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NOTE

Reproductive and stress-related hormones in whiskers from two North Pacific phocids: Harbor and ringed seals

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Several populations of North Pacific pinnipeds are currently listed as depleted under the marine mammal protection act, endangered under the Endangered Species Act, or with unknown status, highlighting the need for new methods to assess the reproductive rates of these populations. Most phocids are annual breeders with estrus and parturition occurring on terrestrial or ice platforms. In phocids, serum progesterone concentrations remain elevated during late gestation, supporting identification of pregnancy after implantation (Gardiner, Boyd, Follett, Racey, & Reijnders, 1999; Gardiner, Boyd, Racey, Reijnders, & Thompson, 1996; Mellish & Iverson, 2005; Reijnders 1990). However, current sampling methods based on blood and feces only provide a snapshot of reproductive status. Recently, methods were developed to measure cortisol in whiskers (Karpovich, Skinner, Kapronczai, Smith, & Janz, 2019), highlighting the potential to measure reproductive hormones in whiskers from free-ranging phocid seals. Unlike other tissues currently used for determining reproductive status, whiskers do not require special storage or handling, which can be challenging in remote field conditions. More importantly, the potential to use whiskers to

measure reproductive hormones may alleviate problems associated with a single sample by capturing reproductive hormone concentrations sequentially along the length of the whisker, allowing for the examination of hormone concentrations over the course of one year for phocids (Greaves, Hammill, & Eddington, 2004; Hirons, Shell, & St. Aubin, 2001; Lübcker, Condit, Beltran, Bruyn, & Bester, 2016; Zhao & Schell, 2004). Given the potential utility of measuring reproductive hormones in phocid whiskers, our objectives were to (1) validate enzyme immunoassays (EIA) to measure reproductive and stress-related steroid hormones in phocid whiskers, (2) compare the patterns of multiple steroid hormones along the length of whiskers to evaluate the retention of steroid hormones in phocid whiskers, (3) apply immunohistochemistry (IHC) methods to explore deposition of progesterone and cortisol along harbor seal whiskers, and (4) investigate the influence of age class (i.e., adult vs. subadult) and reproductive state on hormone concentrations in whiskers.

Phocid whiskers grow continuously until most are shed during the annual molt; however, the nonlinear growth rate

complicates assigning season to specific areas within a whisker (McHuron et al., 2016; McHuron, Williams, Costa, & Reichmuth, 2020; Hirons et al., 2001; Smith, Karpovich, & Horstmann, 2019). We used archived whiskers for this study. During field research between late May and early July, 20 female harbor seals (16 adult, 4 subadult) were captured by entanglement in monofilament gill nets, disentangled from the net, and transferred to the primary research vessel. Seals were sedated with Diazepam (intravenous 0.25 mg/kg) and manually restrained for sample collection. Researchers noted whether females were visibly pregnant (distended nipples, visible lump of fetus), lactating, or observed with a pup prior to sampling. The longest whisker was removed with pliers and stored in Whirl-Paks or paper envelopes at room temperature until further processing. Female ringed seals (seven adult, two subadult) were sampled during subsistence hunts and whole cheek pads and reproductive tracks were collected and frozen. Reproductive tracks were thawed, and ovaries were thinly sectioned (~2 mm) and visually inspected for follicles, corpora lutea, and corpora albicans and uterine horns were inspected for the presence of an implanted fetus. Ringed

seals were classified as pregnant and implanted, pregnant and not implanted, or not pregnant. Further, uterine scars were recorded, and females were classified as nulliparous, primiparous, or multiparous. The longest whisker was collected and stored in a Whirl-Pak or paper envelope at room temperature until further processing.

Whiskers were sonicated for 10 min in deionized water, the outer and inner root sheaths were removed when present, and whiskers were cleaned with a 2:1 chloroform methanol solution to remove surface contaminants and left to dry overnight (Rea et al., 2015). Each whisker was weighed (±0.1 mg) and length measured on a flat surface using a ruler (±0.05 cm) before sectioning. Starting at the proximal end of the whisker (root end), 0.25–3.2 cm of whisker tissue was sectioned using a hand chisel. The length of each segment was dependent on the mass of the segments, with a minimum target mass of 2.5 mg and length of 0.25 cm. Whisker segments were further sectioned into smaller pieces and placed into a 2 ml polypropylene tube (Type I, Sarstedt) with two 5 mm steel ball bearings and pulverized at 30 KHz for 12 min using a Retsch MM 400 mixer mill with adapters

for 10 vials (Verder Scientific Inc., Newtown, PA). Once powdered, 1 ml of 100% methanol was added to each vial and the samples were rotated slowly on a benchtop tube rotator for 24 hr at room temperature to extract the hormones (Hunt et al., 2014; Macbeth, Cattet, Stenhouse, Gibeau, & Janz, 2010). Samples were then centrifuged at 10,500 × g, 10°C for 13 min. The supernatant was transferred to a new polypropylene tube and the pellet was rinsed with 0.2 ml methanol, agitated, centrifuged, and the supernatant combined with the previously removed supernatant. Methanol extracts were stored at ≤−80°C until assayed. Methanol extracts were centrifuged, and a subsample was transferred to a borosilicate glass tube, dried under forced air, and reconstituted in assay buffer specific for each hormone EIA kit.

EIA kits from Arbor Assay (Ann Arbor, MI) were used to quantify the concentration of three steroid hormones: progesterone (K025), 17β-estradiol (KB30), and cortisol (K003). Standard methods including recovery of added mass, parallelism and dilution linearity were used for the laboratory validations (Hunt et al., 2014). Four additional whiskers from four ringed seals, which were not the longest whisker, were used for the

validation of the cortisol EIA. All samples were run in duplicate per manufacturer's instructions and all assays included a full standard curve, two controls, nonspecific binding wells and "zero" (blank) wells.

Published assay sensitivities are 17.3 pg/ml for cortisol, 47.9 pg/ml for progesterone, and 2.21 pg/ml for 17β-estradiol. Interassay coefficients of variation were ≤16% for all assays and intraassay coefficients of variation were as follows: ≤12% for cortisol, ≤20% for progesterone, and ≤13% for 17β-estradiol. To determine if the rapid decline in cortisol concentrations previously reported in phocid whiskers (Karpovich et al., 2019) was associated with where steroid hormones are deposited in phocid whiskers, we applied IHC methods to one whisker from four female harbor seals. Whiskers were incubated at 37°C in 1.0×10^{-2} M Cleland's reagent for 2–10 days until softened, with stiffer whiskers requiring a longer incubation period. Once softened, the whiskers were air dried, and cut on a transverse plane into 25 μm segments, starting from the root and moving toward the tip. Each segment was then cut on a cross-section using a sliding stage microtome with a freezing stage at 7 μm.

The whiskers ranged from having 4–10 segments depending on the original length and each segment produced between 7 and 20 sections. Following sectioning, serial sections were placed in microcentrifuge tubes with 10× citrate buffer solution (FisherSci, Waltham, MA). The centrifuge tubes were placed in an autoclave for 15 min at high pressure. Once cooled, the samples were removed from the centrifuge tubes and blocked for 30 min in 1% bovine serum albumin (BSA solution, FisherSci). Segments were rinsed three times in 0.1 M phosphate buffer solution (PBS) and placed on 1% gelatin coated slides and allowed to dry, and then circled with a PAP pen. Each slide was incubated with 200 µl of primary antibodies, either anti-cortisol or antiprogesterone (Sigma-Aldrich). Two parafilm bridges were placed on either side of a wax barrier and a coverslip was placed over each slide. Slides were incubated overnight at 2°C in a dark humidity chamber. The following morning the coverslips were removed, and the slides were rinsed with PBS for 30 min and then again for 5 min. Slides were incubated with 200 µl of the secondary antibody (Anti-Rabbit IgG, Sigma-Aldrich) in the dark humidity chamber overnight at 2°C with new parafilm bridges placed next to the

wax barrier. The slides were then rinsed with PBS for 30 min and 5 min. Next, 3,3′-Diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) was pipetted onto the slides for 25 min, and then rinsed three times with PBS. A counterstain of hematoxylin & eosin (H&E) was used for 45 s and 15 s, respectively, with a DH_20 rinse in between. Following H&E staining, the samples were soaked in 90% glycerin 10% PBS for approximately 72 hr and then mounted in glycerin onto 5% gelatin coated slides. The slides were processed and the best sample section from each whisker segment was photomicrographed using a SPOT Pursuit camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon SMZ 1500 stereoscope. The micrographs were scored on a scale of 0–5 by three different readers, and the mean score for each section was calculated. Zero represented no staining and 5 represented the highest amount of staining.

Data were analyzed with Systat 13 (Systat Software, Inc., Point Richmond, CA). Hormone concentrations are expressed as means plus or minus standard deviation in pg/mg of whisker (herein, pg/mg) and descriptive statistics by species are reported in Table 1. Cortisol and 17β-estradiol concentrations

were low requiring larger sections of whiskers to ensure the concentrations were above the detection limit of the EIA kits (discussed below). We therefore limited the number of whiskers analyzed for the concentrations of cortisol and 17β-estradiol and only report descriptive statistics (Table 1). Similarly, the limited ringed seal whiskers that were analyzed in our study did not support statistical comparisons and only descriptive statistics are reported (Table 1). Sufficient data were available for progesterone concentrations to compare age classes (adult, subadult) and reproductive state in adult harbor seals (pregnant, nonpregnant). We took two approaches, first a repeated measures analysis of variance (ANOVA) was used to evaluate progesterone concentrations among whisker sections and either age class or reproductive state in adult harbor seals. Secondly, we used two-tailed unpaired *t* tests to compare the progesterone convention for whole whiskers between age classes (adult, subadult) or reproductive state in adult harbor seal (pregnant, nonpregnant). An alpha less than 0.05 was considered significant.

For analytical validations, pools of extracted methanol

were made for each species and consisted of multiple segments from multiple whiskers. Serially diluted pools were used to determine linearity and parallelism to the standard curves. We used *F* tests to compare the slope of the serial dilution pools and the standard curves and found no significant difference for progesterone ($p \geq .354$), 17β-estradiol ($p \geq .243$), or cortisol ($p \geq 0.247$). Accuracy tests were within the accepted slope range for all three hormones ($R^2 \geq 0.989$) and indicate no matrix interference with binding in the EIAs.

In both phocids species, whiskers were thicker near the root and became thinner toward the tip, requiring longer segments to have enough tissue for analyses and reducing resolution in the distal portion of the whiskers. Harbor seal whiskers were on average longer and heavier than ringed seal whiskers (Table 1). Whiskers were sectioned based on the required minimum mass for detection of progesterone, and methanol extracts from adjacent whisker segments were combined for 17β-estradiol and cortisol and the combined mass and length of whisker segments used to measure each hormone are reported in Table 2. This sampling approach allowed the ratio of the tissue

mass to methanol volume to be consistent while allowing for multiple hormones to be measured. However, the sampling design provides fewer measurements of 17β-estradiol and cortisol concentrations along the whiskers, leading to loss of resolution in these hormones.

Progesterone was measurable in all whiskers of both species regardless of age class and reproductive status. Harbor seal whiskers (n = 20) provided between 7 and 35 segments per whisker. Adult female harbor seals had greater variation in concentrations of progesterone, with females noted as pregnant or lactating having elevated concentrations near the root of the whisker while concentrations in nonpregnant adult and subadult harbor seals were fairly even or had slightly lower values towards the root of the whiskers (Figure 1a–c). When comparisons were made between age classes using repeated measures ANOVA, subadult females had lower progesterone concentrations compared to adults (p = .012) while no difference was found among whisker segment (p = .060) or age class and segment interaction (p = .242). For adult female harbor seals that were lactating (n = 9) or had a visible fetal lump (n = 1), progesterone concentrations

were greater compared to nonlactating adult females (Figure 1a,b; p = .040) and concentrations varied among whisker segments (p = .044) while the interaction between reproductive state and whisker segment was not significant (p = .064). When we averaged progesterone concentrations for the entire whiskers, concentrations were lower in subadult harbor seals (Figure 2a; p = .012). Whole whisker progesterone concentration in adult females that were lactating or had a visible fetal lump had on average 35.3 ± 7.2 pg/mg (n = 10) compared to 27.0 ± 3.5 pg/mg in nonlactating adult females (Figure 2b; n = 5; p = .051).

Adult ringed seals had higher progesterone concentrations with variability between segments, though no obvious pattern was found (Figure 3a). Whereas, progesterone concentrations in the two subadult ringed seals were lower with little difference between segments, though the small whiskers in these seals only allowed for 2–3 segments across each whisker (Figure 3a). Like harbor seals, progesterone concentrations for whole whiskers were lower in subadult seals compared to adults (Figure 3b). It is of note that while both subadult females had progesterone concentrations lower than those of the adults, one of the

subadults was 4 years old and primiparous with an implanted fetus (PH15SH011), and displayed a very slight increase in progesterone near the root, while the second subadult was 2 years old with no reproductive information.

For both species, progesterone concentrations were higher in adult seals compared to subadults, with greater variability in progesterone concentrations along the whiskers in adults. Progesterone concentrations in serum have been used to determine sexual maturity in phocid seals (Gardiner et al., 1996, Zhang et al., 2014). As with serum concentrations, progesterone concentrations in whiskers could likely determine sexual maturity in phocid seals; however, a larger study is needed with a broader range of age classes and with seals of known age to define a threshold for sexual maturity and to determine if we can distinguish immature seals from nonpregnant adult seals.

We measured 17β-estradiol in the whiskers of four harbor seals and two ringed seals which required combining the methanol extracts from up to three adjacent segments to ensure estradiol was detectable in the EIA kit. Estradiol concentrations for the two phocid species had similar concentrations (Table 1). For

each harbor seal whisker, four or five estradiol concentrations were measured and the concentrations were higher near the tip and slightly decreased and flattened out towards the root, whereas one whisker showed a sharper decline from tip towards the root of the whisker (Figure 4a). The two ringed seal whiskers provided three estradiol concentrations each and the concentration of estradiol displayed opposite patterns in the two whiskers (Figure 4b). For a subset of whiskers, we measured both progesterone and 17β-estradiol concentrations. Both reproductive hormones showed variation along the whiskers but there was not an obvious pattern or relationship between the two hormones (Figure 4c,d). For example, in one harbor seal whisker the two reproductive hormones had similar patterns, whereas in the ringed seal whisker, estradiol and progesterone had opposing concentrations (Figure 4c,d). It was not surprising that concentrations of 17β-estradiol in whiskers did not provide a discernable pattern given the length of the whisker segments and the highly transient nature of estradiol during estrous. In fact, the peak 17β-estradiol in serum associated with estrous is often missed (Mellish & Iverson, 2005), even in captive

facilities when samples were collected every 5–7 days (Kiyota, Yamaguchi, Nishikawa, & Kohyama, 1999; Sattler & Polasek, 2017). Given our initial results, we limited the number of whiskers that were analyzed for 17β-estradiol.

We were able to measure two reproductive hormones in harbor and ringed seals. Progesterone concentrations were higher during late gestation, as indicated by elevated concentrations near the root in pregnant or lactating female harbor seals, compared to progesterone concentrations remaining low in nonpregnant adult and subadult harbor seals. These findings support the use of harbor seal whiskers to measure these hormones to investigate reproductive states and potentially identifying sexual maturity, but further studies are needed with larger sample sizes. However, the progesterone concentration in phocid whiskers is likely influenced by the nonlinear growth rates and the annual molting of phocid whiskers (Beltran et al., 2015; Greaves et al., 2004; Hirons et al., 2001; Lübcker et al., 2016), leading to the majority of hormones associated with active gestation to be in a small segment of the whisker near the root. Therefore, caution is needed as most phocid seals molt their whiskers

annually and the timing of sample collection needs to be carefully considered (Smith, Karpovich, Breed, & O'Brien, 2018). More importantly, collection of whiskers from live seals required capture and restraint, during which we were able to determine pregnancy or recent birth based on the presence of distended nipples, active lactation, or a fetal lump. In these cases, visual inspection or measurement of progesterone concentrations in serum may be easier to determine pregnancy during late gestation in phocids (Gardiner et al., 1996). Measuring progesterone in phocid whiskers is feasible and could be useful for archived samples when reproductive information is not available.

The analysis of cortisol was not exhaustive, but rather specific to determining if the rapid decline in cortisol concentrations previously observed in phocid whiskers by Karpovich et al. (2019) was replicated in our laboratory and to compare to patterns observed in reproductive hormones. We sectioned and measured cortisol concentrations in segments of whiskers from three harbor seals and two ringed seals. Cortisol was measurable in the whiskers of both species (Table 1), with

higher concentrations being found near the root followed by rapid decrease towards the tip of some whiskers (Figure 5 a,b) as previously reported (Karpovich et al., 2019). This pattern was largely consistent in the few whiskers we examined. There was quite a difference between individuals within each species in cortisol concentrations for segments near the root whereas the individual differences were less pronounced in segments near the distal part of the whiskers (Figure 5a,b). The declining cortisol concentrations paired with thinning of whiskers moving away from the root required larger segment lengths to meet the mass needed to ensure samples were above the detection limit of the cortisol EIA kit. This required the methanol extracts from multiple segments within each whisker to be combined (Table 2), leading to loss of some temporal resolution as sampling moved towards the tip of the whiskers. The cortisol pattern led us to explore hormone deposition in whiskers using IHC staining.

The IHC staining provided similar patterns as observed in the concentration of cortisol and progesterone in harbor seal whiskers (Figure 6). All whisker segments had some degree of staining for progesterone and cortisol. Though the sample size

was small, the two pregnant females had slightly higher scores for progesterone near the root of the whisker compared to the two nonpregnant seals. There was either little change in cortisol score along the whiskers or cortisol scores were slightly higher near the root of the whisker and decreased toward the tip. For staining both cortisol and progesterone were diffused throughout the whiskers with some variation in the location of the staining; however, when paired sections stained for cortisol and progesterone were compared, no obvious difference between the locations of the stains were observed, suggesting progesterone and cortisol are deposited similarly within phocid whiskers.

Karpovich et al. (2019) proposed explanations for the rapid decrease in the concentration of cortisol near the root including: the nonlinear growth rates found in phocid whiskers, water immersion or leaching of cortisol from of the whisker, or inclusion of nonkeratin tissues near the root. While we also found cortisol concentrations higher near the root followed by a rapid decrease towards the tip of whiskers, this pattern was not observed in the concentrations of progesterone or 17β-estradiol.

If the pattern observed in cortisol was solely due to the nonlinear growth of phocid whiskers, we would expect to find similar patterns in the other hormones, which we did not observe. Rather, reproductive state seems to explain the increase in progesterone concentrations near the root observed in pregnant seals (Figure 1a). Similarly, while steroid hormones are hydrophobic and likely not readily leached out in water, if exposure to water or ultraviolet light did alter the stability or retention of cortisol in the distal portion of whiskers, we would expect a similar pattern across steroid hormones, unless cortisol was deposited in locations of the whisker more at risk to abrasion or loss. However, IHC staining did not show cortisol deposition to be more peripheral than progesterone in harbor seal whiskers (data not shown). While our sample size is small, taken together, it seems more likely that the pattern found in cortisol in phocid whiskers may be due to the inclusion of nonkeratin tissues near the root. Rea et al. (2015) found the isotope signatures near the root of Steller sea lion (*Eumetopias jubatus*) whiskers were depleted and suggested it was due to additional tissue, beyond keratin, being present in the most

proximal portion of the root. However, our study did not include isotope analysis and we did not attempt to investigate the type of tissue was present at the root. A larger question is whether cortisol concentrations in keratinous tissue such as whiskers represents circulating concentrations. Cortisol is also produced within the epidermal and dermal components of the skin, as well as being present in secretions from glands (Sharpley, McFarlane, & Slominski, 2012) and Keickeis et al. (2012) suggested local production of glucocorticoids contributes more than circulating levels to concentrations in hair. Future studies are needed to investigate the relationship between circulating cortisol concentrations and cortisol deposition in whiskers.

Whiskers have proven useful for supporting studies assessing dietary stable isotope signatures and migration in phocids (Lerner et al., 2018; Lowther, Fisk, Kovacs, & Lydersen, 2017; Newland et al., 2011). In this study, we report a novel method to measure reproductive hormones in phocid whiskers, providing another tool for future studies. We found hormones are deposited throughout the length of a phocid whisker, helping to validate the utility of whiskers as a reliable matrix for

measuring reproductive hormones. We found differences in whisker progesterone concentration between adults and subadults from two phocid species and between pregnant and nonpregnant adult harbor seals. There may be other reproductive steroid hormones, such as testosterone, that may also be useful for assessing age class. The timing of the whisker collection for both phocid species may influence detection of the rise in progesterone in the whisker likely associated with active gestation. Nevertheless, phocid whiskers contain hormones incorporated during the estimated years' worth of growth (Beltran et al., 2015, Hirons et al., 2001, Lübcker et al., 2016). Analysis of hormone concentrations from whiskers could be beneficial in accruing long-term physiological data from keratinized tissues in phocids. Further, whiskers in archived collections from museums, stranding networks, and government agencies could serve as a reservoir of samples to perform retrospective studies on phocid reproductive and stress physiology, helping to understand how future environmental changes may impact phocid physiology.

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TABLE 1 Mean (± standard deviation) concentrations, ranges, and *n* (number of whiskers) are reported by species for cortisol, progesterone, and 17β-estradiol.

	Whole whisker length (cm)	**Whole whisker mass (mg)**	**Progesterone (pg/mg)**	**17β-estradiol (pg/mg)**	**Cortisol (pg/mg)**
Harbor seal	10.7 ± 1.5 (7.7–13.0) *n* = 19	64.7 ± 15.1 (28.5–92.0) *n* = 19	31.2 ± 14.8 (2.6–87.4) *n* = 20	0.8 ± 0.4 (0.4–2.1) *n* = 19	2.7 ± 2.0 (1.0–6.6) *n* = 17
Ringed seal	8.6 ± 0.7 (7.4–9.8) *n* = 9	29.3 ± 12.5 (20.2–59.2) *n* = 9	22.2 ± 17.8 (6.7–54.2) *n* = 7	1.7 ± 1.1 (0.5–3.5) *n* = 6	3.2 ± 3.3 (0.84–8.5) *n* = 7

TABLE 2 Mean (± standard deviation) and ranges of mass and length of whisker segments used to measure cortisol, progesterone, and 17β-estradiol by species.

	Progesterone	**17β-estradiol**	**Cortisol**
Harbor seal			
Whisker segment mass (mg)	4.1 ± 2.6 (0.9–11.9)	12.4 ± 4.7 (5.6–23.3)	11.1 ± 8.9 (4.6–33.2)
Whisker segment length (cm)	0.7 ± 1.1 (0.2–12.1)	2.1 ± 1.0 (1.0–4.3)	1.8 ± 1.5 (1–5.7)
Ringed seal			
Whisker segment mass (mg)	5.6 ± 2.1 (2.2–10.5)	8.3 ± 2.7 (3.8–11.4)	11.5 ± 5.1 (4.8–20.3)
Whisker segment length (cm)	1.6 ± 0.8 (1.0–4.7)	3.0 ± 0.6 (2.0–3.5)	2.5± 0.6 (2.0–3.4)

FIGURE 1 Harbor seal progesterone concentrations along the length of whiskers from (a) pregnant or lactating adults, (b) nonlactating adults, and (c) subadults. Different symbols within each panel denotes individual seals.

FIGURE 2 Box plot of whole whisker concentration of progesterone for (a) subadult and adult female harbor seals and (b) lactating and nonlactating adult harbor seals. * denotes significant difference.

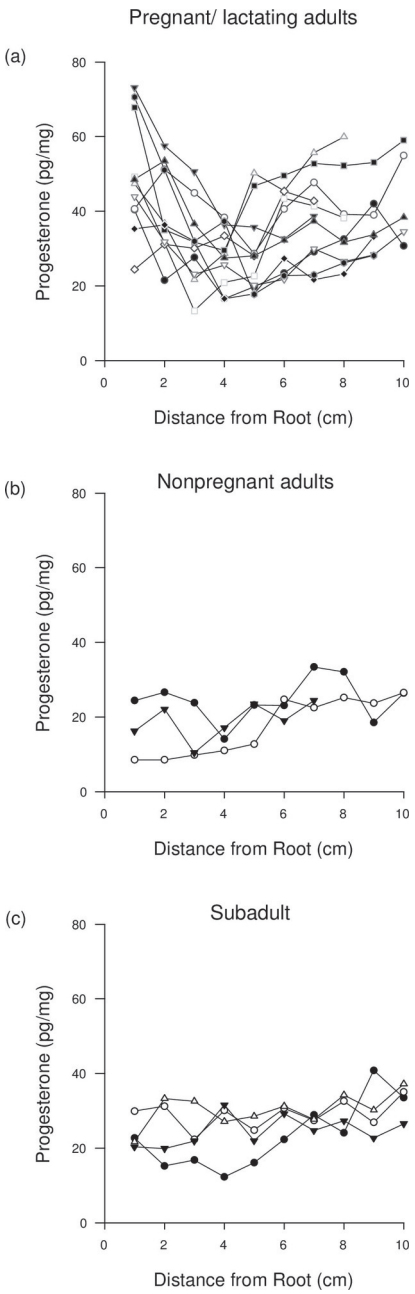
FIGURE 3 Ringed seal progesterone concentrations (a) along the length of whiskers from five adult and two subadult females and (b) progesterone concentrations of whole whiskers from the seven whiskers presented in (a). Note: subadults have white symbols and bars, primiparous adult females have grey symbols and bars, and multiparous adult females have black symbols and bars.

FIGURE 4 Concentrations of 17β-estradiol along the length of the whisker from (a) three harbor seals and (b) two ringed seals. Comparison of 17β-estradiol and progesterone concentrations along the length of one example whisker from (a), (c) harbor seal, and (d) ringed seal.

FIGURE 5 Concentrations of cortisol along the length of two

whiskers from (a) harbor seals and (b) ringed seals with different symbols in each panel denoting individual seals. Concentrations of cortisol (open circles) and progesterone (filled circles) along the length of one whisker from (a), (c) harbor seal, and (d) ringed seal.

FIGURE 6 Scoring for immunohistochemistry staining for (a) progesterone and (b) cortisol in four harbor seal whiskers. Different color bars denote segments within each whisker.



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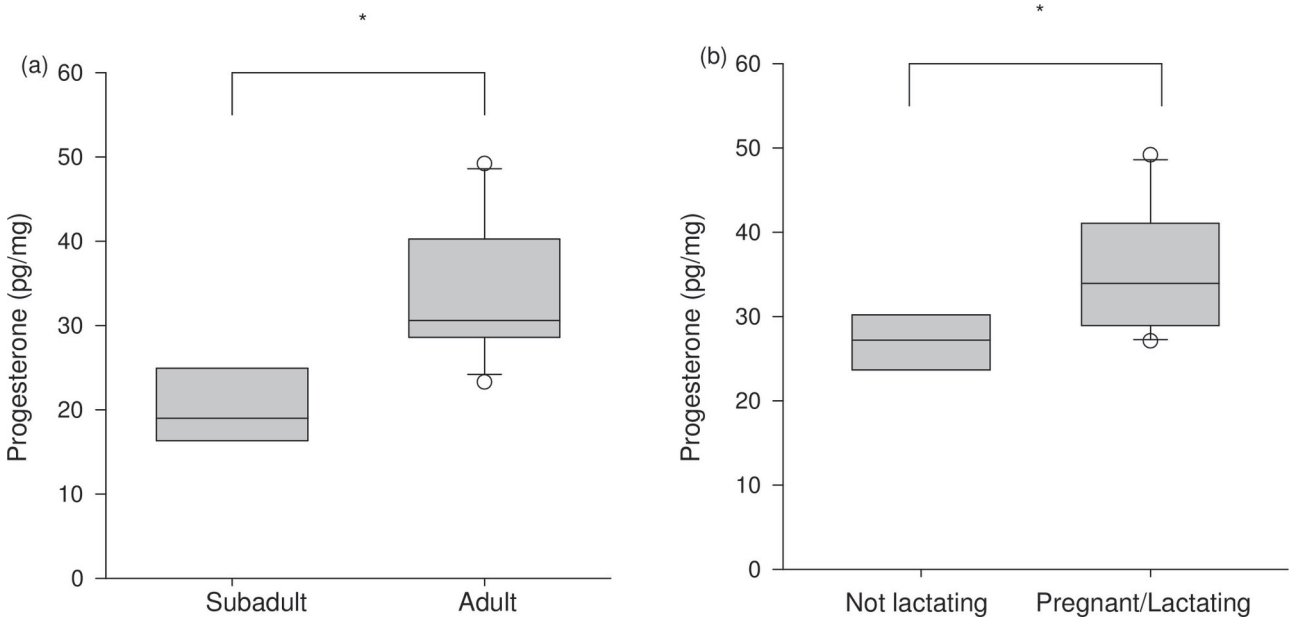

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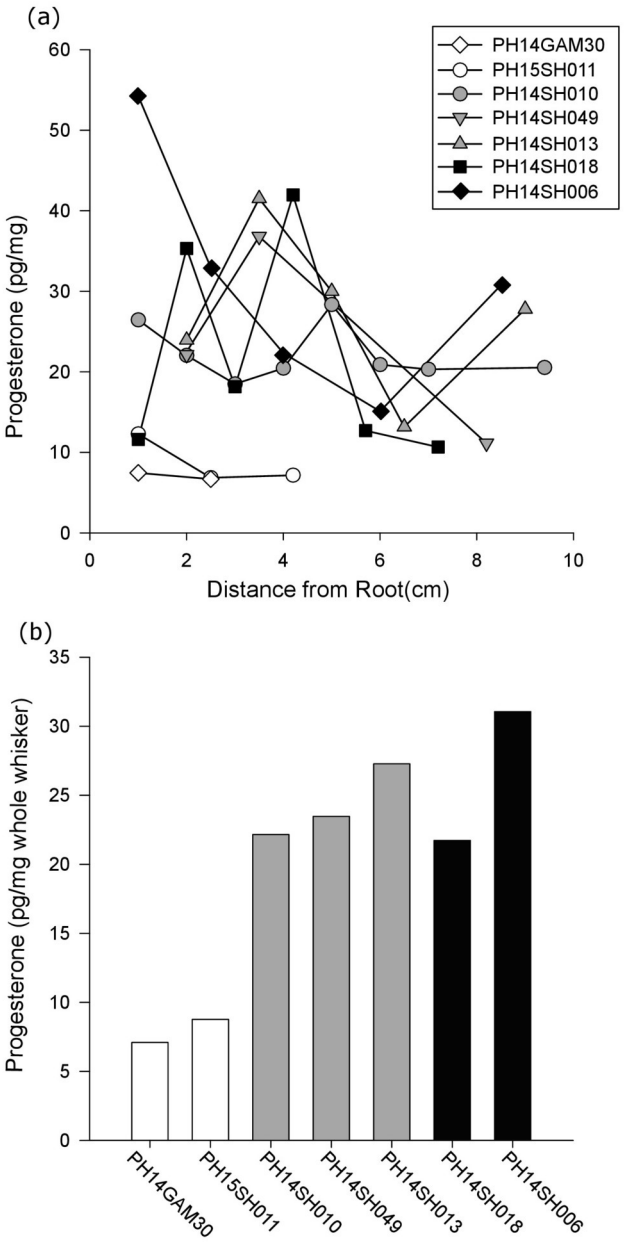

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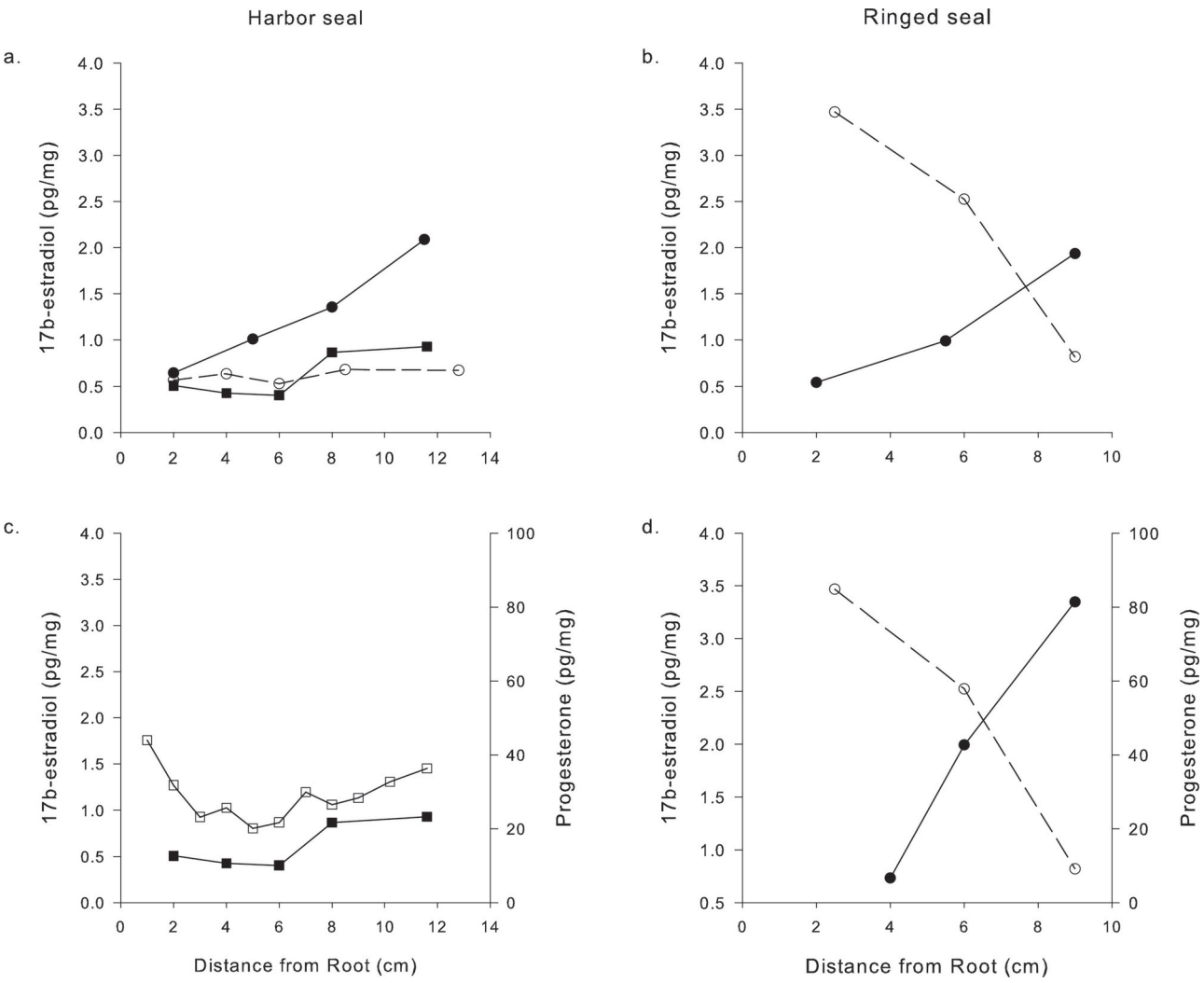

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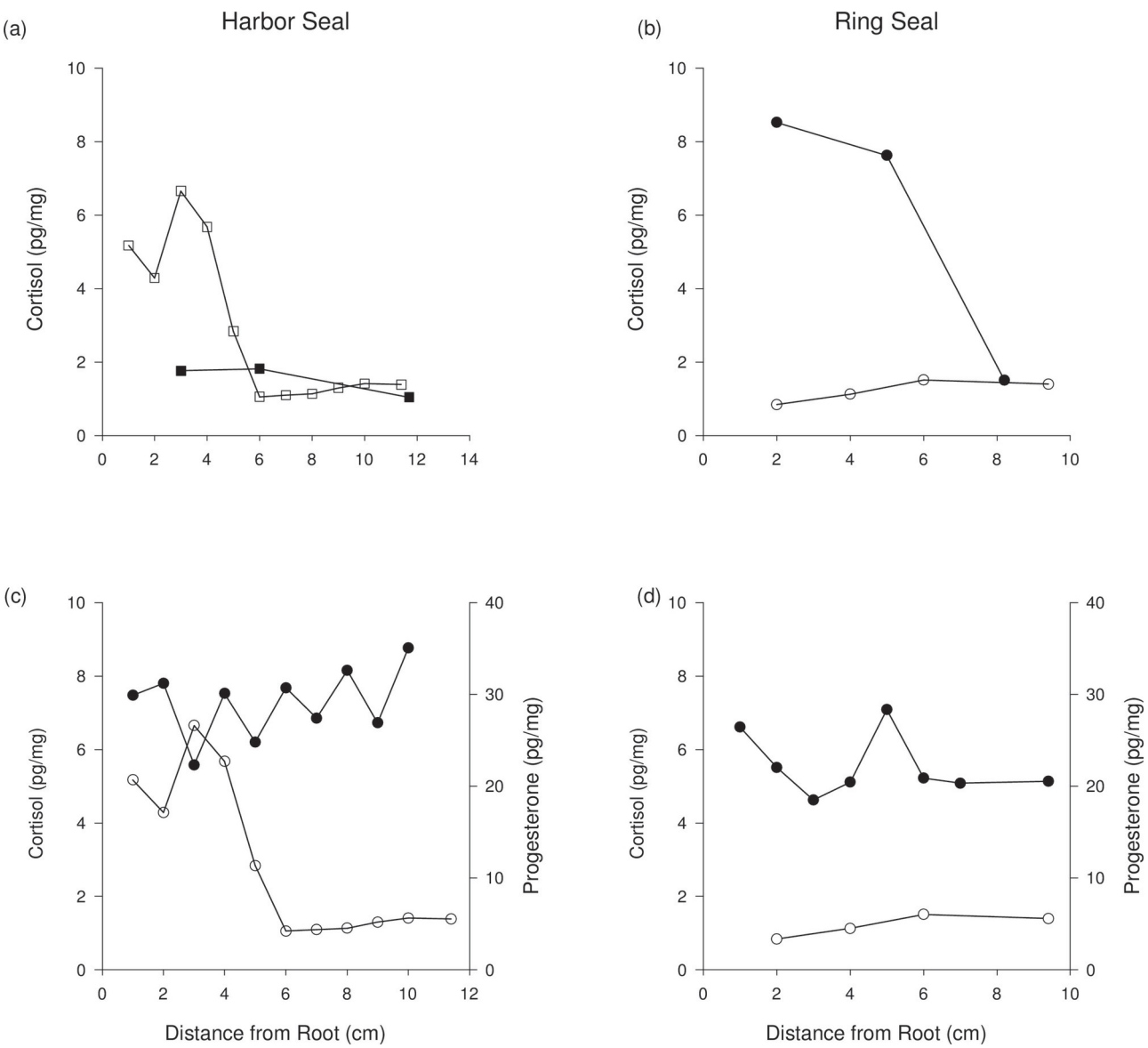

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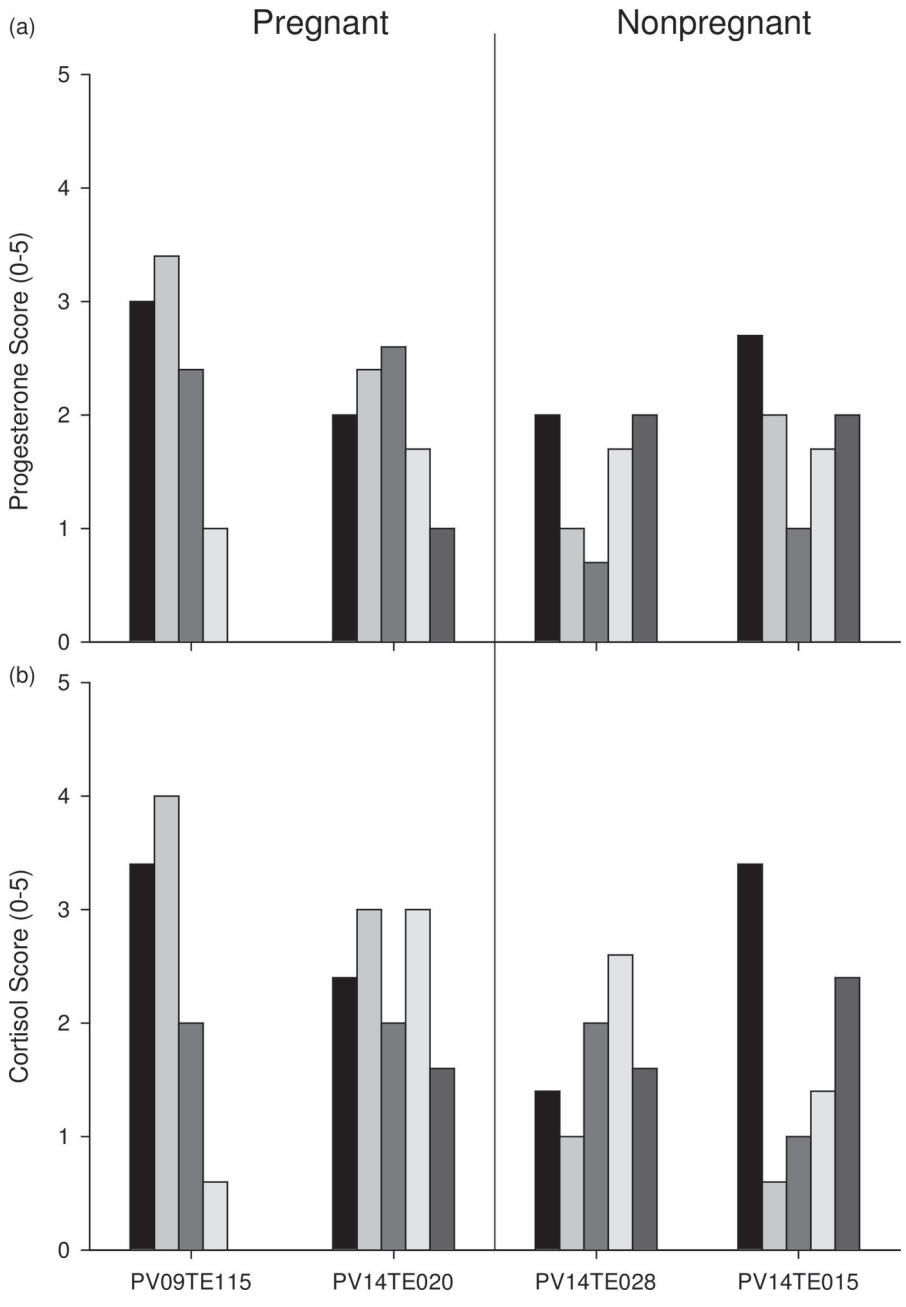

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