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Short Note

Cortisol Levels for Pregnant and Non-Pregnant Steller Sea Lions (*Eumetopias jubatus*) in Human Care

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While a considerable amount of research has gone into investigating stress responses in wild populations or in controlled laboratory experiments (van Raaij et al., 1996; Reeder & Kramer, 2005; Cockrem, 2013; Gong et al., 2015), animals housed in artificial habitats, such as those in zoos and aquaria, are exposed to a myriad of potentially stress-inducing stimuli, yet little is known about stress responses in these environments. Stressful stimuli may be abiotic (i.e., artificial lighting, unnatural sounds, odors, or temperatures) or confinement-specific (i.e., maintenance in abnormal social groups, restrictions on movement and breeding behaviors, and accessible retreat space; Morgan & Tromborg, 2007). An acute stress response can result in behavioral alterations in locomotion (Frankel & Clark, 2000), increased alertness, and vigilance (Perry et al., 2002; Granquist & Sigurjonsdottir, 2014) or physiological symptoms such as increased breathing, heart rate (Gärtner et al., 1980), and release of glucocorticoid isomers (e.g., cortisol; Gong et al., 2015). Additionally, many zoo and aquarium animals are involved in research and undergo routine veterinary health exams, both of which often require repeat sampling, potentially further compounding a stress load. The capture and restraint techniques used in these sampling events can have different impacts on individual serum or plasma cortisol levels—measures that are commonly used as an index of acute stress response in mammals in relation to capture and handling (Desportes et al., 2007; Petrauskas et al., 2008; Cook et al., 2012; Pennington et al., 2013).

Steller sea lions (*Eumetopias jubatus*) are the largest of the otariid pinnipeds, and there are currently fewer than 20 individuals in human care in North America. Though several of these individuals have participated in extensive research on energetics and nutrition under a collaboration between the Vancouver Aquarium and the University of British Columbia and on reproductive physiology at the Alaska SeaLife Center partnering with the University of Alaska Fairbanks, there is little information on baseline cortisol levels associated with research activities, breeding, or pregnancy. Therefore, having reference information, even from a small sample, can be valuable for the continued care, directed research, and health of marine mammals under human care.

For pinnipeds, there are a number of capture-and-restraint methods available to facilitate routine veterinary care and research that ensures animal welfare and human safety, including positive reinforcement (Greig et al., 2007), chemical restraint (Baylis et al., 2014), and physical restraint (Gerlinsky et al., 2018). These techniques have varying impacts on stress responses. For example, serum cortisol levels were three-fold greater in harbor porpoises (*Phocoena phocoena*) that were removed from the water for blood sampling as opposed to individuals that were trained via positive reinforcement to participate in voluntary blood collection from the side of the pool (Desportes et al., 2007). Chemically immobilized grizzly bears (*Ursus arctos horribilis*) also displayed significantly higher serum cortisol than bears trained for voluntary blood draws (Joyce-Zuniga et al., 2016).

Voluntary blood draws are not always feasible; and while efforts to use minimal to non-invasive sampling (i.e., scat, hair, and saliva) to measure animal health and to conduct research studies are rising, the need to occasionally capture and handle animals will likely never be obsolete. However, alterations to other abiotic or confinement-specific stressful stimuli through positive reinforcement training may provide reductions in stress responses. Therefore, our objectives for this study were (1) to quantify serum cortisol levels over multiple years in a captive breeding population of Steller sea lions and (2) to characterize the variation in cortisol levels across various sampling contexts.

From 2012 to 2015, we quantified serum cortisol levels for three adult females (EJ00004, EJ00005, and EJ06006), one subadult male (EJ09007), and one adult breeding male (EJ93001) housed at the Alaska SeaLife Center (ASLC) in Seward, Alaska (Table 1). These animals were concurrently involved in a captive breeding program designed to characterize the reproductive endocrinology of adult female Steller sea lions. During this period, blood was collected from the females every other month (April through December) and from males at varying intervals as part of routine health checks (Sattler & Polasek, 2017). Only the adult breeding male was trained to perform voluntary blood draws from his rear flipper. Due to the frequency of animal handling required for the female sea lions in other concurrent research objectives, voluntary blood draws were not possible. Non-voluntary blood sampling occurred under isoflurane inhalation anesthesia delivered through a modified rubber traffic cone connected to an anesthesia machine. Following a period of positive reinforcement training to desensitize the study animals to the sampling and anesthesia equipment, blood was collected in four contexts:

1. *Habitat Only* – Under stimulus control, the Steller sea lion placed their face in the anesthetic mask in a routine portion of their habitat, outside of a restraint cage for anesthesia.

2. *Cage Only* – Steller sea lions entered the restraint cage, assumed a prone body position, and placed their face into the mask for anesthesia. The restraint cage was designed for Steller sea lions at the University of British Columbia and has been previously used in sample collections at the ASLC and Vancouver Aquarium to facilitate marine mammal research (Mashburn & Atkinson, 2004; Gerlinsky et al., 2014; Spitz et al., 2015; Sattler & Polasek, 2017). The stainless steel restraint cage is 1.06 m in H × 1.09 m in W × 2.74 m in L with two interlinked side panels. When the panels are raised, an adult female or subadult male would be capable of making a 360° turn; but when lowered, the animal is restrained in a prone position.

3. *Cage Press* – Steller sea lions entered the restraint cage under behavioral control. Once inside, if the animal refused the anesthesia mask, cage bars were lowered to apply restraint, and the animal was involuntarily masked for anesthesia. In general, sampling under a Cage Press context occurred early in the study before animals were reliably trained for voluntary anesthesia.

4. *Cage Diazepam* – Following the Steller sea lion entering the restraint cage under behavioral control, 0.4 to 0.5 mg/kg diazepam was delivered orally, concealed inside one thawed capelin (*Mallotus villosus*), 19 to 101 min prior to the initiation of anesthesia and 35 to 116 min before the subsequent blood draw. Diazepam is a low potency benzodiazepine that has been reported to decrease activity in the hypothalamic-pituitary-adrenal axis (HPA-axis), a neuroendocrine system that activates in individuals experiencing some degree of stress (Hommer et al., 1986; Petraglia et al., 1986; Roy-Byrne et al., 1988). While an animal in this sampling context entered the restraint cage voluntarily, the time for them to voluntarily place their face in the anesthesia mask varied and resulted in considerable variation in the time from the administration of diazepam to voluntarily undergoing anesthesia and the successful collection of blood.

Table 1. Animal ID, sex, year of birth (YOB), age class, range of sample collection dates, and the number of samples per Steller sea lion (*Eumetopias jubatus*) in each sampling context per season (CP = Cage Press, CO = Cage Only, CD = Cage Diazepam, and HO = Habitat Only). The adult male (EJ93001) had all samples taken under voluntary sampling (breeding season: $N = 2$; non-breeding season: $N = 9$).

					Breeding				Non-breeding			
Animal ID	Sex	YOB	Age class	Sample collection	CP	CO	CD	HO	CP	CO	CD	HO
EJ00004	F	2000	Adult	Oct. 2012–Dec. 2015	4	2	--	3	2	16	5	2
EJ00005	F	2000	Adult	April 2012–Dec. 2015	2	9	--	3	2	12	7	3
EJ06006	F	2006	Adult	July 2013–June 2015	4	3	5	--	--	2	1	--
EJ09007	M	2009	Subadult	Feb. 2013–Dec. 2015	--	--	1	--	5	--	2	--

Each animal's exposure to the different sampling contexts varied in frequency and by season/reproductive status (breeding: June-August; non-breeding: September-May; Table 1). Within the non-breeding season, samples were further classified by whether they were from a pregnant or non-pregnant female.

Blood draws (N = 107) occurred under ASLC veterinary supervision and were approved via the ASLC Internal Animal Care and Use Committee (R12-03-02) and National Marine Fisheries Service (Permit No. 18534). Steller sea lions were fasted a minimum of 15 h prior to blood collection, and, in general, sampling was conducted in the morning between 0900 to 1200 h (N = 87). The time between the start of anesthesia (i.e., gas on) and blood draw ranged from 7 to 101 min. Blood samples were collected via venipuncture from the caudal gluteal or rear flipper interdigital veins into non-gel clot activator red top tubes (Greiner Bio-One, Monroe, NC, USA), were allowed to clot for no less than 30 min, and were spun at 3,500 rpm for 5 min in an Eppendorf 5804 centrifuge.

Serum was extracted and stored at -80° C until analysis. Serum cortisol from healthy animals (e.g., standard blood hematology parameters and serum chemistries assessed by the veterinary team at the ASLC) was quantified using a commercially produced, enzyme-linked immunosorbent assay kit (ELISA; Alpco, Salem, NH, USA) developed for human serum, with a detectable range of 0.5 to 60 µg/dl on a Spectramax Plus 384 microplate reader with *Softmax Pro* software (Molecular Devices, Sunnyvale, CA, USA). Alpco enumerated cross reactivity as cortisol (100%), prednisolone (13.6%), corticosterone (7.6%), deoxycorticosterone (7.2%), progesterone (7.2%), cortisone (6.2%), deoxycortisol (5.6%), pednisone (5.6%), and dexamethasone (1.6%). Tests of parallelism and accuracy were conducted to validate the assay for Steller sea lions. Parallelism was assessed by serially diluting pooled sea lion sera with kit buffer assayed alongside the standard curve. Lines were parallel; and the slope of the diluted sea lion sera was -0.0368 ($y = -0.0368x + 0.283$; $R^2 = 0.99$), and the slope of the standard curve was -0.0693 ($y = -0.0693x + 0.5371$; $R^2 = 0.95$). Accuracy was tested by comparing a four-parameter nonlinear curve generated from 100 µl samples comprised of 50 µl of pooled serum and 50 µl of one of the 2, 10, or 30 µg/dl kit-provided standards to the standard curve generated solely from 50 µl of kit-provided standards. Curves were parallel between 2 and 30 µg/ml, with the slope of pooled plus standards being -0.0295 and standards being -0.069. A high and low control was run in six separate assays and resulted in an inter-assay variation of 16% (39.24 ± 6.28 SD) and 15% (14.81 ± 2.25 SD). Intra-assay variation calculated from 20 samples equaled 7%.

Kit-provided standards, controls, and sea lion sera were assayed in triplicate. The mean absorbance enumerated from a four-parameter nonlinear regression was used to calculate samples' cortisol concentration. Samples were accepted if (1) the coefficient of variation of all assay standards were < 10%, (2) the standard curve's R^2 was > 97%, and (3) the coefficient of variation about the sample's mean hormone concentration was < 10%.

Our results show that in our captive breeding Steller sea lion population, female cortisol was highest during the breeding season (mean = 26.2 µg/dl ±15.1 SD; N = 31), but means were similar across pregnant and non-pregnant females in the non-breeding season (pregnant: 17.2 µg/dl ± 5.5 SD, N = 26; non-pregnant: 17.0 µg/dl ± 11.9 SD, N = 30). We found no correlation between cortisol levels and the lag time between start of anesthesia and blood draw ($p = 0.68$; $R^2 = 0.002$). While derived from a small sample of individuals, these data are novel for Steller sea lions and complement previous findings of elevated cortisol during the breeding season in other marine mammals (Orlov et al., 1991; Bartsh et al., 1992; Gardiner & Hall, 1997).

Females that underwent positive reinforcement training to the restraint cage and anesthesia mask in general exhibited lower serum cortisol levels than those restrained (i.e., Cage Press). Average cortisol was highest for samples collected from Cage Press (29.4 µg/dl ± 16.1 SD; N = 14), followed by Cage Diazepam (20.3 µg/dl ± 17.2 SD; N = 18), Cage Only (18.1 µg/dl ± 8.3 SD; N = 44), and Habitat Only (17.9 µg/dl ± 7.8 SD; N = 11) contexts. For the subadult male, average cortisol under the Cage Press context was similar to the adult female value (27.61 µg/dl ± 4.3 SD; N = 5). These findings are similar to reports from studies that suggest northern elephant seals (*Mirounga angustirostris*) exhibit increased cortisol release during physical restraint and reduced stress when chemically sedated (Champagne et al., 2012). The general pattern of similarity across positive reinforcement sampling contexts could suggest that positive reinforcement training to the anesthesia mask itself provided a signal of predictability and control (Bassett & Buchanan-Smith, 2007), and, therefore, the context of restraint cage or habitat was potentially less influential on the stress response.

As a minor tranquilizer and not a sedative, diazepam can be, and sometimes is, used as a component in a chemical restraint cocktail for immobilizing pinnipeds (Harcourt et al., 2010; Champagne et al., 2012; Haulena, 2014) but is not used as a chemical restraint by itself. Thus, literature assessing the effect of diazepam alone on stress markers for marine mammals is scant. In our dataset, we expected diazepam contexts to have the lowest

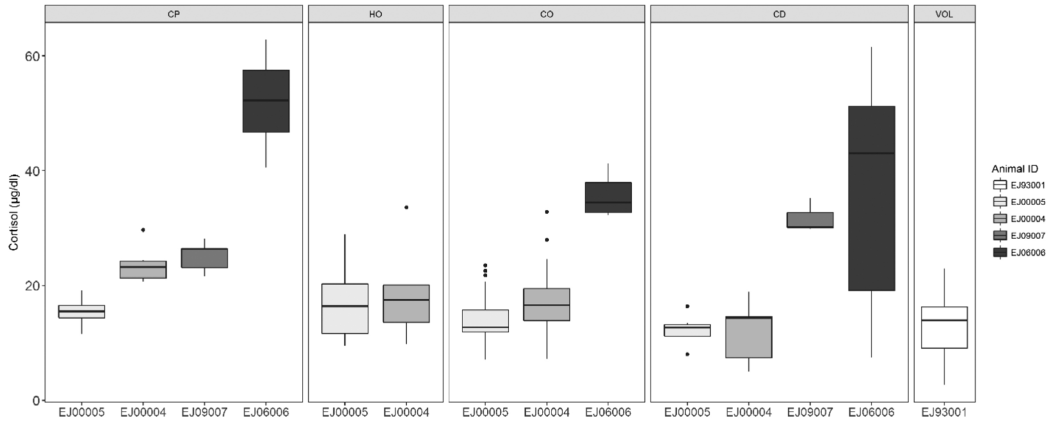

Figure 1. Individual variation in median cortisol levels (µg/dl) across sampling context (CP = Cage Press, CO = Cage Only, HO = Habitat Only, CD = Cage Diazepam, and Vol = Voluntary) for three female Steller sea lions (*Eumetopias jubatus*) (EJ00005, EJ00004, and EJ06006), one subadult male (EJ09007), and one breeding adult male (EJ93001)

cortisol levels; however, cortisol levels under diazepam in general were higher than the Cage Only and Habitat Only contexts for adult females, and all of the subadult male's cortisol values under diazepam were higher than any collected under restraint (mean = 31.77 µg/dl ± 3.0 SD, *N* = 3; Figure 1). A review of training and behavioral notes leading up to blood draws revealed that in the majority of the cases involving diazepam, EJ06006 and EJ09007 had refused when asked into the restraint cage and were given time outs between reattempting. When they eventually went into the restraint cage, they initially would not place their faces in the anesthesia masks. In these instances, it seems probable that the animals were already triggered to the impending procedure, and the repeated attempts to facilitate collections increased stress levels, potentially beyond the effectiveness of diazepam at the dose prescribed. While difficult to tease apart in this dataset due to sample sizes, these data provide some support for the use of diazepam; however, further exploration into the factors influencing the effectiveness of this tool could provide important information for minimizing stress and managing pinnipeds in breeding and research contexts.

Finally, we observed individual variation in the ranges and mean cortisol levels across individuals within sample contexts (Figure 1). These differences could reflect behavioral confounds that we did not control in this study (e.g., activity prior to presentation of the anesthesia cone or entry into the restraint cage); however, animals were generally maintained in the same holding area prior to procedures, providing some level of behavioral consistency across sampling events. Alternatively, it is now widely recognized that individuals within numerous wild animal populations exhibit different behavioral phenotypes and different coping styles, defined as suites of behavioral and physiological responses, to stressors (Sgoifo et al., 1997; Koolhaas et al., 1999, 2010; Estes et al., 2003; Dingemanse & Réale, 2005; Twiss et al., 2012; Cockrem, 2013; Monestier et al., 2016). The two recognized coping styles are *proactive* when individuals are less responsive to stimuli and have lower glucocorticoid responses, and *reactive* when individuals are flexible and have higher glucocorticoid responses to stimuli (Koolhaas et al., 1999; Cockrem, 2013). Therefore, further work investigating the inter-individual differences in stress responses will be important for animal welfare during sampling and for understanding the population consequences of disturbance in both wild and managed populations (Lusseau et al., 2007; King et al., 2015).

In conclusion, these data demonstrate the value of continuing to investigate how stress-relieving chemicals and behavioral, physiological, and medical markers together can be used to determine the appropriate sampling context instead of applying a one-size-fits-all approach to this and other species in human care.

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