Establishing Endocrine and Behavioral Parameters of Reproduction in Pacific Walrus

(*Odobenus rosmarus divergens*)

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Abstract

Establishing Endocrine and Behavioral Parameters of Reproduction in Pacific Walrus (*Odobenus rosmarus divergens*)

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Reproduction of walrus (*Odobenus rosmarus*) in zoos and aquaria has met with limited success. While basic information on reproductive characteristics is available, there is limited knowledge of reproductive physiology and endocrinology for walrus. The overall goal of the research was to monitor reproductive events and seasonal changes that occur in *ex situ* walrus of North America by utilizing a variety of methods. To track changes in males, longitudinal hormonal analysis of saliva samples, changes in testicular size, presence or absence of spermatozoa and seasonal fluctuations in behavior were examined. Saliva samples were collected from six male walrus for periods of 12 to 36 months. Measurements of testicular length and width were collected on a weekly basis. Baseline testosterone values for each animal were established using an iterative process that eliminated elevated values. Elevations were defined as values ≥ 2 times the individual's baseline (range 0.838-4.228 ng/ml). The mean combined baseline testicular measurement for individuals (R linear length + R linear width + L linear length + L linear width) was 54.81±2.13 cm while the mean combined increased testicular measurement (an increase of 15% or more over minimum values) was 65.38±1.71. Three of six animals displayed the typical rise and fall of testosterone concentrations observed in seasonal breeders in the wild and elevations were coincident with reproductive behaviors. Behaviors included visual and vocal courtship displays, pursuit of females, clasping and penetration. Sperm presence in a single walrus during two years coincided with increased testicular measurements and mean duration of spermatozoal presence was 131±3 days. Endocrine profiles varied extensively among animals, demonstrating within-animal decline in testosterone levels over time, a lack of testosterone elevations coincident with the wild walrus breeding season and a blind male with a unique derivation in his endocrine profile, hormone elevations directly opposite the wild walrus breeding season.

To track female reproductive cycles, longitudinal hormonal analysis of saliva samples and seasonal fluctuations in behavior were examined. Saliva samples were collected from six female walrus at least three times a week for periods of 12 to 55 months. Salivary estradiol-17β was deemed an unreliable indicator of the follicular phase because hormone concentrations did not display a rise in estrogen concentrations prior to a rise in progesterone concentrations nor increased estrogen concentrations coincident with estrus behaviors. The most commonly reported signs of behavioral estrus were soliciting male attention, clasping and penetration. In general, behavioral observation also was not a consistently reliable method for detecting impending estrus. Salivary progesterone was found to be unsuitable for monitoring the functional capacity of the corpus luteum because profiles were difficult to interpret biologically or physiologically. Progesterone profiles did not appear to coincide with behavioral or seasonal changes. However, periods of elevated progesterone concentrations were demonstrated in two of six females and were most likely representative of consecutive pseudopregnancies (mean 204.27

days). An inconsistency elucidated in this study involved a blind female with a unique derivation in her endocrine profile, inconsistent with the wild walrus breeding season and varying in time frame each year.

Issues with walrus reproduction elucidated by this study included: within-animal decline in testosterone levels over time, unsynchronized cycles between animals residing together and the inconsistent timing of reproductive events in blind animals. To increase breeding success of the *ex situ* walrus population the following changes are proposed: a) increase the number of walrus in the population or consolidate existing walrus into larger social groups; and b) separate the genders during the non-breeding season or move animals to different facilities. Facility collaboration and continued monitoring of reproductive functioning via endocrine, anatomical and behavioral parameters would allow evaluation of the efficacy of the proposed changes.

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Introduction

The suborder Pinnipedia within marine mammals consists of three families: true seals (Phocidae), sea lions and fur seals (Otariidae) and walrus (Odobenidae). The wild Pacific walrus (*Odobenus rosmarus divergens* [Illiger 1851]) population is estimated at approximately 129,000 (95% confidence limits 55,000-507,000) (Speckman *et al*. 2011). In contrast, the current zoo and aquarium population in the United States consists of only 19 individuals (8 males and 11 females) housed at seven facilities (Oland 2012). Of these, 16 were wild-caught and three were born in zoos. Since the 1930s, walrus have been maintained in U.S. zoos and aquaria with limited reproductive success, with 18 walrus calves born of which only six have lived beyond 12 months (Genrich 1984, Oland 2012). This population is currently in a demographic crisis (i.e. population is not self-sustaining). Only seven offspring have been born in the past 10 years, and of these animals only one has survived to adulthood (Oland and Marti 2011). As a result, maintaining and increasing the *ex situ* population of walrus depends upon obtaining stranded animals or collecting wild walrus. This is problematic as stranded animals only occur sporadically and the capture of wild walrus is politically and logistically difficult and expensive. Therefore, zoos and aquaria are becoming increasingly interested in creating a self-sustaining population via breeding.

Marine mammals are popular in zoos and aquaria as most visitors find them engaging and charismatic, and rarely have an opportunity to view them in the wild. In particular, *ex situ* walrus stimulate conservation consciousness and action for their wild counterparts (Clayton *et al.* 2009) especially in recent times given the recognition of trends in global-scale climate change (Tynan and DeMaster 1997, Moore and Huntington 2008, Ragen *et al*. 2008, Walsh 2008). The current decline in Arctic sea ice is expected to have increasingly negative impacts on wild populations (Cooper *et al*. 2006, Burek *et al*. 2008, Laidre *et al*. 2008, Moore and Huntington 2008, Ragen *et al.* 2008, Huntington 2009, Garlich-Miller *et al*. 2011). Walrus depend upon the pack ice for feeding, breeding, parturition, nursing, resting and molting, and sea ice may serve an important function in facilitating productivity of walrus prey populations. A significant decrease of critical sea ice habitat may result in lost access to feeding grounds, interruption of breeding activities, reduced prey production, increased calf mortality and increased anthropogenic disturbances (Rausch *et al.* 2007, Center for Biological Diversity 2008, Laidre *et al*. 2008, Ragen *et al*. 2008, Huntington 2009). In 2011, the Pacific walrus was listed by the United States Fish and Wildlife Service (USFWS) as a candidate species for classification as threatened or endangered as defined by the U.S. Endangered Species Act of 1973 as amended (16 United States Code §§1531-1543 et seq.; ESA) based on projected changes in sea ice habitats associated with global climate change (Garlich-Miller *et al*. 2011). Like the wild population, the zoo-based population is vulnerable because of low numbers and limited productivity. In the short term, managers of *ex situ* walrus must focus on breeding the current founders (genetically unrelated individuals) and increasing the reproductive rate with the goal of increasing the effective zoo-based population size. Meeting the long term goals of maintaining genetic diversity in the *ex situ* environment will require additional founders and supplementary holding facilities to expand the population size.

An essential first step toward enhancing the propagation of a species is to understand the fundamental attributes of its reproductive biology. Walrus reproductive physiology is poorly understood. Particularly lacking is a comprehensive understanding of both female and male reproductive cycles. Achieving successful breeding in *ex situ* walrus may depend on developing a database of reproductive biological norms. In seasonally breeding species, concentrations of estrogen, progesterone and testosterone differ between periods of reproductive activity and non-activity, and these differences can be used to determine reproductive status (Schwarzenberger *et al.* 1996, Atkinson 1997, Graham 2004).

Analysis of hormones to evaluate the reproductive status of individuals has become a valuable tool for animal management (Lasley and Kirkpatrick 1991, Schwarzenberger *et al.* 1996, Graham 2004, Amaral 2010). It is anticipated that this tool will be useful in walrus to examine patterns of estrous cyclicity and/or the onset of estrus to optimally time breeding and determine the effects of seasonality on reproductive function. Determining steroid hormone profiles through collection of daily serum samples would be logistically difficult and potentially stressful for the animals. While urinary and fecal hormone analyses are routinely used to monitor many terrestrial species (Lasley and Kirkpatrick 1991, Schwarzenberger *et al.* 1996, Brown 2006, Bateman *et al*. 2009), the aquatic nature of marine mammals, often not trained to urinate or defecate on cue, provides obvious logistical challenges. In the last several decades, saliva has become a widely accepted sample source for the analysis of human steroids. Specifically, saliva has shown good correlation with unbound reproductive steroid hormones in serum (Riad-Fahmy *et al*. 1987, Rilling *et al.* 1996, Ostatnikova *et al.* 2002, Groschl 2008). The study of steroid hormone values in the saliva of female and male marine mammals has been accomplished in several species, including bottlenose dolphins (*Tursiops truncatus* – testosterone; Hogg *et al.* 2005), Hawaiian monk seals (*Monachus schauinslandi* - estrone sulfate, progesterone, total androgens; Pietraszek and Atkinson 1994, Theodorou and Atkinson 1998) and Steller sea lions

(*Eumetopias jubatus* – progesterone and testosterone; Harmon 2001). Because of its success in other marine mammals, salivary steroid monitoring is likely to be a useful tool for monitoring walrus reproductive hormones.

Odobenid Wild Population and Distribution

The family Odobenidae is comprised of three subspecies including the Atlantic walrus (*Odobenus rosmarus rosmarus*), the Pacific walrus (*O. r. divergens*) and the Laptev walrus (*O. r. laptevi*). Walrus are circumpolar, but the sub-species are concentrated in several geographically separated areas with little or no probability of interbreeding (Figure 1). Pacific walrus inhabit the Bering and Chukchi Seas along the coastlines of Alaska and Russia. Atlantic walrus range from the central Canadian Arctic eastward to the Kara Sea. Laptev walrus reside in the Laptev Sea which is located north of Siberia, Russia. The Laptev Sea is part of the Arctic Ocean and neighbors the Kara Sea and East Siberian Sea.

As it is difficult to survey the wild Pacific walrus population, the size of the population has never been known with certainty. Based on large harvests in the 18th and 19th centuries, Fay (1957) hypothesized that the pre-exploitation population was approximately 200,000 animals. Since that time the population size has fluctuated in response to varying levels of human exploitation. The Pacific walrus population was severely depleted in the mid-20th century by overharvesting for commercial purposes (Fay 1982), reducing the population to an estimated 50,000-100,000 animals in the mid-1950s (Fay *et al.* 1997). The killing of walrus in Alaska, except for local use by natives, was prohibited by the U.S. Department of Commerce regulation in 1937 and by the Congressional “Walrus Act” of 1941 (48 United States Code §248). In 1957, the Soviet Ministry of Fisheries placed rigid regulations on walrus harvested in Russia. During this time period both the Soviet Union and the state of Alaska provided protection of walrus herds from hunting on or near major land based haul-outs. In 1972, walrus gained protection under the U.S. Marine Mammal Protection Act (16 United States Code §§ 1361-62, 1371-84, and 1401-07 [supp. IV 1974]; MMPA), which made it illegal to hunt or harass any marine mammal in U.S. waters. As a result, the population appeared to increase rapidly in size during the 1960s and 1970s and Fay *et al.* (1989, 1997) suggested that the population was approaching, or had exceeded, the carrying capacity of its environment in the early 1980s.

Between 1975 and 1990, aerial surveys were carried out by the United States and Russia at 5-year intervals, producing population estimates ranging from 201,039 to 290,000 walrus (Johnson *et al*. 1982, Fedoseev 1984, Gilbert *et al*. 1992, Udevitz *et al*. 2001). However, due to differences in methodologies, comparisons of estimates are not reliable.

The most recent survey of the Pacific walrus population was conducted in 2006 by U.S. and Russian researchers, using thermal sensors and aerial photography to detect walrus hauled out on sea ice and satellite transmitters to account for walrus in the water (Speckman *et al.* 2011). Due to limitations imposed by weather conditions only half the range was surveyed. The estimate in the counted area was 129,000 animals (95% confidence limits 55,000-507,000). In response to a variety of factors such as the population estimate generated from the 2006 survey, significant reductions in recent summer sea ice habitats and large numbers of mortalities reported at coastal haulouts in Russia, Russian resource managers have reduced harvest quotas in recent years based on perceptions that the population is in decline (Garlich-Miller *et al.* 2011).

The International Union for the Conservation of Nature (IUCN) classifies the walrus as "data deficient" because of lack of current and specific population information, but reports that climate change will most likely have an adverse effect on the population (Lowry *et al*. 2008). In February 2011, the United States Fish and Wildlife Service (USFWS) listed the Pacific walrus as a candidate species for classification as a threatened or endangered species under the ESA based upon projected changes in sea ice habitats associated with global climate change (Garlich-Miller *et al*. 2011). The report states – "The Pacific walrus is experiencing habitat modification due to a warming climate and loss of summer sea ice that has not occurred for several thousand years. Our review and analysis of potential threats suggests that the intensity of stressors will continue to increase in the future and will likely result in a population decline. The time frame over which population changes are likely to occur and the magnitude of population level impacts are uncertain. Continued monitoring and evaluation of population status and trends, as well as habitat assessment (availability and quality) will be critical to evaluate our assumptions, make adjustments as we gain increased understanding, and make direct links among the threats and population performance." (Garlich-Miller *et al*. 2011).

Odobenid History in United States Facilities

Walrus have been maintained in zoos and aquaria throughout the world since the early 1600s. The first recorded collection for zoological display occurred in the year 1608, when an English walrus hunting expedition brought a pair of calves to the London Zoo. The first housing of walrus in U.S. facilities occurred in the 1930s (Table 1). In these early years survival was short-lived, lasting only a year or two. Repeated attempts at display were made by various facilities in the 1940s and 50s but real success, defined as increased survival rate of more than just a couple years, was not achieved until the 1960s. However, it was not until the 1980s that walrus were held in larger numbers (several new facilities began housing walrus). The first zoo birth occurred in 1975, the most recent in 2011. In total, 18 walrus calves have been born in U.S. facilities with only six surviving past a year of age (Table 2; Genrich 1984, Oland 2012). Calves have been born in the months of March through August with a peak in May and June (Figure 2).

Since 1932, approximately 90 walrus have been housed in U.S. facilities (Oland 2012). As of November 2013, there were eight male and 11 female walrus in seven U.S. locations, including the Indianapolis Zoo, Indianapolis, Indiana; New York Aquarium, Brooklyn, New York; Point Defiance Zoo & Aquarium, Tacoma, Washington; Six Flags Discovery Kingdom, Vallejo, California; Sea World California, San Diego, California, Sea World Florida, Orlando, Florida; and Sea World Texas, San Antonio, Texas.

Walrus have been observed to live into their early 40s in the wild (Fedoseev and Gol'tsev 1969, Fay 1982, Fay 1985). As of 2012, the oldest recorded *ex situ* male is still living at 34 years old and the oldest recorded female is still living at 35 years old. Both of these individuals were wild-caught. The youngest female to give birth was approximately 10 years of age, the youngest male to sire offspring approximately 9 years. The oldest males and female to successfully breed were 28 years of age. Currently there is no evidence of reproductive senescence in the *ex situ* population (Oland and Marti 2011).

The zoo-based Pacific walrus is designated as a Red Program under the Association of Zoos and Aquariums (AZA). Red programs involve zoo-based populations of a given species that are comprised of fewer than 50 individuals and are recommended for cooperative management among AZA facilities. A regional studbook is maintained for each subject population, containing the pedigree and demographic history of each component individual. Minimal acceptable guidelines for the standards of care provided for walrus in zoos and aquaria are detailed in the Guidelines from the U.S. Department of Agriculture (USDA) Code of Regulations - subpart E.

Reproduction in Odobenids

General

The Pinnipedia evolved from arctoid carnivore ancestors approximately 30 million years ago. There are currently 14 species of otariids, 18 species of phocids, and only one extant odobenid species, although odobenids are diverse in the fossil record for pinnipeds. It is difficult to study walrus in the wild due to their remote location and inhospitable environment. In addition, mating season occurs during winter when most breeding sites are only accessible by costly means such as with icebreaker vessels. As such, the majority of historical knowledge has been obtained from harvested animals.

The reproductive cycle of the walrus has many features in common with both the otariids and phocids, such as an annual mating season and delayed implantation of similar duration (several months). However, both birth and mating periods of walrus are very different from other pinnipeds. The most profound difference between the walrus and other pinnipeds is that gestation in the walrus is 15-16 months and therefore breeding occurs no more frequently than biennially, with birthing occurring triennially (Garlich-Miller and Stewart 1999, Born 2001). In otariids and phocids all species except the Australian sea lion (*Neophoca cinera*) have gestation constrained so that the reproductive cycle lasts a single year. At the population level, walrus breeding occurs annually. However, individual walrus females do not give birth each year and mating does not occur postpartum as in the phocids and otariids, instead occurring approximately nine months after parturition. Thus, individual female walrus breed every 24 months on average, although reproductive events in walrus populations occur during the same season each year.

Sexual dimorphism is appreciably less in walrus than it is in most other polygynous pinnipeds (Bartholomew 1970, Fay 1982). Wild adult Pacific males are approximately 18% longer and 45% heavier than females, weighing from 878 to 1,652 kg (non-breeding season weights) and measuring 2.7 to 3.7 m in length. Wild adult females weigh 400 to 1,247 kg and are 2.3 to 3 m long (Fay 1982). Males attain full size and secondary sexual characteristics by age 15, which is about five to six years after they are fertile and some seven to eight years after the females are sexually mature (Fay 1982).

Pacific walrus display lek-like mating characteristics (Fay *et al*. 1984) which are not generally associated with pinniped mating systems and have been reported in only four (California sea lions, *Zalophus californianus*; harbor seals, *Phoca vitulina*; Australian sea lions, walrus) of the 34 species of pinnipeds to date (Heath and Francis 1983, Fay *et al.* 1984, Hayes *et al.* 2004, Boness *et al.* 2006, Soto and Trites 2011). The minimal criteria to distinguish classical lek species from those with alternative mating systems are 1) absence of paternal care; 2) aggregation by males in a mating area and defense of small mating territories that do not fill the entire habitat used by females and where most mating occurs; 3) mating grounds with no critical resources required by females; 4) absence of constraints on females in choosing among potential partners (Höglund and Alatalo 1995, Soto and Trites 2011).

During most of the year the adults are segregated by gender and live in small groups (Fay 1982). In the fall, walrus that summered in the Chukchi Sea typically begin migrating south in advance of the sea ice while males that summered in the Bering Sea begin migrating north (Jay and Hills 2005). Large numbers of animals congregate at winter feeding grounds where mating takes place (Fay 1982). Locations of specific feeding and breeding areas vary annually depending on ice conditions. However, three general breeding areas of Pacific walrus exist: a southeast aggregation located south of Nunivak Island; a northern aggregation just southwest of St Lawrence Island; and a smaller western group located south of the Chukotka Peninsula in the Gulf of Anadyr (Figure 3; Fay 1982, Burn *et al*. 2009, Speckman *et al.* 2011).

Mating is aquatic and occurs in areas of unconsolidated, drifting pack ice or polynyas (areas of open water surrounded by stable landfast ice) from December to March (Fay 1982). During the breeding season, female walrus and calves haul out on ice pans or rest in the water while one or more adult males station themselves in the water alongside a herd and perform both visual and vocal displays (Fay *et al*. 1984, Sjare and Stirling 1996). Only the largest, most experienced and dominant males actually breed. The vocal portion of the courtship displays include teeth clacking, clanging, bell-like sounds and whistles. These displays may have two functions: an advertisement of the males' ostensible sexual proclivity directed to females and as a claim of dominance among competitive males nearby (Fay *et al*. 1984, Sjare 1993). Adult male walrus exhibit some of the longest song bouts ever recorded among mammals, lasting from a few hours to more than 48 hours (Sjare 1993). When more than one male attends a herd of females, they remain spaced about 5-10 meters apart and vocalize from fixed locations (Fay 1982). When displaying males are present, sub-adult males are scarce or absent. Sjare (1993) found that in Atlantic walrus, young adult males and less successful mature males tend to engage in three alternative reproductive strategies. Some males spent most of their time either as a silent herd member or as a vocal satellite male, while others alternated between the two positions. Silent herd members could sometimes "steal" (copulate with a female that a dominant, displaying male is attending) copulations and some satellite males appear to mimic the behaviors of the displaying males to practice courtship behavior (Sjare and Stirling 1996). In the Pacific subspecies, a greater number of males display and no single individual monopolizes access to the herd (Fay 1982, Fay *et al*. 1984). Males actively defend small display territories near the herd, and the frequency of fighting seems to be higher than that observed in the Atlantic subspecies. Thus, the female Pacific walrus may have the opportunity to assess and then choose one of the displaying males as her mate. Actual copulation has only been observed a few times in the wild. During one such observation, the male had his hips curled around the female's hind-quarters while his fore flippers were around the female's mid-section. The male mounted the female for 30-150 seconds, 8-10 times during a two hour period, vocalizing the entire time (Sjare 1993).

In the spring, post-breeding, thousands of males remain in the Bering Sea during the summer months, foraging in Bristol Bay and the Gulf of Anadyr, while the rest of the population migrates north to feeding grounds in the Chukchi Sea (Jay and Hills 2005). It is during this northward migration, in mid-April to mid-June, that females give birth on ice (Fay 1982). A female generally gives birth to a single calf, but twins have been observed (Fay *et al*. 1991). Newborn calves weigh 45-75 kg and are 0.9-1.2 m long. Young walrus are gradually weaned during their second year of life (Fay 1982). Some may nurse even longer if the mother does not have a new calf.

Endocrinology

Mammalian reproduction is regulated by a series of neurological and hormonal feedback mechanisms involving the hypothalamus, pituitary gland and gonads (hypothalamic-pituitary-gonadal [HPG] axis; Figure 4). The hypothalamus releases gonadotropin releasing hormone (GnRH) which acts on the pituitary gland to regulate the production and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are responsible for the development of the gonads and gameteogenesis. Estrogens, progesterone (P) and testosterone (T) are steroid hormones produced by the gonads and function to maintain the reproductive organs, elicit reproductive behavior and physiologically prepare an animal for mating and pregnancy. Seasonal breeders are reproductively quiescent during the non-breeding season with relatively inactive gonads as a result of decreased frequency of GnRH secretion from the hypothalamus, and hence a decreased secretion of FSH and LH from the pituitary gland. During the breeding season, increased frequency of GnRH secretion leads to increased secretion of FSH and LH and activates gonadal function for seasonal breeders.

The pineal gland, a small endocrine gland in the vertebrate brain, is involved in regulating reproduction. It produces melatonin, a hormone that affects the modulation of seasonal fluctuations in seasonal breeders. Melatonin can affect the HPG axis by inhibiting secretion of LH and FSH from the pituitary gland. The reproduction of short-day breeders is stimulated by melatonin while the reproduction of long-day breeders is repressed by melatonin. The most common environmental factors that influence the HPG axis of pinnipeds are photoperiod, nutrition and climate (Boyd 1991). Photoperiodism is the ability of organisms to assess and use the length of day as an anticipatory cue to time seasonal events (Bradshaw and Holzapfel 2007). This principal proximate factor will be addressed in further detail below.

Male Reproductive Biology

General

The first estimates of testicular and bacular growth rates, seasonal development and regression of testes and other reproductive patterns in male walrus stemmed from anatomical observations of harvested walrus (Mansfield 1958, Fay 1982). Most male walrus reach sexual maturity at 8-10 years of age but probably are not successful at breeding until 15 years of age, when they attain full physical size and are capable of competing for females (Fay 1982). Secondary acceleration of growth of the testes, baculum and body size begins at eight to 10 years, as well as a change in the rate of tusk growth and in the development of the thick bosses in the skin of the neck and shoulders. Secondary sexual characteristics of adult males include the shape of the tusks (males diverge more and females converge more), the development of the shoulder and neck muscles and the extent of blubber deposition in the upper neck region (Fay 1982). Full sexual maturity is likely not attained until secondary growth of body, baculum and testes has been completed, allowing males to be competitive with other adult bulls for mates (Fay 1982). Limited evidence exists regarding reproductive senescence (a decrease in the reproductive rate or breeding probability with increasing age post-maturity) in the male walrus. However, Fay (1982) found spermatozoa in the corpora epididymides of individuals up to 37 years of age.

The walrus, like all pinnipeds, possess a penis bone, the os penis or baculum. The baculum of the adult Pacific walrus is the largest in any living mammal. Fay (1982) studied the baculum of 273 specimens and determined that the growth in length is most rapid in the first 10 years of life and continues until at least the 15th year. In adults (>15 years), the length ranged from 47.6-62.2 cm.

The testes are situated within the subcutaneous blubber outside the muscular abdominal wall, lateral to the base of the penis and are oblong, with length being three to four times the diameter. The length of adult male non-spermatogenic testes ranges from 135-240 mm with a mean of 188 mm (Fay 1982). Fay (1982) examined 181 specimens and Born (2003) examined 171 specimens, and found that incomplete spermiogenesis may begin as early as five to six years old, but complete spermatogenic cycles ending in production of spermatozoa seem not to occur until the animals are seven to eight years old. Most males are potent by the time they are 10 years old. The principal period of breeding is approximately two to three months long and coincides with peak testicular size (Fay 1984). Adult males (>15 years) were most often fertile (spermic) from November to February. Younger adults (12-16 years) were spermic less often and over a longer period and subadults (5-11years) were least often spermic and their highest fertility occurred about two months later than for mature males. The cycle in testis size suggests a peak in development in December or January for the older males and in February and March for the younger individuals (Born 2003).

Annual Development and Regression of Testes

Spermatogenesis in pinnipeds, walrus included, generally follows normal mammalian patterns

(Boyd 1991) and can require up to three months (Setchell 1978). In the majority of seasonal breeders during the breeding season, the testes undergo the following: recrudescence, maximum testosterone production and spermatogenesis. During the non-breeding season, the testes regress, testosterone concentrations decline and testes are aspermic (Frick *et al*. 1977, Gardiner *et al*. 1999). In wild male walrus, there is a profound annual cycle of testicular recrudescence and regression. Fay (1982) recognized three stages of the annual testis cycle of the walrus: recrudescence, spermiogenesis and retrogression. Recrudesence is the prolonged, initial part of spermatogenesis, when the spermatagonia undergo premeiotic divisions being eventually transformed to spermatids. Spermiogenesis is the final part of spermatogenesis, beginning with spermatids and ending with the last release of spermatozoa. The last part is retrogression which is a period of testicular quiescence. Retrogression begins when the secondary germ cells degenerate, leaving only the spermatagonia and Sertoli cells. In a single *ex situ* walrus, spermatogenesis was induced using an exogenous hormone (human chorionic gonadotropin - hCG) (Muraco *et al*. 2012). Morphologically intact sperm were first observed 27 days after initiation of treatment, however, sperm concentrations remained low until day 56. In walrus, during spermiogenesis, the testes increase in weight by about 40% and length by about 15% (Fay 1982). This increase in size is due to the increased seminiferous tubule dimension and the increase in size of the epidydmis and the Leydig cells (de Krester 1984). Typically, the season of fertility in male walrus seems to be somewhat longer than the seasonal period of estrus in females and some extra-seasonal ovulations apparently result in a number of successful pregnancies as evidenced by records of newborns (Born 2001, 2003).

Endocrinology

In mammals, the production of normal numbers of spermatozoa (spermatogenesis) is governed by endocrine regulation of the testis via the hypothalamic-pituitary-gonadal axis (HPG) (de Kretser 1984) (Figure 5). Spermatozoa production relies on the interaction of GnRH from the hypothalamus, FSH and LH from the pituitary gland and reproductive steroid secretion from the gonads. The discharge of GnRH from the hypothalamus occurs in frequent, intermittent bursts during the breeding season. These bursts last just a few minutes and cause the discharge of LH and FSH. FSH promotes spermatogenesis via the Sertoli cells in the testes while LH acts on the Leydig cells to stimulate testosterone production. Increased testosterone and inhibin (secreted from the Sertoli cells) in the blood eventually exert a negative feedback on the production of GnRH in the hypothalamus, therefore reducing levels of LH and FSH (Karsch 1984). Two aspects of LH production are important: the frequency of the pulses and the amount of LH released in each pulse (de Kretser 1984, Karsch 1984). When secretion rate is maximal, both the frequency and amplitude of pulses are at their greatest. When secretion rate is minimal, the frequency and amplitude are minimal. It is this change in LH production throughout the year that is primarily responsible for regulating seasonal testosterone production. A species' physiological responsiveness to LH changes seasonally. In seasonally breeding animals near the breeding season, a small amount of LH has greater stimulatory effects on testosterone than the same amount would produce during the non-breeding season (de Kretser 1984, Karsch 1984).

Testosterone is responsible for spermatogenesis and for stimulating the aggressive behavior necessary for establishment and maintenance of territories (Nelson *et al*. 1990). Concentrations of testosterone can therefore be used to evaluate the onset of the breeding season (Noonan *et al*.

1991, Atkinson and Gilmartin 1992, Bartsh *et al*. 1992, Gardiner *et al*. 1999). The information on testosterone concentrations in pinnipeds is from studies on phocids and otariids, with the exception of one peer-reviewed walrus article. There is little reason to believe that such patterns are significantly different in walrus.

Annual Changes in Body Mass

In many mammal species weight gain in both *in situ* and *ex situ* males, given adequate access to increased food rations, signifies they are ready to breed. This has been termed the "fatted" male phenomenon (DuMond and Hutchison 1967). Large size at the beginning of the breeding season not only improves fighting ability but also permits males to fast for protracted periods and, therefore, hold territories longer (Boyd and Duck 1991, Lidgard *et al*. 2003). Territorial holding behavior is fueled by using energy stored in the form of blubber that is accumulated prior to the start of the mating period (Anderson *et al*. 1975, Boness and James 1979). Males that are largest at the beginning of the breeding season have the greatest apparent reproductive success (Anderson and Fedak 1985, Walker and Bowen 1993, Deustch *et al.* 1990, Galimberti *et al.* 2007). In pinnipeds, male-biased sexual dimorphism in size is evident. This pattern is much more likely to have evolved if breeding receptivity in females is strongly seasonal, and if, during the breeding season, receptive females are aggregated in space (Bartholomew 1970).

During the breeding season, males typically do not consume water, and either fast or feed at reduced rates for as long as two to three months. A male that leaves his "breeding station" to feed may provide opportunities for preemptive activities by other males, resulting in loss of high-ranking status in prevailing dominance hierarchies, thereby potentially losing opportunities for mating and fertilization. Accumulating energy reserves prior to rut (a recurrent period of mammalian reproductive activity) is paramount to male breeding success in many pinniped species. Prime examples are the two species of elephant seal (northern, *Mirounga angustirostris*; southern, *M. leonina*; Deustch *et al.* 1990, Galimberti *et al.* 2007). During the pre-breeding season, bulls undertake a period of intensive foraging and energy acquisition (hyperphagia). This is followed by fasting and lack of water consumption during the breeding season, leaving the bulls weakened by the end, losing the majority of their blubber reserves and up to 25.8% of their pre-breeding body mass (Galimberti *et al*. 2007).

Many pinniped species exhibit depressed metabolic rates to conserve energy stores during periods of fasting (Castellini and Rea 1992, Markussen *et al.* 1992, Rea and Costa 1992, Worthy *et al*. 1992, Nordøy *et al*. 1993, Rosen and Renouf 1998, Kumagai *et al*. 2006). This is thought to help minimize energy output and conserve energy stores during periods of low food intake (Keys *et al*. 1950). High rates of energy expenditure can be maintained despite hypophagia by using seasonal blubber deposits as an energy reserve. Resting metabolic rate can be depressed during the pre-breeding period, despite extreme hyperphagia (Rosen and Renouf 1998). This period of increased energy intake is associated with increased body mass in preparation for the demands of the breeding period (Rosen and Renouf 1998). Since walrus are seasonal breeders, there is little reason to believe that the patterns of male walrus would be significantly different from other species that undergo the “fatted” male phenomenon.

Female Reproductive Biology

General

The first estimates of ovulation rates and other reproductive patterns in female walrus stemmed from observations of follicles, corpora lutea and corpora albicans in the ovaries of harvested individuals (Mansfield 1958, Fay 1982). Most female walrus reach sexual maturity at about 10 years of age (Fay 1982). Using 205 specimens, Fay (1982) suggested that at least 10% of females ovulated for the first time at four years of age; about 68% ovulated at least once by the time they were six years old and 100% were fertile by the age of 10 years. These findings were similar to Atlantic walrus that experience first ovulation at about 6.1 years of age on average (Born 2001). Reproductive senescence in female walrus may occur by age 30 (Fay *et al*. 1997).

Based on 205 specimens, Fay (1982) discovered several anatomical characteristics regarding reproductive anatomy of female walrus. The reproductive tract lies in the posterior third of the abdomen between the colon and the bladder. The urogenital vestibule is 15-20 cm in length. The vaginal canal is 20-25 cm long and about 6 cm in diameter with walls that are 2 cm thick. The vagina is partially divided by a median dorsal fold that extends 3-5 cm leading to the separate orifices of the didelphic (double uterus and two separate cervices) uterus. The cervices and corpora uteri are 10-15 cm long and uterine horns are about 25 cm long and 4 cm in diameter. Adjacent to the end of each uterine horn are the ovaries. The ovaries of adults are 5-8 cm long and 2-4 cm in diameter, being essentially oval and smooth. The partly septate vagina and wholly didelphic uterus are unique among pinnipeds (Fay 1982).

Female walrus have a synchronous, triennial calving and reproductive cycle (Garlich-Miller and Stewart 1999, Born 2001), biennial ovulation (unless they did not conceive the year before) and are believed to be functionally seasonally monoestrous (Fay 1982). Fay (1982) reported a phase of postpartum follicular activity which peaked in July and August. However, he found that this follicular activity did not appear to result in a postpartum ovulation. About six months later, females undergo a lactational estrus when males are actively producing sperm and fertilization can occur (Fay *et al.* 1984). Non-pregnant wild female walrus may go into estrus sometime between December and June but most ovulate in February (Fay 1982, Fay *et al*. 1984). Females in estrus will choose a displaying male and join him in the water where mating will take place (Sjare and Stirling 1996). In many pinnipeds, if a female fails to conceive after a successful mating, a period of pseudopregnancy (a condition resembling pregnancy, marked by the persistence of the corpus luteum [CL]) may occur in which the CL produces progesterone for a length of time equivalent to or longer than embryonic diapause (Reijnders 1990, Working Group 7 1993, Renouf *et al.* 1994, Greig *et al.* 2007). Gestation for the walrus lasts 15 to 16 months, including a period of delayed implantation lasting four to five months (Fay 1982). Most births occur in mid-April to mid-June. Ovulation may be suppressed until the calf is weaned, raising the birth interval to three years or more (Garlich-Miller and Stewart 1999). The lowest rates of successful conception and gestation (40-60%) tended to occur in the youngest and oldest age classes of fertile females while the highest rates (>80%) occurred between the ages of eight and 15 years (Born 2001). Data suggest that failure to conceive may be greater than 10% per year. After conception, about 5% of the implanted embryos failed to result in live births. Birth rates may be density dependent (i.e. inverse relationship between population size and per capita reproductive rate) (Fay *et al.* 1989, Fay *et al.* 1997, Garlich-Miller *et al.* 2006).

Endocrinology

In mammals, the female reproductive cycle is governed by endocrine regulation of the ovaries via the HPG axis (Karsch 1984, Short 1984) (Figure 6). This cycle could not occur without adequate secretion of GnRH from the hypothalamus, FSH and LH secretion from the pituitary gland and gonadal secretion of steroids. The estrous cycle consists of a follicular phase and a luteal phase. The discharge of GnRH causes the anterior pituitary gland to release FSH which promotes follicular growth in the ovary. The follicular phase is dominated by the hormone estradiol from ovarian follicles. Estradiol initiates sexual receptivity and behavior (Nelson *et al*. 1990). After estrogens reach a threshold level, a discharge of high amplitude, high frequency pulses of GnRH occurs (Karsch 1984, Short 1984). This event causes the discharge of LH which in turn causes ovulation. The luteal phase begins with ovulation and the formation of the CL. The CL, a transient endocrine gland, is formed at the site of the recently ruptured follicle and primarily secretes progesterone, which is essential for the establishment and maintenance of pregnancy. LH is responsible for stimulating the CL to produce progesterone. Mating behavior often ceases with the onset of corpus luteum activity (Nelson *et al*. 1990). The luteal phase ends with CL regression.

Ovarian Cycle

During the follicular phase of the estrous cycle, walrus ovaries contain growing follicles ranging in size between 1-28 mm (Fay 1982). Estrus occurs nine months post-partum, during lactation and the winter breeding season. Healthy walrus CLs range in size from 17-50 mm (Fay 1982). Successive ovulations occur more often from alternate ovaries than from the same one, indicating that follicular development is usually suppressed for a year or more in the ovary that contained the CL of the previous pregnancy (Garlich-Miller and Stewart 1999, Born 2001). The ability to ovulate in successive years appears usually to be the result of unsuccessful fertilization, implantation or gestation the year before. The numbers of corpora lutea in walrus ovaries are not reliable indices of fecundity as they do not persist indefinitely (Mansfield 1958, Krylov 1966, Fay 1982). Regression of the corpora lutea in the walrus occurs many months after implantation.

Delayed Implantation

Gestation in pinnipeds occurs in two stages: delayed implantation and active fetal growth (Boshier 1981, Daniel 1981). Delayed implantation or embryonic diapause was recognized in pinnipeds as early as 1940 (Harrison 1968). All female pinnipeds undergo this obligatory seasonal delay, in which the ovum is fertilized but remains dormant for a period of time (Boshier 1981, Renfree and Calaby 1981, Sandell 1990). Delayed implantation is an adaptation that allows mating and parturition to occur in optimal times of the year. The optimal time for mating is when the animals are in their prime condition, whereas parturition should be timed so that rearing coincides with environmental conditions favorable to neonate survival and the condition of the lactating mother (Sandell 1990, Lopes *et al.* 2004). Following fertilization, a period of delayed implantation occurs when the fertilized egg divides into a ball of undifferentiated cells called a blastocyst. The blastocyst stops growing and remains free-floating in the uterus for four to five months (Fay 1982). After reactivation, the blastocyst implants in the uterine wall and active gestation begins. Fetal development ensues for 10 to 12 months. The time of implantation is likely late June to late July (Fay 1982). The fetus develops in the uterine horn adjacent to the ovary with the CL.

Pregnancy/Parturition

The CL of walrus pregnancy generally makes up ⅓ to ½ of the ovary (Fay 1982). It is unknown if the CL persists for the entire pregnancy. In a longitudinal study of two pregnant *ex situ* walrus, progesterone levels decreased prior to implantation and rose again at the time of implantation (Siembieda 2005).

The birthing range, as indicated by data, is about 55 days from April 15th to at least June 8th (Fay 1982). Most births take place on ice during the female's spring migration from mid-April to mid-June but calves are born occasionally in other months of the year (Fay 1982). The placenta, which weighs about 7 kg, is expelled soon after birth and is usually consumed by scavengers. A short piece of the umbilical cord typically remains attached to the calf.

Walrus cows with calves tend to gather in herds separate from the bulls and other females. The nursery herds normally include 20-50 individuals with maximum numbers approaching 200 (Fay 1982). Mothers and newborn calves stay on ice floes until calves develop sufficient energy reserves for thermoregulation. The social bond between the cow and calf is very strong and can take many forms. Cows may shelter calves under the chest between fore-flippers or protect calves by herding them first into the water before entering themselves (Burns 1965). Calves may also ride on the mothers' backs in the water. Young walrus are believed to be dependent entirely on the cow's milk (~30% fat) for at least the first six months after birth and are gradually weaned during the second year (Fay 1982). Some calves may nurse longer if not supplanted by a new calf. Nursing takes place both aquatically and terrestrially. Estimated survival rates through the first year of life range from 50-90% (Fay *et al.* 1997). After weaning, young males may stay in the same herd as the cow for an additional two to three years before joining an all-male herd, while young females tend to stay with the adult females continuously (Fay 1982).

Pseudopregnancy

Pseudopregnancy is defined as a prolonged period of high concentrations of progesterone in the absence of a fetus (Renouf *et al*. 1994). The purposes of pseudopregnancies in both proximate and evolutionary contexts are unknown. During pseudopregnancy, progesterone concentrations remain high for at least the normal time span from conception to implantation (Yoshida *et al*. 1977, Reijnders 1990, Renouf *et al*. 1994) and possibly longer (Noonan 1989). Although the process of ovulation and CL formation occurs across pinniped species, the functional life of the CL in non-pregnant pinnipeds seems to vary between species. The occurrence of pseudopregnancy has been observed several times in the walrus (Kinoshita *et al.* 2012, Muraco *et al*. 2012). High circulating levels of serum progesterone have been recorded for periods up to 9 months in *ex situ*, non-pregnant walrus (Kinoshita *et al.* 2012, Muraco *et al*. 2012). Fay (1982) found that the CL of pseudopregnancy resembles the CL of pregnancy, except that it is no more than half as large and shows retrogressive signs that are greatly out of phase with the CL in the ovaries of pregnant females.

Seasonalit and Photoperiod

Seasonal breeding is an important strategy for maximizing reproductive success. There is an optimal time to give birth for each species, determined largely by the time of maximum resources available for weaned calves and lactating mothers. Animals are dependent on environmental cues, often called proximate factors, to tell them when to begin the reproductive process (Short 1984). The principal proximate factor thought to control reproductive cycles in pinnipeds is photoperiod (Temte 1985, Boyd 1991, 1996). Photoperiodism is the ability of organisms to assess and use the length of day as an anticipatory cue to time seasonal events (Bradshaw and Holzapfel 2007).

It is important that males and females respond appropriately to the same factors so they can reach reproductive maturity and viability simultaneously each year. Both males and females must begin their reproductive development (folliculogenesis and spermatogenesis) in advance of the breeding season because both of these processes require significant time in advance of copulation. The requisite time in anticipation of copulation varies by species (Setchell 1978).

In seasonally breeding mammals, a circannual endogenous rhythm is likely to generate periods of sexual activity alternating with periods of sexual quiescence throughout the year (Short 1984). The specific roles of short days and long days in timing reproductive activity vary among species. Short photoperiods have been shown to stimulate, and long photoperiods inhibit, testicular size, spermatogenesis and the occurrence of estrus in species ordinarily breeding in the winter during short days (i.e. sheep, goats, roe deer) (Lincoln and Short 1980, Short 1984). The opposite effects of photoperiod have been observed for long day breeders (i.e. golden hamsters, horses) (Palmer *et al*. 1982, Short 1984).

In mammals, the pineal gland is responsible for the neuroendocrine communication of photoperiod to the rest of the body via melatonin (Hansen 1985, Ebling and Barret 2008, Morgan and Hazlerigg 2008). Species vary in their response to photoperiod because responses of target organs to a given pattern of melatonin differ. Photoperiodic control of gonadal function is probably largely a result of changes in gonadotropin secretion. Light exposure can regulate gonadotropin secretion by altering responsiveness of the HPG axis to negative feedback actions of gonadal steroids. The major photoreceptor in all mammals studied is the eye (Dixit *et al.* 1977, Gravis 1978). The photic signals are received by the retina and transmitted to the pineal gland via the suprachiasmatic nucleus in the hypothalamus. The effect of the pineal gland on reproductive processes depends on both the prevailing photoperiod and the species. In both short and long day breeders, exposure to inhibitory photoperiods causes a decline in pituitary blood levels of LH and FSH, while exposure to stimulatory day lengths causes opposite effects (Lincoln and Short 1980, Short 1984).

There are apparent effects of photoperiod on the synchronization of pinniped reproductive cycles (Daniel 1981). Evidence for photoperiodic influence on reproduction has been found in the Cape fur seal (*Arctocephalus pusillus*; Stewardson *et al.* 1998), southern elephant seal (Griffiths 1984a), Northern fur seal (*Callorhinus ursinus*; Temte 1985), California sea lion (Temte 1993, Temte and Temte 1993) and harbor seal (Bigg and Fisher 1975, Temte 1991, 1993, 1994). A clear example of the variation in the reproductive cycle with a change in latitude is the harbor seal (long day breeder) of the North American Pacific coast, from Baja California, Mexico to Vancouver Island, British Columbia, Canada (Temte 1991, 1993, 1994). In this case, the pupping season occurs as early as March in Mexico and as late as September in Canada (Temte *et al.* 1991). In addition, the timing of estrus in *ex situ* harbor seals can be experimentally altered by the manipulation of photoperiod (Bigg and Fisher 1975, Temte 1994). Short day lengths delay and long day lengths advance the timing of estrus. Sea surface temperature (Coulson

1981) and nutritional conditions (Stewart *et al*. 1989, Temte 1994, Boyd 1996, Pitcher *et al*. 2001) may also contribute to the reproductive synchrony of pinnipeds.

Evidence against photoperiodic influence on reproduction in pinnipeds is limited. Spotte and Adams (1981) demonstrated that photoperiod could be altered substantially from that of the natural range without affecting the timing of reproductive events in Northern fur seals. The authors suggest that the importance of photoperiod on reproduction via the pineal gland may have been overemphasized in the past.

Measurement of Hormones

Analysis of hormones to evaluate the reproductive status of individuals has become a valuable tool for animal management. In the last several decades, saliva has become a widely accepted sample source for the analysis of human steroids. Specifically, saliva has shown good correlation with unbound steroid hormones in serum (Riad-Fahmy *et al*. 1987, Rilling *et al*. 1996, Ostatnikova *et al.* 2002, Groschl 2008). Because of its success in association with various marine mammal species, salivary steroid monitoring is likely to be a useful tool for tracking reproductive hormone levels in walrus.

In humans, most saliva originates from three pairs of salivary glands – parotid (by the ear; serous fluid), submandibularis (under the mandible; sero-mucous secrete) and sublingualis (under the tongue; mucous saliva) (Aps and Martens 2005). However, these glands can vary between different mammalian species in number, size and location.

Progesterone, estrogen and testosterone exists in human serum in three states – free, albumin-bound and sex hormone binding globulin (SHBG)-bound (Westphal 1971). Passive diffusion through the salivary gland epithelium is the primary entry pathway of the steroid into the saliva (Quissell 1993). Steroid hormones, which are not bound strongly to serum proteins such as albumin or SHBG, are relatively small in size, and most are fatty, non-polar compounds, so they tend to pass relatively easily by diffusion through the lipid bi-layer of the cell membrane to enter cells (Rosner 1990, Petra 1991).

Albumin and SHBG do not allow the bound fraction of the hormones to get into saliva due to their molecular weights. Therefore, salivary steroids are thought to reflect the concentration of unbound serum steroids, or the serum free fraction. Thus, salivary concentration represents a valuable marker for the free, unbound and bioactive fraction of the particular steroid (Ostatnikova *et al*. 2002, Arregger *et al.* 2007). Since it is the free concentration of the hormone in the plasma that dissociates and passes through the salivary gland during saliva formation (Quissell 1993), the concentrations of steroids in saliva are only a fraction of those found in the blood. Saliva/plasma ratios of reproductive hormones have been reported to range between 2-10% of the total plasma concentration (Riad-Fahmy *et al*. 1982). Immunoassays have been widely used for analyzing salivary hormones because they are relatively simple to use, require small sample volumes (≤100 uL), and are analytically sensitive (Groschl 2008).

Because of demonstrated successful application to hormonal studies in other marine mammal species, salivary steroid monitoring is likely to be a useful tool for tracking walrus reproductive hormones and events. Longitudinal monitoring will allow the definition of endocrine patterns of estrous cycles and rut as well as pseudopregnancy through the measurement of reproductive hormones.

Rationale/Objectives

The U.S. *ex situ* walrus population has met with limited reproductive success with only seven calves born in the past 10 years, and of these animals only one has survived to adulthood. This presents a problem for the long-term maintenance of the population as stranded animals are identified only sporadically and the capture of wild walrus is politically and logistically difficult. With a limited number of breeding-aged animals, the long-term existence of the *ex situ* population is at risk. Upon recommendation by the walrus AZA Red Program, research into the reproductive physiology of this species has been initiated to move closer to successful breeding. In-depth knowledge of the basic reproductive endocrinology of *ex situ* walrus is limited to results from only a few published studies (Siembieda 2005, Kinoshita *et al*. 2012, Muraco *et al*. 2012).

In response to concerns from the *ex situ* walrus community, the Point Defiance Zoo & Aquarium (PDZA) proposed to conduct collaborative, non-invasive endocrine studies that would not only increase the knowledge regarding the basic reproductive physiology of both male and female walrus but potentially elucidate problems regarding reproductive success. Previous studies have shown that long-term evaluation of reproductive steroids in saliva is effective for evaluating ovarian and testicular activity in marine mammals. Compared to blood sampling, saliva monitoring permits longitudinal, non-invasive studies of steroid excretion and allows for a more complete description of reproductive events for a given species. Baseline data collected from the study will assist in elucidating the reproductive mechanisms in walrus and could be used to improve reproductive management of the species through natural or assisted breeding.

The research for this thesis has several main objectives, all of which relate to the primary goal of defining endocrine and behavioral parameters of reproduction in U.S. *ex situ* Pacific walrus.

1. Develop a non-invasive technique for monitoring ovarian and testicular steroidogenic activity in walrus by validating a protocol to measure progesterone, etradiol-17β and testosterone by enzyme immunoassay;
2. Establish a database of endocrine norms for U.S. male and female walrus;
3. Define endocrine patterns of estrous cycles (females) and rut (male reproductive season) through the longitudinal measurement of estrogen, progesterone and testosterone in the saliva of walrus;
4. Determine seasonal changes in testicular size;
5. Determine seasonal presence and absence of spermatozoa in the male PDZA walrus;
6. Define behavioral changes in male and female walrus associated with seasonal hormonal fluctuations;
7. Evaluate seasonal weight loss and gain in the male PDZA walrus;
8. Evaluate the effect of seasonality and photoperiod on walrus.

There is a need to develop strategies to accurately assess the reproductive parameters of *ex situ* walrus. The determination of reproductive status of individuals is essential to successful zoo-based breeding programs, and efforts to use assisted reproductive techniques depend on the knowledge of the basic reproductive physiology of the species. Each of the above goals has great potential as valuable tools towards the management of the U.S. walrus population and would improve the reproductive success for the species.

Because one or more of the main chapters of this thesis may be submitted for publication separate from the other chapters, some repetition of material among chapters within the thesis is necessary.

Table 1. History of facilities housing walrus in the United States

Facility	Location	Year Began	Intervening Years	Year Ended	Births	Year of "Birth"	# Calves
San Diego Zoo	San Diego, CA	1932	1933, 1951-1952, 1960-1961, 1969-1972	1972	no	NA	0
Brookfield Zoo	Chicago, Illinois	1937	1938, 1940-1941, 1962-1988, 1991-2006	2006	yes	1999, 2002	2
Smithsonian Natl Zool Park	WA, DC	1938	1938	1938	no	NA	0
Marineland of the Pacific	Palos Verdes, CA	1961	All	1987	yes	1971, 1975,1978, 1978, 1981, 1982, 1985	8
St. Louis Zoo	Saint Louis, MO	1961	All	1976	no	NA	0
Sea World	4 locations*	1978	All	present	yes	1987, 1993, 2003, 2005, 2006	6
Cincinnati Zoo	Cincinnati, OH	1978	All	2000	yes	1988, 1989, 1991	3
Point Defiance Zoo & Aq	Tacoma, WA	1982	All	present	no	NA	0
New York Aquarium	Brooklyn, NY	1956	All	present	yes	2007	1
Six Flags Discovery Kingdom	Vallejo, CA	1994	All	present	yes	2011	1
Indianapolis Zoo	Indianapolis, IN	1995	All	present	no	NA	0
Mystic Aquarium	Mystic, CN	2001	All	2002	no	NA	0
*San Diego, CA; Orlando, FL; Aurora, OH; San Antonio, TX							21

Table 2. History of walrus calves “born” in the United States

Year of Birth	**Month of Birth**	**Facility**	**Location**	**Gender**	**Survival**	**Comments**
1971	Dec	Marineland of the Pacific	Palos Verdes, CA	M	aborted	8 kg
1975	May	Marineland of the Pacific	Palos Verdes, CA	F	> 1 year	
1978	June	Marineland of the Pacific	Palos Verdes, CA	M	adulthood	died at 21 years of age
1978	July	Marineland of the Pacific	Palos Verdes, CA	F	adulthood	died at 19 years of age
1981	June	Marineland of the Pacific	Palos Verdes, CA	M	same day	
1982	May	Marineland of the Pacific	Palos Verdes, CA	F	~ 1 month	
1985	March	Marineland of the Pacific	Palos Verdes, CA	unknown	full term	twin; dam died from a uterine torsion
1985	March	Marineland of the Pacific	Palos Verdes, CA	unknown	full term	twin; dam died from a uterine torsion
1987	June	SeaWorld San Diego	San Diego, CA	F	adulthood	still alive
1988	May	Cincinnati Zoo	Cincinnati, Ohio	F	same day	
1989	August	Cincinnati Zoo	Cincinnati, Ohio	unknown	same day	
1991	May	Cincinnati Zoo	Cincinnati, Ohio	F	~ 2 months	born rear flippers first
1993	June	SeaWorld San Diego	San Diego, CA	M	adulthood	still alive
1999	May	Brookfield Zoo	Chicago, IL	M		died during delivery
2002	April	Brookfield Zoo	Chicago, IL	F	~ 2 days	
2003	May	SeaWorld San Diego	San Diego, CA	F	adulthood	still alive
2005	April	SeaWorld San Diego	San Diego, CA	M	~ 2 weeks	
2006	March	SeaWorld San Diego	San Diego, CA	F	stillborn	Twin
2006	March	SeaWorld San Diego	San Diego, CA	F	~ 2 days	Twin
2007	June	New York Aquarium	Brooklyn, NY	M	~ 2 years	
2011	May	Six Flags Discovery Kingdom	Vallejo, CA	M	stillborn	

Information in the table is based on Genrich 1984, Oland 2012 and personal communication.

Figure 1. Range map of wild walrus populations.

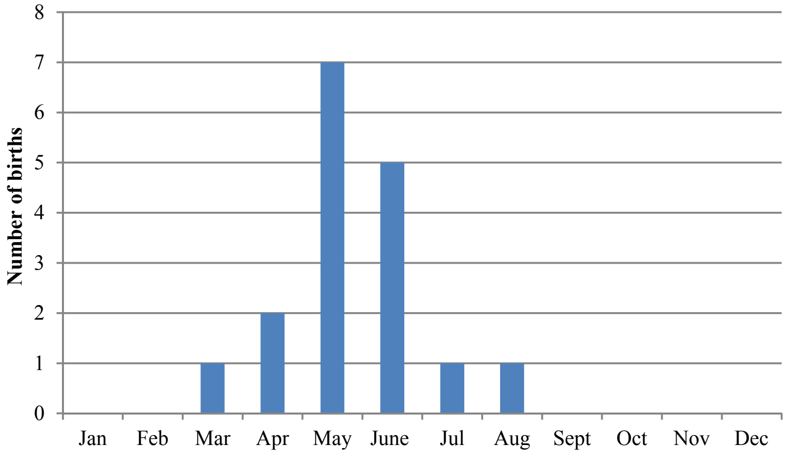


Figure 2. Number of walrus births per month for U.S. *ex situ* population (Genrich 1984, Oland 2012 and personal communication).

Figure 3. Breeding locations of Pacific walrus: south of Nunivak Island, southwest of St Lawrence Island and south of the Chukotka Peninsula in the Gulf of Anadyr.

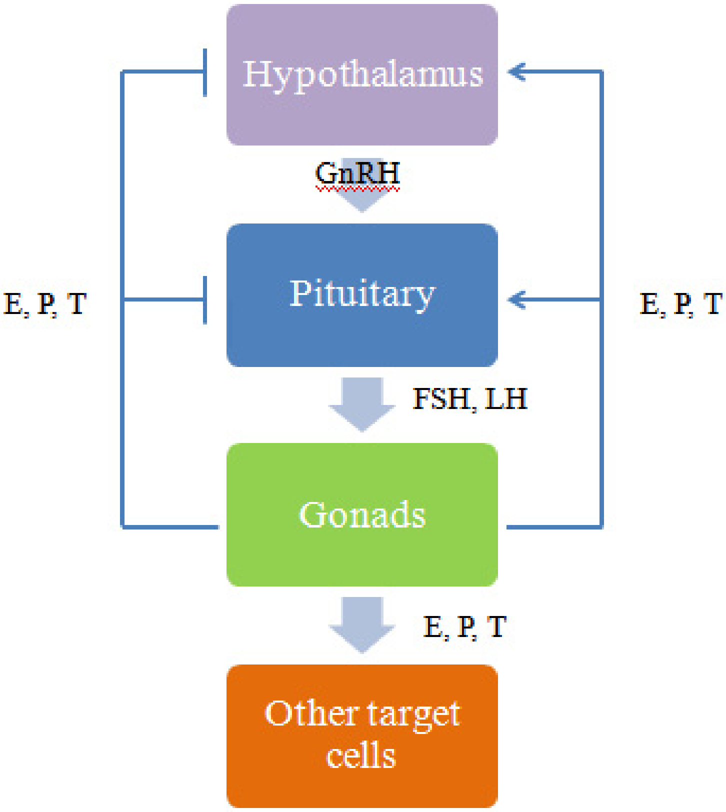


Figure 4. The general hypothalamic-pituitary-gonadal axis as an example of a complex endocrine control system. Arrows indicate stimulation (+ feedback) and blunt-ended lines represent inhibition (– feedback). GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone ; LH, luteinizing hormone; E, estrogens; P, progesterone; T, testosterone.

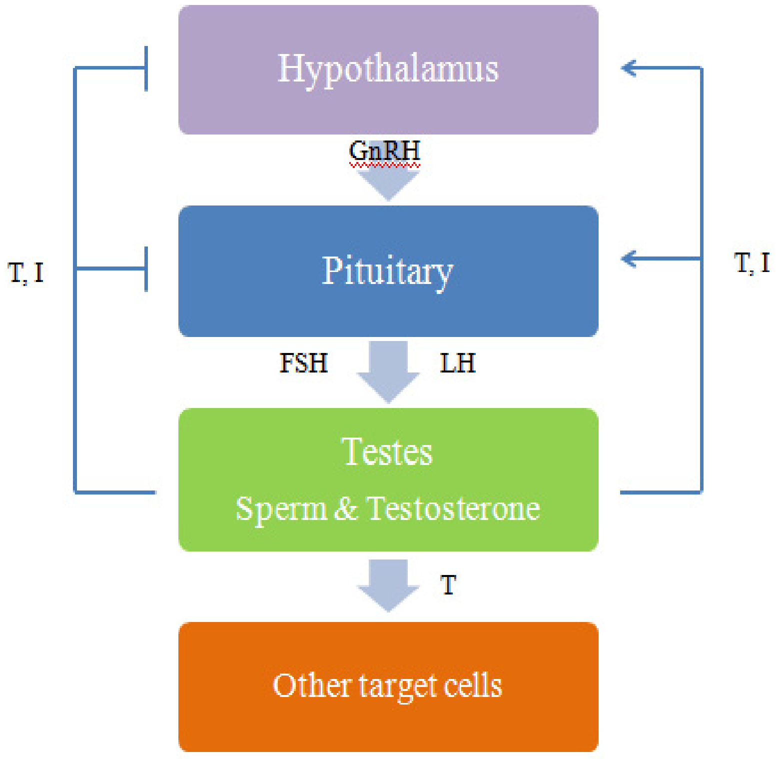


Figure 5. The general male hypothalamic-pituitary-gonadal axis as an example of a complex endocrine control system. Arrows indicate stimulation (+ feedback) and blunt-ended lines represent inhibition (- feedback). GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone; I, inhibin.

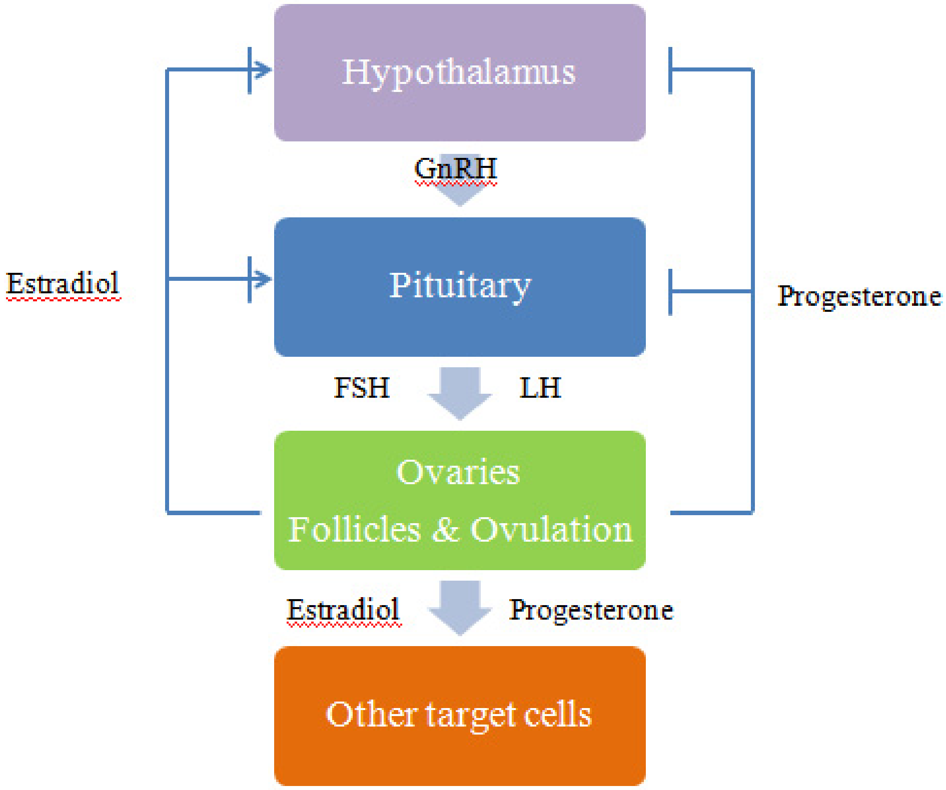


Figure 6. The general female hypothalamic-pituitary-gonadal axis as an example of a complex endocrine control system. Arrows indicate stimulation (+ feedback) and blunt-ended lines represent inhibition (- feedback). GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

Parameters of Reproduction in Male Odobenids

Introduction

Since the 1930s, walrus have been maintained in U.S. zoos and aquaria with limited reproductive success, with 18 walrus calves born of which only six have lived past a year of age (Genrich 1984, Oland 2012). As a result, the *ex situ* population is in a demographic crisis (Oland and Marti 2011). Accurate information about the reproductive biology of a species is necessary for the effective management of animals in zoos and aquaria. Determination of the reproductive status is one of the most important factors for effective management, and efforts to use assisted reproductive techniques depend on the knowledge of a species' basic reproductive physiology (Lasley and Kirkpatrick 1991, Wildt *et al*. 2001, Graham 2004).

Hormones are involved in all aspects of reproduction and defining normal endocrine patterns associated with reproduction is an important first step in characterizing the reproductive biology of any species. Due to the fact that male sperm cell production and hormonal secretion are less tightly coupled than those of female oocyte maturation (Lasley and Kirkpatrick 1991), there are fewer reports describing non-invasive reproductive hormone monitoring in males. The walrus is no exception. Particularly lacking in the zoo and aquaria walrus population is a comprehensive understanding of the endocrine patterns of the male reproductive cycle, the interaction between testosterone concentrations and spermatogenesis, seasonal testicular recrudescence and regression, photoperiodic effects on reproduction cycles and male reproductive senescence.

The overall goal of the study is to non-invasively define endocrine and behavioral parameters of reproduction in walrus with the following specific objectives: 1) develop a non-invasive technique for monitoring testicular steroidogenic activity; 2) validate a protocol to measure testosterone by enzyme immunoassay; 3) establish a database of endocrine norms for male walrus in U.S. facilities; 4) define the endocrine patterns of rut (male reproductive season) through the longitudinal measurement of salivary testosterone; 5) determine the seasonal changes in testicular size; 6) determine the seasonal presence and absence of spermatozoa; 7) define behavioral changes in males associated with hormonal fluctuations; 8) evaluate seasonal changes in body mass; and 9) evaluate the effect of seasonality and photoperiod on reproduction. The knowledge gained has great potential in guiding development of valuable tools towards the management of the zoo based U.S. walrus population and would improve the reproductive success for the species.

Materials and Methods

Subjects

Study animals consisted of six male Pacific walrus (ages 6-28; Table 3). Prior to the study, two of the males (NYA and SWSD) had sired offspring. Study animals were trained to allow voluntary saliva collection and testicular measurements. All animals were housed outdoors with exposure to natural fluctuations in photoperiod. PDZA, NYA and SWSD maintain natural sea water systems, while SFDK and IZ produce salt water. Water temperatures in the various facilities ranged from 7° - 21°C (Table 3). All five facilities fed a diet of capelin (*Mallotus villosus*), herring (Pacific – *Clupea pallasii* or Atlantic – *Clupea harengus*), and various clam species. In addition, three of the five facilities fed squid (*Loligo opalescens;* PDZA, NYA, IZ), while one facility supplemented with Pacific mackerel (*Scomber japonicas;* PDZA) and another facility added sardines (*Sardinops sagax;* SWSD).

Saliva Collection

Salivary samples were collected from male walrus for periods ranging from 6 to 37 months. Saliva sampling occurred at least weekly from each animal during the period 2007-2010. Prior to the first feed (between 8:00 and 10:30 am local time), saliva was collected from the animals' mouths using three sites: under the tongue, base of the tongue and the sides of the mouth. The cotton-tipped applicators were then placed in 5 ml sealed cryotubes and frozen at a minimum of -20°C until analyzed. All males were maintained in a "breeding situation" (mixed-gender conditions) throughout the study period.

Serum Collection

Serum samples were collected from the PDZA male walrus opportunistically from October 2007 to October 2010. Blood was obtained from the epidural intravertebral sinus using a 16 gauge, 5.5 inch (14 cm) spinal needle or the proximal rear flipper, from a branch of the lateral saphenous vein, using a 21 gauge, 1.5 inch (3.8 cm) needle, and transferred to a tube with serum separator gel. Blood was centrifuged at 3,500 rpm for 5 minutes and the resulting sera poured into a cryotube and stored at a maximum of -20 °C until analyzed.

Assay Procedures

Sample Extraction and Dilution

Reproductive steroid hormones were extracted from saliva samples prior to analysis to concentrate the hormones. For extractions, one ml of 90% methanol was added to each saliva tube. Samples were then placed on a microplate shaker for one hour and subsequently air dried with a drying manifold to remove methanol. Each sample was reconstituted by adding 0.5 ml of assay buffer into the cryotube. A sample was refrigerated at 4°C if it was to be assayed within the next few days, or if not, stored in a freezer at a maximum of -20°C until analyzed.

All samples were assayed in duplicate and estimates of raw hormone concentrations were generated by a Tecan Sunrise microplate reader at a wavelength of 405 nm (Tecan Group Limited, Männedorf, Switzerland) utilizing a four parameter logistic curve fitting program (Tecan Sunrise, MagellanCE V 3.0). The final hormone concentrations were calculated as: (raw hormone concentration) x (ml assay buffer used to reconstitute sample) x (dilution factor). All salivary data were expressed on a per gram wet weight basis (ng/ml).

Testosterone Assay

Saliva samples were analyzed utilizing testosterone competitive enzyme immunoassay (EIA) kits (Enzo Life Sciences International, Inc., [previously Assay Designs, Inc.] Farmingdale, New York, USA, ADI-900-065). Cross-reactivity, sensitivity and precision data were provided by Enzo Life Sciences EIA testosterone kits. The testosterone antibody cross reacted 100% with testosterone, 14.64% with 19-hydroxytestosterone, 7.2% with androstendione, 0.72% with dehydroepiandrosterone, 0.40% with estradiol and <0.001% with dihydrotestosterone, estriol, aldosterone, corticosterone, cortisol, cortisone, estrone, progesterone and pregenolone. The sensitivity (the minimum concentration the assay can detect) was 5.67 pg/mL. Intra-assay and inter-assay indices of precision were determined by taking samples containing low, medium and high concentrations of testosterone and running the samples multiple times through the assay. Intra-assay coefficients of variation were 10.8, 10.0 and 7.8%, respectively. Inter-assay coefficients of variation were 14.6, 11.3 and 9.3%, respectively.

Assay Validation

The testosterone EIA used in the study was subjected to validation in walrus by assessing EIA parallelism against the standard curve, direct correlation of testosterone in serum and saliva and the relationship of measured hormone levels to observable anatomical and physiological events.

The appropriateness of the testosterone assay for walrus saliva was tested by determining parallelism of serially diluted samples to the standard curve. Walrus saliva samples were taken before, during and after breeding season to provide material containing a mean value for testosterone. Pooled samples were serially diluted (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) and analyzed (inspection of plots and determination of slopes of linear portions of plots) with EIA kits for testosterone to determine whether the concentration-response relationship for the "unknown" was similar to that for the standard analyte (standard curve). Similarities of relationships were interpreted to indicate that the assay could reliably measure steroids in the sample over the entire range of the assay kit standards.

Endocrine Profiles/Reproductive Patterns

Basal and peak levels of testosterone were determined for each individual by an iterative process, in which the average concentration of all samples was calculated and elevated values were excluded if they exceeded the mean plus two standard deviations (Brown *et al.* 1994, Graham *et* *al*. 2000, Bateman *et al*. 2009). The average was then recalculated and the elimination process repeated until no values exceeded two standard deviations above the mean. The average of the remaining values was considered baseline for the animal while values greater than two times the baseline were considered elevated. The length of an elevated testosterone period was calculated from the initial testosterone increase (i.e. at least two consecutive samples with elevated testosterone) until testosterone levels returned to basal values (for three or more consecutive samples below elevated level) (Pelican *et al*. 2005, Bateman *et al*. 2009). Animals not demonstrating seasonal elevations were considered to indicate a lack of substantial elevation in testosterone for a period of six or more weeks (estimated time period of spermatogenesis; Senger 2003, Muraco *et al*. 2012).

Testicular Measurements

Testicular measurements were obtained from five male walrus on a weekly basis, in conjunction with saliva collection, for periods ranging from 6 to 36 months. Each walrus was trained, via operant conditioning, to lay in dorsal recumbency for voluntary testicular measurements. Measurements were taken using a soft plastic tape measure placed across the animal's scrotum at its widest portion (center) to measure the linear width of each testis. The linear length of each testis was determined by measuring from cranial to caudal (Figure 7).

Elevated levels of testicular size, as indexed by the measurements described above, were determined for each individual by examining the change in growth and regression. The minimum and maximum combined measurements (right linear length + left linear length + right linear width + left linear width) were determined and elevations for each individual were established by taking into account the findings of Fay (1982). Based on Fay's findings, walrus testes increase in length by approximately 15% during spermiogenesis. The elevated level of combined testicular size for each individual was determined by an equation that took the "unknown elevated level of combined testicular size for each individual" ("e"), adding 15% of "e" and setting the equation equal to the individual's maximum combined measurement. The equation was then solved for "e" to establish the individual's combined testicular elevation (i.e. maximum = 74.0; $e + 0.15e = 74$; $e = 64.348$). Seasonal changes in testes size were documented in all participating males. Combined testicular measurements were used since these measurements provided profiles that demonstrated larger changes in measurements than individual lengths or widths. When the data allowed, mean combined testicular measurements for individuals were compared between seasons.

Anatomical Changes versus Reproductive Endocrinology

The process of spermatogenesis in mammals typically takes five to eight weeks (Senger 2003, Muraco *et al*. 2012) from the onset of hormonal changes until the first mature spermatozoa are released in ejaculate. Testosterone levels, in theory, should begin to rise prior to testicular recrudescence. Therefore, correlations of these two variables were measured to determine significance in two ways: without offsetting the data and with an offset of minus six weeks. An example of offset data: a testicular measurement taken in the first week of October would be paired with a salivary testosterone concentration from mid-August. All correlation tests were conducted at a significance level of $p \leq 0.05$.

Spermatozoa

Sperm presence or absence from one male walrus (PDZA) was determined opportunistically for a period of 23 months. The walrus was trained to lie in a lateral position on land, present his penis and allow milking of the penis. A microscope slide was then touched against the urethral opening to acquire three droplets. The slide was examined with a light microscope under 100x and 400x magnification (Nanyang City Srate Optical Instrument Manufactory, Nanyang City, China) for the presence or absence of sperm, and to determine if any sperm observed were motile.

Behavior

Behavioral changes between the breeding and non-breeding seasons for one male walrus (PDZA) were evaluated over six time periods from 2007 to 2009. Behavioral data were collected by instantaneous scan sampling during December-March (breeding period for wild walrus) and June-September (non-breeding period) for a total of 158 and 145 observational hours, respectively. The observation sessions were 20 minutes long, divided into 30 second sample intervals (Appendix 2). Days were broken into four 2-hour time blocks (0730-0930, 1000-1200, 1230-1430, and 1500-1700) with samples taken randomly but equally dispersed among the periods. All observations occurred while the animals were in the main exhibit (Figure 8). Behaviors recorded included: reproductive behavior (male courtship display, pursuit of females, clasping and penetration), general activity, location and vocalizations (Appendix 3). Independent variables were month, within-day time period, season (breeding and non-breeding) and year.

All statistical analyses for behavioral data were conducted using R (R version 2.12.2, The R Foundation for Statistical Computing, Vienna, Austria). Two-way ANOVAs were utilized to test the influence of the independent variables on behavior, determine the main effect of contributions of each independent variable and identify if any significant interaction effects between the independent variables existed. A Tukey post-hoc comparison was used to decipher which aspects of each variable were significant. T-tests were used when only one variable (i.e. season) was being examined for effect when that variable only had two treatments (breeding vs. non-breeding).

Staff at participating facilities recorded behavioral notes on a weekly basis regarding breeding behavior during the time periods of each facility's participation in the study.

Seasonality

Walrus are considered seasonal breeders, with the wild population breeding during the short days of winter (Figure 9). Day lengths, at each location of subject walrus residence, were compared to testosterone concentrations and testicular measurements of individual males to evaluate effects of photoperiod via correlation analysis. Day lengths for specific latitudes were acquired from an internet source (http://www.timeanddate.com). Testicular measurements and testosterone levels were paired with day length that occurred six weeks prior. This resulted in higher correlations (day lengths from six weeks prior were more effective predictors of testicular size than the day length on the day of the measurement.) Once the data were offset, correlation tests were conducted to determine significance at the $p \leq 0.05$ level.

Body Mass

The body mass (in kilograms) of one male walrus (PDZA) was determined for a period of 36 months, from October 2007 to October 2010. The walrus was trained to position his body on a 5 by 10 foot scale for weight measurement (GSE Scale Systems, model 350, 5,000x1, Novi, Michigan, USA). Body mass data were collected at least monthly, with the exception of February and September 2010. Basal and elevated levels of weight were determined by examining the percentage change in mass. The minimum and maximum masses were determined and baseline was established as values below minimum mass + 25% of the difference in change in mass. Values greater than minimum mass + 25% of the difference in change in mass were considered elevated (Anderson and Fedak 1985, Walker and Bowen 1993, Galimberti *et al*. 2007). Mass measurements were examined over two complete baseline periods and three complete elevation periods and means compared.

Results

Assay Validation

The curve generated from the serially diluted pooled saliva from male walrus was parallel to the standard curve for testosterone (Figure 10). The testosterone dilution curve displayed dilution linearity to the standard curve, indicating that the sample hormone cross-reacts with the antibody in a manner immunologically similar to the standard hormones. The linear portions of the curves (standards - 31.25 to 2,000 ng/ml; dilutions - 1:1 to 1:128) were parallel. The slope of the dilutions was -0.0387 and the slope of the standards was -0.0368. Sample dilutions were determined by the 50% binding rate (T=1:4). A 1:4 dilution at the 50% binding rate indicates that there is sufficient sensitivity in the testosterone EIA for salivary testosterone.

Direct correlation of testosterone in serum and saliva was found to be significant ($r=0.793$; $p<0.013$) (Figure 11). In addition, the assay was validated via anatomical findings (testicular measurements and sperm production) and physiological findings (by collecting samples before and after a known physiological event – breeding season). Salivary hormone profiles also were evaluated relative to observed rut and testicular measurements showing strong correspondence and providing additional validation of the EIA in walrus. Testicles recrudesced and spermatozoa were present with the onset of increased salivary testosterone concentrations and testicles regressed and spermatozoa were absent with decreased concentrations (Figure 25).

Endocrine Profiles/Reproductive Patterns

Individual male endocrine profiles were not combined to create a species average because there were extreme differences in hormonal patterns and seasonal changes. Salivary testosterone demonstrated erratic amplitude throughout all seasons as well as elevations during the "non-breeding" seasons.

PDZA

Saliva collection from the male at PDZA occurred for a period of 37 months (Table 3). During the first weeks of collection, salivary testosterone concentrations were at basal levels and remained at lower levels (average 0.15 ± 0.025 ng/ml) until December 2007 (Table 4; Figure 12). In December, testosterone began to rise and remained elevated for 5+ months (24 weeks) (average 1.55 ± 0.17 ng/ml) returning to basal levels in the beginning of June 2008. Basal levels lasted for approximately 3+ months (16 weeks) (average 0.65 ± 0.06 ng/ml), until the end of September 2008, and then rose again for 3+ months (14 weeks) (average 1.19 ± 0.15 ng/ml), from the end of September until the end of December 2008. Following the two distinct periods of elevations, the male's salivary testosterone cycle ceased to exhibit set patterns throughout the collection period for the study.

SWSD

Saliva collection from the male at SWSD occurred for a period of 12 months (Table 3). The SWSD male exhibited elevated levels of testosterone from June through October averaging 2.04 ± 0.20 ng/ml (Table 4; Figure 13). Salivary testosterone levels for the remaining months of the study were at baseline levels, averaging 0.44 ± 0.03 ng/ml.

IZ – 1

Saliva collection from the first male at IZ occurred for a period of 19 months (Table 3). The IZ-1 male displayed elevated levels of testosterone (Table 4; Figure 14) during the months of June through October, averaging 1.29 ± 0.10 ng/ml. This trend may have continued, however samples were discontinued in November. Concentrations were at baseline levels from September 2009 through March 2010 averaging 0.54 ± 0.04 ng/ml. The IZ male exhibited lower levels of salivary testosterone, both overall and individually when compared to the PDZA and SWSD males (Table 4).

IZ – 2/SFDK/NYA

Saliva collection from males IZ-2 and SFDK occurred for a period of 12 months (Table 3). Due to the death of the NYA male, participation lasted 6 months. Elevated concentrations of testosterone did not occur in any of these males (Figures 15-17). The three males exhibited lower levels of salivary testosterone, both overall and individually when compared to the PDZA and SWSD males (Table 4).

Testicular Measurements

Individual male testicular profiles were not combined to create a species average because there were extreme differences in measurements and seasonal changes. All males exhibited increased testicular size at some point during the study (Table 5).

PDZA

Testicular measurements from the male at PDZA occurred for a period of 36 months (Table 5; Figure 18; Appendix 4). The elevation threshold was determined to be 64.35 cm and above. The PDZA male had elevated testicular measurements of comparable pattern and magnitude between mid-autumn and late spring in three successive years. In addition, periods of increased measurements coincided closely with the wild walrus breeding season.

Combined testicular measurements were examined over two complete baseline periods and three complete elevation periods and means compared. Overall mean baseline measurements were 60.70±0.26 cm while mean elevations were 67.90±0.33. The means were significantly different (t=-17.159, df=116, p<1.287E-33). This male displayed the "expected" recrudescence and regression in testicular growth patterns during the three cycles. Testicular growth coincided with the wild walrus breeding season.

SWSD

During the first several months of measurements, combined measurements were mostly elevated (Table 5; Figure 19; Appendix 5). Measurements then declined below baseline during the months of January through June with a couple of sporadic elevated measurements in January. Recrudescence began in July 2008, when measurements began to increase. Testicular growth did not coincide with the wild walrus breeding season.

IZ – 1

The 20 month time series of data for the IZ-1 animal experienced a gap in sample collection. There were three periods of elevated testicular measurements in the IZ-1 animal during the sampling period (Table 5; Figure 20; Appendix 6). Two of the three periods of testicular elevations coincided with the wild walrus breeding season. The third period of elevation was abnormal in its timing and could have been an unexplained continuation of the first period of testicular elevation that coincided with the wild walrus breeding season. Testicular measurements were discontinued due to animal illness and subsequent death.

IZ – 2

The second male at Indianapolis Zoo did not participate in the testicular measurement portion of the study.

SFDK

During the sampling period of 18 months, there were three periods of elevated testicular measurements in the SFDK male (Table 5; Figure 21; Appendix 7). One of the three periods partially overlapped the end of the wild walrus breeding season. The other two periods of elevation were temporally anomalous in their timing, and in both cases were relatively modest in terms of magnitude and duration of elevation

NYA

Testicular measurements from the male at the New York Aquarium began in Dec 2007 and concluded in April 2008 (due to death; Table 5; Figure 22; Appendix 8). During the initial months of data collection combined measurements began to rise. Testes then remained enlarged during the months of January through April 2008. Testicular measurements were discontinued due to illness and eventual death of the animal in June 2008. Testicular growth did coincide with the wild walrus breeding season.

Anatomical Changes versus Reproductive Endocrinology

Saliva collection and testicular measurements were taken concurrently from male walrus for a period of 5 to 36 months. Testicular measurements were not collected from the IZ-2 male. Therefore, he was not included in this portion of the study. Correlation analysis was used to compare data with and without offsets (Table 6). All correlations were found to be non-significant at the $p \leq 0.05$ significance level.

Spermatozoa

Sperm presence or absence from the male walrus at PDZA was determined opportunistically beginning in November 2008 and concluding in October 2010 (Figure 23; Figure 24; Appendix 9). Sperm began appearing in the pre-ejaculate fluid in December of 2008 and continued to be observed through May 2009, a period of approximately 128 days. Once sperm was no longer present (three consecutive slides; collection normally occurred once per week) collection discontinued until prior to the next anticipated breeding season. Sperm began appearing in the pre-ejaculate fluid again in December 2009 and was observed consistently until April 2010, a period of approximately 134 days. Pre-ejaculate fluid was a useful way to assess the presence or absence of spermatozoa in this male walrus. Sperm presence in this single walrus coincided with increased testicular measurements. The approximate mean duration of spermatozoan presence in pre-ejaculate fluid was 131±3 days.

Behavior

Formal Observations

PDZA

In depth behavioral data were collected from the male walrus at PDZA from 2007 through 2009 during both breeding and non-breeding seasons (Table 7). As expected, rut display and clasping were significantly more frequent during the breeding season. However, clasping was only observed during the 2008 breeding season. Copulation, vocals and self-stimulation did not demonstrate a significant incidence by season. The researcher's observation location only allowed for minimal visualization of copulations, likely resulting in a negative bias for frequencies of copulation-related behaviors.

Informal Observations

PDZA

The PDZA male displayed changes in behavior from mid-autumn to late winter or early spring during the three years of observation, with slight variations in initiation and termination dates among years (Figure 25). His rut behaviors during time periods of changes in behavior included: bell and whistle vocalizations, "Chewbacca" vocalizations, flipper slapping on the water's surface, flipper slapping through the water, self-stimulating, chasing or corralling the females, clasping and penetration. The PDZA male intermittently displayed the following behaviors year round: self-stimulation, flipper slapping, belling and whistling.

SWSD

The SWSD male displayed changes in behavior consistent with rut from November 2007 through the beginning of February 2008 and then again in July 2008 through September 2008 (Figure 26). His rut behaviors during both periods included: "hammer" and whistle vocalizations, high pitched squeal vocalizations, flipper slapping on the water's surface, rubbing the fore flipper over face in a fast motion, self-stimulating, chasing or corralling the females, clasping and penetration. The SWSD male intermittently displayed rut behaviors (hammer, whistle) during the non-breeding season.

IZ – 1

The IZ-1 male displayed changes in behavior consistent with rut from January through April in 2008 and 2010 (Figure 27). His rut behaviors during the breeding seasons included: whistling and roar vocalizations, weed eater sound, bell vocalization (rare), self-stimulation, flipper slapping, bubble blowing, periods of inappetence, chasing or corralling the female, clasping and penetration. The IZ-1 male intermittently displayed the following behaviors year round: self-stimulation, occasionally chasing or grasping the female and other male.

IZ – 2

The IZ-2 male exhibited rut type behaviors but not in a consistent fashion (i.e. not during a concentrated time period but instead interspersed throughout the year). Behaviors included: whistling vocalization, self-stimulation, flipper slapping on the surface of the water, chasing or corralling the female, clasping, attempted penetration, rubbing against the female in a "sexual manner" and penetration.

SFDK

The SFDK male displayed changes in behavior consistent with rut beginning in mid-February and concluding in May 2008 (Figure 28). His rut behaviors included: "knocking" vocalization, "tinging" vocalization, striking his front flippers together (hard), striking the underwater viewing windows with tusks, self-stimulation, decreased interest in training sessions and food, chasing or corralling the females and attempted penetration. The SFDK male tended to not display behaviors consistent with rut outside of the breeding season.

NYA

The NYA male displayed changes in behavior consistent with rut during the months of January through April 2008 (Figure 29). His rut behaviors included: whistling, flipper slapping on the water surface, chasing the female, self-stimulation and not always participating in sessions. The

NYA male also intermittently displayed rut behaviors (self-stimulation and flipper waving through the water) throughout the year.

Seasonality

Testosterone

Three out of six walrus demonstrated the expected inverse relationship of overall salivary testosterone with day length (Table 8; Figures 30-35A). However, only one of the correlations was significant. Surprisingly, the SWSD, IZ-1 and IZ-2 males exhibited a significant positive correlation between day length and testosterone.

Testicular Measurements

In regards to testicular measurements versus day length, three out of six males displayed the expected inverse relationship (Table 9; Figures 30-35B). Of the three that were not significant, one had no testicular data collected. Correlations were inconsistent, with some negative and others positive.

Body Mass

Overall body mass of the PDZA male ranged from 1,426 to 1,936 kg (Figure 36; Appendix 10). Weights reached a peak in January 2008 (absolute value – 1,678 kg; seasonal average – 1,649.40±10.08), December 2008 (1,740 kg; 1,672.27±15.45) and December 2009 (1,936 kg; 1,763.83±35.63). Weights hit a nadir in July 2008 (1,521 kg; 1,535.85±2.32), May 2009 (1,474 kg; 1,521.25±7.65) and May 2010 (1,533 kg). Differences in weight between seasons/years ranged from 156 to 463 kg (156, 218, 266, 463 and 404) with a mean of 301.45 kg. Overall mean baseline measurements were 1,530.45±3.40 kg while mean elevations were 1,675.36±18.08 kg. The means were significantly different ($t=-7.87117$, $df=35$, $p<2.977E-09$). The PDZA male displayed the “expected” gain and loss in mass during the three cycles.

Discussion

This study represents the first comprehensive assessment of endocrine and behavioral parameters of reproduction utilizing non-invasive techniques in *ex situ* male walrus. Saliva samples, testicular measurements and behavioral data were collected from more than half of the *ex situ* male walrus maintained in U.S. facilities. Findings have shown that saliva is an informative non-invasive technique for monitoring testicular steroidogenic activity in male walrus. Furthermore, results have demonstrated that testicular measurements are a useful method for examining testicular recrudescence and regression. Successful monitoring of the male breeding season can be accomplished through behavioral observation, while collection of spermatozoa in pre-ejaculate can be an effective means to track spermatogenesis. When salivary testosterone concentrations are used in conjunction with other data, the reproductive stage (rut vs. non-rut) can be determined quite readily.

Saliva

The results in the study demonstrate the valuable use of saliva sampling for noninvasive reproductive monitoring of testosterone in male walrus. The assay was validated via parallelism, with correlations of testosterone in serum and saliva found to be significant and with testosterone present in walrus saliva as displayed by the EIA and mass spectrometry (Appendix 11). Even though serum is considered the standard of choice for reproductive hormone monitoring, saliva was chosen due to its non-invasive nature, ease of collection, relative stability (Lipson and Ellison 1989) and successful use in other species. Collections of feces and urine are also non-invasive. However, such collections are not easy to complete with walrus. Feces of different individuals tend to get mixed while animals sleep together on land and urine collections require trained cooperation by subject animals. Training of saliva collection with operant conditioning was accomplished without difficulty, allowing multiple facilities to participate in this study thus providing a larger sample size. Assay Designs EIA kits can be reliably used to measure salivary and serum testosterone in male walrus. The positive correlation between serum and saliva indicates that saliva can be used for testosterone analysis, providing an effective, less invasive alternative to blood.

In the last several decades, saliva has become a widely accepted and non-invasive sample source for the analysis of human steroids. Specifically, salivary hormones have shown good correlation with unbound steroid hormones in serum (Riad-Fahmy *et al.* 1987, Rilling *et al.* 1996, Ostatnikova *et al.* 2002, Groschl 2008). This study demonstrated a significant correlation between testosterone concentrations in serum and saliva of male walrus (r=0.793; p=0.013) and findings were consistent with serum and saliva correlations found in other male marine mammals (Steller sea lions, *Eumetopias jubatus*, r=0.840, Harmon 2001; Hawaiian monk seal, *Monachus schauinslandi*, r=0.902, Theodorou and Atkinson 1998). The assay for walrus was also validated via anatomical findings (testes measurements and sperm production) and physiological findings (by collecting samples before and after a known physiological event – mating behavior/rut).

The use of cotton to collect saliva has shown mixed results. Some research has demonstrated decreased concentrations of reproductive hormones (Höld *et al*. 1995, Groschl and Rauh 2006), while others have demonstrated increased concentrations of reproductive hormones (Dabbs 1991, Shirtcliff *et al*. 2001, Strazdins *et al*. 2005). Regardless, the methodology for the research was conducted in a consistent manner, similar to the female portion of the project, so the issue should not affect resulting overall patterns or their interpretations.

Salivary testosterone concentrations are known to show rapid, highly fluctuating levels differing by as much as 95% in consecutive samples. Testosterone release is cyclical and salivary testosterone is a momentary snapshot (unlike fecal and urinary testosterone) so concentrations vary widely (Walker *et al*. 1980). To keep fluctuations to a minimum, salivary testosterone samples were always taken in the mornings.

Testosterone

Study males exhibited both similarities and differences in testosterone concentration patterns when compared to other pinnipeds. Male pinnipeds exhibit seasonal fluctuations in circulating concentrations of testosterone with increasing concentrations coinciding with the onset of rut (Hawaiian monk seals - Atkinson and Gilmartin 1992, Weddell seals, *Leptonychotes wedellii* - Bartsh *et al*. 1992). In species with a short, tightly synchronized pupping and breeding season, testosterone concentrations increase for periods of one to three months at the start of the breeding season, but decrease to baseline concentration before breeding behavior ends (Griffiths 1984, Noonan *et al*. 1991, Bartsh *et al*. 1992). In hooded seals (*Cystophora cristata*) and southern elephant seals (*Mirounga leonina*), plasma testosterone levels are elevated for approximately one month prior to the onset of breeding behavior but then decline, or reach basal levels before breeding season ceases (Griffiths 1984, Noonan *et al.* 1991).

In the males observed in the study, salivary testosterone analysis demonstrated substantial differences among animals, with mature animals showing patterns similar to those found in male pinnipeds and younger animals displaying no seasonal pattern in testosterone secretion. In mature animals, salivary testosterone concentration was substantially elevated over basal levels for several months. It is likely that the protracted period of male fertility ensures that a high fraction of females become pregnant and may improve probabilities of successful copulations by males within a given breeding season. The exact timing of peak values in testosterone varied with animals and did not correlate with geographic latitude or photoperiod. Results are not consistent with findings in other seasonally reproducing species in which latitude and the associated differences in day length and other environmental factors act to control seasonality (Bronson 1985, Boyd 1991).

It is generally believed that photoperiod is a proximate control factor in the annual cycle of pinniped reproduction (Boyd 1991, 1996). Synchronized breeding is mediated through the pineal–pituitary axis and gonadotropic action varies according to species and latitude (Daniel 1981, Griffiths and Bryden 1981). The pineal gland is responsible for the neuroendocrine communication of photoperiod to the rest of the body via melatonin and plays a key role in regulating an animal’s response to photoperiod (Hansen 1985, Ebling and Barret 2008, Morgan and Hazlerigg 2008). Walrus possess a large pineal gland (Turner 1888, Tilney and Warren 1919). Therefore, photoperiod most likely plays a role in walrus reproduction. Wild walrus are short day breeders, with peak breeding occurring from December to March (Fay 1982). Wild walrus cue the start of spermiogenesis with a decrease in day-light hours ensuring the completion of spermatogenesis for the breeding season. A change in latitude can affect the seasonal variability of reproduction (Hansen 1985, Boyd 1991, Bradshaw and Holzapfel 2007). Photoperiod at higher latitudes experiences a greater overall change within a year than at lower latitudes. Animals adapted to high-latitudes, like walrus, have shorter, well-defined breeding seasons. As latitude diminishes, breeding seasons tend to increase in duration. It is quite possible that the difference in latitude of the three primary breeding locations of wild Pacific walrus - 60°05'N at Nunivak Island, 63°24'N at St Lawrence Island and 64°40'N at the Gulf of Anadyr, or the range of locations during the non-breeding season (ranging from 71°17'N at Barrow, Alaska along the Chukchi Sea to 58°61'N at Round Island, Alaska in Bristol Bay) versus the *ex situ* walrus in the study (47°18'N at PDZA, 40°34'N at NYA, 39°46'N at IZ, 38°08'N at SFDK and 32°45'N at SWSD) may account for a change in photoperiod cues and therefore a change in temporal patterns of reproduction (Figure 9).

Three out of the six males (four out of nine seasons) displayed salivary testosterone elevations consistent with seasonal breeding. The onset and length of rut was consistent with rutting behavior and timing of wild walrus proposed by Fay (December-March; 1982). Possible explanations for the three males that did not display elevations consistent with seasonal breeding seasons are varied. The IZ-2 male was most likely still sexually immature at six years of age. The NYA male died during the study. However, the male successfully sired offspring in 2007. The NYA male's subsequent acute impaired health may have accounted for the lack of normal testosterone levels during the collection period. When fighting illness, the reproductive system becomes a fairly low priority for the body, which needs to focus instead on vital organs. The SFDK male, at 13 years of age, underwent rut as evidenced by testicular measurements and behavior, from mid-February through May. However, testosterone concentrations were not consistent with occurrence of rut. Low salivary levels during the study were consistent with overall decreased serum testosterone levels for the SFDK male both during and after the study (Muraco *et al*. 2012). Due to the animal's shifted rut and low serum testosterone levels, the male was subsequently treated with exogenous human chorionic gonadotropin (hCG) prior to the 2010 breeding season in an attempt to increase testosterone levels in synchrony with the female's annual ovulation. The male went on to impregnate the female and sire a still born calf.

One male in the study (PDZA), presented with with-in animal decline in testosterone concentrations over multiple breeding seasosn. This trend also continued after the study (Triggs, unpublished data). One possible explanation for the varied concentrations is based on a study of testosterone levels in Weddell seals. Bartsh *et al.* (1992) compared testosterone levels in non-territorial, territorial and transitional males. Non-territorial males demonstrated lower initial serum testosterone concentrations. If Weddell seals are like other reported seasonal breeders, then the peak in circulating testosterone occurs before inter-male sexual competition begins during the breeding season. Circulating testosterone concentrations decline from the seasonal peak concurrently with sexual competition among males. However, most *ex situ* walrus do not encounter male competition during the breeding season. Lack of male-male competition may contribute to less prominent maxima in testosterone levels, with potential consequences for breeding behavior and breeding success. To test these suggestions, baseline monitoring of singularly housed males (with females) followed by multiple male housing (with females) followed by the comparison of breeding behavior, T levels and sperm development patterns during both conditions, would elucidate whether changes in social structure had an effect on reproductive parameters.

Another possible explanation for the findings of the PDZA male involves the process referred to as biological complacency, in which continued exposure to a familiar female can result in habituation (decreased levels of reproductive hormone production; Fowler and Whalen 1961, McGill 1963, Michael and Zumpe 1978, Coquelin and Bronson 1979). The pulse generating system for luteinizing hormone (LH) is susceptible to habituation. LH secretion upon initial exposure to a female is high but the size of the pulse can decrease with repeated exposure to the same female. Exposure to a new female restores the response to the magnitude observed initially (Coquelin and Bronson 1979). The habituation of the LH response in the Coquelin and Bronson study parallels the pattern of behavioral arousal seen in other mammalian species (Fowler and Whalen 1961, McGill 1963, Michael and Zumpe 1978) generally referred to as the Coolidge effect (Wilson *et al*. 1963). Males allowed to copulate with a female repeatedly are eventually satiated. However, if a new female is introduced, the male usually demonstrates renewed sexual interest and copulates with the new female. Thus, both LH responsiveness and behavioral arousal habituate with repeated exposure to the same female and are re-aroused by a novel female. Biological complacency or the Coolidge effect could explain the decreasing testosterone levels in the PDZA male walrus over time. Keeping males and females in close proximity continually in facilities may be detrimental to promoting elevated T levels. The result may be decreased courtship behavior, inadequate sperm production and reproductive failure in subject animals. Testing the hypothesis would require continued monitoring of the population to establish baseline knowledge regarding sperm morphology, concentration and motility and continued monitoring of T levels, followed by separation of genders during the non-breeding season. Continued in-depth monitoring of sperm and T concentrations could then be evaluated during the separations, introductions and transfers to evaluate effectiveness.

Testes and Sperm

Subject male walrus exhibited both similarities and differences in testicular recrudescence and regression patterns compared with those found in wild walrus and other male pinnipeds. In seasonal breeders, testicular mass, seminiferous tubule dimensions and the rate of spermatogenesis begin to increase in advance of the breeding season. In wild walrus, the principal period of mating is just after the peak of the testis cycle of mature males (Harrison 1969, Fay 1982). Changes in linear dimensions and mass of pinniped testes coincide with the onset and cessation of spermatogenesis (Boyd 1991). Therefore, testicular measurements have been useful as indicators of sperm production in a variety of animals (Southern elephant seal, Griffiths 1984; wapiti, *Cervus canadensis,* Haigh *et al.* 1984; bison, *Bison bison,* Helbig *et al.* 2007). Since testicular recrudescence and regression are expected in seasonal breeders, it was anticipated that subject males would exhibit increased testicular size several weeks prior to and during the wild walrus breeding season, which is typically December through March. The pattern was observed in all subject males that participated in the testicular measurement portion of the study. However, some male patterns were less pronounced than others. Possible explanations for the observed mixed patterns involve a variety of factors. Testicular measurements were not always taken by the same person, such that measurement error among data collectors may have been a factor. Due to scheduling constraints, measurements were made by one to three different people at the participating facilities. In addition, during periods of regression, the testes were less easily palpated, possibly further exaggerating differences in accuracy of measurement among data collectors. An alternative hypothesis involves consistency in positioning among subject animals at the time of measurement. Depending on how the walrus presented, measurements could be altered anatomically by the baculum, rear flippers or gravity.

The subject male at SWSD presented a unique temporal pattern in his breeding cycle, almost the seasonal opposite of the wild walrus breeding season. The SWSD male arrived at the study location in 1997 at 10 years of age. From 1997 through 2007, he typically displayed rut behavior in late October through January for a period of 3-3.5 months. Offspring were sired in 2003 and 2005. During the summer of 2008, his cycle shifted and he began displaying behaviors consistent with rut from July through September. The temporally shifted rut continued in 2009 and 2010. In 2011, the SFDK animal began rut behavior in April and continued into early July, another shift. In 2012, rut behavior began at the end of March and continued into May. This animal started developing cataracts and had been blind for several years at the time of the study (M. Synnott, pers. comm. 2013). A similar situation occurred with a female PDZA walrus (female portion of study). Seasonally breeding species respond to specific environmental cues, in particular photoperiod, via the pineal gland to regulate hypothalamic activity and consequently, testicular growth and recrudescence (Lincoln 1981). It is possible that the SFDK male was not correctly responding to photoperiodic cues due to blindness. In mammals, photoperiodic input is perceived through the eyes then transmitted via a multi-synaptic pathway to the pineal gland, which transduces the photic signal into a chemical signal by synthesizing and secreting melatonin. Blind or pinealectomized animals can still undergo annual breeding seasons, although they do not necessarily begin or end at the expected time of year (Czyba *et al*. 1964, Herbert 1972, Herbert *et al.* 1978, Lincoln *et al*. 1989). Both the eyes and the pineal gland are necessary if the breeding season for an individual is to be synchronized with the time of year in which breeding normally occurs on a population scale. However, neither the eyes nor the pineal gland are required for the periodic occurrence of breeding seasons. Annual changes in day length, nutrition, temperature and social factors are all features of the environment which may interact to influence the seasonal reproductive cycle (Sadleir 1969, Bronson 1985, 1988). Interference with the pineal gland specifically blocks the reproductive responses to changes in day length without necessarily impairing the responses to other environmental factors (Lincoln *et al*. 1989). Effects of photoperiod manipulation on reproductive processes have not been studied in walrus.

Some animals demonstrated periods of elevated testicular measurements that were not preceded by or did not coincide with periods of elevated testosterone levels. Among male pinnipeds, spermatogenosis usually lags behind testosterone production by one to three months, as activity and steroidogenesis of the Leydig cells is necessary for germ-cell differentiation in the seminiferous tubules (Griffiths 1984). During the non-breeding season the testes regress, the epididymides are aspermic and testosterone concentrations decline to baseline (Frick *et al*. 1977, Gardiner *et al*. 1999). In addition, the seminiferous tubules undergo involution, resulting in a decrease in both testicular dimension and mass (Griffiths 1979, 1984). Some subject males experienced recrudescence without substantial elevations in testosterone.

Longitudinal monitoring of testicular and seminal traits simultaneously in one male walrus found that seasonal changes in testicular measurements coincided with patterns of spermatozoan presence. Spermatozoa were present during the breeding season and absent the rest of the year. During periods of sperm presence, testicular measurements were also increased. The findings are consistent with those of Fay (1982) and Born (2003). However, during the third year of the study, the PDZA male experienced recrudescence and spermatogenesis without substantial elevations in testosterone.

Current knowledge supports a role for follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone in initiation of spermatogenesis. However, the relative roles of each are not well defined (McLachlan *et al.* 2002). Generally, spermatogenesis occurs during the period when peripheral testosterone concentrations are high. However, in southern elephant seals (Griffiths 1984) and hooded seals (Noonan *et al.* 1991), concentrations of plasma testosterone only increased near the onset of the breeding season. Generally, during the non-breeding season, testosterone release is inhibited and spermatogenic activity is low (Sinha *et al*. 1977).

Seasonal changes have been reported in peripheral testosterone concentrations of several pinniped species (Noonan *et al*. 1991, Seely and Ronald 1991, Bartsh *et al*. 1992, Gardiner *et al*. 1999, Harmon 2001) and spermatogenic activity changes (via histology) have been reported in the crabeater seal (*Lobodon carcinophaga*), leopard seal (*Hydrdrurga leptonyx*, Sinha *et al*. 1977), southern elephant seal (Griffiths 1984) and walrus (Fay 1982). The crabeater seal, leopard seal (Sinha *et al*. 1977), Weddell seal (Bartsh *et al*. 1992) and southern elephant seal (Griffiths 1984), spermatogenic activity initiates one to two months before the breeding season and full spermatogenesis occurs during the breeding season. The level of spermatogenic activity in the subject walrus indicates a tendency similar to that of other pinnipeds.

A possible explanation for recrudescence and spermatogenesis without substantial elevations in testosterone could be that salivary testosterone represents only the free or unbound levels of testosterone in blood. Bound and total serum testosterone levels generally are much higher than salivary concentrations. Even though androgens are essential for spermatogenesis in mammals (Weinbauer *et al*. 2000, McLachlan *et al*. 2002) and elevated serum testosterone levels generally indicate spermatogenesis, the intratesticular levels of testosterone needed for spermatogenesis are significantly higher than serum levels. Secretion of testosterone by the Leydig cells ensures that the seminiferous tubules are exposed to concentrations of androgens approximately 100 times greater than circulating levels. Several studies have shown that spermatogenesis can proceed normally at levels of testosterone considerably less than those found within the testis but the levels required are still fivefold higher than peripheral circulating levels (Cunningham and Huckins 1979, Sun *et al*. 1989). There is an absolute need for androgens in spermatogenesis in all species except for the unique model of the photoperiod-inhibited Djungerian hamster, in which FSH alone appears sufficient (Lerchl *et al*. 1993). It is also clear from rat studies that testicular testosterone levels of 10-20% of normal are sufficient to maintain spermatogenesis (Boccabella 1963, Cunningham and Huckins 1979). The minimum salivary testosterone levels indicating required blood and testicular levels necessary to initiate testicular recrudescence and maintain sperm production may also be low in walrus. Validation of the suggestion requires in-depth monitoring of T levels in saliva, in circulation and within the testes, in conjunction with analysis of sperm concentration, morphology and motility.

An alternative hypothesis concerns the lack of knowledge regarding the dominant androgens in walrus serum and saliva. Testosterone is regarded as the “active” androgen in supporting spermatogenesis in the normal testis. However, testosterone can be metabolized within the testis by 5α-reductase type 1 or 2 isoenzymes to the more potent androgen dihydrotestosterone (DHT), which has a higher affinity for androgen receptors and forms a more stable complex (Grino *et al*. 1990, Zhou *et al*. 1995). The EIA kit used had a low cross reactivity (<0.001%) with DHT. It is possible, though unlikely, that testosterone is not the dominant androgen in walrus saliva and serum. To test the theory, samples of walrus saliva and serum could be evaluated by high performance liquid chromatography to determine the dominant androgen.

Only sperm presence and absence were examined in the study. Ejaculate volume, motility, morphology, concentration, and acrosomal integrity were not evaluated. There is definitive evidence that spermatogenesis is impaired or never commences in the presence of severe androgen deficiency (McLachlan *et al.* 1995). When the decline in LH and testosterone levels is not so severe, spermatogenesis proceeds to completion but is quantitatively impaired resulting in low sperm counts. Low testosterone levels in walrus could be causing reproductive failure via impaired sperm quality or function. Validation of the suggestion requires continued longitudinal monitoring of T levels as well as in-depth analysis of sperm morphology, concentration and motility.

Behavior

The mature subject males performed stereotypical vocal and visual displays during rut. Observed displays were consistent with other *in situ* and *ex situ* studies of walrus that have demonstrated similar courtship displays during the breeding season (Fay 1982, Fay *et al*. 1984, Genrich 1984, Stirling *et al*. 1987, Sjare and Stirling 1996). Four of six subject males also displayed various rut behaviors outside of the breeding season. The extra-seasonal behavior is consistent with observations of *in situ* and *ex situ* males (Miller 1975, Gehnrich 1984, Triggs pers. obs.). Miller (1975) found that the summer displays of males were incomplete and non-stereotypical, even though they included behaviors and vocalizations similar to those used by mature bulls in their displays during the breeding season. In a study conducted by Genrich (1984), the *ex situ* males displayed more frequently during the breeding season than at other times (10.1 displays/hour versus 3.1 displays/hour). The displays during the breeding season were more stereotypical, included more behaviors per display and were more intense than the displays outside the breeding season. Overall, behavioral observations from subject males support previously published indications that Pacific walrus undergo a breeding season lasting approximately three to four months (Mansfield 1958, Fay 1982, Born 2003).

Actual penetrations and copulations were not commonly observed during formal observations but occurred more often during the informal portion. Walrus copulations have rarely been observed in the wild. In a nine year study, Sjare and Stirling (1996) observed probable copulation only once. However, courtship and mounts were observed often. During copulation, the male mounted the female for 30-150 seconds, 8-10 times during a two hour period, singing the entire time. During PDZA's formal observations, the observer's location allowed for documentation of courtship and clasping behaviors but not penetrations. During informal observations, probable copulations took place in the water, with the male clasping the female ventrum to ventrum or ventrum to dorsum. During one such day of observation, the male penetrated the female 11 times during a 56 minute period.

In six out of eight breeding seasons observed, walrus rut behavior coincided with elevated testosterone levels or elevated testicular measurements. In the male in which presence of sperm was monitored, behaviors also coincided with sperm presence. Behaviors consistent with rut generally lasted several months.

Successful walrus reproduction in zoos and aquaria remains low, even though *ex situ* males displayed behaviors consistent with rut. The determinants of breeding success are not known with certainty. One of the challenges of zoo and aquaria animal husbandry and management is to understand behavioral patterns and their significance, and to match them as closely to wild patterns as possible to enhance reproduction. A significant result of living in a zoo or aquarium, compared to the wild, is the reduction in the ratio of males to females. It has been demonstrated that the social environment of animals and the conditions under which they mate can affect how males function in groups (Tilbrook and Cameron 1990). However, we do not yet know enough about conditions ideal for eliciting optimum reproductive performance from male walrus. Increased competition between breeding males of other polygynous mammal species seems to encourage breeding success (Bartholomew 1970, Galimberti *et al*. 2007, Harcourt *et al*. 2007, Mainguy and Cote 2008) and may facilitate successful reproduction in walrus.

Historically, U.S. facilities managing multiple male walrus with females have separated mature males during the breeding season to help alleviate male-male aggression. In the wild, mature walrus are segregated by gender most of the year; coming together only during the breeding season. During rut, males compete with one another for females. While thus untested in walrus, housing genders separately except during the breeding season has been successful in other species (Markham 1990, Caro 1993). To mimic the wild social structure and facilitate male breeding competition, it may be beneficial to house more than one male at an individual facility. In the 1970s and 80s, Marineland of the Pacific (MLP) housed two male walrus with only auditory and olfactory contact. Each of the males was housed with a female and the male and female pairs were separated by a physical barrier that prevented visual contact. During rut, when one male began to display, the second male would begin displaying within seconds. MLP had seven pregnancies in a 15 year period (Gehnrich 1984, Triggs 2006, Oland 2012).

Body Mass

The adult male walrus at PDZA gained and lost mass consistent with the "fatted" male phenomenon (DuMond and Hutchison 1967). Mass increased in the months preceding the breeding season and decreased during the breeding season. While in rut, the male consumed less food than at other times of the year. Mass results were consistent with previous findings in other *ex situ* walrus (Gehnrich 1984, L. Oland pers. comm. 2013, J. Paschke pers. comm. 2013, M. Synnott, pers. comm. 2013).

Fay (1982) found a decrease in food intake of wild male walrus during the breeding season. Reduced consumption by adult males of other pinnipeds during the breeding season also has been reported. Pinniped species include northern fur seals (*Callorhinus ursinus*; Bartholomew and Hoel 1953), Steller sea lions (Spalding 1964), southern elephant seals (Ling and Bryden

1981) and grey seals (*Halichoerus grypus*; Bonner 1982). Large body size enables breeding bulls to display for extended periods (by metabolizing extensive blubber stores), and also provides a direct advantage in competitive interactions (Bartholomew 1970).

Wild male walrus may need to fast for long periods of time to maintain their mobile territories near the estrous females (Fay *et al.* 1984), whereas the PDZA male had no such necessity. Nonetheless, intake was still decreased for several months during the breeding season. Following the period of reduced intake, consumption increased steadily until the period prior to the breeding season, resulting in increased body mass. Findings were consistent with wild male walrus which also feed intensively throughout the summer and autumn and tend to be fattest in early winter just prior to the breeding season (Fay 1982).

Based on a combination of salivary testosterone, testicular measurements, sperm production and behavior, six out of nine breeding seasons coincided with the wild walrus breeding season. When examining historical studbook data, births in U.S. zoos and aquaria occurred during March (1), April (2), May (7), June (6), July (1) and Aug (1) for a total of 18 live births (Oland 2012). Since walrus gestation is 15-16 months, extrapolating back, fertilization most likely occurred in the following time frames (allowing some room for variable periods of delayed implantation): March birth = November/December fertilization, April = December/January, May = January/February, June = February/March, July = March/April and August = April/May. Thus, approximately 82% of the calves were conceived during the wild walrus breeding season, 12% were conceived on the outer edge of the breeding season and 6% were conceived outside the breeding season of wild Pacific walrus. There are several possibilities for the three *ex situ* cycles that did not coincide with the wild cycle. The IZ-2 male was most likely not sexually mature at six years of age. Another male, SFDK, underwent rut as evidenced by testicular measurements and behavior, from mid-February through May. His timing is consistent with subsequent research regarding the SFDK animal. Muraco *et al.* (2012) reported that the SFDK male underwent a seasonal rut routinely occurring in late Feb through May with a serum testosterone peak in March. Muraco *et al.* hypothesized that photoperiod was the primary reason that the male's rut timing differed from the adult cycle in wild walrus of December through March resulting in reproductive failure in the group of *ex situ* walrus residing at SFDK (Vallejo, California - 38°08'N). However, the hypothesis is inconsistent with the successfully breeding pairs of walrus that lived even further south at Marineland of the Pacific (Palos Verdes, California - 33°73'N) and Sea World (San Diego, California - 32°45'N and San Antonio, Texas - 29°46"N). Collectively, walrus at the aforementioned facilities experienced 12 pregnancies (Genrich 1984, Oland 2012, Wilcox, pers. comm.). Fay's research (1982) found adult males (>15 years) were most often fertile from November to February. Younger adults (12-16 years) were fertile less often and over a longer period. Subadults (5-11years) were least often fertile and their highest fertility occurred about two months later than for mature males. The cycle in testes size suggests a peak in development in December or January for the older males and in February and March for the younger individuals (Born 2003). Fay (1982) also suggested that wild male walrus in the same breeding location have variations in the timing of rut associated with age and degree of sexual maturity. It is likely that the SFDK male, in his younger years, may have been experiencing a cycle consistent with wild young adults or simply a variation in the timing of rut consistent with Fay's findings.

Conclusion

This study represents the first comprehensive assessment of behavioral, testicular and reproductive endocrine traits in *ex situ* U.S. male walrus. The thesis demonstrates that male walrus secrete measurable amounts of testosterone in their saliva and those concentrations can be used along with other anatomical and physiological processes to better understand and track reproductive cyclicity. The application of endocrine monitoring combined with testicular measurements, sperm presence or absence and behavior has proven useful for the initial characterization of the reproductive biology of male walrus, and findings may have important implications for improving population management for *ex situ* walrus.

To increase breeding success of the zoo-based population, the following changes are suggested: a) increase the number of walrus in the population or consolidate existing walrus into larger social groups to better mimic the wild social structure; and b) separate animals by gender during the non-breeding season or move animals to new facilities to combat biological complacency (Coolidge effect). Group participation and continued monitoring of reproductive functioning via endocrine and behavior parameters would allow evaluation of the changes. Further studies addressing seasonal influence on reproductive activity, reproductive senescence, seasonal changes in testicular steroidogenesis and spermatogenesis, and the influence of social conditions on the reproductive performance of *ex situ* male walrus via longitudinal endocrine monitoring, sperm evaluation, testicular measurements, behavioral observations and ultrasonography would greatly improve our understanding of reproduction in the species.

Table 3. Description of male walrus used in study

Male	Facility	Latitude	Water temp. (°C)	Studbook#	Birth date	Sample period	Age*	w/ female	Sired offspring
PDZA	Point Defiance Zoo & Aq	47° 18'N	7-13	31	1982	6/2007-10/2010	25	yes	no
SWSD	Sea World San Diego	32° 45'N	12-14	38	1987	11/2007-11/2008	20	yes	yes
IZ-1	Indianapolis Zoo	39° 46'N	10-13	39	1987	11/2007-11/2008 9/2009-4/2010	20	yes	no
IZ-2	Indianapolis Zoo	39° 46'N	10-13	71	2003	9/2009-9/2010	6	yes	no
SFDK	Six Flags Discovery Kingdom	38° 08'N	7-13	52	1994	11/2007-11/2008	13	yes	yes#
NYA	New York Aquarium	40° 34'N	7-21	49	1994	12/2007-6/2008	13	yes	yes

All walrus were wild born.

* Age at start of study

sired offspring post-study (2011)

Table 4. Male walrus salivary testosterone concentrations and iterative statistics for baseline and elevated levels.

	Overall statistics (ng/ml)				Iterative Statistics (ng/ml)	
Animal	**n**	**min**	**max**	**mean**	**Baseline**	**≥Elevation**
PDZA	183	0.06	4.23	0.75	0.49	0.99
SWSD	51	0.14	4.00	1.16	0.42	0.84
IZ-1	78	0.13	2.30	0.81	0.62	1.23
IZ-2	50	0.10	1.54	0.52	0.37	0.73
SFDK	52	0.07	1.67	0.36	0.27	0.53
NYA	26	0.20	1.79	0.60	0.53	1.06

Table 5. Measurement data for the testes of male Pacific walrus

			Length (cm)			Width (cm)			Combined (cm)		
Walrus	**N**	**Time period**	**Mean**	**Range**	**% change**	**Mean**	**Range**	**% change**	**Mean**	**Range**	**% change**
PDZA (1*)	200	7 Oct 2007-17 Oct 2010	21.69	18.5-25.5	27.45%	10.45	9.0-12.5	28.00%	64.26	57.0-74.0	22.97%
SWSD (3*)	61	22 Aug 2007-14 Nov 2008	16.74	13.0-21.0	38.10%	10.63	8.0-14.0	42.86%	54.73	48.0-64.0	25.00%
IZ-1 (3*)	76	10 Nov 2007-27 Nov 08 2 Sept 2009-21 Apr 2010	20.12	15.0-27.0	44.44%	9.31	6.5-13.0	50.00%	58.84	48.0-71.5	32.87%
IZ-2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
SFDK (1*)	77	19 May 2007-30 Nov 2008	21.74	20.0-25.0	20.00%	10.12	9.0-12.0	25.00%	63.71	59.0-71.0	16.90%
NYA (1*)	17	11 Dec 2007-15 Apr 2008	20.37	15.0-25.0	40.00%	9.57	8.0-13.0	38.46%	59.88	51.5-67.5	23.70%

N = total number of weekly measurements
Combined = R linear length + R linear width + L linear length + L linear width
*number of personnel that took measurements

Table 6. Salivary testosterone concentration versus combined testicular measurements (no offset and offset)

	Season							
No offset	**2007/2008**		**2008/2009**		**2009/2010**		**Overall**	
Animal	**r**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**
PDZA	0.270	0.058	0.015	0.917	-0.046	0.751	-0.146	0.0641
SWSD	0.051	0.730	NA	NA	NA	NA	0.051	0.730
IZ-1	-0.067	0.648	NA	NA	-0.024	0.923	-0.012	0.920
IZ-2	NA	NA	NA	NA	NA	NA	NA	NA
SFDK	0.045	0.75	NA	NA	NA	NA	0.045	0.750
NYA	0.100	0.693	NA	NA	NA	NA	0.100	0.693

	Season							
Offset	**2007/2008**		**2008/2009**		**2009/2010**		**overall**	
Animal	**r**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**
PDZA	0.286	0.060	-0.224	0.149	0.180	0.243	-0.063	0.433
SWSD	-0.220	0.132	NA	NA	NA	NA	-0.220	0.132
IZ-1	-0.215	0.171	NA	NA	0.275	0.285	-0.040	0.756
IZ-2	NA	NA	NA	NA	NA	NA	NA	NA
SFDK	-0.119	0.395	NA	NA	NA	NA	-0.119	0.395
NYA	0.029	0.928	NA	NA	NA	NA	0.029	0.928

Table 7. Male behavioral data - formal observations (PDZA)

2007/2008/2009 combined (p-values)

	Water	Land	Swim	Pattern A	Pattern B	Walk	Forage	Inactive	Flipper suck
Season	0.231	0.231	**<2.2E-16***	**5.58E-12**	**<2E-16***	0.959	**8.986E-05***	**0.007**	**6.89E-14**
Year	0.197	0.197	**1.54E-05**	**0.0249**	0.313	0.831	0.885	0.454	0.528

	Mouth manip	Self stim	Object	Rut	Clasp	Copulation	Non-visible	Other	Vocal
Season	**1.88E-05**	0.106	**0.007***	**2.608E-05***	**0.004***	0.330	**4.927E-06***	**4.80E-15***	0.361
Year	0.0569	0.747	0.478	0.0581	**0.000**	0.375	0.192	**0.047**	0.172

* more frequent during breeding season

	Season	breeding and non-breeding		
	Year	**A=2007**	**B=2008**	**C=2009**
		B-A	**C-A**	**C-B**
Year (p-values)	**Swim**	0.088	**0.000**	**0.021**
	Pattern A	0.564	**0.018**	0.199
	Clasp	**0.001**	0.999	**0.001**
	Other	**0.035**	0.472	0.444

Table 8. Day length versus salivary testosterone concentration (offset)

	Season									
	2006/2007		**2007/2008**		**2008/2009**		**2009/2010**		**Overall**	
Animal	**R**	**p-value**	**R**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**
PDZA	NA	NA	-0.432	**0.001**	-0.092	0.519	.003	0.986	-0.219	**0.006**
SWSD	NA	NA	0.710	**5.34E-09**	NA	NA	NA	NA	0.710	**5.34E-09**
IZ-1	NA	NA	0.298	**0.040**	NA	NA	0.390	**0.036**	0.417	**0.000**
IZ-2	NA	NA	NA	NA	NA	NA	0.308	**0.030**	0.308	**0.030**
SFDK	NA	NA	-0.111	0.439	NA	NA	NA	NA	-0.111	0.439
NYA	NA	NA	-0.244	0.244	NA	NA	NA	NA	-0.244	0.240

Table 9. Day length versus combined testicular measurements (offset)

	Season									
	2006/2007		2007/2008		2008/2009		2009/2010		Overall	
Animal	**R**	**p-value**	**R**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**
PDZA	-0.876	**<2.2E-16**	-0.776	**2.22E-11**	-0.931	**<2.2E-16**	-0.876	**<2.2E-16**	-0.807	**<2.2E-16**
SWSD	NA	NA	0.253	0.053	NA	NA	NA	NA	0.253	0.053
IZ-1	NA	NA	-0.549	**2.53E-05**	NA	NA	-0.467	**0.033**	-0.506	**4.89E-06**
IZ-2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
SFDK	NA	NA	-0.334	**0.003**	NA	NA	NA	NA	-0.334	**0.003**
NYA	NA	NA	-0.088	0.737	NA	NA	NA	NA	-0.088	0.737

(A)

(B)

Figure 7. Locations of linear testicular measurements: (A) width and (B) length.

1 – tunnel

2 – public viewing window

3 – spot

4 – peninsula

5 – main public viewing

6 – main beach

7 – underwater viewing windows

8 – public viewing window by seal

9 – island

10 – observer location

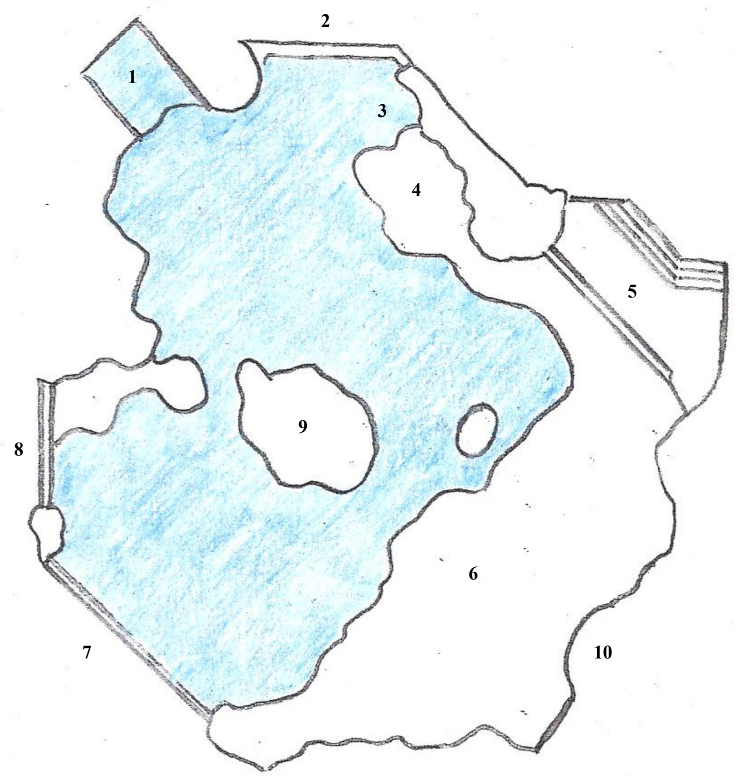


Figure 8. Overview of Point Defiance Zoo & Aquarium's main walrus habitat.

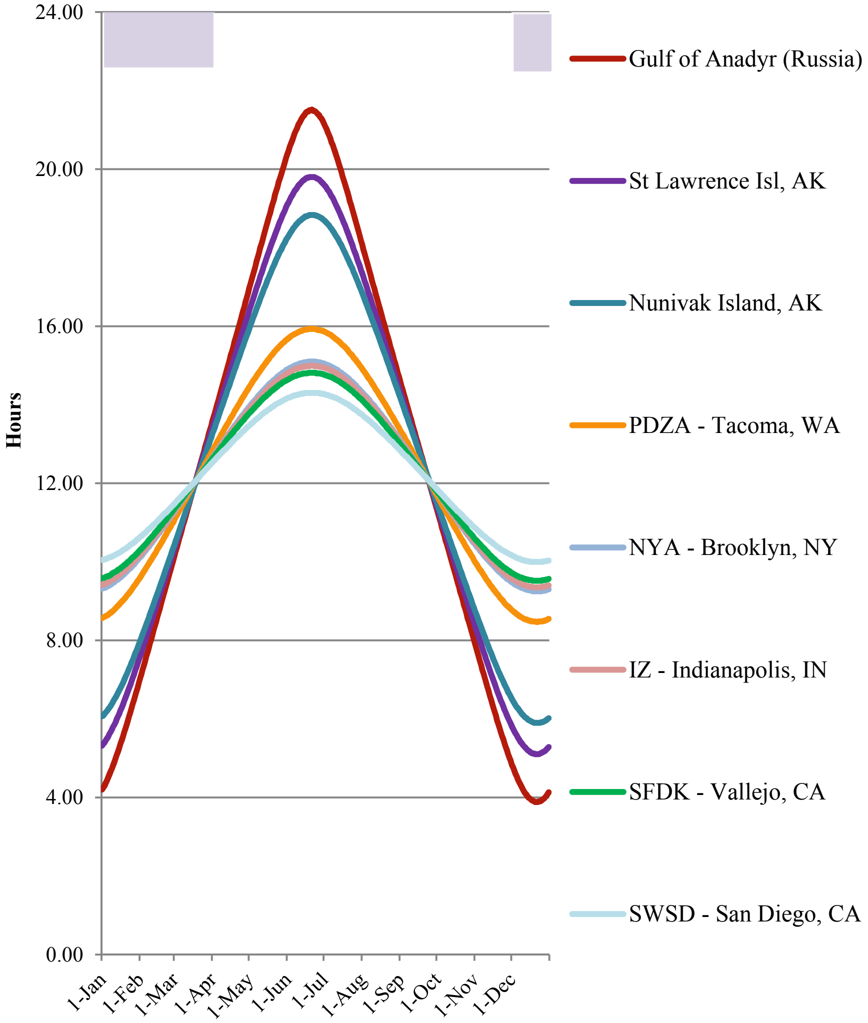


Figure 9. Day length of the Pacific walrus wild population. (Gulf of Anadyr, Russia ———,St Lawrence Island, AK ———, Nunivak Island, AK———), and at the facilities involved in study, during the breeding season. Shaded area represents breeding season of wild population.

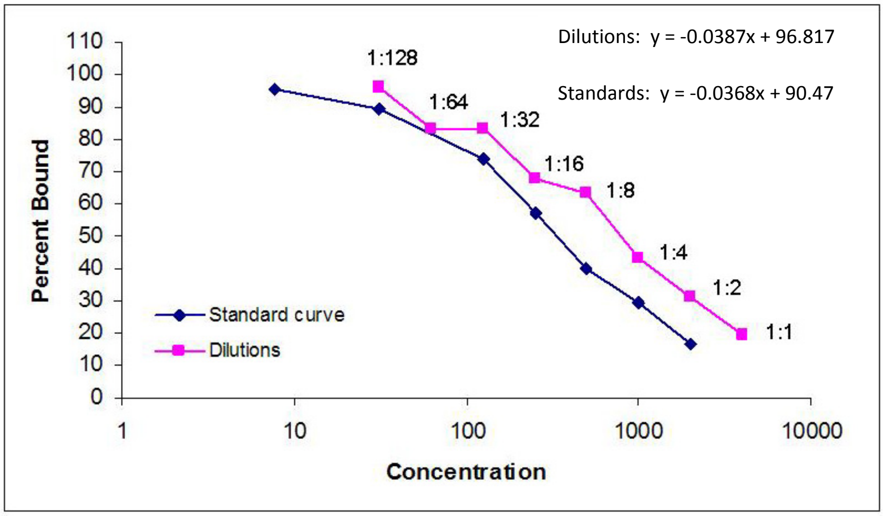


Figure 10. Parallelism curve of serially diluted standards and pooled saliva samples for testosterone. Line equations are for the linear portion of the curves (standards: 31.25 to 2,000 ng/ml; dilutions: 1:1 to 1:128).

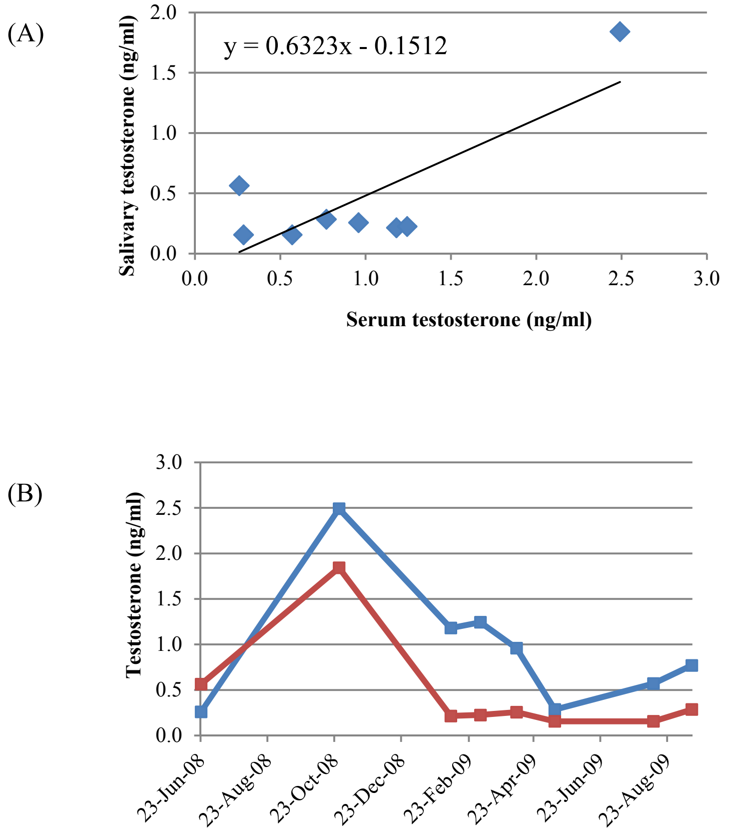


Figure 11. Correlation of testosterone in serum (———) and saliva (———) for male Pacific walrus.

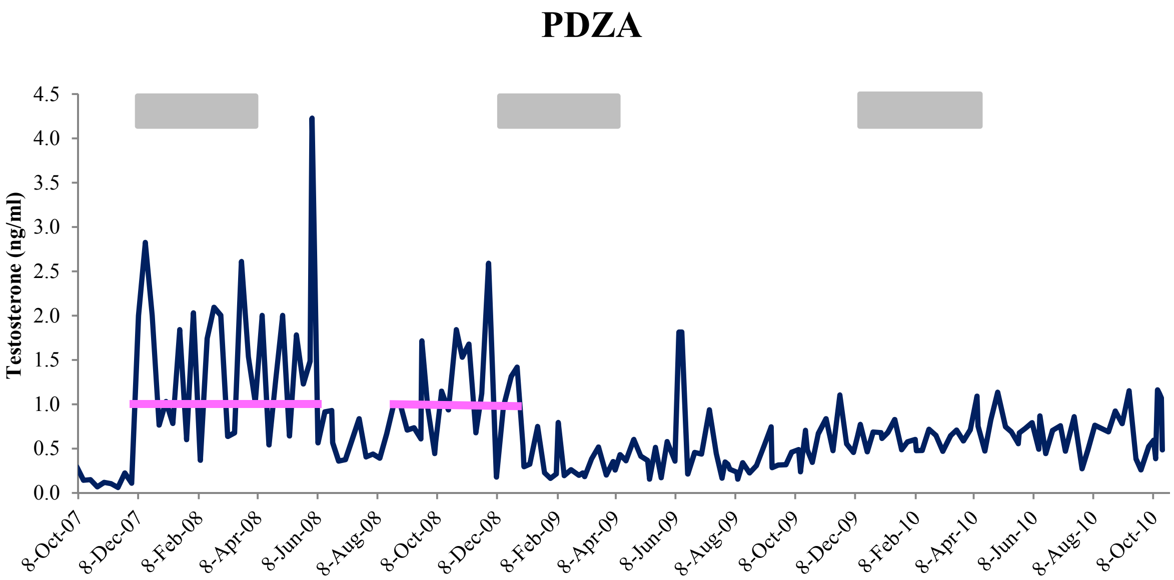


Figure 12. Endocrine profile from the longitudinal measurement of testosterone (———) from a 25 year old male Pacific walrus (PDZA) from October 2007 to October 2010. The pink lines (———) denote periods of elevated testosterone levels (≥0.99). The gray shaded bars denote the wild walrus breeding season.

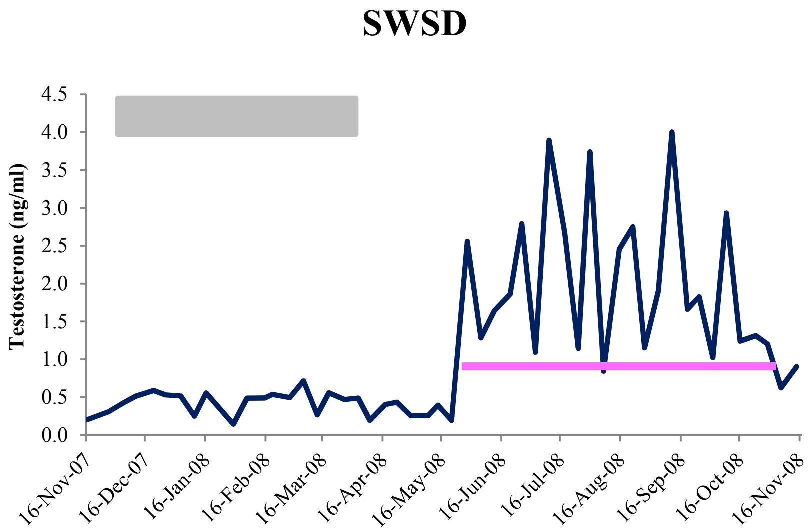


Figure 13. Endocrine profile from the longitudinal measurement of testosterone (——) from a 20 year old male Pacific walrus (SWSD) from November 2007 to November 2008. The pink line (——) denotes periods of elevated testosterone levels (≥0.84). The gray shaded bar denotes the wild walrus breeding season.

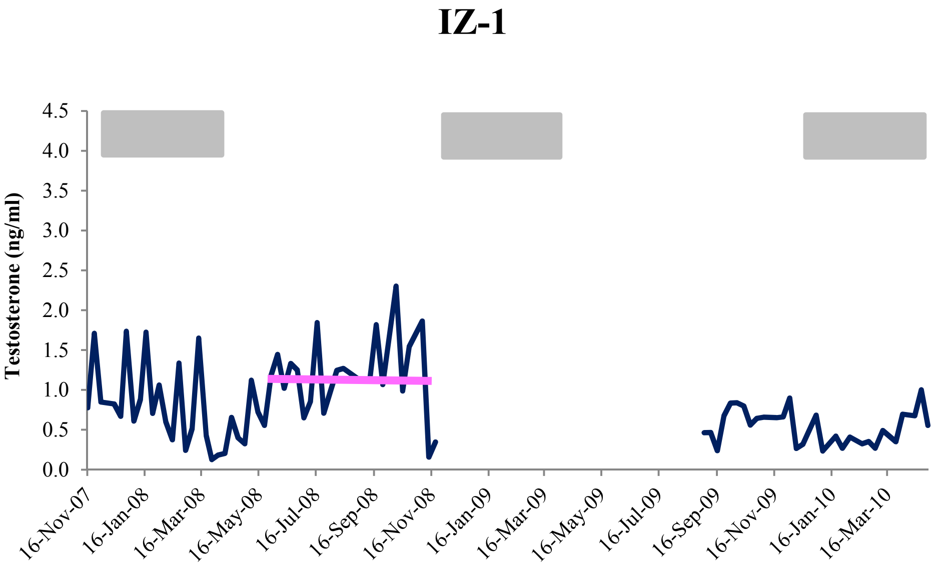


Figure 14. Endocrine profile from the longitudinal measurement of testosterone (———) from a 20 year old male Pacific walrus (IZ-1) from November 2007 to November 2008 and September 2009 to April 2010. The pink line (———) denotes periods of elevated testosterone levels (≥1.23). The gray shaded bars denote the wild walrus breeding season.

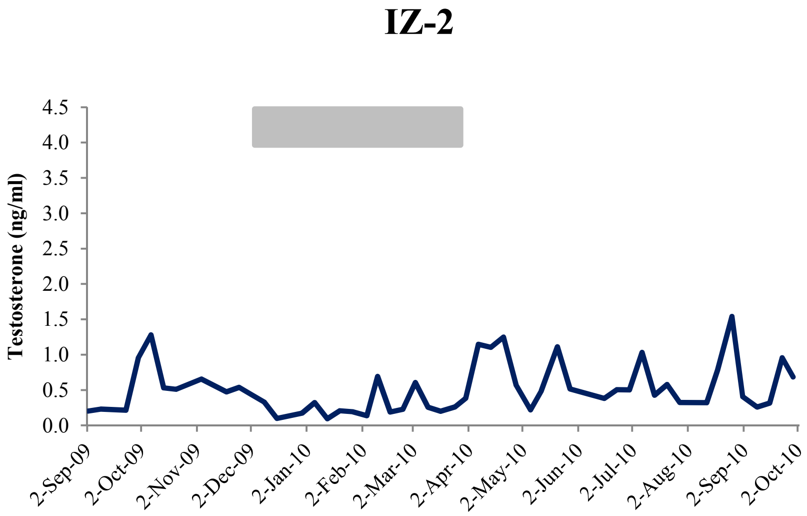


Figure 15. Endocrine profile from the longitudinal measurement of testosterone (———) from a 6 year old male Pacific walrus (IZ-2) from September 2009 to September 2010. The gray shaded bar denotes the wild walrus breeding season. No elevations greater than six weeks in length for this animal (≥0.73).

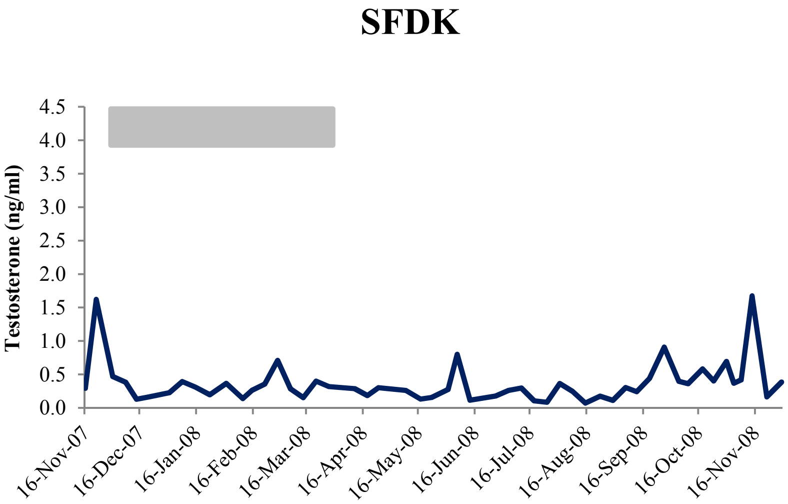


Figure 16. Endocrine profile from the longitudinal measurement of testosterone (———) from a 13 year old male Pacific walrus (SFDK) from November 2007 to November 2008. The gray shaded bar denotes the wild walrus breeding season. No elevations greater than six weeks in length for this animal (≥0.53).

NYA

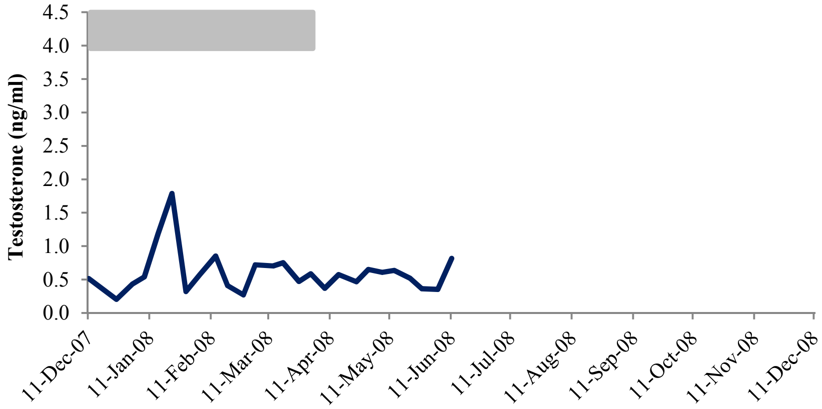


Figure 17. Endocrine profile from the longitudinal measurement of testosterone (———) from a 13 year old male Pacific walrus (NYA) from December 2007 to April 2008. The gray shaded bar denotes the wild walrus breeding season. No elevations greater than six weeks in length for this animal (≥1.06).

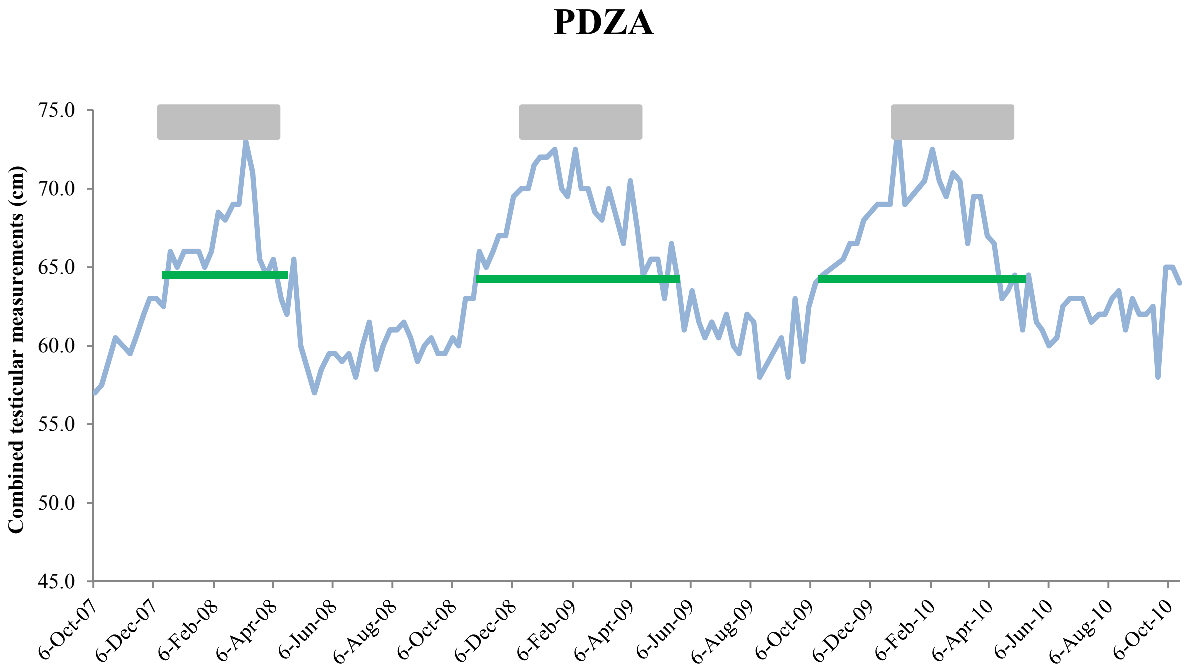


Figure 18. Testicular recrudescence and regression profile from the longitudinal measurement of testes combined measurements (——) and periods of significant elevations (——) (≥64.35) (any blue marks above the green line) for a male Pacific walrus (PDZA) from October 2007 to October 2010. The gray shaded bars denote the wild walrus breeding season.

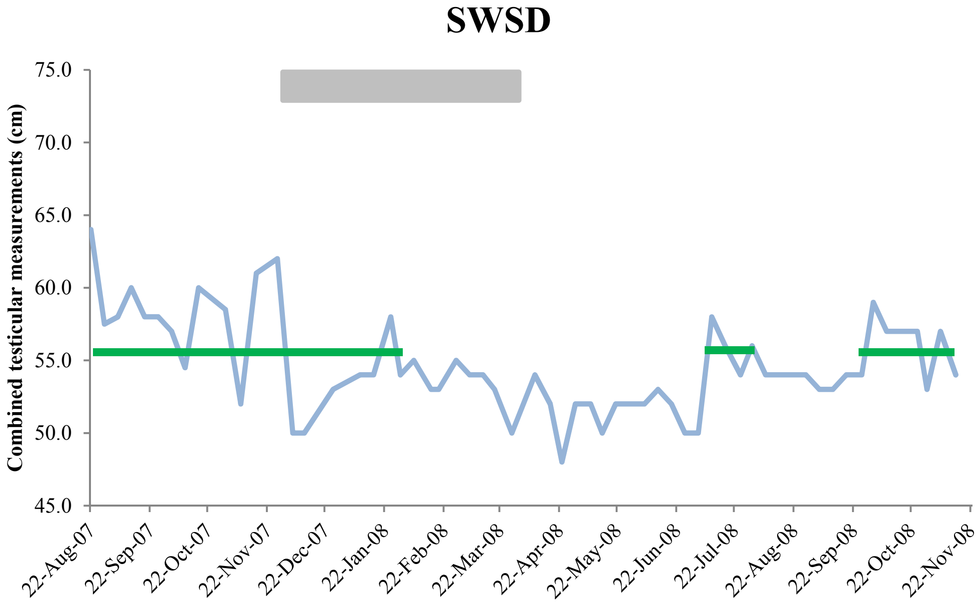


Figure 19. Testicular recrudescence and regression profile from the longitudinal measurement of testes combined measurements (———) and periods of significant elevations (———) (≥55.65) (any blue marks above the green line) for a male Pacific walrus (SWSD) from August 2007 to November 2008. The gray shaded bar denotes the wild walrus breeding season.

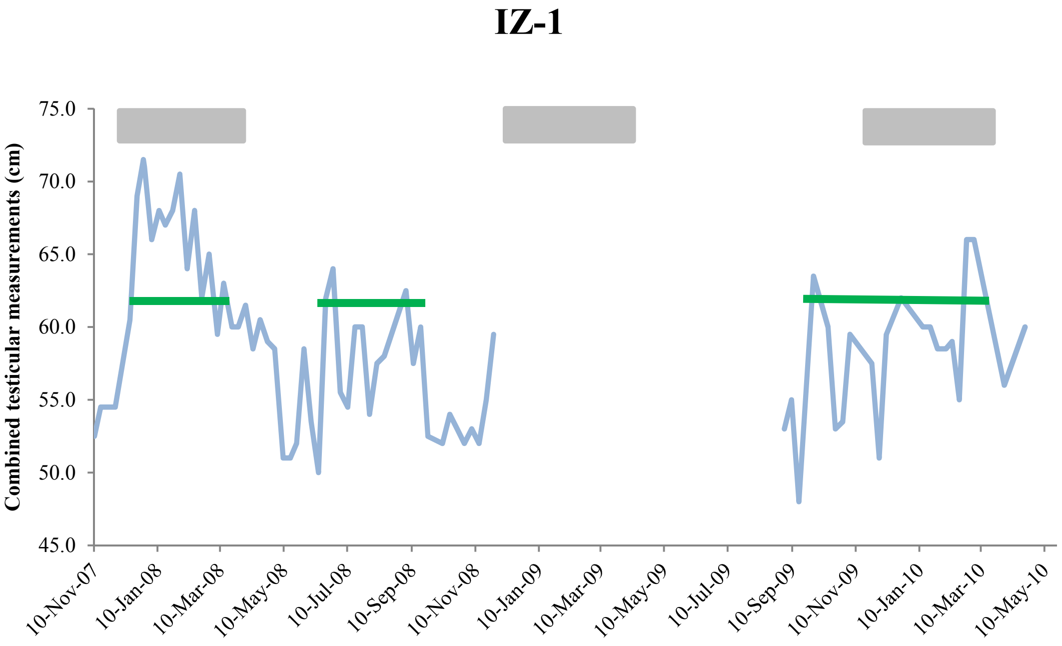


Figure 20. Testicular recrudescence and regression profile from the longitudinal measurement of testes combined measurements (——) and periods of significant elevations (——) (≥62.17) (any blue marks above the green line) for a male Pacific walrus (IZ-1) from November 2007 to November 2008 and September 2009 to April 2010. The gray shaded bars denote the wild walrus breeding season.

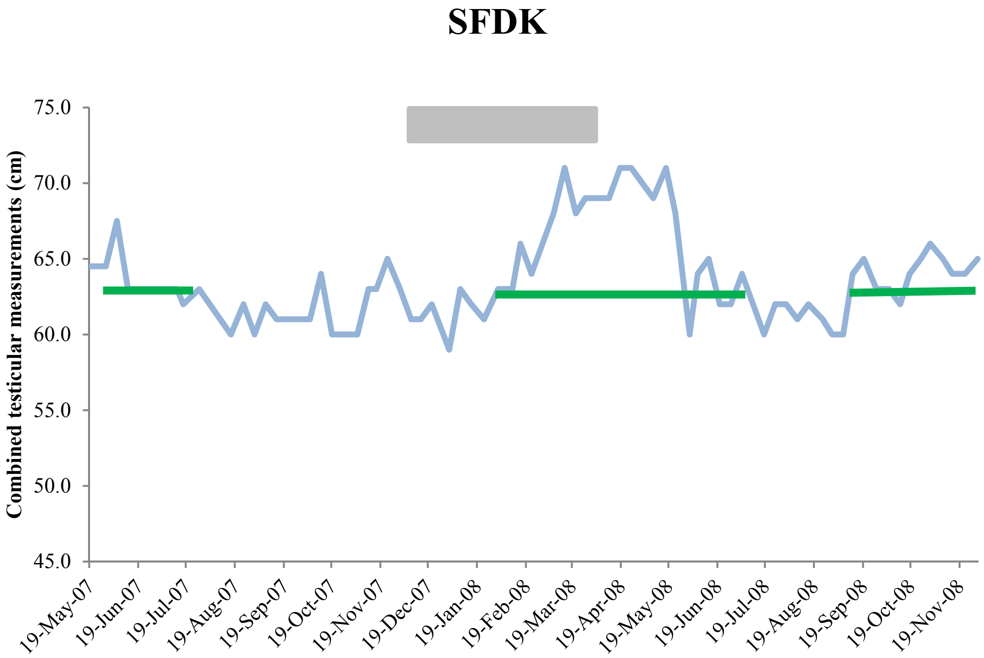


Figure 21. Testicular recrudescence and regression profile from the longitudinal measurement of testes combined measurements (———) and periods of significant elevations (———) (≥61.74) (any blue marks above the green line) for a male Pacific walrus (SFDK) from May 2007 to November 2008. The gray shaded bar denotes the wild walrus breeding season.

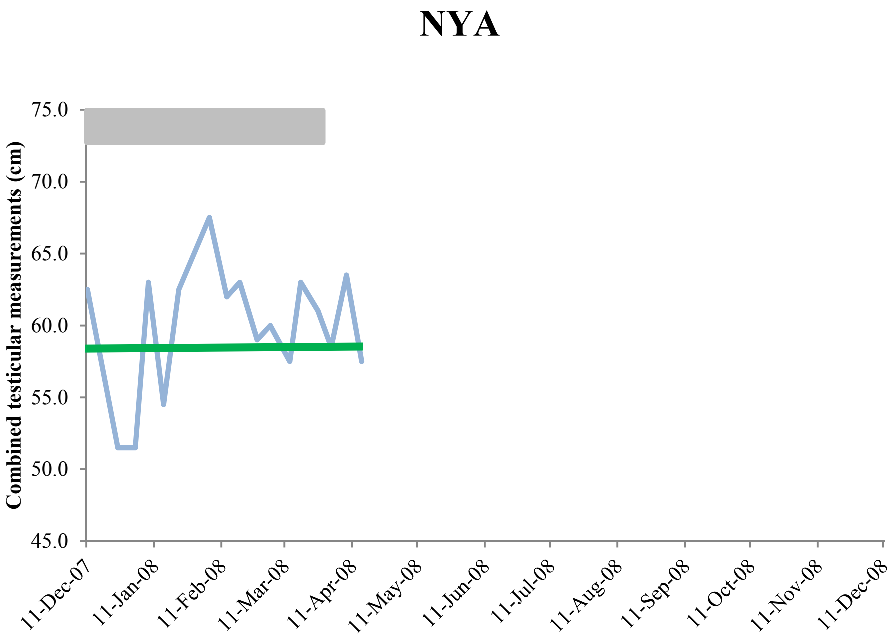


Figure 22. Testicular recrudescence and regression profile from the longitudinal measurement of testes combined measurements (——) and periods of significant elevations (——) (≥58.7) (any blue marks above the green line) for a male Pacific walrus (NYA) from December 2007 to April 2008. The gray shaded bar denotes the wild walrus breeding season.

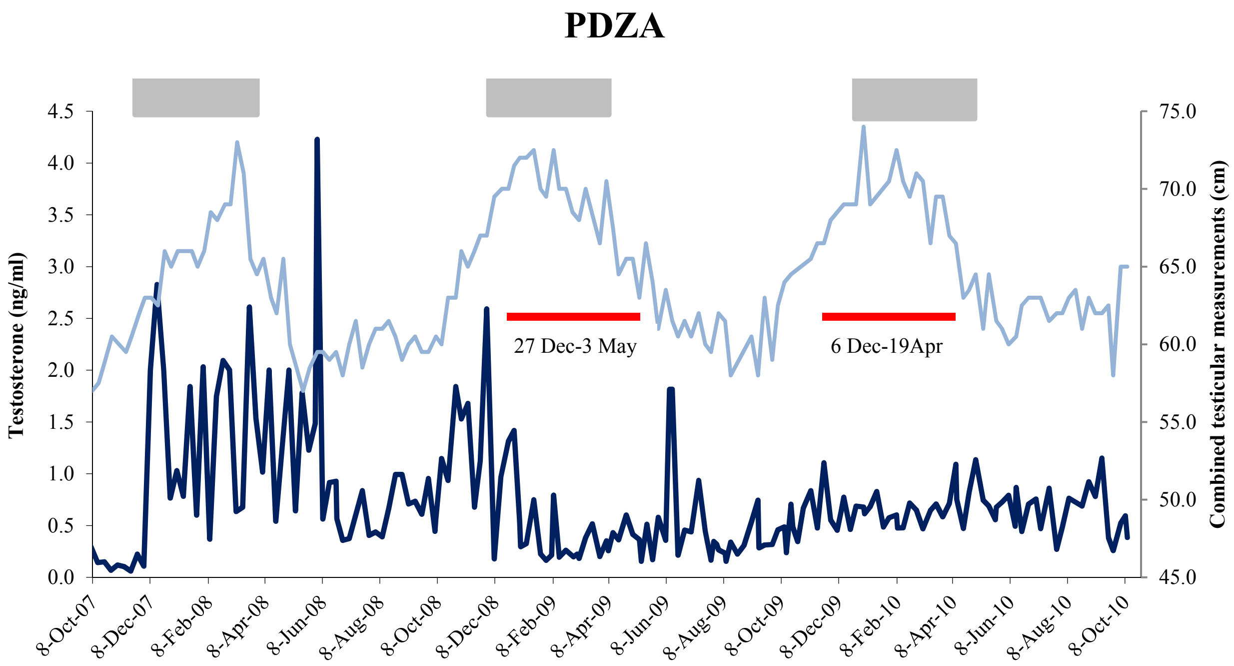


Figure 23. Sperm presence and absence (——) combined with the testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——) and seasonal testosterone levels (Testosterone ——) for a 24 year old male Pacific walrus (PDZA) from October 2007 to October 2010. Sperm was not looked for from October 2007 to October 2008. The gray shaded bars denote the wild walrus breeding season.

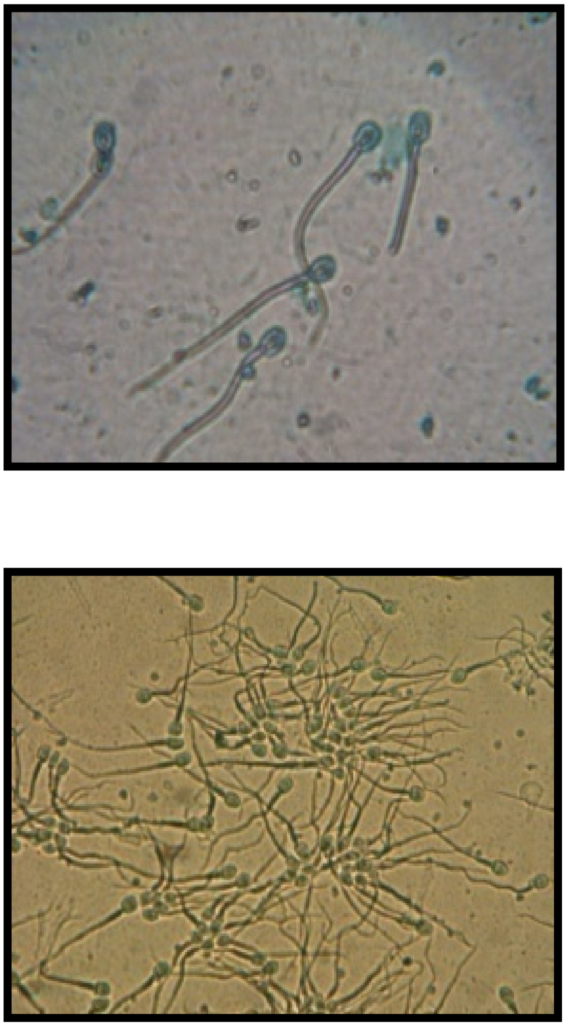


Figure 24. Spermatozoa found in the pre-ejaculate of *ex situ* Pacific walrus.

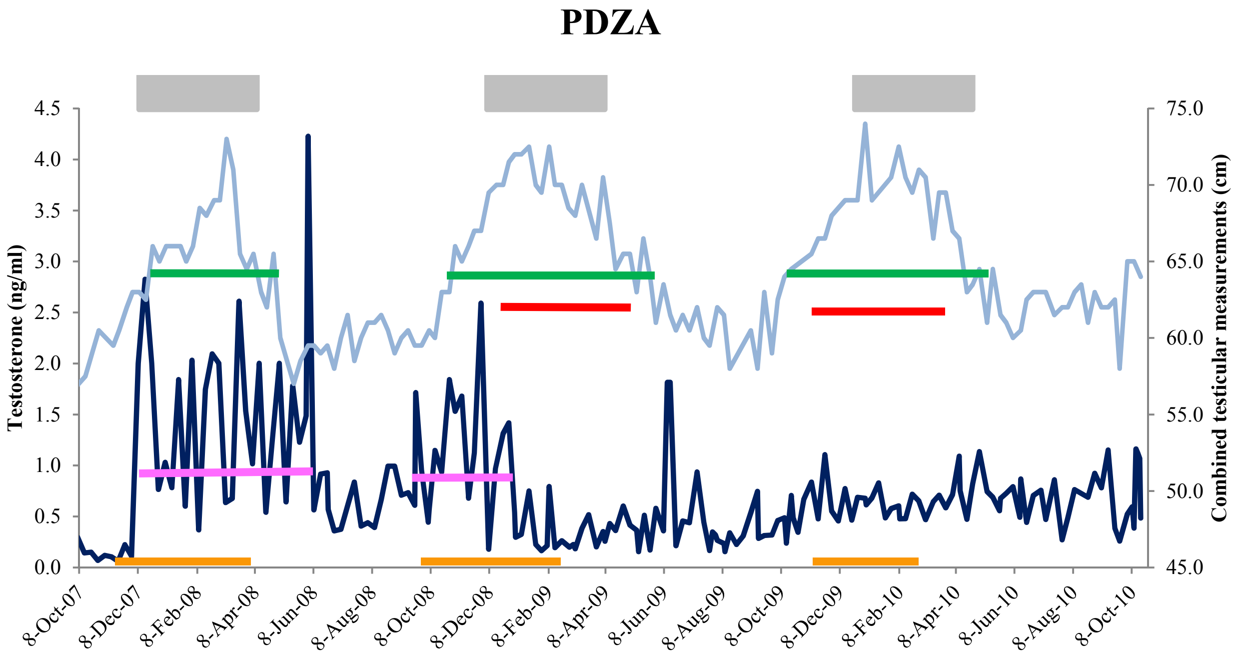


Figure 25. Testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——), seasonal testosterone levels (Testosterone ——), sperm presence and absence (——) and periods of behavioral change (——) for a 24 year old male Pacific walrus (PDZA) from October 2007 to October 2010. The pink lines (——) denote periods of elevated testosterone levels. The green lines (——) denote periods of elevated combined testicular measurements. The gray shaded bars denote the wild walrus breeding season.

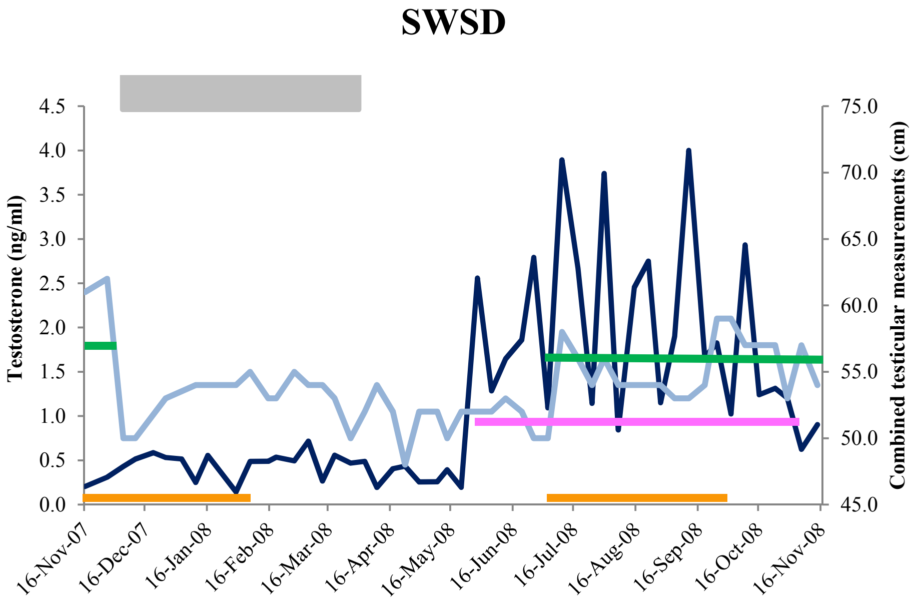


Figure 26. Testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——), seasonal testosterone levels (Testosterone ——) and periods of behavioral changes (——) for a 20 year old male Pacific walrus from August 2007 to November 2008. The pink line (——) denotes periods of elevated testosterone levels. The green line (——) denotes periods of elevated combined testicular measurements. The gray shaded bar denotes the wild walrus breeding season.

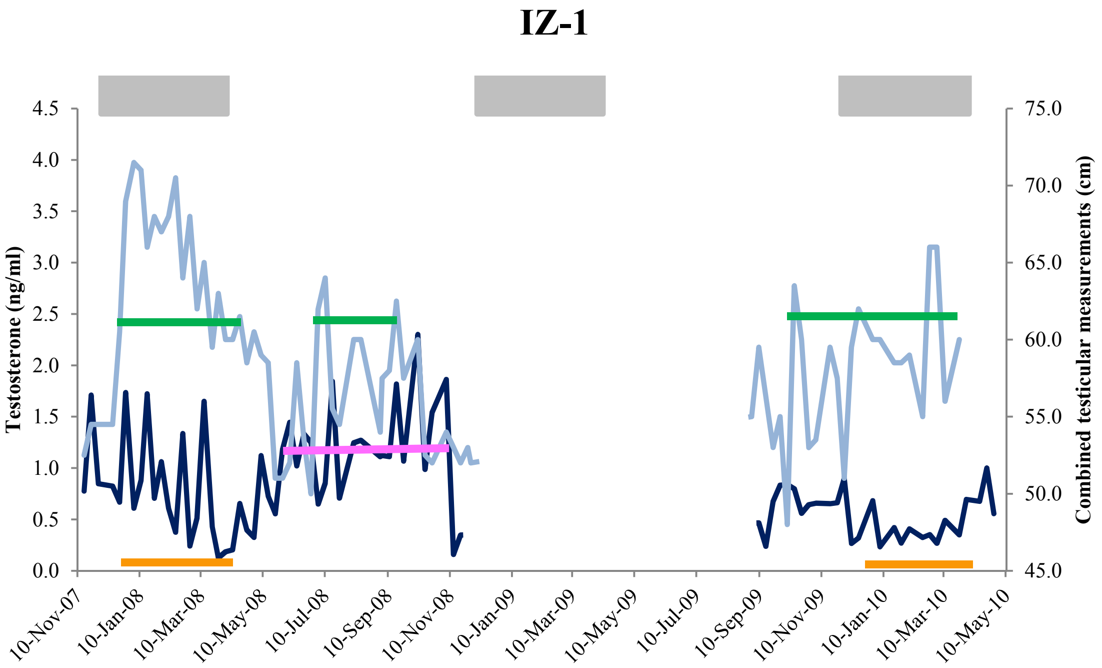


Figure 27. Testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——), seasonal testosterone levels (Testosterone ——) and periods of behavioral changes (——) for a 20 year old male Pacific walrus (IZ-1) from November 2007 to November 2008 and September 2009 to April 2010. The pink line (——) denotes periods of elevated testosterone levels. The green lines (——) denote periods of elevated combined testicular measurements. The gray shaded bars denote the wild walrus breeding season.

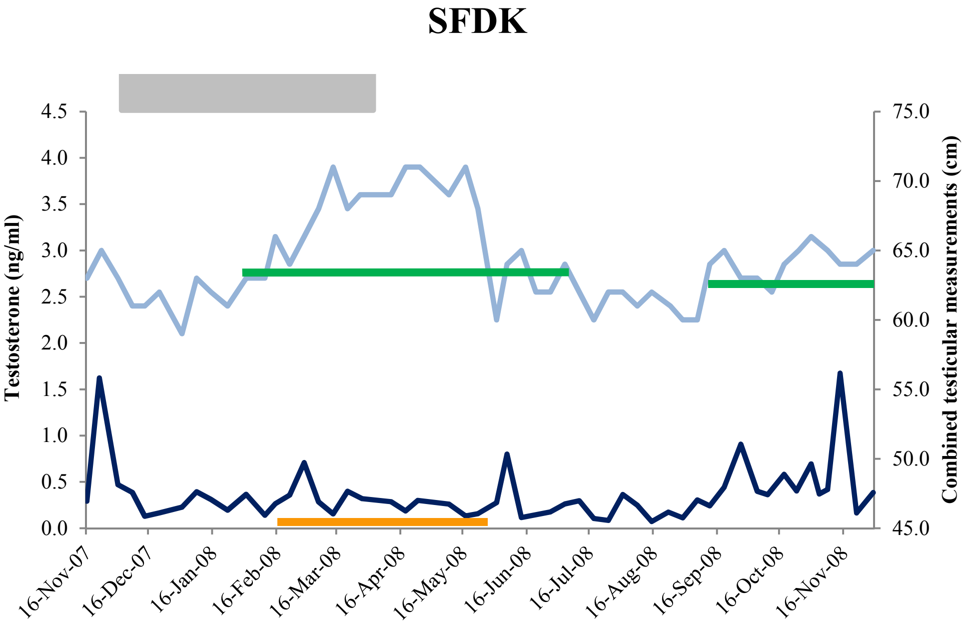


Figure 28. Testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——), seasonal testosterone levels (Testosterone ——) and periods of behavioral changes (——) for a 13 year old male Pacific walrus (SFDK) from May 2007 to November 2008. No testosterone elevations greater than six weeks in length for this male (≥0.53). The green line (——) denotes period of elevated combined testicular measurements. The gray shaded bar denotes the wild walrus breeding season.

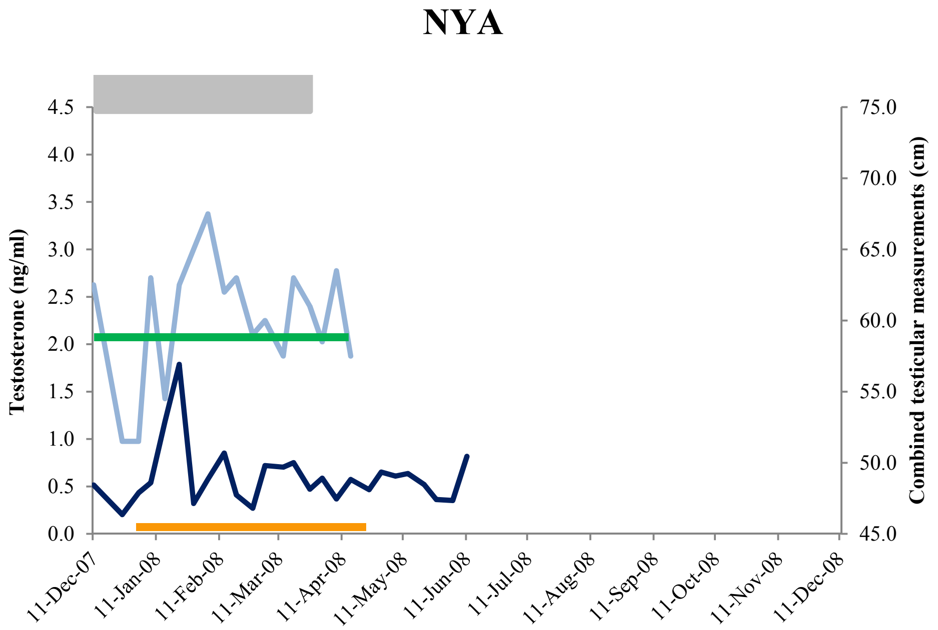


Figure 29. Testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——), seasonal testosterone levels (Testosterone ——) and periods of behavioral changes (——) for a 13 year old male Pacific walrus (NYA) from December 2007 to April 2008. No testosterone elevations greater than six weeks in length for this male (≥1.06). The green line (——) denotes period of elevated combined testicular measurements. The gray shaded bar denotes the wild walrus breeding season.

PDZA

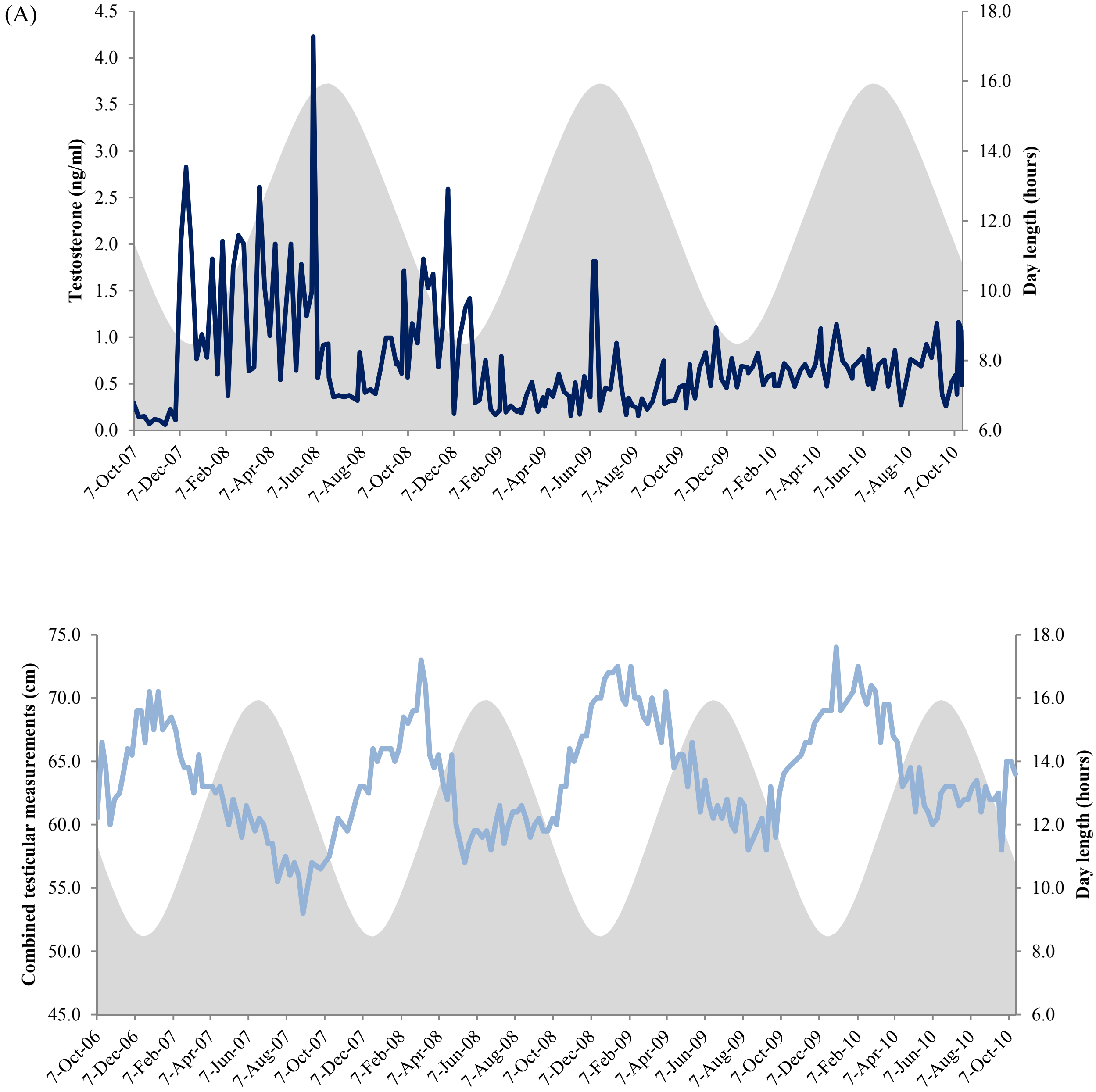


Figure 30. Day length (▨) versus (A) testosterone (—) concentrations and (B) combined testicular measurements (—) for the PDZA (47°18'N) male walrus.

SWSD

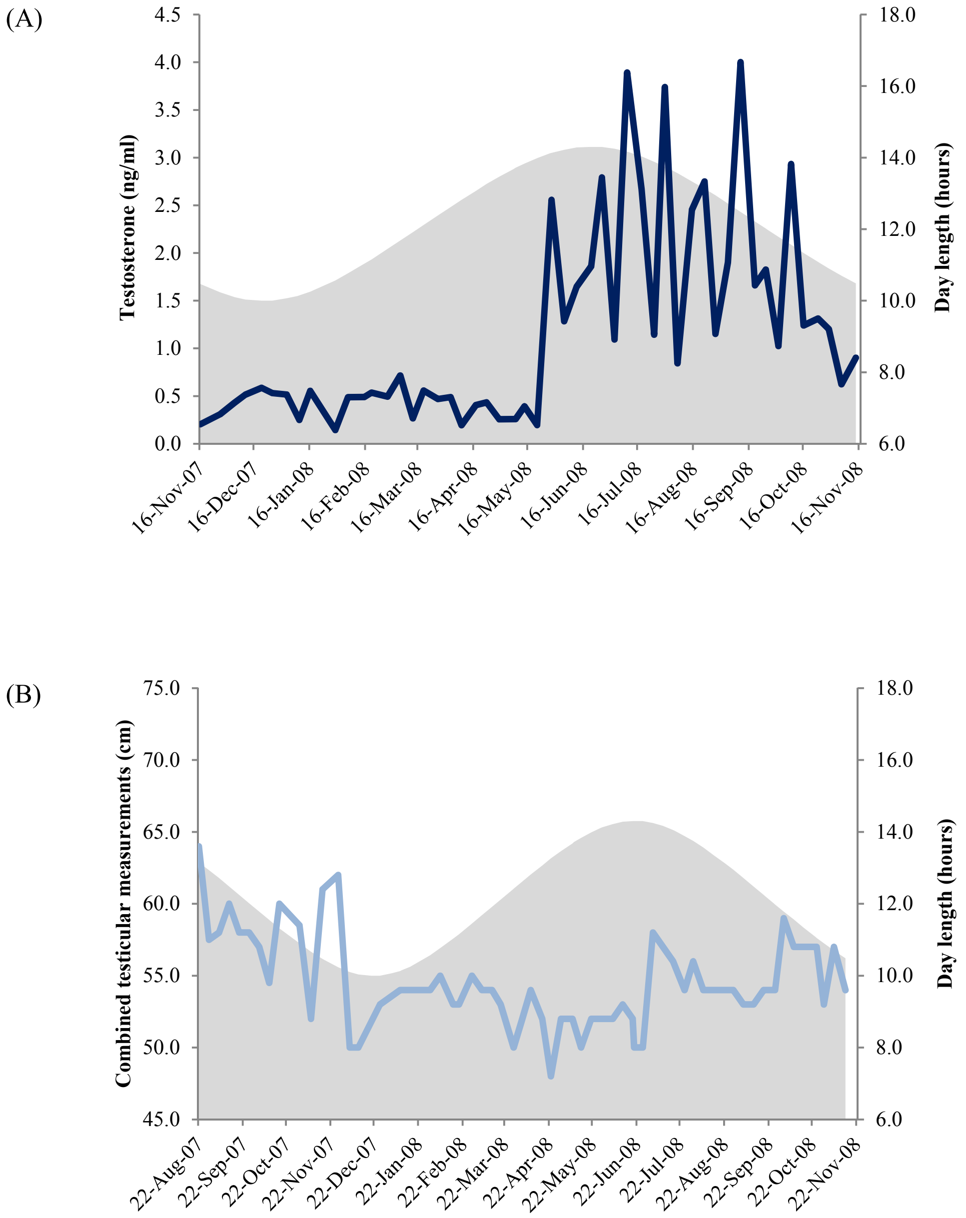
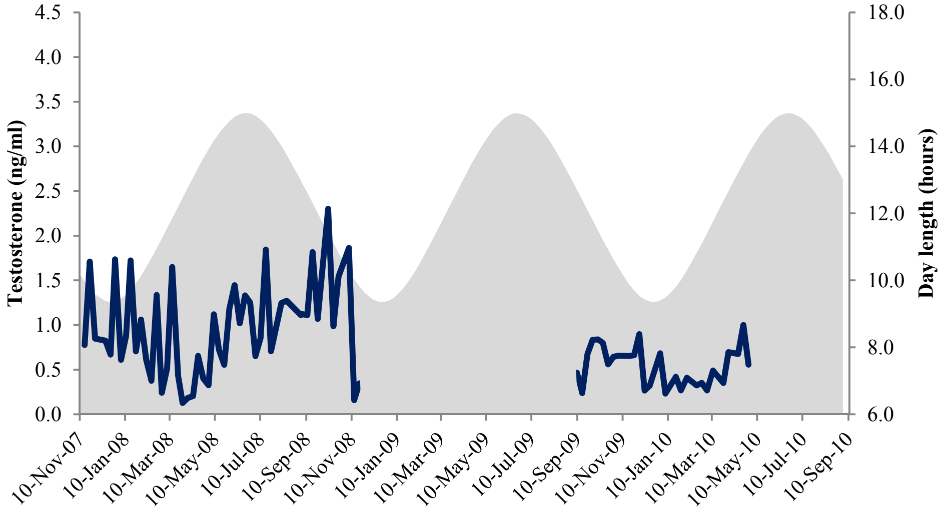


Figure 31. Day length (▨) versus (A) testosterone (—) concentrations and (B) combined testicular measurements (—) for the SWSD (32°45'N) male walrus.

IZ-1

(A)



(B)

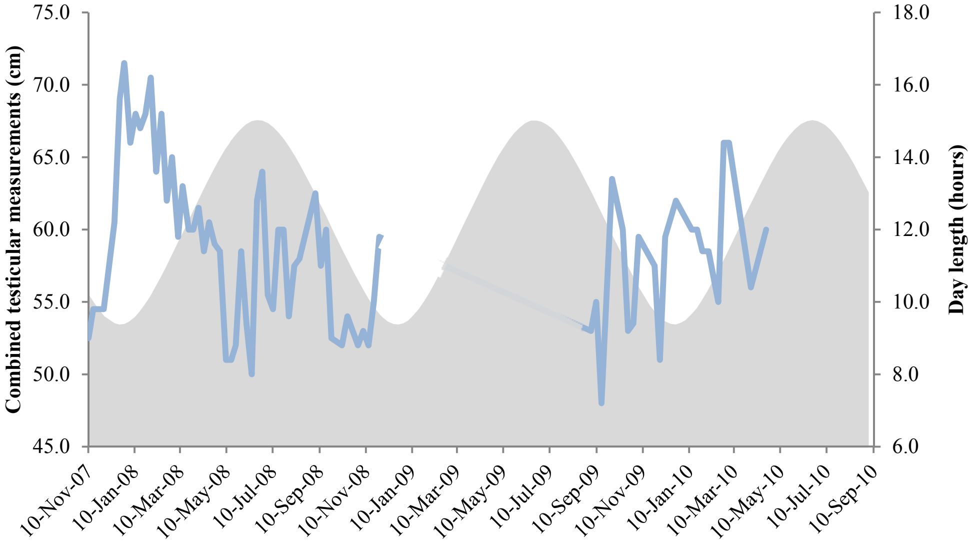


Figure 32. Day length (▨) versus (A) testosterone (——) concentrations and (B) combined testicular measurements (——) for the IZ-1 (39°46’N) male walrus.

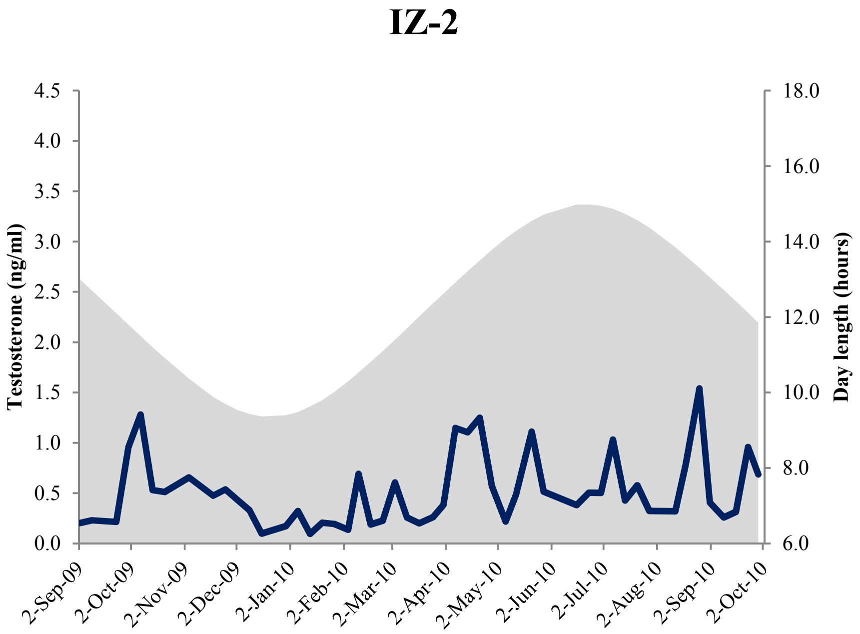


Figure 33. Day length (▨) versus testosterone (——) concentrations for the IZ-2 (39°46'N) male walrus.

SFDK

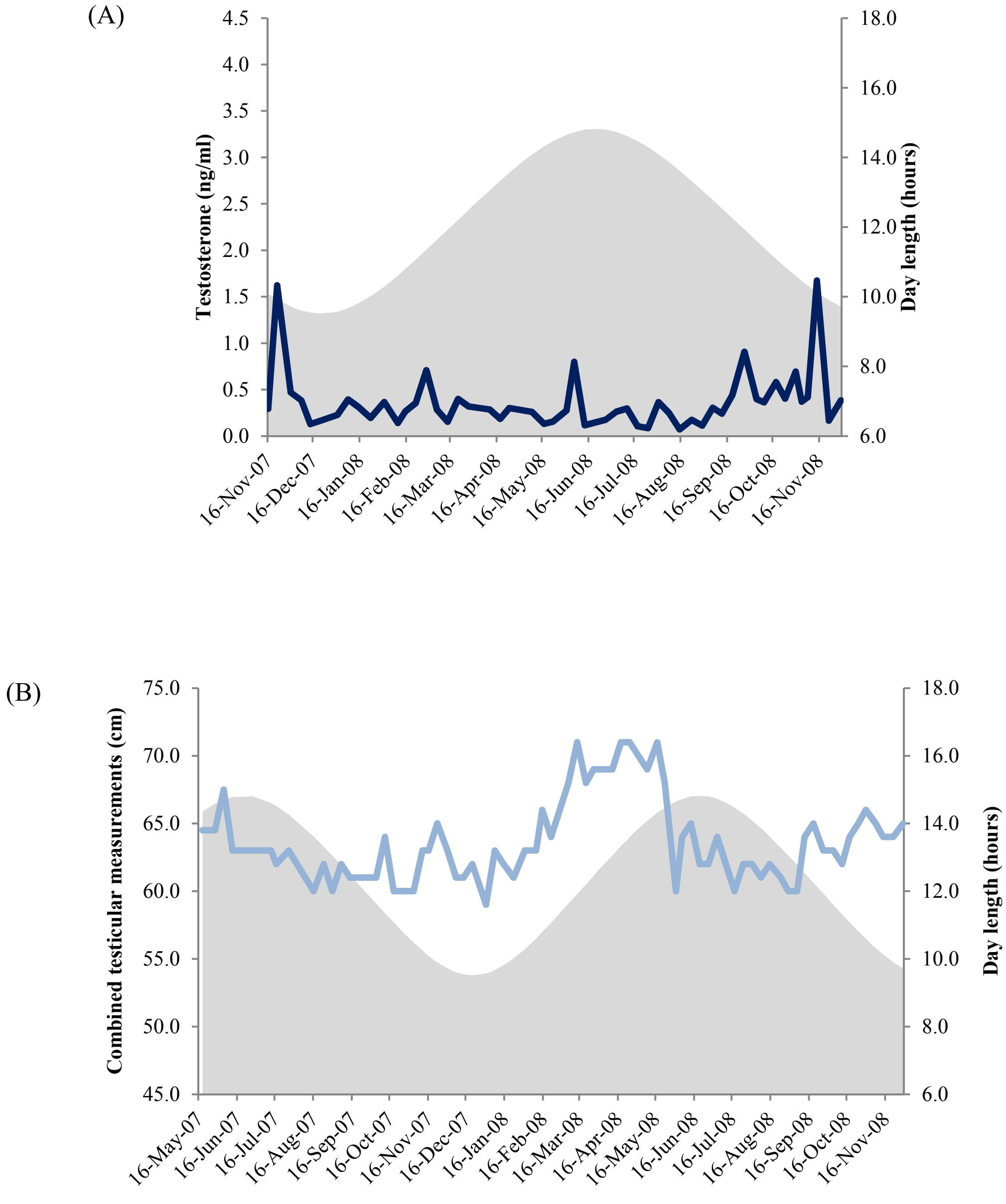


Figure 34. Day length (▨) versus (A) testosterone (—) concentrations and (B) combined testicular measurements (—) for the SFDK (38°08'N) male walrus.

NYA

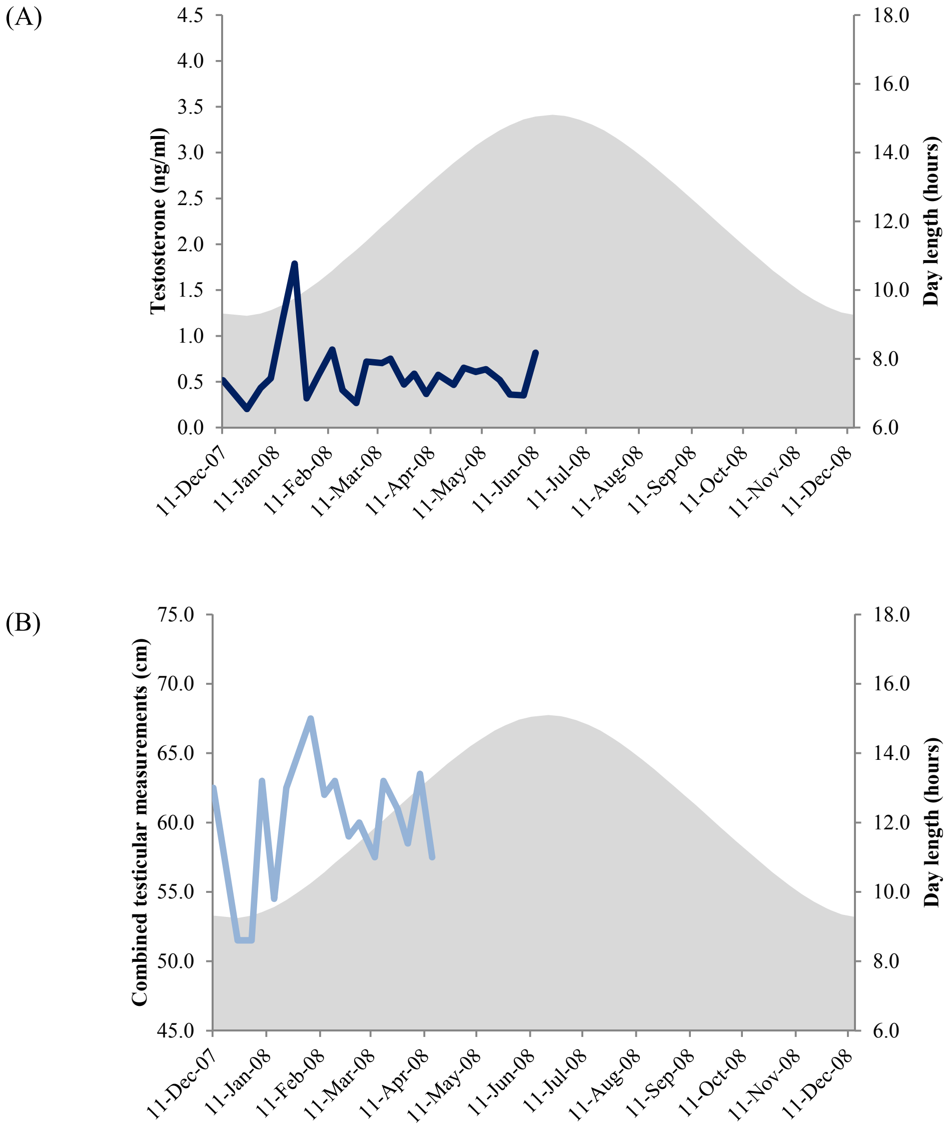


Figure 35. Day length (▨) versus (A) testosterone (—) concentrations and (B) combined testicular measurements (—) for the NYA (40°34’) male walrus.

PDZA

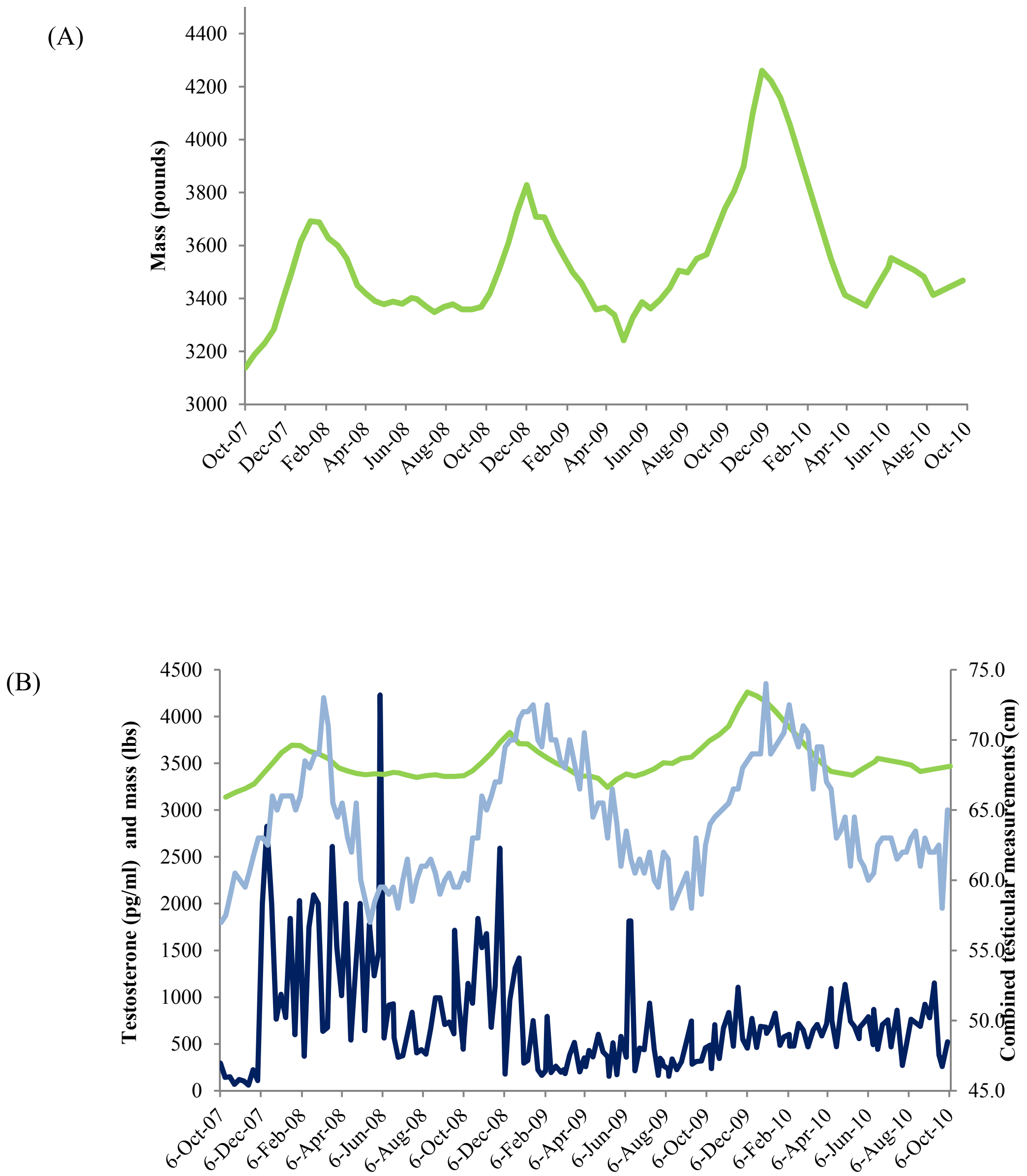


Figure 36. (A) Body mass measurements (——) and (B) body mass measurements combined with the testicular recrudescence and regression profile from the longitudinal measurement of testes (Combined testicular measurements ——) and seasonal testosterone levels (Testosterone ——) for the PDZA male walrus from October 2007 to October 2010.

Parameters of Reproduction in Female Odobenids

Introduction

Walrus in U.S. facilities have had limited reproductive success and the population is currently not self-sustaining. Population management of zoo and aquaria species requires an understanding of basic reproductive biology to elucidate abnormalities and improve reproductive success. Assessing reproductive status is important to the effective management of *ex situ* populations to evaluate sexual maturity, fertility and reproductive status to provide guidance for breeding recommendations or separations and the overall success of a breeding plan. Hormones are involved in all aspects of reproduction and defining normal reproductive endocrine patterns is an important step in characterizing the reproductive biology of any species. Particularly absent in the zoo and aquaria walrus population is a comprehensive understanding of the endocrine patterns of the female reproductive cycle. Specifically lacking are the interactions between progesterone concentrations and pregnancy and pseudopregnancy, estrogens and estrus, and the effects of photoperiod on reproductive cycles and female reproductive senescence.

This research project represents a compilation of physiological data as well as behavioral observations for *ex situ* walrus. Assessing the presence or absence of ovarian cyclicity can assist in determining the appropriate individual animals to be included in a breeding plan and what individuals are candidates for treatment of infertility. In addition, the diagnosis of pregnancy before parturition can greatly improve the survival of neonates by giving animal care providers an opportunity to prepare appropriate husbandry and medical conditions.

The overall goal of the study was to non-invasively define endocrine and behavioral parameters of reproduction in female walrus, with the following specific objectives: 1) develop a non-invasive technique for monitoring ovarian steroidogenic activity; 2) validate a protocol to measure progesterone and estradiol by enzyme immunoassay; 3) establish a database of endocrine norms for female walrus in U.S facilities; 4) define endocrine patterns of estrus (female reproductive receptivity) through the longitudinal measurement of salivary estradiol; 5) define the endocrine patterns of pregnancy and pseudopregnancy through the longitudinal measurement of salivary progesterone; 6) define behavioral changes in females associated with hormonal fluctuations; and 7) evaluate the effects of seasonality and associated photoperiod on reproduction. The knowledge gained has great potential as valuable tools towards the management of the zoo-based U.S. walrus population and could improve the reproductive success for the species.

Materials and Methods

Subjects

Study animals consisted of six female Pacific walrus (ages 12-27; Table 10). Prior to the study, one female at the Point Defiance Zoo & Aquarium (PDZA) had calved twice and two, one at the New York Aquarium (NYA) and one at Six Flags Discovery Kingdom (SFDK), were primiparous. Study animals were trained to allow voluntary saliva and blood collection. Five of the six animals were maintained in a “breeding situation” (mixed-gender conditions) throughout the study period. All animals were housed outdoors with exposure to natural fluctuations in photoperiod. PDZA and NYA maintained natural sea water systems, while SFDK and the Indianapolis Zoo (IZ) produced artificial salt water. All four facilities fed diets of capelin

(*Mallotus villosus*), herring (Pacific – *Clupea pallasii* or Atlantic – *Clupea harengus*), and various clam species. In addition, three of the four facilities fed squid (*Loligo opalescens;* PDZA, NYA, IZ) and one facility supplemented with Pacific mackerel (*Scomber japonicas;* PDZA).

Saliva Collection

Saliva samples were collected from the six subject female walrus for 12 to 55 months. Saliva sample collection occurred daily from October through April (breeding season and buffers) and twice weekly during the months of May through September (non-breeding season) during the period 2007-2011. Prior to the first feed (between 8:30 and 10:30 am local time), saliva was collected from the subjects' mouths using three sites: under the tongue, base of the tongue and the sides of the mouth. The cotton-tipped applicators were then placed in 5 ml sealed cryotubes and frozen at a minimum of -20°C until analyzed. Five of six females were maintained in a "breeding situation" (mixed-gender conditions) throughout the study period.

Serum Collection

Serum samples were collected from the two PDZA female walrus opportunistically (generally on a monthly basis) from June 2007 to December 2011. Blood was obtained from the epidural intravertebral sinus using a 16 gauge, 5.5 inch (14 cm) spinal needle or the proximal rear flipper, from a branch of the lateral saphenous vein, using a 21 gauge, 1.5 inch (3.8 cm) needle, and transferred to a tube with serum separator gel. Blood was centrifuged at 3,500 rpm for five minutes and the resulting sera poured into a cryotube and stored at a maximum of -20 °C until analyzed.

Assay Procedures

Sample Extraction and Dilution

Reproductive steroid hormones were extracted from saliva samples prior to analysis to concentrate the hormones. For extractions, one ml of 90% methanol was added to each saliva tube. Samples were then placed on a microplate shaker for one hour and subsequently air dried with a drying manifold to remove methanol. Each sample was reconstituted by adding 0.5 ml of assay buffer into the cryotube, and then refrigerated at 4°C if it was to be assayed within the next few days, or if not, stored in the freezer at a maximum of -20°C until analyzed.

All samples were assayed in duplicate and estimates of raw hormone concentrations were generated by a Tecan Sunrise microplate reader at a wavelength of 405 nm (Tecan Group Limited, Männedorf, Switzerland) utilizing a four parameter logistic curve fitting program (Tecan Sunrise, Magellan CE V 3.0). The final hormone concentrations were calculated as: (raw hormone concentration) x (ml assay buffer used to reconstitute sample x dilution factor). All salivary hormone data were expressed on a per gram wet weight basis (ng/ml).

Progesterone Assay

Saliva samples were analyzed utilizing progesterone competitive enzyme immunoassay (EIA) kits (Enzo Life Sciences International, Inc. [previously Assay Designs, Inc.] Farmingdale, New York, USA, ADI-900-008 and ADI-900-011). Cross-reactivity, sensitivity and precision data were provided by Enzo Life Sciences EIA progesterone kits. The progesterone antibody cross reacted 100% with progesterone and 5α-pregnane-3,20-dione, 3.46% with 17-OH-progesterone, 1.43% with 5-pregnen-3β-ol-20-one, 0.77% with corticosterone, 0.28% with 4-androstene-3,17-

dione, 0.056% with deoxycorticosterone, 0.013% with DHEA and <0.001% with 17β-estradiol, estrone, estriol, testosterone, hydrocortisone, 5α-pregnana-3α,20α-diol and danazol. The sensitivity of the progesterone assay was 8.57 pg/mL. Intra-assay and inter-assay indices of precision were determined by taking samples containing low, medium and high concentrations of progesterone and running the samples multiple times through the assay. Intra-assay coefficients of variation were 7.6, 5.4 and 4.9%, respectively. Inter-assay coefficients of variation were 6.8, 8.3 and 2.7%, respectively.

Estradiol-17β Assay

Saliva samples were analyzed utilizing estradiol-17β competitive EIA kits (Enzo Life Sciences International, Inc., [previously Assay Designs, Inc.] Farmingdale, New York, USA, ADI-900-008 and ADI-900-011). Cross-reactivity, sensitivity and precision data were provided by Enzo Life Sciences EIA estradiol-17β kits. The estradiol-17β antibody cross reacted 100% with estradiol-17β , 4.64% with estrone, 0.53% with estriol, 0.36% with estrone-3-sulfate, 0.06% with progesterone, 0.02% with testosterone, <0.2% with 17α-ethynylestradiol, <0.02% with 19-hydroxytestosterone and 17α-estradiol, and <0.001% with dehydroisoandrosterone and dehydroisoandrosterone-3-sulfate. The sensitivity of the estradiol-17β assay was 28.5 pg/mL. Intra-assay and inter-assay precision were determined by taking samples containing low, medium and high concentrations of estradiol-17β and running the samples multiple times through the assay. Intra-assay coefficients of variation were 8.4, 8.1 and 9.2%, respectively. Inter-assay coefficients of variation were 7.4, 6.1 and 5.2 %, respectively.

Assay Validation – Progesterone and Estradiol-17β

The progesterone and estradiol-17β EIAs used in the study were subjected to validation in walrus by three techniques: assessing EIA parallelism of pooled, serially diluted samples against the standard curve, direct correlation of hormones in serum and saliva and the relationship of measured hormone levels to physiological events.

To test parallelism, walrus saliva samples were taken before, during and after breeding season to provide material containing a mean value for hormones. Pooled samples were serially diluted (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) and analyzed (inspection of plots and determination of slopes of linear portions of plots) with EIA kits for respective hormone to determine whether the concentration-response relationship for the "unknown" was similar to that for the standard analyte (standard curve). Similarity was interpreted to indicate the assay could reliably measure steroids in the sample over the entire range of the assay kit standards.

Endocrine Profiles/Reproductive Patterns

Basal and peak levels of progesterone were determined for each individual by an iterative process, in which the average concentration of all samples was calculated, then high values were excluded if they exceeded the mean plus 1.75 standard deviations (Pelican *et al*. 2005, Bateman *et al*. 2009). The average was then recalculated and the elimination process repeated until no values exceeded 1.75 standard deviations above the mean. The average of the remaining values was considered baseline for the animal while values greater than baseline were considered elevated. The length of an elevated progesterone period was calculated from the initial progesterone increase (i.e. at least three consecutive samples with elevated progesterone) until progesterone levels returned to basal values (15+ days below elevated level) (Brown, pers. comm.). To adequately describe follicular and luteal phases of the estrous cycle, at least twice weekly sampling was required. Gaps in data were defined as six or more days between successive samples. Luteal phases were defined as the interval between periods of elevated progesterone concentrations.

Behavior

Behavioral changes between the breeding and non-breeding seasons for two female walrus (PDZA) were evaluated over six time periods from 2007 to 2009. Behavioral data were collected by instantaneous scan sampling during December-March (breeding period for wild walrus) and June-September (non-breeding period) for a total of 158 and 145 observational hours per female, respectively. The observation sessions were 20 minutes long, divided into 30 second sample intervals (Appendix 2). Days were broken into four 2-hour time blocks (0730-0930, 1000-1200, 1230-1430, and 1500-1700) with samples taken randomly but equally dispersed among the periods. All observations occurred while the animals were in the main exhibit (Figure 37). Behaviors recorded included: reproductive behavior (female solicitation of male, clasping and penetration), general activity, location and vocalizations (Appendix 12). A female was deemed to be in estrus if she solicited the male and then allowed clasping or penetration. Independent variables were month, within-day time period, season (breeding and non-breeding) and year.

All statistical analyses of behavioral data were conducted using R (R version 2.12.2, The R Foundation for Statistical Computing, Vienna, Austria). Two-way ANOVAs were utilized to test the influence of the independent variables on behavior, determine the main effect of contributions of each independent variable and identify if any significant interaction effects between the independent variables existed. A Tukey post-hoc comparison was used to decipher which aspects of each variable were significant. T-tests were used when only one variable (i.e. season) was being examined for effect when that variable only had two treatments (breeding vs. non-breeding).

Staff at participating facilities recorded behavioral notes on a weekly basis regarding breeding behavior during the time periods of each facility's participation in the study.

Seasonality

Walrus are considered seasonal breeders, with the wild population breeding during the short days of winter (Figure 38). Day lengths, at each location of subject walrus residence, were compared to progesterone and estradiol-17β levels of individual females to evaluate effects of photoperiod via correlation analysis. Day lengths for specific latitudes were acquired from an internet source (http://www.timeanddate.com). Correlation tests were conducted to determine significance at the $p \leq 0.05$ level.

Results

Assay Validation

Progesterone

The curve generated from the serially diluted pooled saliva from female walrus to assess parallelism against the standard curve for progesterone was parallel to its respective standard curve (Figure 39). The progesterone dilution curve displayed dilution linearity to the standard curve, indicating that the sample hormone cross-reacts with the antibody in a manner immunologically similar to the standard hormone. The linear portions of the curves (standards: 125 to 2,000 ng/ml; dilutions: 1:1 to 1:32) were parallel. The slope of the dilutions was -0.0386, and the slope of the standards was -0.0374. Sample dilution was determined by the 50% binding rate (P=1:6). A 1:6 dilution at the 50% binding rate indicates that there is sufficient sensitivity in progesterone EIA for salivary progesterone.

Correlation of progesterone in serum and saliva was found to be significant (PDZA-1: $r=0.543$; $p<4.136E-05$; PDZA-2: $r=0.188$, $p<0.0002$) (Figure 40A; Figure 41A). Saliva samples were pooled by week for each of the PDZA female walrus. Salivary progesterone values tended to be erratic in the dynamic profiles and pooling helped decrease the “noise” in samples. The assay was difficult to validate via physiological findings (by collecting samples before and after a known physiological event – breeding season). Salivary hormone profiles also were evaluated relative to observed behavioral estrus showing some similarities (Figure 40B; Figure 41B).

Estradiol-17β

The curve generated from the serially diluted pooled saliva from female walrus to assess parallelism against the standard curve for estradiol-17β was parallel to its respective standard curve (Figure 42). The estradiol-17β dilution curve displayed dilution linearity to the standard curve, indicating that the sample hormone cross-reacts with the antibody in a manner immunologically similar to the standard hormone. The linear portions of the curve (standards: 234.375 and 3,750 ng/ml; dilutions: 1:1 to 1:16) were parallel. The slope of the dilutions was

-0.0129, and the slope of the standards was -0.0110. Sample dilution was determined by the 50% binding rate (E_2=1:1). A 1:1 dilution at the 50% binding rate indicates that there is not a lot of sensitivity in the estradiol-17β EIA assay for E_2.

Direct correlation of estradiol-17β in serum and saliva was found to be low (PDZA-1: $r=0.455$, $p<0.001$; PDZA-2: $r=0.147$, $p<0.001$) (Figure 43). Salivary hormone profiles were also evaluated relative to observed behavioral estrus and were found to be unsuitable indicators of the follicular phase due to a lack of correspondence between E_2 and estrus (Figure 44; Figure 45). Estradiol-17β did not display the typical mammalian pattern of a rise in estrogen concentrations, with developing follicles/ovulation, prior to a rise in progesterone concentrations (from the corpus luteum). Therefore, the results from the estradiol-17β EIA assays were deemed unreliable.

Endocrine Profiles/Reproductive Patterns

Individual female endocrine profiles were not combined to create a species average because there were extreme differences among individuals in hormone concentration patterns. Salivary progesterone tended to be noisy in this dynamic situation demonstrating a pulsatile pattern throughout all seasons as well as elevations during the "non-luteal" phase.

PDZA-1

Saliva collection from female #1 at Point Defiance Zoo & Aquarium (PDZA-1) occurred for a period of 55 months (Table 10). There was one significant gap in data collection. The PDZA-1 female exhibited an increase in salivary progesterone during each year of the study (Table 11;

Figure 44). Initial elevations of progesterone were annually inconsistent, occurring during the summer or fall seasons each year. Levels returned to baseline values during the following summer or fall seasons. The extended elevated progesterone levels are most likely representative of the luteal phase of the estrous cycle and may represent consecutive pseudo-pregnancies (non-pregnant luteal phases). Average luteal phase for the PDZA-1 subject was 221.6 days (range 85-360 days). Average inter-luteal (baseline) length was is 56.2 days (range 21-104 days). Initial progesterone elevations coincided with the wild walrus breeding season (Fay 1982) for one out of five cycles.

PDZA-2

Saliva collection from female #2 at Point Defiance Zoo & Aquarium occurred for a period 55 months (Table 10). One gap in data collection occurred for the individual. Increased levels in salivary progesterone were demonstrated during each year of the study (Table 11; Figure 45). Initial elevations of progesterone occurred most often during the months of the wild walrus breeding season (Fay 1982). However, luteal phases were inconsistent in length. The average luteal phase lasted 189.33 days (range 22-356 days). Progesterone levels returned to baseline during various times of the year. Average baseline length was 36.5 days (range 16-67 days).

IZ

Saliva samples were collected from the female walrus at the Indianapolis Zoo for 12 months (Table 10). Three gaps in data collection occurred for female IZ (Figure 46). Gaps ranged from 6-9 days with an average gap of 7.0 days. Gaps may affect an understanding of the reproductive profile. The IZ female exhibited an increase in salivary progesterone at the end of October 2008 that continued through the end of the study. The sustained elevation lasted at least 371 days (1.53±0.13 ng/ml; Table 11; Figure 46).

NYA

Saliva collection from the female at the New York Aquarium occurred for 12 months (Table 10). There were nine gaps in data collection for the NYA female (Figure 47). Gaps ranged from 6-19 days with an average gap of 9.0 days. The gaps most likely limit a clear understanding of the reproductive profile of NYA. An initial increase in salivary progesterone was displayed in mid-February 2010 that lasted approximately 208 days (2.41±0.17 ng/ml) (Table 11; Figure 47).

SFDK-1

Salivary samples were collected from the first female at Six Flags Discovery Kingdom for a period of 36 months (Table 10). Twenty gaps in data occurred for SFDK-1 (Figure 48). Gaps ranged from 6-21 days with an average gap of 7.45 days. The gaps most likely affect the interpretation of the reproductive profile. SFDK-1 female exhibited several increase in salivary progesterone during the study concurrent with the wild walrus breeding season (Fay 1982; Table 11; Figure 48). Average luteal phase and baseline length were not determined for this individual due to the amount of noise and gaps in the subject's profile.

SFDK-2

During the 36 months of salivary collection, from the second female at Six Flags Discovery Kingdom, seventeen gaps in data occurred (Table 10; Figure 49). Gaps ranged from 6-15 days with an average gap of 7.06 days. The understanding of the reproductive profile of SFDK-2 is mostly likely affected by the gaps in data. Several increases in salivary progesterone were demonstrated in subject SFDK-2 during the study (Table 11; Figure 49). The animal had a confirmed pregnancy which resulted in a stillborn in May 2011. The extended periods of elevated progesterone levels are believed to be representative of the luteal phase of the estrous cycle and may represent pseudopregnancies and a pregnancy in the SFDK-2 female. The progesterone concentrations during the period of pregnancy in this individual are lower than the period of pseudopregnancy.

Behavior

In depth behavioral data were collected from the two female walrus at PDZA from 2007 through 2009 during both breeding and non-breeding seasons. Informal observations were subjectively documented by walrus staff during the timeframe of the study.

Formal observations

PDZA-1

Estrus, clasp and copulation behaviors were never observed during formal observations (Table 12). However, the researcher's observation location only allowed for minimal visualization of copulations, likely resulting in a negative bias for frequencies of copulation-related behaviors.

PDZA-2

As expected, clasping ($p<0.005$) was significantly more frequent during the breeding season (Table 13). Estrus and clasp were only observed during the 2008 breeding season and clasp was found to be significant ($p<0.0002$). Copulation and estrus did not demonstrate a significant incidence by season. As stated above, the researcher's observation location likely resulted in a negative bias for frequencies of copulation-related behaviors.

Informal observations

PDZA-1

During the first year of the study (2006/2007), female PDZA-1 did not display changes in behavior consistent with estrus (soliciting male's attention, following male, clasping, permitting penetration/copulation) (Figure 44). During the second breeding season (2007/2008), the female allowed clasping and penetrations at the end of February/beginning of March 2008. During the third season (2008/2009), behaviors consistent with estrus occurred in mid-November 2008. Through the fourth season (2009/2010) the PDZA-1 female displayed changes in behavior during two separate time periods. The first period occurred at the end of July/beginning of August 2009 and entailed toleration of advances made by the male. The second time period occurred at the end of December 2009 and involved following the male, clasping and allowing penetrations. Season five (2010/2011) behaviors consistent with estrus started at the beginning of November 2010. The final season of observations, season six (2011/2012), the female left feeds, followed the male, clasped and allowed penetrations in mid-September. Based on behavioral observations, the PDZA-1 female did not display a consistent seasonal pattern of estrous behavior. Behavioral estrus coincided with the wild walrus breeding season (Dec through March) in two of five possible breeding seasons, and the onset of elevated progesterone concentrations in one out of five possible cycles.

PDZA-2

During the first year of the study (2006/2007), the PDZA-2 female displayed behaviors consistent with estrus during the beginning of January (Figure 45). These behaviors included: following the male, clasping and allowing penetration. During the second year (2007/2008), a change in behavior was displayed during two separate time periods. The first period occurred in mid-November 2007 and involved following the male and allowing penetrations. The second time period occurred mid-January 2008 and entailed initiating interactions and allowing penetrations. During the third year of the study (2008/2009), the female followed the male and allowed clasping and penetrations in mid-January 2009. The fourth season (2009/2010), behaviors consistent with estrus occurred at the beginning of February. Through the fifth season (2010/2011), the PDZA-2 female did not display changes in behavior consistent with estrus. The subject was thought to be pregnant based on early ultrasound findings in September 2011. However, the suspected pregnancy did not develop. During the final year of observations, season six (2011/2012), the female had a decreased appetite, followed the male and allowed penetrations in mid-January 2012. Based on behavioral observations, a consistent seasonal pattern of estrous behavior occurred in January. The PDZA-2 female displayed behaviors consistent with estrus during the wild walrus breeding season in five of six potential breeding seasons. However, behaviors consistent with behavioral estrus were also displayed at other times of the year. Behavioral estrus coincided with the onset of elevated progesterone concentrations in three out of four possible cycles.

IZ

Behavioral observations were subjectively documented by the walrus staff at Indianapolis Zoo beginning in October 2008 and concluding in October 2009. The IZ female displayed changes in behavior consistent with estrus during the beginning of March 2009 for several days (Figure 46). Estrus behaviors involved soliciting the male and a decreased appetite. Behaviors consistent with estrus were displayed during the period of wild walrus breeding. Since the progesterone profile was questionable for the IZ female, behavioral estrus was not evaluated in relation to progesterone.

NYA

Behavioral observations were subjectively documented by the walrus staff at the New York Aquarium for 12 months. The NYA female displayed changes in behavior consistent with estrus from 20-25 February 2011 (Figure 47). Her estrous behaviors during this period included vocalizing and pattern swimming during feeds and a decreased appetite. She was not housed with a male during the study period. The female displayed behaviors consistent with estrus during the wild walrus breeding in one of two possible breeding seasons.

SFDK-1

Staff at Six Flags Discovery Kingdom subjectively documented behavioral observations for a period of 38 months. During the first season (2008/2009), in the beginning of February 2009, her swim pattern changed (Figure 48). She was inappetant from February 9-10 and solicitation of the male was observed through the 15th. The next year (2009/2010), the female was transferred to SWSD in October 2009 for the 2009/2010 breeding season. No estrous or breeding behaviors were noted during this time period. She was transferred back to SFDK in May 2010. The following year (2010/2011), behaviors consistent with estrus were observed during the end of January 2011. Sexual interactions were observed from January 25-31. During the final year (2011/2012), the female displayed inappetance from January 14-17. On January 15th, she began pattern swimming and soliciting the male. Behavior lasted through the 18th. Based on behavioral observations, a consistent seasonal pattern of estrous behavior occurred in January/February. Behaviors consistent with estrus were displayed during three of four possible wild walrus breeding seasons.

SFDK-2

During the first year (2008/2009), female solicitation of the male was observed during the beginning of February (Figure 49). The following year (2009/2010), the female was observed soliciting the male at the end of October 2009. The third year (2010/2011), the SFDK-2 female did not display changes in behavior consistent with estrus. Pregnancy was confirmed via ultrasound in November 2010 and the cow gave birth to a still-born calf in May 2011 (Muraco *et al*. 2012). During the last year (2011/2012), solicitation of the male occurred at the end of December 2011. Based on behavioral observations, the female displayed a varied seasonal pattern of estrous behavior. Behaviors consistent with estrus were displayed during two of four potential wild walrus breeding seasons.

Seasonality

Progesterone

Overall salivary progesterone versus day length, for individual females, failed to demonstrate the expected significant inverse relationship (Table 14; Figures 50-55A). Only one of six animals displayed a significant relationship but the relationship consisted of a positive correlation. The remaining five females displayed non-significant relationships demonstrating inconsistent correlations, some were positive and others negative.

When broken down by seasons, salivary progesterone concentrations versus day length, demonstrated a significant correlation in 6 out of 16 seasons (Table 14). Correlations were inconsistent, lacking a seasonal pattern.

Estradiol 17-β

For individual females, overall salivary estradiol-17β versus day length demonstrated the expected significant inverse relationship in one of six animals (Table 15; Figures 50-55B). Correlations for the remaining five animals displayed variable relationships between E_2 and day length.

A significant correlation was displayed in 8 out of 16 seasons when salivary E_2 versus day length was broken into individual seasons (Table 15). However, similar to progesterone, correlations were inconsistent.

DISCUSSION

This research represents the first comprehensive assessment of endocrine and behavioral parameters of reproduction utilizing non-invasive techniques in *ex situ* female walrus. Results demonstrate that salivary progesterone and estradiol-17β are not the best methods for monitoring female walrus hormone cycles. In addition, behavioral observation was not a consistently reliable way to detect estrus or impending ovulation.

Saliva

To monitor reproductive hormones in walrus, non-invasive saliva collection was chosen due to its ease of training, simplicity of collection, relative stability (Lipson and Ellison 1989) and successful use in other species. The aforementioned factors allowed more subjects to participate in the study.

However, the results of the study demonstrate that the use of salivary progesterone for noninvasive reproductive monitoring of luteal function in female walrus is ineffective. Even though the assay was validated via parallelism and correlations of progesterone in serum and saliva were found to be significant, progesterone profiles were difficult to interpret biologically and physiologically. There appears to be some physiological relevance in the progesterone profiles of the two PDZA females, but the pattern is less evident in the other study females. Some females displayed increases of progesterone coincident with behavioral estrus suggestive of the beginning of a luteal phase, but the pattern was not consistent. Profiles were often times noisy with no clear cessation of the luteal phase.

Progesterone appears to be present in walrus saliva as displayed by EIA and mass spectrometry (Appendix 11). However, progesterone was found to be unsuitable for monitoring the functional capacity of the corpus luteum using the assay or methodology in the study. In general, salivary progesterone has shown good correlation with unbound steroid hormones in serum (Riad-Fahmy *et al*. 1987, Ellison 1993, Lipson and Ellison 1996, Lu *et al*. 1999, Gröschl 2008) and reflects biological activity of the ovaries (Vinning and McGinley 1987, Mandel 1993) making its measurement in saliva successful in humans. However, good correlations are not always displayed in other species. In a study of false killer whales, salivary secretions failed to show seasonal or cyclic trends (Atkinson *et al*. 1999). In the case of the Indian rhinoceros, researchers were unable to assess luteal function via saliva using a variety of EIA and RIA procedures but eventually found a commercially available kit made specifically for saliva that produced accurate results for estradiol (Gomez *et al*. 2004). The methodology used in the male portion of the study was similar to the female portion and yielded useful results for the males, so it is unlikely that methodology was responsible for the inaccuracy of progesterone results. Interfering matrix effects were an unlikely explanation for the difficulties with data from females since the parallel displacement curves were successfully generated. Progesterone and estradiol-17β lack a circadian rhythm (Riad-Fahmy *et al*. 1987) so timing of samples was not likely the cause of a lack of utility of salivary progesterone data.

A possible explanation for the lack of usefulness of results for both progesterone and estradiol-17β could be interference due to sample contamination by oral bacteria, blood, feces or food debris. Intermittent dilution by salt water could cause inaccurate results. However, staff had no control over whether walrus open their mouths in water prior to feeds. Lack of immediate refrigeration after sample collection can cause degradation (Riad-Fahmy *et al*. 1987). Interference by peroxidases, which are known to be present in saliva (Dogon *et al*. 1970) and act as binding proteins for estradiol (making levels lower and thus more difficult to assay (Riad-Fahmy *et al*. 1987) could cause concentration inaccuracy.

Use of cotton to collect saliva is another possible cause of the lack of utility of the salivary data for progesterone and estradiol-17β assessment. Some research has demonstrated decreased concentrations of hormones (Hold *et al*. 1995, Groschl and Rauh 2006), while others have demonstrated increased concentrations of hormones (Dabbs 1991, Shirtcliff *et al*. 2001, Strazdins *et al*. 2005) in samples collected with cotton versus samples collected by other techniques. Regardless, the methodology for the female portion of the research was conducted in a consistent manner and similarly to the male portion so the use of cotton to collect saliva should not affect the overall patterns or their understanding.

The results of the study demonstrate the lack of usefulness of salivary estradiol-17β for non-invasive reproductive monitoring of follicular activity in female walrus. Even though assay validation was successful, there was not a lot of sensitivity in the assay for E_2. In addition, correlations of estradiol-17β in serum and saliva were low and profiles were found to be unsuitable indicators of the follicular phase. Estradiol-17β did not display the typical mammalian pattern of a rise in estrogen concentrations, with developing follicles/ovulation, prior to a rise in progesterone concentrations. Therefore, the results from the estradiol-17β EIA assays were deemed unreliable. The particular assay system or methodology is inappropriate for walrus.

Estradiol-17β appears to be present in walrus saliva as displayed by EIA and mass spectrometry although at very low levels (Appendix 11). However, estradiol-17β was found to be an unsuitable indicator of follicular activity. In general, E_2 tends to have low salivary concentrations precluding its precise measurement in saliva, due to a lack of high sensitivity in assays. As a result, estradiol data are relatively rare (Vining *et al*. 1983, Lu *et al*. 1999). Generally, concentrations of reproductive hormones found in saliva represent active plasma free concentrations. Concentrations are therefore low, from 0.2% (Worthman *et al*.1990) to 7.9% (Belkien *et al.* 1985). In the case of Indian rhinoceros, researchers attempted five different methods to measure salivary estrogens before discovering an effective protocol (Gomez *et al*. 2004). Reduced levels of estradiol in animals have also been found in serum. In Asian (*Elephas maximas*) and African (*Loxodonta africana*) elephants, serum estradiol concentrations were found to be low and fluctuated without any discernible pattern (Chappel and Schmidt 1979, Brannian *et al.* 1988, Brown *et al*. 1999). For a reliable analysis of the preovulatory estrogen peak in walrus saliva, more rigorous extraction and sensitive assays may be necessary.

In some species, reproductive hormones other than estradiol-17β have been useful in tracking the follicular phase. Testosterone serves as a precursor for estradiol. Therefore salivary androgens can and have been used to monitor the follicular phase in Indian rhinoceros (Schwarzenberger *et al*. 2000, Gomez *et al*. 2004). Estrone sulfate was used successfully to track the follicular phase in female Hawaiian monk seals (Pietraszek and Atkinson 1994). Mass spectrometry results for walrus saliva revealed higher levels of estrone when compared to estradiol (Appendix 11). It is possible that estrone is the dominant estrogen in walrus saliva. Further investigation is necessary for identification of the appropriate hormone for tracking of the follicular phase in walrus.

The route of excretion of a hormone is species dependent and varies considerably among species as well as between steroids within the same species (Schwarzenberger *et al*. 1996, Graham 2004). For example, most steroids in felids (Brown *et al*. 2001) are voided into feces, whereas the same hormone products are excreted through the kidneys into the urine in cervids (Monfort *et al*. 1990). The feces of elephants contain the majority of the progestagens, but only approximately 10% of the estrogens (Brown 2000). In the Sumatran rhinoceros (*Dicerorhinus sumatrensis)* progesterone is mostly voided in the feces (Heisteremann *et al.* 1998) while in the white rhinoceros (*Ceratotherium simum simum*) it is excreted via the urine (Hindle and Hodges 1990). Therefore, the choice of a matrix is crucially species-dependent. The primary route of reproductive steroid excretion in the walrus is still unknown. Saliva may not be the best matrix to assess estrogens and progestagens. Further analyses of walrus urine and feces using injection of radiolabeled steroid hormones would likely determine the major route of excretion. However, because of the dangers inherent in using the procedure, radiolabeled hormones are not suggested for use in the *ex situ* walrus population. For now, serum remains the best way to monitor estrogens and progestagens in female walrus (Siembieda 2005, Kinoshta *et al*. 2012, Muraco *et al*. 2012).

The progesterone profiles were very noisy with prolonged elevations making finding a suitable iterative process for data difficult. The iterative process standard to progesterone data to elucidate baseline and elevated levels, entails taking the average concentration of all samples then excluding high values that exceed the mean plus two standard deviations. The average is then recalculated and the elimination process repeated until no values exceed two standard deviations above the mean (Brown, pers. comm.). However, using the typical procedure for this data set would have caused most values to fall out leaving no discernible way to distinguish between baseline and elevations. The iterative process chosen for the progesterone data allowed periods of elevated progesterone levels consistent with a luteal phase to be elucidated and clustering of levels was demonstrated in two out of six animals. It is most likely that the successive elevations in progesterone representated consecutive pseudopregnancies in the PDZA-1 and PDZA-2 animals. Pseudopregnancies have been recorded in both wild (Mansfield 1958, Fay 1982, Lipton and Fay 1993) and *ex situ* walrus (Kinoshita *et al*. 2012, Muraco *et al.* 2012). Lengths of elevated progesterone levels in two females in the study averaged 204.27 days. The findings are consistent with high circulating levels of serum progesterone for periods of up to nine months found in *ex situ*, non-pregnant walrus (Kinoshita *et al*. 2012, Muraco *et al.* 2012).

Even though estradiol-17β results were determined to be an unreliable indicator of the follicular phase, E_2 profiles from the two PDZA animals warrant further consideration. Both PDZA females displayed estradiol-17β elevations coincident with initial progesterone elevations during November 2007 and October 2008. The patterns were not subsequently observed. A possible explanation for the findings involves the biological process referred to as the "male effect", in which females are stimulated by the presence of a male after a period of gender separation (Shelton 1960, Ianson and Guinness 1985, Delcroix and Mauget 1990, Lindsay 1996). A common characteristic of species with a known "male effect" is that all are animals that naturally congregate in groups in which genders live separately for some part of the year. The sexes come together just before the breeding season, meeting suddenly after a relatively long period of separation (Short 1980). Wild walrus exhibit a similar social structure. However, in zoos and aquaria, males and females are typically housed together throughout the year. In 2006, the PDZA females were introduced to the male for the first time. It is possible that the initial exposure caused "synchronous" ovulation in the two females and that over time the constant exposure led to abnormal cycles and decreased breeding success, ultimately with reproductive failure. Validation of the suggestion requires continued monitoring of the population to establish baseline knowledge regarding female cycles, followed by separation of genders during the non-breeding season. Continued in-depth monitoring of endocrine cycles could then be evaluated during the separations and re-introductions.

Successful walrus reproduction in zoos and aquaria remains low even though some females undergo periods of elevated progesterone levels indicating functional corpora lutea from ovulation. However, the determinants of breeding success are not known with certainty. A significant result of living in a zoo or aquarium compared to the wild is the possible reduction in the ratio of males to females. Modeling zoo social groups after wild populations can facilitate animal health and welfare, and therefore reproduction (Price and Stoinski 2007). For some species, being housed in inappropriate social groups can lead to an inability to perform necessary reproductive behaviors (Tilbrook and Cameron 1990, Price and Stoinski 2007). Although untested in walrus, the absence of male-male competition may be detrimental to natural courtship behaviors in both genders. Increased competition between breeding males of other polygynous mammal species seems to encourage breeding success (Bartholomew 1970, Galimberti *et al.* 2007, Harcourt *et al.* 2007, Mainguy *et al.* 2008) and may facilitate successful reproduction in walrus. Male-male competition in walrus warrants further investigation using more controlled experimental conditions.

The PDZA-1 female presented a unique pattern in her breeding cycle, inconsistent with the wild walrus breeding season. The female subject arrived at the study location in 2006 at 22 years of age. Offspring were born in May 1999 and April 2002. With a gestation of 15 to 16 months fertilization most likely took place in January/February 1998 and December/January2000/2001, respectively. This is within the range of the wild walrus breeding season. However, based on elevated progesterone levels during the study, the animal has experienced estrus both within and outside the wild walrus breeding season. In addition, post study estrus/ovulation, based on serum progesterone levels, occurred in July 2012 and June 2013 (Triggs, unpublished data). The PDZA-1 female began experiencing a loss of visual function in her teens and was completely blind by 2002. A similar situation occurred with the SWSD male walrus (male portion of study). As previously discussed in the male chapter, photoperiod is a proximate control factor in the annual cycle of pinniped reproduction (Boyd 1991, 1996). It is possible that the PDZA-1 female, due to blindness, was not correctly responding to photoperiodic cues and therefore experiencing a continued derivation in her annual breeding cycle.

Behavior

Mature females in the study often displayed behaviors consistent with estrus concurrent with the wild walrus breeding season (December-March). However, periods of behavioral estrus did not always precede an increase in salivary progesterone concentrations. *Ex situ* females in estrus will become more receptive to breeding attempts by a male, and will often become aggressive to avoid breeding during non-estrous periods (Triggs 2006). In zoos and aquaria, females' appetites may decrease and the animals may become more preoccupied with each other than with caretakers or training sessions. The behaviors observed in the study included: increased vocalizations, increased directional swimming, pattern swimming, inappetance, soliciting male attention, pursuing male, clasping and permitting penetration/copulation. The most reliable signs of estrus were soliciting male attention, allowing clasping or mutual clasping and allowing penetration, but behaviors were not observed in every cycle. Behaviors consistent with estrus generally lasted several days.

There are several difficulties with using behavior to identify females in estrus. Behaviors are not always consistent between females, signs may change from one year to the next, signs can be easily missed by staff, behaviors may occur outside the breeding season, and animals can undergo a “silent estrus” (ovulation occurring in the absence of estrous behavior).

Behavioral signs of estrus were not consistent between females. For example, some females increased or changed the pattern of their swimming while others decreased their pattern swimming and increased their directional swimming. The increase in directional swimming could be to follow a male, find the shortest distance between two points to catch up with the male, while the increase or change in pattern swimming could be an expression of “excess energy”. It has been demonstrated that females of many species appear to show a marked increase in general physical activity when they come into estrus and the elevated physical activity is generally manifested by increased locomotion (Kiddy 1977, Donovan 1985, Moline and Albers 1988, Arney *et al*. 1994, Ranasinghe *et al*. 2010).

Behavioral signs of estrus can change from one year to the next in an individual. For example, some females in the study showed periods of inappetance during behavioral estrus during some years but ate well during others. In addition, females actively solicited and engaged the male in some years, whereas in other years the female was passive but participated in breeding behaviors with the male.

Since estrus lasts for only a short period, as opposed to rut, behavioral signs can easily be missed by staff. Animal care staff is limited in their time and availability to observe the animals under their care. Normal work schedules only cover eight to 10 hours a day. Unless facilities employ overnight staff or use video cameras, signs of estrus could be missed. In addition, new staff may not be familiar enough with walrus behavior to know what signs are important to note.

Behaviors consistent with estrus also can occur outside of the breeding season making it difficult to know if ovulation is truly occurring during the time. The PDZA-1 female in the study displayed behavioral signs of estrus during the months of July, August and September. The female even continued the irregular pattern post-study. It has been suggested that the PDZA-1 female's reproductive cycle was off due to her blindness. Some females displayed signs consistent with behavioral estrus during other times of the year. Similar behavior has been recorded in other mammals (Asa 1986, Odell *et al*. 1995).

The phenomenon known as "silent ovulation" can also make it difficult to use behavior to pinpoint ovulation. Silent ovulation (also referred to as "silent estrus" or "silent heat") has been reported in a variety of mammals (Yuthasastrakosol *et al*. 1975, Foster *et al*. 1985, Ranasinghe *et al*. 2010). In such cases, the animal ovulates without overt behavioral signs. Thus, specific behaviors, or lack thereof, may be unreliable indicators of ovulation.

Seasonality

The walrus in this study were housed at varying latitudes (from 47°18’N to 32°45’N), which could account for the inconsistent seasonal differences in reproductive hormone concentrations. However, the seasonal patterns were inconsistent even among walrus housed together at the same institution or facilities at the same approximate latitude. Findings presented by Fay (1982) and Fay *et al*. (1984), suggest that estrus in some wild walrus in a single breeding location may take place as early as December or as late as June but most ovulate in February.

It is generally believed that photoperiod is a proximate control factor in the annual cycle of pinniped reproduction (Boyd 1991, 1996). However, there also is evidence that photoperiod may not be as responsible as other factors for temporal variation in the pattern of walrus reproduction. Successful breeding pairs of walrus lived in lower latitudes at Marineland of the Pacific (Palos Verdes, California, 33°73’N) and Sea World (San Diego, California, 32°45’N and San Antonio, Texas, 29°46’N). Collectively, walrus at the three facilities have experienced 12 pregnancies (of the 18 recorded for walrus in U.S. zoos and aquaria; Genrich 1984, Oland 2012, Wilcox, pers. comm.). Wild female walrus may go into estrus sometime between December and June but most ovulate in February (Fay 1982, Fay *et al*. 1984). Evidence of wild Atlantic walrus ovulations in July and November (Born 2001) and August and November (Mansfield 1958) were also found. Fay (1982) suggested that wild female walrus in a given breeding location have variations in the timing of ovulation based upon age and sexual maturity. Born (2001) suggested that extra seasonal ovulations may be associated with approaching sexual maturity in females. This could account for the differences in ovulation timeframes among *ex situ* walrus.

Conclusion

This study represents the first attempt at the comprehensive assessment of behavioral and reproductive endocrine traits in *ex situ* female walrus in the United States. The results of the study demonstrate that use of saliva for noninvasive reproductive monitoring of the follicular phase in female walrus is unreliable. Correlations of estradiol-17β in serum and saliva were low and profiles were found to be unsuitable indicators of the follicular phase. The particular assay system is not appropriate for walrus and other assays or approaches need to be explored.

Salivary progesterone did display some relevance to physiological events. Overall, endocrine profiles varied with some females displaying increases of progesterone coincident with behavioral changes (estrus) suggestive of the beginning of a luteal phase. However, progesterone profiles were not consistently informative indices of luteal phase initiation or cessation. Conducting further studies to determine which assay system is best for non-invasive monitoring in female walrus is needed.

In general, behavioral observation was not a consistently reliable method for detecting impending estrus. Behavioral signs were not always consistent between females. Signs varied among cycles, signs may have been missed, behaviors occurred outside the breeding season and animals may have undergone silent estrus. Behavior should not be used as the only indicator of ovulation in walrus.

For zoo and aquaria management to increase breeding success of the *ex situ* population, an effort to mimic the wild walrus structure is recommended. The same methodological changes as in the male chapter are suggested: a) increase the number of walrus in the population or consolidate existing walrus into larger social groups; and b) separate individuals by gender during the non-breeding season or move animals to different facilities to combat the “male effect”. Continued zoo and aquaria participation and monitoring of reproductive functioning via endocrine and behavioral parameters would allow evaluation of the benefits of the proposed changes. Further studies addressing seasonal influence on reproductive activity, reproductive senescence, seasonal changes in behavior and the influence of social conditions on the reproductive performance of *ex situ* walrus via longitudinal serum endocrine monitoring, behavioral observations and ultrasonography would greatly improve our understanding of reproduction in female walrus.

Table 10. Description of female walrus used in study

Female	Facility	Latitude	Water temp. (°C)	Studbook#	Birth date	Sample period	Age*	w/ male	Repro history
PDZA-1	Point Defiance Zoo & Aq	47° 18'N	7-13	35	1984	6/2007-12/2011	23	yes	2 calves
PDZA-2	Point Defiance Zoo & Aq	47° 18'N	7-13	58	1995	6/2007-12/2011	12	yes	nulliparous
IZ	Indianapolis Zoo	39° 46'N	10-13	56	1995	10/2008-10/2009	13	yes	nulliparous
NYA	New York Aquarium	40° 34'N	7-21	51	1994	2/2010-2/2011	16	no	1 calf
SFDK-1	Six Flags Discovery Kingdom	38° 08'N	7-13	55	1994	1/2009-1/2012	15	yes	nulliparous
SFDK-2	Six Flags Discovery Kingdom	38° 08'N	7-13	54	1994	1/2009-1/2012	15	yes	1 calf (stillborn)

All walrus were wild born.

* Age at start of study

Table 11. Female walrus salivary progesterone concentrations and iterative statistics for baseline and elevated levels

		Overall statistics (ng/ml)			Iterative Statistics (ng/ml)	
Animal	**n**	**min**	**max**	**mean**	**Baseline**	**>Elevation**
PDZA-1	837	0.09	16.51	2.07	1.30	1.30
PDZA-2	855	0.16	20.97	1.77	1.22	1.22
IZ	144	0.27	10.72	1.51	1.06	1.06
NYA	120	0.80	10.86	2.17	1.51	1.51
SFDK-1	352	0.37	17.46	1.76	1.22	1.22
SFDK-2	395	0.16	14.78	1.63	1.04	1.04

Table 12. Female behavioral data - formal observations (PDZA-1)

2007/2008/2009 combined (p-values)

	Water	**Land**	**Swim**	**Pattern**	**Walk**	**Forage**	**Inactive**	**Whisker**
Season	0.119418	0.119418	**2.35E-06***	**0.014904**	0.339	0.251	**0.03864***	0.08629
Year	**0.005745**	**0.005745**	0.3144	**0.004026**	0.4092	**6.31E-14**	0.63573	0.119

	Estrus	**Object**	**Clasp**	**Copulation**	**Non-visible**	**Other**	**Vocal**
Season	NA	NA	NA	NA	0.06314	**1.058E-05***	0.5297
Year	NA	NA	NA	NA	0.64948	0.4624	0.8629

* more frequent during breeding season

	Season	breeding and non-breeding		
	Year	**A=2007**	**B=2008**	**C=2009**
		B-A	**C-A**	**C-B**
Year (p-values)	**Water**	0.8817515	**0.0262798**	**0.0078537**
	Land	0.8817515	**0.0262798**	**0.0078537**
	Pattern	0.6253658	**0.0031549**	0.0531331
	Forage	0.6564079	**0.00000**	**0.00000**
		2008-2007	**2009-2007**	**2009-2008**

Table 13. Female behavioral data – formal observations (PDZA-2)

2007/2008/2009 combined (p-values)

	Water	**Land**	**Swim**	**Pattern**	**Walk**	**Forage**	**Inactive**	**Whisker**
Season	**6.53E-08**	**6.53E-08***	**2.82E-07***	**1.04E-07**	0.11921	**0.01508**	**1.45E-07***	0.08554
Year	**1.22E-05**	**1.22E-05**	**6.76E-11**	**3.38E-08**	0.07418	**5.18E-11**	**<2.2E-16**	0.65136

	Estrus	**Object**	**Clasp**	**Copulation**	**Non-visible**	**Other**	**Vocal**
Season	0.1984	0.44505	**0.0050017***	0.3389	0.06374	**2.13E-11***	0.44
Year	0.1646	**0.01969**	**0.0001918**	0.3685	**0.009677**	**0.002233**	0.6407

* more frequent during breeding season

	Season	breeding and non-breeding		
	Year	**A=2007**	**B=2008**	**C=2009**
		B-A	**C-A**	**C-B**
Year (p-values)	**Water**	**0.0075145**	**0.0000101**	0.2081084
	Land	**0.0075145**	**0.0000101**	0.2081084
	Swim	**0.0008435**	**0.00000**	**0.0029723**
	Pattern	**0.0424566**	**0.00000**	**0.0015311**
	Forage	**0.00000**	**0.0000073**	0.1658498
	Inactive	**0.00000**	**0.00000**	0.1765339
	Object	**0.0296342**	0.9899501	0.0549305
	Clasp	**0.000778**	0.999601	**0.0012383**
	Non-vis	**0.007549**	0.1893315	0.4672645
	Other	**0.0018415**	0.0626066	0.5300763
		2008-2007	**2009-2007**	**2009-2008**

Table 14 . Day length versus salivary progesterone concentration

	Season											
	2007/2008		**2008/2009**		**2009/2010**		**2010/2011**		**2011/2012**		**Overall**	
Animal	**r**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
PDZA-1	0.1212	0.0660	-0.1603	**0.0293**	0.0426	0.5649	0.0118	0.8827	NA	NA	-0.0319	0.3571
PDZA-2	0.3073	**2.0290E-06**	-0.1046	0.1622	0.0884	0.2278	0.2975	**7.7500E-05**	NA	NA	0.0700	**0.0407**
IZ	NA	NA	0.0377	0.6536	NA	NA	NA	NA	NA	NA	0.0377	0.6536
NYA	NA	NA	NA	NA	NA	NA	0.1222	0.1835	NA	NA	0.1222	0.1835
SFDK-1	NA	NA	NA	NA	-0.2091	**0.0135**	0.2487	**0.0113**	0.2136	**0.0251**	-0.0295	0.5818
SFDK-2	NA	NA	NA	NA	-0.0861	0.2715	0.1431	0.1305	-0.0005	0.9955	-0.0346	0.4928

Table 15 . Day length versus salivary estradiol concentration

	Season											
	2007/2008		**2008/2009**		**2009/2010**		**2010/2011**		**2011/2012**		**Overall**	
Animal	**r**	**p-value**	**r**	**p-value**	**R**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
PDZA-1	-0.1727	**-0.0120**	-0.3181	**8.6160E-06**	0.3710	**2.0130E-07**	0.1606	**0.0431**	NA	NA	-0.1337	**0.0001**
PDZA-2	0.0213	0.7483	-0.1278	0.0796	0.3291	**3.9960E-06**	-0.0584	0.4478	NA	NA	-0.0479	0.1599
IZ	NA	NA	0.2994	**0.0002**	NA	NA	NA	NA	NA	NA	0.2994	**0.0002**
NYA	NA	NA	NA	NA	NA	NA	0.0073	0.9370	NA	NA	0.0073	0.9370
SFDK-1	NA	NA	NA	NA	-0.0591	0.4722	0.1623	0.1015	-0.1995	**0.0367**	-0.0096	0.8547
SFDK-2	NA	NA	NA	NA	-0.2670	**0.0005**	0.0656	0.4902	-0.0359	0.7010	-0.0519	0.3037

1 – tunnel

2 – public viewing window

3 – spot

4 – peninsula

5 – main public viewing

6 – main beach

7 – underwater viewing windows

8 – public viewing window by seal

9 – island

10 – observer location

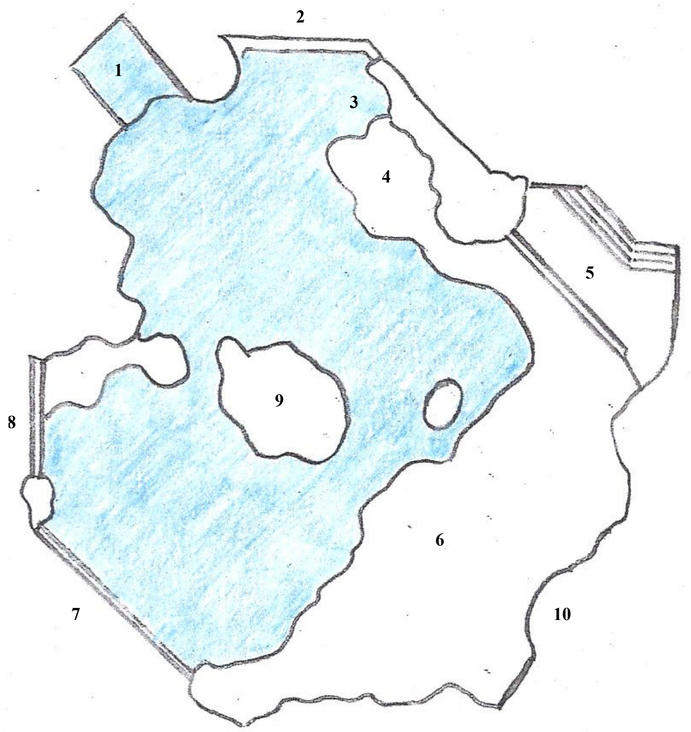


Figure 37. Overview of Point Defiance Zoo & Aquarium's main walrus habitat.

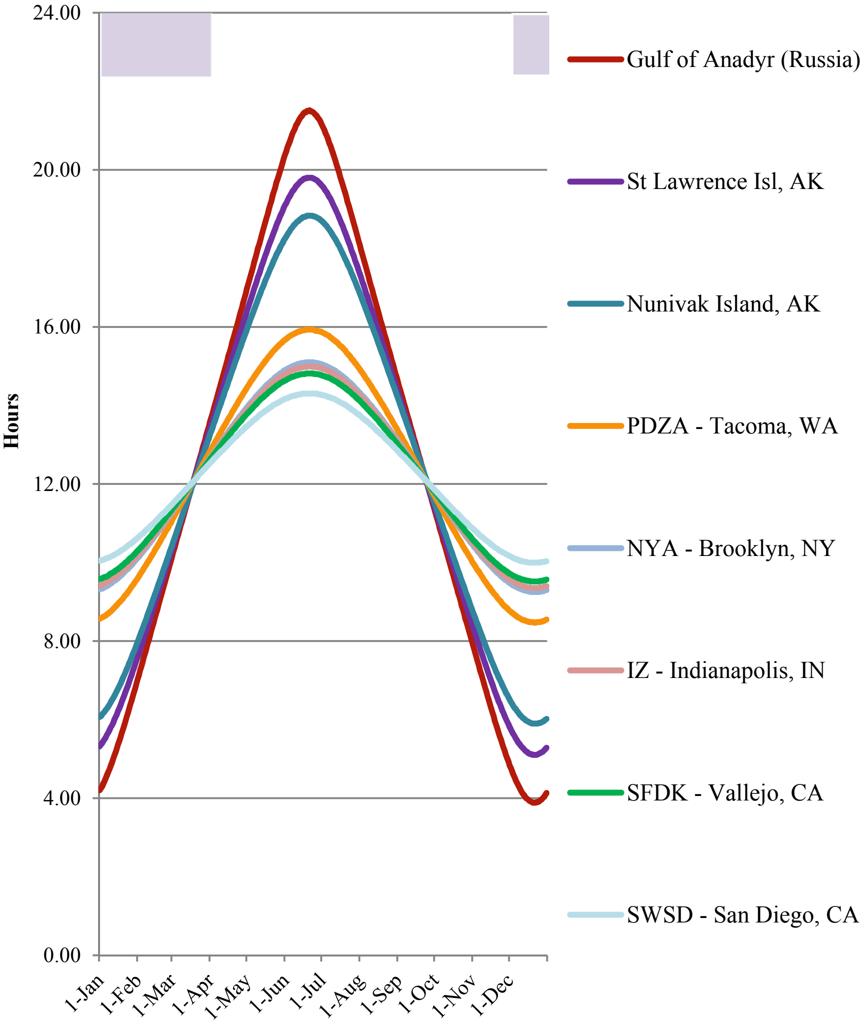


Figure 38. Day length of the Pacific walrus wild population. (Gulf of Anadyr, Russia ——, St Lawrence Island, AK ——, Nunivak Island, AK ——), and at the facilities involved in study, during the breeding season. Shaded area represents breeding season of the wild population.

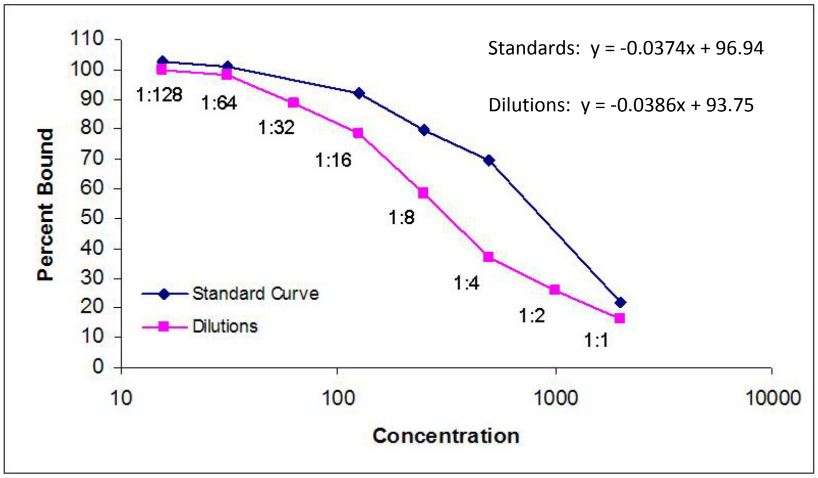


Figure 39. Parallelism curve of serially diluted standards and pooled saliva for progesterone. Line equations are for the linear portion of the curves (standards: 125 to 2,000 ng/ml; dilutions: 1:1 to 1:32).

PDZA-1

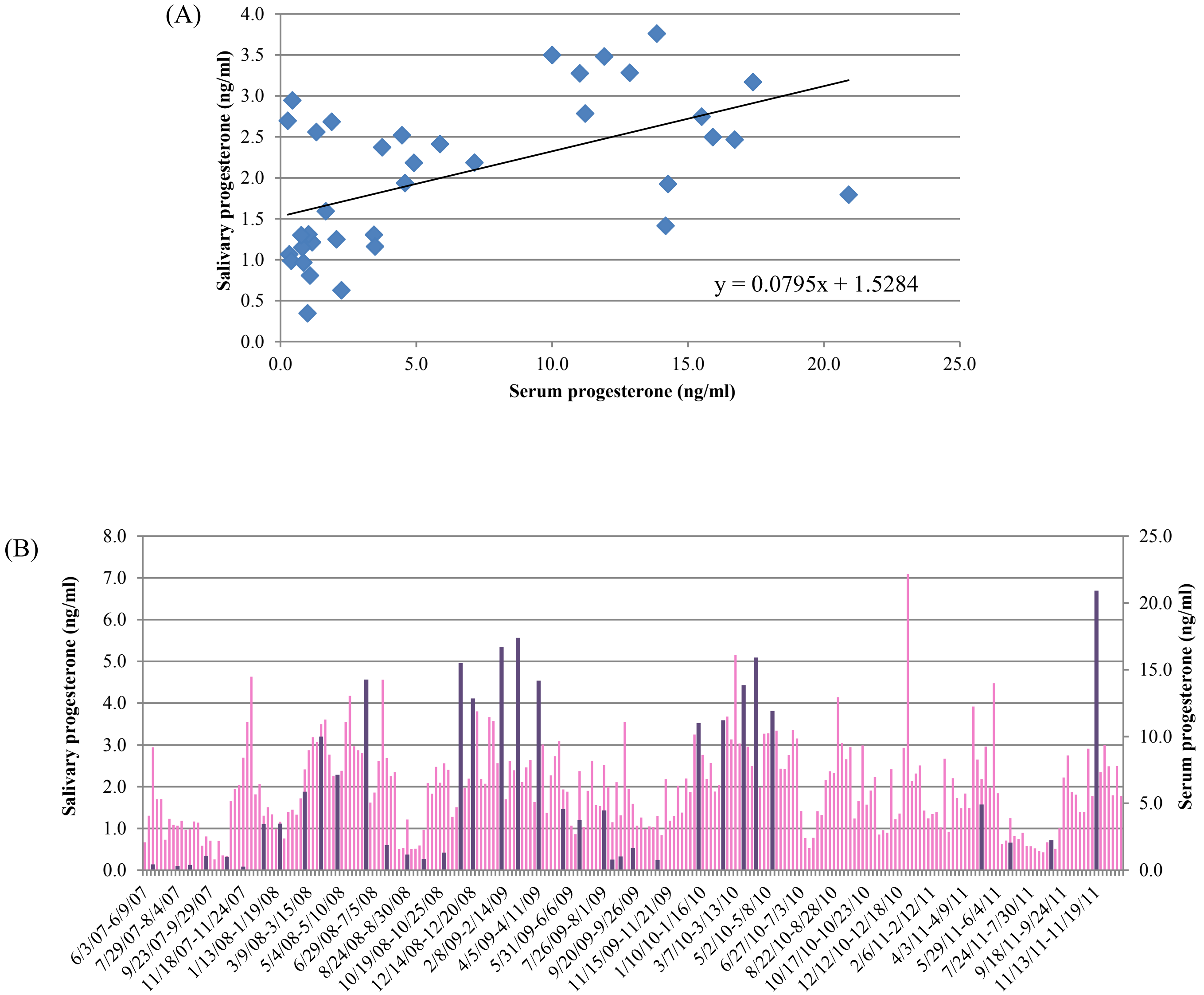


Figure 40. (A) Correlation of progesterone in serum and pooled and averaged weekly saliva and (B) histogram of serum (———) and weekly pooled and averaged saliva (———) for female PDZA-1 Pacific walrus.

PDZA-2

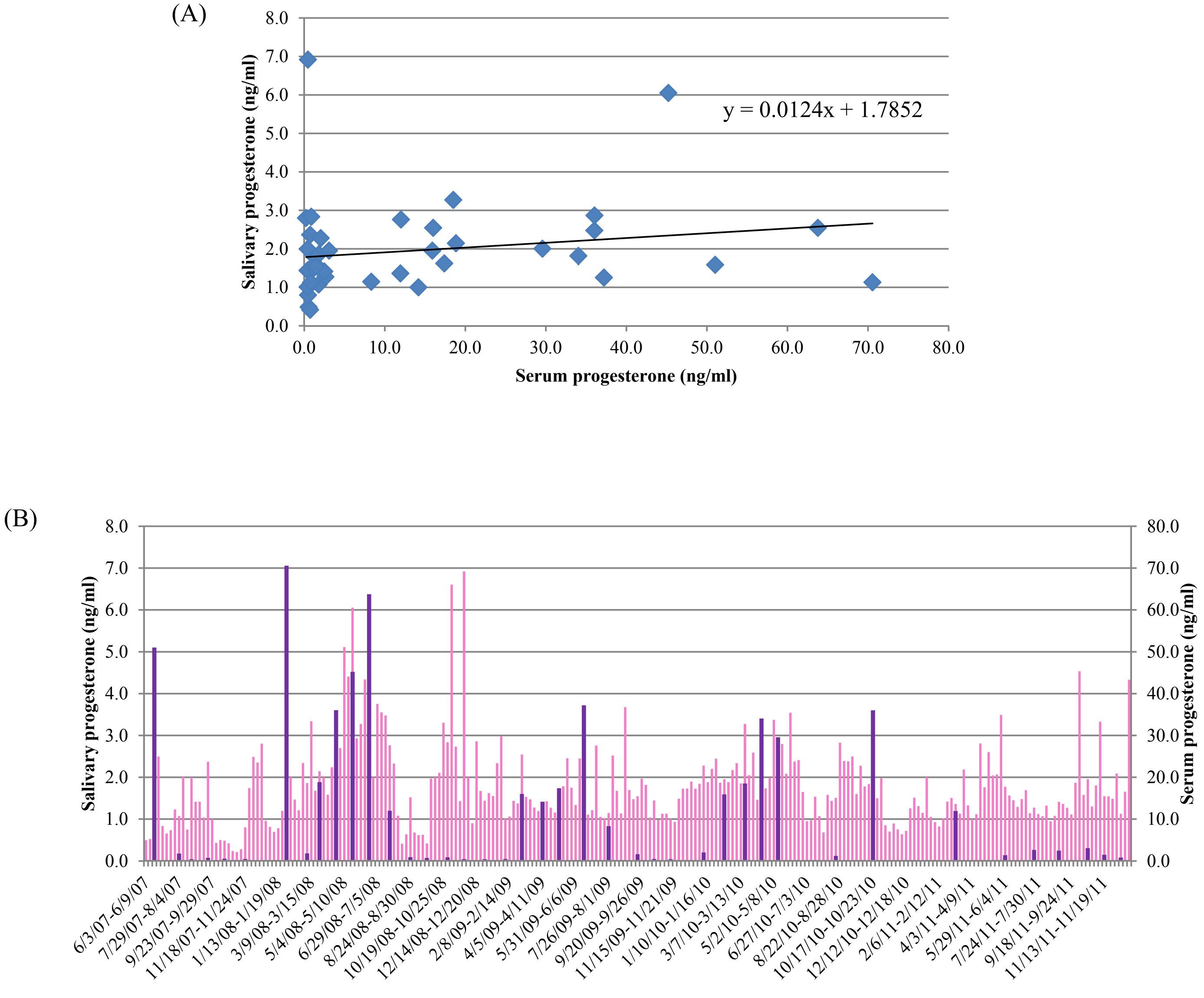


Figure 41. (A) Correlation of progesterone in serum and pooled and averaged weekly saliva and (B) histogram of serum (——) and weekly pooled and averaged saliva (——) for female PDZA-2 Pacific walrus.

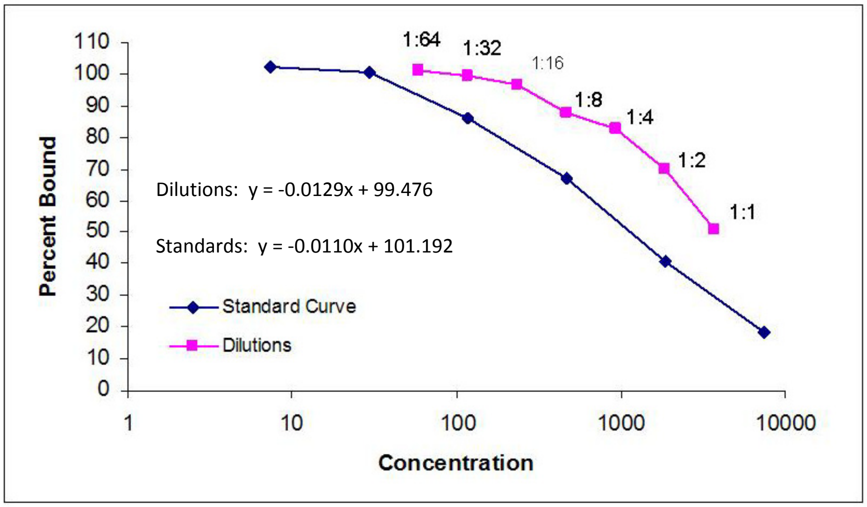


Figure 42. Parallelism curve of serially diluted standards and pooled saliva for estradiol-17β. Line equations are for the linear portion of the curves (standards: 234.375 to 3,7500 ng/ml; dilutions: 1:1 to 1:16).

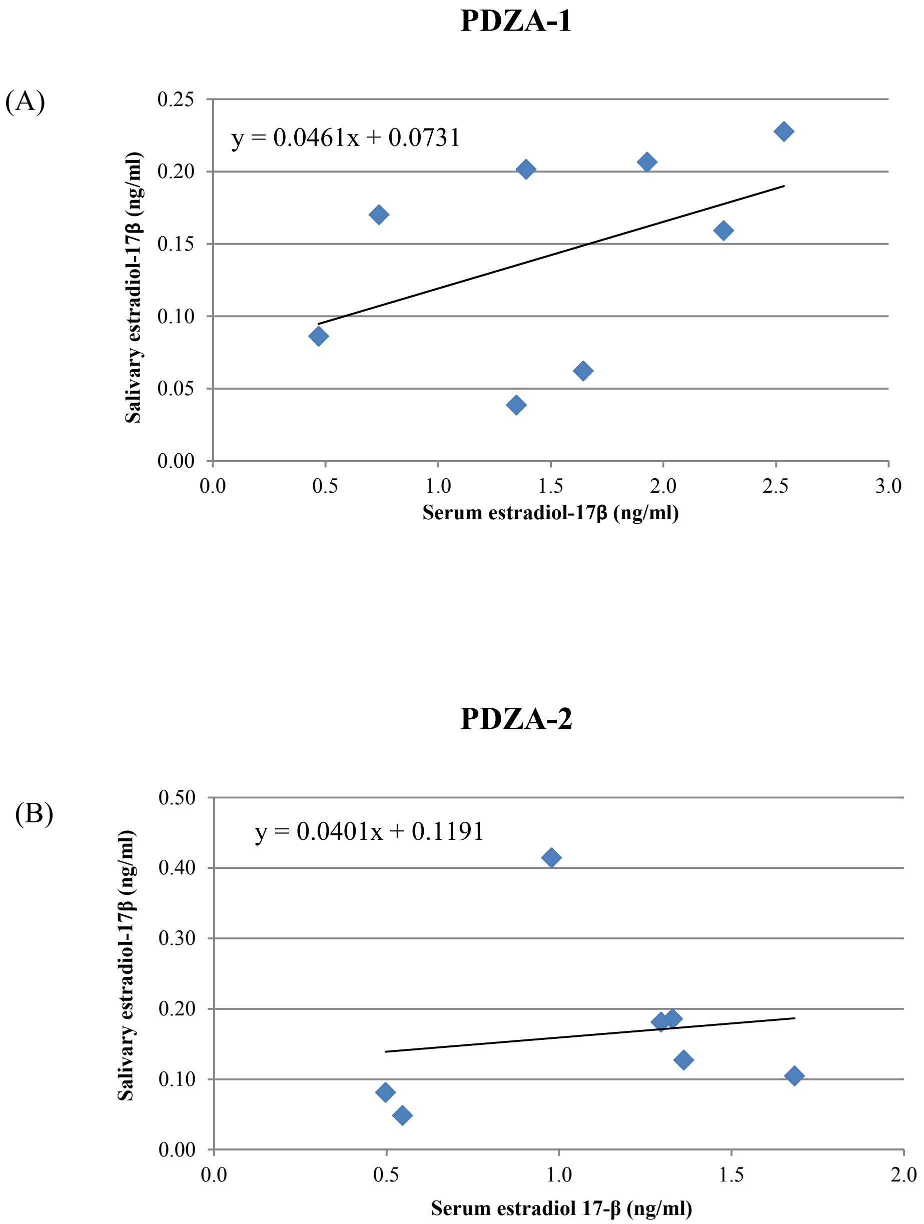


Figure 43. Correlation of estradiol-17β in serum and saliva and for female Pacific walrus (A) PDZA-1 and (B) PDZA-2.

PDZA-1

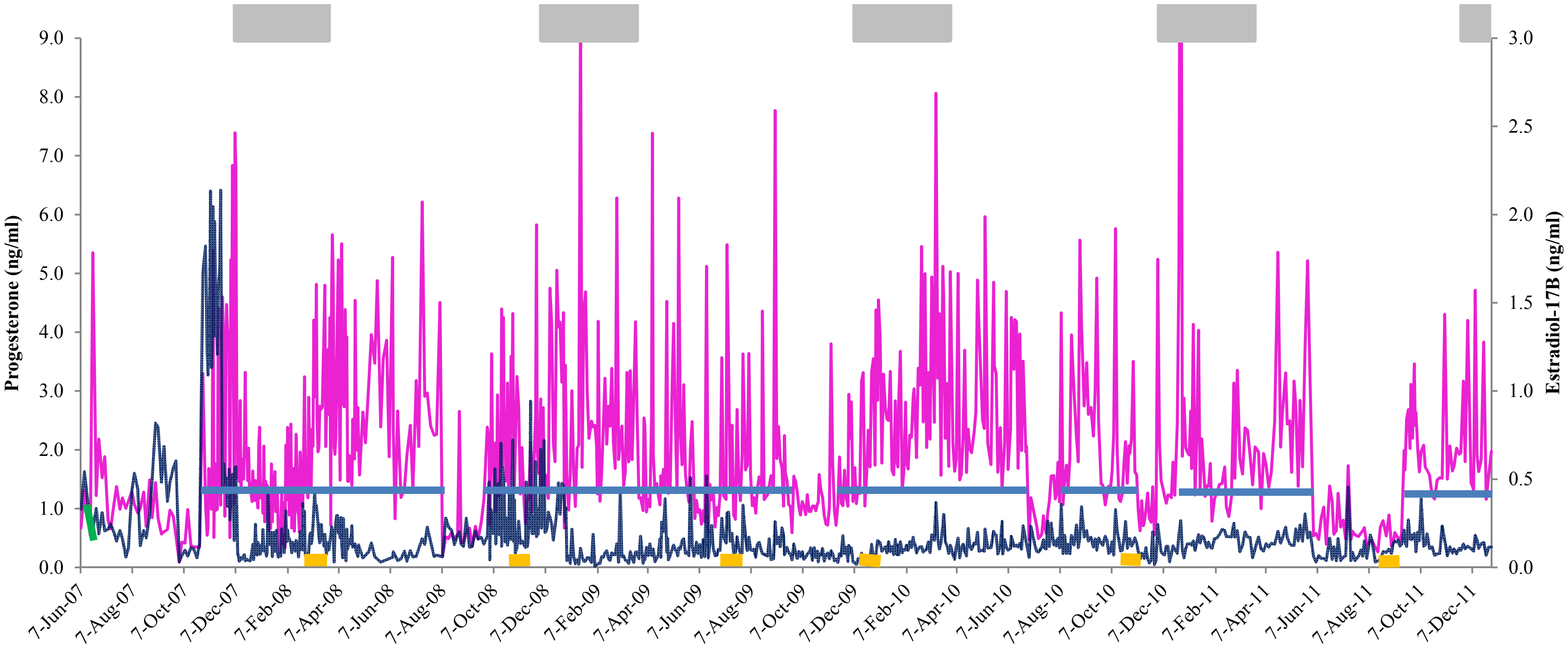


Figure 44. Endocrine profile from the longitudinal measurement of progesterone (———), estradiol-17β (———) and periods of behavioral changes (———) for a 23 year old female Pacific walrus (PDZA-1) from June 2007 to December 2011. The blue lines (———) denote periods of elevated progesterone levels (>1.222). The gray shaded bars denote the wild walrus breeding season. Green lines (———) denote gaps in data collection of more than 1 week.

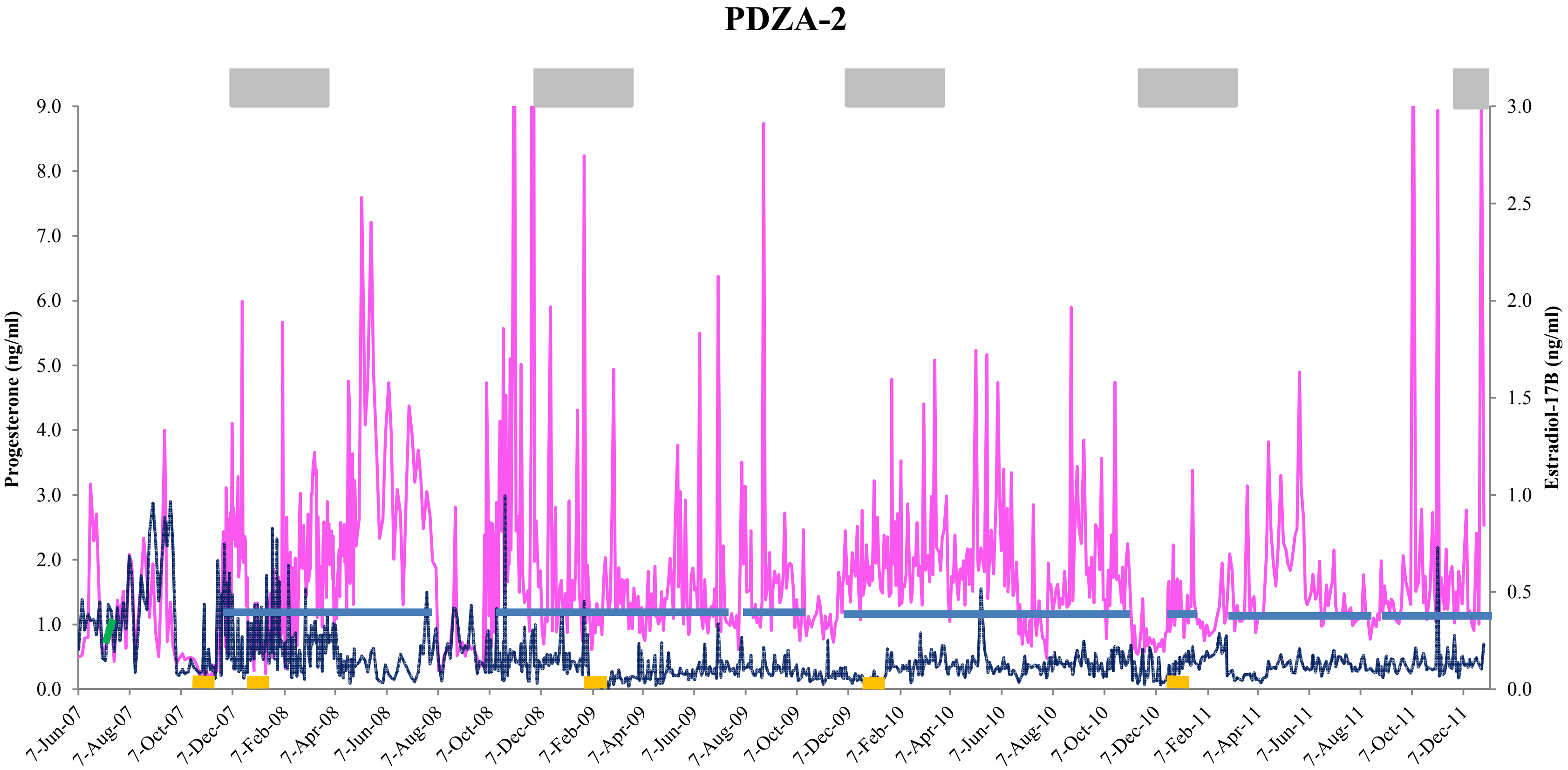


Figure 45. Endocrine profile from the longitudinal measurement of progesterone (———), estradiol-17β (———) and periods of behavioral changes (———) for a 12 year old female Pacific walrus (PDZA-2) from June 2007 to December 2011. The blue lines (———) denote periods of elevated progesterone levels (>1.057). The gray shaded bars denote the wild walrus breeding season. Green lines (———) denote gaps in data collection of more than 1 week.

IZ

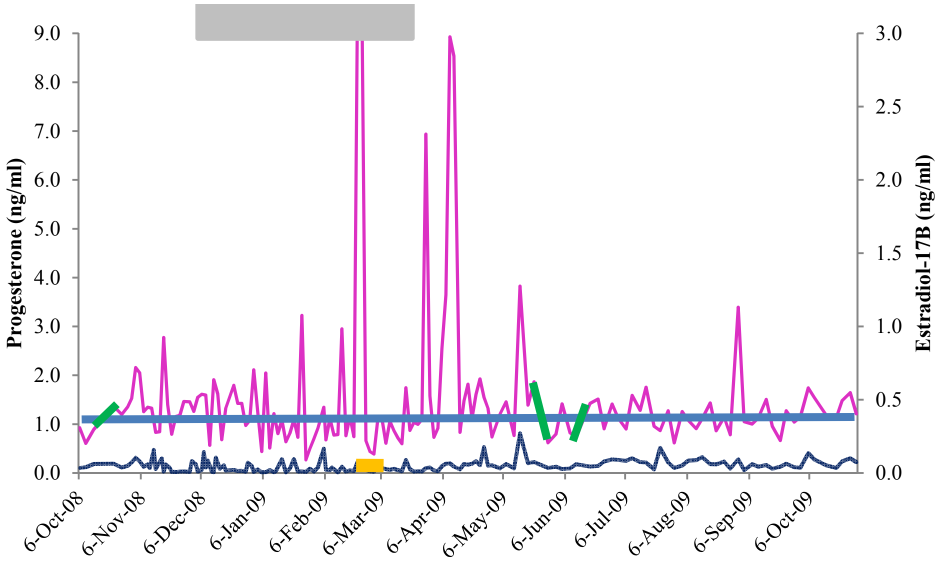


Figure 46. Endocrine profile from the longitudinal measurement of progesterone (——), estradiol-17β (——) and periods of behavioral changes (——) for a 13 year old female Pacific walrus (IZ) from October 2008 to October 2009. The blue lines (——) denote periods of elevated progesterone levels (>1.505). The gray shaded bars denote the wild walrus breeding season. Green lines (——) denote gaps in data collection of more than 1 week.

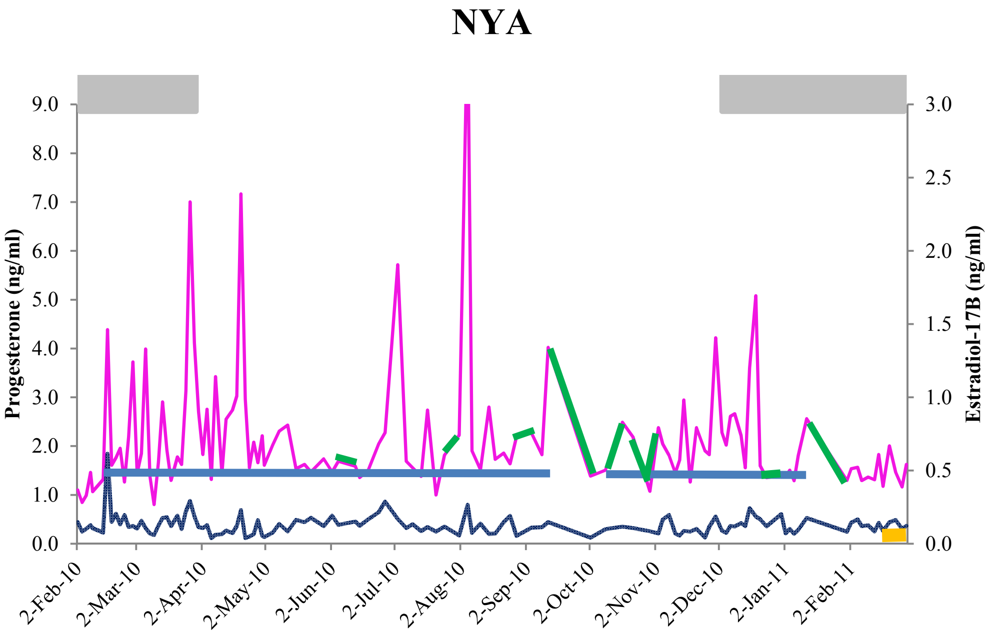


Figure 47. Endocrine profile from the longitudinal measurement of progesterone (———), estradiol-17β (———)and periods of behavioral changes (———) for a 16 year old female Pacific walrus (NYA) from February 2010 to February 2011. The blue lines (———) denote periods of elevated progesterone levels (>1.220). The gray shaded bars denote the wild walrus breeding season. Green lines (———) denote gaps in data collection of more than 1 week.

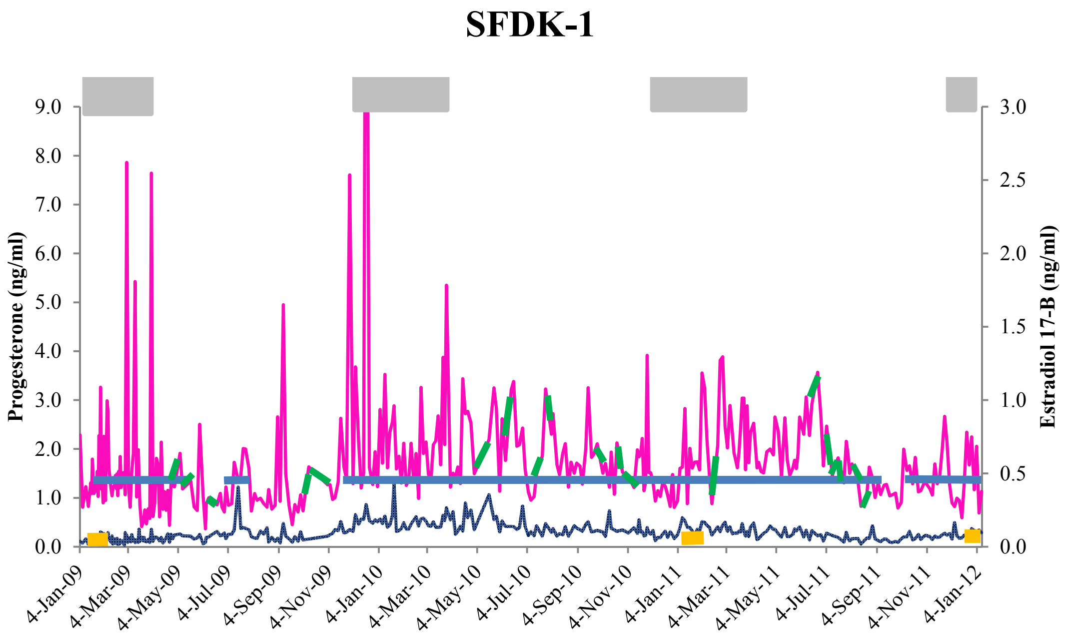


Figure 48. Endocrine profile from the longitudinal measurement of progesterone (——), estradiol-17β (——) and periods of behavioral changes (——) for a 15 year old female Pacific walrus (SFDK-1) from January 2009 to January 2012. The blue lines (——) denote periods of elevated progesterone levels (1.044). The gray shaded bars denote the wild walrus breeding season. Green lines (——) denote gaps in data collection of more than 1 week.

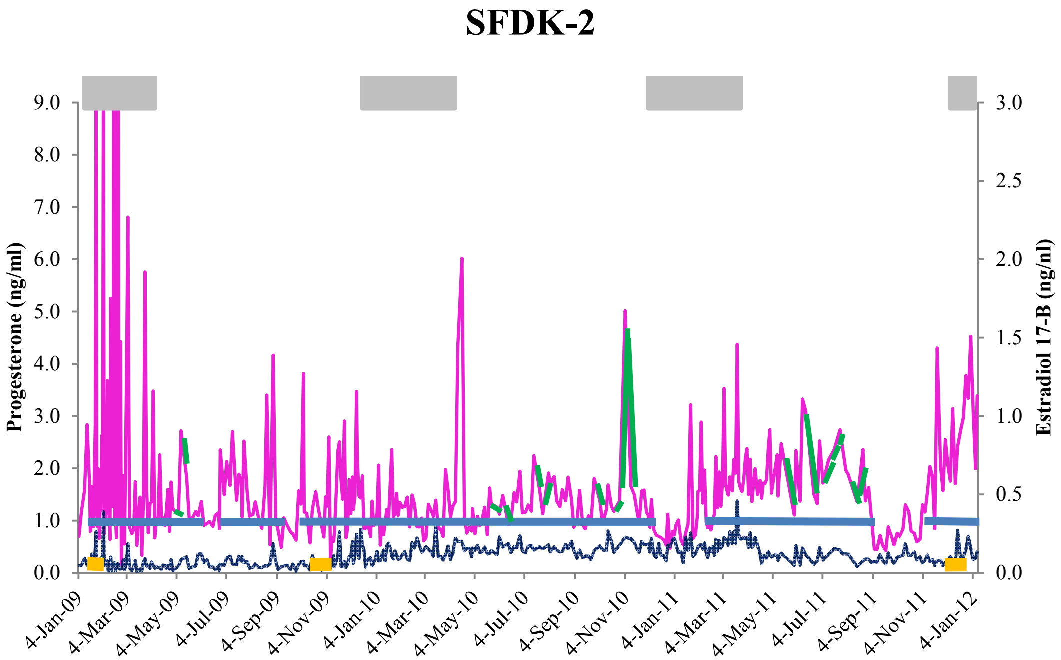


Figure 49. Endocrine profile from the longitudinal measurement of progesterone (——), estradiol-17β (——) and periods of behavioral changes (——) for a 15 year old female Pacific walrus (SFDK-2) from January 2009 to January 2012. The blue lines (——) denote periods of elevated progesterone levels (>1.044). The gray shaded bars denote the wild walrus breeding season. Green lines (——) denote gaps in data collection of more than 1 week.

PDZA-1

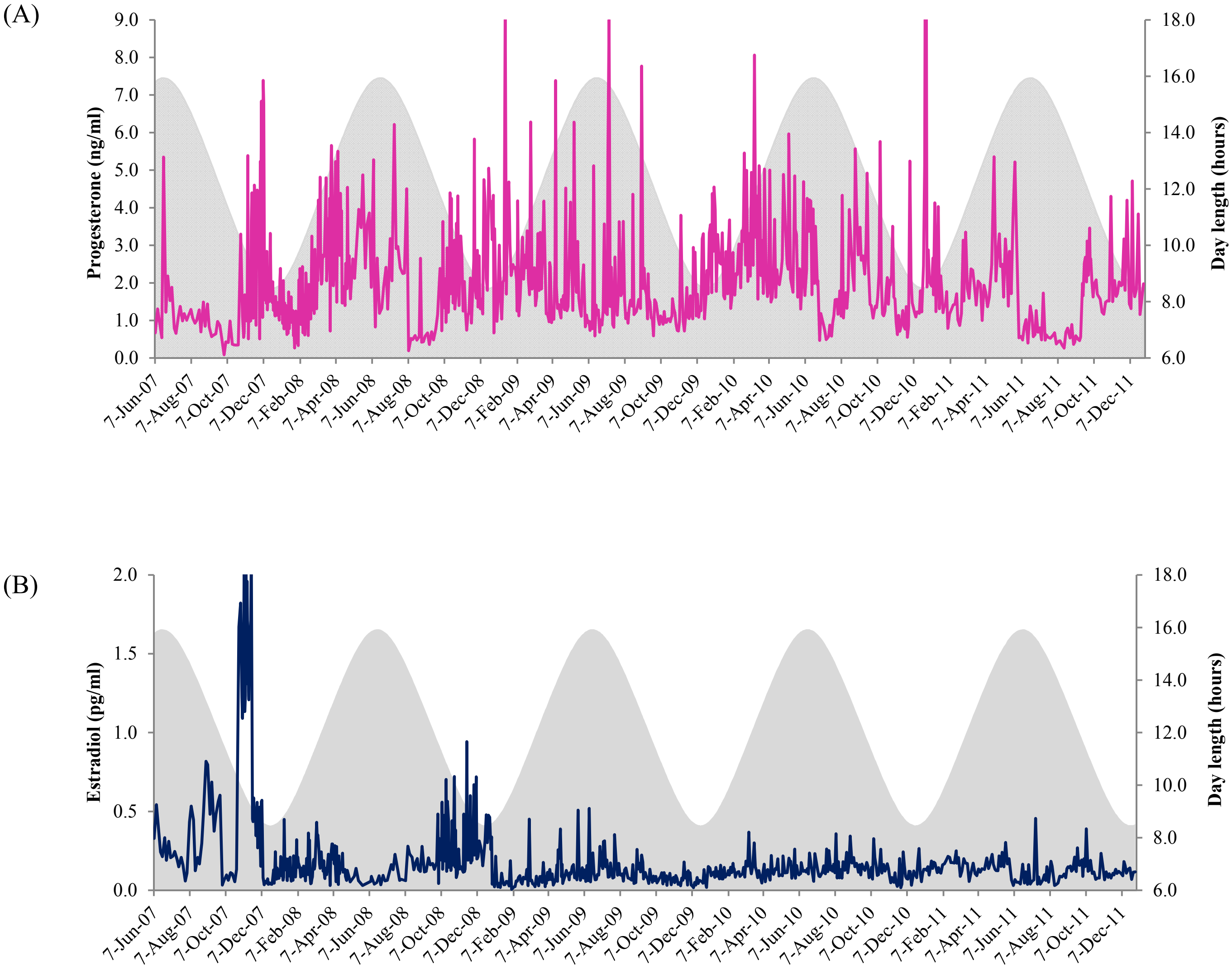


Figure 50. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) concentrations for the PDZA-1 (47°18’) female walrus.

PDZA-2

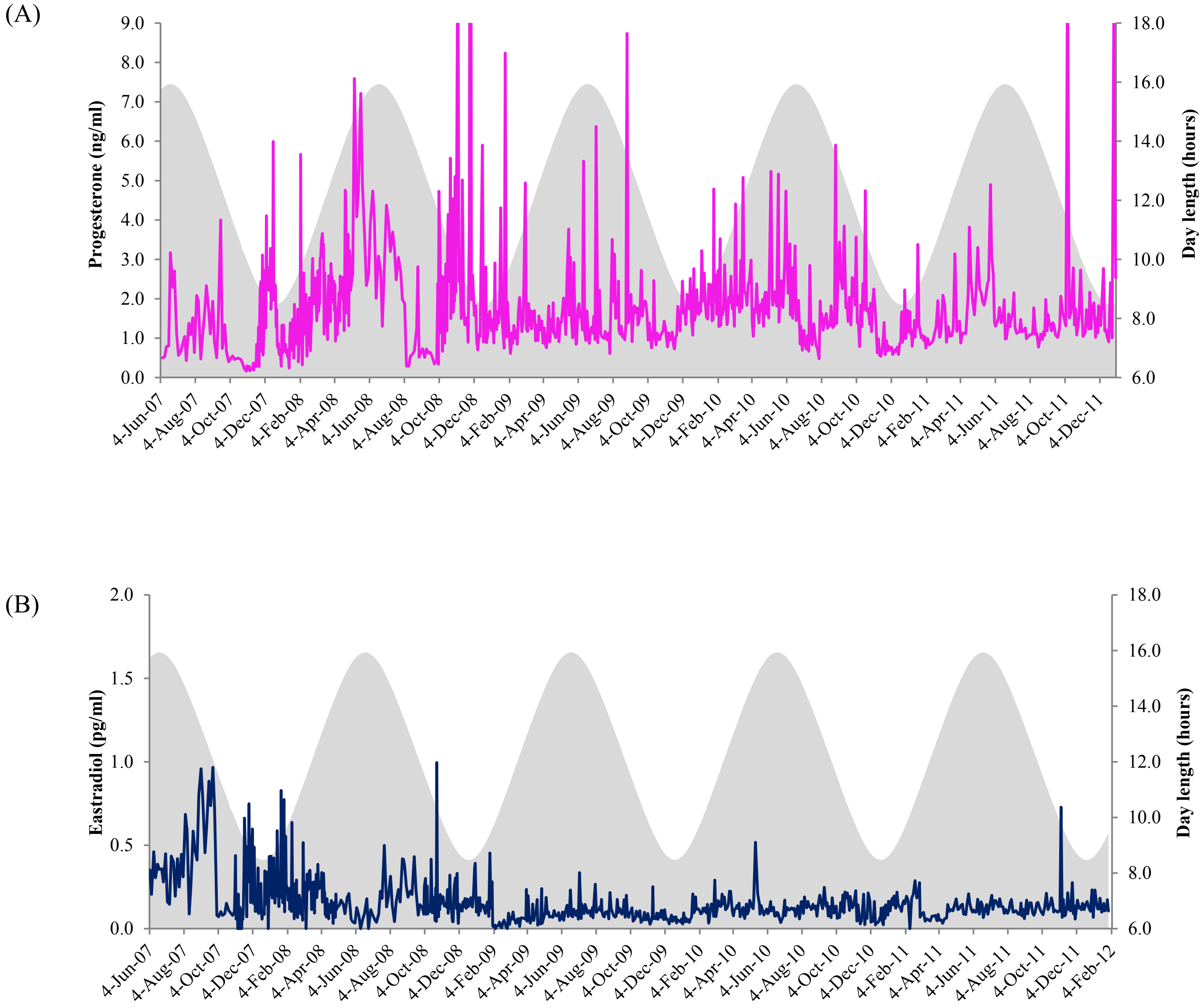


Figure 51. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) concentrations for the PDZA-2 (47°18') female walrus.

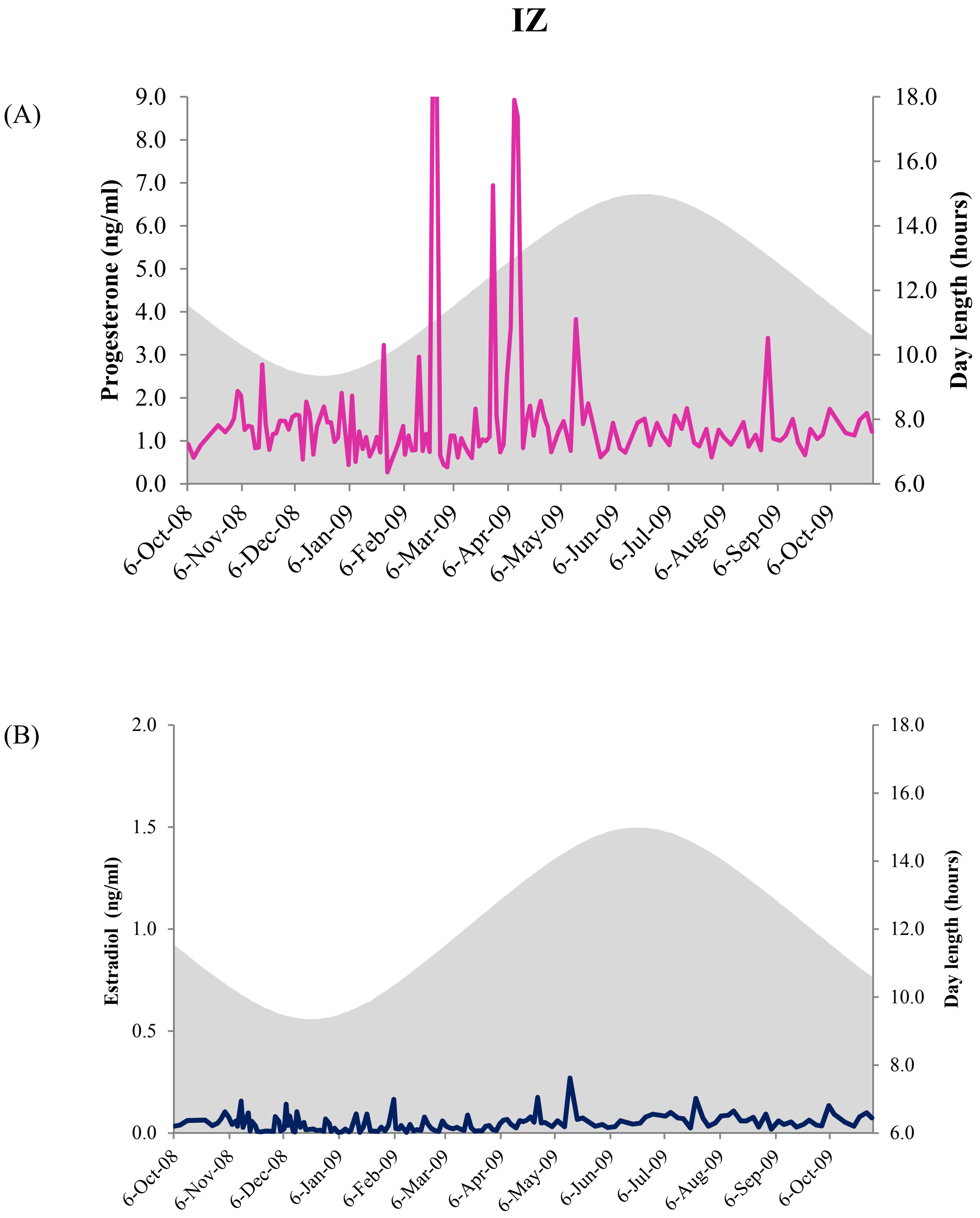


Figure 52. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) concentrations for the IZ (39°46') female walrus.

NYA

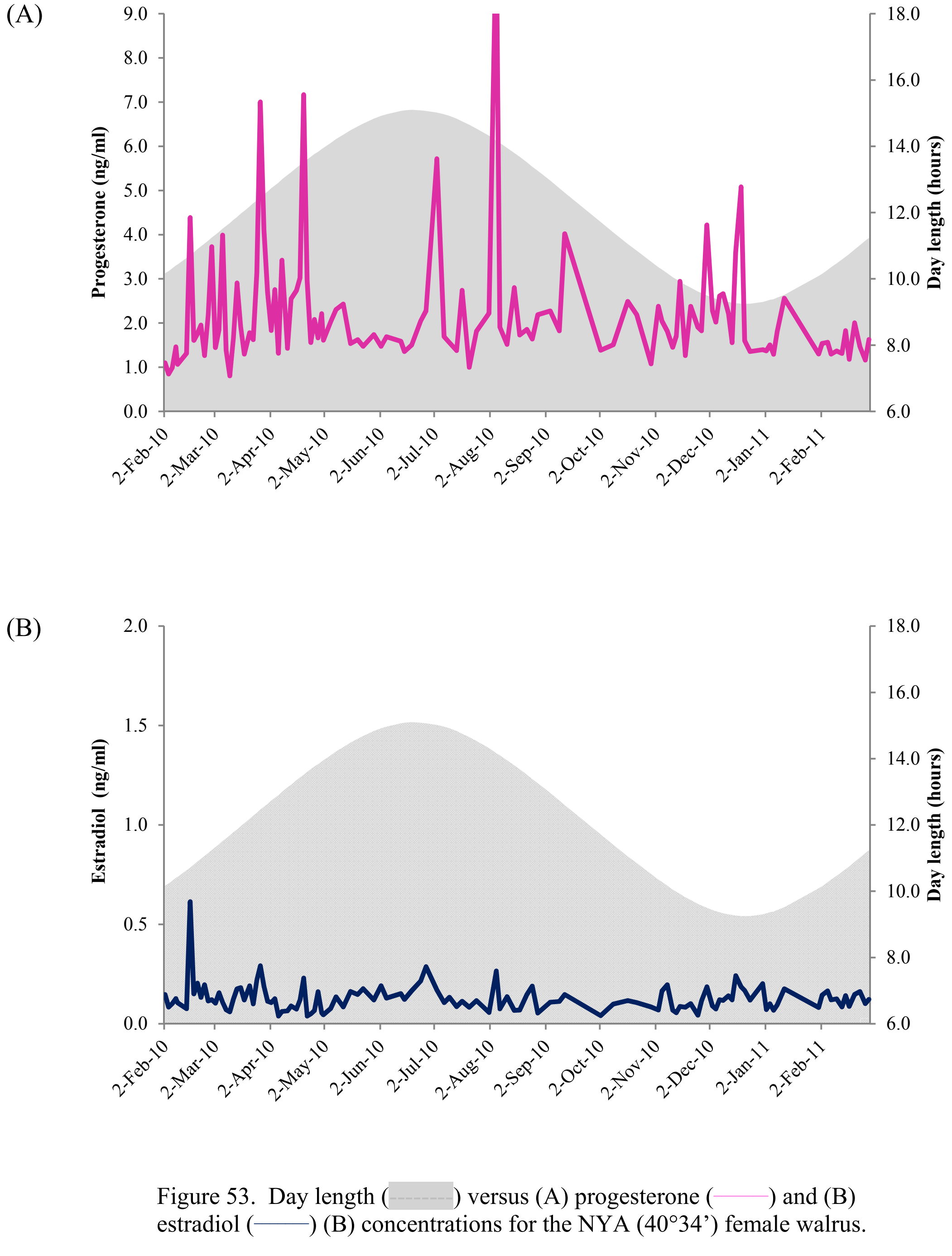


Figure 53. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) (B) concentrations for the NYA (40°34') female walrus.

SFDK-1

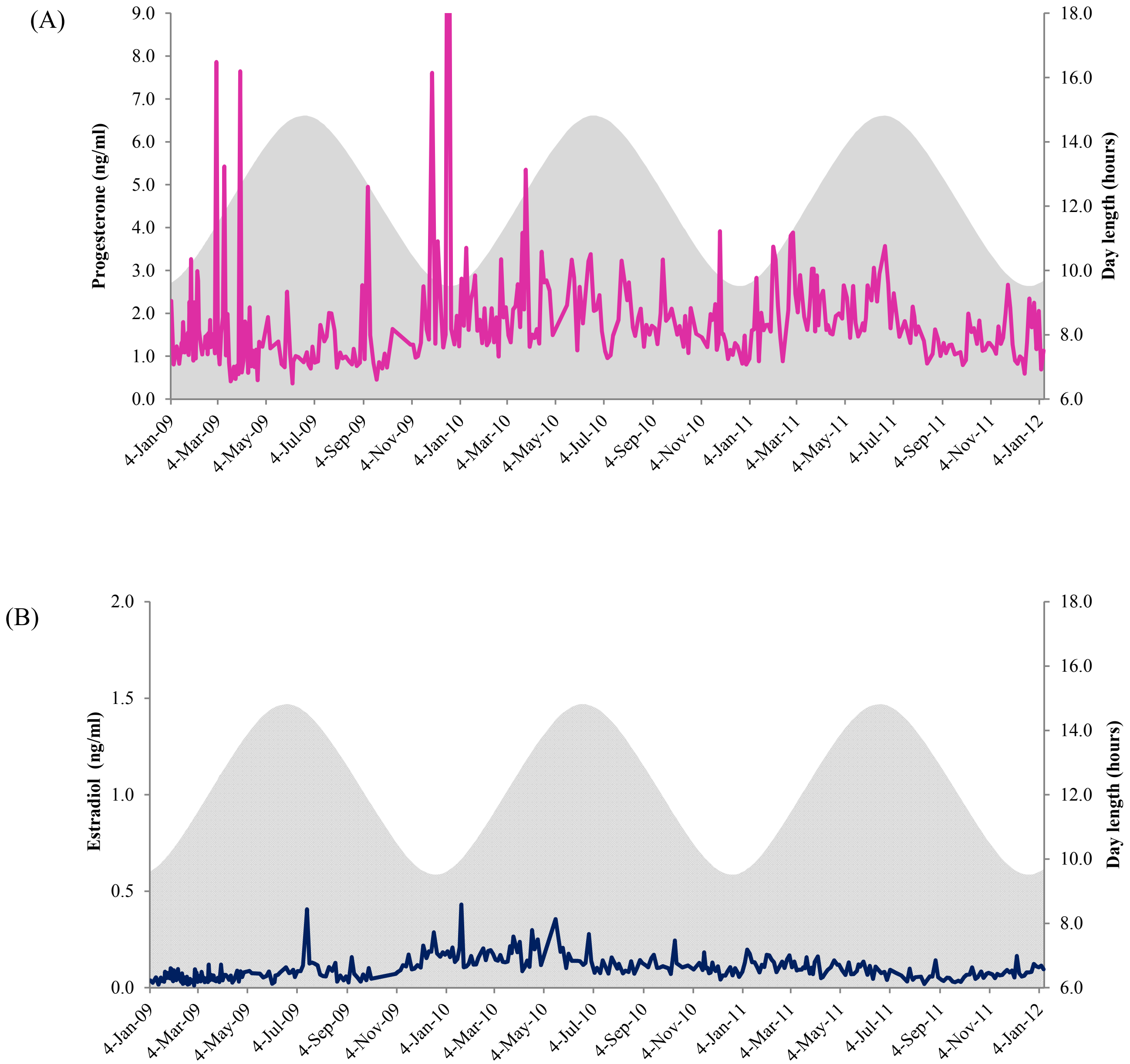


Figure 54. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) for the SFDK-1 (38°08’) female walrus.

SFDK-2

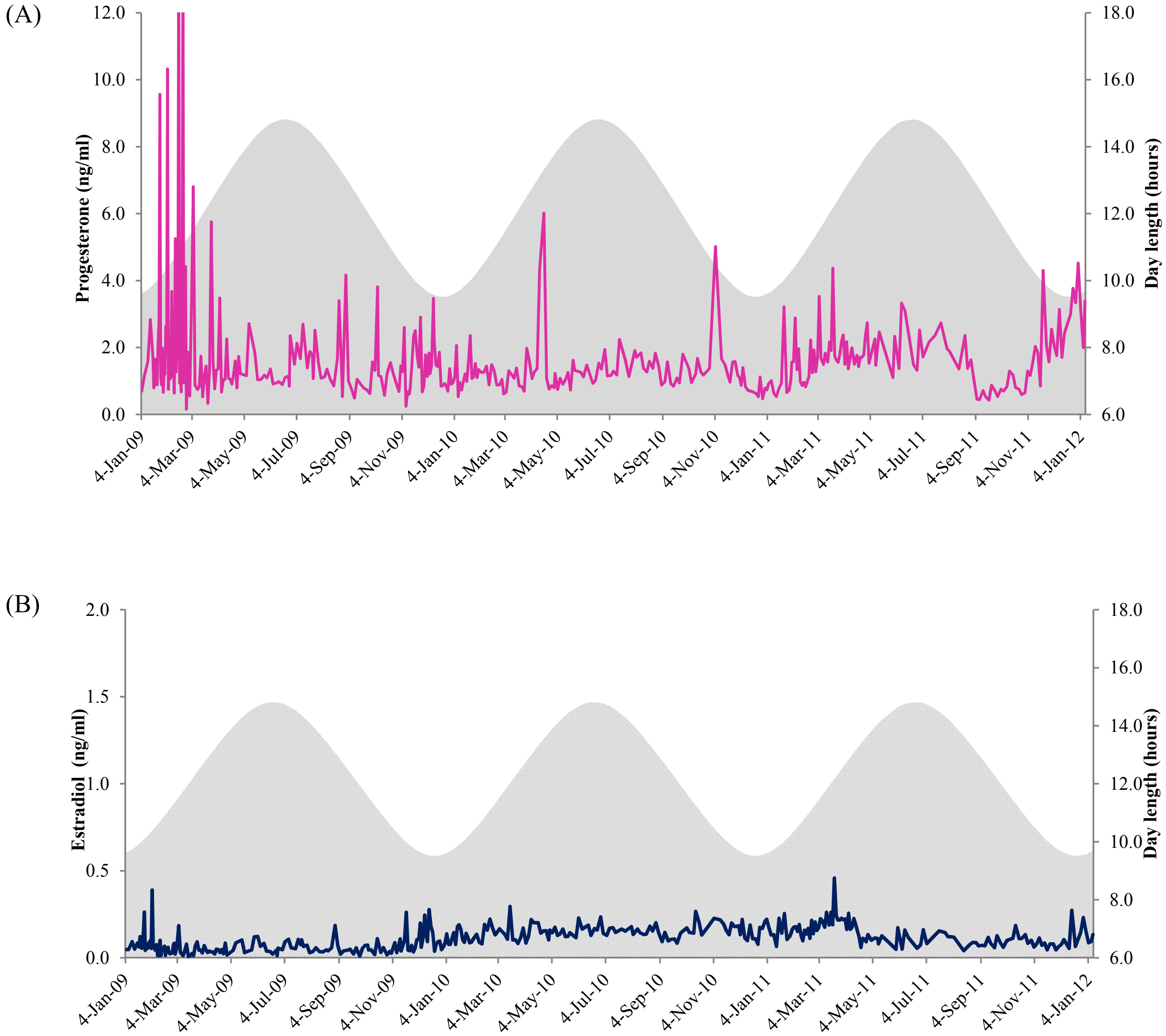


Figure 55. Day length (▨) versus (A) progesterone (—) and (B) estradiol (—) concentrations for the SFDK-2 (38°08') female walrus.

Summary and Future Research

The U.S. *ex situ* walrus population has met with limited reproductive success with only seven calves born in the past 10 years, and of these only one has survived to adulthood (Appendix 13). This presents a problem for the long-term maintenance of the zoo-based population of walrus in the U.S., as stranded wild animals are identified only sporadically and the capture of wild walrus is politically and logistically difficult. In response to concerns from the zoo-based walrus community, PDZA initiated a collaborative, non-invasive endocrine study of *ex situ* walrus that would not only increase the knowledge regarding the basic reproductive physiology of both male and female walrus but potentially elucidate problems regarding reproductive success. In depth knowledge of the basic reproductive endocrinology of *ex situ* walrus utilizing serum is limited to a few studies (Siembieda 2005, Kinoshita *et al*. 2012, Muraco *et al*. 2012). In addition, no research had been conducted utilizing salivary reproductive hormones in walrus. Previous saliva studies in other marine mammal species have shown that long-term evaluation of reproductive steroids is effective for evaluating ovarian and testicular activity (Hogg *et al*. 2005, Pietraszek and Atkinson 1994, Theodorou and Atkinson 1998*,* Harmon 2001). Saliva was chosen for the study for a variety of reasons including: non-invasive sample collection, relative stability, successful use in other species, and ease of training and collection allowing more facilities to participate in the study and thus providing a larger sample size.

Males

Walrus reproductive events and seasonal changes were monitored in 12 *ex situ* walrus in five facilities in North America utilizing a variety of methods. To track reproductive patterns in males, longitudinal hormonal analysis of saliva samples, changes in testicular size, presence or absence of spermatozoa and seasonal fluctuations in behavior were examined. The study demonstrates for the first time that male walrus secrete measurable amounts of testosterone in their saliva and resulting concentrations can be used along with other anatomical and physiological processes to better understand and track reproductive cyclicity. Based on a combination of salivary testosterone, testicular measurements, sperm production and behavior, six out of nine breeding seasons for subject animals in the study coincided with the wild walrus breeding season.

Baseline testosterone values for each animal were established using an iterative process. Elevations were defined as values ≥ 2 times mean "baseline" values (elevation range 0.838-4.228 ng/ml). Endocrine profiles varied extensively among animals. Mature animals displayed elevated concentrations for several months while young animals displayed no seasonal pattern in secretion. The timing of peak values in testosterone varied with animals and did not correlate with geographic latitude or photoperiod. Other patterns involved a mature male with decreasing hormone concentrations in successive years as well as a male with increased hormone concentrations out of phase with the wild walrus breeding season.

Mean baseline testicular measurements for individuals (R linear length + R linear width + L linear length + L linear width) were 54.81±2.13 cm while mean increased testicular measurements were 65.38±1.71 cm consistent with the findings of Fay (1982). All males in the study exhibited periods of testicular recrudescence and regression. However, some animals demonstrated periods of elevated testicular measurements that were not preceded or did not coincide with periods of elevated testosterone levels.

Longitudinal, simultaneous monitoring of testicular and seminal traits in one male demonstrated that seasonal changes in testicular measurements coincided with sperm presence or absence. Mean duration of spermatozoal presence was 131±3 days.

Three out of six animals displayed the typical rise and fall of testosterone concentrations observed in seasonal breeders and elevations were consistent with reproductive behaviors. Behaviors most indicative of rut included visual and vocal courtship displays, pursuit of females, clasping and penetration. In six out of eight breeding seasons, walrus rut behavior coincided with elevated testosterone levels or increased testicular measurements. Behaviors consistent with rut generally lasted several months.

Females

Hormonal analysis of longitudinally collected saliva samples and seasonal fluctuations in behavior were examined to track female reproductive cycles. The results of the study demonstrate the lack of effectiveness of use of salivary estradiol-17β for non-invasive reproductive monitoring of follicular activity in female walrus. Therefore, the results from the estradiol-17β assays were deemed unreliable.

Salivary progesterone levels were found to be unsuitable for monitoring the functional capacity of the corpus luteum in female walrus. Even though the assay was validated via parallelism and correlations of progesterone in serum and saliva were found to be significant, progesterone profiles were difficult to interpret biologically or physiologically. The particular assay system or methodology is not the most appropriate for walrus making data inconclusive for females.

Despite the results for progesterone profiles several findings were reported. Periods of elevated progesterone levels were demonstrated in two out of six animals and most likely represent consecutive pseudopregnancies (mean 204.27 days). The timing of peak values in progesterone concentrations varied with animals and did not correlate with geographic latitude or associated photoperiod. An inconsistency elucidated in the study involved a female with a unique derivation in her endocrine profile, inconsistent with the wild walrus breeding season and varying in time frame each year.

Mature females in the study displayed behaviors consistent with estrus more often than not concurrent with the wild walrus breeding season. Soliciting male attention, allowing clasping or mutual clasping and allowing penetration were the signs most indicative of estrus, but were not observed in every cycle. In general, behavioral observation was not a consistently reliable method for detecting impending estrus.

Reproductive Issues

The main issues concerning walrus reproduction elucidated by the study included: within-animal decline in hormone concentrations over time, unsynchronized cycles between animals residing together and inconsistent timing of reproductive events in blind animals.

Historically in zoos and aquaria, genders have been housed together continuously throughout the year. The lack of breeding success may be due in part to biological complacency in the male (Fowler and Whalen 1961, McGill 1963, Michael and Zumpe 1978, Coquelin and Bronson 1979). In addition, a significant result of living in a zoo or aquarium compared to the wild is the reduction in group size. For some species, being housed in inappropriate social groups can lead to an inability to perform necessary reproductive behaviors (Price and Stoinski 2007). While thus untested in walrus, the absence of male competition and the reduction in group size may be detrimental to promoting natural courtship behavior in both genders. Increased competition between breeding males of other polygynous mammal species seems to encourage breeding success (Bartholomew 1970, Galimberti *et al*. 2007, Harcourt *et al*. 2007, Mainguy and Cote 2008) and increased group size or an improved sociosexual environment may facilitate successful reproduction in walrus.

To increase breeding success of the *ex situ* population, the following changes are suggested: a) increase the number of walrus in the population or consolidate existing walrus into larger social groups to better mimic the wild social structure; and b) separate the genders during the non-breeding season or move animals to new facilities. Group participation and continued monitoring of reproductive functioning via endocrine, anatomical and behavior parameters would allow evaluation of the changes.

Future Research

Additional research into walrus reproduction is needed to achieve a nationally successful breeding program in *ex situ* walrus. Initial efforts should focus on conducting additional non-invasive studies to determine the main routes of hormone excretion in walrus and establishing the dominant reproductive hormones in serum, saliva, feces and urine allowing for the determination of assay systems most appropriate for monitoring gonadal function in walrus species. Further studies addressing seasonal changes in reproductive activity, reproductive senescence, gonadal steroidogenesis, folliculogenesis, spermatogenesis, and the influence of social conditions on the reproductive performance of walrus via longitudinal endocrine monitoring, sperm evaluation, testicular measurements, behavioral observations and ultrasonography would greatly improve our understanding of reproduction in the species.

Continued longitudinal monitoring and assessment of *ex situ* walrus reproductive processes presents a number of potentially valuable applications for animal management. Specifically, by monitoring reproductive hormones over time, the reproductive status and synchrony of males with females can be evaluated before pairing for the breeding season. Peak testicular measurements can be identified for the precise timing of semen collection procedures to ensure recovery of sufficient samples. Semen collection would allow the evaluation of male reproductive potential by correlating testicular growth with sperm production and testosterone levels with sperm morphology, concentration and motility. Successful sperm preservation could potentially allow artificial insemination given that the shipping of sperm is logistically easier and less costly than shipping walrus. The characterization of the follicular cycle via ultrasound would be useful to pinpoint ovulation and correlate the event to hormonal and behavioral changes. In addition, ultrasound could be used to define the normal reproductive tract and facilitate identification of abnormalities or species specific reproductive problems that could be contributing to reduced reproductive success. It is evident that there is more to breeding walrus than just housing the species together. The study is just the tip of the iceberg in elucidating issues with *ex situ* walrus reproduction and serves as a reference for future studies.

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Appendix 1.

Glossary of terms.

Baculum – a bony support in the penis of certain mammals.

Bicornate uterus - a uterus consisting of two distinct uterine horns.

Blastocyst – an early embryo consisting of an inner cell mass, a blastocoele and a trophoblast.

Corpus luteum (CL) – an orange to yellow colored transient endocrine structure formed after ovulation from granulosal and thecal cells of the ovarian follicle. The corpus luteum is responsible for producing progesterone. It also secretes estrogen to inhibit the further release of gonadotropin releasing hormone (GnRH) and thus secretion of lutenizing hormone (LH) and follicle stimulating hormone (FSH).

Delayed implantation – a period of dormancy in the development of a fertilized ovum beginning with attainment of the blastocyst and concluding with implantation in the uterine wall.

Didelphic uterus – a double uterus with two separate cervices.

Enzyme immunoassay (EIA) – a method of detecting and quantifying hormones utilizing an enzyme-linked antibody that produces an identifying color in the presence of the appropriate substrates.

Epididymis – an elongated mass of convoluted efferent tubules adjacent to the testis, functioning as a transport, storage and maturation site for spermatozoa.

Estradiol-17β (E_2) – the most potent naturally occurring ovarian and placental estrogen in mammals; prepares the uterus for implantation of the fertilized ovum and promotes the maturation of and maintenance of the female accessory reproductive organs and secondary sexual characteristics.

Estriol (E_3) – a steroid hormone secreted in significant amounts by the placenta during pregnancy.

Estrogen – a class of steroid hormones that exerts physiologic effects on the female reproductive and mammary systems.

Estrone (E_1) – a steroid hormone secreted by the ovary and by adipose tissue.

Estrus – the period of sexual receptivity in the female.

Follicle stimulating hormone (FSH) – a glycoprotein hormone secreted by the anterior lobe of the pituitary in response to GnRH. FSH promotes follicular development in the female and Sertoli cell (sperm production) function in the male.

Follicular phase – the phase of the estrous cycle characterized by the presence of a dominant follicle or follicles that produces E_2. Females display behavioral estrus and ovulate during the follicular phase.

Folliculogenesis – the process whereby ovarian follicles develop from primary follicles into secondary follicles and eventually into antral follicles that become competant for ovulation.

Gonadotropin – the hormones (FSH and LH) of anterior pituitary origin that stimulate gonadal function.

Gonadotropin releasing hormone (GnRH) – a decapeptide released from terminals of neurons in the surge and tonic centers of the hypothalamus that causes the release of gonadotropins from the anterior lobe of the pituitary.

Hypothalamic-pituitary-gonadal (HPG) axis – three endocrine glands that work cooperatively to play a critical part in the development and regulation of the body's reproductive system.

Hypothalamus – basal portion of the forebrain, linking the nervous system to the endocrine system via the pituitary gland; acts as an endocrine gland by producing hormones, including the releasing factors that control the hormonal secretions of the pituitary gland.

Inhibin – a glycoprotein hormone produced by the Sertoli cells in the male and granulose cells in the female that specifically inhibits the release of follicle stimulating hormone (FSH) from the anterior lobe of the pituitary.

Lek – a breeding system in which males assemble during the mating season and engage in competitive displays intended to attract females as breeding partners.

Leydig cells – cells found in the interstitial compartment of the testes that produce testosterone.

Luteal phase – the phase of the estrous cycle that begins with ovulation and the formation of the corpus luteum and ends with luteal regression.

Lutenizing hormone (LH) – a glycoprotein hormone secreted by the anterior lobe of the pituitary that causes ovulation and subsequent development and maintenance of the corpus luteum in the female. In the male, LH causes Leydig cells to produce testosterone.

Luteal-placental shift – an interval of time during mammalian gestation when the primary production of progesterone switches from the corpus luteum to the placenta.

Luteolysis – the process whereby luteal tissue (corpus luteum) undergoes regression and cell death.

Monoestrous – the characteristic of having only one period of sexual receptivity (estrus) during a year.

Ovum – the female reproductive cell or gamete.

Photoperiodism – the ability of organisms to assess and use the length of day as an anticipatory cue to time and synchronize seasonal, physiological and behavioral events.

Pineal gland – a neural structure on the dorsal surface of the midbrain that secretes melatonin in response to changing photoperiods.

Pituitary gland – an endocrine gland found at the base of the brain.

Polyestrous – the characteristic of having multiple estrous cycles uniformly distributed throughout the year without marked seasonal influence.

Progesterone – a steroid hormone produced by corpora lutea and the placenta that is required for the maintenance of pregnancy.

Prostate gland – an exocrine gland of the male reproductive system in most mammals.

Pseudopregnancy – prolonged period of high concentrations of progesterone in the absence of a fetus.

Recrudescence – redevelopment of the testes; the prolonged, initial part of spermatogenesis.

Reproductive senescence – a decrease in the reproductive rate with increasing age post-maturity.

Rut – the seasonally recurrent period of male mammalian reproductive activity.

Seminiferous tubules – the highly convoluted tubules within the testes that produce spermatozoa.

Septate vagina – a vagina that is divided, longitudinally, to create a double vagina.

Sertoli cells – somatic cells in the seminiferous epithelium that are believed to govern spermatogenesis. Setoli cells contain FSH receptors and produce a wide variety of materials and hormones.

Sex hormone binding globulin (SHBG) – a glycoprotein that binds to sex hormones (androgens and estrogens) in the bloodstream.

Spermatogenesis – the process whereby spermatozoa are formed.

Spermiogenesis – a subcategory of spermatogenesis during which spermatids undergo morphological transformation into highly specialized spermatozoa. Spermiogenesis consists of the Golgi phase, the cap phase, the acrosomal phase and the maturation phase.

Testosterone – the male sex hormone and the most potent naturally occurring androgen produced by the Leydig cells in the testes

Appendix 2.

Behavioral data collection form (male and females)

Date **Start time** **Observer**

	Location		Activity							Interaction/Manipulation												
Int	**W**	**L**	**S**	**pa**	**pb**	**pc**	**w**	**F**	**i**	**FS**	**MM**	**SS**	**WM**	**OM**	**RD**	**ED**	**CL**	**C**	**NV**	**O**	**Vocals**	**Comments**
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Date **Start time** **Observer**

	Location		**Activity**							**Interaction/Manipulation**												
Int	**W**	**L**	**S**	**pa**	**Pb**	**pc**	**w**	**f**	**i**	**FS**	**MM**	**SS**	**WM**	**OM**	**RD**	**ED**	**CL**	**C**	**NV**	**O**	**Vocals**	**Comments**
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ET																						
Joan																						
Basa																						

Appendix 3.

Male walrus behavioral ethogram (formal observations)

Location
W = Water: Animal has more than 50% of its body in the water
L = Land: Animal has more than 50% of its body on the land

Activity
s = non-pattern swim: locomotion in the water with no distinguishable pattern or from point A to point B
pa = pattern swim A: locomotion in the water in any one of three recorded patterns starting in a counterclockwise direction and originating at "spot" and terminating at "spot"

- swims ventrally to under water viewing windows (UVW)→ swims dorsally to main beach → swims dorsally to "spot" at the surface or underwater
- swims ventrally to under water viewing windows → swims dorsally to "spot" (returns counter-clockwise)
- swims ventrally to whale viewing window → swims dorsally to spot (returns counter-clockwise)

pb = pattern swim B: locomotion in the water in the following specified pattern

- starting in a clockwise direction and originating at tunnel and terminating at tunnel, swims ventrally to middle of main beach → swims dorsally from middle to whale viewing window → swims ventrally from whale viewing to middle (counter clockwise) → swims dorsally from middle to tunnel

w = walk: locomotion on land
f = foraging: locomotion in the water along horizontal surface (bottom of exhibit) with mystacial pad
i = inactive: remains in a stationary (non-swimming or non-propulsion) position either on land or in the water

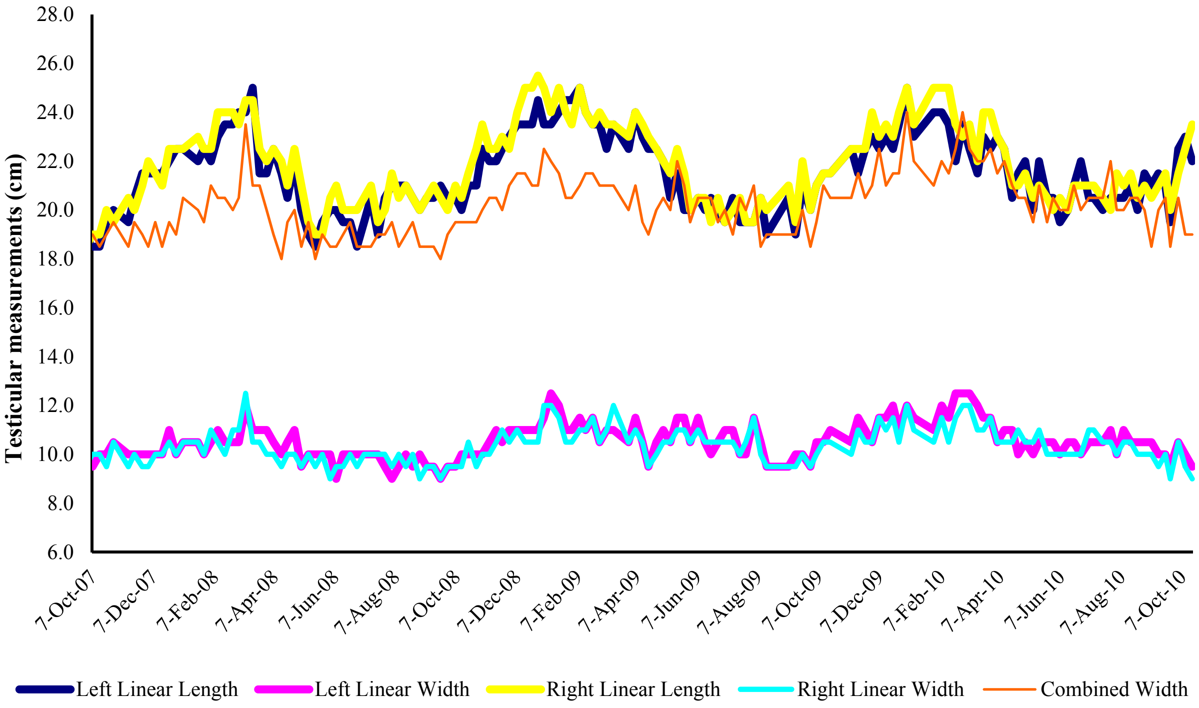
Interaction/Manipulation (I/M)
FS = Flipper Suck: 5th digit of right flipper in mouth
MM = Mouth Manipulation: stimulation of mouth with the tongue/lips
SS = Self Stimulation: manipulation of genital area with flippers, mouth, object or substrate
WM = Whisker Manipulation: movement of mystacial pad on vertical surface (may or may not be accompanied by food regurgitation)
OM = Object Manipulation: moves or touches object with any part of body other than the genital area
RD = Rut Display: displays any of the following behaviors associated with rut – pectoral flipper slapping, bubble blowing from mouth or nostrils, penis extended without self stimulation
CL = Clasping: holding onto other walrus with both front flippers
C = Copulation: successful penetration with con-specific (male inserts penis into female's vagina)

Not Visible (NV)
NV = Not Visible: data are unobtainable because animal is partially or totally out of sight

Other
Other: any behavior that is not defined

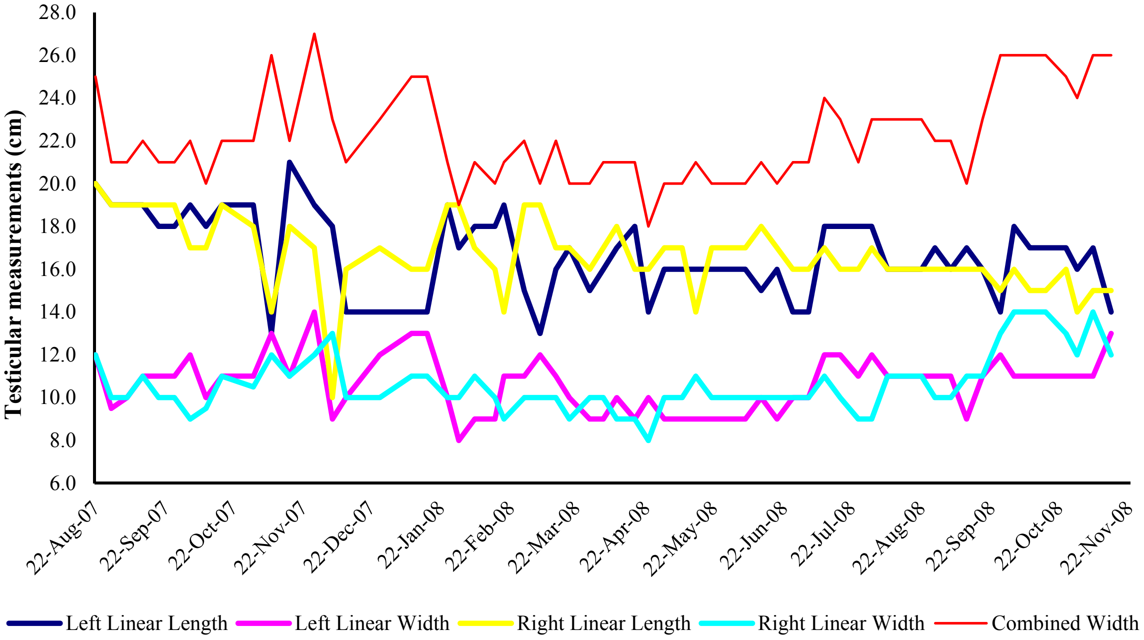
Vocals
Vocals: any sound production from vocal cords or air sacs

Appendix 4.



Testicular recrudescence and regression profile from the longitudinal measurements of testes for a male Pacific walrus (PDZA) from October 2007 to October 2010.

Appendix 5.

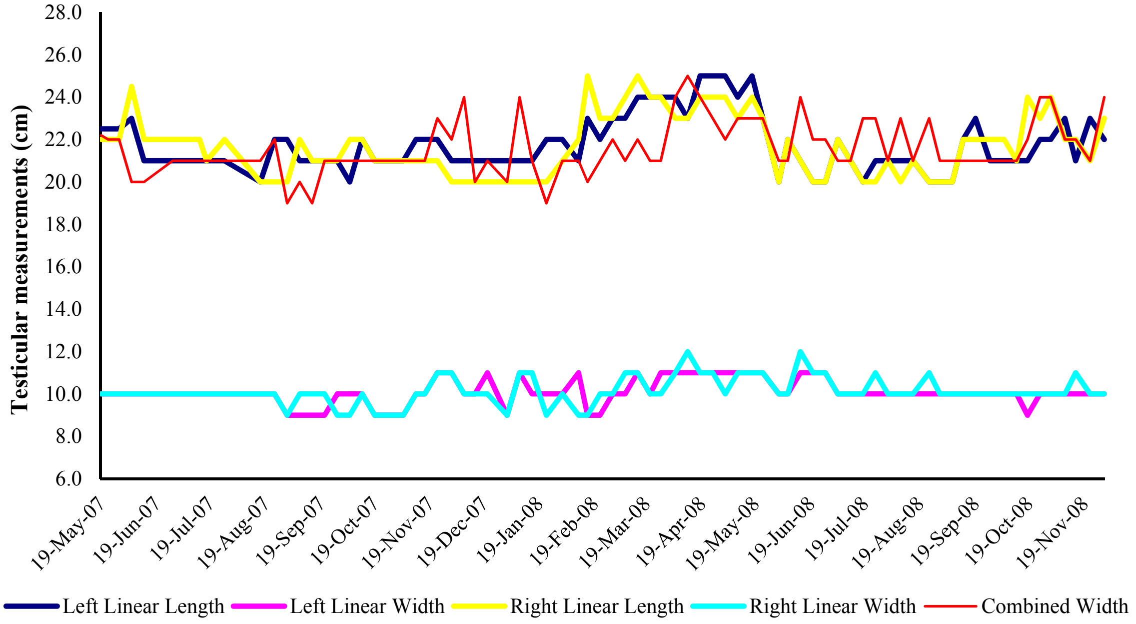


Testicular recrudescence and regression profile from the longitudinal measurements of testes for a male Pacific walrus (SWSD) from August 2007 to November 2008.

Appendix 6.

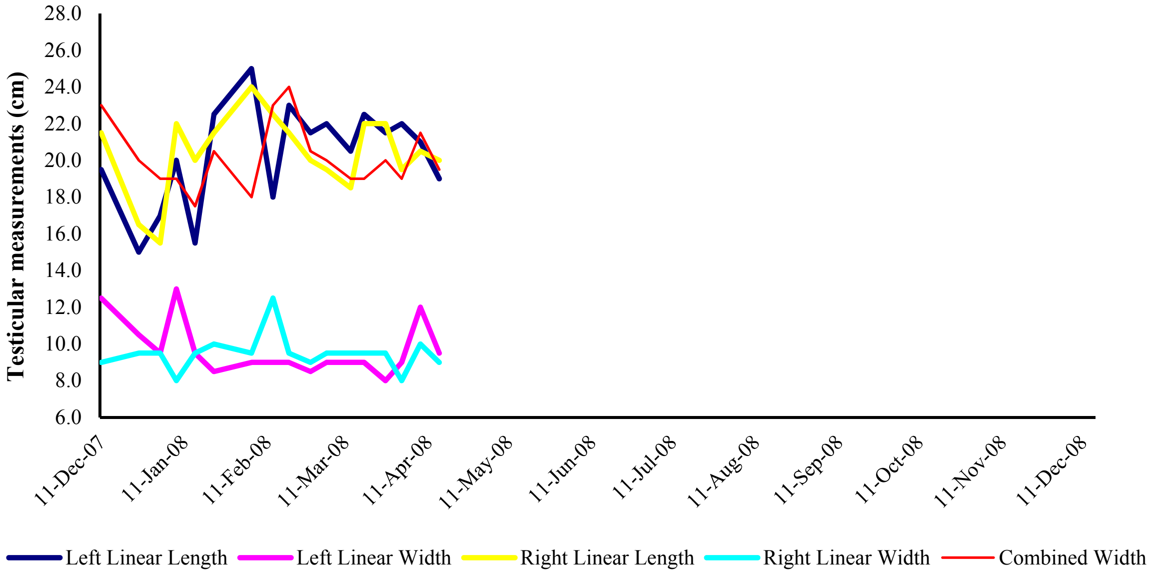
Testicular recrudescence and regression profile from the longitudinal measurements of testes for a male Pacific walrus (IZ-1) from November 2007 to November 2008 and September 2009 to April 2010.

Appendix 7.



Testicular recrudescence and regression profile from the longitudinal measurements of testes for a male Pacific walrus (SFDK) from May 2007 to November 2008 and September 2009 to April 2010.

Appendix 8.



Testicular recrudescence and regression profile from the longitudinal measurements of testes for a male Pacific walrus (NYA) from December 2007 to April 2008.

Appendix 9.

Sperm presence or absence (PDZA)

	Sperm		Locomotion	
Date	Present	Absent	Moving	Sedentary
1-Nov-08		X		
22-Nov-08		X		
30-Nov-08		X		
10-Dec-08		X		
20-Dec-08		X		
27-Dec-08	X			X
3-Jan-09	X			X
10-Jan-09	X		X	
13-Jan-09	X		X	
23-Jan-09	X			X
24-Jan-09	X		X	X
25-Jan-09	X		X	X
27-Jan-09	X		X	X
31-Jan-09	X		X	X
1-Feb-09	X		X	X
14-Feb-09	X		X	X
3-Mar-09	X		X	X
14-Mar-09	X		X	X
29-Mar-09	X			X
16-Apr-09		X		
18-Apr-09	X		X	X
25-Apr-09		X		
3-May-09	X			X
24-May-09		X		
28-May-09		X		
30-May-09		X		

	Sperm		Locomotion	
Date	Present	Absent	Moving	Sedentary
10-Oct-09		X		
11-Oct-09		X		
18-Oct-09		X		
8-Nov-09		X		
15-Nov-09		X		
29-Nov-09		X		
6-Dec-09	X		X	
13-Dec-09	X			X
20-Dec-09	X			X
26-Dec-09	X		X	
3-Jan-10	X		X	
10-Jan-10	X		X	X
30-Jan-10	X		X	X
7-Feb-10	X			X
14-Feb-10	X		X	X
21-Feb-10	X			X
28-Feb-10		X		
12-Mar-10	X			X
15-Mar-10	X			X
21-Mar-10	X		X	X
28-Mar-10	X			X
4-Apr-10	X		X	X
11-Apr-10	X		X	X
19-Apr-10	X			X
25-Apr-10		X		
2-May-10		X		
10-May-10		X		
16-May-10		X		
7-Oct-10		X		
10-Oct-10		X		
12-Oct-10		X		
17-Oct-10		X		

Appendix 10.

Body mass measurements (PDZA)

Date	Kilograms	Date	Kilograms
14-Oct-07	1426	22-Feb-09	1590
28-Oct-07	1449	07-Mar-09	1572
12-Nov-07	1468	29-Mar-09	1526
26-Nov-07	1492	12-Apr-09	1530
09-Dec-07	1540	26-Apr-09	1517
23-Dec-07	1590	10-May-09	1474
06-Jan-08	1643	24-May-09	1513
21-Jan-08	1678	07-Jun-09	1539
03-Feb-08	1676	20-Jun-09	1528
17-Feb-08	1649	04-Jul-09	1543
02-Mar-08	1636	19-Jul-09	1564
16-Mar-08	1614	02-Aug-09	1593
01-Apr-08	1568	15-Aug-09	1590
14-Apr-08	1554	29-Aug-09	1614
28-Apr-08	1541	13-Sep-09	1621
11-May-08	1535	28-Sep-09	1664
25-May-08	1540	10-Oct-09	1700
08-Jun-08	1536	25-Oct-09	1730
22-Jun-08	1546	08-Nov-09	1772
30-Jun-08	1545	22-Nov-09	1863
13-Jul-08	1533	06-Dec-09	1936
27-Jul-08	1522	20-Dec-09	1919
10-Aug-08	1531	03-Jan-10	1890
24-Aug-08	1535	17-Jan-10	1845
07-Sep-08	1526	21-Mar-10	1613
21-Sep-08	1526	04-Apr-10	1569
06-Oct-08	1531	11-Apr-10	1551
19-Oct-08	1555	13-May-10	1533
02-Nov-08	1595	26-May-10	1560
16-Nov-08	1640	16-Jun-10	1599
29-Nov-08	1692	20-Jun-10	1615
14-Dec-08	1740	26-Jul-10	1594
28-Dec-08	1685	09-Aug-10	1583
10-Jan-09	1685	23-Aug-10	1551
25-Jan-09	1646	07-Oct-10	1576
08-Feb-09	1617		

Appendix 11.

Mass Spectometry

Salivary samples with low and elevated concentrations of hormones were submitted to a mass spectrometry lab (University of Washington, Seattle, WA) for quantitative analysis of testosterone, progesterone and estradiol-17β. This was the first time this lab had worked with saliva from any species.

Two separate sample series were analyzed qualitatively. Samples from both series were run against internal known standards (d4-Cortisol-oxime, d4-Estrone-oxime and d9-Progesterone-oxime). The first series used derivatized and non-derivatized samples. Derivatized samples provided more sensitivity. The second series only examined underivatized samples. Only one of the samples in series one was run in duplicate; the other samples did not provide enough volume. Each of the seven samples in series two was run with 8 concentrations each in duplicate.

Chemicals and reagents used were as follows: 2-Androsten-17α-ol-3-one (epitestosterone; epiTST), 5β-pregnan-3α,11β,17,21-tetrol-20-one (tetrahydrocortisol), 5β-androsten-3β-ol-17-one (epietiocholanolone), 5-androsten-3β-ol-17-one (dehydroepiandrosterone, DHEA), 5α-androstan-3β-ol-17-one (transandrosterone), 4-pregnen-3,20-dione (progesterone), 5α-androstan-3α-ol-17-one (androsterone), 1,3,5(10)-estratrien-3-ol-17-one (estrone), 1,3,5(10)-estratrien-3-ol-17-one-2,4,16,16-d4 (d4-estrone), 4-pregnen-11β,17,21-triol-3,20-dione (cortisol), 4-pregnen-11β,17,21-triol-3,20-dione-9,11,12,12-d4 (d4-cortisol), 5α-pregnan-3α,11β,17,21-tetrol-20-one (allotetrahydrocortisol), 5α-androstan-17α-ol-3-one (epiallodihydrotestosterone), 5β-androstan-3α,11β-diol-17-one (11β-hydroxyetiocholanolone), 5β-andtostan-3α-ol-11,17-dione (11-

oxoetiocholanolone), 5β-pregnan-3α,17,21-triol-11,20-dione (tetrahydrocortisone), 4-androsten-17β-ol-3-one (testosterone) were purchased from Steroids, Inc (Newport, Rhode Island, USA). Rtiocholan-3α-ol-17-one (eticholanone) and hydroxyamine hydrochloride were purchased from Sigma-aldorich (St Louis, Missouri, USA), and 4-pregnen-3,20-dione-2,2,4,6,6,17,21,21,21-d9 (d9-progesterone) was from Cerilliant (Round Rock, TX).

The following instrumentation and methods were used for liquid chromatography procedures. The ultra-pressure liquid chromatography (UPLC) system consisted of a quatenary pump, degasser and autosampler (Agilent 1290 infinity; Agilent Technologies Palo Alto, California, USA). A Poroshell 120 EC-C18 column (150X2.1mm, 2.7μm, Agilent Technologies) was used with a guard column equipped with a C4 security guard cartridge (widepore C4, 4X20mm ID; Phenomenex, Torrance, California, USA). The column was maintained at 50˚C and the autosampler tray was kept at 6˚C. The mobile phase was (A) aqueous 10mM formic acid solution with (B) acetonitrile containing 10mM formic acid, and mobile phase (B) was 30% initially. The flow rate was 0.5 mL/min throughout. The gradient of (B) was initiated as follows: from 30% to 44% for 7 minutes, maintained at 44% for 0.5 minutes, from 44% to 60% for 8 minutes. After washing with 80% (B), the column was equilibrated at the initial condition for 4 minutes.

The following equipment and methods were used for mass spectrometric procedures. An Agilent 6460 triple Quad LC/MS equipped with electron spray ionization was used (Agilent Technologies). Nitrogen (N_2) was used as the drying, nebulizer and collision gas. The steroids were analyzed in positive ion mode, and drying gas temperature and flow were 350˚C and

10L/min, respectively. Nebulizer pressure was 40psi and capillary voltage was 3000V. Sheath gas heater and flow were 300˚C and 10L/min, respectively. Dynamic multiple reaction monitoring (dMRM) was used, and the delta electron multiplier voltage was 300V. Dwell time was 500ms and cell accelerator energy was 7V.

Preparation of standards and calibration were done as follows. Individual steroids were dissolved in methanol to prepare 1.0mg/mL stock solution and stored at -20˚C. To prepare a mixture of internal standard (IS), d4-estrone, d4-cortisol and d9-progesterone were diluted in methanol at 1µg/mL as a IS stock. All steroids were prepared as follows. Working standard solutions were prepared in methanol by sequential dilution at the concentration of 0.50, 1.0, 5.0, 10, 50, 100, 500, 1000ng/mL. Ten µL of working standard solutions were mixed with 490µL of phosphate buffer saline; thus, the final concentrations of calibrations were 0.01, 0.02, 0.1, 0.2, 1, 2, 10 and 20ng/mL. To determine the accuracy of the concentration of standards, two different batches were prepared as quality control.

Samples were prepared in the following manner. Saliva samples were obtained from male and female Pacific walrus, and the collected saliva samples were stored at -80˚C until analyzed. The extraction derivatization procedures followed the studies previously reported with modifications (Hauser *et al*. 2008; Keski-Rahkonen *et al*. 2011). In brief, 500 µL of saliva or standard solutions were spiked with 5µL of the IS stock, and the pH was adjusted to 9.5-10 for better extraction efficiency. Four mL of methyl t-butyl ether (MTBE) were added to the saliva or standard samples, and vortexed for 10min. The samples were then tightly sealed and spun at 3500rpm at 4˚C for 10min using a Thermo Allegra centrifuge (Thermo Scientific, Asheville, North Carolina, USA). After storage at -20˚C overnight, the organic layer was decanted to a glass tube and dried under a stream of N_2 gas. The extracts were reconstituted in 50µL of methanol and transferred into a vial containing 50µL of 0.5M hydroxyammonium chloride solution. The reconstituted samples were incubated at 65˚C for 2 hours.

Salivary samples submitted for quantitative analysis of testosterone, progesterone and estradiol (series one)

Sample	Gender	EIA Testosterone (ng/ml)	EIA Progesterone (ng/ml)	EIA Estradiol-17β (ng/ml)	Comments
2284	male	1.01	not run	not run	
2302	female	not run	10.79	0.13	
2324	female	not run	max	0.73	sensitivity issues

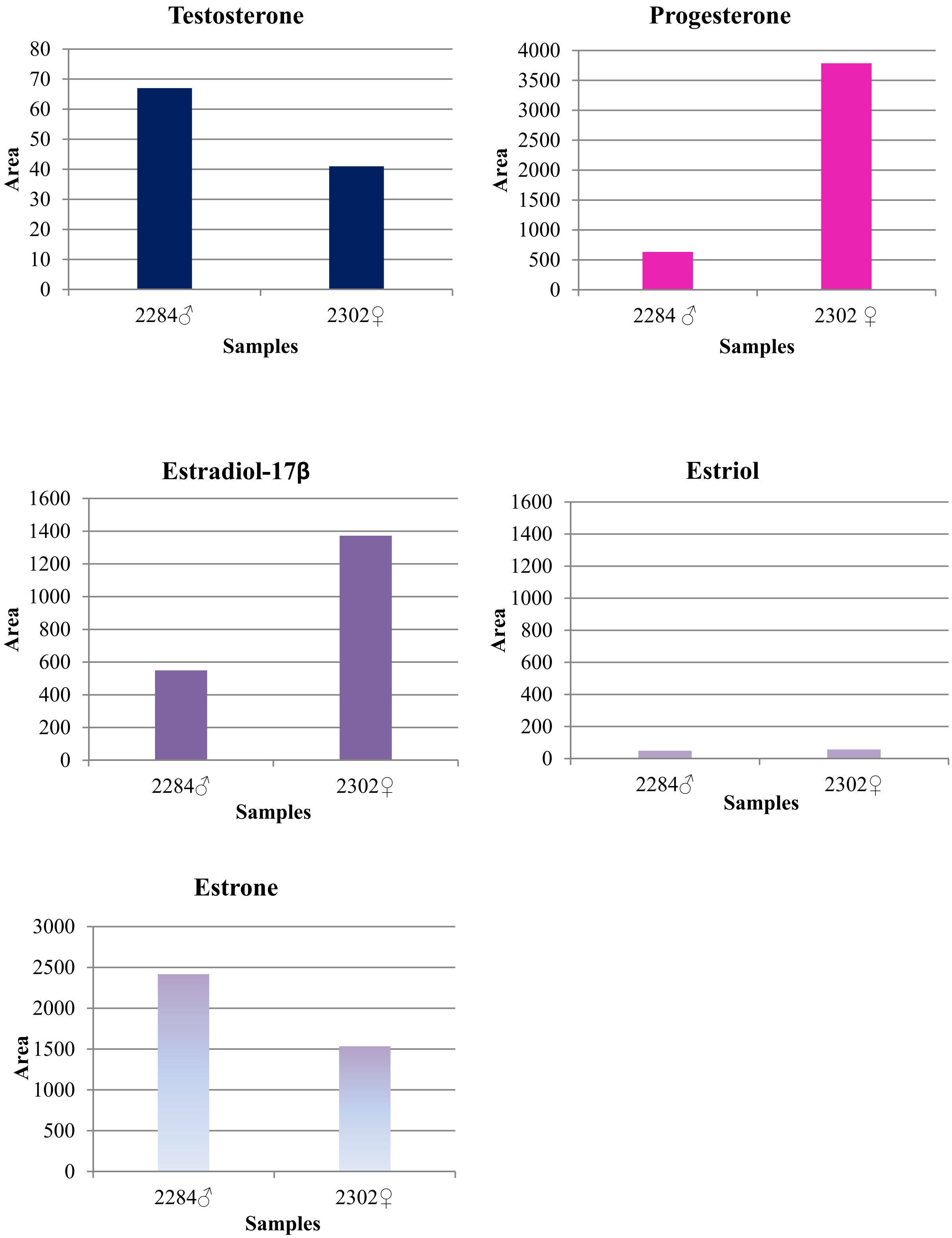
Results from mass spectrometry quantitative saliva analysis for testosterone, progesterone and estradiol-17β samples (series one).

Sample (series one)			Progesterone-Amines Results			Testosterone-Amines Results		
Sample	**Gender**		**Area**	**Height**	**Retention Time**	**Area**	**Height**	**Retention Time**
2284A deriv	male	1st peak	487	87	8.905	78	11	5.421
2284B deriv	male	1st peak	405	70	8.872	56	7	5.480
2302 deriv	female	1st peak	2372	419	8.881	41	5	5.580

Samples (series one)			Progesterone-Amines Results		
Sample	**Gender**		**Area**	**Height**	**Retention Time**
2284A deriv	male	2nd peak	932	169	9.214
2284B deriv	male	2nd peak	714	124	9.189
2302 deriv	female	2nd peak	5199	909	9.197

Non-Derivitized Samples

Samples (series one)			17b-Estradiol Results			Estriol Results			Estrone Results		
Sample	**Gender**		**Area**	**Height**	**Retention Time**	**Area**	**Height**	**Retention Time**	**Area**	**Height**	**Retention Time**
2284A non-deriv	male		643	81	4.930	53	12	1.280	2707	456	6.531
2284B non-deriv	male		456	58	5.005	46	16	1.280	2128	345	6.696
2302 non-deriv	female		1373	215	5.047	57	25	1.221	1531	279	6.716



Mass spectrometry quantitative saliva analysis results (in area) for testosterone, progesterone, 17β-estradiol, estriol and estrone (sample series one); note differences in scale

Salivary samples submitted for quantitative analysis of testosterone, progesterone and estradiol (series two)

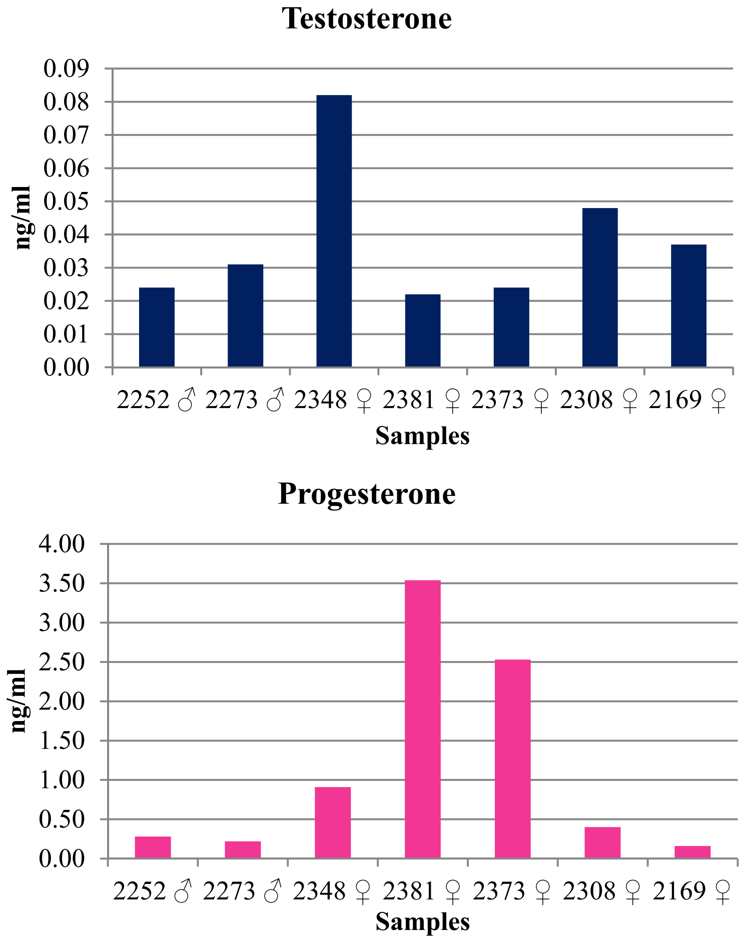
Sample #	Gender	Testosterone (ng/ml)	Progesterone (ng/ml)	Estradiol-17β (ng/ml)
2252	male	1.06	not run	not run
2273	male	1.03	not run	not run
2348	female	not run	9.45	0.10
2381	female	not run	5.20	not run
2373	female	not run	4.71	0.18
2308	female	not run	1.97	0.39
2169	female	not run	1.73	0.46

Results from mass spectrometry quantitative saliva analysis for elevated testosterone and progesterone samples (series two).

Samples (series two)				Testosterone-oxime Results		
Sample	Gender	EIA Testosterone (ng/ml)	EIA Progesterone/Estradiol (ng/ml)	Area	Retention Time	Mass-spec cnc (ng/ml)
2252	Male	1.06	not run	2008	5.9104	0.024
2273	Male	1.03	not run	2729	5.9104	0.031
2348	Female	not run	9.45 P/0.10 E	6564	5.9186	0.082
2381	Female	not run	5.21 P/not run E	2286	5.9104	0.022
2373	Female	not run	4.71 P/0.18 E	1897	5.8938	0.024
2308	Female	not run	1.97 P/0.04 E	1337	5.9104	0.048
2169	Female	not run	1.73 P/ 0.05 E	921	5.9021	0.037

Sampls (series two)				Progesterone-oxime Results		
Sample	Gender	EIA Testosterrone (ng/ml)	EIA Progesterone/Estradiol (ng/ml)	Area	Retention Time	Mass-spec cnc (ng/ml)
2252	Male	1.06	not run	3873		0.275
2273	Male	1.029	not run	5424		0.216
2348	Female	not run	9.45 P/0.10 E	17777		0.906
2381	Female	not run	5.21 P/not run E	61376		3.543
2373	Female	not run	4.71 P/0.18 E	33404		2.526
2308	Female	not run	1.97 P/0.04 E	4134		0.402
2169	Female	not run	1.73 P/ 0.05 E	1032		0.162

Samples (series two)				Esterone-oxime Results		
Sample	**Gender**	**EIA Testosterone (ng/ml)**	**EIA Progesterone/Estradiol (ng/ml)**	**Area**	**Retention Time**	**Mass-spec cnc (ng/ml)**
2252	Male	1.06	not run	-2	4.617	ND
2273	Male	1.029	not run	2	4.609	ND
2348	Female	not run	9.45 P/0.10 E	49	4.609	ND
2381	Female	not run	5.21 P/not run E	18	4.609	ND
2373	Female	not run	4.71 P/0.18 E	102	4.609	ND
2308	Female	not run	1.97 P/0.04 E	10	4.609	ND
2169	Female	not run	1.73 P/ 0.05 E	-4	4.609	ND



Mass spectrometry quantitative saliva analysis results for testosterone and progesterone; estradiol-17β and estriol were not tested for and esterone was not detectable (sample series two); note differences in scale

Appendix 12.

Female walrus behavioral ethogram (formal observations)

Location
W = Water: Animal has more than 50% of its body in the water
L = Land: Animal has more than 50% of its body on the land

Activity
s = non-pattern awim: Locomotion in the water with no distinguishable pattern or from point A to point B
pc = pattern swim C = locomotion in the water in repeated clockwise or counterclockwise swimming pattern
w = walk: locomotion on land
f = foraging: locomotion in the water along horizontal surface (bottom of exhibit) with mystacial pad
i = inactive: remains in a stationary (non-swimming or non-propulsion) position either on land or in the water

Interaction/Manipulation (I/M)
WM = Whisker Manipulation: movement of mystacial pad on vertical surface (may or may not be accompanied by food regurgitation)
OM = Object Manipulation: moves or touches object with any part of body other than the genital area
ED = Estrus Display: displays any of the following behaviors associated with estrus – presenting rear end, soliciting male attention, following/chasing male
CL = Clasping: holding onto other walrus with both front flippers
C = Copulation: successful penetration with con-specific (male inserts penis into female's vagina)

Not Visible (NV)
NV = Not Visible: data are unobtainable because animal is partially or totally out of sight

Other
Other: any behavior that is not defined

Vocals
Vocals: any sound production from vocal cords or air sacs

Appendix 13.

History of calves born in U.S. since 2002

Birth locale	Cow/studbook#	Parturition*	Bull/studbook#	Insemination$	Calf birth	Breeding situation	Survival	Gender	Calf/ studbook #	Comments
BZ^	Basilla #35	18	Unknown	NA	April-02	breeding loan	2 days	female	no name #69	
SWSD	Kitkatska #29	22	Obie #38	~15	May-03	intro. in 2000 then lived tog. cont.	still alive	female	Kaboodle #70	
SWSD	Seahook #22	28	Obie #38	~17	Apr-05	intro. in 2000 then lived tog. cont.	15 days	male	no name #78	
SWSA	Takena #41	19	Iliayak #23	~28	Mar-06	intro. in 2000 then lived tog. cont.	stillborn	female	no name #80	twin
SWSA	Takena #41	19	Iliayak #23	~28	Mar-06	intro. in 2000 then lived tog. cont.	2 days	male	no name #81	twin
NYA	Kulusiq #41	13	Ayveq #49	~12	Jun-07	lived together continuously	2 years	male	Akituusaq #82	
SFDK	Uquq #54	17	Sivuqaaq #52	~16	May-11	lived together continuously	stillborn	male	no name #86	exogenous hormones%

Information according to AZA Regional Studbook 2012

^Brookfield Zoo

*age (yrs) of female at parturition

$age (yrs) of male at insemination

%used exogenous hormones to synchronize male rut with female cycle