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Aging and Seasonal Serum Cortisol Concentrations in Captive Spotted Seals (*Phoca largha*) from the Liaodong Bay Colony

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Abstract

Cortisol, a regulator of both energy intake and storage, changes with an animal's physiological state and is often used as an index of body fitness and stress response. To obtain a baseline concentration of cortisol in spotted seals, we analyzed serum cortisol concentrations using electro-chemiluminescence immunoassay in captive spotted seals (*Phoca largha*) (individual seals could be sampled multiple times with 64 cortisol samples from females and 81 from males) of different ages and in different reproductive seasons from the Liaodong Bay colony. Serum cortisol concentration first dropped steadily from 359 (334 to 463) nmol/L at 1 year of age to 254 ± 81 nmol/L at 6 years of age, and then increased steadily to 387 ± 55 nmol/L at age 12. This cortisol concentration pattern appears to experience a nadir concomitant with sexual maturity. We found significant serum cortisol concentration differences in the varying reproductive seasons, with cortisol concentration in the pre-breeding season (November to January; 341 ± 88 nmol/L) and the breeding-molt season (February to March; 355 ± 108 nmol/L) higher than in the other season (April to October; 212 [155 to 267] nmol/L; because the data for the other season is skewed, the interquartile range was presented). We did not detect serum cortisol concentration differences between male and female seals or between immature and mature seals. The present study provides a normal serum cortisol concentration profile related to age, sex, and reproductive season for captive spotted seals from the Liaodong Bay colony, which can provide helpful reference material for wild counterparts of this polar species.

Key Words: spotted seal, *Phoca largha*, cortisol, Liaodong Bay, glucocorticoid

Introduction

Cortisol is the major glucocorticoid in mammals (Liberzon et al., 1997) and is demonstrated as one of the regulators of both intake and storage of energy (Djurhuus et al., 2002). High glucocorticoid concentration can increase gluconeogenesis and protein catabolism (Sapolsky et al., 2000). Cortisol is released when an animal encounters a stressor (Sapolsky et al., 1986). Stressor stimuli usually come in two forms: (1) internal stimuli are the anticipated physiological stressors during typical life history events such as breeding, gestation, and lactation (Engelhard et al., 2002; Champagne et al., 2006; Burgess et al., 2013; Kershaw & Hall, 2016); and (2) external stimuli are environmental stressors such as human impacts and climate changes (Morton et al., 1995; Bechshøft et al., 2013). Baseline concentrations of cortisol are increasingly used as physiological indices of relative condition or health of individual animals and populations (Wikelski & Cooke, 2006; Cooke & O'Connor, 2010; Madliger & Love, 2014).

Some studies have shown that glucocorticoid or cortisol concentrations may indicate condition or fitness of an individual and maybe even a population (Bonier et al., 2009; Burgess et al., 2013). For example, Macbeth et al. (2013) showed that polar bear (*Ursus maritimus*) cortisol concentrations were negatively associated with their growth indices. Another case study on an endangered Grévy's zebra (*Equus grevyi*) reported that cortisol concentrations could reflect an animal's stress during translocation and acclimation (Franceschini et al., 2008). Glucocorticoid or cortisol also increased and was used to monitor the physiological state of marine mammals. For example, through analysis of glucocorticoid in feces, scientists monitored adrenal activity and reproductive condition of North Atlantic right whales (*Eubalaena glacialis*; Hunt et al., 2006), and they found that ship noise was associated with chronic stress in these whales (Rolland et al., 2012). Through analysis of cortisol concentrations in both plasma and blubber, scientists suggested that blubber cortisol concentrations had the potential to be used as physiological state indicators in phocid seals (Kershaw & Hall, 2016).

In pinnipeds, background cortisol concentration changes with an animal's physiological state such as breeding, molting, and onset of reproductive maturity (du Dot et al., 2009; Myers et al., 2010; Kershaw & Hall, 2016). For example, cortisol concentrations increase in lactating fur seals (*Arctocephalus tropicalis*) and fasting northern elephant seals (*Mirounga angustirostris*) (Guinet et al., 2004; Champagne et al., 2006); cortisol concentrations are correlated with the molt in harbor seals (*Phoca vitulina*), spotted seals (*P. largha*), and fur seals (Ashwell-Erickson et al., 1986; Atkinson et al., 2011; Kershaw & Hall, 2016); and high concentrations of cortisol were noted in the breeding season in Weddell seals (*Leptonychotes weddelli*; Bartsh et al., 1992), and at onset of sexual maturity in Steller sea lions (*Eumetopias jubatus*; Myers et al., 2010).

The spotted seal is the only pinniped species that reproduces in the wild in China (Rugh et al., 1997), and they currently face many challenges that result in a trending population decline due to human influences such as overfishing and environmental pollution (Dong, 1991; Han et al., 2010). Conservation of spotted seals in Chinese waters has been on the agenda of local scientific and conservation communities since the 1980s. Studies relevant to the conservation of spotted seals in the Liaodong Bay colony have been conducted on several topics such as population dynamics, population genetics, migration, reproductive physiology, and acoustics (Wang et al., 2008; Han, 2009; Han et al., 2010, 2013; Gao et al., 2013; Zhang et al., 2014a, 2014b, 2016; Yang et al., 2017; Ma et al., 2018; Yan et al., 2018), but the physiological status of wild seals in the Liaodong Bay colony has not yet been reported. In addition, it is not currently possible to monitor the fitness and condition of the wild seals in Liaodong Bay due to the rigorous environment and lack of logistics/resources.

In order to investigate background cortisol concentrations of spotted seals in the Liaodong Bay colony, in the present study, we collected blood samples from a captive seal population in the Dalian Sun Asia Aquarium (DSAA) that was rescued from the Liaodong Bay colony; through this collection, we were able to examine cortisol concentrations throughout the entire reproductive cycle. Through analysis of serum cortisol concentrations in spotted seals in DSAA, we investigated differences in serum cortisol between reproductive stages and changes with animal age. Our results should allow for examination of potential patterns in baseline cortisol concentration throughout the year in spotted seals, and should also provide background information to help scientists better monitor the physiological status and fitness of both captive and wild spotted seals.

Methods

Animals and Blood Sampling

The animals in this study were long-term captive individuals from DSAA. Spotted seals were either rescued at less than 1 y old from the Liaodong Bay colony in China between 2000 and 2006 or they were born in human care at the DSAA between 2005 and 2011. The total potential sample population included 62 seals of which 54 seals were part of this study, and individuals could be sampled more than once a year. We obtained seal age from the studbook or historical resighting data, and spotted seal age ranged between 1 and 12 y. During a 1-y period (July 2012 to June 2013), we sampled an average of 12 (range 1 to 28) randomly selected individuals each month. Pregnant females were not involved in this study and, thus, were not sampled. We obtained a total of 145 samples (female: n = 64; male: n = 81). Approximately 3 mL of blood was collected for each sample. It should be noted that these numbers represent the sample number rather than the animal number. Between 0700 and 0900 h, blood samples were collected from hind flippers using plain syringes while the animals were restrained on a V-shaped bench. It took approximately 5 to 10 min from first restraint to finish sampling one individual. Blood samples were stored in medical biochemical tubes with coagulation gel for serum extraction. Sera were separated by centrifugation (1,500× g for 15 min) after collection and stored at 4°C. Assays were conducted within 6 h.

Serum concentrations (20 µL per serum sample) of cortisol were measured for both sexes via electro-chemiluminescence immunoassay (ECLIA), using a Cobas® e411 (Roche Diagnostics, Mannheim, Germany) and cortisol assay kits (Cobas®), following the manufacturer's instructions. A Tris (2,2'-bipyridyl)ruthenium(II) complex was used as a label in these assays. The cortisol kit (ovine-antibody, Intra-Assay: coefficient variation [CV] 5.6%; Inter-Assay: 5.1%; n = 12) had an analytical sensitivity range of

0.5 to 1,750 nmol/L and functional sensitivity of < 8.5 nmol/L (the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of 20%). The total cross-reactions of the cortisol antiserum were corticosterone, 5.8%; 11-deoxycortisol, 4.1%; 17-α-hydroxyprogesterone, 1.5%; 11-deoxycorticosterone, 0.69%; and progesterone, 0.35%. The kits used in this study were validated by running serial dilutions of reconstituted pools of serum from spotted seals, and results were compared to those of the standard (see Appendix Table 1).

After each calibration and during each run for routine testing, Elecsys PreciControl Universal one and two from the Roche Diagnostics GmbH were used for internal quality control. External quality assessment of the assays was continuously performed and certified by the accredited Reference Institute for Bioanalytics (RfB, Bonn, Germany; www.dgkl-rfb.de).

Data Analysis

Trend line was used to assess cortisol concentrations from the spotted seals at different ages for both sexes plotted via package "ggplot2" (https://ggplot2.tidyverse.org). Seals of 1 to 3 y of age were considered as sexually immature, and seals older than 4 y of age were treated as mature as reported by Zhang et al. (2014b). Seasons were defined into three sections: (1) pre-breeding (November to January), (2) breeding-molt (February to March), and (3) other (April to October) according to spotted seal reproductive behaviors observed in the Liaodong Bay colony (Boveng et al., 2009). Data obeying normal distribution were described as mean ± standard deviation (SD), while skewed distributions were described as median (interquartile range). Cortisol concentrations comparison among different seasons was conducted using one-way ANOVA in *SPSS*, Version 20.0 (SPSS Inc., Chicago, IL, USA). Cortisol concentrations comparison between sexually immature and mature seals was conducted using two-factor ANOVA with "age" and "season" as factors; and comparison between seals with different sex was conducted using two-factor ANOVA with "sex" and "season" as factors. A p value of less than 0.05 was set as the level of significance.

Results

The age of sampled spotted seals ranged between 1 and 12 y. Serum cortisol concentrations dropped steadily from 350 ± 130 nmol/L at 1 y to 254 ± 81 nmol/L at 6 y, and then increased steadily to 387 ± 55 nmol/L at 12 y of age, forming a typical "V" shaped trend line with the trough located at 6 y (Figure 1). We did not find significant cortisol concentration differences between seals 0 to 6 y of age and 7 to 12 y of age (two-factor ANOVA, $p > 0.05$). Serum cortisol concentration values for seals of different sex, age class, and reproductive seasons are shown in Table 1.

Serum cortisol concentrations from the three identified reproductive seasons were significantly different (one-way ANOVA, $p < 0.05$), with mean cortisol concentrations in the pre-breeding season (341 ± 88 nmol/L, ± SD) and the breeding-molt season (355 ± 108 nmol/L) higher than in the other season, 212 (155 to 267) nmol/L (interquartile) (Table 1; Figure 2). Although trends in serum cortisol concentrations between sexually immature and mature individuals changed as seals aged (i.e., cortisol concentrations decreased in sexually immature individuals but increased in mature individuals; Figure 1), no statistically significant difference of cortisol concentrations was detected between immature and mature animals (two-factor ANOVA, $p > 0.05$; Table 1).

Serum cortisol concentrations for males and females in different seasons are shown in Table 1. No significant serum cortisol concentration difference was detected between male and female spotted seals (two-factor ANOVA, $p > 0.05$).

Discussion

In the present study, a clear age-related cortisol concentration pattern was observed in spotted seals (Figure 1). This pattern is believed to be representative of normal changes of cortisol concentration as seals aged and is driven by internal stimuli (physiological stressors); this interpretation is supported by previous studies (Engelhard et al., 2002; Champagne et al., 2006; Burgess et al., 2013; Kershaw & Hall, 2016), which showed that greater cortisol responses were necessary for juveniles to preserve body homeostasis and body composition to the same level as subadults in Steller sea lions and dugongs (*Dugong dugon*) (du Dot et al., 2009; Burgess et al., 2013). Frost & Burns (2018) showed that spotted seals reached sexual maturity at 3 to 5 y old, while physical maturity was reached at 6 to 9 y old. Herein, we observed an inflection point in the cortisol concentration trend at 6 y old, which coincides with the time of physical maturity in spotted seals. Other studies reported that animals in the process of reproduction had higher glucocorticoid concentrations, implying that increased cortisol concentration is associated with a higher chance of reproduction (Bartsh et al., 1992; Lidgard et al., 2008). Therefore, we propose that the increase in cortisol with age, after age 6, is associated with an increasing chance of reproductive success.

This study showed a strong seasonal effect on cortisol concentrations in spotted seals and that

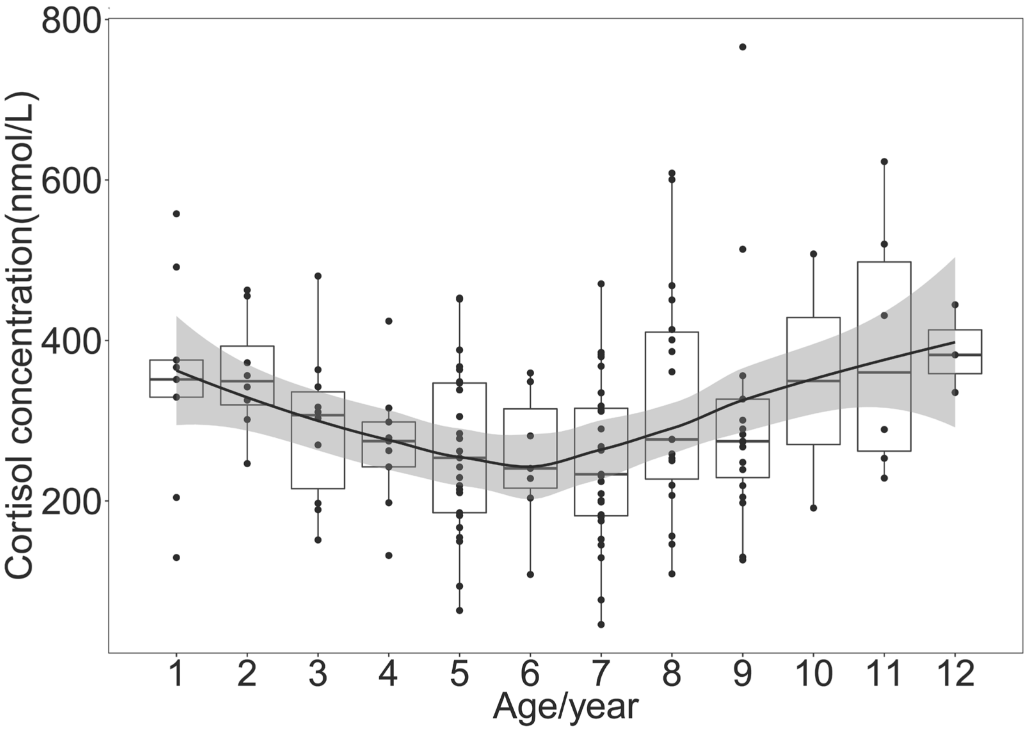

Figure 1. Scatterplots of serum cortisol concentrations in spotted seals (*Phoca largha*) of different ages. The dots represent cortisol concentrations, the solid line represents the trend of mean values, and the grey area indicates 95% confidence interval.

Table 1. Serum cortisol concentrations (nmol/L) of studied spotted seals (*Phoca largha*) in different categories from Liaodong Bay colony

Sex	Mature status	Pre-breeding season	Breeding-molt season	Other season
Male and female	Sex immature	343 ± 79	359 ± 71	193 (146-412)**
	Sex mature	340 ± 93	310 (272-456)**	217 (156-264)**
	Both	341 ± 88	355 ± 108	212 (155-267)**
Male	Sex immature	366 ± 58	357 ± 87	558*
	Sex mature	331 ± 86	345 ± 129	245 ± 135
	Both	337 ± 82	348 ± 120	229 (168-304)**
Female	Sex immature	323 ± 93	361 ± 62	206 ± 92
	Sex mature	366 ± 112	370 ± 102	213 ± 85
	Both	347 ± 103	367 ± 88	212 ± 84

Note: Data obeying normal distribution are described as mean ± standard deviation; and data obeying a skewed distribution are described as median and interquartile (indicated with a **).

*Only one sample was obtained in this category.

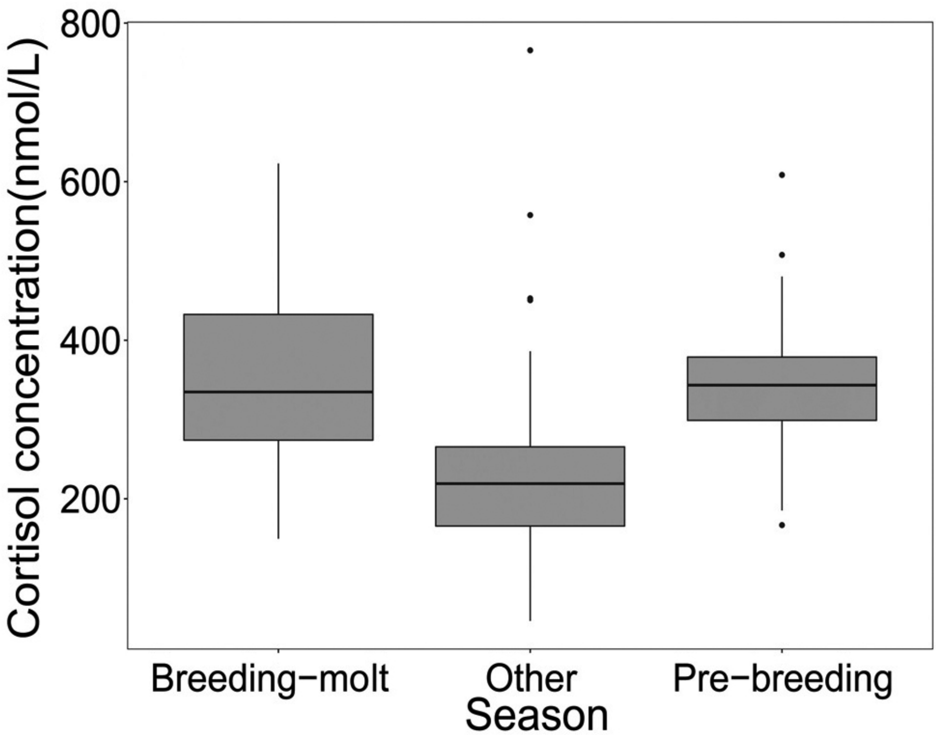

Figure 2. Boxplots of serum cortisol concentrations in spotted seals in different reproductive seasons from the Liaodong Bay colony

cortisol concentrations were starting to rise in the pre-breeding season. One possible reason suggested by our data for this rise is that the pre-breeding season implies a seal's energy demands will increase during migration. Or, said another way, there will be an increased motivation to feed as they fatten up for breeding. From October to December each year, wild spotted seals start their journey back to the Liaodong Bay colony for reproduction (Won & Yoo, 2004). Under pressures related to the lack of food and long distance migration, the seals would need to rely on their self-storage to provide energy (Kershaw & Hall, 2016). High levels of cortisol in the pre-breeding season could contribute to an increase in mobilization of protein that would improve their energy supply (Bennett et al., 2013) as well as facilitate fat storage (Bennett et al., 2017). We believe the second possible reason for the rise in cortisol concentration may be related to a drop in the environmental temperature since cortisol can contribute to production of metabolic heat as a byproduct of gluconeogenesis (Oki & Atkinson, 2004). Studies in spotted seals, harbor seals, and dugongs showed that they had significantly higher cortisol concentrations in winter than in summer (Ashwell-Erickson et al., 1986; Gardiner & Hall, 1997; Burgess et al., 2013).

In the breeding-molt season, spotted seals shed and regrow their epidermis and pelage annually (molting). Molt usually occurs immediately after breeding (Ashwell-Erickson et al., 1986; Burns, 2009), and completion of molt is marked by decreases in cortisol concentration (Ashwell-Erickson et al., 1986). This study found cortisol concentrations decreased in April, so most of the seals in the Liaodong Bay colony could be predicted to finish their molt before April. High concentrations of cortisol were previously reported to be associated with molt in pinnipeds (Ashwell-Erickson et al., 1986; Atkinson et al., 2011), and this study found that cortisol concentration in spotted seals reached the highest levels in the breeding-molt season, possibly due to food and temperature stress. For example, weight loss in gray seals and southern elephant seals (*Mirounga leonina*) suggested increased energy expenditures during molt (Hindell et al., 1994; Boily, 1996), while harbor seals were reported to spend most of their time ashore during the molt to minimize energy consumption and facilitate hair regeneration to avoid thermal stress (Paterson et al., 2012).

The present study provides the first age and seasonal serum cortisol concentration profiles of spotted seals, which is a useful baseline to predict fitness of wild seals, and, also, according to which natural or anthropogenic stressors can be assessed. However, we want to mention that all current results were obtained from animals under human care. Thus, these results should be used with caution when interpreting the physiology and behavior of spotted seals in the wild until data are available from these seals for corroboration.

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Literature Cited

Ashwell-Erickson, S., Fay, F. H., Elsner, R., & Wartzok, D. (1986). Metabolic and hormonal correlates of molting and regeneration of pelage in Alaskan harbor and spotted seals (*Phoca vitulina* and *Phoca largha*). *Canadian Journal of Zoology*, *64*(5), 1086-1094. https://doi.org/10.1139/z86-163

Atkinson, S., Arnould, J. P. Y., & Mashburn, K. L. (2011). Plasma cortisol and thyroid hormone concentrations in pre-weaning Australian fur seal pups. *General and Comparative Endocrinology*, *172*(2), 277-281. https://doi.org/10.1016/j.ygcen.2011.03.014

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*(4), 680-692. https://doi.org/10.1139/z92-102

Bechshøft, T. Ø., Sonne, C., Rigét, F. F., Letcher, R. J., Novak, M. A., Henchey, E., Meyer, J. S., Eulaers, I., Jaspers, V. L. B., Covaci, A., & Dietz, R. (2013). Polar bear stress hormone cortisol fluctuates with the North Atlantic Oscillation climate index. *Polar Biology*, *36*(10), 1525-1529. https://doi.org/10.1007/s00300-013-1364-y

Bennett, K. A., Robinson, K. J., Moss, S. E., Millward, S., & Hall, A. J. (2017). Using blubber explants to investigate adipose function in grey seals: Glycolytic, lipolytic and gene expression responses to glucose and hydrocortisone. *Scientific Reports*, *7*(1), 7731. https://doi.org/10.1038/s41598-017-06037-x

Bennett, K. A., Fedak, M. A., Moss, S. E., Pomeroy, P. P., Speakman, J. R., & Hall, A. J. (2013). The role of glucocorticoids in naturally fasting grey seal (*Halichoerus grypus*) pups: Dexamethasone stimulates mass loss and protein utilisation, but not departure from the colony. *Journal of Experimental Biology*, *216*(6), 984-991. https://doi.org/10.1242/jeb.077438

Boily, P. (1996). Metabolic and hormonal changes during the molt of captive gray seals (*Halichoerus grypus*). *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, *270*(5), R1051-R1058. https://doi.org/10.1152/ajpregu.1996.270.5.R1051

Bonier, F., Martin, P. R., Moore, I. T., & Wingfield, J. C. (2009). Do baseline glucocorticoids predict fitness? *Trends in Ecology & Evolution*, *24*(11), 634-642. https://doi.org/10.1016/j.tree.2009.04.013

Boveng, P. L., Bengtson, J. L., Buckley, T. W., Cameron, M. F., Dahle, S. P., Kelly, B. P., Megrey, B. A., Overland, J. E., & Williamson, N. J. (2009). *Status review of the spotted seal* (Phoca largha) (NOAA Technical Memorandum NMFS-AFSC-200). National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

Burgess, E. A., Brown, J. L., & Lanyon, J. M. (2013). Sex, scarring, and stress: Understanding seasonal costs in a cryptic marine mammal. *Conservation Physiology*, *1*(1). https://doi.org/10.1093/conphys/cot014

Burns, J. J. (2009). Harbor seal and spotted seal: *Phoca vitulina* and *P. largha*. In W. F. Perrin, B. Würsig, & J. G. M. Thewissen (Eds.), *Encyclopedia of marine mammals* (2nd ed., pp. 533-542). Academic Press. https://doi.org/10.1016/B978-0-12-373553-9.00126-7

Champagne, C. D., Houser, D. S., & Crocker, D. E. (2006). Glucose metabolism during lactation in a fasting animal, the northern elephant seal. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, *291*(4), R1129-R1137. https://doi.org/10.1152/ajpregu.00570.2005

Cooke, S. J., & O'Connor, C. M. (2010). Making conservation physiology relevant to policy makers and conservation practitioners. *Conservation Letters*, *3*(3), 159-166. https://doi.org/10.1111/j.1755-263X.2010.00109.x

Djurhuus, C. B., Gravholt, C. H., Nielsen, S., Mengel, A., Christiansen, J. S., Schmitz, O., & Møller, N. (2002). Effects of cortisol on lipolysis and regional interstitial glycerol levels in humans. *American Journal of Physiology-Endocrinology and Metabolism*, *283*(1), E172-E177. https://doi.org/10.1152/ajpendo.00544.2001

Dong, J. (1991). Estimates of historical population size of harbor seal (*Phoca largha*) in Liaodong Bay. *Marine Sciences*, *3*, 26-31.

du Dot, T. J., Rosen, D. A., Richmond, J. P., Kitaysky, A. S., Zinn, S. A., & Trites, A. W. (2009). Changes in glucocorticoids, IGF-I and thyroid hormones as indicators of nutritional stress and subsequent refeeding in Steller sea lions (*Eumetopias jubatus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *152*(4), 524-534. https://doi.org/10.1016/j.cbpa.2008.12.010

Engelhard, G., Brasseur, S., Hall, A., Burton, H., & Reijnders, P. (2002). Adrenocortical responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific handling. *Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology*, *172*(4), 315-328.

Franceschini, M. D., Rubenstein, D. I., Low, B., & Romero, L. M. (2008). Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. *Animal Conservation*, *11*(4), 263-269. https://doi.org/10.1111/j.1469-1795.2008.00175.x

Frost, K. J., & Burns, J. J. (2018). Spotted seal: *Phoca largha*. In B. Würsig, J. G. M. Thewissen, & K. M. Kovacs (Eds.), *Encyclopedia of marine mammals* (3rd ed., pp. 928-931). Academic Press. https://doi.org/10.1016/B978-0-12-804327-1.00244-2

Gao, X. G., Han, J. B., Lu, Z. C., Li, Y. F., & He, C. B. (2013). De novo assembly and characterization of spotted seal *Phoca largha* transcriptome using Illumina paired-end sequencing. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *8*(2), 103-110. https://doi.org/10.1016/j.cbd.2012.12.005

Gardiner, K. J., & Hall, A. J. (1997). Diel and annual variation in plasma cortisol concentrations among wild. *Canadian Journal of Zoology*, *75*(11), 1773-1780. https://doi.org/10.1139/z97-806

Guinet, C., Servera, N., Mangin, S., Georges, J. Y., & Lacroix, A. (2004). Change in plasma cortisol and metabolites during the attendance period ashore in fasting lactating subantarctic fur seals. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *137*(3), 523-531. https://doi.org/10.1016/j.cbpb.2003.11.006

Han, J. B. (2009). *Studies on population resources and molecular genetic characteristics of spotted seals* (Phoca largha) *in Chinese waters* (Unpub. doctoral dissertation). Qingdao Institute of Oceanology, the Chinese Academy of Sciences, Qingdao, China.

Han, J. B., Sun, F. Y., Gao, X. G., He, C. B., Wang, P. L., Ma, Z. Q., & Wang, Z. H. (2010). Low microsatellite variation in spotted seal (*Phoca largha*) shows a decrease in population size in the Liaodong Gulf colony. *Annales Zoologici Fennici*, *47*(1), 15-27. https://doi.org/10.5735/086.047.0102

Han, J. B., Lu, Z. C., Tian, J. S., Ma, Z. Q., Wang, Z., Yang, Y., Wang, Q., Song, X., & Peng, Z. (2013). Release studies on spotted seals (*Phoca largha*) using satellite telemetry tracking technique. *Acta Theriologica Sinica*, *33*, 300-307. https://doi.org/10.16829/j.slxb. 2013.04.002

Hindell, M. A., Slip, D. J., & Burton, H. R. (1994). Body mass loss of moulting female southern elephant seals, *Mirounga leonina*, at Macquarie Island. *Polar Biology*, *14*(4), 275-278. https://doi.org/10.1007/BF00239176

Hunt, K. E., Rolland, R. M., Kraus, S. D., & Wasser, S. K. (2006). Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). *General & Comparative Endocrinology*, *148*(2), 260-272. https://doi.org/10.1016/j.ygcen.2006.03.012

Kershaw, J. L., & Hall, A. J. (2016). Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol – A novel indicator of physiological state? *Scientific Reports*, *6*, 21889. https://doi.org/10.1038/srep21889

Liberzon, I., Krstov, M., & Young, E. A. (1997). Stress-restress: Effects on ACTH and fast feedback. *Psychoneuroendocrinology*, *22*(6), 443-453. https://doi.org/10.1016/S0306-4530(97)00044-9

Lidgard, D. C., Boness, D. J., Bowen, W. D., & McMillan, J. I. (2008). The implications of stress on male mating behavior and success in a sexually dimorphic polygynous mammal, the grey seal. *Hormones and Behavior*, *53*(1), 241-248. https://doi.org/10.1016/j.yhbeh.2007.10.003

Ma, Z., Han, J., Lu, Z., Tian, J., & Wu, J. (2018). Quantitative statistics and behavioral observation of spotted seals in waters around Shixian Island of Jinzhou Bay in Bohai Sea. *Fisheries Science*, *37*(5), 684-688. https://doi.org/10.16378/j.cnki.1003-1111.2018.05.017

Macbeth, B. J., Cattet, M. R. L., Obbard, M. E., Middel, K., & Janz, D. M. (2013). Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears. *Wildlife Society Bulletin*, *36*(4), 747-758. https://doi.org/10.1002/wsb.219

Madliger, C. L., & Love, O. P. (2014). The need for a predictive, context-dependent approach to the application of stress hormones in conservation. *Conservation Biology*, *28*(1), 283-287. https://doi.org/10.1111/cobi.12185

Morton, D. J., Anderson, E., Foggin, C. M., Kock, M. D., & Tiran, E. P. (1995). Plasma cortisol as an indicator of stress due to capture and translocation in wildlife species. *The Veterinary Record*, *136*(3), 60-63. https://doi.org/10.1136/vr.136.3.60

Myers, M. J., Litz, B., & Atkinson, S. (2010). The effects of age, sex, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (*Eumetopias jubatus*). *General and Comparative Endocrinology*, *165*(1), 72-77. https://doi.org/10.1016/j.ygcen.2009.06.006

Oki, C., & Atkinson, S. (2004). Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. *General and Comparative Endocrinology*, *136*(2), 289-297. https://doi.org/10.1016/j.ygcen.2004.01.007

Paterson, W., Sparling, C. E., Thompson, D., Pomeroy, P. P., Currie, J. I., & McCafferty, D. J. (2012). Seals like it hot: Changes in surface temperature of harbour seals (*Phoca vitulina*) from late pregnancy to moult. *Journal of Thermal Biology*, *37*(6), 454-461. https://doi.org/10.1016/j.jtherbio.2012.03.004

Rolland, R. M., Parks, S. E., Hunt, K. E., Castellote, M., Corkeron, P. J., Nowacek, D. P., Wasser, S. K., & Kraus, S. D. (2012). Evidence that ship noise increases stress in right whales. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1737), 2363-2368. https://doi.org/10.1098/rspb.2011.2429

Rugh, D. J., Shelden, K. E., & Withrow, D. E. (1997). Spotted seals, *Phoca largha*, in Alaska. *Marine Fisheries Review*, *59*(1), 1-18.

Sapolsky, R. M., Krey, L. C., & McEwen, B. S. (1986). The neuroendocrinology of stress and aging: The glucocorticoid cascade hypothesis. *Endocrine Reviews*, *7*(3), 284-301. https://doi.org/10.1210/edrv-7-3-284

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, *21*(1), 55-89. https://doi.org/10.1210/er

Wang, P. L., Han, J. B., & Ma, Z. Q. (2008). Status survey of spotted seal (*Phoca largha*) in Bohai and Yellow Sea. *Chinese Journal of Wildlife*, *29*(1), 29-31.

Wikelski, M., & Cooke, S. J. (2006). Conservation physiology. *Trends in Ecology & Evolution*, *21*(1), 38-46. https://doi.org/10.1016/j.tree.2005.10.018

Won, C., & Yoo, B-H. (2004). Abundance, seasonal haul-out patterns and conservation of spotted seals *Phoca largha* along the coast of Bak-ryoung Island, South Korea. *Oryx*, *38*(1), 109-112. https://doi.org/10.1017/S0030605304000171

Yan, H. K., Wang, N., Wu, N., & Lin, W. N. (2018). Maritime construction site selection from the perspective of ecological protection: The relationship between the Dalian offshore airport and spotted seals (*Phoca largha*) in China based on the noise pollution. *Ocean & Coastal Management*, *152*, 145-153. https://doi.org/10.1016/j.ocecoaman.2017.11.024

Yang, L. L., Xu, X. M., Zhang, P. J., Han, J. B., Li, B., & Berggren, P. (2017). Classification of underwater vocalizations of wild spotted seals (*Phoca largha*) in Liaodong Bay, China. *The Journal of the Acoustical Society of America*, *141*(3), 2256-2262. https://doi.org/10.1121/1.4979056

Zhang, P. J., Song, X. R., Han, J. B., Wang, L. M., & Yang, Y. (2014a). Milk composition, milk consumption, and growth rate of a captive spotted seal (*Phoca largha*) pup from Liaodong Bay, China. *Canadian Journal of Zoology*, *92*(5), 449-452. https://doi.org/10.1139/cjz-2013-0295

Zhang, P. J., Lu, J. J., Li, S. H., Han, J. B., Wang, Q. G., & Yang, L. L. (2016). In-air vocal repertoires of spotted seals, *Phoca largha*. *The Journal of the Acoustical Society of America*, *140*(2), 1101-1107. https://doi.org/10.1121/1.4961048

Zhang, P., Yang, Y., Han, J., Lu, Z., Wang, L., Tian, J., & Wang, Q. (2014b). Serum testosterone, progesterone, and estradiol concentrations and sexual maturation in spotted seals (*Phoca largha*). *Theriogenology*, *82*(3), 475-480.e4. https://doi.org/10.1016/j.theriogenology.2014.05.013

Appendix

Dilution Test

The cortisol kit showed recovery results from 89 to 111% deriving from 34 samples in the concentration range of 83 to 764 nmol/L according to the manufacturer's instructions. However, the manufacturer did not provide a specific recovery value on each concentration, so we could not conduct a strict parallel examination through a dilution test.

In our present study, the recovery results are from 87.4 to 100% in the concentration range of 26.5 to 242.4 nmol/L, which is consistent with the expected recovery values aforementioned. Details are shown in the table below:

Appendix Table 1. Dilution results of cortisol kit when assaying spotted seal sera

Dilution fold	Results (nmol/L)	Recovery percentage (%)
1	242.4	100.0
2	113.8	93.9
4	54.4	89.8
8	26.5	87.4