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Evaluating suitability of saliva to measure steroid concentrations in grey seal pups

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Summary

Measurement of steroids in wild pinnipeds can facilitate assessment of breeding, nutritional and stress status, and is useful in understanding behavioral responses. Even in young animals, sex steroids may be important in behavioral interactions and immune modulation. Use of saliva can avoid the large fluctuations seen in some steroids in plasma, and can negate the need for venipuncture, making it a potentially useful matrix in the wildlife. However, its utility in estimating steroid levels in wild young pinnipeds has not been evaluated. Here, we investigated the suitability of saliva for steroid hormone analysis in wild grey seal pups during their suckling and post-weaning fast periods. We collected saliva (n = 38) and plasma (n = 71) samples during the breeding season on the Isle of May, Scotland, 2012. We investigated success of sample collection, ease of preparation, accuracy and precision of analysis, and, where possible, comparability of measurements (n = 27) from saliva and plasma. Plasma sampling was rapid, whereas sampling saliva took up to five times longer. Analytical performance criteria (parallelism, accuracy, and precision (intra and inter assay co-efficient of variation (% CV)) of commercial ELISA kits to measure estradiol, testosterone and cortisol in both matrices were assessed. Estradiol and cortisol assays performed well and can be used in plasma and saliva. However, we could not confidently validate testosterone for either matrix. Saliva estradiol correlated with levels in plasma. Saliva sample preparation was faster and simpler than plasma preparation because it did not require extraction. However, given the additional time taken to obtain saliva in the wild, the possibility of blood contamination from oral damage and the lower success rate in obtaining sufficient sample for analysis, we recommend that this matrix only be used as an alternative to plasma sampling measurement in pinnipeds when animals are anaesthetized, tolerate mouth swabbing, or have been trained to accept saliva sampling in captivity.

Key words: steroids, testosterone, estradiol, cortisol, grey seal, saliva, ELISA, juveniles, validation

1. Introduction

Steroids play a major part in successful reproduction and appropriate stress responses in mammals (Ruiz-Cortes, 2012). Gonadal steroids are also important for neural development, immune system function, energy balance and behavior in mammals (see Klein (2000) for review). Glucocorticoids indicate nutritional and stress status (Bryan et al., 2015), and facilitate understanding of behavioral responses (French et al., 2013), immune function and regulation of energy balance in young animals (Bennett et al., 2013), as well as in adults (Lidgard et al., 2008).

Sex steroid levels are lower in pre-pubertal individuals than adults, but are detectable and are important mediators of behavior and energy partitioning (Bell, 2018). Measurement of circulating sex steroids in young animals may thus be important in understanding behavioral and physiological differences within or between sexes (Bell, 2018). Sample collection methods and hormone analysis procedures should be refined to reduce animal handling time and stress. Blood samples are often used as the matrix of choice for analysis of steroid samples in pinnipeds (Bennett et al., 2013; Gardiner and Hall, 1997; Lidgard et al., 2008). However, cortisol levels change rapidly in response to a range of stressors. Lipemia, and hemolysis can interfere with colorimetric enzyme linked immunosorbent assays (ELISA) (Holder et al., 2010). Proteins can also negatively affect target availability (Holder et al., 2010). Differences in adrenal cortex activity means neonates can also produce additional steroid metabolites that cross-react with some immunoassay antibodies (Makin et al., 2010). Some alternatives to plasma sampling (e.g. urine (Constable et al., 2006), faeces (Petrauskas and Atkinson, 2006), blubber (Kershaw and Hall, 2016) and hair (Keogh et al., 2020)) for steroid analysis have been tested in captive and wild pinnipeds, but require substantial processing time before analysis.

Saliva sampling can avoid the need for venipuncture, is a simpler and more stable matrix that can be kept at ambient temperatures for many hours (Wood, 2009), steroid measurements appear unaffected by salivary flow rate (Vining et al., 1983) and may show less rapid and lower amplitude changes than plasma levels (Vincent and Michell, 1992), making them suitable to obtain baseline levels. Salivary samples from captive pinnipeds have been used to measure testosterone (Theodorou and Atkinson, 1998), estrone sulfate and progesterone (Pietraszek and Atkinson, 1994). However, wild animals may have oral injuries or infection, or may struggle during collection, and blood-contaminated saliva leads to elevated steroid values (Granger et al., 2004). Thus, it is important to evaluate the feasibility, quality and effectiveness of saliva sample collection against the more usual blood sampling from wild pinnipeds. Moreover, it is important to assess whether alternative approaches to obtaining material for hormone assays can offer better welfare and logistical advantages while retaining sampling success rate.

Grey seals (*Halichoerus grypus*) are large marine top predators, whose population status is indicative of the health status of the wider marine environment (Bäcklin et al., 2011; Jüssi et al., 2008), and, although globally rare, are regionally important in the North East Atlantic and Baltic Seas (SCOS, 2020). Although cortisol has been measured in post weaned grey seal pups (Bennett et al., 2013, 2012), there have been no studies on androgens and estrogens in either dependent or weaned pups of this species, and information is sparse of other young pinnipeds (Ortiz et al., 2003).

In this study, we assessed the suitability of saliva for analysis of three steroid hormones – estradiol (E), testosterone (T) and cortisol (C) – in wild grey seal male and female pups during their early development. We examined a) success of sample collection including approximate volume obtained, b) ease of sample preparation and analysis in the laboratory, c) performance of commercially available ELISA kits for salivary measurements and d) where available, comparability of hormone levels measured in contemporaneous saliva and plasma samples taken from pups at different nutritional stages in their first six weeks of life.

2. Materials and methods

2.1 Study location and animals

Samples were collected from grey seal pups as part of a long term study of grey seal reproduction during the breeding season (October-December) of 2012 on the Isle of May, Scotland (56◦11'N, 02◦33'W) on up to four occasions (nutritional stages – S1 – early suckling, S2 – late suckling, W1 – early weaning, W2 – late weaning), when animals were 4 to 38 days old (see Supplementary Table 1 and Supplementary Fig. 1). Samples were taken at approximately 10 day intervals (mean ± SD = 9.86 ± 1.54 days) and no significant difference was found in the length of time between sample occasions (Kruskal Wallis test (KW), $p > 0.05$).

All sample collection was performed by personal license holders/ designated competent personnel under UK Home Office license PPL 60/4009. This work received ethical approval from the University of St Andrews Animal Welfare and Ethics Committee (AWEC) and was performed in compliance with Animal (Scientific Procedures) Act (ASPA) 1986 and the EU directive on the protection of animals used for scientific purposes (2010/63/EU).

2.2 Sample collection

Blood sample collection. Samples (n = 71) were collected during daylight between 10:50 to 17:15 GMT (no significant difference was found between time of the day during different nutritional stages KW with Bonferoni correction, $p > 0,05$). Mothers were anesthetized using intramuscular injection from a blow dart (Zoletil $_{100}$, Virbac, France) and capture took place once the anaesthesia had taken effect approximately 10 min. later. Mothers and

pups were then captured simultaneously. Pups were manually restrained, the blood sampling area cleaned and then anesthetized before they were weighed and sampled. Pups received approximately 0.1 mg/kg, i.v. of anesthetic (Zoletil $_{100}$, Virbac, France) on all occasions (n = 65), a low risk dose for animals of this age class and effective for the sampling regime, to obtain blubber samples (Bennett et al., 2015). Four animals were not anesthetized because only blood samples were collected. Blood was collected from the extradural vein into a sterile 10 ml potassium ethylenediaminetetraacetic acid (EDTA) Vacutainer (Becton Dickinson, Oxon, UK).

Saliva sample collection. Saliva sampling was attempted during every blood sampling (n = 65) when animals were anaesthetized. Saliva samples were taken with inert polymer, cylindrical swabs (8 mm x 125 mm) designed for saliva sample collection in humans (Salimetrics, UK). Anesthetized pups were not aware or responsive, which allowed sample collection without additional restraint. The saliva swab was placed in the cheek where it would pose no threat to the anaesthetized animal and removed prior to returning the pup to it's mother or when rejected by the animal on recovery from anesthesia. Before removal, if possible, the swab was used to collect saliva from the corners of the mouth. No samples were collected when signs of blood or abrasions were visible inside the mouth or around the gums. Any other particles of grass or dirt, if present, were not rinsed away to avoid diluting the saliva sample. The sample was discarded if any traces of blood on the polymer swab were observed during collection. The swab was then transferred to a 2 mm sterile syringe, which was used to squeeze the saliva out of the swab into a 2 ml collection tube (Salicap, IBL International, Germany). Saliva volume recovered was estimated to the nearest 0.1 ml using gradations on the collection tubes. Blood and saliva samples were stored at ambient temperatures after collection for an average of 2 hours 9 min. (from 25 min. to 8 hours 49 min.) before they were processed.

2.3 Duration and success of sample collection

Plasma was collected within 2 minutes as in Bennett et al. (2012). Saliva sampling took approximately 10 min., since this is the typical duration of anesthesia (Moss, pers. obs.). Sufficient plasma was obtained for all assays.

Success of saliva sample collection was assessed by comparing the volume collected with the minimum volume necessary for analysis of three steroids (150 µl for plasma and 250 µl for saliva). 'Fully collected' saliva samples contained the minimum volume of 250 µl (defined as possible to use for analysis of all three steroids), 'partially collected' samples had a volume around 100-250 µl (defined as possible to use for analysis of one or two steroids), and samples were considered 'not collected' when the saliva volume was too low for steroid analysis or sample was contaminated with blood.

2.4 Sample preparation for analysis

Blood samples were centrifuged in a swing-out bench top centrifuge at 2000 g for 15 min. The plasma was then drawn off using glass Pasteur pipettes, divided in 1.5 ml aliquots, immediately frozen and stored at -20° C until extraction and analysis. Plasma samples were extracted according to the Steroid Liquid Sample Extraction Protocol provided by Arbor Assays® (Michigan, USA), which is recommended by ELISA kit various manufacturers (Kumar, 2020) and validated for prepubertal human serum using EIA and RIA (Ankarberg-Lindgren et al., 2001; Norjavaara et al., 1996; Raivio et al., 2001). The collected material was evaporated in room temperature using a vacuum Eppendorf concentrator 5301 (Hamburg, Germany) and kept at -20^{0}C prior to analysis. On the day of the ELISA the sample was thawed, reconstituted in 300 µl of steroid free serum (Ibl-International, Hamburg, Germany) and vortexed for 30 min. at room temperature, centrifuged for 10 min. at 3000 g and the supernatant used for ELISA. Saliva samples were heated at 60˚C for 1 h then left to cool to room temperature and centrifuged at 3000 g for 10 min. prior to ELISA.

2.5 Kit validation and sample analysis

Commercially available ELISA kits (IBL-International, Hamburg, Germany, codes for products: TB - RE52151, TS - RE52631, EB - RE52041, ES - RE52601, CB - RE52061, CS - RE52611) were used according to the manufacturer's protocol. We attempted to validate each kit for use with saliva and plasma from grey seal pups (see Table, Mean ± SD) as follows: pooled plasma and pooled saliva samples were each diluted with the zero standard provided by the manufacturer to 0.75, 0.5 and 0.25 of neat concentration to ensure the measurements remained within the limits of the standard curve. The concentrations in the dilutions were then compared with expected concentrations from the neat pooled samples. The pooled extracted and reconstituted saliva and plasma samples were spiked 1:1 with low and high standard provided by the manufacturer to obtain recovery values. All standards and samples were analyzed in duplicate within any given plate.

Assay validation and sample measurements for plasma samples were performed across two plates, which were analyzed in two consecutive years, 2014 (Plate 1) and 2015 (Plate 2). Inter-assay CV of plasma was estimated using two samples, each measured in duplicate on each of the two plates. Mean and standard deviation concentrations were calculated for each sample in each plates and the standard deviation expressed as a percentage of the mean to give inter assay CV. The average inter-assay CV of the two samples is reported here. All saliva samples were measured within one plate; thus only intra-assay CV is reported. Intra-assay CV was calculated from the mean and standard deviation of the duplicates of each sample in the same plate and the mean intra-assay CV calculated.

2.6 Prioritization of hormone assays for analysis

A total number of plasma (for different plates) and saliva samples is provided in Table 1 (and in Supplementary Table 2 for more detail). When a partial saliva volume was collected and was below 100 µl, the cortisol assay was prioritized since it required less sample (2 × 25 µl) than the estradiol kit (2 × 50 µl). When partial saliva volume collected allowed analysis of two steroids, testosterone was chosen as the second option, because, like cortisol, it required a lower volume of saliva (2 × 25 µl) than the estradiol kit (2 × 50 µl). As a result, the number of samples analyzed differs between steroids and is lowest for estradiol.

2.7 Statistics

Data analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and statistical differences considered to be significant when $p < 0.05$. Parallelism was determined by visually investigating the graphs in which optical density of standard curve and pooled saliva and plasma samples were plotted against dilution (Supplementary Fig. 3-5). Accuracy of plasma kits was determined by performing linear regression of diluted pooled samples (n = 6) of two plates comparing observed versus expected measurements, which should show a positive linear relationship with a slope of approximately 1.0 (a slope of 0.8-1.2 and $r^2 > 0.95$ was considered acceptable) (Hunt et al., 2014; Kershaw and Hall, 2016). Due to the low number of diluted samples we were only able to inspect recovery rates for saliva. Expected and observed concentrations in high and low spiked saliva and plasma samples were compared using a paired t-test. Dilution values were considered acceptable if they were within 30 % of the expected concentrations for low concentrations, and recoveries of spiked samples if they were within 20 % of expected values in both saliva and plasma (Yan, 2018). Precission of intra- and inter-assay CV was considered acceptable when < 20 %.

Spearman rank correlation was used to evaluate whether the saliva sample volume obtained was related to steroid (ES, TS, CS) concentration. Testosterone was excluded from subsequent comparability analysis due to extremely high interassay CV. Even though the interassay CV of CB was within an acceptable range, it was removed from comparability analysis because we were unable to determine how much disturbance animals perceived prior to sampling, preventing us from reliably comparing CS and CB levels as a result of the potential stress response.

Linear regression analysis was used to examine the relationship between estradiol concentrations in saliva and plasma. Positively skewed ES values were log transformed using natural logarithm prior to regression analysis.

A generalized linear mixed model (GLMM) was used to evaluate the possible effect of nutritional stage, pup sex, body mass, age, and time of the day on EB and ES comparable samples (n = 27). A normal distribution model and a linear link function was used for EB and a gamma distribution model with a log link function was used for ES.

Scaled identity covariance structure for nutritional stage as repeated measures for every pup (ID as a Subjects) and Satterthwaite approximation for estimating degrees of freedom was used (Zuur et al., 2009). Corrected Akaike information criteria (AICc) was used to select the best model. Residuals plots were inspected to check model fit.

3. Results

3.1 Success of sample collection

Based on previous equivalent sampling (Bennett et al., 2012) the duration of blood collection was up to 5 times shorter (< 2 min.) than that for taking saliva (< 10 min.). We collected 38 useable saliva samples, which represents ~54 % of all 71 sampling occasions (Table 1). Unsuccessful saliva sampling events (N = 33) were due to limited saliva production, which did not provide the minimum amount of saliva during anesthesia, or because samples were contaminated with blood, and thus had to be discarded. The manufacturer states that a minimum of 500 µl of saliva is recommended for each ELISA analysis (requiring in this case 1,5 ml for all three steroids), but we never obtained 1.5 ml from any animal (Supplementary Fig. 2). However, the saliva analysis of all three steroids requires a minimum of 250-300 µl (TS and CS – 50 µl and ES 25 µl for each well) and we obtained this in 31 (82 %) cases. From samples that were collected, we had 7 (18 %) partial samples (five samples when only two steroids and three samples when only one steroid was analyzed). Therefore, we obtained enough saliva to analyze all three steroids in 44 % of all the animal sampling events. There were also three samples (two from nutritional stage S1 and one from S2) of TS that were above the limit of detection, and we did not have sufficient sample or reagent to perform a subsequent dilution. These high samples were not included in further statistical analysis.

3.2 Validation of ELISA kits for different matrices

Plasma (EDTA) validation. We successfully validated plates for estradiol and cortisol, as they showed intra-assay CV < 10 % and spike recovery rates within 20% of expected (Table 1). Expected vs. observed spiked concentrations were not different for both steroids (CB: $t = 0.24$, $df = 3$, $p = 0.83$, EB: $t = 0.7$, $df = 3$, $p = 0.54$). Dilution curves were visually parallel to standard curves for CB and EB (Supplementary Fig. 3 and Fig. 5). Inter-assay CV was 8 % for EB and 25 % for CB. Expected vs. observed concentrations of diluted samples showed a linear relationship with a slope close to 1 for both cortisol ($r^2 = 0.95$, $df = 5$, $p < 0.01$, slope – 0.95) and estradiol ($r^2 = 0.97$, $df = 5$, $p < 0.001$, slope – 0.92). TB intra-assay CV was < 11 % and average spike recovery was 106 % (Table 1). Expected vs. observed spiked TB concentrations were not significantly different ($t = -0.642$, $df = 3$, $p = 0.57$). Dilution curves were visually parallel to the standard curve (Supplementary Fig. 4) and dilution performance was good with a slope

close to 1 (r^2 = 0.99, df = 5, p < 0.001, slope – 0.99). However, inter-assay CV was unacceptably high (86 %), thus we did not successfully validate this ELISA for plasma testosterone.

Saliva validation. Dilution curves were visually parallel to standard curves for all steroids in saliva (Supplementary Fig. 3-5). TS ELISA overestimated the lowest diluted values up to 18 % and the CS kit by up to 15 %. The ES kit provided the poorest dilution performance with the lowest diluted values being underestimated by around 40 %, however such low values were not observed in the samples analyzed and recovery rate was above 80 % for ES values typically measured. Intra-assay CV was < 10 % and average recovery of spiked samples ranged from 90 % for ES to 95 % for CS and 111 % for TS (Table 1). Expected vs. observed spiked concentrations were not different for all steroids (ES: t = -0.89, df = 1, p = 0.54; TS: t = -0.99, df = 1, p = 0.5; CS: t = 1.4, df =1, p = 0.4). Saliva sample volume showed a significant moderate negative correlation with TS (Spearman r = -0.35, p = 0.047), while there were no significant correlations between saliva volume and ES (Spearman r = -0.32, p = 0.08) or CS (Spearman r = -0.03, p = 0.86).

Table 1 should be inserted here

3.3 Correlations between steroid concentrations in saliva and plasma

It was possible to compare the relationship of estradiol concentrations between 27 saliva and plasma samples. There was a significant positive relationship between log normalized ES and EB concentrations (r = 0.53, r_{adj}^2 = 0.28, $F_{1,25}$ = 9.64, p < 0,01) (Fig. 1) showing broad agreement between the two matrices.

Fig. 1 should be placed here.

3.4 Sex and nutritional stage effects in different matrices

The best model to describe variation in EB included two significant variables (AICc = 260.61 vs. $AICc_0$ = 314.83). EB was significantly different between nutritional stages ($F_{3,22,}$ = 7.18, p < 0.01) and higher in females ($F_{1,22}$ = 4,42, p = 0,047). EB was highest during the early suckling period, remained lower and stable during late suckling – early post-weaning fast periods and dropped again during the late post-weaning fast (Fig. 2A). No significant interactions were observed. Individual ID as a random intercept factor was removed, since it was not significant in any of the models (more details in Supplementary Table 3). Nutritional stage was the only significant effect in the model explaining variation in ES (AICc = 30.64 vs. $AICc_0$ = 56.23; $F_{3,23}$ = 14.44, p < 0.001): higher concentrations occurred during early and late suckling compared with the postweaning fast (Fig. 2B).

Fig. 2 should be placed here.

4 Discussion

4.1 Ease and success of saliva sample collection and sample preparation

Saliva sample preparation was faster by up to one day and simpler because it required less equipment, chemicals and steps of extraction, than preparation of plasma samples. However, while saliva may appear to be an attractive option for measuring E and C in grey seals, there are a number of caveats. First, saliva samples take substantially longer (up to five times) to collect than plasma samples in wild grey seal pups, even when, as here, the animals are anaesthetized. As this type of anesthesia still requires venipuncture, the involvement of a trained professional is required to administer the anesthetic agent and obtain the sample and there is risk of extended apnea from the anesthesia. In unanesthetized animals handling time and stress, and the risk of oral damage and bleeding if the animal struggles can be substantially greater. Thus this method cannot be considered non-invasive for this species. In addition, it was often difficult to obtain enough sample for all three steroids to be analyzed: we were only able to obtain sufficient saliva to use in 44 % of sampling events (full samples), compared to 100 % for blood collection. We only obtained 0.5 ml or more – the volume recommended by the manufacturer for one of the steroid assays - in 32 % of saliva samples collected. We did not obtain an overall 1.5 ml, which was recommended by the manufacturer for all three assays to be performed, from any of the animals. Saliva may therefore be more appropriate for use in pinniped species that will tolerate mouth swabbing, in captive individuals that have been trained to accept saliva sampling, where anesthesia is deemed appropriate and safe for the species and age group, and if sufficient sample is guaranteed to be obtained for the analysis. These conditions are less likely to be met in a wild context. Our study shows that sufficient and uncontaminated saliva samples are unlikely to be collected with sufficient success to complete an intended analysis in grey seals, even when animals are anesthetized and when adequate time is given for saliva collection.

4.2 Validity of commercial ELISA kits for steroid analysis from grey seal saliva and plasma

This study demonstrates that estrogen can be measured with acceptable accuracy in both plasma and saliva of grey seal pups. Commercial ELISA kits may thus be suitable for estimating estradiol in grey seal pup plasma, however efforts need to be made to minimize between plate variation and ensure inter-plate confounds are minimized, because small biologically important differences might be too small to detect, especially when intra and inter assay variability are high. Saliva and plasma estradiol concentrations measured here were comparable with those reported previously in other marine mammals (Amaral et al., 2015; Theodorou and Atkinson, 1998). However, the range of steroid concentrations in blood vary more between studies than concentrations reported in saliva. Plasma estradiol was higher than concentrations reported in harbor seal young and adults by Lydersen & Kovacs (2005), northern

fur seals (*Callorhinus ursinus*) (Dierauf and Gulland, 2001) and southern elephant seals (*Mirounga leonina*) (Ferreira et al., 2005), but was similar to concentrations in harbor seal reported by Reijnders (1990).

For CB, lack of specificity due to high cross reactivity with progesterone and its metabolites could be one of the reasons for high CV. While T assay dilution performance for saliva and blood was from acceptable to excellent, the repeatability of TB concentrations was very poor. It is hard to identify what could have caused the differences between plates analyzed at different times. To the best of our knowledge this assay has not been validated for other wildlife in either saliva or plasma so it is difficult to establish whether the performance issues here were assay, age class or species specific.

4.3 Steroid concentration differences between sex and nutritional stages

Saliva provided similar detail on estradiol differences between nutritional states compared with plasma. However, no sex differences were observed in saliva, whereas females consistently had higher estradiol than males in the matched plasma samples. These early sex differences have not been previously reported. The lack of detectable sex differences in saliva estradiol may limit its utility for measuring sex steroids in young animals. Higher concentrations of steroids during suckling than fasting may result from a reduction in metabolic processes after weaning when animals need to conserve energy (Reilly, 1991), are not metabolized rapidly after birth and still resemble concentrations transferred from mother *in utero* or are transferred to pups through mother's milk (Pundir et al., 2020). Steroid levels may track changes seen in the mother, which have high concentrations of estradiol at birth followed by a drop postpartum and increasing levels during the second half of lactation, possibly due to estrus (Mellish and Iverson, 2005). Higher concentrations of estradiol during the suckling period are related to immune system activity and cause sex-associated resistance to helminth parasite infection (Guzmán et al., 2009). Differences in steroid concentrations between nutritional categories and sex may thus have consequences for pups' behavior, immunity (Hall et al., 2002), energy balance (Bennett et al., 2013) and sensitivity to endocrine disrupting chemicals (Troisi et al., 2020) and may contribute to reported sex differences in their survival (Hall et al., 2001).

5 Conclusions

This study is a first attempt to validate assays to measure sex steroids in grey seal pups in two different matrices. Estradiol and cortisol assays performed well and can be used in plasma and saliva, even in young grey seal pups. However, we could not confidently validate testosterone for both matrices. Although saliva is a promising analytical matrix for some steroids in some wildlife species, saliva collection is time consuming compared to rapid blood collection and easily affected by blood from gums or tongue in grey seals. The need for longer restraint and

anesthesia of animals during sampling compared with blood collection, and the lack of reliability in obtaining sufficient sample volume means this method is not 'non-invasive' and is not justifiable in this context for this study species. More importantly, saliva does not provide the same resolution of detail provided by plasma estradiol concentrations, since differences between sexes and the pattern of change between developmental states is not observed in saliva. We therefore recommend that salivary sampling is not performed in wild grey seals, unless animals are anesthetized for another reason; saliva can be obtained during that time in a way that minimizes blood and other contamination; and a large enough volume is obtained for the intended analysis. Saliva samples could be appropriate for analysis of estrogen and cortisol when collection occurs in captivity in trained animals that will tolerate mouth swabbing, where the stress effect of disturbance can be quantified and minimized, and where sufficient sample can be obtained, but may not be a good matrix for testosterone analysis, at least in young individuals.

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Literature Cited

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). Reprod. Fertil. Dev. 27, 1065–1071. https://doi.org/10.1071/RD13334

Ankarberg-Lindgren, C., Elfving, M., Wikland, K.A., Norjavaara, E., 2001. Nocturnal application of transdermal estradiol patches produces levels of estradiol that mimic those seen at the onset of spontaneous puberty in girls. J. Clin. Endocrinol. Metab. 86, 3039–3044. https://doi.org/10.1210/jc.86.7.3039

Bäcklin, B.M., Moraeus, C., Roos, A., Eklöf, E., Lind, Y., 2011. Health and age and sex distributions of Baltic grey seals (Halichoerus grypus) collected from bycatch and hunt in the Gulf of Bothnia. ICES J. Mar. Sci. 68, 183–188. https://doi.org/10.1093/icesjms/fsq131

Bell, M.R., 2018. Comparing postnatal development of gonadal hormones and associated social behaviors in rats, mice, and humans. Endocrinology 159, 2596–2613. https://doi.org/10.1210/en.2018-00220

Bennett, K.A., Fedak, M.A., Moss, S.E.W., 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. J. Exp. Biol. 216, 984–91. https://doi.org/10.1242/jeb.077438

Bennett, K.A., Hughes, J., Stamatas, S., Brand, S., Foster, N.L., Moss, S.E.W., Pomeroy, P.P., 2015. Adiponectin and Insulin in Gray Seals during Suckling and Fasting: Relationship with Nutritional State and Body Mass during Nursing in Mothers and Pups. Physiol. Biochem. Zool. 88, 295–310. https://doi.org/10.1086/680862

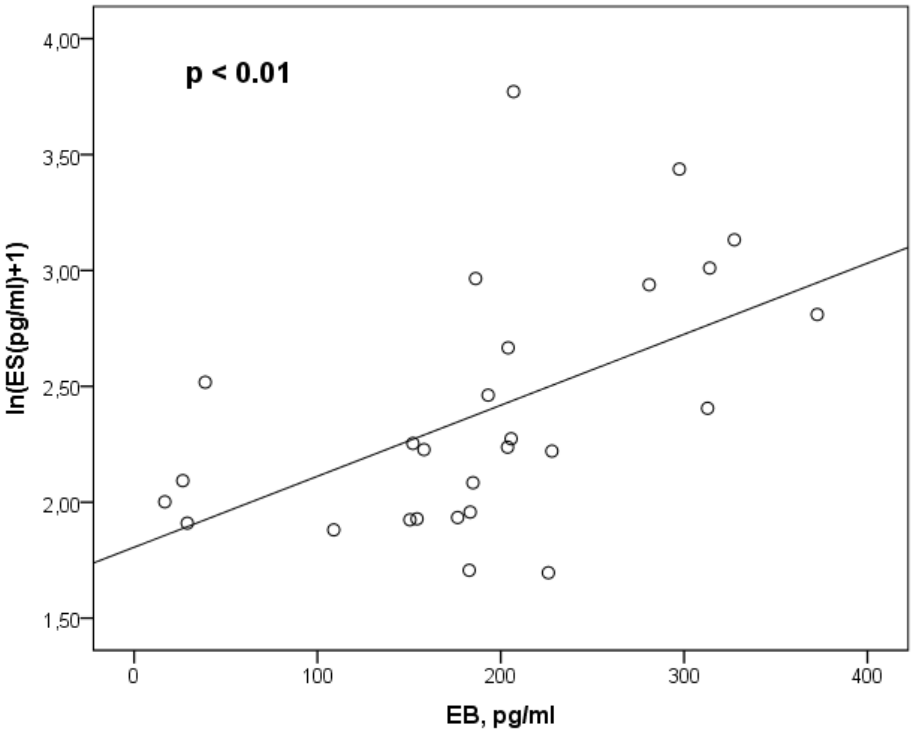
Bennett, K.A., Moss, S.E.W., Pomeroy, P., Speakman, J.R., Fedak, M.A., 2012. Effects of handling regime and

sex on changes in cortisol, thyroid hormones and body mass in fasting grey seal pups. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 161, 69–76. https://doi.org/10.1016/j.cbpa.2011.09.003
Bryan, H.M., Smits, J.E.G., Koren, L., Paquet, P.C., Wynne-Edwards, K.E., Musiani, M., 2015. Heavily hunted wolves have higher stress and reproductive steroids than wolves with lower hunting pressure. Funct. Ecol. 29, 347–356. https://doi.org/10.1111/1365-2435.12354
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 Biol. 25, 137–144. https://doi.org/https://doi.org/10.1002/zoo.20088
Dierauf, L.A., Gulland, F.M.D., 2001. CRC Handbook of Marine Mammal Medicine. 2nd Edition, 2nd ed. CRC Press, New York.
Ferreira, A.P.S., Martínez, P.E., Colares, E.P., Robaldo, R.B., Berne, M.E. a, Miranda Filho, K.C., Bianchini, A., 2005. Serum immunoglobulin G concentration in Southern elephant seal, Mirounga leonina (Linnaeus, 1758), from Elephant island (Antarctica): Sexual and adrenal steroid hormones effects. Vet. Immunol. Immunopathol. 106, 239–245. https://doi.org/10.1016/j.vetimm.2005.02.024
French, J.A., Mustoe, A.C., Cavanaugh, J., Birnie, A.K., 2013. The influence of androgenic steroid hormones on female aggression in “atypical” mammals. Philos. Trans. R. Soc. B Biol. Sci. 368. https://doi.org/10.1098/rstb.2013.0084
Gardiner, K.J., Hall, A.J., 1997. Diel and annual variation in plasma cortisol concentrations among wild and captive harbor seals (Phoca vitulina). Can. J. Zool. 75, 1773–1780. https://doi.org/10.1139/z97-806
Granger, D.A., Shirtcliff, E.A., Booth, A., Kivlighan, K.T., Schwartz, E.B., 2004. The ‘“ trouble ”’ with salivary testosterone 1229–1240. https://doi.org/10.1016/j.psyneuen.2004.02.005
Guzmán, C., Camacho-Arroyo, I., De León-Nava, M.A., Morales-Montor, J., 2009. Neonatal exposure to estradiol induces resistance to helminth infection and changes in the expression of sex steroid hormone receptors in the brain and spleen in adult mice of both sexes. Brain. Behav. Immun. 23, 709–715. https://doi.org/https://doi.org/10.1016/j.bbi.2009.02.014
Hall, A.J., McConnell, B.J., Barker, R.J., 2002. The effect of total immunoglobulin levels, mass and condition on the first-year survival of Grey Seal pups. Funct. Ecol. 16, 462–474. https://doi.org/10.1046/j.1365-2435.2002.00649.x
Hall, A.J., McConnell, B.J., Barker, R.J., 2001. Factors Affecting First-Year Survival in Grey Seals and Their Implications for Life History Strategy. J. Anim. Ecol. 70, 138–149.
Holder, G., Makin, H.L.J., Bradlow, H.L., 2010. The Measurement of Estrogens, in: Makin, H.L.J., Gower, D.B. (Eds.), Steroid Analysis. Springer, London, New York, pp. 605–705.
Hunt, K.E., Rolland, R.M., Kraus, S.D., 2014. Detection of steroid and thyroid hormones via immunoassay of North Atlantic right whale (Eubalaena glacialis) respiratory vapor. Mar. Mammal Sci. 30, 796–809. https://doi.org/10.1111/mms.12073
Jüssi, M., Härkönen, T., Helle, E., Jüssi, I., 2008. Decreasing ice coverage will reduce the breeding success of Baltic grey seal (Halichoerus grypus) females. Ambio 37, 80–85. https://doi.org/10.1579/0044-7447(2008)37[80:DICWRT]2.0.CO;2
Keogh, M.J., Gastaldi, A., Charapata, P., Melin, S., Fadely, B.S., 2020. Stress-related and reproductive hormones in hair from three north Pacific otariid species: Steller sea lions, California sea lions and northern fur seals. Conserv. Physiol. 8, 1–13. https://doi.org/10.1093/conphys/coaa069
Kershaw, J.L., Hall, A.J., 2016. Seasonal variation in harbour seal (Phoca vitulina) blubber cortisol - A novel indicator of physiological state? Sci. Rep. 6, 1–9. https://doi.org/10.1038/srep21889
Klein, S.L., 2000. The effects of hormones on sex differences in infection: From genes to behavior. Neurosci. Biobehav. Rev. 24, 627–638. https://doi.org/10.1016/S0149-7634(00)00027-0
Kumar, A., 2020. Are sample extraction methods necessary For ELISA? [WWW Document]. https://www.enzolifesciences.com/science-center/technotes/2020/october/are-sample-extraction-methods-necessary-for-elisa?/.
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. Horm. Behav. 53, 241–248. https://doi.org/10.1016/j.yhbeh.2007.10.003
Lydersen, C., Kovacs, K.M., 2005. Growth and population parameters of the world’s northernmost harbour seals Phoca vitulina residing in Svalbard, Norway. Polar Biol. 28, 156–163. https://doi.org/10.1007/s00300-004-0656-7
Makin, H.L.J., Honour, J.W., Shackleton, C.H.L., Griffiths, W.J., 2010. General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry, in: Makin, H.L.J., Gower, D.B. (Eds.), Steroid Analysis. Springer Science+Business Media B.V, pp. 163–282.

https://doi.org/10.10 / 23 b135931_3
Mellish, J.E., Iverson, S.J., 2005. Postpartum dynamics of reproductive hormones in gray and hooded seals. Mar. Mammal Sci. 1, 162–168. https://doi.org/10.1111/j.1748-7692.2005.tb01217.x
Norjavaara, E., Ankarberg, C., Albertsson-Wikland, K., 1996. Diurnal rhythm of 17beta-estradiol secretion throughout pubertal development in healthy girls: evaluation by a sensitive radioimmunoassay. J. Clin. Endocrinol. Metab. 81, 4095–4102.
Ortiz, R.M., Houser, D.S., Wade, C.E., Ortiz, C.L., 2003. Hormonal changes associated with the transition between nursing and natural fasting in northern elephant seals (Mirounga angustirostris). Gen. Comp. Endocrinol. 130, 78–83. https://doi.org/10.1016/S0016-6480(02)00572-5
Petrauskas, L.R., Atkinson, S.K., 2006. Variation of fecal corticosterone concentrations in captive steller sea lions (Eumetopias jubatus) in relation to season and behavior. Aquat. Mamm. 32, 168–174.
Pietraszek, J., Atkinson, S., 1994. Concentrations of estrone sulfate and progesterone in plasma and saliva, vaginal cytology, and bioelectric impedance during the estrous cyckle of the Hawaiian monk seal (Monachus shauinslandi). Mar. Mammal Sci. 10, 430–441.
Pundir, S., Gridneva, Z., Pillai, A., Thorstensen, E.B., Wall, C.R., Geddes, D.T., Cameron-Smith, D., 2020. Human Milk Glucocorticoid Levels Are Associated With Infant Adiposity and Head Circumference Over the First Year of Life. Front. Nutr. 7, 1–8. https://doi.org/10.3389/fnut.2020.00166
Raivio, T., Palvimo, J.J., Dunkel, L., Wickman, S., Jänne, O.A., 2001. Novel assay for determination of androgen bioactivity in human serum. J. Clin. Endocrinol. Metab. 86, 1539–1544. https://doi.org/10.1210/jc.86.4.1539
Reijnders, P.J.H., 1990. Progesterone and oestradiol-17b concentration profiles throughout the reproductive cycle in harbour seals (Phoca vitulina). Journals Reprod. Fertil. 90, 403–409.
Reilly, J.J., 1991. Adaptations to prolonged fasting in free-living, weaned gray seal pups. Am. J. Physiol. 260, 267–272.
Ruiz-Cortes, Z.M., 2012. Gonadal Sex Steroids: Production, Action and Interactions in Mammals, in: M, O.S. (Ed.), Steroids: From Physiology to Clinical Medicine. pp. 3–44.
SCOS, 2020. SCOS-BP 20/02, Scientific advice on matters related to the menagement of seal populations: 2020.
Theodorou, J., Atkinson, S., 1998. Monitoring total androgen concentrations in saliva from captive Hawaiian monk seals (Monachus schauinslandi). Mar. Mammal Sci. 14, 304–310.
Troisi, G.M., Barton, S.J., Liori, O., Nyman, M., 2020. Polychlorinated Biphenyls (PCBs) and Sex Hormone Concentrations in Ringed and Grey Seals: A Possible Link to Endocrine Disruption? Arch. Environ. Contam. Toxicol. 78, 513–524. https://doi.org/10.1007/s00244-020-00716-z
Vincent, I.C., Michell, A.R., 1992. Comparison of cortisol concentrations in saliva and plasma of dogs. Res. Vet. Sci. 53, 342–345. https://doi.org/10.1016/0034-5288(92)90137-q
Vining, R.F., Mcginley, R.A., Maksvytis, J.J., 1983. Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. Ann Clin Biochem 20, 329–335.
Wood, P., 2009. Salivary steroid assays - Research or routine? Ann. Clin. Biochem. https://doi.org/10.1258/acb.2008.008208
Yan, M., 2018. Dilutional Linearity, Parallelism, Spike-and-Recovery in ELISA: How to QC Your Results? [WWW Document]. URL https://www.enzolifesciences.com/science-center/technotes/2018/february/dilutional-linearity-parallelism-spike-and-recovery-in-elisa-how-to-qc-your-results/
Zuur, A.F., Ieno, E.N., Walker, J.N., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Lideks and Extensions in Ecology with R, Mixed Effects Models and Extensions in Ecology with R. Springer, New York.

Table 1. Descriptive statistics of ELISA analysis of three steroid hormones – testosterone, estradiol and cortisol – measured in plasma and saliva from suckling and fasting grey seal pups.

Hormone	**Testosterone**			**Estradiol**			**Cortisol**		
Matrix	Plasma (ng/ml)		Saliva (pg/ml)	Plasma (pg/ml)		Saliva (pg/ml)	Plasma (ng/ml)		Saliva (µg/dl)
Plate number (year of analysis)	Plate 1 (2014)	Plate 2 (2015)	Plate 1 (2014)	Plate 1 (2014)	Plate 2 (2015)	Plate 1 (2014)	Plate 1 (2014)	Plate 2 (2015)	Plate 1 (2014)
N of samples of grey seal pups analyzed	32	29	32	32	29	32	32	29	37
Mean ± SE	0.7 ± 0.02	0.2 ± 0.04	131.37 ± 14.23	233.45 ± 12.98	98.9 ± 12.12	11.77 ± 1.44	107.62 ± 4.95	49.4 ± 4.13	0.212 ± 0.02
Min – Max	0.51 – 1.12	0.05 – 1.26	31.07 – 313.42	150.43 – 385.9	16.7 – 236.88	4.45 – 42.45	63.97 – 161.25	16.56 – 99.98	0.065 – 0.479
Obtained/expected % of sample dilution:									
1 is the measured concentration of pooled sample	1 = 0.56	1 = 0.18	1 = 111.80	1 = 105.43	1 = 49.43	1 = 11.61	1 = 224.47	1 = 141.49	1 = 0.25
0.75 is 75% sample mix	95.63 %	105.84 %	100.69 %	95.05 %	82.13 %	83.97 %	97.45 %	102.10 %	92.31 %
0.5 is 50 % sample mix	99.55 %	87.57 %	116.63 %	83.18 %	95.07 %	66.17 %	73.05 %	92.59 %	114.62 %
0.25 is 25 % sample mix	72 %	72.32 %	118.36 %	71.23 %	109.79 %	59.28 %	81.87 %	81.49 %	106.15 %
RR % low spiked	115.58 %	91.30 %	119.74 %	89 %	119.37 %	104.59 %	104.68 %	100.02 %	91.97 %
RR % high spiked	122.56 %	92.61 %	101.57 %	94.14 %	99.26 %	76.13 %	103.8 %	90.69 %	98.21 %
Intra-assay CV (%)	8.54 %	11.04 %	3.89 %	5.15 %	2.84 %	4.45 %	6.35 %	3.93 %	8.35 %
Inter-assay CV (%)	86.24 %		N/A	8.49 %		N/A	25.22 %		N/A



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434 **Fig. 1.** A linear relationship between the concentrations of lognormalized values of ES and concentrations of EB.

435 Line indicates linear relationship.

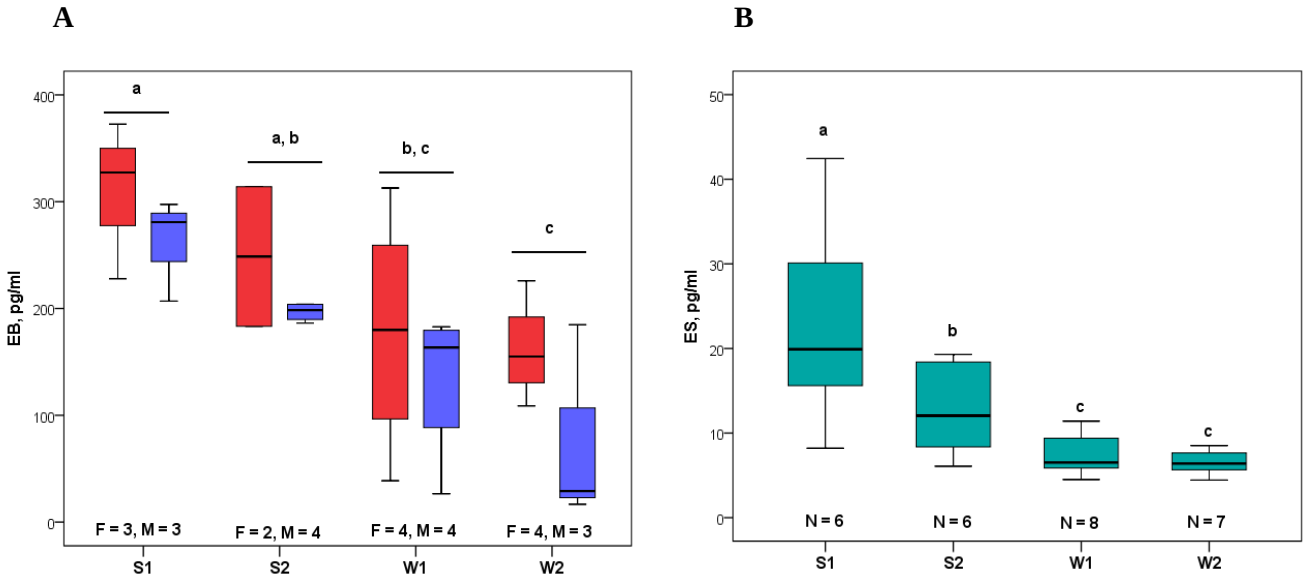

Fig. 2. Concentrations of EB (A) and ES (B) from grey seal pups during different nutritional stages (S1, S2, W1, W2). Red boxes denote EB concentrations of female, blue – of male grey sea pups. Letters indicate significant ($p < 0.05$) differences between steroid concentrations during different nutritional stages. Middle line denotes median, outer box – interquartile range; whiskers – 95 % confidence intervals of steroid levels. Sample size (N) for each nutritional stage is given below each bar in the graphs.

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