Coastal Marine Science 44(1): 1–6, 2021

Effect of diet on faecal testosterone metabolite levels in a northern fur seal (*Callorhinus ursinus*) expressed as ash-free dry weight

Mayuko Otsuki[1]*, Kaoru Kohyama[2], Wataru Goshima[2], Motoki Kobayashi[1], Yuya Hasegawa[3], Yuka Morita[4], Shigeho Ijiri[3] and Yoko Mitani[1]

[1] *Field Science Center for Northern Biosphere, Hokkaido University, 20–5 Benten-cho, Hakodate, Hokkaido 040–0051, Japan*
[2] *Izu Mito Sea Paradise, 3–1 Nagahama Uchiura Numazu, Shizuoka 410–0224, Japan*
[3] *Graduate School of Fisheries Sciences, Hokkaido University, 3–1–1 Minato-cho, Hakodate, Hokkaido 041–8611, Japan*
[4] *Institute for East China Sea Research, Nagasaki University, 1551–7 Tairamachi, Nagasaki, Nagasaki 851–2213, Japan*
* *Corresponding author: motsuki@ees.hokudai.ac.jp*

Recieved 23 Aplil 2020; Accepted 3 December 2020

Abstract — The northern fur seal (*Callorhinus ursinus*) inhabits the North Pacific Ocean and Bering Sea, where it feeds on a variety of prey. In this study, we studied a captive male to determine how different prey affect the composition of and testosterone metabolites in the faeces. Faecal steroid metabolite levels are often expressed per unit dry weight (DW) of faeces, but dry faeces contain both organic and inorganic matter, so we also measured the ash-free dry weight (AFDW), which can more accurately express the levels in samples with organic matter. The mean organic component of faeces was lower when the seal fed on fish than when it fed on squid. Testosterone metabolite levels in the AFDW and serum testosterone levels showed a significant relationship in faeces collected one day after the blood withdrawal. When the seal fed on squid, differences in testosterone metabolite levels between DW and AFDW were small. These results suggest that faecal testosterone metabolite levels expressed in quantities per unit of AFDW are more useful than DW to understand the reproductive physiology of seals.

Key words: Faecal composition, ash-free dry weight, steroid hormones

Introduction

Steroid hormone measurements are valuable because they can be used as indices of health and reproductive physiology. Methods have been developed in various species to measure steroid hormone metabolites in faeces rather than using the more invasive technique of blood sampling. Steroid hormones in blood are metabolized in the liver and intestines (Möstl and Palme 2002, Touma and Palme 2005). Steroid metabolites retain most of their original skeletal structure in the faeces (Macdonald et al. 1983) and can be measured. Steroid hormone metabolites are organic compounds, but in faeces, steroid metabolite levels are generally expressed per unit of dry weight (DW) of the faeces (Palme et al. 2013). Steroid metabolite levels expressed in DW can be influenced by the ratio of organic to inorganic matter in faeces, which can lead to errors in the estimation of these levels. For this reason, it is important to remove bone fragments or undigested fibrous materials when preparing sample materials for the analysis of faecal steroid hormones using DW techniques (Ganswindt et al. 2012). In addition, faecal steroid metabolite levels can also change based on the type of diet (Goymann 2012). If faeces contain large amounts of inorganic matter, faecal steroid metabolite levels per unit DW may less accurately show hormone concentrations.

An alternative method that eliminates this problem is to measure steroid metabolite levels in quantities per unit of ash-free dry weight (AFDW). This method was used by Ganswindt et al. (2012) who expressed the steroid metabolite levels in faeces of aardwolves (*Proeles cristata*), which ingest soil during feeding, per unit AFDW after eliminating water and the inorganic content. Although the qualitative interpretations of the hormone metabolite levels did not change, they demonstrated that steroid metabolite levels in AFDW showed less individual variation, which allowed it to detect biologically relevant differences.

Northern fur seals (*Callorhinus ursinus*) alter their diet depending on the abundance and distribution of prey within their habitat (Kajimura 1984). They often feed on Pacific mackerel (*Scomber japonicus*), Japanese sardine (*Sardinops melanostictus*), myctophids and squids (Yonezaki et al. 2008). A recent study found that they also consume Okhotsk atka mackerel (*Pleurogrammus azonus*) and walleye pollock (*Gadus chalcogrammus*) (Horimoto 2015). The diet of northern fur seals differs widely among individuals (Mori et al.

2001, Yonezaki et al. 2003). This diverse diet can lead to variation in the ratio of organic and inorganic matter in faeces. Changes in faecal steroid metabolites can be more accurately measured by removing inorganic materials in faeces when animals vary their diet seasonally. However, the ways in which faeces composition can influence faecal steroid metabolite levels have received little scientific attention.

Testosterone can be used as an indicator of male reproductive physiology (Kita et al. 1999). Testosterone metabolite levels in faeces can be used as such indices, but it is important to understand how these levels are affected by the seals' diet. In this study, we examined how diet affected the composition of faeces in a captive male northern fur seal and demonstrate the relationship between faecal testosterone metabolite levels per unit AFDW and serum testosterone levels. We also examined how the diet composition influences faecal testosterone metabolite levels using DW and AFDW.

Materials and Methods

This study examined an 8-year-old male (mean body weight±SD=86.3±4.1 kg) at the Izu Mito Sea Paradise aquarium (Numazu, Japan) between April 2017 and January 2018. Sampling was conducted twice a month. Blood (5 mL) was withdrawn from the hind flipper (Day 0), and faeces were collected the following three mornings (Day 1 to 3) (Otsuki et al. 2020). The faeces were collected from a pool using a dip net with 4-mm mesh.

Feeding experiments were conducted over four days each month from October 2017 to January 2018 during the non-breeding season. This season was chosen to avoid the large variations that occur in serum testosterone levels during the breeding season. Before each experiment, the seal was fed daily a diet of Pacific mackerel, but one day before each experiment, no food was given. The experimental diets comprised Japanese flying squid (*Todarodes pacificus*) in October and January, Okhotsk atka mackerel in November, and walleye pollock in December. The seal was fed 2.4–6 kg whole fish or squid each day. Faeces were collected on the second to fourth day, as the first day of feeding provided a buffer for the change in prey species. Serum and faecal samples were stored frozen at −40°C and shipped to the Field Science Center for Northern Biosphere, Hokkaido University, for analyses.

In the laboratory, all faeces from the feeding experiments were analyzed along with seven randomly selected faecal samples collected while the seal was fed Pacific mackerel. Each sample was dried at 60°C to remove water content and homogenized (Otsuki et al. 2020). A 500 mg quantity of each was burned in an electrical muffle furnace (Motoyama Co., Ltd., Japan) based on the method in JIS:M:8812:2006. The temperature was increased from 25°C to 500°C over 1 h and then increased to 815°C over 30 min. The sample remained at 815°C for one hour. The mass of the ash was measured before and after additional heating for another 30 min at 815°C to ensure that the mass of the ash was consistent. The AFDW was calculated by subtracting the mass of inorganic content (ash) from the DW.

Steroid metabolites were extracted from 10 mg of faeces with 80% methanol. Steroid extraction was performed according to the method in Otsuki et al. (2020). Faecal testosterone metabolite levels were analyzed by time-resolved fluoroimmunoassay (DEL-FIA®, PerkinElmer, Waltham, MA) based on Yamada et al. (1997) and Otsuki et al. (2020).

The weight percentage of organic content in faeces was determined by dividing the AFDW of faeces by the DW. We averaged the AFDW percentages of the seven faecal samples collected during feeding with Pacific mackerel to convert faecal metabolite levels in DW to AFDW. For faeces containing squid, Okhotsk atka mackerel and pollock, the AFDW percentage for sample was calculated separately. Faecal testosterone metabolite levels in AFDW were computed by dividing the testosterone metabolite levels by the mass of organic content in the faeces. Faecal testosterone metabolite levels in AFDW with Pacific mackerel were computed using the average AFDW percentage of the seven samples. Those with other prey species were computed using each AFDW percentage. The faecal testosterone metabolite levels of Days 1, 2 and 3 per unit AFDW, including the samples collected during the feeding experiments, was fitted with serum testosterone levels using a generalized linear model (gamma error distribution with a log link). Serum testosterone levels in the seal were obtained from Otsuki et al. (2020). Significance of the models was determined with a null model using an analysis of deviance (Bradshaw et al. 2004). Monthly changes in faecal testosterone metabolite levels on Day 1 expressed as per unit AFDW were plotted and compared with the levels in DW and serum testosterone levels using the data of Otsuki et al. (2020). Furthermore, the time series data of all the faecal testosterone metabolite levels in DW and AFDW on Days 1 through 3 were plotted to show how the diet composition influenced the measurement of these levels. The level of statistical significance was set to α=0.05. The software used for this analysis was R 3.5.1 (R Core Team 2019), and figures were created using the ggplot2 package (Wickham 2016).

Results

For the dietary comparison, seven Pacific mackerel faeces collected on Days 1 and 2 were used. During the feeding experiment with squid, we found faeces on Day 1 in October and Day 3 in January. Similarly, faeces during Okhotsk atka mackerel feeding were collected only on Days 1

and 3. We collected faecal samples from pollock feeding on all three days. The mean±SD of AFDW percentages when the seal fed on Pacific mackerel, squid, Okhotsk atka mackerel and pollock was 44.1±4.0, 60.6±30.2, 41.5±2.3, and 42.5±2.9%, respectively (Fig. 1). The organic content of faeces collected on Day 3 when the seal fed on squid was 81%. This was much greater than the organic content of faeces collected on Day 1 and of faeces in the experiments using fish as prey.

Serum testosterone levels on Day 0 was a significant predictor of faecal testosterone metabolite levels in AFDW on Day 1 (Estimate=0.3845, SE=0.1246, p=0.0087, Fig. 2). An analysis of deviance revealed significant support for the correlation between these two levels (Deviance=0.98, F(1,13)=8.20, p=0.0133). On the other hand, serum testosterone levels did not predict faecal testosterone metabolite levels expressed in AFDW on Day 2 (Estimate=0.1729, SE=0.1878, p=0.3754). Serum testosterone was a marginally significant predictor of the faecal metabolite levels in AFDW on Day 3 (Estimate=0.5075, SE=0.2314, p=0.0507).

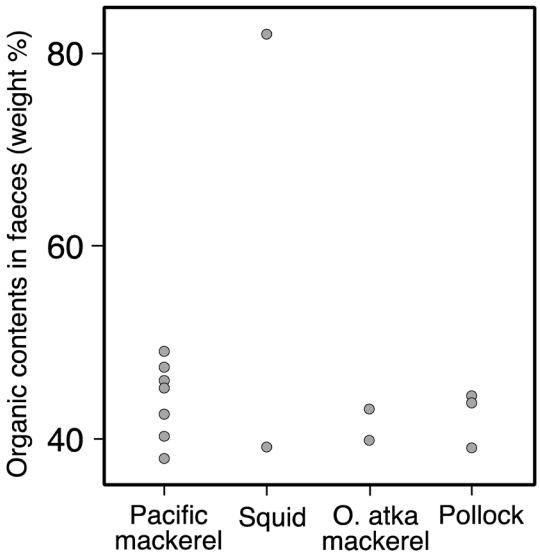

Fig. 1. Organic content in faeces expressed as a percentage of ash-free dry weight in a captive male northern fur seal. The seal was fed four diets consisting of a single prey species: Pacific mackerel, Japanese flying squid (squid), Okhotsk atka mackerel (O. atka mackerel), and Walleye pollock (pollock).

Monthly faecal testosterone metabolite levels expressed as AFDW on Day 1 ranged from 0.30 to 2.15 $\mu g\,g^{-1}$ (Fig. 3). Faecal testosterone metabolite levels expressed in AFDW showed a similar trend to serum testosterone levels. Faecal testosterone metabolite levels peaked in July in both DW and AFDW. The variation of faecal testosterone metabolite levels in AFDW was larger compared to those expressed as DW. We show a time series of faecal testosterone metabolite levels in DW and AFDW on Day 1 through 3 to compare the effect of the four prey species (Fig. 4). In addition to squid on Day 1 in October, when the seal fed on Pacific mackerel, Okhotsk atka mackerel, and pollock, the differences in the metabolite levels between the two analytical techniques (AFDW and DW) were large. When squid was fed on Day 3 in January, however, the variations in the levels between DW and AFDW were minimal.

Discussion

We examined the composition of faeces when the seal fed on different prey species and measured how changes in faecal composition influenced faecal testosterone metabolite levels. In all feeding experiments, the organic content of faeces was below 50%, except for Day 3 in January when the seal fed on squid. One reason for this difference is that the initial organic matter found in squid is greater than that of fish. For example, the organic content of squids such as

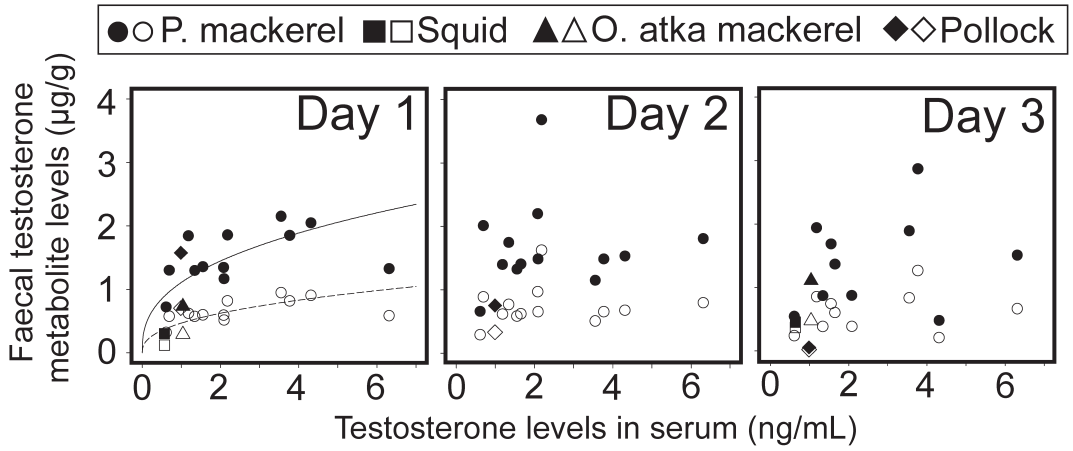

Fig. 2. Relationships between faecal testosterone metabolite levels (expressed in $\mu g\,g^{-1}$ of ash-free dry weight (AFDW): solid symbols, and dry weight (DW): open symbols) and serum testosterone levels in a male northern fur seal on Days 1, 2 and 3 of feeding experiments. Symbol shapes indicate prey species. The broken line shows a fitted line based on DW (Otsuki et al. 2020) while the solid line is a fitted line from a GLM model based on the levels in AFDW. No lines on Days 2 and 3 indicate no significant relationships.

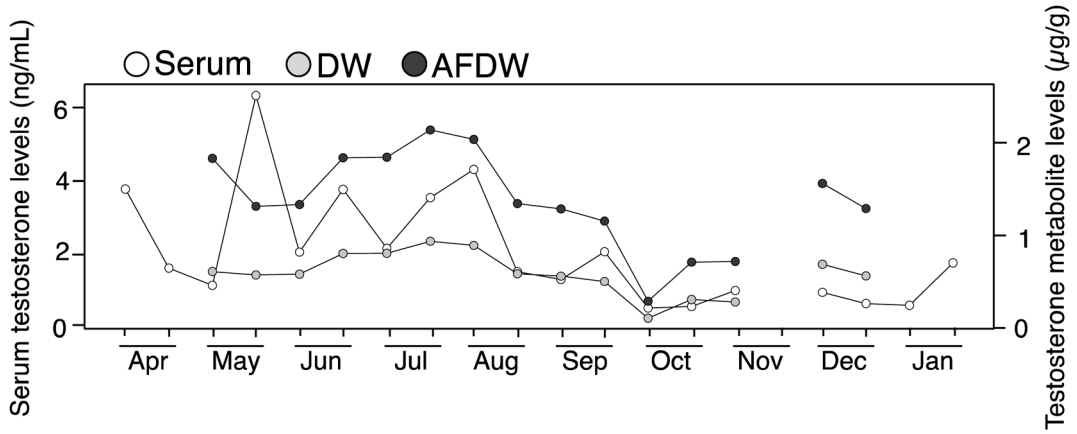

Fig. 3. Monthly changes in serum testosterone levels and faecal testosterone metabolite levels in a male northern fur seal on Day 1 expressed as per unit DW and AFDW. Grey and black filled circles represent the faecal testosterone metabolite levels expressed as DW and AFDW, respectively. Open circles represent serum testosterone levels based on the results from Otsuki et al. (2020).

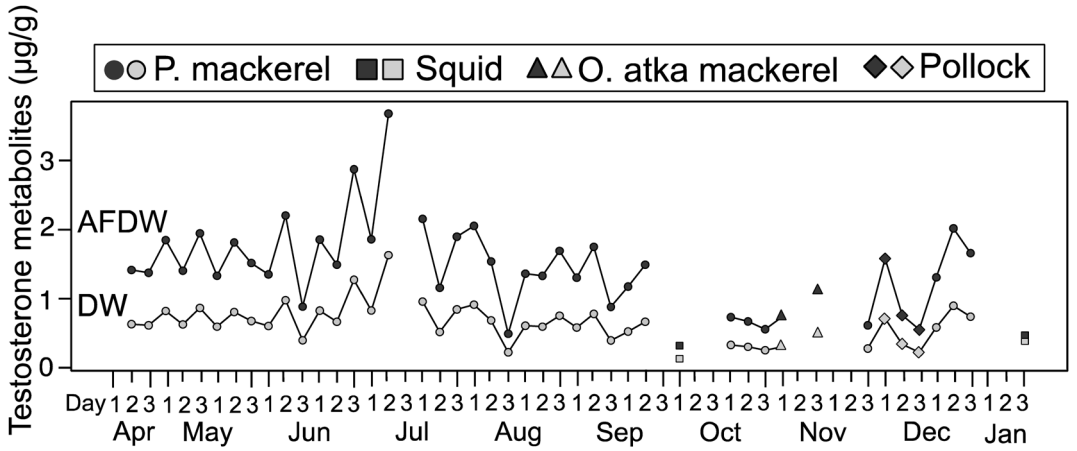

Fig. 4. Faecal testosterone metabolite levels expressed as per unit DW and AFDW on Day 1 through Day 3 between April 2017 and January 2018. Symbols indicate prey species. Light and dark grey circles represent the levels in DW and AFDW, respectively. Each tick mark on the x-axis indicates one sampling day during the study.

Gonatidae and *Illex sp.* is about 92%, whereas it is 88% in Pacific mackerel (Steimle and Terranova 1985, Van Pelt et al. 1997) and approximately 83% in Okhotsk atka mackerel and pollock (Van Pelt et al. 1997). In addition to the organic content of the prey species itself, switching between prey species might have changed the bacterial composition of the faeces. For instance, the organic portion of faeces in humans is about 25–54% bacteria, while the inorganic content comes from remaining food (Rose et al. 2015). Since the amount and kind of faecal microbiota in animals are influenced by prey choice (Ley et al. 2008, Nelson et al. 2013, Banks et al. 2014), this seal's microbiota (which composed a portion of the organic content of its faeces) is likely to have changed by switching prey species to squid. The composition of microbiota can change within 24 h of diet switching in humans (Wu et al. 2011). Furthermore, although the feeding experiment using squid was conducted in a different month, the large variations in the faecal composition within the squid diet on Days 1 and 3 may also come from gradual changes in the microbiota composition as the prey species was switched. In humans who consumed an animal-based diet, the changes in diversity of microbiota was maximized on the fourth day after diet switching (David et al. 2014). Since the diet of northern fur seals includes various fish and squid species (Kajimura 1984, Horimoto 2015), faecal steroid metabolite levels may change according to differences in diet. Different prey types, therefore, likely influenced the composition of microbiota in faeces and possibly resulted the alternation of faecal composition in the northern fur seal.

The faecal testosterone metabolite levels per unit AFDW were related to the serum testosterone levels on Day 1, but not on Day 2, and only marginally related on Day 3. These tendencies were consistent with faecal testosterone metabolite levels expressed in DW (Otsuki et al. 2020). This significant relationship on Day 1 is also in line with the relationship in other mammals (Schwarzenberger et al. 1996).

Although few studies have examined how diet influences faecal steroid metabolite levels, the differences in consumption of fat amount can alter serum testosterone levels (Hämäläinen et al. 1984). It is possible that diet can alter faecal steroid metabolites levels not only faecal composition but also changes in serum steroid levels. Because of this, changes in testosterone levels may not be detected even in serum. Removal of inorganic contents in faeces may eliminate this problem. Although this study was limited by its small sample size, steroid metabolite levels expressed per unit AFDW has the potential to correct variations caused by the diet.

Monthly faecal testosterone metabolite levels in AFDW and serum testosterone levels showed similar changes. The increase in faecal testosterone metabolite levels in July suggests a physiological response during the breeding season between June and August (Gentry 1998), and thus the metabolite levels expressed in AFDW might represent the reproductive physiology of this species. Although the metabolite levels in AFDW and DW showed a similar trend, the variance between the two expressions was greater when the seal fed on fish and smaller when it fed on squid in January. Hence, the metabolite levels in DW and AFDW were affected by the prey species consumed. The consumption of fish generated large differences in the levels between DW and AFDW, whereas that of squid resulted in small differences due to the very small amount of inorganic matter in this prey species. These outcomes come from the composition of faeces as the consumption of squid on Day 3 in January resulted in a greater organic content in the faeces. The conventional method of using DW may not be able to detect changes in steroid hormonal levels, and thus AFDW can be useful when prey species are unknown and animals feed on various species of prey. Our sample size was small in terms of both animals studied and number of faeces collected. Future studies of long-term monitoring of faecal testosterone metabolites should examine a larger number of animals. Our results suggest that changes in the physiology of animals is more accurately determined by expressing faecal hormone metabolite levels as quantity per unit AFDW. The improvement of accuracy in faecal steroid hormone levels can benefit studies of free-ranging northern fur seals especially when they haul out on land during the breeding season. This technique can also be useful for pinniped species that feed opportunistically and haul out on land. In captivity, it is important to feed the same diet to an animal over the period of faecal steroid metabolite monitoring.

In summary, it can be helpful to represent faecal steroid metabolite levels by AFDW when the animal's diet is unknown. Thus, when analyzing faecal steroid levels among individuals or when sampling intermittently, it is useful to represent the levels in AFDW. Although further studies such as challenge tests and a long-term feeding experiment will be necessary, converting faecal testosterone metabolite levels into quantity per unit AFDW is preferred, since free-ranging northern fur seals feed on wide variety of prey species.

Acknowledgements

We thank the staff of the Izu Mito Sea Paradise aquarium, especially Naoki Yoshikawa, for helping collect samples. Chak Aranyakanont, Megumi Tada, and Yukino Hirakawa assisted during sample preparation and measurements. We thank Brianna Wright and John Bower for proofreading our manuscript and providing useful comments. We also thank two anonymous referees for their constructive suggestions to improve our manuscript. This research was partially supported by the Cooperation Research Program of Wildlife Research Center, Kyoto University (2017-A-30) and KAKENHI 15H05709.

References

Banks, J. C., Cary, S. C. and Hogg, I. D. 2014. Isolated faecal bacterial communities found for Weddell seals, *Leptonychotes weddellii*, at White Island, McMurdo Sound, Antarctica. Polar Biol. 37: 1857–1864.

Bradshaw, C. J. A., Higgins, J., Michael, K. J., Wotherspoon, S. J. and Hindell, M. A. 2004. At-sea distribution of female southern elephant seals relative to variation in ocean surface properties. ICES J. Mar. Sci. 61: 1014–1027.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J. and Turnbaugh, P. J. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505: 559–563.

Ganswindt, A., Muilwijk, C., Engelkes, M., Muenscher, S., Bertschinger, H., Paris, M., Palme, R., Cameron, E. Z., Bennett, N. C. and 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. Physiol. Biochem. Zool. 85: 194–199.

Gentry, R. L. 1998. Behavior and ecology of the northern fur seal. Princeton University Press, Princeton, NJ.

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 Ecol. Evol. 3: 757–765.

Hämäläinen, E., Adlercreutz, H., Puska, P. and Pietinen, P. 1984. Diet and serum sex hormones in healthy men. J. Steroid Biochem. 20: 459–464.

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

Kajimura, H. 1984. Opportunistic feeding by the northern fur seal, *Callorhinus ursinus*, in the eastern North Pacific Ocean and eastern Bering Sea. Page 49. NOAA Technical Report National Marine Fisheries Service.

Kita, S., Yoshioka, M. and Kashiwagi, M. 1999. Relationship between sexual maturity and serum and testis testosterone concentrations in short-finned pilot whales *Globicephala macrorhynchus*. Fish. Sci. 65: 878–883.

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. and Gordon, J. I. 2008. Evolution of mammals and their gut microbes. Science 320: 1647–1651.

Macdonald, I., Bokkenheuser, V., Winter, J., McLernon, A. and Mosbach, E. 1983. Degradation of steroids in the human gut. J. Lipid Res. 24: 675–700.

Mori, J., Kubodera, T. and Baba, N. 2001. Squid in the diet of northern fur seals, *Callorhinus ursinus*, caught in the western and central North Pacific Ocean. Fish. Res. 52: 91–97.

Möstl, E. and Palme, R. 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23: 67–74.

Nelson, T. M., Rogers, T. L. and Brown, M. V. 2013. The gut bacterial community of mammals from marine and terrestrial habitats. PLoS One 9: e99562.

Otsuki, M., Kohyama, K., Goshima, W., Kobayashi, M., Hasegawa, Y., Morita, Y., Ijiri, S. and Mitani, Y. 2020. Non-invasive monitoring of faecal testosterone metabolte concentrations in a northern fur seal (*Callorhinus ursinus*). Jpn. J. Zoo. Wildl. Med. 25: 29–34.

Palme, R., Touma, C., Arias, N., Dominchin, M. and Lepschy, M. 2013. Steroid extraction: get the best out of faecal samples. Wien. Tierärztl. Monat—Vet. Med. Austria 100: 238–246.

R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rose, C., Parker, A., Jefferson, B. and Cartmell, E. 2015. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. Crit. Rev. Environ. Sci. Technol. 45: 1827–1879.

Schwarzenberger, F., Mostl, E., Palme, R. and Bamberg, E. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci. 42: 515–526.

Steimle, F. and Terranova, R. J. 1985. Energy equivalents of marine organisms from the continental shelf of the temperate Northwest Atlantic. J. Northwest Atl. Fish. Sci. 6: 117–124.

Touma, C. and Palme, R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Ann. N. Y. Acad. Sci. 1046: 54–74.

Van Pelt, T. I., Piatt, J. F., Lance, B. K. and Roby, D. D. 1997. Proximate composition and energy density of some North Pacific forage fishes. Comp. Biochem. Physiol. A Physiol. 118: 1393–1398.

Wickham, H. 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag, New York.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D. and Lewis, J. D. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science 334: 105–108.

Yamada, H., Satoh, R., Yamashita, T., Kambegawa, A. and 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*). Gen. Comp. Endocrinol. 106: 181–188.

Yonezaki, S., Kiyota, M. and Baba, N. 2008. Decadal changes in the diet of northern fur seal (*Callorhinus ursinus*) migrating off the Pacific coast of northeastern Japan. Fish. Oceanogr. 17: 231–238.

Yonezaki, S., Kiyota, M., Baba, N., Koido, T. and 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. Mamm. Study 28: 97–102.