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Initial Validation of Blubber Cortisol and Progesterone as Indicators of Stress Response and Maturity in an Otariid; The California Sea Lion (*Zalophus californianus)*

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Abstract

Chronic stress can have detrimental effects on an individual's health and reproductive success. The use of cortisol quantification as an indicator of stress in free-ranging cetaceans and phocids is increasing but no studies have applied this technique on blubber in otariids. We measured cortisol concentrations in blubber samples obtained from California sea lions, *Zalophus californianus*, stranded in San Diego County and those incidentally killed in the California drift gillnet fishery. We also measured progesterone concentrations to assess female reproductive status and, in males, as a potential secondary measure of adrenal steroid production. Blubber cortisol and progesterone values were compared across demographic groups (sex and maturity), season and proportion blubber lipid extracted. Stranded animals (247.3 ± 70.767_{SE} ng/g blubber) had significantly higher cortisol concentrations compared to fishery bycaught (8.1 ± 2.108_{SE} ng/g blubber) animals. These findings are likely driven by inherent differences in the cause of death and associated nutritional state coupled with the mean duration of expiration for these two groups of animals (i.e. the duration from an animal's initial perception of the threat-to-self until death). The duration of transition from healthy state to death in stranded animals is on the order of many hours to weeks while in fishery bycaught animals, this transition occurs much more rapidly (i.e., seconds to tens of minutes). The presumed longer duration of the mortality event in stranded animals gives sufficient time for elevated cortisol to diffuse into the blubber. No significant differences between demographic groups, or season were found. However, blubber cortisol declined inversely with proportion blubber lipid extracted, suggesting utility in assessing long-term nutritional status. Blubber progesterone was significantly higher in mature females than immature females (153.8 ± 54.546_{SE} ng/g blubber and 9.7 ± 3.60_{SE} ng/g blubber respectively), containing on average 15 times more progesterone, irrespective of pregnancy state. Additionally, a significant relationship between mean cortisol and progesterone was found in males with >35% blubber lipid ($p < 0.0001$). This study is an initial step in validating blubber cortisol and progesterone concentrations as a potential marker of stress response and reproductive state, respectively, in otariids. Especially when paired with dart biopsying, this

approach could represent a relatively rapid way to assess baseline stress, nutritional status and reproductive states in otariids while minimizing the effects of sampling.

1. Introduction

There are an increasing number of both anthropogenic and natural threats; climate change, pollutants, harmful algal blooms, ship traffic, oil and gas exploration/drilling and emerging diseases that create stressful environments for and pose threats to the reproduction and viability of marine organisms (Fair et al. 2014, Harvell et al. 1999, Jenssen 2006; Schwacke and Wells 2016). Distinguishing between chronic and acute stress is important when extrapolating overall population health from individual stress levels. Stress is often defined as a state of physiological challenge to homeostasis that has activated the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of glucocorticoids (GC) (Delehanty and Boonstra 2008). While acute changes in GCs is normal, chronic stress and release of GCs for extended periods of time can have detrimental effects on an individual's health. Growth, reproduction, immune function, disease resistance and fitness of an individual can all be negatively impacted by the effects of persisting stress (Romero et al 2009; Sheriff et al. 2011).

Measuring stress hormones, such as cortisol, is more frequently being used as an indicator of stress in free-ranging marine mammals but little work has been done on otariids (sea lions and fur seals). Most studies on pinnipeds have measured hormones from blood serum or plasma samples collected from anesthetized animals (Sheriff et al., 2011; Champagne et al. 2015; Greig et al. 2007). However, a common problem with studying stress levels in a wild population is the imposed stress of capture and handling of the animals (Delehanty and Boonstra 2008). Collection of any samples other than feces or hair from a free-ranging otariid requires capture, handling, restraint and, a majority of the time, anesthetizing the animal. Physical stressors (such as capture/handling) stimulate the HPA axis resulting in elevated adrenal steroids such as cortisol into the blood (Ortiz et al. 2000). Additionally, sedation has been shown to reduce the stress response in phocids (Champagne et al. 2012) although the time delay between capture and sedation is reflected in the circulating cortisol concentration post sedation (Kershaw and Hall 2016). In vertebrates, the adrenal cortex can secrete GCs well above basal levels within 3-5 minutes resulting in measurable differences in GC concentrations in the blood (Sheriff et al. 2011). It is very difficult for a researcher to collect a blood sample within 5 minutes of perceived threat by the animal (presence of sampler, chase, capture, and sampling); therefore, an alternative sampling method can be helpful when looking for chronic stress effects without sampling artefacts.

In addition to blood, feces, urine, and hair have been used to evaluate stress levels in free-ranging populations and many studies have shown the ability to detect hormones such as cortisol in a variety of these matrices (Sheriff et al. 2011). However, not all of these matrices are equal in their capacity to be collected and they also represent different temporal windows of physiological integration. A study on Steller sea lions found adrenal response was clearly reflected in a fecal corticosterone peak 32 hours following a stimulus and was cleared within 52 hours indicating that the feces integrated and cleared the corticosterone (Mashburn and Atkinson 2004). A comparative sample matrix study on bowhead whales concluded the most likely path of recently produced progesterone levels are reflected in the blood, then the urine, and finally the blubber (Kellar et al. 2013). Assuming cortisol follows the same clearing/absorption path as progesterone, an acute stress-induced elevation in cortisol could be measured in blood and only

if the stress response was relatively intense or prolonged for sufficient time would an elevated concentration be measurable in the blubber.

Blubber cortisol, because it lags behind the signal measured in blood, could provide a more accurate indication of chronic stress in an individual for two reasons: 1) its temporal lag relative to the blood has the effect of minimizing handling artefacts and 2) it integrates the signal of cortisol production over an extended period (Kellar et al. 2013). As cortisol is circulated through the body in the blood it begins to passively diffuse into the adipose tissue (blubber) (Kershaw and Hall 2016, Kellar et al. 2015). A lag time between blubber and blood concentrations have been seen in bottlenose dolphins as well as in harbor seals (Kershaw and Hall 2016; Schwacke and Wells 2016). With the assumption that otariids have a similar lag time to those seen in cetaceans, it is believed that the use of blubber to obtain GC levels in otariids may better reflect the prevailing stress state of the animal and not elevated (acute) stress state due to sampling especially when combined with dart biopsy (Hoberecht et al. 2006). Dart biopsy techniques allow collection of a small blubber sample without the elevation of cortisol due to capture or need to administer a sedative/tranquilizer prior to capture (Baylis et al. 2015).

Though typically associated with pregnancy, it is common for males and immature females to have measurable circulating progesterone levels. One reason is that progesterone is a metabolic precursor to cortisol and can readily permeate into the circulatory system in times of high stress. An adrenocorticotropic hormone (ACTH) challenge on male guinea pigs found this with an elevation in both cortisol and progesterone plasma levels (Fenske 1997). Ovariecotmized cows with normal adrenocortical function also had a significantly high positive correlation between cortisol and progesterone in an ACTH challenge (Yoshida and Nakao 2005). This study indicated that even without the female gonads present and producing progesterone, progesterone concentrations increase along with cortisol in the event of a stressor (ACTH challenge).

Progesterone levels have been repeatedly studied in marine mammals as they can depict pregnancy status as well as indicate maturity, both of which can inform aspects of population demographics. Gardiner et al. (1999) showed a distinction between progesterone levels in mature and immature harbor seals with mature animals having significantly higher concentrations of progesterone. Pregnancy has also been successfully determined from high progesterone levels in the blubber of cetaceans (Kellar et al. 2006; Mansour et al. 2006) and in the serum of harbor seals (Gardiner et al. 1996). Greig et al. (2007) found that generally, serum progesterone concentrations were distinct between pregnant and non-pregnant California sea lions but that the concentrations were lower in stranded animals, all of which were underweight, compared to captive animals. Reproduction can be impacted by chronic stress, thus it is important to understand the relationship between these physiological processes (Sheriff et al. 2011).

Here we measured cortisol and progesterone concentrations in blubber samples taken from California sea lions, *Zalophus californianus*, that were 1) stranded dead on the beaches in San Diego county, 2) live stranded in San Diego county and transported to a rehabilitation facility prior to expiration, or 3) incidentally killed in the California drift gillnet fishery. The purpose of this study was to validate blubber cortisol concentrations as a marker of stress response in these animals by assessing variation with respect to life-history state, season (breeding, gestation, etc.), mortality type (dead-strand, live-stranded, or fishery bycaught) and nutritional condition. Here we did not asses molting as a life history event because unlike phocids that go through an annual, catastrophic molt replacing the entire epidermis and pelage in approximately 1 month, otariids gradually molt over several months up to a year which reduces the significance of molting as a life history event in this family (Berta et al. 2006; Champagne et

al. 2012; Yochem and Stewart 2002). Progesterone concentrations were measured to determine reproductive state of females based on their presumed maturity class and with respect to the above mentioned life-history states and nutritional condition, and in males, as a potential secondary indicator of adrenal steroid production and stress response.

2. Methods

2.1 Ethics statement

All samples used in this study were collected postmortem after carcasses were recovered by the National Marine Fisheries Service (NMFS) Southwest Regional Marine Mammal Stranding Network the California Drift Gillnet Fishery Observer Program, or Sea World San Diego Marine Mammal Rescue Team. No animals were directly targeted and killed for this or any other associated study. Specimens collected by the observer program were incidentally killed in fishery nets. Incidental bycatch of non-threatened marine mammals is permitted through the NMFS Marine Mammal Authorization Program under the Marine Mammal Protection Act (MMPA) (16 U.S.C. 1371(a)(5)). Response to and sample collection from dead, stranded animals by NMFS is covered under the MMPA (16 U.S.C. 1421). Sample collection of animals which expired at SeaWorld San Diego after live-stranding is covered under SeaWorld's research permit RR2016-07.

2.2 Samples

Three sources of blubber tissue were used in this study. The first composed of blubber tissue samples that were taken from California sea lions incidentally killed in the California Drift Gillnet Fishery (N=55) and collected by California/Oregon Gillnet Observer Program, between 1991 and 2011. The second group of blubber tissue samples were composed of dead-stranded animals (N=18) collected from the beaches of San Diego county by the Southwest Fisheries Science Center Stranding Program between 2002 and 2016. The third group of blubber samples were taken from animals that live-stranded on the beaches of San Diego county and were transported to and expired at SeaWorld San Diego, the Southern California designated rehabilitation center (N=14). Each individual carcass was given a decomposition code at the time of necropsy, where code 1 = live stranding, code 2 = fresh dead stranding, code 3 = moderate decomposition, code 4 = advanced decomposition, and code 5 = mummified carcass (Rowles et al. 2001). All of the specimens were code 2 with the exception of three code 3 dead-stranded animals. No specimen with a code 4 or 5 was used in this study and none of the stranded animals showed signs of human interaction, including fishery entanglement at the time of stranding. All blubber samples were taken from the ventral midline of the abdominal region of each animal. For each sample, the epidermis and muscle layer was removed and the remaining portion of the dermis and hypodermis was used for hormone processing (all of the remaining tissue will be referred to as blubber). Specimen sex, total length, maturity status, pregnancy state, and lactation status were also recorded. For the majority of specimens, no gonadal inspection was possible and therefore total length was used as a measure of maturity when no other evidence was available.

Ten of the specimens in this study had only a curvilinear length recorded, not a standard (straight) length from the tip of the nose to the tip of the tail. Therefore, a correction factor of $7.9\% \pm 0.23\%_{SE}$ was determined empirically from the thirty-five individuals which had both a standard straight length and curvilinear measurement taken in the lab based on the average percent difference between straight and curvilinear lengths. To estimate straight length, 7.9% of

the curvilinear length from the curvilinear length of each individual was used to create a standard straight length (heretofore referred to as "total length"). No significant trend relative to total length was observed in the residuals after this correction was made.

To date, there is no standardized way to classify California sea lions as mature/immature based on total length due to the amount of variability of length-at-age. Female reproductive status categories outlined by Perrin and Donovan (1984) could not be applied in the present study because few to no specimens had ovaries collected nor were the presence/absence of corpus luteum's (CL) recorded. Males as young as 4 years of age have been seen copulating (Odell, 1975) suggesting that males at this age are sexually mature and reproductively active but not yet socially mature, making their reproductive success at this age unknown (Kastelein et al. 2000). Laake et al. (2016) designated length-at-age groups for assumed sexual maturity: females between age 2.5 and 3.5 years had a total length range of 134.4-144.3cm and males between 4 and 7.5 years had total lengths of 162.3-195.1cm. Animals in this study were considered sexually mature when males were a total length ≥162 cm and females were a total length ≥135cm. Although on the low end of the length-at-age given by Laake et al. (2016) for sexually mature females, one of our confirmed pregnant females with a fetus was 135cm in total length.

Pregnancy and lactation states were determined in the field by presence of conceptus/fetus and milk respectively. Due to the life history stages of female California sea lions, mature females found without a fetus (nominally non-pregnant) between mid-July to mid-October were considered in a state of either delayed implantation (embryonic diapause) or pseudo-pregnancy (Odell 1975), here meaning a non-fertilized cycle with an obligate CL retention seen in many species with delayed implantation (Robeck et al. 2001). A mature female physiologically does not recognize pregnancy until after implantation occurs; therefore pregnant and non-pregnant animals physiologically are identical until after implantation (Boyd et al. 1999). Mature females found to be without a fetus inside of the October-June range were considered not pregnant.

2.3 Blubber hormone extraction

The blubber hormone extractions followed the methods described in Kellar et al. (2015) modified for use on pinnipeds based on physiological differences in the blubber tissue. Approximately 0.075 g - 0.15 g of blubber were homogenized six times at a speed of 5 m/s for 45-second intervals. The contents of the homogenization tube were pipetted into a glass tube (T1), the homogenization tube was then rinsed with 500 µL of ethanol, and the washed contents were transferred into T1. The homogenate:ethanol solution was then separated from the grinding media and placed into a new glass tube (T2). The remaining contents of T1 (with grinding media) were rinsed again with another 500 µL of ethanol and combined with the homogenate in T2. This step was repeated once more. Each sample was vortexed individually after the addition of 500 uL ethanol. Extended vortex times were required for some samples. These samples had a sticky consistency and required more time on the vortexer to retrieve the entire sample from the tube. The homogenate/rinse solution combination was combined with 2 mL of 4:1 ethanol:acetone. The resulting solution was hand vortexed until the entire sample was in solution. This is an additional step to the methods in Kellar et al. (2015) as we noticed a different texture, a more sticky consistency, with sea lion samples than what is normally seen for cetacean samples. This is likely due to the differences in fatty acid composition in the adipose tissue of sea lions compared to cetaceans. Once every sample had been vortexed individually, the samples were placed on a plate shaker and vortexed for five minutes and then centrifuged at 5000 rpm for

15 min. The supernatant was transferred and evaporated. 2 mL of diethyl ether was added to the evaporated contents, vortexed, and centrifuged again. The supernatant was collected and evaporated. The tubes used in this step were weighed gravimetrically prior to adding the diethyl ether supernatant and after evaporation to obtain a blubber lipid weight. The diethyl ether residue was then suspended in 1.5 mL of acetonitrile, vortexed, and 1.5 mL of hexane added to the mixture. After the solution was vortexed and centrifuged again, the acetonitrile layer was aspirated into a new tube and the process was repeated with another 1.5 mL of hexane. The final portion of acetonitrile was collected and evaporated. The remaining residue was centrifuged at 5000 rpm for five minutes to bring all remaining sample together in a pellet at the bottom of the tube and was then stored at -20ºC.

Proportion extracted lipid was calculated for all specimens that had a lipid weight available (N=75). Proportion extracted lipid was calculated by: Proportion extracted lipid = blubber lipid weight (g)/total blubber weight (g) of each individual sample used.

2.4 Cortisol and Progesterone Enzyme Immunoassay

To prepare the samples for the enzyme immunoassay (EIA), they were suspended in 250 μL of 1M phosphate buffered saline and then vortexed in the multi-tube vortex for 15 min.

For cortisol, we used an EIA kit 8 K003-H1 (Arbor Assays, Ann Arbor, MI, USA) that has 100% reactivity with cortisol, 18.8% reactivity with dexamethasone, 7.8% reactivity with prednisolone (1-Dehydrocortisol), and 1.2% reactivity with both corticosterone and cortisone. For progesterone, we used an EIA kit ADI-900-011 (ENZO Life Sciences, Farmingdale, NY, USA) that has 100% reactivity with progesterone and 1a-Pregnane-3,20-dione, 3.46% reactivity with 10-OH-Progesterone, 1.43% reactivity with 5-Pregnen-3B-o1-20-one, 0.77% reactivity with Corticosterone, 0.28% reactivity with 4-Androstene-3,17-dione, 0.056% reactivity with Deoxycorticosterone and 0.013% reactivity with DHEA. Cross-reactivity of progesterone in the cortisol assay and of cortisol in the progesterone assay were reported by the manufacturers as <0.01% and <0.001% respectively. Additionally, there was no measurable cross-reactivity in our spiked samples of the non-targeted hormone when compared with their non-spiked counterparts.

Samples that exceeded the range of the assay had to be diluted further to be accurately measured. These samples were diluted further depending on their original EIA measurements such that the final measurements would fall within the range of the control samples. 36 samples were run in duplicate to produce the mean inter-assay coefficient variation (CV) (cortisol = 15.2% and progesterone = 8.8%) and the mean intra-assay CV between 12 samples (cortisol = 13.1% and progesterone = 8.3%).

We determined the extraction efficiency using spiked samples as described by Kellar et al. (2015). The extraction control samples were spiked with 200 ng of cortisol or 150 ng of progesterone. The extraction efficiency was calculated as the amount of quantified cortisol/progesterone (via enzyme immunoassay analysis) of the spiked samples minus the quantified amount in the non-spiked samples, all divided by the original amount of cortisol/progesterone added (spiked) before the extraction. An estimated extraction efficiency for blubber cortisol (0.73) and progesterone (0.69) was used as a correction factor and was applied to all measurements within this study.

2.5 Parallelism and Matrix Effects Analyses

Two quality control tests, parallelism (Figure 1) and matrix interference (Figure 2), were used to assess the quality of hormone measurements. Both tests were run on both cortisol and

progesterone. The first was parallelism, which uses a pool of sample extracts serially diluted and run with the standard controls of the assay to determine if the decrease in concentration of the pooled sample is in-line with the standard curve, an indication that the assay is measuring the antigens in the samples as it is in the standards. Extracts from four individuals for cortisol and five individuals for progesterone were pooled together to obtain a representative sample across our sampled individuals. The pooled sample concentrations were made by diluting seven times from the pooled preparation to 1/128 decreasing by a factor of two. Each dilution was run three times, and the resulting curve of the detection metric (optical density of the sample/optical density when no sample is added (B/Bo)) as a function of the dilution state was then compared to the standard curve using an ANCOVA (interaction effects model) of the log/logit transformations as recommended by Plikaytis et al. (1994).

The second quality assessment examined the potential matrix interference, the effect of the blubber extract itself on the measurement of cortisol or progesterone. The test consists of a pooled sample extract and a hormone standard of a known concentration. The pooled sample extract consisted of five samples for cortisol and six samples for progesterone producing a final volume of 495uL and 1000uL respectively. The hormone standard had a final concentration of 200pg/mL for cortisol and 1000pg/mL for progesterone. The pooled sample was serially diluted by a factor of two for a total of nine times and combined with a straight hormone standard. The nine serially diluted samples combined with the hormone standard, a straight hormone standard and straight pooled sample extract were run in duplicate. The concentration of cortisol or progesterone contributed from the pooled sample (neat = ± 520pg/ml for cortisol, neat = ± 240pg/ml for progesterone) was subtracted from each sample-spiked measurement so its contribution would be factored out of the assessment. A linear regression was used to determine if there was a significant relationship between deviations from expected concentration and increasing level of spiked blubber extract concentration.

2.6 Statistical Analysis

All samples were compared based on fatality type: dead-stranded, live-stranded (rehabilitation) and fishery-bycaught animals. Those types were further broken down by demographic group: immature female, mature female non-pregnant, mature female pregnant, immature male and mature male. From there, each sample was categorized into presumed life history state which will be referred to as season for males and reproductive state for females. The season or reproductive state was decided based on date of stranding. The female annual reproductive cycle includes parturition (~May through end of June), embryonic diapause (~end of June through mid-October), and active placental gestation (~October through June) most commonly, though we also included conception or mating (~June) as a reproductive state (Greig et al. 2007; Boyd et al. 1999; Williams et al. 2007). We grouped the study females as follows: conception (mid-June to mid-July), delayed implantation/pseudo-pregnancy (mid-July to early-October), gestation (mid-October to mid-May), parturition (mid-May- to mid-June). For males: mating (June through July), and foraging (August through May).

Due to the variation in hormone concentrations within each of the fatality types, the data were log transformed to reduce heteroscedastic variation. For all samples that were run in duplicate on either cortisol/progesterone immunoassays, the mean of all concentrations for each sample was calculated and used in the following statistical analyses. Cortisol and progesterone concentrations were compared across all three fatality types, demographic group, and season using an ANOVA and a post hoc Tukey test for multiple comparisons of means. An ANCOVA

(interaction effects model) was used to examine the relationship between blubber cortisol concentration and proportion blubber lipid extracted across all three fatality types. A linear regression was used to assess the relationship between blubber progesterone and cortisol in males. All data analyses were performed in R Studio (RStudio Team, 2015) and all means are reported +/- SE.

3. Results

3.1 Hormone Validation

3.1.1 Parallelism

Serial dilutions of extracts show parallelism with the standards of the cortisol and progesterone EIA assay kits (Figure 1). There is no significant difference seen between the slopes of the pooled blubber extracts and the cortisol ($t=1.856$, $p=0.089$) or progesterone ($t=0.284$, $p=0.684$) standards.

3.1.2 Matrix Interference

There were no significant trends in measurements of constant cortisol ($r^2 = 0.0035$, $p = 0.817$) and progesterone standard ($r^2 = 0.0625$, $p = 0.316$) solutions with increasing pooled blubber extract volume proportion; findings that are consistent with no to limited matrix interference (Figure 2) of blubber extracts on either EIA assays.

3.1.3 Decomposition and storage effects on cortisol

The animals within this study had nearly identical decomposition codes. Of the 87 animals, 84 were code 2 (fresh) carcasses the other 3 were code 3 but they were all dead stranded. There was no significant difference in cortisol concentration between these three code 3 carcasses and the other 15 dead-stranded code 2 animals ($t=1.796$, $p=0.093$). To examine the impact storage time might have on cortisol levels in blubber, we looked at the cortisol concentration by year of the 55 fishery bycaught animals which were caught over a 20 year period of 1991 through 2011. We found no significant trend here ($F_{1,54}=2.1$, $p=0.15$, $r^2=0.04$). This leads us to believe that storage time and decomposition state had minimal effect on the blubber hormone concentrations of these animals.

3.2 Cortisol

3.2.1 Fatality type- (expiration duration)

Blubber cortisol levels were significantly different between fatality types ($F_{2,68} =62.2$, $p<0.0001$). Fishery bycatch animals (8.1 ± 2.1 ng/g blubber) had significantly lower blubber cortisol concentration compared to both dead-stranded (256.3 ± 115.7, $p<0.0001$) and live-stranded animals (235.8 ± 68.5, $p<0.0001$) (Figure 3). There was no significant difference in cortisol concentration found between the dead-stranded and live-stranded animals ($p=0.743$).

3.2.2 Life history status

After controlling for fatality type, no significant differences were observed among demographic group ($p=0.509$) or season/reproductive state ($p=0.784$).

3.2.3 Nutritional status – Proportion extracted lipid

There was a significant negative association of proportion extracted lipid in blubber with blubber cortisol across all fatality types (t= -2.82, p = 0.0063). When this relationship was used to control for the effects of nutritional status, there was still a strong difference between fatality types ($F_{3,71}$ =35.8, p < 0.0001) with fishery bycatch animals having lower blubber cortisol than the other types (p < 0.05) (Figure 4).

3.3 Progesterone

3.3.1 Stress response precursor

Progesterone concentration was examined as an indicator of stress in males. There were no observed significant differences in progesterone concentration between immature (4.2 ± 2.216 ng/g blubber) and mature (7.2± 2.22 ng/g blubber) males (p=0.329) or between fatality types ($F_{2,25}$- =2.131,p=0.108). Also, there appears to be no significant relationship between cortisol and progesterone concentration (r^2 = 0.082, p=0.084, n=26). However, within this dataset there were three animals with low proportion extracted lipid concentrations (<35%). Given the relationship between cortisol and proportion of lipid, these three animals were removed from this analysis to minimize cortisol signals due to differences in nutritive state; this resulted in a substantially stronger statistical relationship between cortisol and progesterone (r^2 = 0.671,p < 0.0001, n=23). Note that we inadvertently failed to record proportion lipid measurement of 7 specimens, reducing the sample set for this analysis to 23 individuals (Figure 5). This large change in the statistical relationship between cortisol and progesterone when the three low percent lipid animals are removed could be important relative to the potential driving mechanisms of the measured cortisol levels, i.e., acute versus chronic cortisol production.

3.3.2 Female reproductive state

We found a significant difference ($F_{3,53}$ =18.0, p=<0.0001) between immature and mature females with mature females (153.8 ± 54.5 ng/g blubber) having 15 times more blubber progesterone than immature females (9.7 ± 3.6 ng/g blubber). A stepwise increase in progesterone from immature females (having the lowest mean concentration) up to mature non-pregnant animals and again up to mature pregnant animals was seen (Table 1). Progesterone concentration follows the expected increase of presumed reproductive state based on time of year for mature females from parturition having the lowest concentration to active gestation having the highest mean concentration of progesterone (Figure 6).

4. Discussion

This study represents the first major step in validating the use of blubber cortisol concentrations in an otariid, the California sea lion, and evaluating its variation relative to fatality type, demographic group, season, and nutritional state (proportion lipid extracted) as indicators of physiological stress. Cortisol quantification from blubber samples is a promising alternative approach for determining stress response in free-ranging otariids.

The apparent lack of relationship between storage conditions and blubber cortisol levels is consistent with previous literature in cetaceans. Kellar et al. (2015) found no significant relationship between cortisol concentration and storage time at -20°C and Kellar et al. (2006) found the same with progesterone concentrations in delphinids. Additionally, delphinid blubber samples kept at ambient temperature for 52 hours showed physical signs of decay (odor and yellowing) but did not have significant differences in progesterone concentrations from those

kept in the freezer indicating that exposure to the elements (condition code) does not impact hormone concentrations (Kellar et al. 2006).

Here we measured significantly lower mean blubber cortisol concentrations in fishery bycaught animals compared to stranded animals (Table 1). Unlike stranded animals, the cause of death of fishery-bycaught animals is known and the duration of each death is relatively constant across all animals examined. The significantly lower cortisol concentrations seen in fishery-bycaught animals are likely because those animals would have expired within minutes, not allowing time for the elevated cortisol released into the bloodstream to diffuse and accumulate into the adipose tissue. Kershaw and Hall et al. (2016) saw no significant relationship between blubber cortisol concentrations and capture time within a 5 hour period in phocids whereas cetacean data demonstrate blubber cortisol levels being substantially elevated in association with serum cortisol concentration with detectably elevated cortisol concentrations in the blubber after a 60-90 minute period (Schwacke and Wells 2016). Assuming the lag time for otariids is similar to phocids and cetaceans, the fishery bycaught animals would not have had sufficient time for any increase in blood cortisol to diffuse into the blubber. For this study, the fishery bycaught animals were presumed predominantly healthy at the time of death (i.e., had they not died within the fishing gear that their survival rates would have been similar to the general population), and as such they represent the general population's baseline level. The supposition here is that these animals generally expired before there was a biologically significant increase in their blubber cortisol levels though it is assumed that during most of their deaths, cortisol was being produced from their adrenals at much higher levels but had insufficient time to accumulate significantly in the blubber. As such, it is important to note that here the use of the word 'baseline' is not assumed to be the physiological basal level, but a reference point for the population at large with minimal sampling artefact. We acknowledge that these animals likely engaged a profound dive response (i.e., peripheral vasoconstriction) in response to entanglement; although for this study we assume that there was insufficient time for substantial hormone accumulation in the blubber prior to death and such a response would have no effect. Therefore, the concentrations seen in the blubber of the fishery bycaught animals is likely much closer to those of the animals prior to the onset of the stressor (entrapment) and death. The fact that after controlling for fatality type, there were no significant differences in cortisol concentrations among demographic groups or seasonal/reproductive states further indicates fatality type as the primary driving factor of differences seen in the blubber cortisol concentration.

A directed study to understand the temporal dynamics of blubber cortisol accumulation relative to changes in serum concentrations is needed for otariids. Stranded animals did not show significant differences in cortisol concentration between the groups (live-, dead-), though both were significantly higher than fishery bycaught animals (Table 1). We also saw variation in cortisol concentrations within the stranded animals which is likely due to the differences in the cause and duration of their deaths. The stranded animals represented those affected by longer term stress (on the order of many hours to weeks, perhaps longer) toward "chronic" stress and it was expected that these animals would have a higher cortisol concentration because they expired due to illness, malnutrition, injury, etc. In other words, in both cases (fishery and stranding) there was likely a substantive stress response and cortisol production at the time of death, but in the stranded animals, there was sufficient time for elevated blood cortisol levels to increase the blubber cortisol concentrations substantially as the two reached or began to reach dynamic equilibrium. A study of Steller sea lions found that, after the onset of a stressor, serum cortisol

concentrations were at a detectably elevated level within 30 minutes, and remaining elevated after 150 minutes (Mashburn and Atkinson, 2008).

We did not find a significant increase in blubber cortisol concentration during the breeding season for either males or females. Similarly, impacts of pregnancy or lactation on blubber cortisol were not evident. However, the inverse relationship between blubber cortisol and proportion blubber lipid extracted (Figure 4) is consistent with the role that cortisol release plays in modifying substrate metabolism and serum cortisol patterns reported previously in fasting pinnipeds. This pattern suggests that nutritional status and variation in the need to mobilize stored fatty acids may be an important driver of serum and blubber cortisol concentrations in California sea lions.

Many pinnipeds go through prolonged periods of food deprivation (fasting) simultaneously with energetically costly activities such as breeding, lactation, and molting (Champagne et al. 2012). Cortisol increases with fasting duration in many phocids (e.g. Ortiz et al. 2003) and natural variation in cortisol has been identified as a major driver of lipolysis in pinnipeds (Crocker et al., 2014); (Fowler et al., 2016). Acute elevation of cortisol in response to ACTH challenges causes rapid mobilization and increased availability of stored fatty acids (Champagne et al. 2015). In contrast, the well-established effect of cortisol on protein mobilization and commitment of amino acids to gluconeogenesis seems reduced in some phocids (Ensminger et al., 2014); (Khudyakov et al., 2015) and some studies have suggested failure to elevate cortisol in response to extreme fasting may facilitate protein sparing (Crocker et al., 2012). Studies on phocid seals have also revealed dramatic transient elevations in serum cortisol at the onset of the molt (Champagne et al. 2015; Kershaw and Hall 2016).

Reported patterns of serum cortisol in otariids have been more variable. Female otariids undergo shorter duration intermittent fasts during lactation, but newly weaned pups and territorial males can undergo extended fasts that rival those of phocids (Champagne et al. 2012). In some species, short duration fasts during lactation are associated with dramatic elevations in cortisol, suggesting a role in enhancing nutrient mobilization as body condition declines and a potential role as a refeeding signal (Guinet et al., 2004). In other species, natural fasts up to 50 days were not associated with changes in serum cortisol levels (Verrier et al., 2012). Similar to phocids, otariid pups undergo a molt of the natal pelage that is associated with transient increases in cortisol concentrations (Atkinson et al., 2011). However, adult otariids molt much more gradually than phocids. In California sea lions, the molt may be spread across several months following breeding (Williams et al. 2007) and the variation of serum cortisol with molting is not well established. Sea lions are lactating and molting simultaneously which contributes to changes in lipid stores and the insulating blubber layer during this period making differentiation of the effects of molting difficult to discern (Williams et al. 2007). However, the energetic cost of molting is still likely significant.

The lack of consistent seasonal changes or effects of life history states on blubber cortisol in the current study may simply reflect the more transient and individually variable nature of natural fasting in otariids and the stronger nutritional impacts of short term foraging success in income breeders. This idea is supported by the evident effects of individual nutritional status on blubber cortisol. The lipid composition of blubber is lower and more variable in otariids compared to phocids and this variability has direct impacts on thermal conductivity and the energy cost of thermoregulation in cold water (Liwanag et al., 2012). At this time, no studies have specifically focused on the relationship of blubber layers and hormone concentration in otariids. This would be an interesting future study and would significantly add to our

understanding of pinniped blubber. The distribution of lipid contents in our sample was highly bimodal with some individuals exhibiting lipid proportions below 35%, indicating severe nutritional stress. These animals had higher blubber cortisol concentrations compared to the animals with higher lipid contents. The few animals that fell between these modes were mostly live-stranded animals which were being cared for at a rehabilitation center prior to death. This bimodality potentially reflects the difference between animals that actually starved to death and those that die for other reasons before starvation occurs. The marked elevation in blubber cortisol in these animals suggests utility for assessing long-term nutritional status in sea lions.

We found that progesterone concentrations did not exceed 32.4ng/g blubber for males, 50.3ng/g blubber for immature females, and a majority of both immature females and males had concentrations below 7ng/g blubber. This is in comparison with mature (pregnant and non-pregnant) females which had progesterone concentrations up to 1299.8ng/g blubber with a mean concentration of 153.8ng/g blubber. When individuals with a proportion lipid extracted below 35% were removed, a significant positive correlation was seen between blubber cortisol and progesterone in males (Figure 5). Immature females were not included in this analysis because the progesterone concentrations for this analysis was intended to be isolated, removing any progesterone values related to reproductive state including early endocrine signals associated with sexual maturation. To fully exclude those situations, no female was included in the progesterone versus proportion lipid extracted or progesterone versus cortisol analyses. In other words, by decoupling the effects of nutrition (with a longer temporal scale) on blubber cortisol and similarly decoupling the effects of reproduction/maturation on blubber progesterone, we can focus on the relationship between cortisol and progesterone during what we believe is more acute HPA activation. This large change in the statistical relationship between cortisol and progesterone in males, when the low proportion lipid extracted animals (<35%) are removed could be important relative to the potential driving mechanisms of the measured cortisol levels, i.e., acute versus chronic cortisol production. Progesterone is a metabolic precursor to cortisol and has been seen to be positively associated with cortisol during a stressful event in both males and females (Yoshida and Nakao 2005)[1]. As seen in Figure 4, animals with a low proportion lipid extracted have high cortisol concentrations though this same correlation is not seen with progesterone (Figure 7). The animals with a proportion blubber lipid below 35% are assumed to be in nutritional distress (a form of chronic impairment) which decouples the relationship between cortisol and progesterone seen in acute stressful situations. The stress-associated progesterone secretion is thought to be a result of rate-limiting enzymes along the pathway that converts progesterone to cortisol which become sporadically overwhelmed during rapid increases in cortisol secretion with stress and allows some progesterone to be shuttled into circulation (Chretien and Seidah 1981; Kellar et al. 2013). Meaning as cortisol is rapidly being produced in response to stress, some of the progesterone precursor will be released prior to being converted to cortisol therefore increasing the progesterone concentration along with cortisol. If this is the case, we expect a positive correlation between progesterone and cortisol during an acute stressful event, such as a perceived threat to life, and less so with chronic stress. This would allow for the distinction between acute stress (high cortisol, high progesterone, and high proportion blubber lipid), non-nutritional chronic stress (high cortisol, low progesterone, and high proportion blubber lipid) and nutritional chronic stress (high cortisol, low progesterone, and low proportion blubber lipid) in males. The significant positive correlation between cortisol and

[1] Obviously for females, progesterone variation is often dominated by its role in reproductive functions. This is much less so in males.

progesterone concentrations along with significant inverse relationship between blubber cortisol and proportion blubber lipid suggests that within each death modality, nutritional stressors do play an important modifying role in the resulting observed cortisol concentrations and that elevated blubber cortisol reflects a longer duration serum elevation rather than an acute response leading to associations with serum progesterone.

We also evaluated blubber progesterone concentrations with respect to reproductive status in female California sea lions, although at this time identifying pregnant animals based on progesterone concentrations is difficult. We found that during the presumed time of parturition/conception (end of May to beginning of July) progesterone concentrations were lowest and then peaked during active gestation (October to May) (Figure 6). We also found a stepwise increase in progesterone concentration from 1) immature females to 2) mature non-pregnant to 3) mature pregnant animals. Reproduction in the California sea lion includes an annual cycle of parturition, estrus, pregnancy (embryonic diapause and active placental gestation), and lactation for mature females (Greig et al. 2007). Greig et al. (2007) found that progesterone and estrogen serum concentrations both increased at the end of diapause and start of active placental gestation. This is in line with our expectations of otariids, which are annual reproducers and have obligate pseudo-pregnancy/delayed implantation after ovulation. We expect that many of the mature females that were found without a visible embryo/fetus during time when the population is undergoing delayed implantation were in fact maintaining a viable conceptus (i.e., they were pregnant). This would explain the apparent disconnect between pregnancy state (as determined by visual inspection of the reproductive tract) and blubber progesterone concentration. A similar increase in systemic progesterone post conception has been seen in pregnant and pseudopregnant cats (Verhage et al. 1976) and ferrets (Heap and Hammond 1974). During pregnancy and pseudopregnancy, progesterone levels rise at equivalent rates until implantation occurs, after which, pseudopregnant animals' progesterone levels decline compared to those of pregnant animals (Verhage et al. 1976). Heap et al. (1974) found the lifespan and growth of CLs is similar in both pregnant and pseudopregnant ferrets in addition to progesterone concentrations. Thus, in species with a delay in implantation after copulation, regardless of fertilization, CLs will continue to develop with a commensurate increase in progesterone concentration until the point of implantation when CLs of non-fertile cycles regress. At this time progesterone concentrations will continue to increase in pregnant females whereas non-pregnant animals with regressing CL show a decline in progesterone. In cetaceans, ovaries are commonly collected from dead-stranded or fishery bycaught animals and are used to determine reproductive and pregnancy status based on the number and size of the CLs (Perrin and Donovan 1984). Unfortunately, for the specimens used in this study, very few had ovaries collected and even fewer had records of presence/absence of CLs. A better understanding of basal progesterone levels of females during different reproductive stages is needed.

5. Conclusions

A common problem for studies of stress in wild vertebrates is that the stress of capture/handling often prevents researchers from obtaining a "true baseline" stress profile (Sheriff et al. 2011). There have been many studies examining cortisol concentration by way of matrices other than blubber (i.e. blood, feces, hair, saliva, and feathers). Blood (plasma or serum) is currently one of the most commonly used substances for looking at hormone levels in wildlife. Blubber cortisol concentrations appear to more accurately reflect those of the chronic state of the

animal and not those of an acute stressful event because 1) the integration of the cortisol into the blubber occurs over hours to days and 2) the amount of time it takes for stress hormones to accumulate in the blubber reduce the artefactual signal due to sampling (Schwacke and Wells 2016; Houser et al. 2016; Champagne et al. 2016). This indicates blubber may be particularly useful for measuring physiological stress and facilitates acquisition of baseline values in otariids.

Blubber cortisol concentrations were strongly impacted by fatality type and blubber lipid proportions indicating a potential proxy for nutritional status. Given that there is a lag between adrenal release and the appearance of cortisol in the blubber, we interpret these values as being suggestive of both longer term perceived threat to life and nutritional stress (occurring on the order of hours or greater). Progesterone concentrations were detectable in both male and female California sea lions. Maturity state was reflected in female progesterone concentrations whereas males showed a positive correlation between progesterone and cortisol. The implication of these findings is the potential to differentiate between acute and chronic stress, especially chronic stress of a nutritive nature. In males, a correlation of high progesterone and high cortisol indicate an acute stress signal while high cortisol and low progesterone indicate chronic stress and is nutritionally significant in nature if associated with low proportion blubber lipid extracted. To our knowledge, this is the first attempt at validating measurements of blubber cortisol and progesterone concentrations for an otariid and they show promise as physiological indicators less influenced by the act of sampling.

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Figures

Table 1

		Sample Size	Cortisol Mean Concentration (ng/g) ±SE		Progesterone Mean Concentration (ng/g) ±SE	
Fatality Type	Fishery Bycaught	55	8.1 ±2.1		97.3 ±37.7	
	Dead-Stranded	18	256.3 ±115.7	247.3 ±70.7	10.61 ±3.1	21.42 ±5.4
	Live Stranded	14	235.8 ±68.5		34.74 ±10.4	
Demographic Group	Immature Females	20	124 ±60.6		9.68 ±3.6	
	Mature Females (NP)	20	216.5 ±103.5	133.11 ±62.82	50.0 ±14.4	153.8 ±54.5
	Mature Females (P)	14	13.99 ±6.5		302.0 ±122.4	
	Immature Males	16	41.1 ±14.7		4.2 ±2.2	
	Mature Males	17	41.0 ±17.9		7.2 ±2.2	

Table 1: Mean cortisol (ng/g blubber) and progesterone (ng/g blubber) concentrations for the different fatality types (fishery bycaught, dead stranded, live stranded) and demographic groups (immature female, mature female non-pregnant (NP), mature female pregnant (P), immature male, mature male). Concentrations are shown for 'stranded animals', a combined dataset of dead- and live- stranded animals. Concentrations also shown for 'mature females' regardless of pregnancy state. These mean values are shown for the demographic groups indicated without controlling for other factors (i.e. fatality type).

Figure 1

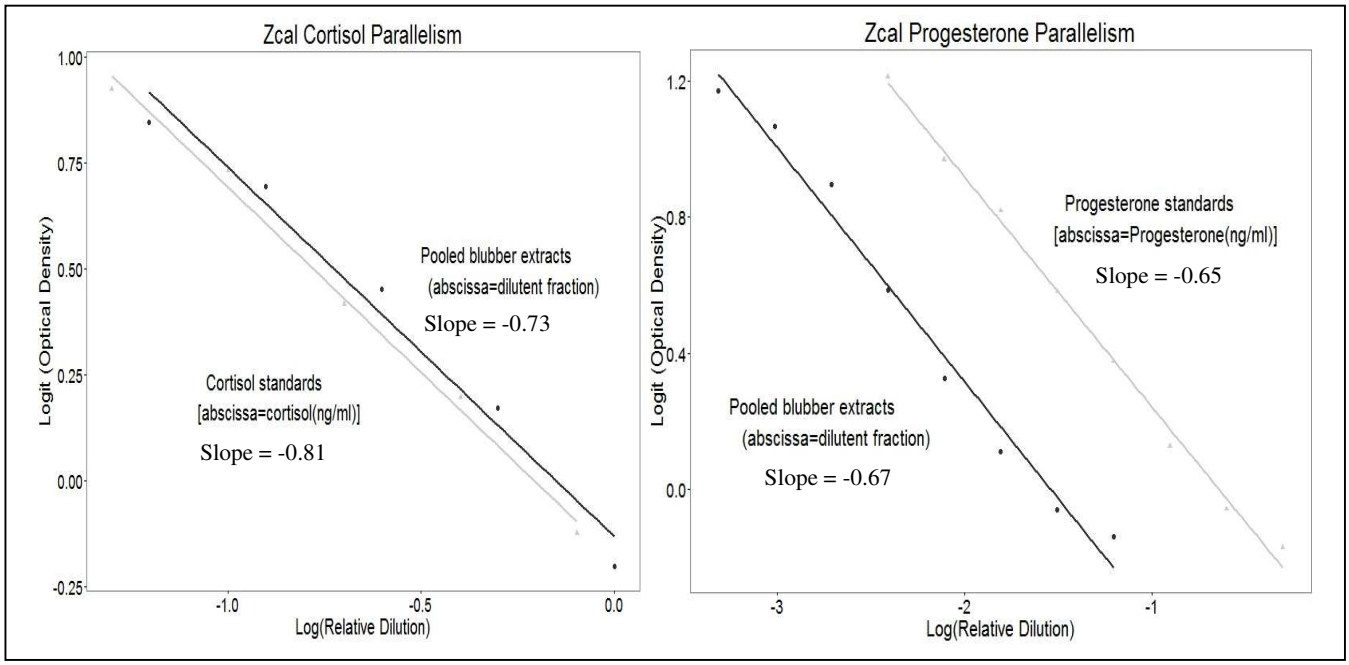

Figure 1: Linearity assessment of cortisol (left) and progesterone (right) enzyme immunoassay (EIA) with blubber tissue extracts. Logit(optical density) is the transformation used to linearize the sigmodal optical density data produced as part of the ELISA assay and the Log(relative dilution) is the natural log of the dilution coefficient used to prepare the hormone standards and the serial dilutions of the pooled extracts. Serial dilutions of extracts (black circles) show parallelism (i.e., non-significantly different slopes) with the standards (gray triangles) of the cortisol (p=0.089) and progesterone (p=0.684) kits. This is an indication that the assay is measuring the same antigens in the blubber as in the standards and therefore is suitable for the use with California sea lion blubber tissue extracts. All p-values were calculated using non-averaged data, only for visualization purposes was data averaged prior to graphing.

Figure 2

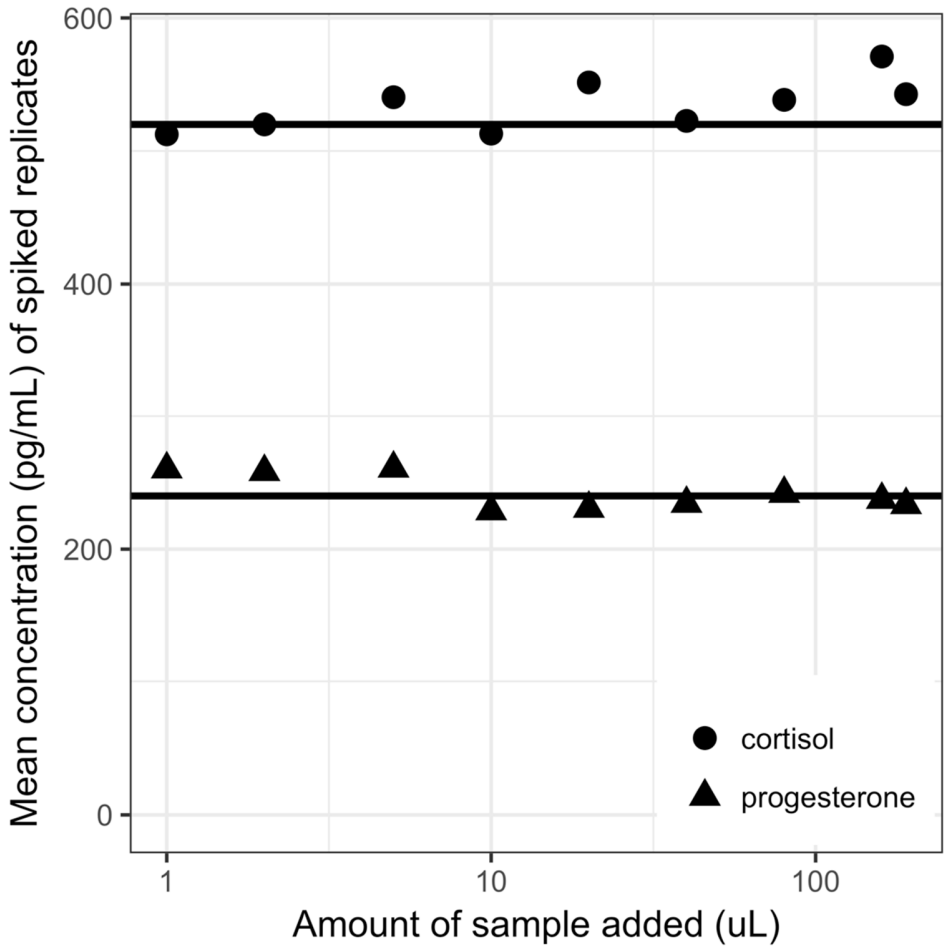

Figure 2: Matrix interference assessment of cortisol (top) and progesterone (bottom). A standard solution (final concentration 520pg/mL for cortisol, 240pg/mL for progesterone) was spiked with either phosphate-buffered saline or a set of serial dilutions of a pooled sample ("uL of sample added") composed of blubber cortisol extracts from five individuals for cortisol and 6 individuals for progesterone to make a final equivalent volume of 495uL for cortisol and 1000uL for progesterone. The concentration of either cortisol or progesterone contributed to the pooled sample was subtracted from each sample-spiked measurement so its contribution would be factored out of the assessment. The solid lines indicate the known concentrations of the standard solutions (520pg/mL for cortisol, 240pg/mL for progesterone); the y-intercepts are the regression model's prediction of the concentrations at zero sample added (523pg/mL for cortisol, 247pg/mL for progesterone). There is no evidence of a statistical relationship between the measured standard concentrations and the pooled extract that was added ($p_{cortisol} = 0.817$ and $p_{progesterone} = 0.316$).

Figure 3

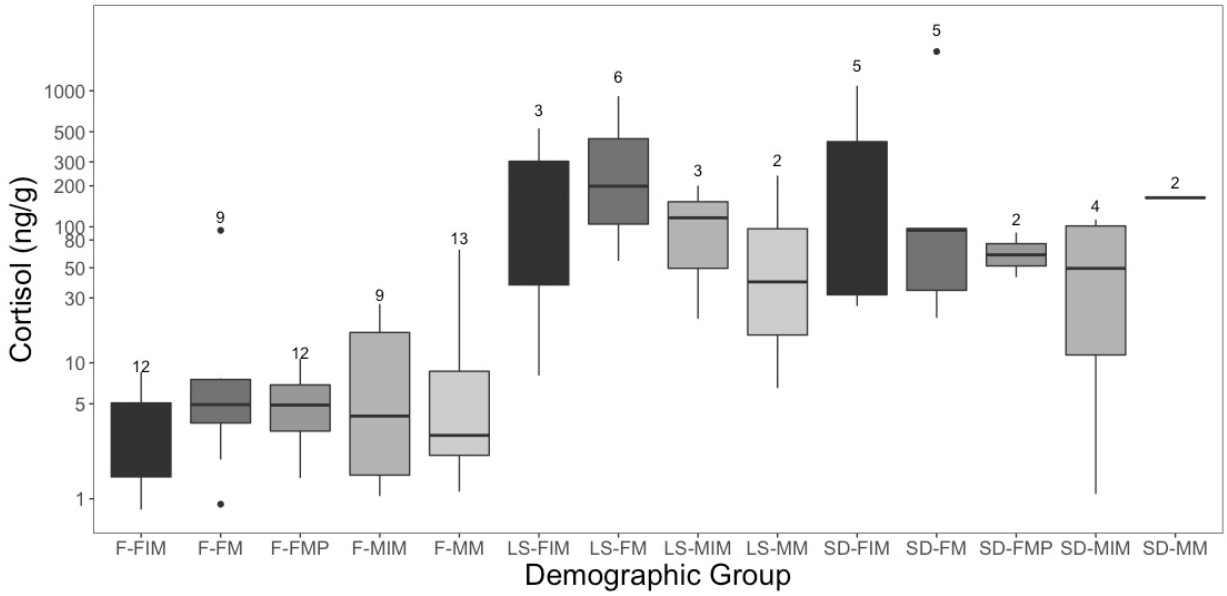

Figure 3: Cortisol concentration by demographic group and fatality type. Fishery (F-), live-stranded (LS-), dead-stranded (SD-) animals; female immature (-FIM), female mature non-pregnant (-FM), female mature pregnant (-FMP), male immature (-MIM), male mature (-MM). Sample size above the corresponding box. Significant difference seen between the fishery bycaught (8.1ng/g blubber) and dead-/live-stranded (274.3ng/g blubber) animals (p=4.436e-16). Horizontal box lines represent the lower quartile, median, and upper quartile values. Whiskers lines indicate range of concentrations.

Figure 4

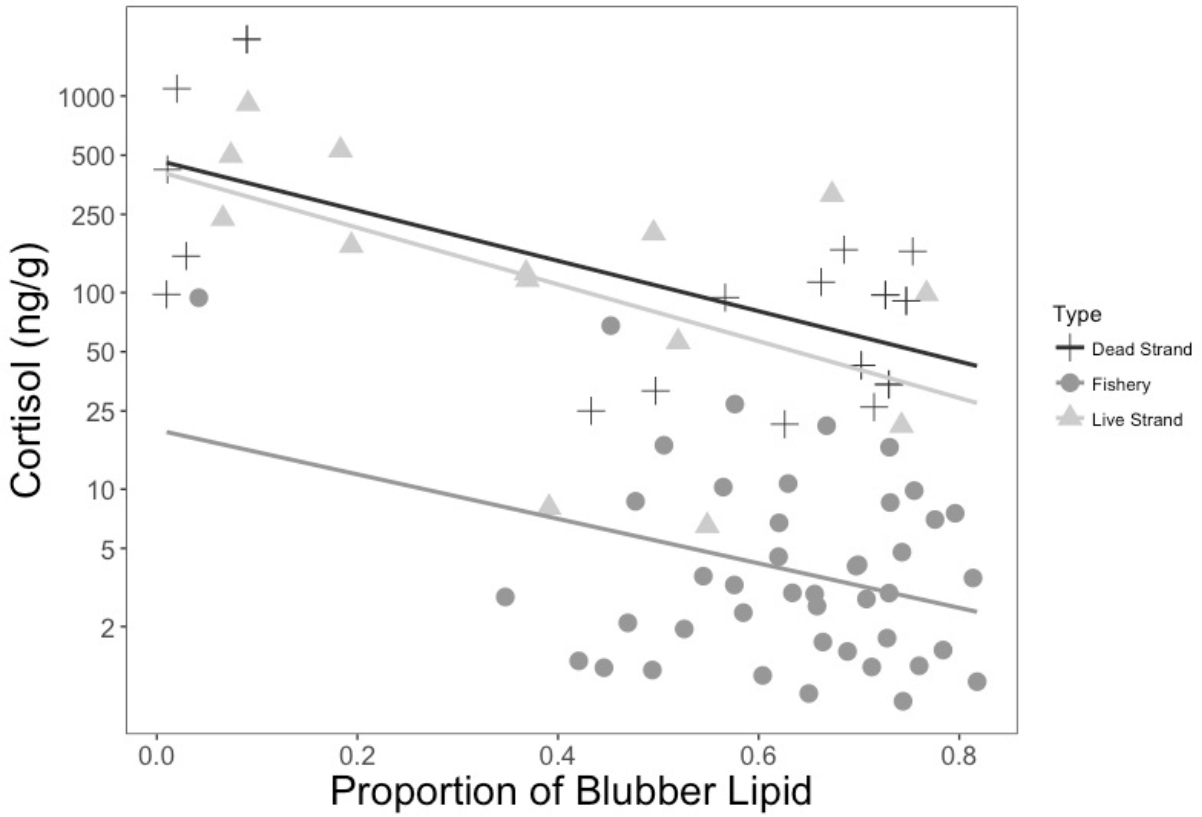

Figure 4: Cortisol concentration (ng/g blubber) varied significantly with respect to proportion of blubber lipid extracted (r2=0.77, $p < 0.0063$) for all three fatality types combined: fishery bycaught (circle), dead-stranded (plus-sign), live-stranded (triangle) animals. When corrected for proportion lipid extracted, significant differences were found between fatality types ($F_{5,68}=35.75$, $p < 0.0001$), with fishery bycaught differing from both dead-/live-stranded animals ($p=<0.05$); no significant difference seen between the dead- and live-stranded animals ($p=0.833$). Moreover, no significant difference between slopes (combined estimated slope = -1.36 ($\log(ng_{cortisol}/g)$)/(%lipid)) of the three fatality types (t-stat<0.5, $p<0.05$) was detected. Note that a single dead-stranded outlier with low cortisol and high %lipid was removed; its value was more than a magnitude lower that the next closest dead stranded animal but similar to the fishery bycatch animals.

Figure 5

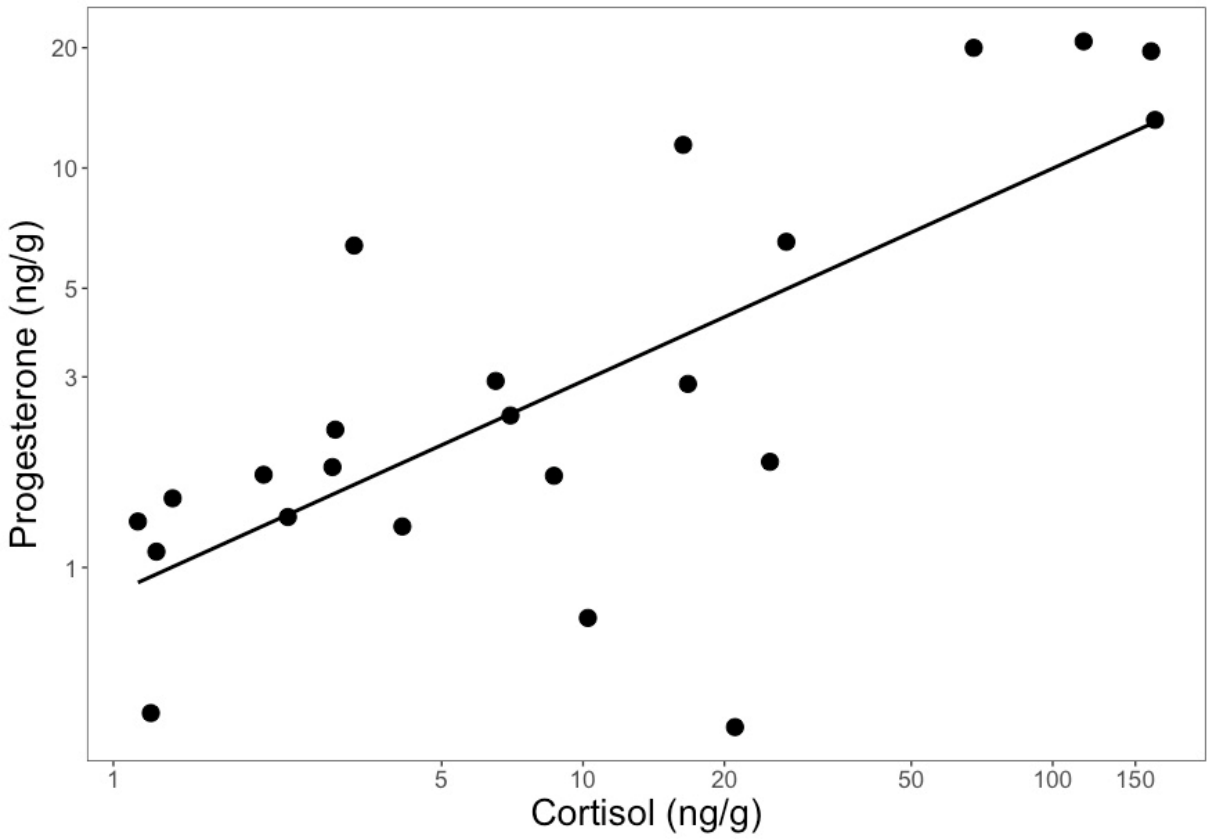

Figure 5: The relationship of progesterone concentration (ng/g blubber) to cortisol concentration (ng/g blubber) of male sea lions with greater than 35% blubber lipid across all fatality types. Values for both axes are depicted in log space. There was a positive correlation between cortisol and progesterone in males ($r = 0.671$, $p < 0.0001$, $n=23$).

Figure 6

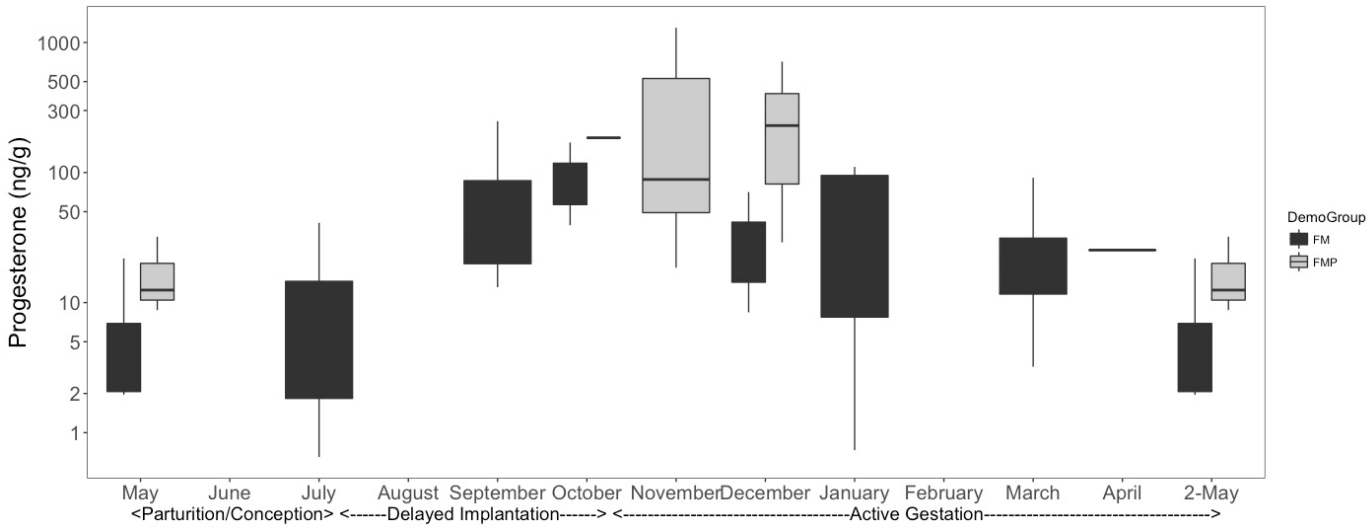

Figure 6: Mean Progesterone concentration (ng/g blubber) of females with a total straight length ≥135cm throughout the year. Presumed mature non-pregnant females (black) and confirmed pregnant females (gray). All females fall under the non-pregnant female category between the months of June and mid-October due to their obligate delayed implantation or pseudopregnancy. During this time pregnancy cannot be confirmed and is therefore unknown. Presumed reproductive state by month outlined along the x-axis. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whiskers lines indicate range of concentrations.

Figure 7

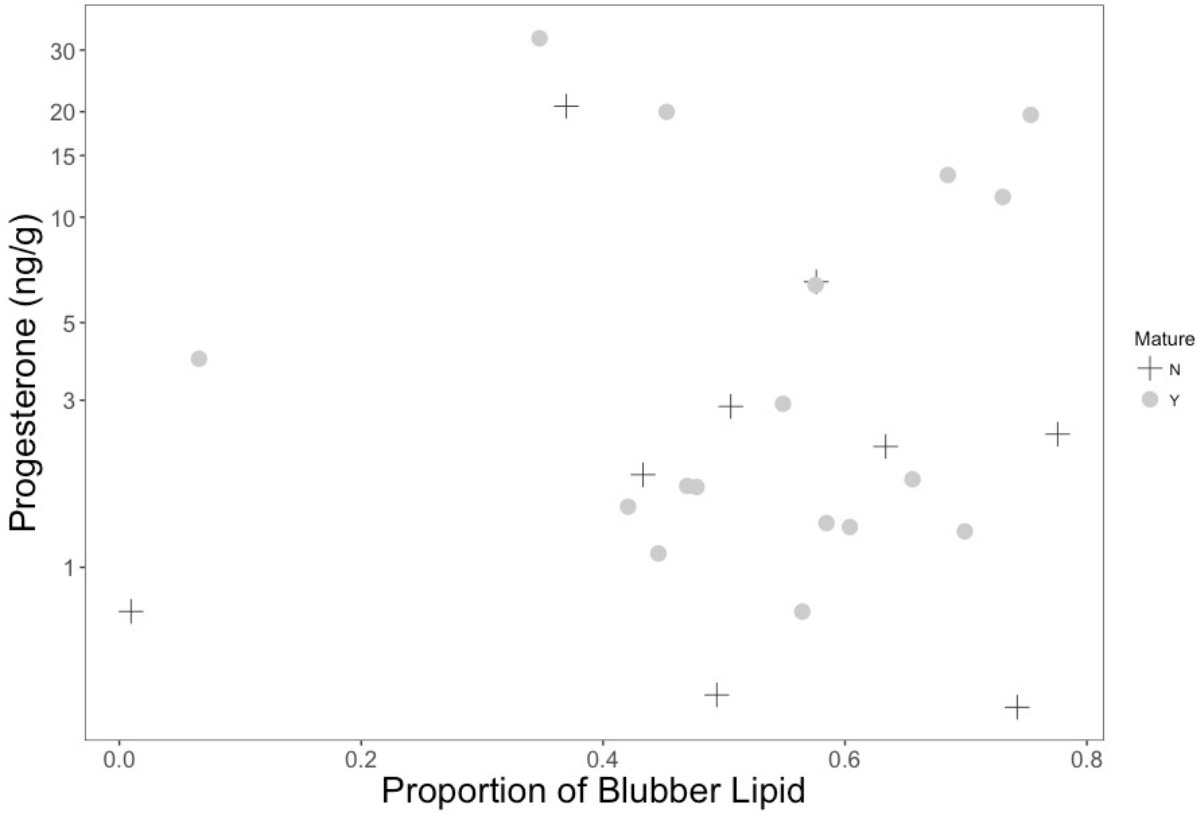

Figure 7: Progesterone (ng/g blubber) as a factor of proportion of blubber lipid extracted of males (immature and mature). Mature males are depicted by a gray circle, immature males are depicted by a black plus-sign. No significant difference found between progesterone concentration and proportion blubber lipid in males or fatality type.