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SEASONAL CHANGES IN CIRCULATING PROGESTERONE AND ESTROGEN CONCENTRATIONS IN THE CALIFORNIA SEA LION (*ZALOPHUS CALIFORNIANUS*)

DENISE J. GREIG,* KENDALL L. MASHBURN, MATTHEW RUTISHAUSER, FRANCES M. D. GULLAND, TERRIE M. WILLIAMS, AND SHANNON ATKINSON

The Marine Mammal Center, 1065 Fort Cronkhite, Sausalito, CA 94965, USA (DJG, FMDG)
Alaska SeaLife Center, 301 Railway Avenue, Seward, AK 99664, USA (KLM, SA)
Long Marine Laboratory, University of California, Santa Cruz 100 Shaffer Road, Santa Cruz, CA 95060, USA (MR, TMW)

The measurement of circulating reproductive hormone levels provides a relatively noninvasive method for assessing reproductive status. We assessed seasonal changes in concentrations of progesterone and total estrogens from serum samples collected from live stranded ($n = 199$) and captive ($n = 5$) California sea lions (*Zalophus californianus*). Progesterone concentrations increased in the fall (September–November) in both pregnant and nonpregnant animals, with no significant difference associated with pregnancy. Progesterone concentrations were greater in pregnant animals in the spring (February–April) than in nonpregnant animals. Circulating estrogen concentrations in captive, nonpregnant sea lions increased in July and November, correlating with periods of estrus and implantation. These data provide a baseline for studying reproduction in California sea lions.

Key words: delayed implantation, embryonic diapause, estrogen, hormones, pregnancy, progesterone, reproduction, *Zalophus californianus*

Hormone concentrations in wild mammal populations are increasingly used to estimate demographic parameters such as pregnancy rate and age at 1st reproduction. Blubber progesterone has been used to diagnose pregnancy in cetaceans (Kellar et al. 2006; Mansour et al. 2002); and serum progesterone has been used to detect pregnancy in harbor seals (*Phoca vitulina*—Gardiner et al. 1996), assess pregnancy rate and reproductive failure in New Zealand fur seals (*Arctocephalus forsteri*—McKenzie et al. 2005), and estimate age at 1st reproduction in Dall's porpoise (*Phocoenoides dalli*—Temte 1991). Hormonal cycles can be used to monitor animal populations remotely, and recently contraceptive programs have been used to manage wild populations of horses and elephants (Fayrer-Hosken et al. 2000; Kirkpatrick and Turner 2002).

As the California sea lion (*Zalophus californianus*) population increases along the west coast of the United States, there is growing interest in understanding reproduction and fertility in this species. From 1975 to 2001, production of young California sea lions increased at an average of 5.4% despite high pup mortality associated with El Niño events (Carretta et al. 2004). High levels of premature parturition also have been documented in this species in association with contaminant exposure (DeLong et al. 1973), infectious disease (Gilmartin et al. 1976), and harmful algal blooms (Brodie et al. 2006). Despite this evidence of reproductive failure, little is known about the hormonal changes associated with reproduction in this species.

Reproductive events in the California sea lion are composed of an annual cycle of parturition, estrus, pregnancy (including embryonic diapause and active placental gestation), and lactation. This species, whose range extends along the west coast of North America from Canada to Mexico, breeds during the summer on the California Channel Islands and offshore islands of Baja California, Mexico (Reidman 1990). After the breeding season, nursing females remain on the rookeries, whereas male sea lions migrate northward, sometimes as far as Alaska (Maniscalco et al. 2004) and reach peak numbers on Vancouver Island, Canada, in February (King 1983). Lactation lasts 6–11 months and females usually remain within 150 km of the breeding areas; however, females radiotagged on San Miguel Island, California, are known to forage as far north as Monterey Bay, California (lactating), and Ano Nuevo Island, California (nonlactating—Melin 2002; Melin and De Long 2000). On San Nicholas Island, California, parturition occurs from approximately 24 May to 23 June, with the greatest number of pups

* *Correspondent: greigd@tmmc.org*

born during the 1st week in June (Odell 1975). Mating takes place about 1 month after parturition and spontaneous estrus is thought to last just a few days (Boyd et al. 1999). Copulations have been observed in mid-July and the end of embryonic diapause is estimated to occur in mid-October (Odell 1975).

Little is known about the hormonal changes that accompany reproductive events in female otariids. The majority of data on reproductive endocrinology are from wild fur seals (Boyd 1991; Guinet et al. 1998; Kiyoto et al. 1999; Temte 1985). In the northern fur seal (*Callorhinus ursinus*), serum concentrations of estradiol slowly decreased during July and August from 50 pg/ml to approximately 10 pg/ml, then surged in October, presumably at the beginning of the active placental phase of pregnancy (Daniel 1974). Progesterone concentrations remained stable around 10 ng/ml during most of the embryonic diapause, and also began to increase in October at the start of active gestation (Daniel 1975). This suggests that circulating reproductive hormone levels can be used to diagnose pregnancy and other changes in reproductive status in otariids once a seasonal baseline has been established. This baseline has not been established for California sea lions despite their ubiquitous presence along the California coast and their common maintenance in captive facilities. The goal of our study was to describe seasonal changes in circulating serum progesterone and estrogen concentrations in female California sea lions.

Materials and Methods

Samples.—We collected serum samples for hormone analysis from live stranded California sea lions admitted to The Marine Mammal Center (Sausalito, California) or held at Long Marine Laboratory (University of California, Santa Cruz, California). At The Marine Mammal Center, single time-point and longitudinal samples were acquired opportunistically from 199 adult female California sea lions that stranded in central California from 1998 through 2000 (Greig et al. 2005). Serum was collected during routine clinical examinations as described by Bossart et al. (2001) and archived at −70°C. The reproductive status (pregnant, nonpregnant, or unknown) was determined based on the time of year, observations of the animals while alive, and necropsy results for those that died ($n = 88$). In live animals, abortions after sampling or detection of a fetal heartbeat by ultrasound indicated pregnancy. Because there is currently no published method for detecting a fertilized blastocyst in sea lions, reproductive status could not be determined from June through October. Animals sampled during this period were therefore assigned to the unknown category unless they had arrived at The Marine Mammal Center before the mating season (July) and were housed without male sea lions, in which case they were considered nonpregnant. During necropsy, gross signs of reproductive status were recorded: presence of a fetus confirmed pregnancy, and lactation or a placental scar on the uterine endometrium and an enlarged uterine horn were indications of a recent pregnancy. Sea lions with reproductive tract lesions such as uterine rupture, prolapse, or torsion, or fetal liquefaction were not included in the study to avoid including any potential changes in circulating hormone concentrations associated with these lesions. During July 2000, 21 focal animals were monitored every 3–4 days for signs of estrus, including swelling of the vulva, mucoid vaginal discharge, or enlargement and congestion of the clitoris. Longitudinal serum samples collected from these animals also were analyzed for hormones. All serum samples were assayed for progesterone, and sera from a representative subset of sea lions with complete histology results ($n = 58$) were tested for estrogen.

Longitudinal serum samples were acquired from 3 nonpregnant, adult female sea lions at Long Marine Laboratory. These females were acquired from Brookfield Zoo (Chicago, Illinois) and trained for voluntary blood sampling. Samples were collected monthly from June 2002 to December 2003.

Two wild, pregnant sea lions were captured on San Nicholas Island, California, on 1 April 2003 and were housed at Long Marine Laboratory until 25 May 2004, when they were released on San Nicholas Island. These females remained untrained for the duration of the study to ensure an effective return to the wild at the end of the study. The animals had just entered the last trimester of pregnancy at the time of capture and gave birth in June 2003. The females nursed their pups from birth until March 2004 when the pups were separated for weaning. Serum was collected monthly from both adult females: the animals were manually or chemically restrained and blood was drawn from the caudal gluteal or flipper vein. Live animal sampling followed guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998) under veterinary supervision and was approved by the designated Internal Animal Care and Use Committee body at University of California, Santa Cruz (the Chancellor's Animal Research Committee).

Hormone analyses.—Duplicate serum samples were assayed for progesterone using a commercial radioimmunoassay kit (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, California). The assay was validated for California sea lions using the standards provided and pooled sera from female sea lions. We tested for linearity by using increasing volumes of a sample of serum: volumes of 25, 50, 100, and 200 µl yielded concentrations of 0.6, 1.6, 3.91, and 6.65 ng/ml ($y = 0.86x - 0.02$, $r^2 = 0.98$). Parallelism was tested by running 50 µl of a low-concentration sample of pooled female serum with 50 µl of each of the standards and comparing that with 100 µl of each of the standards. The lines were parallel between 0.5 and 40 ng/ml: the slope of the serum plus the standards was −0.68, and the slope of the standards was −0.76. The mean percent nonspecific binding was 1.74 ± 0.80 *SE* ($n = 8$), and mean sensitivity was 0.03 ng/ml ± 0.01 *SE* ($n = 8$). Interassay variation of 3 separate controls run in all 8 assays was 11.6% ($\bar{X} = 1.46 \pm 0.17$ *SD*), 10.3% ($\bar{X} = 2.85 \pm 0.29$), and 11.1% ($\bar{X} = 12.05 \pm 1.34$. Ninety-eight percent of intraassay coefficients of variation were <10%.

A double-antibody radioimmunoassay (MP Biomedicals, Costa Mesa, California) for total estrogens was validated for use with California sea lion serum. Radioactivity of bound portion was determined using a gamma counter (Gamma C12;

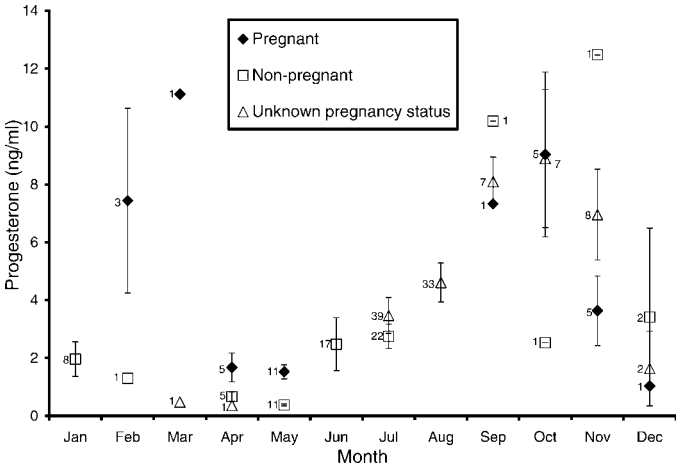

FIG. 1.—Monthly mean serum progesterone concentrations (ng/ml) in 199 live stranded California sea lions. Animals that stranded during July and August were classified as having ''unknown'' reproductive status. Animals that stranded in May or June, but had a sample taken in July, were classified as ''nonpregnant'' because they were in rehabilitation without access to males during the mating season (June–July) and therefore could not be pregnant. Error bars represent *SE* and numbers next to the mean are sample size.

TABLE 1.—Paired mean (*SE*) seasonal serum progesterone (P) and estrogen (E) concentrations in 58 stranded adult female California sea lions.

Reproductive season (month)	Pregnant			Nonpregnant		
	P (ng/ml)	E (ng/ml)	*n*	P (ng/ml)	E (ng/ml)	*n*
Early pregnancy (September–November)	7.42 (1.51)	1.55 (0.17)	5	7.51 (7.04)	2.01 (1.60)	2
Midpregnancy (January–February)	7.44 (2.77)	2.35 (0.61)	3	2.02 (0.42)	1.47 (0.22)	5
Late pregnancy (May)[a]	2.14 (0.16)	0.92 (0.03)	5	0.34 (0.01)	1.50 (0.15)	5
				Unknown status		
Pupping and estrus (June–July)[b]				3.21 (0.15)	1.90 (0.09)	26
Diapause (August)				5.61 (0.40)	1.32 (0.13)	7

[a] All pregnant females sampled in late pregnancy aborted their fetuses within days of sampling.

[b] Some of the females in this category are nonpregnant because they stranded earlier and were housed without access to males, whereas others could potentially have mated in the wild before stranding, but because there was no difference between the 2 groups, concentrations were combined.

Diagnostic Products Corporation, Los Angeles, California). The radioimmunoassays were performed as per manufacturer instructions with the exception that all volumes were halved and an additional standard (one-half the lowest standard) was added to the curve to increase sensitivity. Manufacturer cross-reactivity data were as follows: estradiol 17ß (100.0%), estrone (100.0%), estriol (9.0%), estradiol 17β (7.0%), equilin (2.5%), and 0.01% or below for all other steroids tested.

Serial dilutions of serum pools from both male and female California sea lions (neat to 1:64) yielded displacement parallel to that of the total estrogens standard curve. Recovery of added total estrogens standard (2.5–200 pg/ml) was 96.9% (SD = 12.9 pg/ml coefficient of variation [CV] = 13.4%; y = 0.19 + 0.98x, $r^2 = 1.00$) for females and 91.1% (SD = 15.3 pg/ml; CV = 16.8%; y = −2.4 + 01.09x, $r^2 = 0.99$) for males. All samples were initially run at a dilution of 1:5 in diluent provided by the manufacturer with the more concentrated samples diluted further to 1:10. Interassay variation of 2 separate controls was 9.16% ($\bar{X} = 70.9 \pm 6.50$ pg/ml) and 8.98% ($\bar{X} = 39.9 \pm 3.54$ pg/ml) for a high and a low, respectively. Intraassay coefficients of variation were <5% and assay sensitivity was 3.14 pg/ml. All hormone concentrations were determined after a log-logit transformation of the standard curve.

High-pressure liquid chromatography.—We used high-pressure liquid chromatography (Varian ProStar 210/215; Varian Inc., Walnut Creek, California) to determine immunoreactive estrogenic components in California sea lion serum in the validated radioimmunoassay. Randomly selected sera were used to create 3-ml pools for each sex. Five hundred microliters of phosphate-buffered saline was added to serum pools and all samples were vortexed for 2 min. All pools were analyzed and fractions were collected via high-pressure liquid chromatography using the method of Monfort et al. (1998). Immunoreactivity and radioactivity profiles were obtained from an analysis of collected eluates and expressed per 1-ml fraction collected. High-pressure liquid chromatography of the female serum pool revealed that 13% of the total mass measured in collected fractions coeluted with estradiol and 0.2% coeluted with estrone, with 6 additional immunoreactive individual peaks eluting in fractions not associated with added tracer. High-pressure liquid chromatography of the male serum pool revealed that 2.12% of immunoreactivity coeluted with estradiol, with 1 additional single immunoreactive peak unassociated with added tracer.

RESULTS

Serum progesterone concentrations varied among stranded California sea lions, especially during the end of the embryonic diapause and early in active placental gestation (September–December; Fig. 1). During this period, serum progesterone concentrations were not useful for discriminating pregnant from nonpregnant animals (Table 1). In midpregnancy (February and March), there was a clear separation in progesterone concentrations between pregnant and nonpregnant animals, although small sample sizes precluded a statistical comparison (Fig. 1). In late pregnancy (April and May) among stranded animals, the difference between pregnant and nonpregnant animals was less distinct: although there was a statistical difference in May (independent-samples t-test with equal variances not assumed, $t = 4.720$, $d.f. = 10.45$, $P = 0.001$), the difference was not significant in April ($t = 1.929$, $d.f. = 4.749$, $P = 0.115$). Progesterone concentrations for pregnant and nonpregnant sea lions during April and May were low, ranging from 0.26 ng/ml to 3.37 ng/ml, and all pregnant females aborted fetuses within days of sampling. The trends

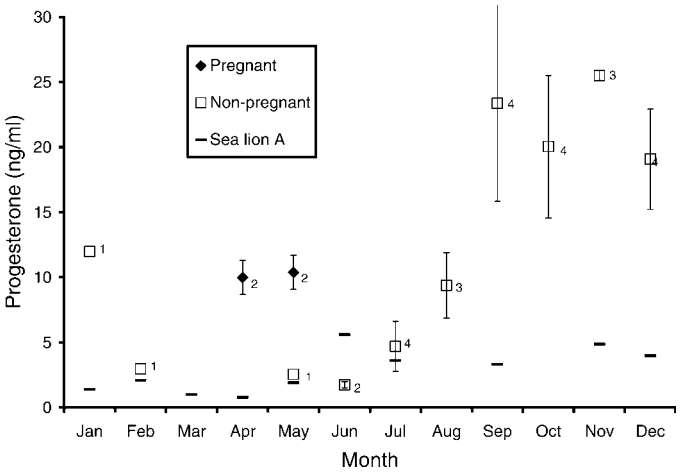

FIG. 2.—Monthly mean (±*SE*) serum progesterone concentrations (ng/ml) in 5 captive, nonpregnant sea lions, and 2 captive, pregnant sea lions (pups were born 21 and 22 June 2003). Results from 1 sea lion (sea lion A) were consistently less than those from the other 4 and are plotted separately. Error bars represent *SE* and numbers next to the mean are sample size.

observed among the stranded animals at The Marine Mammal Center (Fig. 1) were similar to the seasonal pattern observed among the captive sea lions at Long Marine Laboratory (Fig. 2) except that concentrations among the stranded animals were lower overall. Of the sea lions at Long Marine Laboratory, the 2 pregnant, wild-caught females successfully gave birth and were nonpregnant for the rest of the year. The other 3 captive sea lions were nonpregnant and 1 of these had consistently low progesterone concentrations (sea lion A; Fig. 2).

With 1 exception, estrogen concentrations among stranded California sea lions were uniformly low ($\bar{X} = 1.52$ ng/ml ± 0.98 *SD*, $n = 56$) and exhibited no clear pattern associated with date or reproductive status (Fig. 3). The exception was a nonpregnant, stranded animal with a progesterone concentration of

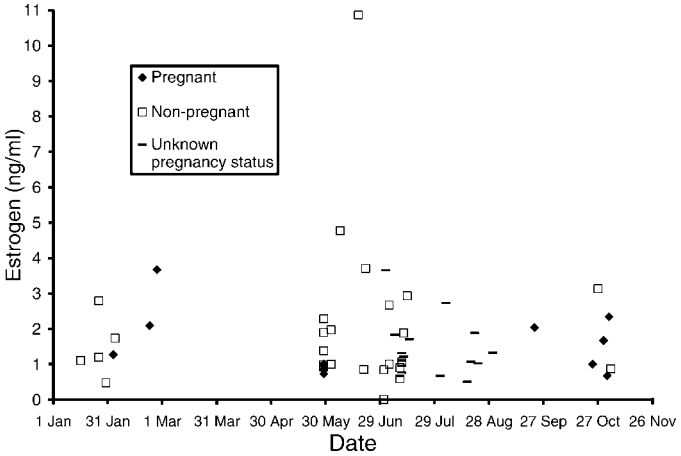

FIG. 3.—Seasonal serum total estrogen concentrations from 57 live, stranded California sea lions. Animals that stranded in May or June, but had a sample taken in July, were classified as ''nonpregnant'' because they were in rehabilitation without access to males during the mating season (June–July) and therefore could not be pregnant.

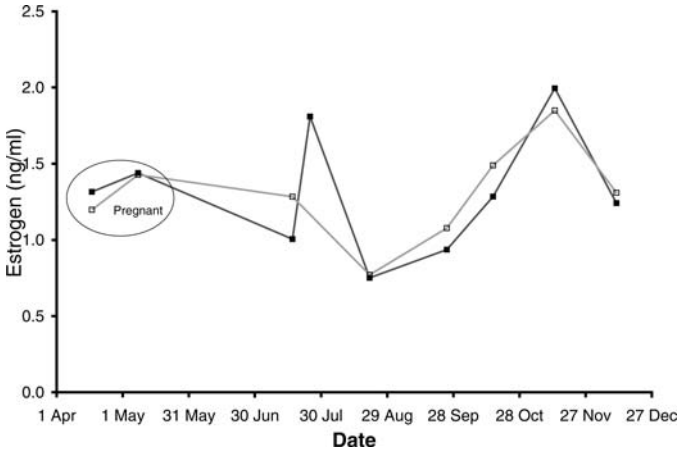

FIG. 4.—Seasonal serum total estrogen concentrations (ng/ml) in 2 wild-caught sea lions housed at Long Marine Laboratory in 2003. Animals were nonpregnant with the exception of the dates circled (17 April 2003 and 8 May 2003—pups were born 21 and 22 June 2003).

0.26 ng/ml on 24 May but a progesterone concentration of 17.48 ng/ml and estrogen concentration of 10.87 ng/ml on 17 June when she died because of intestinal torsion. No changes were detected in any organ on histopathology to indicate the reason for the surge in circulating hormone concentrations.

One of the wild-caught sea lions exhibited 2 estrogen peaks on 25 July and 13 November that may correlate with ovulation and implantation (Fig. 4). It is likely that the sampling regime was not frequent enough to detect a similar ovulatory peak in the 2nd wild-caught sea lion. The captive, nonpregnant sea lions exhibited a similar pattern of circulating total estrogens with an increase in July and October–November (Fig. 5).

Visible signs of estrus were noted in 9 of the 21 actively monitored animals. Swelling of the vulva, mucoid vaginal discharge, and enlargement and congestion of the clitoris were noted between 11 and 22 July. Estrogen and progesterone

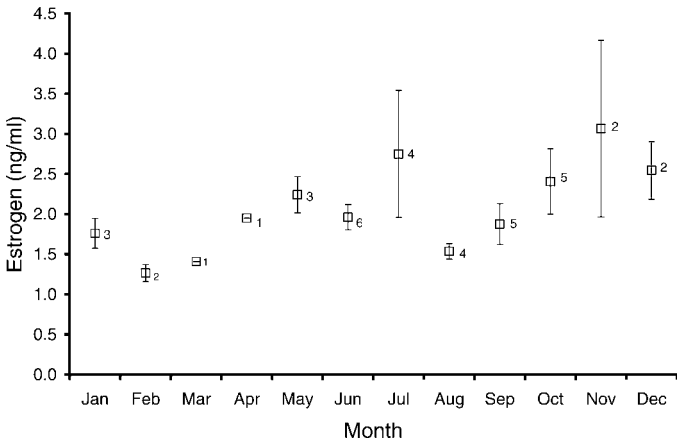

FIG. 5.—Monthly mean serum total estrogen concentrations (ng/ml) for 3 captive, nonpregnant adult female sea lions. Error bars represent *SE* and numbers next to the mean are sample size. Some sea lions were sampled more than once in a given month and values from 2002 and 2003 are combined.

concentrations exhibited considerable variability during this period and no distinct association between estrogen levels and estrus was detected.

Discussion

Relative seasonal changes in serum progesterone concentration in the healthy captive animals and stranded sea lions were similar, although concentrations were lower among stranded animals. With the exception of the animals stranding because of domoic acid exposure, all of the stranded animals were underweight when they were 1st sampled. This difference in hormone levels between stranded and captive sea lions could be due to nutritional differences, as observed in human females who undergo seasonal nutritional stress or increases in workloads. Rural Bolivian Aymara women had progesterone levels that were 71% of those measured from women in Chicago. These lowered hormone levels had no effect on fertility (Vitzthum 2004).

In addition to nutritional differences, lower progesterone levels among the stranded animals may be related to health status or stress. Experimental adrenal stimulation by adrenocorticotrophic hormone in cats (Chatdarong et al. 2006), pigs (Brandt et al., in press), and cattle (Yoshida and Nakao 2005) increased circulating progesterone levels. In cattle, this increase was considered great enough to possibly interfere with estrus. It is thought that pinniped pregnancies are maintained by progesterone produced by the corpus luteum (Ishinazaka et al. 2001), and it is unknown if progesterone can be produced by the adrenal and affect their annual reproductive cycle.

Because the stranded animals could have been chronically stressed due to illness, injury, or malnutrition, this stress could have influenced progesterone production. There were no patterns in hormone concentrations associated with any particular disease, implying that general debilitation is more likely to be the factor influencing hormone levels.

One captive individual (sea lion A) had progesterone concentrations even lower the than the stranded sea lions and did not exhibit an annual cycle. At 25 years old, we suspect she may have been anestrus or senescent. We have never seen signs of senescence among the stranded sea lions at The Marine Mammal Center where the oldest female we have aged was 19 years old.

Serum progesterone levels can likely be used to differentiate pregnant from nonpregnant California sea lions during the 2nd half of gestation (February–June), although a larger sample is needed to confirm this. The difference between progesterone levels in the pregnant and nonpregnant sea lions in April and May was more apparent in the 2 healthy pregnant animals collected from San Nicolas Island than in the stranded pregnant animals. Boyd (1991) found that circulating progesterone concentrations in Antarctic fur seals (*Arctocephalus gazella*) were not detectable 1–2 days before parturition. It is possible that the pregnant animals at The Marine Mammal Center had stopped producing progesterone because all the stranded pregnant animals sampled in April and May (and the pregnant female sampled in December) lost their fetuses within hours or days of being sampled. These animals stranded due to domoic acid toxicosis, likely associated with abortion and premature parturition (Brodie et al. 2006). The mechanism producing fetal loss in this toxicosis is unknown. Despite their otherwise healthy appearance, the progesterone concentrations in these aborting females probably do not represent normal 3rd-trimester hormone levels.

Examination of data from 1 animal suggested that estrogen concentration increased at ovulation similar to in other mammals; however, sampling needs to occur at shorter intervals to better define this increase. Both hormones increased at the end of diapause even during nonconception cycles. The prolonged luteal cycle suggests that sea lions do not use progesterone or estrogen as a hormonal cue to signify pregnancy until active placental gestation has begun.

Our data provide baseline information for the development of diagnostic tools for studying reproduction in California sea lions. The use of wild stranded animals poses considerable limitation on the frequency of sampling and the knowledge of individual reproductive history. Further studies on reproductive hormone changes should focus on more frequent sampling of healthy individuals under controlled conditions.

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