breeding seasons using TR-FIA. We found that the testosterone levels of juvenile seals (< 4 years old) were significantly lower than those of mature seals (≥ 4 years old). Spermatids, the final phase of spermatogenesis, were present in the seals collected between April and June. The seals with spermatids in May showed the highest testosterone levels. Our results demonstrate that the juvenile males can be distinguished from mature males using hair testosterone levels in May, preceding the breeding season. Since hair can be collected using biopsy darts in the field, it will be possible to determine the demography of male northern fur seals in this community.

Data on male reproductive status, thus, enhance our understanding of the ecology such as distribution of the seals off Hokkaido during the non-breeding season.

4.2. Introduction

Hair as an endocrine marker has been used to examine reproductive hormone levels in various species of mammals including carnivores (Accorsi *et al.*, 2008), primates (Kintz *et al.*, 1999; Yamanashi *et al.*, 2013), and ungulates (Ashley *et al.*, 2011; Ventrella *et al.*, 2018). The advantage of hair sampling is that hair can remotely collected in the field using biopsy darts, which reduces handling stress for animals and improve the animal welfare of animals. For example, hair samples collected using remote biopsy darts in pinnipeds have been used in studies of toxicology (Fossi *et al.*, 1997) and DNA (Gemmell and Majluf, 1997). Similarly, hair testosterone can be used to assess the sexual maturity of male animals. Hair testosterone levels in captured and hunted wild bears were used to predict male age class between immature and mature by comparing with the age obtained from a premolar (Cattet *et al.*, 2018). Hair, therefore, is a valuable tool to assess the reproductive status and age class of mammals.

Although hair is a useful matrix to study the maturity of animals, the mechanism how steroid hormones are incorporated into hair is not fully understood. The commonly accepted hypothesis is that the steroid hormones are incorporated into hair via passive diffusion from blood (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012). The hair steroid hormones reflect the integrated free hormone fraction than the total concentration in serum (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012). Hair is known to reflect endocrine activities spanning several weeks or months prior to the time of collection (Koren *et al.*, 2002). The interpretation of steroid hormone levels in hair depends on the timing of hair sampling (Ventrella *et al.*, 2018).

The northern fur seal starts to moult in late July and completes around November, depending on age class (Scheffer, 1962; Scheffer and Johnson, 1963). Hair roots of all seals collected in April and May are dormant, and the hair follicles are resting (Scheffer and Johnson, 1963). Two generations of hair are present in early spring, and both faded by July (Scheffer and Johnson, 1963). Hair steroid hormones in the northern fur seal are likely integrated spanning after moult to almost current hormone levels.

No studies have examined testosterone in pinniped hair to date. When hair testosterone can be used to differentiate the sexual maturity of northern fur seals, it allows us to enhance our understanding of fur seal ecology, such as demography of the male seals off Hokkaido during the non-breeding season. Since the increase in testosterone levels initiate spermatogenesis from spermatogonia to spermatozoa (Atkinson and Gilmarti, 1992), spermatogenesis is an important paramter to assess the maturity of the male northern fur seal. The objective of this study is to assess the sexual maturity of male northern fur seals during the non-breeding season off Hokkaido using hair testosterone levels. We compared the monthly testosterone levels with sampling months, age class and spermatogenesis. We predicted that hair testosterone levels would be greater in preceding the breeding season, socially mature males, and seals with spermatozoa.

4.3. Materials and Methods

4.3.1. Study animals

Fifty-seven stranded, bycaught, and caught northern fur seals between 2011 and 2018 were collected along Hokkaido, Japan (Figure 3.1). The description of seals collected is listed in Table 4.1. The furs were kept at –20°C until analysis. The seals were aged by the number of growth layers in tooth sections (Horimoto *et al.*, 2017). The age of those without teeth was determined from the body length based on Trites and Bigg (1996). The ages of the seals were classified into three categories, juvenile (age < 4 years old), mature (4 ≤ age < 8 years old), and socially mature seals (≥ 8 years old) (Gentry, 1998; Horimoto *et al.*, 2017). The right testes of the seals were collected, and used for the analyses since the left testes were marked with a cut of a knife. The testes were preserved in 10% formaldehyde and weighted (Hirakawa et al. *in prep*). Spermatogenesis in the cross sections of seminiferous tubules was identified using an optical microscope (Hirakawa *et al. in prep*). The testis and spermatogenesis data are based on the results of Hirakawa *et al.* (*in prep*). The spermatogenesis was determined by the presence of spermatogonia, primary spermatocytes, round and elongated spermatids and spermatozoa in the seminiferous tubules of the seals. When no spermatocytes but spermatogonia were observed in the cross section, we defined as SG. When no spermatids but primary spermatocytes were observed, we defined as

SC. Finally, when round and elongated spermatids and spermatozoa were present in seminiferous tubules, we assigned them to SP.

4.3.2. Sample preparation for assay

The furs were thawed, and hair (*i.e.*, guard hair and underfur) was cut along the skin. The hair was washed with distilled water until the water become clear. Then hair was shaken with 70% isopropanol for 2 min and repeated two more times to remove any steroid hormones coating the hair (Yamanashi *et al.*, 2013). The samples were dried at 40 °C (DKM600, Yamato Ltd., Japan). The hair was pulverized with a ball mill at 1100 rpm for 6 min (Shake Master Auto, BioMedical Science Co. Ltd., Japan). Hair samples (10 mg) were weighted into a test tube, and 100% methanol (1 mL) was added. Hair steroid hormones were extracted by sonicating in water bath at 60 °C for 10 min (CPX2800H-J, Branson Ultrasonics Corp., USA). After the extraction, the samples were centrifuged at 20,400 × *g* for 15 min (MX-307, Tomy Seiko Co., Ltd, Japan). Supernatant (0.6 mL) was dried under a centrifugal evaporator (CC-181, Tomy Seiko Co., Ltd, Japan). The dried extract was reconstituted with the same volume (0.6 mL) of Tris-HCl assay buffer.

4.3.3. Analysis of hair by GC-FID

The presence of testosterone in seal hair was confirmed by using GC-FID. Hair samples (100 mg) were extracted with methanol as previously described. Extracted samples were analyzed by GC-FID (GC 390B, GL Sciences, Japan) using the same methodologies in Chapter 3.

4.3.4. Assay for testosterone metabolites

TR-FIA (DEL-FIA®, PerkinElmer, Waltham, MA) was used to assay the samples (Yamada *et al.*, 1997). The assay methods are the same as Chapter 2 and 3. The intra-assay and inter-assay CVs of control samples were 6.89% (n = 8) and 8.57 % (n = 4), respectively. Extraction efficiency was determined by spiking testosterone standards to a sample prior to extraction. The spiked and unspiked samples were assayed to determine the extraction efficiency. Extraction efficiency was 105.7% (n = 4) by spiking with testosterone and compared with non-spiking samples.

4.3.5. Statistical analysis

A parallelism test between serially diluted testosterone standards and hair samples was conducted by an ANCOVA. Hair testosterone levels of a seal per unit 1 g hair were computed. The testis weight of northern fur seals was compared with the three phases of spermatogenesis. A Kruskal-Wallis test was conducted among the three phases since a deviance from the normal distribution of data set was found. When the test was significant, testis weight of northern fur seals with the phases of spermatogenesis was compared using a Bonferroni correction. Next, hair testosterone levels were analyzed using GLM (gamma error distributions with a log link function). The explanatory variable of each model was either sampling month, age class, or spermatogenesis. Since all the explanatory variables were categorical, all possible groupings of each categorical variable were modeled (Mori *et al.*, 2009). The best model was selected using Akaike's information criterion (AIC) score comparisons. For sampling month, the animals collected between March and June were analyzed since the sample size in January, February, and November was one. The statistically significant level was set to $\alpha = 0.05$. The software used for all the analyses was R 3.5.1 (R Core Team, 2018).

4.4. Results

4.4.1. Validation of hair testosterone assay

The testosterone extracted from a sample of northern fur seal hair was determined by GC-FID and a parallelism test. The peaks of the samples with hair testosterone and that with testosterone standard solution appeared at almost exact retention time 27.88 min and 27.83 min, respectively (Figure 4.1). The parallelism test showed no interaction between serially diluted hair samples and standard concentrations ($F = 0.584$, $p = 0.474$, Figure 4.2).

4.4.2. Monthly hair testosterone levels and maturity

Monthly hair testosterone levels in spring were higher than those in fall and winter (Figure 4.3). The means of testosterone levels in March, April, May, and June were 18.9 ng g^{-1}, 15.5 ng g^{-1}, 100.0 ng g^{-1}, and 57.6 ng g^{-1}, respectively. The model results showed that testosterone levels of the seals collected in May were the greatest between March and June (Table 4.2). The hair testosterone levels in June were lower than those in May. The testosterone levels of the seals collected in March and April were identical, and the levels in March and April were lower than those in May and June.

The testosterone levels in hair of mature and socially mature seals in March and April were low while those in May and June were greater (Figure 4.4). The model using the seals in May showed that the mean testosterone levels in each age class were different (Table 4.3). The means of the testosterone levels in juvenile, mature, and socially mature males are 39.5 ng g^{-1}, 128.3 ng g^{-1}, 94.8 ng g^{-1}, respectively. The testosterone levels in mature males were greater than juvenile and socially mature seals.

4.4.3. Testis weight, spermatogenesis and hair testosterone levels

Cross sections of seminiferous tubules in testes are shown in Figure 4.5. Statistically significant differences in testis weight of seals were obtained among three phases of spermatogenesis (Kruskal Wallis test, $\chi^2(2) = 28.76$, $p < 0.0001$, Figure 4.6). Because of the significance using the Kruskal-Wallis test, the results from a Bonferroni correction showed that the testis weight were significantly different each other among the phases. The mean testis weight of the seals with SG were significantly lower than those with SC ($t = 7.5241$, $p < 0.0001$) and SP ($t = 8.9757$, $p < 0.0001$). The mean testis weight of the seals with SC were significantly lower than those with SP ($t = 3.4596$, $p < 0.0001$).

The model with spermatogenesis in May showed that the seals with SP had higher testosterone levels than those with SG and SC (Table 4.4). The mean concentrations of testosterone levels in the seals with SG and SP were 38.8 ng g^{-1} and 109.5 ng g^{-1}, respectively (Figure 4.7). In addition, the mean testosterone levels in the seals with SC were 42.0 ng g^{-1}.

4.5. Discussion

In this study, it is the first report to present hair steroid hormones in pinnipeds to date. Testosterone extracted from a sample of northern fur seal hair was validated by the retention time of the GC-FID analysis and by parallelism. Testosterone in human hair has been detected by GC-MS (*e.g.,* Kintz *et al.*, 1999; Scherer *et al.*, 1998). Hair testosterone in non-human species has not been studied in chromatography. Hair testosterone levels in various species have been measured only using EIAs (*e.g.,* Bryan *et al.*, 2013a; Terwissen *et al.*, 2014). Moreover, a non-significant interaction of standard and sample solutions from the parallelism test indicates that the assay was validated.

Seasonal changes in the reproductive physiology of animals are tight to the breeding season. Our model showed that hair testosterone levels in May were greater than those in other months. The serum testosterone levels in captive northern fur seals are high preceding the breeding season (Kohyama *et al.*, 1999; Otsuki *et al., under review*). Since socially mature northern fur seals start to return their rookeries as early as in May to build territories (Gentry, 1998), the higher concentrations in testosterone in May reflect the reproductive status of male seals toward the breeding season. It is interesting to note that hair testosterone levels in socially mature animals in June was low even though it is preceding to the breeding season, possibly indicating that these seals do not participate in breeding and return to their rookeries later in the season. 30 % of mature males are excluded from reproduction for their entire lives, and socially mature males are involved in breeding on average for only 1.5 seasons, and (Vladimirov, 1987). Next, testosterone levels in socially mature and mature male hair were high compared to juveniles. This can be explained by differences in serum testosterone levels between juvenile and mature animals. For example, juvenile short-finned pilot whales (*Globicephala macrohynchus*) tend to show lower serum testosterone levels than adult whales (Kita *et al.*, 1999), and non-territorial male Weddell seals (*Leptonychotes weddellii*) tend to show lower testosterone levels (Bartsh *et al.*, 1992). The low testosterone levels during January–April thus represent the non-breeding season in northern fur seals since the serum testosterone levels in a captive fur seal were low during the non-breeding season (Kohyama *et al.*, 1999).

The testis weight of northern fur seals was significantly different among three phases of spermatogenesis. The testis weight increased as spermatogenesis progresses. This also agrees with the seasonal changes in testis weight and spermatogenesis of seasonal breeders such as deer (Blottner *et al.*, 1996) and bears (White *et al.*, 2005). Moreover, the testis weight of elephant seals (*Mirounga leonina*) showed a peak during the breeding season (Griffiths, 1984).

Testosterone levels in northern fur seal hair were also compared with spermatogenesis. The northern fur seals with SP showed significantly higher testosterone levels compared to those with SC and SG. Leydig cells produce testosterone that controls spermatogenesis (Moyes and Schulte, 2016), and testosterone acts on seminiferous tubules to stimulate spermatogenesis (Atkinson and Yoshioka, 2007). The increase in testosterone levels initiate spermatogenesis, which lags 3–8 weeks depending on species (Atkinson and Gilmarti, 1992). The serum testosterone levels of male dolphins were peaked before the breeding season while sperm density is the greatest during the breeding season (Schroeder and Keller, 1989). This time lag indicates an increase in testosterone levels to promote spermatogenesis. The testosterone levels of pilot whales also increased as the diameter of seminiferous tubules and the testis weight increased (Kita *et al.*, 1999). These results coincide with the seasonal changes in the testis weight and spermatogenesis of the northern fur seals.

Although the mechanism how steroid hormones are incorporated into hair is not fully understood, the results indicate that hormones in the northern fur seal are presenting close to the current endocrine activities at the time of sampling. Hair likely reflect the integrated free hormone fraction than the total concentration in serum (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012), and thus, hair reflects endocrine activities integrated over several months (Bryan *et al.*, 2013b; Ventrella *et al.*, 2018). In northern fur seals, moult starts around July and August and lasts until November in males (Scheffer and Johnson, 1963), occurring gradually for 4 or 5 months (Scheffer, 1962; Scheffer and Johnson, 1963). High testosterone levels in May mean the higher levels of serum testosterone in April and May (Kohyama *et al.*, 1999; Otsuki *et al., under review*). Since hair testosterone levels of animals with SP were low in April, it might be possible that testosterone levels in northern fur seal hair reflect the past endocrine activities. Hair testosterone levels of seals, thus, likely reflect past endocrine activities to the time of sampling.

In summary, it is the first to examine hair testosterone levels in pinnipeds to date. The sexual maturity of mature seals (≥ 4 years old) can be distinguished from juvenile seals when hair testosterone levels were greater in May. This non-invasive technique, hair, can advance the science significantly because it can non-invasively supply data that were previously only available by directly capturing animals. These data on male reproductive status enhance our understanding of fur seal ecology, such as distribution of the seals off Hokkaido during the non-breeding season, and clarify the connection between non-breeding and breeding seasons since hair can be biopsied in the field. This method can be especially useful for those that spend time offshore during the non-breeding season.

Table 4.1. The description of northern fur seals analyzed in Chapter 4.

Month	Age	Type	Location	Spermatogenesis	Number	Subtotal
January	9	Caught	Matsumae	SC	1	1
February	8	Stranded	Esashi	SC	1	1
March	6	Caught	Matsumae	SC	1	
	7	Caught	Matsumae	SC	2	
	9	Caught	Matsumae	SC	1	
	11	Caught	Matsumae	SC	1	5
April	5	Caught	Matsumae	SP	1	
	9	Stranded	Zenibako	SC	1	
	10	Caught	Matsumae	SP	1	
	10	Stranded	Senata	SC	1	
	14	Caught	Matsumae	SP	1	5
May	1	Caught	Matsumae	SG	2	
	2	Caught	Matsumae	SG	2	
	2	Caught	Matsumae	SC	1	
	4	Caught	Matsumae	SP	4	
	5	Caught	Matsumae	SP	1	
	6	Caught	Matsumae	SP	3	
	7	Caught	Matsumae	SP	6	
	8	Caught	Matsumae	SP	6	
	9	Caught	Matsumae	SP	4	
	10	Caught	Matsumae	SP	4	
	11	Caught	Matsumae	SP	3	
	12	Caught	Matsumae	SP	1	37
June	8	Caught	Matsumae	SP	3	
	9	Caught	Matsumae	SP	1	
	10	Caught	Matsumae	SP	2	
	11	Caught	Matsumae	SP	1	7
November	14	Bycaught	Matsumae	SP	1	1
			Total number of samples			57

Table 4.2. Sampling months estimated from GLMs. The difference in AIC score (ΔAIC) from the top-ranked grouping is shown.

Order	Grouping	ΔAIC
1	(March + April)(May)(June)	
2	(March)(April)(May + June)	2.7
3	(March)(April)(May + June)	4.4
4	(March + June)(April)(May)	9.0
5	(March)(April + June)(May)	12.0

Table 4.3. Sexual maturity of northern fur seals in May estimated from GLMs. The difference in AIC score (ΔAIC) from the top-ranked grouping is shown.

Order	Grouping	ΔAIC
1	(Juvenile)(Mature)(Socially mature)	
2	(Juvenile)(Mature + Socially mature)	0.8
3	(Juvenile + Socially mature)(Mature)	7.1
4	(Juvenile + Mature)(Socially mature)	12.1

Table 4.4. Spermatogenesis of northern fur seals in May estimated from GLMs. The difference in AIC score (ΔAIC) from the top-ranked grouping is shown.

Order	Grouping	ΔAIC
1	(SG + SC)(SP)	0
2	(SG)(RS)(ES)	2.0
3	(SG)(SC + SP)	2.4
4	(SG + SP)(SC)	9.8

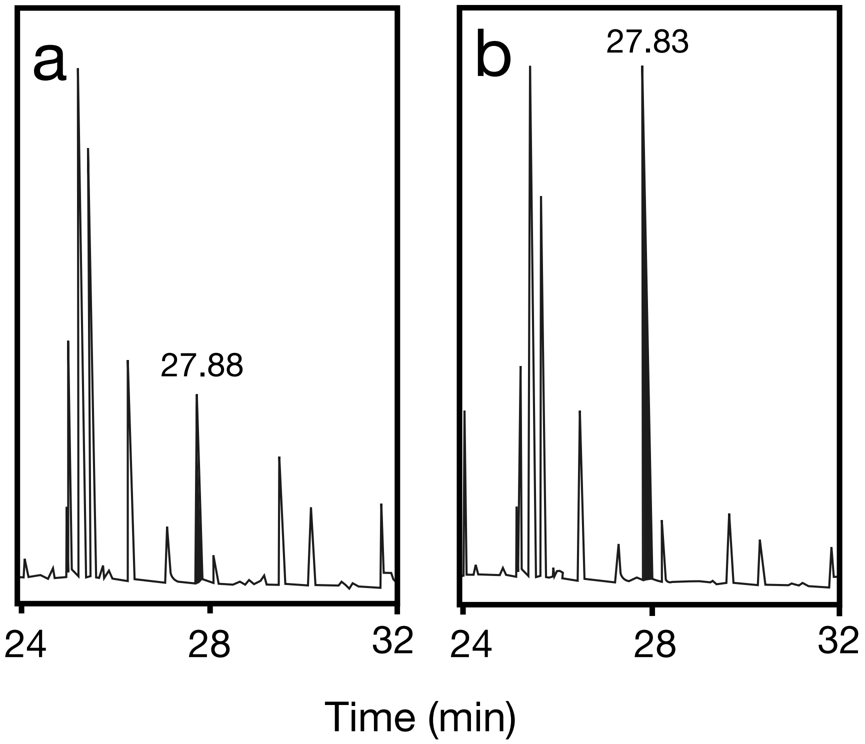

Figure 4.1. Gas chromatograms showing the presence of (a) testosterone and (b) spiked testosterone with standard in a sample of northern fur seal hair analyzed by GC-FID. The numbers in the figures indicate the retention time for testosterone detections.

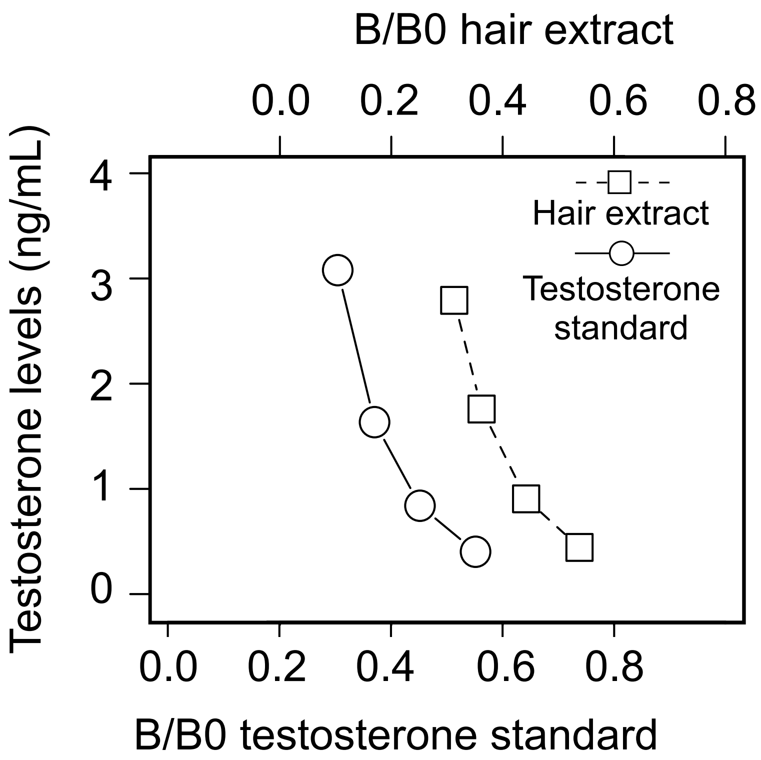

Figure 4.2. Parallelism for testosterone standards tested with serially diluted hair extracts. B/B0 show the ratio of bound.

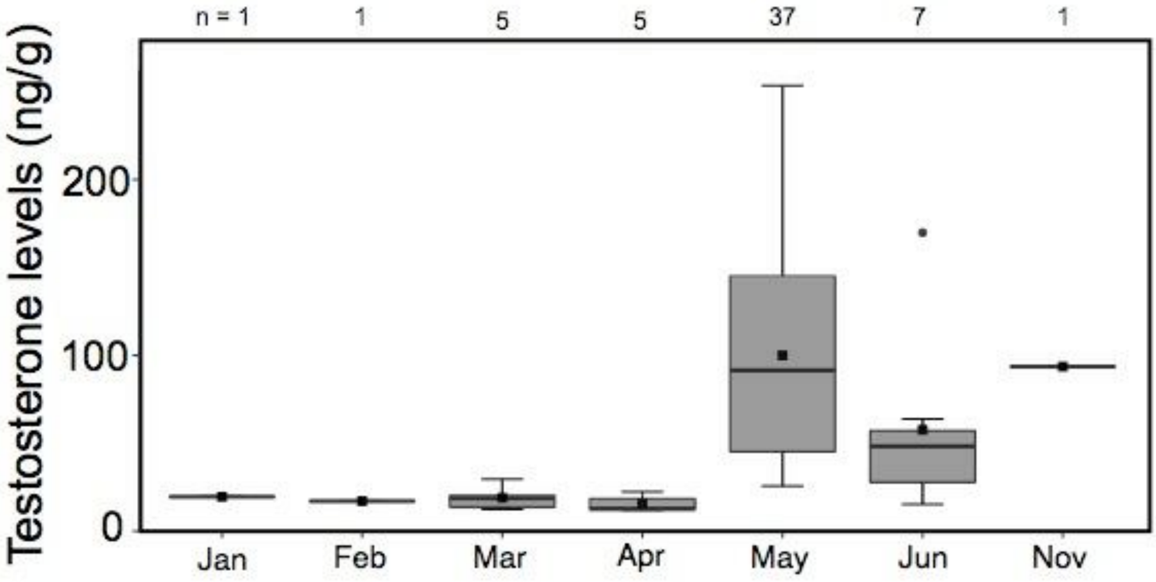

Figure 4.3. Monthly hair testosterone levels of male northern fur seals. The squares in the boxes indicate monthly means of hair testosterone levels.

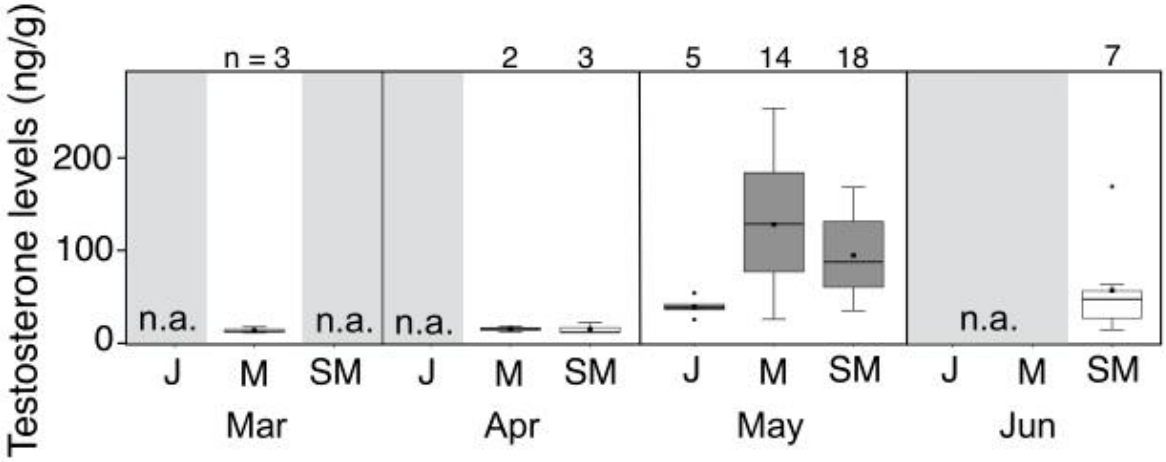

Figure 4.4. Hair testosterone levels of northern fur seals by age class. The squares in the boxes are indicating the mean of testosterone levels in age class. J, M, and SM stands for juvenile, mature and socially mature seals, respectively. The grey shaded boxes in May indicate that modeling was performed.

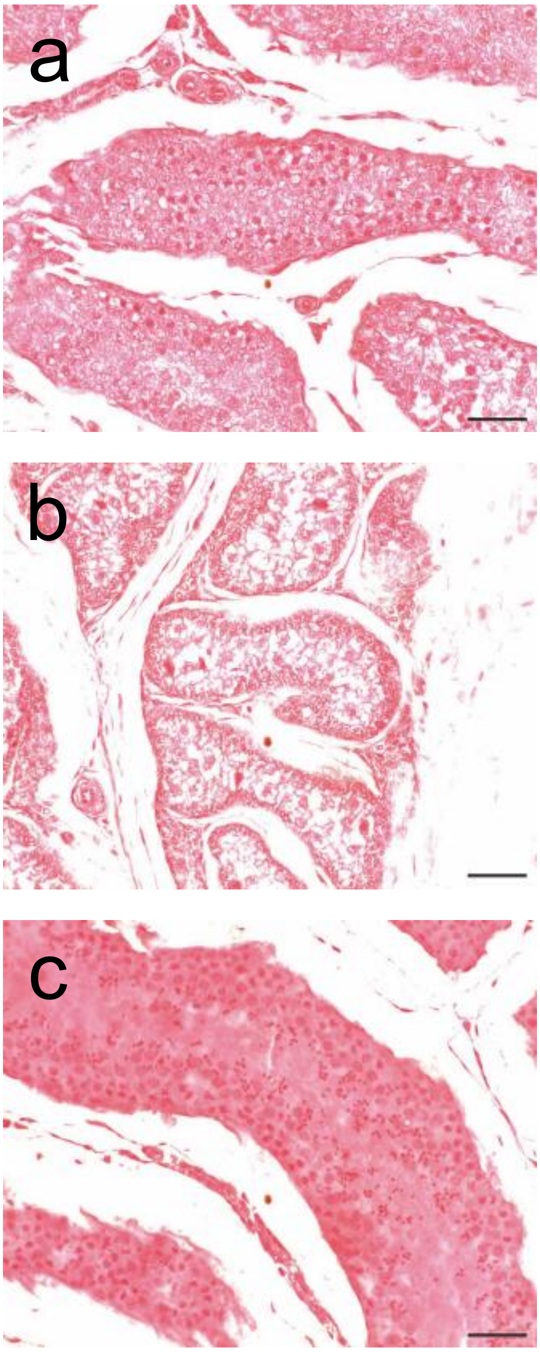

Figure 4.5. Cross section through seminiferous tubules showing (a)spermatogonia, (b) spermatogonia and (c) primary spermatocytes, and spermatogonia, spermatocytes, spermatids and spermatozoa (Hirakawa *et al., in prep*). A black scale bar indicates 100μm.

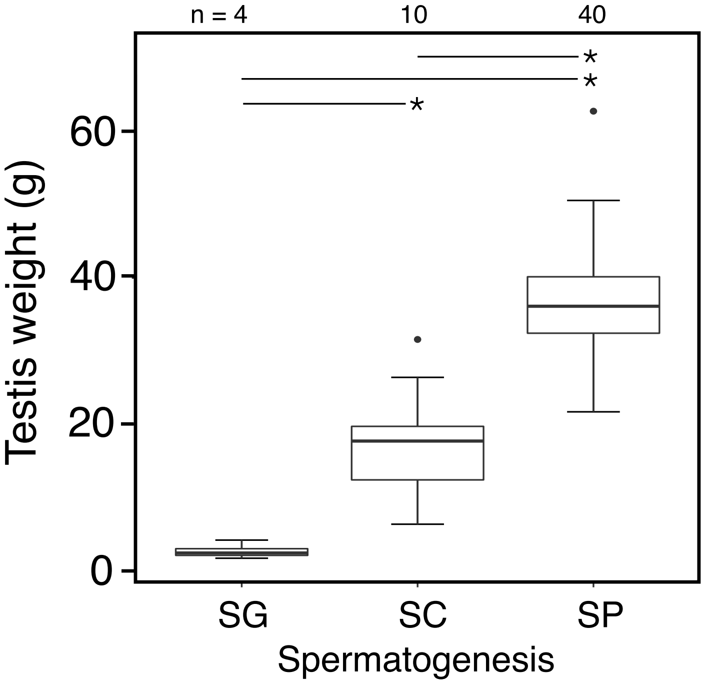

Figure 4.6. Right testis weight and three phases of spermatogenesis of northern fur seals. Asterisks indicate the significant differences among the phases ($p < 0.05$).

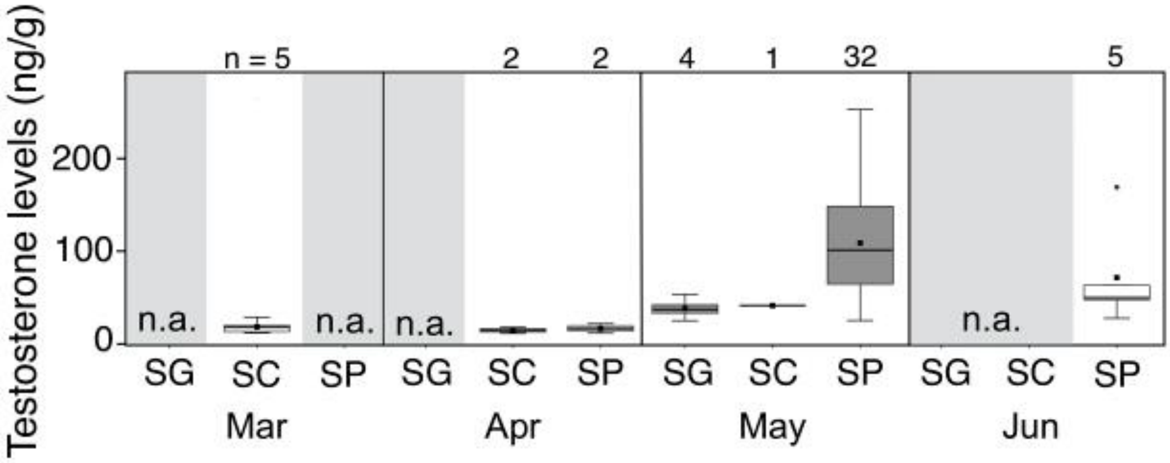

Figure 4.7. Hair testosterone levels of male northern fur seals by three phases of spermatogenesis. The squares at each boxplot are indicating the mean of testosterone levels. The boxplots in May are shaded since these were used in a GLM.

Chapter Five: Conclusion

5.1. General discussion

The overall objective of my research was to develop non-invasive methods of testosterone monitoring in the northern fur seal. In Chapter 2, I compared patterns in serum and faecal testosterone levels of a captive male and examined the effect of prey species and the representation of faecal testosterone metabolites in DW and AFDW. In Chapter 3, I further investigated faecal testosterone metabolite levels in free-ranging males during the non-breeding season. This was to determine the representation of faecal testosterone metabolite levels and to compare sexual maturity of seals with the levels expressed in DW and AFDW. In Chapter 4, I used testosterone levels in free-ranging seal hair to develop non-invasive monitoring of hormone levels. Throughout my research, I have a goal to apply these non-invasive techniques to animals in captivity and the wild. Below I emphasize the most interesting findings from these studies and discuss directions for future research.

5.1.1. Non-invasive monitoring of steroid hormones

Endocrine studies require blood sampling though it is impractical in free-ranging animals (Amaral, 2010) since capture, recapture, and anaesthesia of animals can unfavourably affect animal body conditions and possible death (Arnemo *et al.*, 2006; Cattet *et al.*, 2008). Non-invasive techniques such as analysis of faeces and hair can be an invaluable tool to assess the reproductive status of animals.

In Chapters 2-3, I focused faecal testosterone metabolites of northern fur seals. I found that faecal testosterone metabolites of the captive seal in Chapter 2 were correlated with serum testosterone levels with a time lag of 6 – 22 h. This information becomes crucial when studying longitudinal reproductive status of both captive and free-ranging seals using faecal testosterone metabolites since the time lag is species-specific (Schwarzenberger *et al.*, 1996; Touma and Palme, 2005b). Differences in diet can influence faecal steroid hormones including testosterone (Dantzer *et al.*, 2011; Goymann, 2012); thus, I speculated that removing inorganic component in faeces likely reduce the prey differences in faeces. Despite this prediction, neither captive nor free-ranging seals showed strong evidence to support the use of AFDW for the representation of faecal testosterone metabolite levels. Therefore, representing the metabolite levels in DW is sufficient and saves time for sample preparation.

In the field, there are two ways to collect northern fur seal faeces. Faeces can be collected at rookeries since northern fur seals congregate in summer for breeding. However, it is impossible to tell which animal have defaecated, and knowledge of biological information is missing. This makes it difficult to interpret the results. It is possible to identify the sex of the animals that defaecated with other hormones such as estrogen and progesterone (Barja *et al.*, 2008; Tripovich *et al.*, 2009), though additional concerns exist. The steroid hormones in faeces continue to be metabolized by faecal bacteria after defaecation, showing double increases in testosterone metabolite levels within 72 h though constant levels within 48 h (Webber *et al.*, 2018). Furthermore, it is unknown when faeces in the field are defaecated unless checking the grounds periodically to search for the defaecation. Another way to collect faeces in the field is to capture animals and obtain faeces from the rectum, which can be done at rookeries and open waters. In spite of the additional information about morphological characteristics that can be gathered, animal capture is highly invasive and collecting faeces from rectum requires anaesthesia. Faeces are, therefore, a more useful marker in captive environment where animals are individually enclosed. In this way, which and when animals defaecated can be identified.

It is interesting to note that an order of magnitude of faecal testosterone metabolites in captive and free-ranging fur seal is three. Testosterone levels are much greater and more complex in free-ranging animals than captive animals (Wingfield *et al.*, 1990), which agree with this study. Wingfield *et al.* (1990) argue that the reasons for higher testosterone levels in the field are male-male aggression during territory establishment during the breeding season. Because in this study, free-ranging seal samples were collected during the non-breeding season, their hypothesis is not the case. Although Wingfield *et al.* (1990) examined testosterone in blood, it is possibly environmental cues that affect greater testosterone concentrations in the free-ranging seals. The activity budget toward breeding of free-ranging seals is completely different from captive seals. Free-ranging socially mature northern fur seals migrate a long distance to their natal rookeries for breeding and followed by mature animals (Gentry, 1998). The cost of migration is high for these animals, and very likely results in an increase in their testosterone levels. In addition, testosterone levels of migrant species tend to be greater because of the preparation for the breeding season and the competition for territories and mates (Garamszegi *et al.*, 2008). In captivity where colleagues and I conducted sampling, one male and two mature females were kept together. They do not need to migrate, nor did the male establish a mating territory, which may have resulted in lower faecal testosterone metabolite levels. Another reason is body size. Body size is positively correlated with testis weight (Kenagy and Trombulak, 1986), and testosterone levels in serum are correlated with testis weight and body size (Lincoln, 1998). The mean monthly body weight of the captive seal was 86 ± 7 kg during April–June in this study ($\bar{x} \pm s$, $n = 3$ months), while the mean body weight of seven free-ranging seals at the ages of 7 and 8 years was 130 ± 34 kg ($\bar{x} \pm s$, $n = 7$ animals), which might have resulted in lower testosterone metabolite levels in the captive seal.

In Chapter 4, hair testosterone levels of free-ranging northern fur seals were determined. This is the first study to examine steroid hormones from pinniped hair. I developed the methodologies to extract testosterone from fur seal hair. Although the classification of maturity using hair testosterone levels in the northern fur seal was achieved only in May, it might be possible to distinguish the maturity in other months when more hair samples are collected.

The hardest part of this hair hormone analysis was extraction. Colleagues and I used ultrasound in a warm water bath to extract steroid hormones from fur seal hair. Commonly used methods of extraction from hair are mixing with a rotator (*e.g.*, Terwissen *et al.*, 2014; Yamanashi *et al.*, 2013) and sonication (Koren *et al.*, 2002). The combination of these with warm water temperature was effective. If methodologies of the extraction advance, studies of non-invasive monitoring of steroid hormones of hairy mammals will increase and these studies will help to understand the physiology of wildlife.

Some of the seals in the present study were used in both Chapter 3 and 4. When the relationship between faecal testosterone metabolite and hair testosterone levels of the seals in May was compared, no clear relationship was recognized (Figure 5.1). Although juvenile seals show low testosterone levels in both faeces and hair, some of mature and socially mature seals show low testosterone metabolite levels in faeces but high levels in hair. These trends possibly resulted from the differences in time that steroid hormones reflect in faeces and hair. The time lag between faecal testosterone metabolites and serum testosterone is one-day, while testosterone in hair can reflect integrated free hormone fraction (Russell *et al.*, 2012; Stalder and Kirschbaum, 2012). Furthermore, it might be possible that serum testosterone levels were temporarily low before the collection of animals, though in a longer term, higher testosterone levels in blood have incorporated into hair toward the breeding season. In several other studies, faecal and hair testosterone levels of captive animals were compared. The relationships between faecal and hair

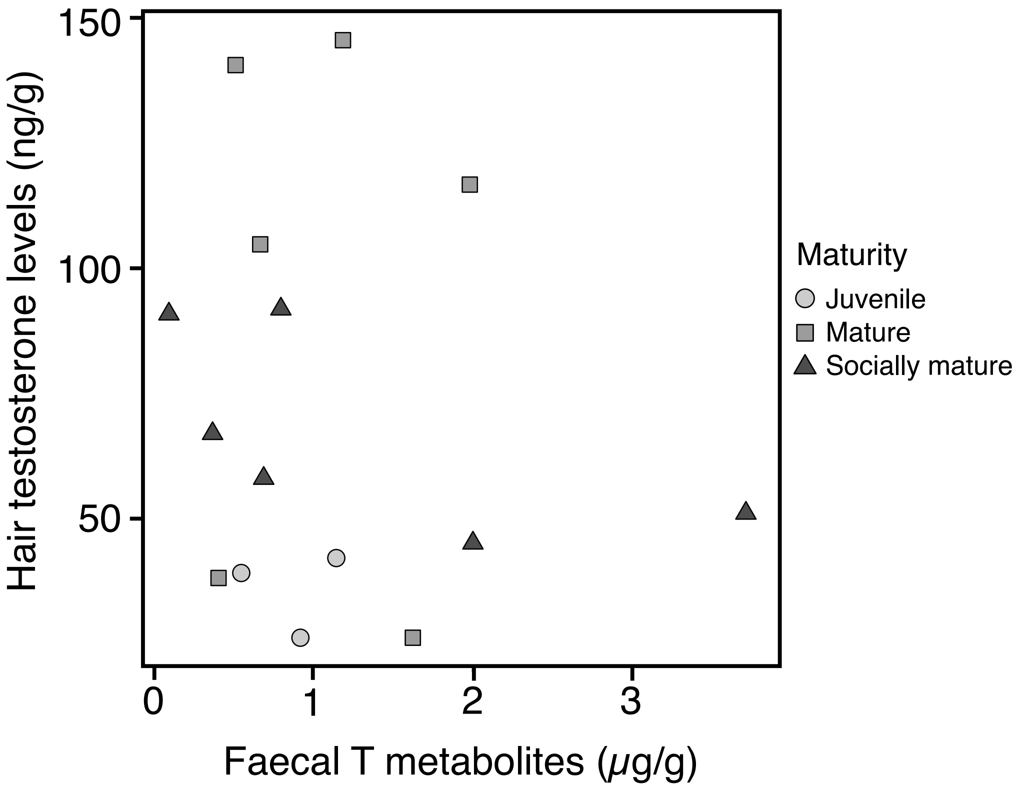

Figure 5.1. The comparison of faecal testosterone metabolite and hair testosterone levels in the northern fur seals in May. These animals were studied in both Chapter 3 and 4.

cortisol levels in cats and dogs were both significantly correlated (Accorsi *et al.*, 2008). On the other hand, that of dogs in another study showed no significance, possibly due to a small sample size (*n* = 7) (Bryan *et al.*, 2013a). Additionally, the relationship between faecal and hair cortisol levels in captive chimpanzees showed no significance (Yamanashi *et al.*, 2013). Although these previous studies examine captive animals, these studies also indicate the difficulties of comparing the faecal and hair steroid hormones.

5.2. Strengths and limitations

The first strength of this study is the use of non-invasive techniques. Faeces can be collected in captivity without disturbing animals. Since husbandry training is unnecessary for faecal sampling, this method is the easiest to study their endocrine physiology. Although it is necessary to house an animal alone to identify its faeces, there is no need to train animals overcomes the serum testosterone monitoring. In addition, hair sampling can be conducted by biopsy darting in open waters, which allows no capture of seals.

Another strength of this study is that samples were mostly obtained from the "*Noxious Organisms Fishery Damage Prevention Program*" supported by the Fisheries Agency of Japan, which allowed me to compare testosterone levels with morphological data such as testis weight and spermatogenesis during the non-breeding season. This information is fundamental to compare with testosterone levels. It does not mean that killing animals for science is favourable since the goal of this study was to develop non-invasive measures of testosterone levels.

One of the limitations of this study was the sample size in Chapter 2. I was unable to compare the faecal testosterone levels of multiple seals, limiting the interpretation of the results. Relating to the problem of a sample size, prey experiment was only conducted once for three species of prey. Since faecal steroid hormones have an effect of diet (Dantzer *et al.*, 2011; Goymann, 2012). Further investigation of longitudinal and comparative studies on other northern fur seals and the effect of prey species will be necessary to understand faecal metabolites of this species.

Another limitation was that faecal steroid metabolites are far more challenging to interpret than hair. For instance, it is sometimes not able to differentiate maturity from faecal testosterone levels (Terwissen *et al.*, 2014) as multiple metabolites from the testosterone are likely present. Furthermore, the captive northern fur seal hair in the present study did not grow outside of moulting season. This indicates that it might not be a suitable matrix for reproductive management as it requires an immediate judgement. In addition to the hair growth, adrenocorticotropic hormone challenge has not revealed the mechanism of hormone incorporation into hair due to a longer span of steroids into hair (Ashley *et al.*, 2011); thus, further determination is necessary. The limitation of non-invasive hormone measurement of free-ranging seals in Chapter 3 and 4 is that samples were obtained once from each individual. The longitudinal data of free-ranging animals were not obtained in this study. Wingfield *et al.* (1990) argue that the changes in hormonal levels are more informative than one point in time.

5.3. Applications of research findings

To apply the methods that have been developed, it is interesting to examine faecal testosterone metabolites in juveniles to compare them with free-ranging animals since the hormones in free-ranging juveniles were high. Although different captive environment such as the length of photoperiods and diet can change the endocrine system (Geyer, 2017; Goymann, 2012), it is possible to compare testosterone levels and other hormones of northern fur seals in captivity. Moreover, it is interesting to examine hair testosterone levels in the northern fur seal in the breeding season and the other months of the non-breeding season, which may be able to reveal the mechanism of steroid hormone incorporation into hair in relation to moult, which is still under debate (Koren *et al.*, 2019; Russell *et al.*, 2012; Stalder and Kirschbaum, 2012).

Another application of this research is that hair samples will be obtained from biopsy darts in the field without manipulating the behaviour before sampling. Biopsy darting is used to study topics such as toxicology and DNA of pinnipeds (Fossi *et al.*, 1997; Gemmell and Majluf, 1997). Although hair samples can be obtained in captivity, the incorporation of hormones into hair requires more time, which might not be practical for captive experiment. As a result, hair is a useful marker to study endocrine system of free-ranging northern fur seals. It is often impractical and unfeasible to repeatedly sample from the same wildlife, especially in marine environment. Analysis of longitudinal hair steroid hormones in seals requires techniques such as tagging. This allows animal identification, and thus it is possible to collect samples from the same animals again, for instance, at rookeries. Satellite tagging of seals can reveal their natal islands (Hirakawa, 2019) and collecting samples from the tagged seals after arriving at the islands might bring to light the longitudinal steroid hormone changes in the free-ranging seals and the incorporation of hormones into hair. To study longitudinal hormonal levels, faecal hormones are more effective, especially in captive environment. For one-point time study, hair might be more useful.

Non-invasive monitoring of steroid hormones can help reveal the mechanism of behaviour of cryptic species. Since the non-invasive monitoring change little in the behaviour of animals when collecting matrices, the mechanism of behaviour can be studied. For instance, the ribbon seal (*Histriophoca fasciata*) is another offshore pinniped species that their endocrine physiology is largely unknown. This species is observed in the close proximity from shore of eastern Hokkaido in winter (Mizuguchi *et al.*, 2016), so biopsy sampling can be conducted. Since their vocalizations lead up to spring breeding season (Otsuki *et al.*, 2018), the relationship between their vocal behaviour and hair reproductive steroid hormone levels can be investigated. This will help to understand their reproduction and the proximate cause of vocalization. Hair steroid hormones, therefore, are suitable for comparing the physiology of offshore species that seasonally observed in nearshore waters.

5.4. Future directions – competition with fisheries

Competition of fisheries in Hokkaido has been observed in several species of pinnipeds such as Steller sea lions and northern fur seals (Hattori *et al.*, 2011; Horimoto *et al.*, 2012). Although the steroid hormone analysis cannot directly prevent the depredation, for healthy resource management of this species requires understanding in the demography of this species since the sexual segregation of this species are observed (Horimoto *et al.*, 2017). The sexual segregation of habitats during the non-breeding season is an important period to maximize the fitness of males (Gentry, 1998; Vladimirov, 1987). Knowledge of the connections of breeding and non-breeding season, therefore, can strength the understanding of the ecology of this species. Long-term monitoring of hair steroid hormones in the wild can reveal the connection between the non-breeding and breeding season in the northern fur seal, providing useful insights into population dynamics.

Hormone levels and environmental factors need to be studied. Free-ranging northern fur seals are opportunistic feeders (Kajimura, 1984), since they depend on the changes in abundance of prey due to oceanographic regime (Kiyota and Yonezaki, 2017). These changes can trigger physiological responses to higher trophic animals. Shifting distribution of prey species might change the endocrine systems of the animals, since the shift alters the distance travelling to and from rookeries and the energy used during predation. Physiological responses to environmental cues can be addressed using non-invasive glucocorticoids analysis. Faecal and hair glucocorticoids are used to assess environmental stress of captive and wild animals (*e.g.*, Koren *et al.*, 2019; Narayan, 2013). Studying the physiological changes in relation to environmental changes requires long-term, probably decades-long, of studies. The hair steroid hormone analyses will help to reveal these changes in the northern fur seal and other pinniped species that spend time offshore.

Literature cited

Accorsi, P.A., Carloni, E., Valsecchi, P., Viggiani, R., Garnberoni, M., Tarnanini, C., Seren, E., 2008. Cortisol determination in hair and faeces from domestic cats and dogs. General and Comparative Endocrinology. 155, 398–402. http://doi.org/10.1016/j.ygcen.2007.07.002.

Amaral, R.D., Rosas, F.C.W., Viau, P., Neto, J.A.D., da Silva, V.M.F., de Oliveira, C.A., 2009. Noninvasive monitoring of androgens in male Amazonian manatee (*Trichechus inungus*): biologic validatoin. Journal of Zoo and Wildlife Medicine. 40, 458–465. http://doi.org/10.1638/2008-0111.1.

Amaral, R.S., 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: A review. Aquatic Mammals. 36, 162–171. http://doi.org/10.1578/am.36.2.2010.162.

Amaral, R.S., Rosas, F.C.W., da Silva, V.M.F., Graham, L.H., Viau, P., Nichi, M., Oliveira, C.A., 2015. Seasonal variation in urinary and salivary reproductive hormone levels in Amazonian manatees (*Trichechus inunguis*). Reproduction Fertility and Development. 27, 1065–1071. http://doi.org/10.1071/rd13334.

Arnemo, J.M., Ahlqvist, P., Andersen, R., Berntsen, F., Ericsson, G., Odden, J., Brunberg, S., Segerström, P., Swenson, J.E., 2006. Risk of capture-related mortality in large free-ranging mammals: experiences from Scandinavia. Wildlife Biology. 12, 109–114. http://doi.org/10.2981/0909-6396(2006)12[109:rocmil]2.0.co;2.

Ashley, N., Barboza, P., Macbeth, B., Janz, D., Cattet, M., Booth, R., Wasser, S., 2011. Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. General and Comparative Endocrinology. 172, 382–391. http://doi.org/10.1016/j.ygcen.2011.03.029.

Atkins, N., Jones, S.M., Edwards, A., 2002. Fecal testosterone concentrations may not be useful for monitoring reproductive status in male blue-tongued lizards (*Tiliqua nigrolutea*: Scincidae). Journal of Herpetology. 36, 106–109. http://doi.org/10.2307/1565811.

Atkinson, S., Gilmarti, W., 1992. Seasonal testosterone pattern in Hawaiian monk seals (*Monachus schauinslandi*). Journal of Reproduction and Fertility. 96, 35-39. http://doi.org/10.1530/jrf.0.0960035.

Atkinson, S., Yoshioka, M., 2007. Endocrinology of reprodution. in: Jamieson, B.G.M., (Ed.), Reproductive biology and phylogeny of Cetacea: whales, dolphins, and porpoises, Science Publishers, Enfield, NH, pp. 171–192.

Banks, J.C., Cary, S.C., Hogg, I.D., 2014. Isolated faecal bacterial communities found for Weddell seals, *Leptonychotes weddellii*, at White Island, McMurdo Sound, Antarctica. Polar Biology. 37, 1857–1864. http://doi.org/10.1007/s00300-014-1567-x.

Barja, I., Silvan, G., Rosellini, S., Pineiro, A., Illera, M.J., Illera, J.C., 2008. Quantification of sexual steroid hormones in faeces of Iberian wolf (*Canis lupus signatus*): a non-invasive sex typing method. Reproduction in Domestic Animals. 43, 701–707. http://doi.org/10.1111/j.1439-0531.2007.00974.x.

Bartsh, S.S., Johnston, S.D., Siniff, D.B., 1992. Territorial behavior and breeding frequency of male Weddell seals (*Leptonychotes weddelli*) in relation to age, size, and concentrations of serum testosterone and cortisol. Canadian Journal of Zoology. 70, 680–692. http://doi.org/10.1139/z92-102.

Beck, C.A., Bowen, W.D., Iverson, S.J., 2003. Sex differences in the seasonal patterns of energy storage and expenditure in a phocid seal. Journal of Animal Ecology. 72, 280–291. http://doi.org/10.1046/j.1365-2656.2003.00704.x.

Bigg, M.A., 1990. Migration of northern fur seals (*Callorhinus ursinus*) off western North America. Canadian Technical Report of Fisheries and Aquatic Sciences, 1764, 64.

Bigg, M.A., Fawcett, I., 1985. Two biases in diet determination of northern fur seals (*Callorhinus ursinus*). in: Beddington, J.R., Beverton, R.J.H., Lavigne, D.M., (Eds.), Marine mammals and fisheries, George Allen and Unwin, London, pp. 284–291.

Blottner, S., Hingst, O., Meyer, H., 1996. Seasonal spermatogenesis and testosterone production in roe deer (*Capreolus capreolus*). Journal of Reproduction and Fertility. 108, 299–305. http://doi.org/10.1530/jrf.0.1080299.

Bradshaw, C.J.A., Higgins, J., Michael, K.J., Wotherspoon, S.J., Hindell, M.A., 2004. At-sea distribution of female southern elephant seals relative to variation in ocean surface properties. ICES Journal of Marine Science. 61, 1014–1027. http://doi.org/10.1016/j.icesjms.2004.07.012.

Bryan, H.M., Adams, A.G., Invik, R.M., Wynne-Edwards, K.E., Smits, J.E., 2013a. Hair as a meaningful measure of baseline cortisol levels over time in dogs. Journal of the American Association for Laboratory Animal Science. 52, 189–196.

Bryan, H.M., Darimont, C.T., Paquet, P.C., Wynne-Edwards, K.E., Smits, J.E., 2013b. Stress and reproductive hormones in grizzly bears reflect nutritional benefits and social consequences of a salmon foraging niche. Plos One. 8, e80537. http://doi.org/10.1371/journal.pone.0080537.

Burgess, E.A., Blanshard, W.H., Barnes, A.A., Gilchrist, S., Keeley, T., Chua, J., Lanyon, J.M., 2013. Reproductive hormone monitoring of dugongs in captivity: Detecting the onset of sexual maturity in a cryptic marine mammal. Animal Reproduction Science. 140, 255–267. http://doi.org/10.1016/j.anireprosci.2013.06.005.

Burgess, E.A., Lanyon, J.M., Keeley, T., 2012. Testosterone and tusks: maturation and seasonal reproductive patterns of live, free-ranging male dugongs (*Dugong dugon*) in a subtropical population. Reproduction. 143, 683-697.

Butterworth, A., Sayer, S., 2017. The welfare impact on pinnipeds of marine debris and fisheries. in: Butterworth, A., (Ed.), Marine mammal welfare: human induced change in the marine environment and its impacts on marine mammal welfare, Springer, Cham, Switzerland, pp. 215–239. http://doi.org/10.1007/978-3-319-46994-2_13.

Cattet, M., Boulanger, J., Stenhouse, G., Powell, R.A., Reynolds-Hogland, M.J., 2008. An evaluation of long-term capture effects in ursids: implications for wildlife welfare and research. Journal of Mammalogy. 89, 973–990. http://doi.org/10.1644/08-mamm-a-095.1.

Cattet, M., Stenhouse, G.B., Boulanger, J., Janz, D.M., Kapronczai, L., Swenson, J.E., Zedrosser, A., 2018. Can concentrations of steroid hormones in brown bear hair reveal age class? Conservation Physiology. 6, coy001. http://doi.org/10.1093/conphys/coy001.

Cockrem, J.F., Rounce, J.R., 1994. Faecal measurements of oestradiol and testosterone allow the non-invasive estimation of plasma steroid concentrations in the domestic fowl. British Poultry Science. 35, 433–443. http://doi.org/10.1080/00071669408417708.

Constable, S., Parslow, A., Dutton, G., Rogers, T., Hogg, C., 2006. Urinary cortisol sampling: a non-invasive technique for examining cortisol concentrations in the Weddell seal, *Leptonychotes weddellii*. Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association. 25, 137−144. http://doi.org/10.1002/zoo.20088.

Dantzer, B., McAdam, A.G., Palme, R., Boutin, S., Boonstra, R., 2011. How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. General and Comparative Endocrinology. 174, 124–131. http://doi.org/10.1016/j.ygcen.2011.08.010.

Darimont, C.T., Reimchen, T.E., Bryan, H.M., Paquet, P.C., 2008. Faecal-centric approaches to wildlife ecology and conservation; methods, data and ethics. Wildlife Biology in Practice. 4, 73–87. http://doi.org/10.2461/wbp.2008.4.7.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. General and Comparative Endocrinology. 147, 255–261. http://doi.org/10.1016/j.ygcen.2006.01.005.

Delport, T.C., Power, M.L., Harcourt, R.G., Webster, K.N., Tetu, S.G., 2016. Colony location and captivity influence the gut microbial community composition of the Australian Sea Lion (*Neophoca cinerea*). Applied and Environmental Microbiology. 82, 3440–3449. http://doi.org/10.1128/aem.00192-16.

Diamond, M.C., Johnson, R.E., Ingham, C.A., 1975. Morphological changes in the young, adult and aging rat cerebral cortex, hippocampus, and diencephalon. Behavioral Biology. 14, 163–174. http://doi.org/10.1016/s0091-6773(75)90161-3.

Fossi, M., Savelli, C., Marsili, L., Casini, S., Jimenez, B., Junin, M., Castello, H., Lorenzani, J., 1997. Skin biopsy as a nondestructive tool for the toxicological assessment of endangered populations of pinnipeds: preliminary results on mixed function oxidase in *Otaria flavescens*. Chemosphere. 35, 1623–1635.

Ganswindt, A., Muilwijk, C., Engelkes, M., Muenscher, S., Bertschinger, H., Paris, M., Palme, R., Cameron, E.Z., Bennett, N.C., Dalerum, F., 2012. Validation of noninvasive monitoring of adrenocortical endocrine activity in ground-feeding aardwolves (*Proteles cristata*): exemplifying the influence of consumption of inorganic material for fecal steroid analysis. Physiological and Biochemical Zoology. 85, 194–199. http://doi.org/10.1086/664591.

Garamszegi, L.Z., Hirschenhauser, K., Bókony, V., Eens, M., Hurtrez-Boussès, S., Møller, A., Oliveira, R., Wingfield, J., 2008. Latitudinal distribution, migration, and testosterone levels in birds. The American Naturalist. 172, 533–546. http://doi.org/10.1086/590955.

Gemmell, N.J., Majluf, P., 1997. Projectile biopsy sampling of fur seals. Marine Mammal Science. 13, 512-516. http://doi.org/10.1111/j.1748-7692.1997.tb00661.x.

Gentry, R.L., 1998. Behavior and ecology of the northern fur seal. Princeton University Press: Princeton, NJ http://doi.org/10.5860/choice.35-6225.

Geyer, C.B., 2017. Setting the stage: the first round of spermatogenesis. in: Oatley, J.M., Griswold, M.D., (Eds.), The biology of mammalian spermatogonia, Springer, New York, pp. 39–63. http://doi.org/10.1007/978-1-4939-7505-1_3.

Goncalves, I.B., Heistermann, M., Santema, P., Dantzer, B., Mausbach, J., Ganswindt, A., Manser, M.B., 2016. Validation of a fecal glucocorticoid assay to assess adrenocortical activity in meerkats using physiological and biological stimuli. Plos One. 11, e0153161. http://doi.org/10.1371/journal.pone.0153161.

Goymann, W., 2005. Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Annals of the New York Academy of Sciences. 1046, 35–53. http://doi.org/10.1196/annals.1343.005.

Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods in Ecology and Evolution. 3, 757–765. http://doi.org/10.1111/j.2041-210X.2012.00203.x.

Graham, L.H., Brown, J.L., 1997. Non-invasive assessment of gonadal and adrenocortical function in felid species via faecal steroid analysis. Zeitschrift Fur Saugetierkunde-International Journal of Mammalian Biology. 62, 78–82.

Griffiths, D., 1984. The annual cycle of the testis of the elephant seal (*Mirounga leonina*) at Macquarie Island. Journal of Zoology. 203, 193–204. http://doi.org/10.1111/j.1469-7998.1984.tb02326.x.

Grocock, C.A., 1979. Testis development in the vole, *Microtus agrestis*, subjected to long or short photoperiods from birth. Reproduction. 55, 423–427. http://doi.org/10.1530/jrf.0.0550423.

Hattori, K., Isono, T., Yamamura, O., 2011. Fisheries dameges by Steller sea lions and the countermeasures. Nippon Suisan Gakkaishi. 77, 126. http://doi.org/10.2331/suisan.77.126.

Hirakawa, Y., 2019. Migration patterns of the northern fur seal found off Hokkaido in the Sea of Japan using C14 in vibrissaes and satellite transmitters [MEnv]. Sapporo: Hokkaido University.

Holekamp, K.E., Talamantes, F., 1991. Seasonal variation in circulating testosterone and oestrogens of wild-caught California ground squirrels (*Spermophilus beecheyi*). Journal of Reproduction and Fertility. 93, 415–425. http://doi.org/10.1530/jrf.0.0930415.

Horimoto, T., 2015. Distribution and foraging ecology of northern fur seals in Russian group during non-breeding season [PhD]. Hakodate: Hokkaido Univeristy.

Horimoto, T., Mitani, Y., Kobayashi, M., Hattori, K., Sakurai, Y., 2017. Seasonal and spatial occurrence of northern fur seals *Callorhinus ursinus* around northern Japan. Mammal Study. 42, 51–56. http://doi.org/10.3106/041.042.0106.

Horimoto, T., Mitani, Y., Kobayashi, Y., Hattori, K., Sakurai, Y., 2012. Distribution and stranding records of the northern fur seal *Callorhinus ursinus* near Tsugaru Strait during winter and spring 2009. Nippon Suisan Gakkaishi. 78, 256–258. http://doi.org/10.2331/suisan.78.256.

Kajimura, H., 1984. Opportunistic feeding by the northern fur seal, *Callorhinus ursinus, i*n the Eastern North Pacific Ocean and Eastern Sering Sea. NOAA Technical Report National Marine Fisheries Service, SSRF-779, 49.

Kellar, N.M., Trego, M.L., Marks, C.I., Chivers, S.J., Danil, K., Archer, F.I., 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science. 25, 507–522. http://doi.org/10.1111/j.1748-7692.2009.00291.x.

Kellar, N.M., Trego, M.L., Marks, C.I., Dizon, A.E., 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science. 22, 1–16. http://doi.org/10.1111/j.1748-7692.2006.00001.x.

Kenagy, G., Trombulak, S.C., 1986. Size and function of mammalian testes in relation to body size. Journal of Mammalogy. 67, 1-22.

Kenyon, K.W., Wilke, F., 1953. Migration of the northern fur seal, *Callorhinus ursinus*. Journal of Mammalogy. 34, 86–98. http://doi.org/10.2307/1375947.

Kersey, D.C., Dehnhard, M., 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. General and Comparative Endocrinology. 203, 296–306. http://doi.org/10.1016/j.ygcen.2014.04.022.

Ketterson, E.D., Nolan Jr, V., Wolf, L., Ziegenfus, C., 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco (*Junco hyemalis*). American Naturalist. 140, 980–999. http://doi.org/10.1086/285451.

Kintz, P., Cirimele, V., Jeanneau, T., Ludes, B., 1999. Identification of testosterone and testosterone esters in human hair. Journal of analytical toxicology. 23, 352–356. http://doi.org/10.1093/jat/23.5.352.

Kita, S., Yoshioka, M., Kashiwagi, M., 1999. Relationship between sexual maturity and serum and testis testosterone concentrations in short-finned pilot whales *Globicephala macrorhynchus*. Fisheries Science. 65, 878–883. http://doi.org/10.2331/fishsci.65.878.

Kiyota, M., Yonezaki, S., 2017. Reconstruction of historical changes in northern fur seal prey availability and diversity in the western North Pacific through individual-based analysis of dietary records. Deep-Sea Research Part Ii-Topical Studies in Oceanography. 140, 25-35. http://doi.org/10.1016/j.dsr2.2017.02.005.

Klasing, K.C., 2005. Potential impact of nutritional strategy on noninvasive measurements of hormones in birds. in: Bauchinger, U., Goymann, W., JenniEiermann, S., (Eds.), Bird Hormones and Bird Migrations: Analyzing Hormones in Droppings and Egg Yolks and Assessing Adaptations in Long-

Distance Migration, New York Acad Sciences, New York, pp. 5–16. http://doi.org/10.1196/annals.1343.003.

Kohn, M.H., Wayne, R.K., 1997. Facts from feces revisited. Trends in Ecology & Evolution. 12, 223–227. http://doi.org/10.1016/s0169-5347(97)01050-1.

Kohyama, K., Furuta, A., Nakajima, M., Baba, N., Kiyota, M., 1999. Serum testosterone level and body weight fluctuations related to he reproductive cycle in a captive adult male northern fur seal, *Callorhinus ursinus*. Journal of Japanese Association of Zoos and Aquariums. 40, 73–78.

Koren, L., Bryan, H., Matas, D., Tinman, S., Fahlman, Å., Whiteside, D., Smits, J., Wynne-Edwards, K., 2019. Towards the validation of endogenous steroid testing in wildlife hair. Journal of Applied Ecology. 56, 547–561. http://doi.org/10.1111/1365-2664.13306.

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Animal Behaviour. 63, 403–306. http://doi.org/10.1006/anbe.2001.1907.

Krone, N., Hughes, B.A., Lavery, G.G., Stewart, P.M., Arlt, W., Shackleton, C.H., 2010. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). The Journal of steroid biochemistry and molecular biology. 121, 496–504. http://doi.org/10.1016/j.jsbmb.2010.04.010.

Lander, R.H., 1981-82. A life table and biomass estimate for Alaskan fur seals. Fisheries Research. 1, 55–70. http://doi.org/10.1016/0165-7836(81)90007-2.

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., Gordon, J.I., 2008. Evolution of mammals and their gut microbes. Science. 320, 1647–1651. http://doi.org/10.1126/science.1155725.

Lincoln, G.A., 1998. Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). Animal Reproduction Science. 53, 87–105. http://doi.org/10.1016/s0378-4320(98)00129-8.

Mansour, A.A., Mkay, D.W., Lien, J., Orr, J.C., Banoub, J.H., ØIen, N., Stenson, G., 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science. 18, 112–120. http://doi.org/10.1111/j.1748-7692.2002.tb01022.x.

Mashburn, K.L., Atkinson, S., 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. General and Comparative Endocrinology. 136, 371–381. http://doi.org/10.1016/j.ygcen.2004.01.016.

Mauget, R., Boissin, J., 1987. Seasonal changes in testis weight and testosterone concentration in the European wild boar (*Sus scrofa L.*). Animal Reproduction Science. 13, 67–74. http://doi.org/10.1016/0378-4320(87)90120-5.

Mizuguchi, D., Mitani, Y., Kohshima, S., 2016. Geographically specific underwater vocalizations of ribbon seals (*Histriophoca fasciata*) in the Okhotsk Sea suggest a discrete population. Marine Mammal Science. 32, 1138–1151. http://doi.org/10.1111/mms.12301.

Moore, I.T., Jessop, T.S., 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Hormones and Behavior. 43, 39–47. http://doi.org/10.1016/s0018-506x(02)00038-7.

Mori, J., Kubodera, T., Baba, N., 2001. Squid in the diet of northern fur seals, *Callorhinus ursinus*, caught in the western and central North Pacific Ocean. Fisheries Research. 52, 91–97. http://doi.org/10.1016/s0165-7836(01)00233-8.

Mori, Y., Nagamitsu, T., Kubo, T., 2009. Clonal growth and its effects on male and female reproductive success in *Prunus ssiori* (Rosaceae). Population Ecology. 51, 175–186.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domestic Animal Endocrinology. 23, 67–74. http://doi.org/10.1016/s0739-7240(02)00146-7.

Moyes, C.D., Schulte, P.M., 2016. Prinicples of animal physiology. Pearson Canada: San Francisco.

Müller, K., Koster, S., Painer, J., Soderberg, A., Gavier-Widen, D., Brunner, E., Dehnhard, M., Jewgenow, K., 2014. Testosterone production and spermatogenesis in free-ranging Eurasian lynx (*Lynx lynx*) throughout the year. European Journal of Wildlife Research. 60, 569–577. http://doi.org/10.1007/s10344-014-0821-4.

Narayan, E.J., 2013. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. Conservation Physiology. 1, cot011. http://doi.org/10.1093/conphys/cot011.

Nelson, T.M., Rogers, T.L., Brown, M.V., 2013a. The gut bacterial community of mammals from marine and terrestrial habitats. Plos One. 9, e99562. http://doi.org/10.1371/journal.pone.0083655.

Nelson, T.M., Rogers, T.L., Carlini, A.R., Brown, M.V., 2013b. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environmental Microbiology. 15, 1132–1145. http://doi.org/10.1111/1462-2920.12022.

Noonan, L., Ronald, K., Raeside, J., 1991. Plasma testosterone concentrations of captive male hooded seals (*Cystophora cristata*). Canadian Journal of Zoology. 69, 2279–2282. http://doi.org/10.1139/z91-319.

Ostner, J., Kappeler, P.M., Heistermann, M., 2002. Seasonal variation and social correlates of androgen excretion in male redfronted lemurs (*Eulemur fulvus rufus*). Behavioral Ecology and Sociobiology. 52, 485–495. http://doi.org/10.1007/s00265-002-0532-9.

Otsuki, M., Akamatsu, T., Nobetsu, T., Mizuguchi, D., Mitani, Y., 2018. Diel changes in ribbon seal *Histriophoca fasciata* vocalizations during sea ice presence in the Nemuro Strait, Sea of Okhotsk. Polar Biology. 41, 451–456. http://doi.org/10.1007/s00300-017-2203-3.

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Animal Reproduction Science. 43, 43–63. http://doi.org/10.1016/0378-4320(95)01458-6.

Palme, R., Touma, C., Arias, N., Dominchin, M., Lepschy, M., 2013. Steroid extraction: get the best out of faecal samples. Wien Tierarztl Monatsschr. 100, 238–246.

Pelletier, F., Bauman, J., Festa-Bianchet, M., 2003. Fecal testosterone in bighorn sheep (*Ovis canadensis*): behavioural and endocrine correlates. Canadian Journal of Zoology. 81, 1678–1684. http://doi.org/10.1139/z03-156.

Plön, S., Ric, B., 2007. Testis, spermatogenesis, and testicular cycles. in: Jamieson, B.G.M., (Ed.), Reproductive biology and phylogeny of Cetacea: whales, dolphins, and porpoises, Science Publishers, Enfield, NH, pp. 215–244. http://doi.org/10.1201/b11001-9.

Pribbenow, S., Wachter, B., Ludwig, C., Weigold, A., Dehnhard, M., 2016. Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*). General and Comparative Endocrinology. 228, 40–47. http://doi.org/10.1016/j.ygcen.2016.01.015.

Putman, R., 1984. Facts from faeces. Mammal Review. 14, 79–97. http://doi.org/10.1111/j.1365-2907.1984.tb00341.x.

R Core Team, 2018. R: A language and environment for statistical computing, http://www.R-project.org/

Roberts, K.P., Zirkin, B.R., 1991. Androgen regulation of spermatogenesis in the rat. Annals of the New York Academy of Sciences. 637, 90–106. http://doi.org/10.1111/j.1749-6632.1991.tb27303.x.

Rolland, R.M., Hunt, K.E., Kraus, S.D., Wasser, S.K., 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. General and Comparative Endocrinology. 142, 308–317. http://doi.org/10.1016/j.ygcen.2005.02.002.

Rose, C., Parker, A., Jefferson, B., Cartmell, E., 2015. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. Critical Reviews in Environmental Science and Technology. 45, 1827–1879. http://doi.org/10.1080/10643389.2014.1000761.

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. Psychoneuroendocrinology. 37, 589–601. http://doi.org/10.1016/j.psyneuen.2011.09.009.

Samuels, T.P., Nedderman, A., Seymour, M.A., Houghton, E., 1998. Study of the metabolism of testosterone, nandrolone and estradiol in cattle. Analyst. 123, 2401–2404. http://doi.org/10.1039/a805531i.

Scheffer, V.B., 1962. Pelage and surface topography of the northern fur seal. U.S. Fish and Wildlife Service North American Fauna, 64, 206, http://doi.org/10.3996/nafa.64.0001.

Scheffer, V.B., Fiscus, C.H., Todd, E.I., 1984. History of scientific study and management of the Alaskan fur seal, *Callorhinus ursinus,* 1786–1964. NOAA Technical Report National Marine Fisheries Service, SSRF-780, 73.

Scheffer, V.B., Johnson, A.M., 1963. Molt in the northern fur seal. U.S. Fish and Wildlife Service Special Scientific Report, 450, 44.

Scherer, C., Wachter, U., Wudy, S., 1998. Determination of testosterone in human hair by gas chromatography-selected ion monitoring mass spectrometry. Analyst. 123, 2661–2663. http://doi.org/10.1039/a805131c.

Scheun, J., Greeff, D., Ganswindt, A., 2018. Non-invasive monitoring of glucocorticoid metabolite concentrations in urine and faeces of the Sungazer (*Smaug giganteus*). Peerj. 6, e6132. http://doi.org/10.7717/peerj.6132.

Schroeder, J.P., Keller, K.V., 1989. Seasonality of serum testosterone levels and sperm density in Tursiops truncatus. Journal of Experimental Zoology. 249, 316–321. http://doi.org/10.1002/jez.1402490310.

Schwarzenberger, F., Mostl, E., Palme, R., Bamberg, E., 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Animal Reproduction Science. 42, 515–526. http://doi.org/10.1016/0378-4320(96)01561-8.

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain Behavior and Immunity. 26, 1019–1029. http://doi.org/10.1016/j.bbi.2012.02.002.

Steimle, F., Terranova, R.J., 1985. Energy equivalents of marine organisms from the continental shelf of the temperate Northwest Atlantic. Journal of Northwest Atlantic Fishery Science. 6, 117–124. http://doi.org/10.2960/j.v6.a11.

Szymanski, D., Gist, D., Roth, T., 2006. Anuran gender identification by fecal steroid analysis. Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association. 25, 35–46.

Teichroeb, J.A., Sicotte, P., 2008. Social correlates of fecal testosterone in male ursine colobus monkeys (*Colobus vellerosus*): the effect of male reproductive competition in aseasonal breeders. Hormones and Behavior. 54, 417–423. http://doi.org/10.1016/j.yhbeh.2008.04.006.

Terwissen, C.V., Mastromonaco, G.F., Murray, D.L., 2014. Enzyme immunoassays as a method for quantifying hair reproductive hormones in two felid species. Conservation Physiology. 2, 1–9. http://doi.org/10.1093/conphys/cou044.

Touma, C., Palme, R., 2005a. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. in: Bauchinger, U., Goymann, W., JenniEiermann, S., (Eds.), Bird Hormones and Bird Migrations: Analyzing Hormones in Droppings and Egg Yolks and Assessing Adaptations in Long-Distance Migration, New York Acad Sciences, New York, pp. 54-74. http://doi.org/10.1196/annals.1343.006.

Touma, C., Palme, R., 2005b. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Annals of the New York Academy of Sciences. 1046, 54–74. http://doi.org/10.1196/annals.1343.006.

Tripovich, J.S., Rogers, T.L., Dutton, G., 2009. Faecal testosterone concentrations and the acoustic behaviour of two captive male Australian fur seals. Australian Mammalogy. 31, 117–122. http://doi.org/10.1071/am09009.

Trites, A.W., Bigg, M.A., 1996. Physical growth of northern fur seals (*Callorhinus ursinus*): Seasonal fluctuations and migratory influences. Journal of Zoology. 238, 459–482. http://doi.org/10.1111/j.1469-7998.1996.tb05406.x.

Tsubota, T., Nagashima, T., Kohyama, K., Maejima, K., Murase, T., Kira, I., 2001. Seasonal changes in testicular steroidogenesis and spermatogenesis in a northern fur seal, *Callorhinus ursinus*. Journal of Reproduction Development. 47, 415–420. http://doi.org/10.1262/jrd.47.415.

Van Pelt, T.I., Piatt, J.F., Lance, B.K., Roby, D.D., 1997. Proximate composition and energy density of some North Pacific forage fishes. Comparative Biochemistry and Physiology Part A: Physiology. 118, 1393–1398. http://doi.org/10.1016/S0300-9629(97)00240-5.

Ventrella, D., Elmi, A., Barone, F., Carnevali, G., Govoni, N., Bacci, M.L., 2018. Hair testosterone and cortisol concentrations in pre- and post-rut roe deer bucks: correlations with blood levels and testicular morphometric parameters. Animals. 8, 113. http://doi.org/10.3390/ani8070113.

Vladimirov, V.A., 1987. Age-specific reproductive behavior in northern fur seals on the Commander Islands. In: Croxall, J.P., Gentry, R.L., (Eds.), Status, biology, and ecology of fur seals, NOAA Technical Report National Marine Fisheries Service, 51, 113–120.

Wasser, S.K., Thomas, R., Nair, P.P., Guidry, C., Southers, J., Lucas, J., Wildt, D.E., Monfort, S.L., 1993. Effects of dietary fiber on fecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). Journal of Reproduction and Fertility. 97, 569–574. http://doi.org/10.1530/jrf.0.0970569

Wearmouth, V.J., Sims, D.W., 2008. Sexual segregation in marine fish, reptiles, birds and mammals: behaviour patterns, mechanisms and conservation implications. Advances in Marine Biology. 54, 107–170. http://doi.org/10.1016/s0065-2881(08)00002-3.

Webber, J.T., Henley, M.D., Pretorius, Y., Somers, M.J., Ganswindt, A., 2018. Changes in African elephant (*Loxodonta africana*) faecal steroid concentrations post-defaecation. Bothalia. 48, a2312. http://doi.org/10.4102/abc.v48i2.2312.

White, D., Berardinelli, J.G., Aune, K.E., 2005. Seasonal differences in spermatogenesis, testicular mass and serum testosterone concentrations in the grizzly bear. Ursus. 16, 198–208. http://doi.org/10.2192/1537-6176(2005)016[0198:sdistm]2.0.co;2.

Wilke, F., 1951. Pelagic fur seal research off Japan in 1950. General Headquarters, Supreme Commander for the Allied Powers, Natural Resources Section, Preliminary Study, 67, 35.

Wingfield, J.C., Ball, G.F., Dufty, A.M., Hegner, R.E., Ramenofsky, M., 1987. Testosterone and aggression in birds. American Scientist. 75, 602–608.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. American Naturalist. 136, 829–846. http://doi.org/10.1086/285134.

Witschi, E., 1961. Sex and secondary sexual characters. Academic Press: New York. http://doi.org/10.1016/C2013-0-12390-2.

Yamada, H., Satoh, R., Yamashita, T., Kambegawa, A., Iwata, M., 1997. Development of a time-resolved fluoroimmunoassay (TR-FIA) for testosterone: Measurement of serum testosterone concentrations after testosterone treatment in the rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology. 106, 181–188. http://doi.org/10.1006/gcen.1996.6861.

Yamanashi, Y., Morimura, N., Mori, Y., Hayashi, M., Suzuki, J., 2013. Cortisol analysis of hair of captive chimpanzees (*Pan troglodytes*). General and Comparative Endocrinology. 194, 55–63. http://doi.org/10.1016/j.ygcen.2013.08.013.

Yonezaki, S., Kiyota, M., Baba, N., 2008. Decadal changes in the diet of northern fur seal (*Callorhinus ursinus*) migrating off the Pacific coast of northeastern Japan. Fisheries Oceanography. 17, 231–238. http://doi.org/10.1111/j.1365-2419.2008.00475.x.

Yonezaki, S., Kiyota, M., Baba, N., Koido, T., Takemura, A., 2003. Size distribution of the hard remains of prey in the digestive tract of northern fur seal (*Callorhinus ursinus*) and related biases in diet estimation by scat analysis. Mammal Study. 28, 97–102. http://doi.org/10.3106/mammalstudy.28.97.

Appendices

Appendix A: Difference in faecal colour of the northern fur seal

Figure A.1. Difference in the colour of dried faeces of northern fur seals. The upper photograph shows the dried faeces of a captive northern fur seal when the seal was fed mackerel. The lower one shows those of free-ranging northern fur seals. Each petri dish contains faeces of different animals.

Appendix B: Study animals in Chapter 3 and 4.

Table B.1. Study animals in Chapter 3 and 4. Type shows the types of fur seal collections. The hyphens indicate no data or no measurements.

ID	Type	Date	Location	Age	Body length (cm)	Spermatogenesis
11FS03	Caught	2011-05-06	Matsumae	10	192.9	SP
11FS06	Caught	2011-05-25	Matsumae	10	191.5	SP
12019	Bycaught	2012-11-14	Matsumae	14	204.0	SP
12FS01	Caught	2012-01-23	Matsumae	9	201.0	SC
12FS02	Caught	2012-03-09	Matsumae	7	172.0	SC
12FS04	Caught	2012-03-09	Matsumae	7	157.0	SC
12FS09	Caught	2012-05-10	Matsumae	1	94.0	SG
12FS14	Caught	2012-05-21	Matsumae	10	181.5	SP
12FS15	Caught	2012-05-21	Matsumae	12	194.0	SP
13002	Stranded	2013-02-02	Esashi	8	164.5	SC
13FS01	Caught	2013-03-04	Matsumae	6	166.0	SC
13FS02	Caught	2013-03-05	Matsumae	11	183.0	SC
13FS04	Caught	2013-05-14	Matsumae	7	193.0	SP
13FS06	Caught	2013-05-15	Matsumae	2	99.5	SG
13FS07	Caught	2013-05-20	Matsumae	7	178.0	SP
13FS08	Caught	2013-05-20	Matsumae	7	174.4	SP
13FS09	Caught	2013-05-20	Matsumae	8	172.9	SP
13FS10	Caught	2013-05-20	Matsumae	10	203.7	SP
13FS12	Caught	2013-05-21	Matsumae	11	188.0	SP
13FS15	Caught	2013-05-25	Matsumae	7	163.0	SP
13FS17	Caught	2013-05-27	Matsumae	6	185.3	SP
13FS18	Caught	2013-05-27	Matsumae	8	178.0	SP
13FS19	Caught	2013-05-28	Matsumae	8	187.5	SP
14001	Stranded	2014-04-07	Otaruzenibako	9	-	SC
14003	Bycaught	2014-05-01	Kombumori	2	123.4	SC
14004	Bycaught	2014-05-09	Ogifushi	7	171.3	SP
14005	Bycaught	2014-06-07	Kombumori	3	123.8	SC
14006	Bycaught	2014-06-12	Kombumori	2	100	SG
14007	Bycaught	2014-06-12	Kombumori	8	177.5	SP
14021	Bycaught	2014-11-10	Hamanaka	2	88.2	SG
14FS02	Caught	2014-03-26	Matsumae	9	191.0	SC
14FS03	Caught	2014-03-27	Matsumae	7	173	SP
14FS05	Caught	2014-04-18	Matsumae	14	198.5	SP

ID	Type	Date	Location	Age	Body length (cm)	Spermatogenesis
14FS06	Caught	2014-04-30	Matsumae	10	183.0	SP
14FS08	Caught	2014-05-01	Matsumae	7	183.0	SP
14FS09	Caught	2014-05-01	Matsumae	4	130.5	SP
14FS10	Caught	2014-05-01	Matsumae	4	135.3	SP
14FS11	Caught	2014-05-01	Matsumae	11	198.2	SP
14FS12	Caught	2014-05-01	Matsumae	6	157.1	SP
14FS13	Caught	2014-05-20	Matsumae	6	173.1	SP
14FS14	Caught	2014-05-20	Matsumae	4	140.3	SP
14FS15	Caught	2014-05-20	Matsumae	9	198.5	SP
14FS17	Caught	2104-06-18	Matsumae	11	186.4	SP
15003	Stranded	2015-04-13	Setana	10	188.5	SC
15004	Caught	2015-05-03	Matsumae	9	191.2	SP
15FS01	Caught	2015-04-30	Matsumae	4	130.5	SP
15FS02	Caught	2015-04-30	Matsumae	4	129	SP
15FS03	Caught	2015-04-30	Matsumae	5	162.5	-
15FS04	Caught	2015-05-18	Matsumae	6	166.9	-
15FS05	Caught	2015-05-27	Matsumae	10	179.4	-
15FS06	Caught	2015-05-27	Matsumae	5	135.5	-
15FS08	Caught	2015-05-30	Matsumae	4	136.2	SP
15FS11	Caught	2015-06-14	Matsumae	10	194.0	SP
15FS12	Caught	2015-06-15	Matsumae	9	184.4	SP
15FS13	Caught	2015-06-16	Matsumae	9	183.7	SP
16FS02	Caught	2016-04-25	Matsumae	9	186	SP
16FS03	Caught	2016-05-01	Matsumae	11	203.5	SP
16FS04	Caught	2016-05-09	Matsumae	2	107.4	SG
16FS05	Caught	2016-05-09	Matsumae	10	205	SP
16FS06	Caught	2016-05-09	Matsumae	5	139.0	SP
16FS07	Caught	2016-05-14	Matsumae	2	96.2	SC
16FS08	Caught	2016-05-19	Matsumae	9	171.2	SP
MO1601	Caught	2016-05-20	Matsumae	8	163.2	SP
17FS01	Caught	2017-05-03	Matsumae	1	84.0	SP
17FS02	Caught	2017-05-03	Matsumae	7	175.2	SP
17FS03	Caught	2017-05-04	Matsumae	10	202.0	SP
17FS04	Caught	2017-05-05	Matsumae	8	180.0	SP
17FS05	Caught	2017-05-12	Matsumae	8	187.2	SP
17FS06	Caught	2017-05-23	Matsumae	8	177.2	SP
17FS08	Caught	2017-05-25	Matsumae	9	180.0	SP
17FS09	Caught	2017-06-01	Matsumae	8	180.4	SP

ID	Type	Date	Location	Age	Body length (cm)	Spermatogenesis
17FS10	Caught	2017-06-13	Matsumae	10	192.6	SP
MO1702	Caught	2017-04-21	Matsumae	6	143.5	SP
18FS01	Caught	2018-04-18	Matsumae	5	149.9	SP
18FS02	Caught	2018-06-04	Matsumae	8	167.4	SP
18FS03	Caught	2018-06-05	Matsumae	8	160.8	SP