

Exxon Valdez Oil Spill
Restoration Project Final Report

Harbor Seal Recovery: Application of New Technologies for Monitoring Health

Restoration Project 03558
Final Report

Shannon Atkinson, Ph.D.
Anne Hoover-Miller
Carolyn Oki
Danielle O'Neil

School of Fisheries and Ocean Sciences
University of Alaska Fairbanks

and

Alaska SeaLife Center
301 Railway Ave. POB 1329
Seward, Alaska 99664

for:

Alaska Department of Fish and Game
Habitat and Restoration Division
333 Raspberry Road
Anchorage, Alaska 99518

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Coordination/Collaboration:

The initial collaborator on this project was Dr. Bobby Middlebrooks from the University of Southern Mississippi, however Part Two of the project also utilized samples collected by Dr. Michael Castellini, University of Alaska, Fairbanks and Dr. Frances Gulland, the Marine Mammal Center, Sausalito CA. Anne Hoover-Miller the ASLC harbor seal program manager has also played a major role in project coordination and collaboration.

Community Involvement/TEK & Resource Management Application:

I have developed and maintained a solid working relationship with the Alaska Native Harbor Seal Commission. Either I or Anne Hoover-Miller, the ASLC Harbor seal program manager, have participated and made presentations at each of the Alaska Native Harbor Seal Commission annual meetings.

Information Transfer:

Two Master's theses were conducted and another two were facilitated through sample analysis supported by this grant:

Primary Projects:

Oki, C. (2001). Cortisol and thyroid hormones secretory patterns and levels in the harbor seal (*Phoca vitulina*) in summer and winter seasons. Master's Thesis submitted to University of Hawaii.

O'Neil, D. (2004). Metabolic hormone levels and immunocompetence of neonatal harbor seals (*Phoca vitulina*) in rehabilitation settings. University of Alaska Fairbanks. In preparation.

To date one manuscript has been published under the following reference:

Oki, C and Atkinson, S. (2004) Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. Gen. Comp. Endo. 136:289-297.

Three additional manuscripts are in preparation or under review:

Stanberry, K., Carpenter, J., and Atkinson, S. (2004) Rate of passage and assimilation efficiency of herring diets containing different fat levels in harbor seals.

Submitted to Comparative Biochemistry and Physiology. In review.

Grieg, D., Burns, J., Harvey, J., and Atkinson, S. (2003) Pregnancy rate and morphology of the harbor seal (*Phoca vitulina*) in Monterey Bay, Ca. In Preparation.

O'Neil, D., Atkinson, S. (2004) Comparison of rehabilitated and wild harbor seal pups (*Phoca vitulina*) using hormone studies, cellular immunity and morphometrics. In Preparation.

Four posters were presented at conferences.

O'Neil, D. and Atkinson, S. (2001) Baseline metabolic hormone levels in neonatal harbor seals (*Phoca vitulina*) admitted to rehabilitation settings. 14th Biennial Conference on the Biology of Marine Mammals. Vancouver BC. November 28 – December 3.

O'Neil, D. and Atkinson, S. (2002) Baseline metabolic hormone levels in neonatal harbor seals (*Phoca vitulina*) admitted to rehabilitation settings. 33rd Annual

- IAAAM (International Association of Aquatic Animal Medicine) Conference. Albuferia, Portugal, May4-8.
- O'Neil, D. and Atkinson, S. (2002) Metabolic hormone levels of neonatal harbor seals (*Phoca vitulina*) in rehabilitation facilities. Marine Science in the Northeast Pacific: Science for Resource Dependent Communities Symposium. Anchorage, Alaska, January 13-17/
- O'Neil, D. and Atkinson, S. (2003) Comparison of hormone levels from permanently captive and rehabilitated harbor seals (*Phoca vitulina*) 34th Annual IAAAM Conference, Waikoloa, Hawaii, May 9-15.

Signature of PI:

Project Web Site Address:

www.alaskasealife.org

Study History:

Harbor seals were one of the resources that were injured by the 1989 *Exxon Valdez* oil spill (EVOS). To date this species is listed as 'not recovering'. Several studies have focused on the general health and metabolism of these seals as it relates to their diet, body condition and habitat (EVOS Projects 001, 341, 371, and 441). This study complimented these investigations as it utilized new techniques to enhance our understanding of the health and physiology of the species. The techniques were combined to develop an indicator of a given animal's health, then these techniques incorporated into the routine assessment and monitoring of harbor seals in the Gulf of Alaska, both free-ranging seals as well as those admitted to rehabilitation.

Abstract:

This project investigated the potential for new technologies to assess and monitor the endocrine and immune systems as diagnostic measures of the health of harbor seals. Analysis of thyroxine (T4), triiodothyronine (T3), and cortisol (primary metabolic and gluconeogenic hormones) and measurement of cellular (lymphocytes and eosinophils) and humoral (IgA) immunity provided a health assessment in both permanently captive seals as well as seals that are brought into the Alaska SeaLife Center (ASLC) or The Marine Mammal Center for rehabilitation. Part one of this project covered the analysis of diurnal patterns of cortisol and thyroid hormones to determine baseline hormonal parameters. Part two of the project established baseline immune profiles as well as provided data on the metabolic hormone concentrations from permanently captive harbor seals, from harbor seals admitted for rehabilitation, and from free-ranging harbor seals. The predictive ability of these tests may be powerful in monitoring the overall health of harbor seals.

Key Words:

Harbor seal, *Phoca vitulina*, circadian patterns, cortisol, thyroid hormones, thyroxine, triiodothyronine, lymphocytes, eosinophils, immunoglobulins.

Project Data:

Data Description: The data from this project include morphometrics data, caloric and biomass intake of harbor seals at ASLC. Data were also obtained from 59 free-ranging harbor seal sera samples provided by Dr. Michael Castellini, University of Alaska Fairbanks, and 32 rehabilitated seals from ASLC and from Dr. Francis Gulland at The Marine Mammal Center. *Format:* The data are recorded in Microsoft EXCEL spreadsheets. *Custodian:* Dr. Shannon Atkinson, School of Fisheries and Ocean Sciences, University of Alaska Fairbanks and Alaska Sealife Center PO Box 1329 Seward, Alaska 99664 phone 907-224-6346 e-mail shannon_atkinson@alaskasealife.org

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Executive Summary:

The population of Alaskan harbor seals has been declining over the last two to three decades. The purpose of this research project was to develop a suite of physiological parameters that may be used to monitor the overall health of free-ranging harbor seals. In the first part of this study, baseline data that reflect seasonal changes needed to be obtained. The suite of parameters that were chosen were the metabolic hormones, cortisol and total and free thyroxine (T₄) and triiodothyronine (T₃) as well as morphometric measurements. The first part focused on determining whether diurnal patterns of cortisol and total and free T₄ and T₃ were present during the summer and winter seasons, and identify how they might alter metabolic rate and/or maintenance of body reserves. This study was carried out at the Alaska SeaLife Center (ASLC) in Seward, Alaska (60.136⁰ N. latitude, 149.429⁰ W. longitude) in June of 2000 and January of 2001. The second part of the study focused on comparing permanently captive harbor seals with apparently healthy free-ranging seals and those admitted to rehabilitation, and demonstrated changes in development, immune profiles and hormone parameters that may be expected with differing ages and coming from different environments. Through collaboration, some of the free-ranging seals in this study were the same individuals as those in EVOS project 341.

Cortisol levels were not different ($P \geq 0.1$) between the summer and winter, but cortisol displayed a circadian rhythm only during the summer. Mean levels of cortisol (51.5 ng/ml \pm 20.3) in the summer hours of *ante meridian* differed ($P \leq 0.05$) from levels in the hours of *post meridian* (28.5 ng/ml \pm 17.4). Neither total and free T₄, nor T₃, displayed a diurnal rhythm in either season. However, total T₄, total T₃, and free T₃ levels were higher in the winter (total T₄, $P \leq 0.05$; total T₃, $P \leq 0.05$; free T₃, $P \leq 0.1$) than in the summer. There was no seasonal effect on free T₄ levels ($P \geq 0.1$). The absence of a circadian rhythm of cortisol during the winter may have been a result of the limited amount of daylight during the winter season as well as the continued need to produce metabolic heat through gluconeogenesis. Higher levels of thyroid hormones in the winter indicate an adaptive mechanism to cope with the low temperatures of winter.

In addition to the baseline diurnal data from captive seals, samples were collected from harbor seals that were admitted to rehabilitation at both Alaska SeaLife Center and the Marine Mammal Center (Sausalito, CA) and compared to apparently healthy free-ranging harbor seals. It is clear that the physical development of rehabilitating harbor seals is retarded and the circulating total thyroxine (TT₄) concentrations are also suppressed. However cortisol concentrations were higher in pre-weaning rehabilitating pups, nevertheless after weaning (and presumed environmental acclimation) all groups of seals had similar cortisol concentrations.

Of the cell-mediated immunity, lymphocytes were higher in the free-ranging pups than the rehabilitating pups, probably reflecting the compromised nature of the rehabilitating pups. While eosinophils were higher in the post-weaning rehabilitating pups, the standard error in those results were also double that of the other groups, possibly indicating that the elevated mean may be due to only a few animals. Immunoglobulin A, a

measure of humoral immunity, was the only immunoglobulin that antisera were found to cross react with, although the molecular weights of IgG, IgM, and IgA were characterized.

This project has demonstrated the use of endocrine and immune profiles to enhance our knowledge of the general health and physiological status of free-ranging harbor seals and how they compare to seals admitted to rehabilitation or in permanent captivity. The predictive ability of these tests may be powerful in monitoring the overall health of harbor seals and warrants further testing.

Introduction:

The population of *Phoca vitulina richardsi*, the subspecies found in the eastern Pacific from Baja California, Mexico to Alaska, is estimated to be at 140,000 (Angliss et al. 2001, Barlow *et al.*, 2001). Since the mid 1970's the population of the harbor seal in the Gulf of Alaska at Prince William Sound has continued to significantly decline. At Tugidak Island, near Kodiak, the Alaskan harbor seal population decreased by 90% since the mid 1970's (Kinkhart et al. 1994). Exact causes of their decline are unknown; however, diseases, environmental instability, commercial fishing, and reduced prey availability have been suspected. The *Exxon Valdez* oil spill in Prince William Sound in 1989 also impacted the seal population; and the harbor seal is still considered an injured species that has yet made little or no progress towards recovery over ten years after the incident (*Exxon Valdez* Oil spill Trustee Council, 2001). Because of the decline of the harbor seal population, it is important that we understand their physiological status and how they maintain their body reserves and regulate their metabolism during different seasons. Poor physiological states likely contributed to the overall health and low survival of individual seals during unfavorable environmental conditions.

The purpose of this research project was to develop a suite of physiological parameters to use in assessing the overall health of a given animal or subpopulation. To do this, baseline concentrations of cortisol and thyroid hormones were measured and the presence of diurnal patterns of cortisol, and total and free T₄ and T₃ in the harbor seal during the summer and winter seasons was determined. The circadian pattern, cycles of night and day, is a biological rhythm that aids in controlling many physiological processes. The biological clock is a neural network located in the suprachiasmatic nucleus (SCN) of the basal hypothalamus and is responsible for the circadian rhythm of hormones in mammals (Reuss, 1996). Studies have shown that if the SCN is damaged, all circadian rhythms of normal biological activity disappear (Zucker, 1980). In mammals there is a direct link between photoperiod and pituitary and pineal gland hormone secretion. Diurnal and seasonal variation found in cortisol and thyroid hormone levels maintain physiological conditions, such as body temperature, from digressing from homeostasis. Both these hormones play an important role in metabolism and in regulating homeostasis of the body.

Cortisol, a glucocorticoid, is released from the cortex of the adrenal gland, which is stimulated by adrenocorticotropin (ACTH) of the anterior pituitary gland. Cortisol is involved mainly in carbohydrate, lipid, and protein metabolism and serves as an indicator of an animal's state of well-being, as its levels increase during times of stress. Its role is to maintain the sympathoadrenal system, which regulates homeostasis of the body (Eckert et al. 1998). Glucocorticoids promote gluconeogenesis, the process of glucose molecules being synthesized mainly from protein and fat conversion in the liver, which in turn increases blood glucose levels. The circadian pattern of hormones in pinnipeds has not been well studied. In a study of Weddell seals in Antarctica, there was no evidence that cortisol followed a circadian rhythm of secretion during months of constant daylight (Barrell and Montgomery, 1998). However, this study did not investigate the hormone secretion pattern, or longitudinal profile, of each individual animal used in the study;

rather it examined numerous individual seals across the field season. In a study a Gardiner and Hall (1997), there were significant differences in cortisol levels due to season in free-ranging mature female harbor seals, with higher levels during the winter months versus the summer or breeding months.

Activity levels also influence the secretion of the two thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). Both T_4 and T_3 are mainly involved in increasing metabolism by increasing the rate of glucose oxidation and additionally, increasing the amount of metabolic heat produced (Nelson, 1995). Thyroid activity in some mammals increases during the winter compared to the summer; however, in some nonhibernating mammals, such as beavers and muskrats, thyroid hormone concentrations are lower during the winter (Legrand, 1986) and during molting of the pelage in mammals (Nelson, 1995).

Thyroid hormones have been shown to display circadian and seasonal secretory patterns, dependent on the species of animal. However, there have been no studies measuring the diurnal rhythm of thyroid hormones in pinnipeds. Compared to terrestrial mammals, aquatic mammals were thought to have a higher metabolic rate to prevent heat loss through convection in cold waters (Whittow, 1987), but other findings have shown that marine mammals such as seals and cetaceans have similar basal metabolic rates (Lavigne et al., 1986; St. Aubin et al., 1996). Factors such as having a smaller surface area to volume ratio, thicker blubber, higher lipid content in blubber, and better insulation quality of the blubber allow marine mammals to better adapt to colder water temperatures (Worthy and Edwards, 1990).

Neonatal marine mammals are more inclined to suffer from immunosuppression due to abandonment, malnourishment and anthropogenic contaminants. A combination of these factors can lead to young animals to fail to thrive in their natural environment. Analysis of hormone concentrations and body condition via morphometrics can give an indication of an animal's physiology. Immunocompetence in an individual animal or species involves a complex series of metabolic and physiological parameters. It is dependent on environmental, nutritional and endocrinological factors. Baseline information on the immune system of target pinniped species is critical to any future field assessment of immunocompetence. The lack of baseline information on the immune system of the harbor seal (*Phoca vitulina*) population in Europe hindered assessment of the role of pollution-induced immunosuppression in the phocid distemper virus outbreak of 1998 (Dietz et al., 1989a; Thomas and Hinsdill, 1978; Vos and Luster, 1989). Studies of levels of immunoglobulins and isotypes of those immunoglobulins have been reported for a few species of pinnipeds. Cavagnolo and Vedros (1979) evaluated IgG, IgM and IgA levels in serum and colostrum of adult and immature northern fur seals (*Callorhinus ursinus*) and found low immunoglobulin levels in the serum of pups during the first four months of life. Baker (1984) found similar results for overall gamma globulin levels in gray seal (*Halichoreus grypus*) pups. Carter et al. (1990) measured specific immunoglobulin isotype levels in the serum and colostrum of the grey seal. Ross et al. (1993) evaluated IgG levels in the harbor seal (*Phoca vitulina*), and also evaluated lymphocyte function in this species by measuring responsiveness to a T-cell mitogen. Several reports of neutralizing antibodies against marine caliciviruses in Steller sea lion sera have appeared

(Akers, et al. 1974; Barlough, et al. 1987a, 1987b) emphasizing the need for immunological assays to monitor disease/infection exposure in target populations of a species. A number of reports have appeared describing ELISA's or other immunoassays measuring pinniped antibody levels against canine distemper virus (e.g. Dietz et al., 1989b; Carter et al. 1990; Bengston et al. 1991; King et al. 1993). It is of note that some of the latter studies utilized antibodies specific for canine immunoglobulins to measure pinniped immunoglobulins, with which they cross-react. In assays such as the ELISA's mentioned above that require the use of anti-immunoglobulin indicator antibodies it is generally preferable to utilize species-specific antisera when available, but such antisera are not available for most species of pinnipeds.

The degree to which an animal is immunocompetent is indicated by how quickly and efficiently its immune system responds to an antigen. Normally upon exposure to an antigen there will be both a T and B-cell mediated response. With regard to the B-cell response, an animal responds by producing immunoglobulins (antibodies) specific for the infecting antigen. In most mammals, there are five classes of isotypes of immunoglobulins, IgG, IgM, IgA, IgE, and IgD. Of these, IgG, IgM, and IgA are the most prevalent serum immunoglobulins. IgA is also important because of its role as a secretory immunoglobulin, and it is found in secretions such as saliva, milk and colostrum, mucus membrane secretions, etc. The various immunoglobulin classes have identifiably separate but overlapping roles or functions in the immune response, and vary in rate of production and relative concentrations in an immune response. Individuals with deficiencies in the production or function of immunoglobulin classes may have health problems directly or indirectly related to these deficiencies; establishment and monitoring of levels can thus be an important indicator of natural or induced humoral immunodeficiencies. It should be clear that since the various immunoglobulin isotypes have half-lives in serum ranging from a few days to about a month, levels do not fluctuate on a moment to moment basis as many other physiologic parameters measurements. Rather levels in samples taken on a particular date reflect sustained conditions that have persisted for weeks or months. These levels may be particularly useful in evaluating the effects of adverse conditions that have persisted from some time.

The potential exists for several environmental factors to impact the biology of harbor seals resulting in poor survival, recruitment and reproductive rates. While the leading hypothesis is that changes in the availability of high quality prey have reduced the carrying capacity of the Gulf of Alaska, a contributing factor to poor survival and reproduction may include exposure to organochlorine contaminants (OCs), with associated endocrine and immune system impairment (Addison, 1989; De Swart et al. 1994, 1996; Ross et al. 1995; Reijnders, 1986). OCs and their by-products are bioaccumulated, biomagnified and transferred through lactation from mother to pup (Beckmen et al. 1999; Gallenberg and Vodienik, 1989; Vreel et al. 1996; Wagemann and Muir, 1984). These contaminants and by-products may continually affect a population of animals even though no major polluting event has occurred. The adverse effects on the physiology of the animal may be subtle or subclinical, or may manifest themselves with symptoms such as, 'failure to thrive' or 'failure to reproduce'. The systems that typically

respond to environmental changes, including contamination or suitable prey, are the endocrine and immune systems.

Objectives:

The overall goal of this project was to develop and test new methods of monitoring the overall health and physiology of harbor seals. In doing so, the project has the following objectives:

1. Determine seasonal and diurnal patterns of total and free triiodothyronine (T₃), thyroxine (T₄), and cortisol in healthy captive harbor seals (Yrs 1 and 2).
2. Develop new antibodies specific to harbor seal immunoglobulin classes (Yr 1 and Yr 2).
3. Determine seasonal patterns of immunoglobulins, in healthy captive harbor seals (Yrs 1 and 2).
4. Begin initial assessments in harbor seals admitted for rehabilitation (years 1-3).

Methods:

Objective 1. Seven harbor seals (3 males, 4 females) housed at the ASLC had monthly blood samples collected to assay for total and free T₄, T₃, and cortisol. In addition, diurnal patterns of these hormones were assessed from the five seals (2 males and 3 females) during the seasonal extremes of the summer and winter solstices, with samples collected at 2 to 3 hourly intervals over a 24 hour period.

This study was carried out at the Alaska Sealife Center (ASLC) in Seward, Alaska (60.136⁰N latitude, 149.429⁰W longitude) in June 2000 and January 2001. In June 2000, blood samples were obtained from the five captive harbor seals, ranging from 4 to 25-years old, that resided at ASLC. All seals were considered to be adults and the females were non-pregnant. Approximately 18-24h before blood samples were obtained, the seals were restrained and catheterized in the extradural vein with a 14 gauge, 13.3 cm angiocath with attached tubing extensions. A neoprene patch was glued to the fur of the seal to secure the catheter and contain the extensions. Cortisol levels were then allowed to return to base levels by allowing the seals to rest for 18-24h following catheterization in a gated outdoor tank (1.8m deep, 9.1 m wide, 6.1 m long). The seals were then bled every 2-3 h over a 24-h period.

During January of 2001, the previously sampled five seals at ASLC were again used for the winter study. The seals were contained in a community cage (3.0m x 6.1m) made of chain link in an indoor-outdoor wet lab. The lab was exposed to natural outdoor lighting, and the indoor artificial lighting was adjusted to correlate to the natural light. The seals were again catheterized 18-24h before blood sampling began. Blood samples were obtained every 3 h over a 24-h period.

Twenty milliliters of blood were drawn at each sampling period and immediately centrifuged. Blood for routine complete blood chemistries (CBCs) was collected and analyzed once during each 24-h period. The plasma samples were then frozen at -80 °C

until analysis by radioimmunoassay methods. For both studies, all seals were offered a combination of herring (*Clupeus harengus pallasii*) and pollock (*Theragra chalcogramma*) during the 24-h period. All seals were confined without access to the tank water during the experiment, to ensure that they were all exposed to the same condition.

In June of 2000 and January of 2001, sunlight intensity was recorded throughout the blood sampling periods by a photo cell light meter. Intensity of the natural light was recorded as a percentage (LI) and converted to units of cad cell resistance, expressed in ohms, using the conversion factor of 986.10 and the following equation:

$$\text{Cad cell resistance (ohms)} = (1 - (\text{LI}/100)) \times 986.10.$$

Air temperature in Seward on June 22nd and 23rd of 2000 and from January 3rd to January 6th, 2001 was obtained from the Western Regional Climate Center of the National Oceanic and Atmospheric Administration (Ashby, personal communication, May 1, 2001).

Weight and blubber thickness measurements were collected during the week of our summer and winter study. Weight was measured in kilograms and blubber thickness was recorded in millimeters taken at the axillary, mid-section, and hip-section of the seals body using a SCANCO Ultrasonic Scanprobe II Model 731C. Blubber measurements during the summer were taken once during the week of June 19th to the 25th of 2000, and winter measurements were taken once during the week of January 1st to 7th of 2001.

During the months of June and December of 2000, the seals were fed a combination of Pacific herring and walleye pollock. Gross energy composition of the herring and pollock fed to the animals at the Alaska Sealife Center were analyzed by Castellini et al. (2001) and Bando (2002) for separate studies. This was used to estimate the average daily gross energy intake by the harbor seals in both summer and winter seasons. The total monthly intake for each season was recorded and the average daily gross energy intake, expressed in magacalories per day, was calculated using gross energy data analyzed by Castellini et al. (2001) and Bando (2002). During the summer season, Castellini et al (2001) obtained mean gross energy data (wet basis) of 9.0kJ/g for herring and 4.7 kJ/g for pollock. For the winter season, gross energy values were calculated based on the analysis by Bando (2002) using the lipid extraction method of chloroform and methanol. Mean gross energy values of 10.8 and 5.5 kJ/g (wet basis) were obtained for herring and pollock, respectively (Bando, 2002).

All hormone concentrations were determined using solid phase radioimmunoassays that were specific for cortisol, total T₄, free T₄, total T₃ and free T₃ (Diagnostic Products, Los Angeles, CA). Assays run in June 2000 had non-specific binding readings for cortisol total T₄, free T₄, total T₃ and free T₃, assays of 0.93, 0.48, 0.56, 1.17, and 0.34%, respectively. Sensitivities for the cortisol, total T₄, free T₄, total T₃ and free T₃, assays were 0.33ng/tube, 4.5ng/tube, 0.02 pg/tube, 0.006 ng/tube, and 0.06 pg/tube, respectively. Intraassay variation was less than 5% for each assay. Assays conducted in January 2001

for cortisol, total T₄, free T₄, total T₃ and free T₃ had non-specific binding readings of 1.49, 1.14, 1.99, 1.72 and 1.25%, respectively. Of the assays conducted in January 2001, sensitivity for the cortisol, total T₄, free T₄, total T₃ and free T₃, assays was 0.06 ng/tube, 1.81 ng/tube, 0.006 pg/tube, 0.11 ng/tube, and 0.25 pg/tube, respectively. Intraassay variation was less than 10% for each assay run during January 2001. In each assay conducted in both the summer and winter study, three quality controls (Diagnostic Products Corporation) were inserted into each hormone assay to measure the interassay variation. Interassay variation for cortisol, total T₄, free T₄, total T₃ and free T₃ was 10.74, 14.56, 19.75, 23.97, and 19.50%, respectively. However, interassay variation was calculated from only two assays, in which one was run each season. The standard curves of each assay were long-logit transformed, enabling linear extrapolation of sample concentrations (Rodbard, 1974).

Objective 2 and 3 The prerequisite for development of heavy chain specific antisera for the major immunoglobulin classes of the harbor seal is the production of purified preparations of each of these immunoglobulin classes. Affinity chromatography methods were used to purify harbor seal immunoglobulins. Pooled serum samples were subjected to 100,000 molecular weight (MW) centrifuge filters in order to remove proteins other than the immunoglobulins making them more accessible to the column matrix. The Protein G column was used to purify IgG. Preliminary tests showed that another possible isotype of IgG was discovered using the Protein A column. The Jacalin column proved useful in purifying IgA from the serum. In order to obtain IgM, an AminoLink column was used in which anti-canine IgM (mu chain, Bethyl) was linked to the matrix.

In order to characterize the eluted proteins from all of previously described columns, SDS-PAGE gels and SigmaGel analysis were used to compare the proteins with immunoglobulins of other species. All four immunoglobulins are similar to those of different species (compared to dog and human) when viewed on a gel. Once the immunoglobulins were purified and characterized, it was possible to test for cross-reactivity with commercially bought antisera (human and dog) to see what reagent could then be used in an enzyme linked immunosorbant assay (ELISA) to determine immunoglobulin levels. Ideally, the desired effect was to find antisera that would react with only the immunoglobulin in question and not the other two as to prevent non-specific binding while testing whole serum samples. Therefore, the antisera was tested against all of the purified immunoglobulins. Several methods were used to test for cross-reactivity including Grabar Williams immunoelectrophoresis, Wester Blot, and ELISA.

It was found that anti-human IgA (SIGMA, developed in rabbit) cross-reacted with only harbor seal IgA. A sandwich ELISA was developed by testing various dilutions of test serum, biotinylated reagent, and ExtrAvidin labeled alkaline phosphatase (eAAP) that produced a standard curve when testing known decreasing concentrations of purified putative harbor seal IgA. The test that was developed was as follows: a NUNC flat 96 well plate was coated with 0.02mg/ml anti-human IgA diluted in coating buffer (pH 9.6, 1.6M Na₂CO₃, 2.9M NaHCO₃, 0.2M NaN₃) in 50µl amounts and incubated at room temperature overnight. The plates were then washed with wash buffer (pH 7.4, 1.14M Na₂HPO₄, 0.2M KH₂PO₄, 8M NaCl, 0.2M NaN₃, 0.05% Tween 20) in a plate washer

(Bio-Tek Instruments, ELx405 Auto Plate Washer). The harbor seal serum was diluted in incubation buffer (pH 7.0, 0.02M Na₂HPO₄, 1.36M KH₂PO₄, 8M NaCl, and 0.05% Tween 20) depending upon the original dilution (usually to make a 1:20 dilution), added to the appropriate wells, and allowed to incubate at 37°C for thirty minutes. A series of wells containing various concentrations of the purified IgA preparation were included to permit development of a standard curve. The plates were then washed again. Next biotinylated anti-human IgA was diluted 1/6000 in incubation buffer, added to the wells, and incubated again at 37°C before washing. eAAP was diluted 1/3000 in incubation buffer, then added to the wells, and incubated at 37°C. After washing, p-Nitrophenyl Phosphate indicator substrate (pNP), diluted in substrate buffer (pH 9.8, 9.7% Diethanolamine, 0.1M MgCl₂, 6H₂O), was added to the plates for a thirty minute incubation period at room temperature. A color change indicated the presence of IgA and the plates were placed in a plate reader (Bio-Tek Instruments, ELx808 Ultra Microplate Reader) and absorbance was read at 405nm. There were six wells per sample dilution, three that were originally coated and three that were not. Using a statistical bootstrapping technique and then comparing those numbers to the standard curve, harbor seal IgA levels were determined.

Objective 4. Two groups of seals were used in this study; 1) seal pups admitted for rehabilitation and; 2) free-ranging seal pups. These rehabilitating seals included pre-wean and post-wean pups, up to the maximum age of 8 months. Harbor seal pups from the Alaska SeaLife Center (ASLC) rehabilitation department (n=26) and the Marine Mammal Center (TMMC) in California (n=16) were used in this study. The pups were any and all harbor seals admitted under the age of approximately 6 months in Alaska, and any extra serum generously donated from The Marine Mammal Center's from their routine diagnostic and clinical veterinary procedures from pups under the approximate age of 8 months. Blood was normally drawn on rehabilitating animals every 10 to 14 days. Blood was normally drawn from the extradural intervertebral vein with 21G x 1.5 inch spinal needles. Average restraint time on animals at ASLC was under 7 minutes. TMMC animals were restrained for under 20 minutes. The pups, depending on age and mass, were minimally physically restrained and without chemical restraint. Chemical restraint was only used in older seal pups, post-wean, if blood collection was accompanied with satellite tagging procedures (n=4). This was normally the last sample collected prior to release. If this was the case, a mass based dose (0.055mg/kg) of Torbugesic (Butorphanol tartate) was used. The serum from both facilities was frozen at -80 degrees C until analysis. However, in some cases when serum was unavailable, plasma was validated and used in lieu of serum (Oki, 2001).

Wild harbor seals pups (n=60) were collected under Alaska Fish and Game permits #1000 and #358-1585. These samples were collected from the years 1997 to 2000 on Tugidak Island and generously donated by Drs. Steven Trumble and Michael Castellini at the University of Alaska, Fairbanks.

Radioimmunoassay Analysis

Hormone concentrations were measured using solid phase radioimmunoassay kits specific for cortisol or total thyroxine (TT4) (Diagnostic Products Corporation, Los

Angeles, CA). Mean non-specific binding for TT4 and cortisol were 1.10% and 1.01%, respectively. Quality control indicators, provided by DPC, were included in each assay to validate hormone range. Pooled samples from young captive harbor seals were run in each assay to determine and track interassay variation. Intraassay variation was less than 5% and interassay variation for all assays was less than 10%. Sensitivity of the assays for TT4 and cortisol were 0.33ng/tube and 4.5ng/tube, respectively. The standard curves of each assay were log-logit transformed, enabling extrapolation of sample concentrations (Rodbard, 1974). Parallelism was calculated using a 25%, 50%, 100% and 200% of pooled sear added to the standard curves for both cortisol and TT4 to ensure that these curves were parallel with the standard curves without serum.

Morphometrics, Aging Techniques and Weaning

Morphometrics were taken at the time of each blood collection for all animals. The free-ranging pups were weighed and measured at capture. The rehabilitating pups were weighed every one to two days upon arrival and during early rehabilitation, however, after a pup had been stabilized, they were weighed and measured only on sample collection (for blood and morphometrics) dates, or less often. Morphometrics consist of standard length (cm), weight (kg), axillary girth, hip and maximum girth measurements (cm). Not all measurements were always taken due to time constraints or the behavior of the animal.

Age was estimated at initial physical examination of the animal upon admittance to the rehabilitation center. Aging techniques consisted of status of dentition, or lack thereof. The presence of an umbilicus and the state of the umbilicus, is also a good indicator of age, those with an umbilicus present are typically neonate animals (1 week or younger). Coat condition referenced whether or not the animal still had any form of lanugo, or neonatal coat, which is normally shed in utero. If so, this is deemed a neonate and is premature. Animals with no dentition, lanugo present, and a pink or even bloody umbilicus present were the youngest animals seen in rehabilitation and were presumed premature status. Older pups had shed their umbilicus, possess a new adult looking pelage and had erupting dentition.

Weaning consisted of taking the pup off of the fat-rich fabricated formula that had been administered via stomach tube onto a solid fish diet. Formula was made up of a mix of Zoologic 50/30, a powdered wild animal milk fabricator with water and salmom oil added to make a milk matrix that closely mimic the fat and nutrient content of wild harbor seal milk. This diet shift was determined by the weight, feces status (lack of dehydration), age, dentition and overall general health of the pup. Harbor seals were weaned anywhere from 4-6 weeks in the wild and rehabilitation centers try to mimic this time range, depending on the health of the pup. Some pups were not as easily weaned and continued on an interim diet of formula and fish, approximately 50% or a 2:2 ratio of each feed type, past the normal documented weaning age.

Results:

Part one

There was no difference in mean cortisol concentrations during winter season ($P > 0.1$, Figs 1, 2 and 3); mean cortisol concentrations in the hours of 12 am to noon during the winter were 51.6 ± 14.3 ng/ml, while concentrations from noon to midnight in the winter season were 46.7 ± 16.3 ng/ml. However, a diurnal rhythm was shown to be present in the summer season (Fig 2) as mean concentrations of cortisol in the hours of *ante meridian* (51.5 ± 20.3 ng/ml) were higher ($P \leq 0.05$) than concentrations in the hours of *Post meridian* of the summer days were significantly lower as levels dropped during the time block of 12pm to 6pm (Fig. 2). Seasons did not have an effect on mean daily cortisol concentrations ($P > 0.1$, Table 1).

Mean concentrations of total T_4 and T_3 , and free T_3 levels were higher in the winter season (total T_4 , $P \leq 0.05$, Fig. 4, total T_3 , $P \leq 0.05$, Fig. 6; free T_3 , $P \leq 0.1$, Fig. 5) than in the summer season. However, there was no seasonal effect on free T_4 levels ($P > 0.1$ Fig. 7). Mean daily thyroid hormone concentrations in each season are shown in Table 1. Samples collected in *ante meridian* and *post meridian* within each of the seasons were not different from each other for any of the measured thyroid hormones ($P > 0.1$), nor was there an apparent diurnal rhythm for any of the thyroid hormones.

All seals were considered to be adults based on size and sexual maturity, despite a distinct age difference between two of the five seals at 4 years old, and the other three at 16, 25, and 27 years of age. Therefore a statistical analysis was performed to ensure there was no age-related difference in hormone concentrations between seasons. We found that only during the summer, in the 6pm to midnight time block, cortisol concentrations were significantly lower ($P < 0.05$) in the younger seals versus the older seals. No other time block nor hormone showed significant differences between the two age groups of seals. Analysis of blood urea nitrogen, glucose, triglycerides, and total protein revealed no significant differences in concentrations between seasons.

Each seal ($n=5$) gained a significant amount of blubber thickness at each body region during the winter (axillary, $P \leq 0.05$; mid-section, $P \leq 0.1$; hip-section $P \leq 0.05$; Table 2). The average daily gross energy intake of herring and pollock was greater for four (PO, PE, SK, and SN) of the five seals during the winter month of our study (Table 3). However, seal SY had a higher daily gross energy intake in the summer, despite an increase in weight and blubber thickness in the winter. Seal SY had an average daily gross energy intake of 3.48 Mcal/d in the summer month compared to 1.87 cal/d in the winter month. The amount of total feed intake, in kilograms, by SY in the winter was approximately 52% less than what was consumed in the summer, although the proportions (70:30) of pollock and herring were similar between seasons.

Air temperature in Seward on the days blood samplings occurred was averaged for each season. The average summer temperature on June 22nd and 23rd was 11.1°C and the winter temperature, averaged from January 3rd to the 6th, was -1.1°C . During the summer study, we had an average of 55% light that the photo cell read at 445 Ω ; and during the

winter, we had an average of 14% light that the photo cell read at 847Ω. Light intensity during the summer remained particularly high between the periods of 7am and 7pm at approximately 67% light, while winter light intensity remained at its highest at 41% light between the time period of 12noon to 3pm.

Part two:

The pre-wean rehabilitating harbor seal pups had the highest mean cortisol (Table 4); they also exhibited the highest standard error. The post wean pups exhibited the lowest mean cortisol of all three groups. There was a significant difference ($p=0.049$) between the wild pups and the post-weaned pups and a significant difference ($p=0.003$) when pre-wean, post-weaned and wild pups were compared using a one-way ANOVA test. The free-ranging pups showed the largest TT4 levels of all three groups, however the pre-wean pups showed the highest standard error (Table 4). There was a significant difference between the pre-wean rehabilitating pups and the free-ranging pup samples ($p=0.017$) in terms of total thyroxine (TT4).

Weaning had mixed results on pups in rehabilitation in terms of both eosinophil and lymphocyte percentages (Table 5). Pre-wean and post-wean rehabilitating pup lymphocyte count means were closer in value and statistically lower than the free-ranging pups, which had a larger lymphocyte percentage compared to the rehab pups. However, pre-wean and wild pups were more closely comparable in eosinophil range than with pre-wean and post-wean rehabilitation pups, although there was quite a large difference between pre-wean and post-wean rehabilitation pup eosinophil percentages (Table 5). Pups from both facilities were compared by charting the two leukocyte groups in terms of; (1) the bleed directly after weaning, and (2) the last bleed before the pup was released from rehabilitation (Table 6). To note, the sample sizes for the lymphocyte and eosinophil comparisons are different due to the fact that not all pups had recorded values for either of the leukocyte groups.

Rehabilitation pups were compared at the bleed directly after weaning and directly before release or death. For efficiency, animals were not further divided into groups based on death or release ($n=3$, dead). One reason for this is the fact that most animals that died during rehabilitation did not have enough data points to be used in the statistical comparisons. The highest percentage of lymphocytes found was in the “high percentage of lymphocyte at last bleed” category (Table 6). The lowest percentage of lymphocytes was found in the “lymphocyte high at weaning” category. Conversely, one of the highest percentages for the eosinophil categories was in the “eosinophil high at weaning” group of animals (Table 6). However, the equally high percentage of eosinophils being high at last bleeds is in agreement with the last bleed group of animals with high lymphocyte percentages. The smallest percentage for eosinophils is in the low at weaning category (Table 6). The high at weaning and high at last bleed category percentages equal (58%) in the eosinophil groups while the low at weaning and low at last bleeds category percentages (47%) equal each other in the lymphocyte groups. A small percentage of animals exhibited both high or low counts of either leukocytes at both weaning and last bleed from both facilities. For example, the largest percentage, 32% of rehab pups, had low lymphocyte counts at both weaning and last bleed categories. However, the

percentages calculated of groups of animals sharing the highest or lowest ranges of each leukocyte group was negligible.

Three immunoglobulins were described for harbor seals. The heavy chain of IgG derived from the Protein G column was found to be 57,386 Daltons. IgA heavy chain was 60,777 Daltons while IgM heavy chain was 82,226 Daltons. The light chains were all at around 28,000 Daltons. Ranges of IgA were determined for rehabilitation and permanently captive seals (Table 7). No IgA values were determined for the free-ranging seals as the samples were taken from the archive of Dr. Michael Castellini. Preliminary organochlorine ranges (sum PCBs, sum toxic equivalents, and sum DDTs,) were also compared between rehabilitated pups and free-ranging subadult seals (Table 7).

Hormone profiles for the permanently captive adults were averaged over a 22 month period (Fig 9). Similarly caloric intake and body mass were plotted on continuation graphs from EVOS project 341 (Figure 10 - 17). IgA was plotted for individual permanently captive seals over approximately 3 years. (Figure 18 - 24).

Discussion:

This study resulted in significantly lower cortisol concentrations in harbor seals during the afternoon, however only in the summer season. Cortisol did not show a diurnal pattern in the winter, indicating that the higher intensity and longer duration of daylight during the summer may serve as the environmental cue for the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which controls the circadian rhythm of hormones. A study by Vondrasova-Jelinkova et al. (1999) suggested that the bright sunlight in the morning hours may be the signal that entrains the cortisol rhythm in humans. In humans, a desynchronization of biological rhythms can occur if they live in latitudes above 37° N and are not exposed to 2500 lux of light during winter seasons (Nelson, 1995). During the summer study, there was a longer duration and higher light intensity compared to the winter. The low intensity and shorter duration of daylight during the winter season may have been insufficient in entraining the seal's biological clock, and cortisol levels became free-running in the winter, such that it was not synchronized with environmental cues.

In the study of captive harbor seals by Gardiner and Hall (1997), cortisol exhibited a diurnal rhythm in which levels peaked during the darkness around 1:00 am and declined in the early afternoon. In humans, the cortisol circadian rhythm of night workers, who worked night hours for at least 2 years, were similar to normal circadian patterns in day workers (Roden et al. 1993). However, in other animals, activity levels at different times of the day have been shown to affect the pattern of cortisol secretion (Nelson, 1995), and in turn, circadian rhythms of blood pressure, circulatory pulse, body temperature, and skin temperature can be affected by activities throughout the day (Hadley, 1992). When a circadian rhythm of plasma cortisol was present in horses, the animals were also accustomed to a daily activity routine of feeding and exercise, which may have set the circadian pattern (Irvine and Alexander, 1994).

One of the functions that cortisol plays is to maintain body temperature in homeotherms as hypothermia and death will occur if the adrenal gland is removed (Hadley, 1992). Although not significant, cortisol concentrations were slightly higher in the winter season and levels remained steady throughout the 24-h cycle. Concentrations did not decrease in the afternoon as they did in the summer season. This could indicate that cortisol needed to be continually produced throughout the day in order to compensate for a higher homeostatic demand on the seals due to the colder environment during the winter. Air temperature during the winter study was approximately 12⁰C colder than the period of the summer study. Body temperatures in humans are known to follow a circadian rhythm as it peaks in the mid-afternoon (Moore-Ede et al. 1982). Assuming this is true in other animals, such as the harbor seal, it may explain the drop in summer cortisol concentrations during the mid-afternoon time period, when body temperatures are rising.

The presence of a diurnal rhythm in thyroid hormones is dependant on the species. In our captive harbor seals, neither Total nor free T₄ and T₃ displayed circadian rhythms in either summer or winter seasons. However, seasons did have a significant effect on total T₄, total T₃ and free T₃ levels as mean concentrations were higher in the winter than in the summer. It is known that thyroid hormones are mainly involved in creating metabolic heat for the body in order to maintain a homeothermic state. Thyroid hormones increase the rate of glucose oxidation and thus increase the amount of metabolic heat that is produced. It is thought that the thyroid hormones can uncouple oxidative phosphorylation, which decreases the efficiency of ATP synthesis increased the quantity of heat released per mole of glucose oxidized (Nelson, 1995).

Triiodothyronine, or T₃, is the physiologically active thyroid hormone, and T₄ is converted to T₃ by the loss of the iodine atom. Although most of the circulating thyroid hormones are bound to transport proteins, it is the free quantities of T₄ and T₃ that carry out their metabolic activity. Our data showed that there was no significant seasonal effect on free T₄ levels, and this may have been due to the conversion of T₄ to T₃. Exposure to cold has been associated with increased rates of deiodination, enhanced biliary excretion of T₄ and T₃, and increased conversion of T₄ to T₃ (Wartofsky and Burman, 1982). Lower thyroid hormone concentrations during the summer indicate that the environmental temperature is one factor that can alter thyroid levels. Lower thyroid levels during the summer prevent overheating due to reduced endogenous heat production (Hudson, 1981). Total and free T₃ concentrations in the winter season of our study were significantly higher possibly due to the demand for elevated concentrations of this metabolically active thyroid hormone. Increased concentrations of T₃ allowed the seals to make the necessary physiological changes in order to effectively adapt to the colder environment.

Blood parameters such as blood urea nitrogen, glucose, triglycerides, and total protein concentrations did not show significant differences between seasons indicating that these seals maintained homeostasis despite the increased thyroid hormone levels in the winter. This supports our hypothesis that increased thyroid hormone concentrations primarily served a thermogenic function, but allowed the seals to maintain metabolic homeostasis.

The seals used in the present study all had greater weight and blubber thickness in axillary, mid- and hip-sections of the body in the winter than in the summer. Their blubber layer helps insulate them while in colder temperatures as well as serves as an energy source when fasting would normally occur. Animals that increase their fat stores seasonally must also decrease their metabolic rate or increase their caloric intake for fat deposition to occur (Hudson, 1981). During times of limited feed intake, metabolism is slower as fat reserves are utilized (Hudson, 1981). The blubber layer of seals is thought to play a role in thermoregulation, energy storage (Slip et al., 1992) and providing nourishment during periods of fasting. Therefore, the maintenance of it during the winter season is very important. Seals should be maintaining a thick layer of blubber during the winter season, accounting for 27-30% of their total body mass (Ridgway, 1972), which helps them to prevent major heat loss. Blubber and a high rate of metabolism both help the harbor seal in regulating its temperature and conserving heat, since the seal's hair provides little or no insulation.

On average, the herring and pollock fed to the seals in the winter season had a greater gross energy value than the summer feed. Four of the five harbor seals (PO, PE, SK, and SN) consumed more biomass in the winter season and had a greater average daily caloric intake during the winter. Interestingly, seal SY had a lower gross energy intake during the winter months despite a gain in weight and significant increase in its blubber thickness during the time of season. The average gross energy intake for seal SY in December of 2000 was approximately half of what was consumed during June of 2000, although the proportions of pollock to herring (70:30) were similar for both seasons. In a study by Renouf and Noseworthy (1990), captive harbor seals gained body mass during the winter despite a decrease in their feed intake. However, their increase in weight negatively correlated with water temperature (Renouf and Noseworthy, 1990). Follow-up studies by Renouf and Noseworthy (1991) have supported their findings that food intake negatively correlated with body mass. In Renouf and Noseworthy's (1991), study their seals could have gained body mass when they were feeding on herring with a higher fat content, despite a total lower intake of herring. Although this was not expected in this particular harbor seal, SY, some hibernating animals will display similar physiological changes as they may continue to gain body mass even though their feed intake is decreased and food is not stored for the hibernation season (Musacchia and Deavers, 1981).

Measurements of free T_4 levels in Renouf and Noseworthy's (1991) male captive harbor seals were found to be significantly lower in the winter as feed intake decreased and blubber mass increased. However, in our harbor seals, total T_4 , total T_3 , and free T_3 levels were higher in the winter than in the summer, and there were no seasonal effect on free T_4 levels. Using these captive animals, food resources were adequate and the seals may not have had to decrease their activity levels and depend on their fat for energy since there was not a limited food supply. For seal SY, our findings of higher thyroid levels in the winter is unusual considering the decrease in that particular seal's feed intake and its increase blubber layer and weight.

A comparison between rehabilitating and free-ranging harbor seals was designed utilizing metabolic hormones cortisol and thyroxine, and cell-mediated immunity factors, lymphocytes and eosinophils. The results show that free-ranging harbor seal pups had the highest concentration of total thyroxine (TT4) of the three groups of pups compared. A reason for these results might be the fact that pups in the wild undergo higher amount of energy expenditure. The pups must forage for their food to stay alive, escape from predators and stay in a thermal neutral zone to maintain favorable metabolism. All of these reasons might lead to a free-ranging pup's metabolic hormones to run a high circulating concentration. However, it is also possible that rehabilitating pups might be euthyroid or hypothyroid. Heat is a by-product of gluconeogenesis and thus, influenced by cortisol (Oki and Atkinson, 2004). The more circulating cortisol, the more heat is produced to maintain body core. This is an attribute to pups living in a cold environment where heat via food intake may be scarce. Thyroxine is the dominant metabolic hormone that controls thermoregulation. TT4 helps to increase the rate of metabolic heat produced by increasing the rate of glucose oxidation by breaking bonds in ATP (Nelson, 1995; Oki and Atkinson, 2004). This may account for the higher concentrations of TT4 in the free-ranging pup samples. Taking into account the use of these two hormones in the metabolic system, the results support the theory that the thyroid hormones are higher in free-ranging pups when compared to the rehabilitating pups due to higher metabolic energy need and higher thermoregulatory need of free-ranging pups. The higher concentrations of cortisol in the pre-weaning pups during rehabilitation reflects the compromised status of the animals upon entrance to the rehabilitation program. The decrease after weaning reflects the return of homeostasis. While pups entering rehabilitation are typically very young and often neonatal, the initial cortisol concentration could also be considered ontogenetic.

The free-ranging pups were larger in mass and longer in length at the same post-weaning age than the pups in rehabilitation. There may be many reasons for this and one main reason might be the high efficiency and complete sustenance of maternal investment. The animals in rehabilitation are fed on a consistent timeframe, a diet at least 10-13% of their weight per day. Their nutrition, however, is not equivalent to the nutrition provided by their mother. Harbor seal milk is approximately 45-48% milk fat throughout lactation with a higher percentage of fat early in lactation, approximately the first and second week (Reidman, 1990). Fabricated milk substitute, even when supplemented with high fat salmon oil, is only approximately 32-35% fat. In addition, rehabilitated harbor seal pups are often sick when admitted to rehab centers, normally including moderate to severe emaciation resulting from malnutrition and abandonment. This compromised state may have contributed to retarded growth that was measured.

Free-ranging harbor seal pups also had the highest mean lymphocyte ranges when compared to rehabilitating pups. There may be a connection between the lower cortisol and elevated total thyroxine levels and the higher lymphocyte ranges. Both cortisol and thyroid hormones, including thyroxine, aid in the development and maintenance of the immune system (Eckert et al., 1998; Hall et al., 1998). If the thyroid hormone levels are elevated for various reason such as thermoregulation and other metabolic demands, it may be possible to attribute the higher lymphocyte ranges to stimulation by higher thyroid hormone concentrations. In other words, if TT4 was increased resulting in

metabolic enhancement, there may be a compensatory increase in all forms of the immune response, whether a pathogen was present or not to illicit an immune reaction. However, the free-ranging pups' eosinophil ranges were not the highest of the three categories of pups. Pre-wean rehabilitating harbor seal pups exhibit the lowest levels of both leukocyte types of all three groups compared. The pre-wean pups also had the highest cortisol concentrations of all three groups of pups compared. However, the pre-wean pups had a higher concentration of total thyroxine than the post-wean pups. This supports the suggestion that chronically high levels of cortisol are immunosuppressive.

Neonate pups have a plethora of metabolic issues to deal within the first two months of life. A blubber layer has been established for the pups, however the layer is not as thick as it needs to be for thermoregulatory success. All organ systems are still developing and the endocrine system is attempting to regulate all hormone concentrations in the body. The immune system may be weak due to lack of maternal immunity, a disease state, or simply that developmentally their immune systems are still immature. Developmental issues such as these obscure diagnostic assessments and interpretation of normal health parameters. To aid in the assessment and diagnostics of neonatal seals additional research is needed to continue contrasting rehabilitating seals with normally reared free-ranging seals.

This study utilized new techniques to enhance our understanding of the health and physiology of harbor seals. Analysis of thyroxine (T4), triiodothyronine (T3), and cortisol (primary metabolic and gluconeogenic hormones) and measurement of cellular (lymphocytes and eosinophils) and humoral (IgA) immunity provided a health assessment in both permanently captive seals as well as seals that are brought into the Alaska SeaLife Center (ASLC) or The Marine Mammal Center for rehabilitation. The predictive ability of these tests may be powerful in monitoring the overall health of harbor seals.

Conclusions:

1. Environmental cues, such as light intensity and air temperature, may be the signal for the presence of a diurnal rhythm of cortisol.
2. Thyroid hormones did not display a circadian rhythm in the captive harbor seals for both seasons.
3. Total T₄, Total T₃, and free T₃ concentrations were significantly higher in the winter months, which allow the seals to metabolically adapt to the colder temperatures.
4. Measurement of cortisol and thyroid hormones in both the summer and winter seasons in Alaska show how these seals physiologically adapt to extreme changes in their environment.
5. Feed intake, blubber thickness, and weight gain are factors that play a significant role in the endocrine concentrations of the harbor seal, however body weight is not necessarily correlated to food intake.

6. Harbor seal pups undergoing rehabilitation have higher cortisol concentrations pre-weaning when compared to either free-ranging pups or post-weaning pups in rehabilitation.
7. Post-weaning harbor seal pups in rehabilitation have similar cortisol concentration to adult permanently captive harbor seals.
8. Total thyroxine concentrations are significantly higher in free-ranging harbor seal pups than in pups during rehabilitation.
9. Elevated total thyroxine concentrations in free-ranging harbor seals are likely due to the metabolic need for thermogenesis.
10. Body weights and length of rehabilitated pups are significantly lower than free-ranging pups. Both lower morphometrics and lymphocytes likely reflect either the compromised health status or lacking diet of pups during rehabilitation.
11. Lymphocyte percentages in harbor seal pups during rehabilitation were significantly lower than free-ranging pups.
12. Eosinophil percentages that were elevated in post weaning pups during rehabilitation remain unexplained.
13. Preliminary IgA and organochlorine ranges were presented for several groups of harbor seals

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Tables

Table 1. Comparison of mean (\pm s.e.) hormone concentrations from permanently captive harbor seals during summer and winter seasons

| | <i>SUMMER</i> | <i>WINTER</i> | <i>P-value</i> |
|------------------------------|-----------------|-----------------|----------------|
| Cortisol (ng/ml) | 41.4 \pm 22.2 | 49.4 \pm 15.3 | > 0.1 |
| Total T ₄ (ng/ml) | 5.2 \pm 4.1 | 8.5 \pm 4.2 | < 0.05 |
| Free T ₄ (pg/ml) | 1.9 \pm 0.9 | 1.9 \pm 1.1 | > 0.1 |
| Total T ₃ (ng/ml) | 0.37 \pm 0.06 | 0.55 \pm 0.07 | < 0.05 |
| Free T ₃ (pg/ml) | 0.25 \pm 0.18 | 0.63 \pm 0.24 | < 0.1 |

Table 2. Sex, age, mean (\pm s.e.) seasonal weight, and blubber measurements (taken during week of June 19-25, 2000 and January 1-7, 2001), of five permanently captive harbor seals.

| Seal ID | Sex | Age ^a (years) | Weight (kg) | | Blubber axillary (mm) | | Blubber mid (mm) | | Blubber hip(mm) | |
|---------|--------|--------------------------|----------------|----------------|-----------------------|--------|------------------|--------|-----------------|--------|
| | | | Summer | Winter | Summer | Winter | Summer | Winter | Summer | Winter |
| SK | Female | 25 | 60.5 \pm 0.0 | 81.0 \pm 0.0 | 16.0 | 30.0 | 22.0 | 33.0 | 21.0 | 38.0 |
| PO | Female | 27 | 56.5 \pm 1.0 | 70.5 \pm 0.0 | 16.0 | 23.0 | 20.5 | 29.0 | 18.0 | 28.0 |
| SY | Female | 4 | 45.8 \pm 0.4 | 55.3 \pm 0.0 | 17.0 | 25.0 | 22.0 | 31.0 | 23.0 | 34.0 |
| SN | Male | 16 | 84.0 \pm 0.0 | 95.5 \pm 0.0 | 20.0 | 33.0 | 21.0 | 30.0 | 22.0 | 34.0 |
| PE | Male | 4 | 42.9 \pm 0.1 | 56.0 \pm 0.0 | 12.0 | 18.0 | 13.0 | 19.0 | 15.0 | 23.0 |

^a Approximate age in 2000

Table 3 Monthly feed intake and average daily gross energy intake of herring and pollock by permanently captive harbor seals in June (summer) and December (winter) 2000.

| Seal ID | Total pollock consumed (kg) | | Total herring consumed (kg) | | Average daily gross energy intake (Mcal/day wet basis) | |
|---------|-----------------------------|--------|-----------------------------|--------|--------------------------------------------------------|--------|
| | Summer | Winter | Summer | Winter | Summer | Winter |
| SK | 17.9 | 16.5 | 45.0 | 53.1 | 3.88 | 5.10 |
| PO | 52.6 | 20.6 | 21.6 | 60.3 | 3.50 | 5.87 |
| SY | 19.1 | 15.5 | 38.6 | 43.7 | 3.47 | 4.28 |
| SN | 42.4 | 40.0 | 43.0 | 40.2 | 4.65 | 5.02 |
| PE | 50.9 | 26.1 | 22.2 | 9.2 | 3.48 | 1.87 |

Table 4. Mean (\pm Se.) hormone concentrations and morphometrics of rehabilitating and wild harbor seal pups, and permanently captive adult harbor seals.

| | n | TT4 (ng/ml) | | Cortisol (ng/ml) | | Weight (kg) | | Length (cm) | |
|-----------------------------|----|-------------|------|------------------|------|-------------|------|-------------|------|
| | | mean | SE | mean | SE | mean | SE | mean | SE |
| Pre wean rehab pups | 32 | 3.08 | 0.31 | 16.4 | 1.83 | 10.2 | 0.29 | 65.7 | 4.59 |
| Post wean rehab pups | 32 | 2.79 | 0.21 | 11.5 | 0.90 | 19.6 | 0.62 | 83.2 | 2.91 |
| Free-ranging pups | 59 | 3.8 | 0.14 | 13.1 | 0.52 | 26.9 | 0.70 | 94.5 | 0.82 |
| Captive adults | 7 | 3.0 | 0.10 | 12.2 | 0.50 | 63.4 | 0.90 | 127.6 | 1.01 |

Table 5. Mean (\pm Se.) leukocyte ranges of rehabilitating and wild harbor seal pups.

| | n | Percent Lymphocytes | | n | Percent Eosinophils | |
|----------------------|----|---------------------|------|----|---------------------|------|
| | | mean | SE | | mean | SE |
| Pre wean rehab pups | 32 | 15.5 | 1.50 | 19 | 1.62 | 0.28 |
| Post wean rehab pups | 32 | 15.9 | 1.50 | 19 | 4.03 | 0.51 |
| Free-ranging pups | 59 | 26.0 | 1.37 | 59 | 2.9 | 0.27 |

Table 6. Leukocyte percentages at weaning and during the last blood sampling prior to release in harbor seal pups (N=whole group studied, n=# of animals exhibiting leukocyte percentage).

| Lymphocytes | N | n | % |
|--------------------|----|----|----|
| High at wean | 32 | 13 | 41 |
| Low at wean | 32 | 15 | 47 |
| High at last bleed | 32 | 16 | 50 |
| Low at last bleed | 32 | 11 | 47 |
| Eosinophils | | | |
| High at wean | 19 | 11 | 58 |
| Low at wean | 19 | 4 | 21 |
| High at last bleed | 19 | 11 | 58 |
| Low at last bleed | 19 | 6 | 32 |

Table 7. Ranges of IgA, sum PCBs, sum toxic equivalents and sum DDT from harbor seals (TEQ) during rehabilitation, permanent captivity or free-ranging.

| | General age | IgA Mg/ml | Tissue type | EPCB (ppb) | ETEQ (ppb) | EDDT (ppb) |
|---------------------------|-------------------|-----------|---------------|------------|------------|------------|
| Rehabilitated Seals | Pups | 0-59.4 | Blood (n=13) | 0.13-1.8 | 1.04 | 0-0.61 |
| Permanently Captive Seals | Adults | 0-28.9 | Blubber (n=1) | 2400* | 14.2 | 1240 |
| Free-ranging seals | Sub adults/Adults | | Blood (n=7) | 0.45-4.3 | 0-0.03 | 0-0.34 |
| | | | Blood (n=2) | 160-170 | 1.8-2.2 | 56-81 |

* note only one sample collected opportunistically upon death

Figures

Figure 1. Mean (\pm Se.) cortisol concentrations in permanently captive harbor seals during summer (blue) and winter (pink).

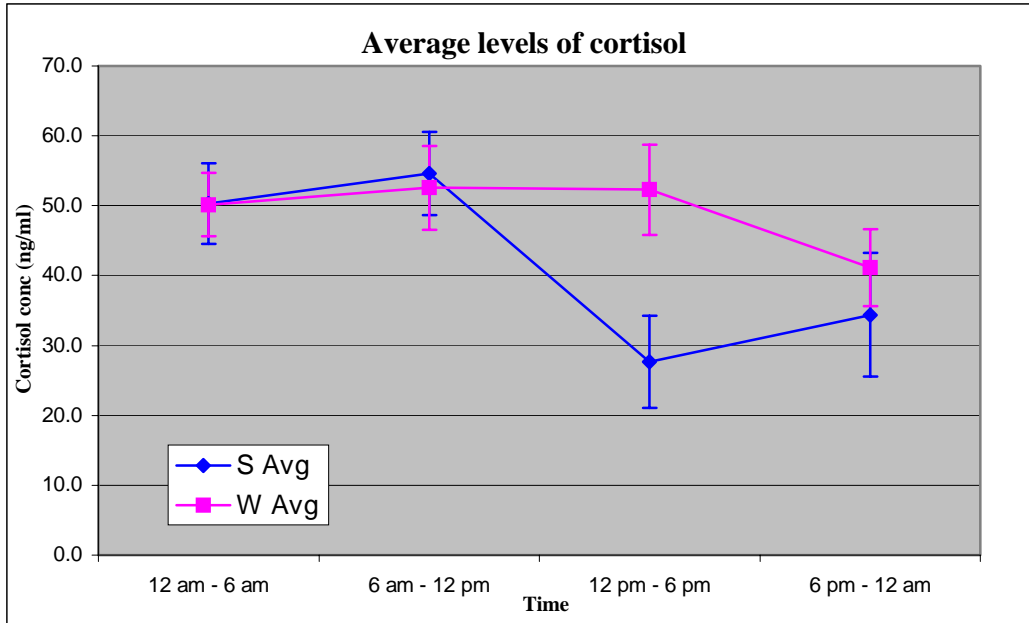


Figure 2. Cortisol concentrations of individual permanently captive seals during summer.

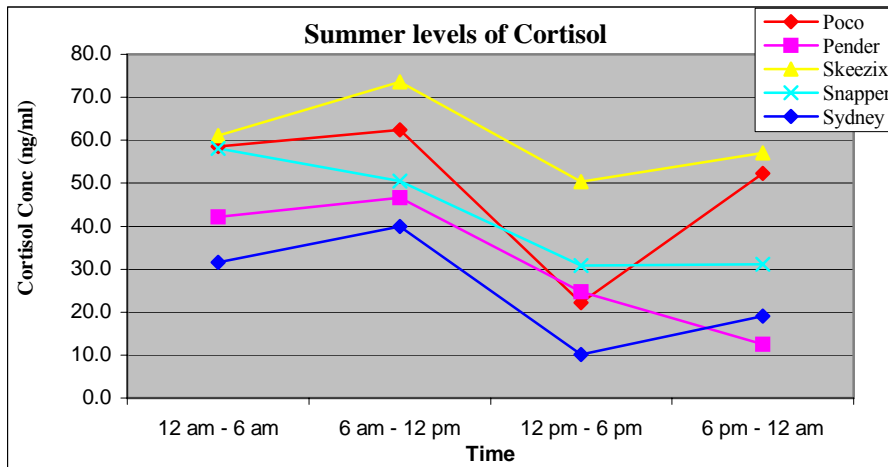


Figure 3. Cortisol concentrations of individual permanently captive seal during winter.

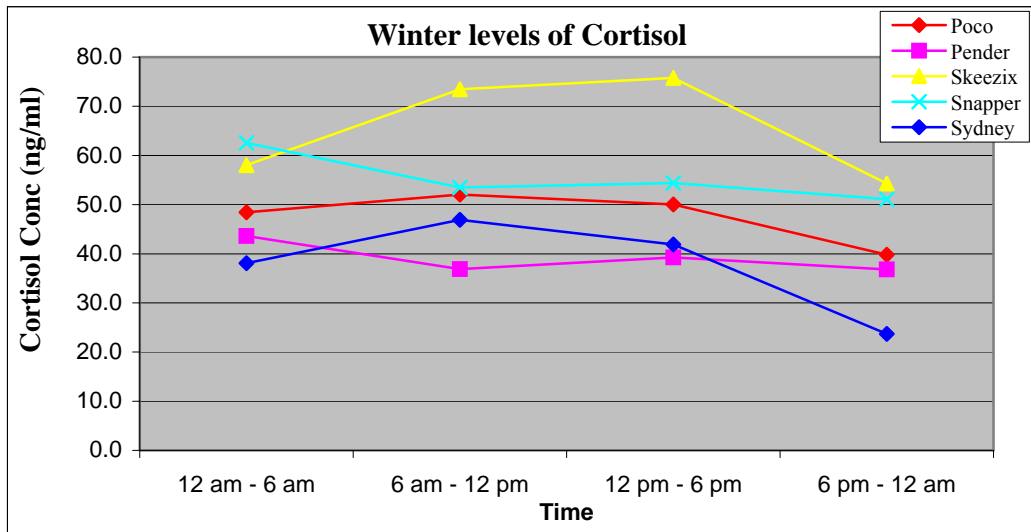


Figure 4. Mean (\pm Se.) Total T₄ concentrations in permanently captive harbor seals during summer (blue) and winter (pink).

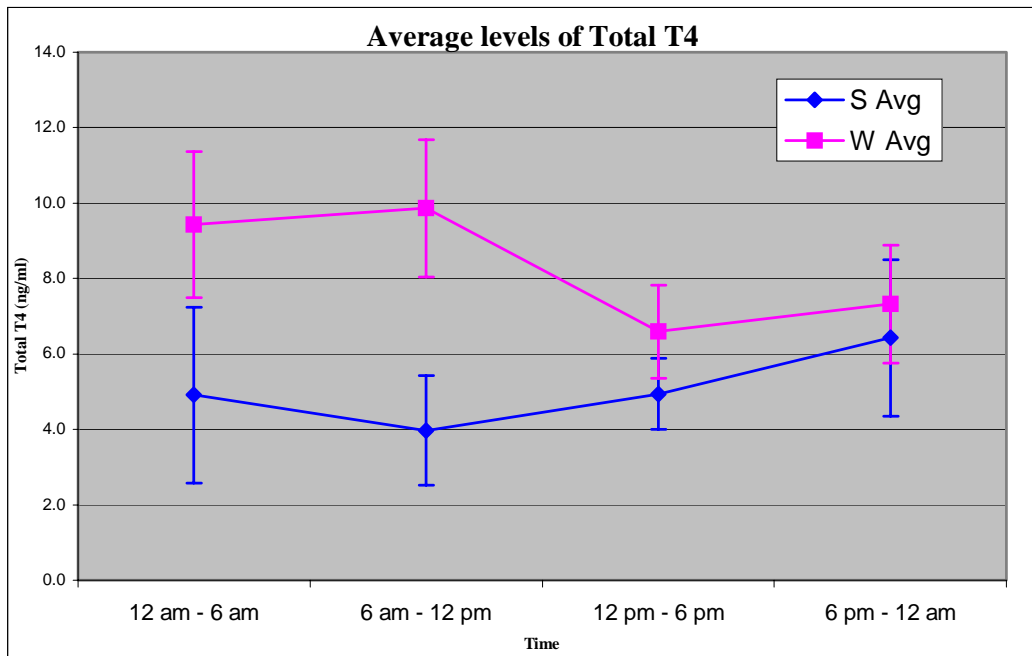


Figure 5. Mean (\pm Se.) Free T₄ concentrations in permanently captive harbor seals during summer (blue) and winter (pink).

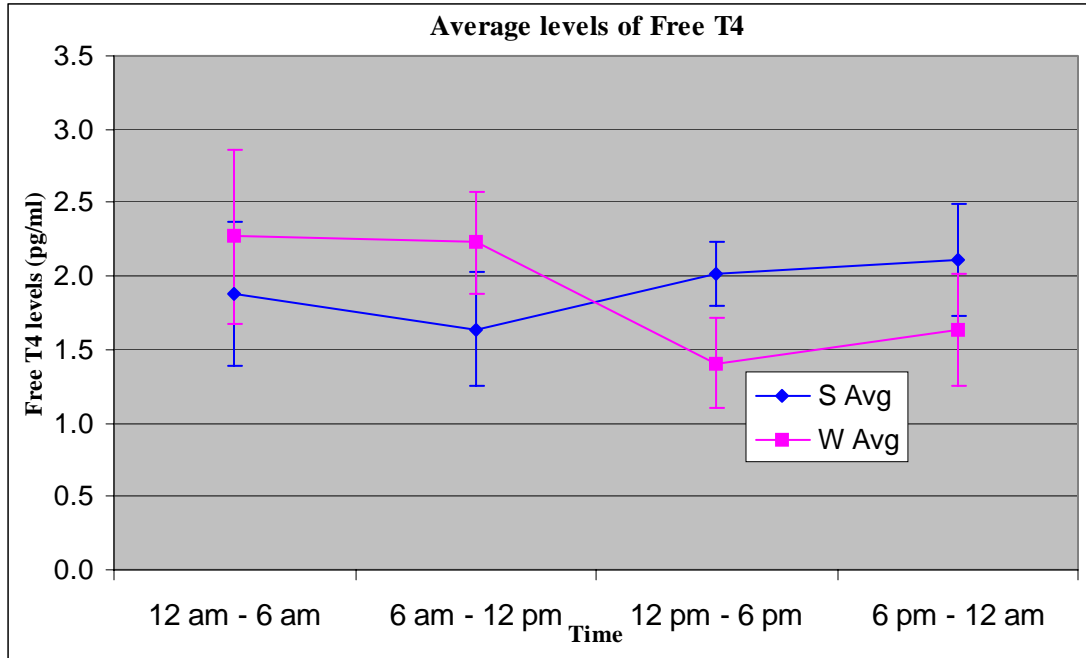


Figure 6. Mean (\pm Se.) Total T₃ concentrations in permanently captive harbor seals during summer (blue) and winter (pink).

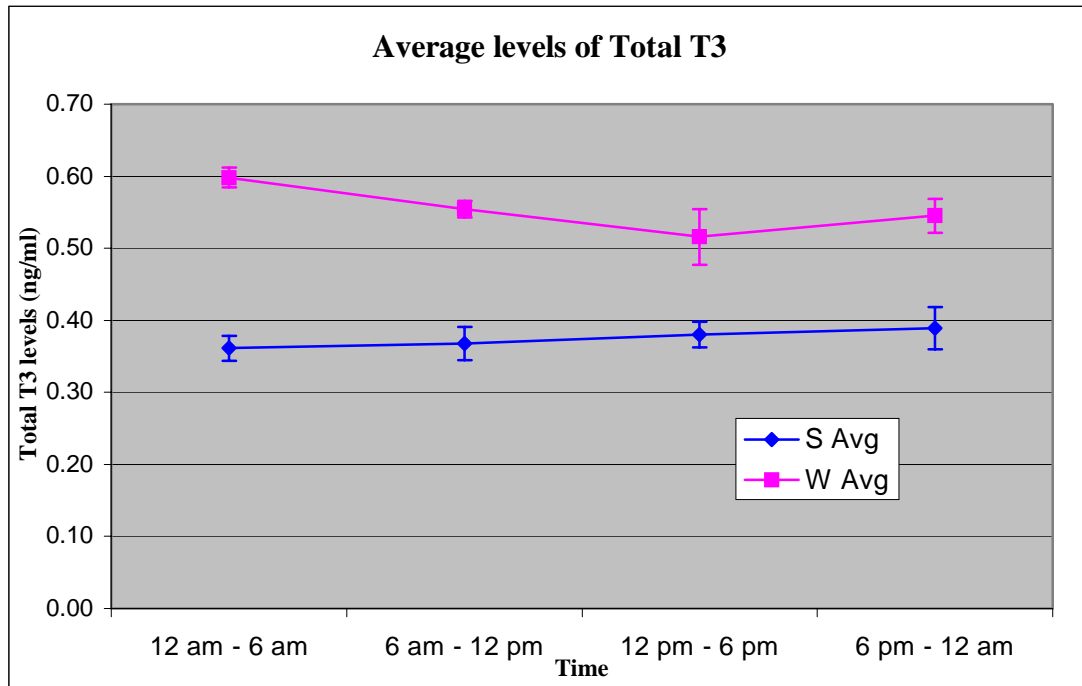


Figure 7. Mean (\pm Se.) Free T₃ concentrations on permanently captive harbor seals during summer (blue) and winter (pink).

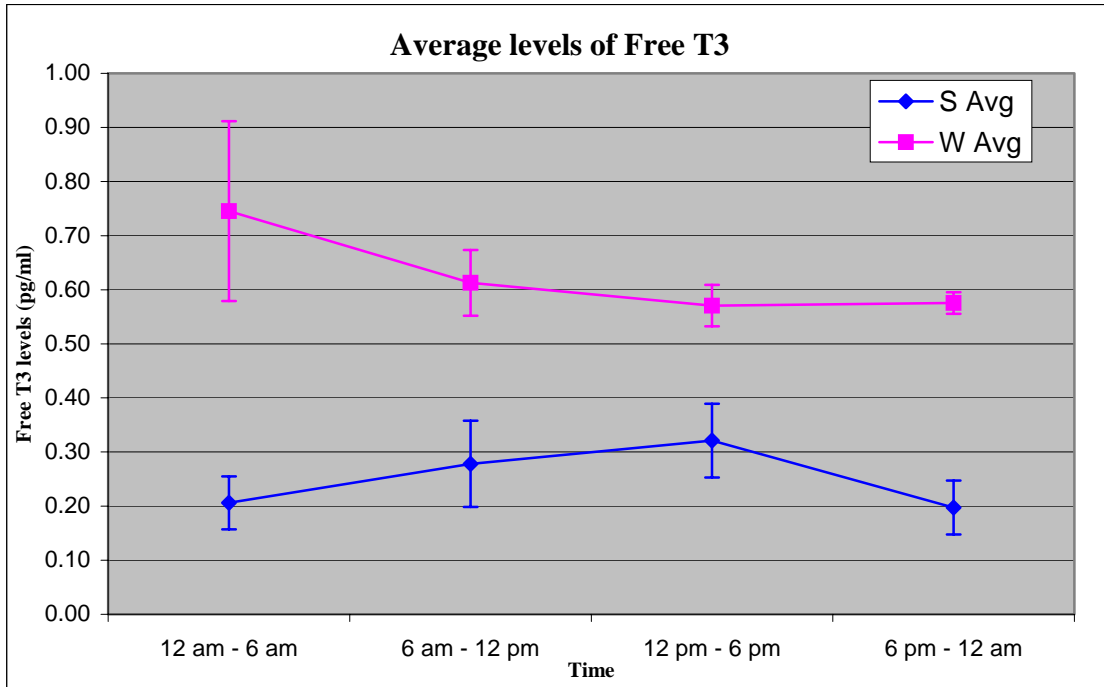


Figure 8 Cortisol and total thyroxine concentrations in a harbor seal pup undergoing rehabilitation, pre and post weaning. Changes in management practices are highlighted on the x-axis.

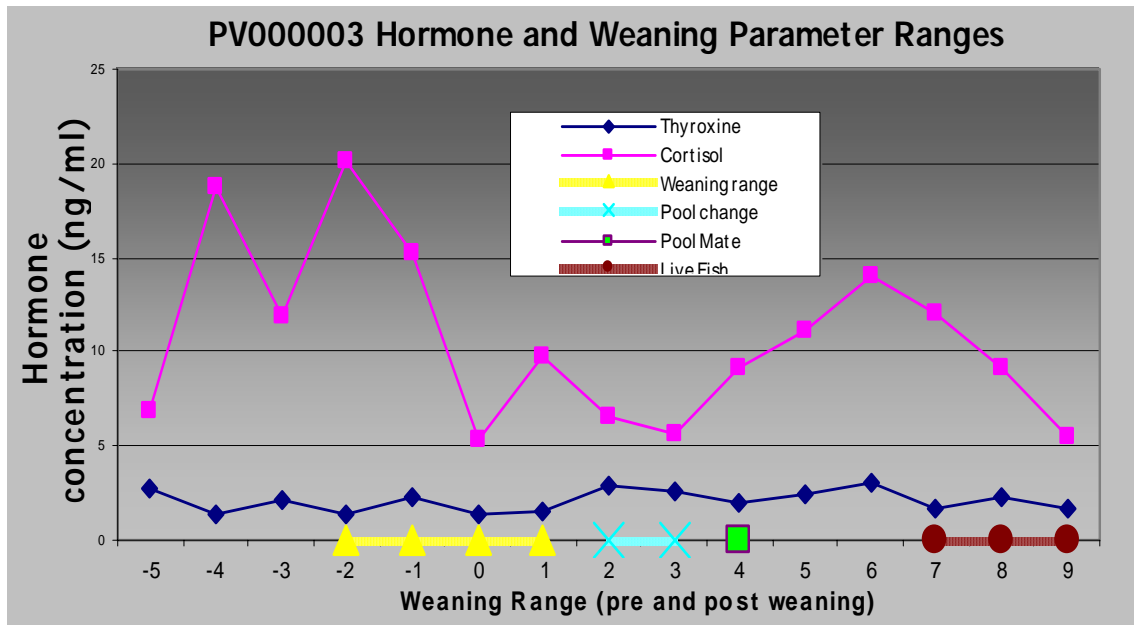


Figure 9 Mean (\pm Se.) metabolic hormone concentrations in permanently captive harbor seals over time.

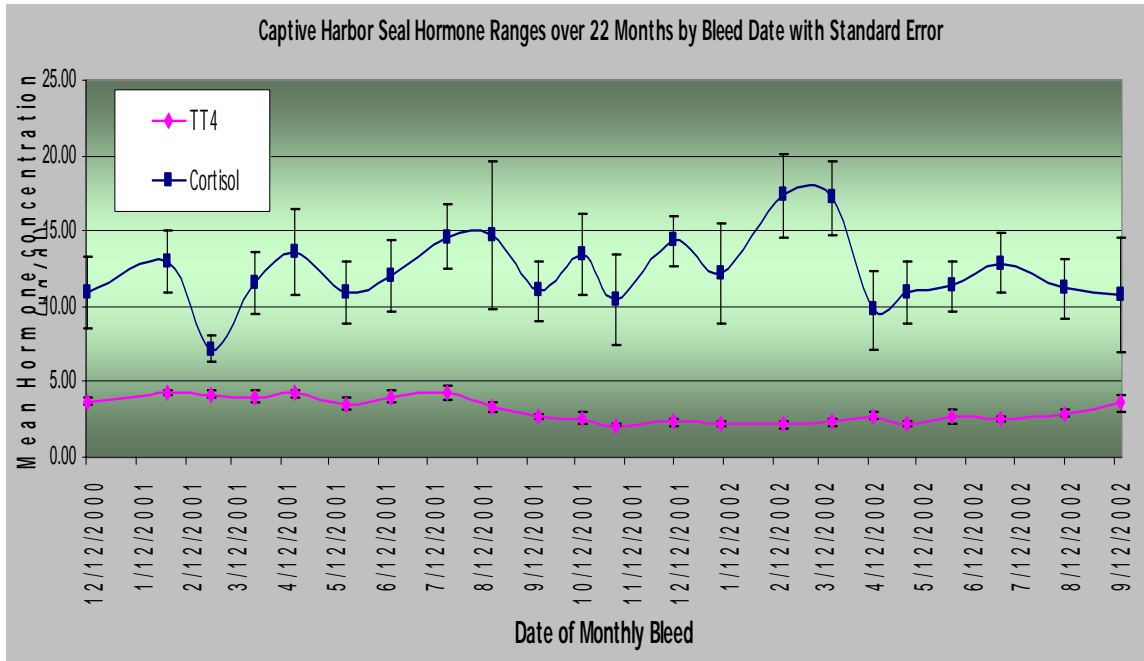


Figure 10 Body mass and caloric intake of permanently captive harbor seal (Cecil) over time.

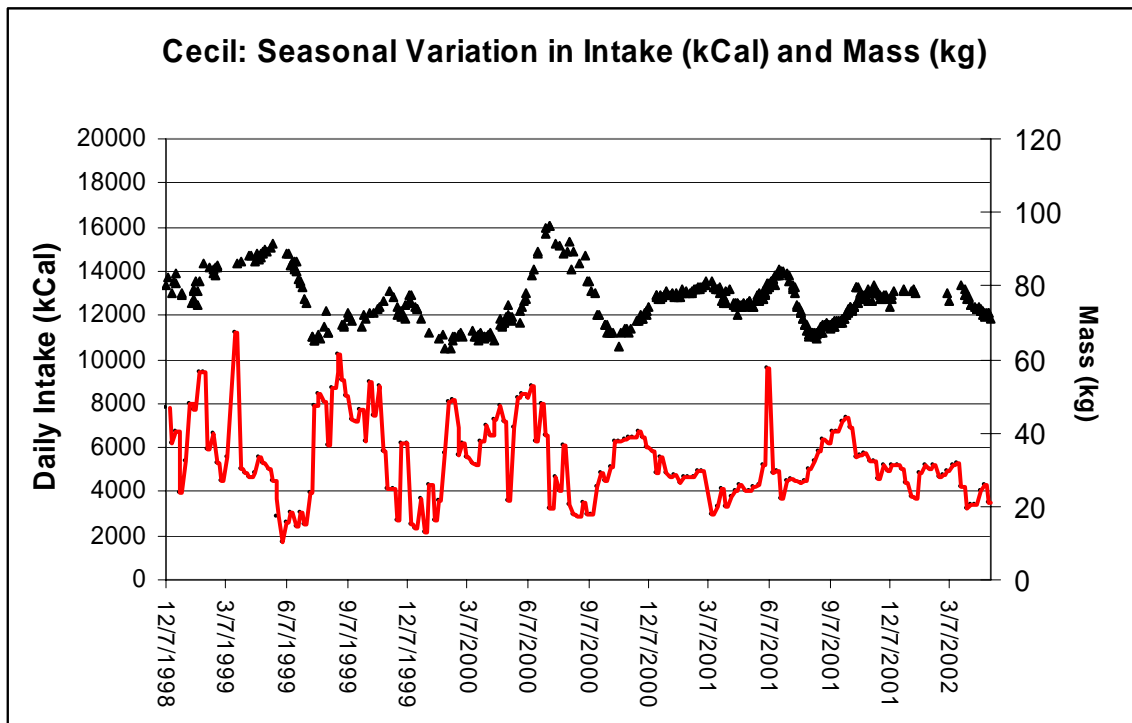


Figure 11 Body mass and caloric intake of permanently captive harbor seal (Pender) over time.

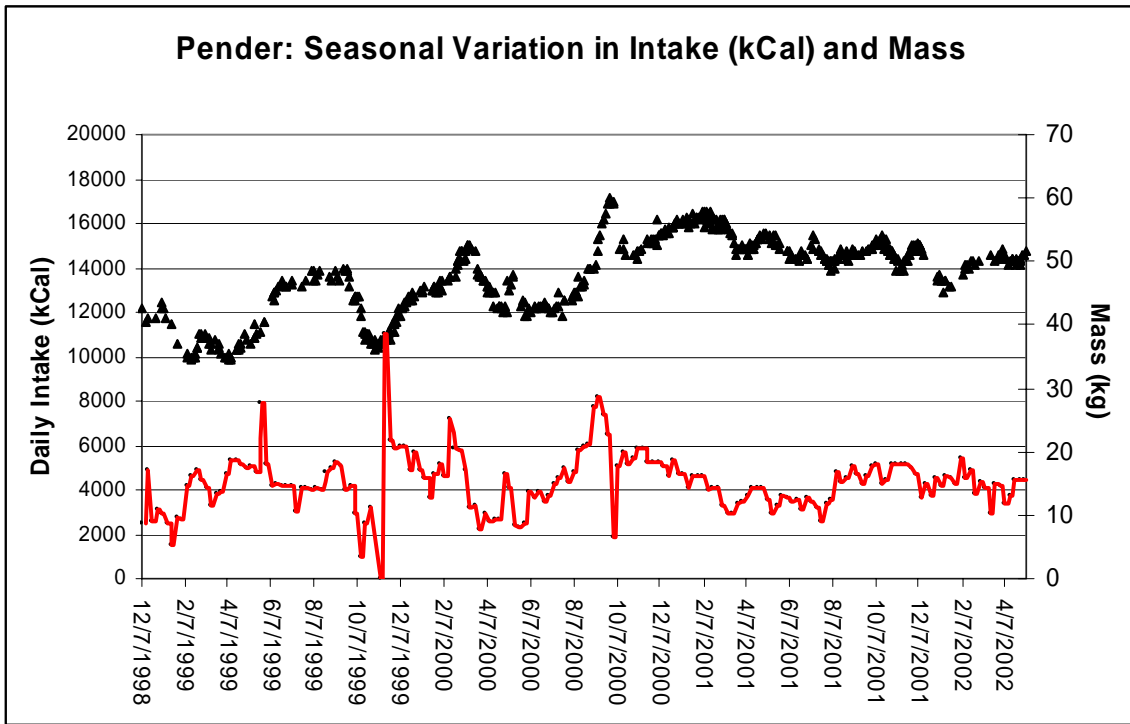


Fig 12 Body mass and caloric intake of permanently captive harbor seal (Poco) over time.

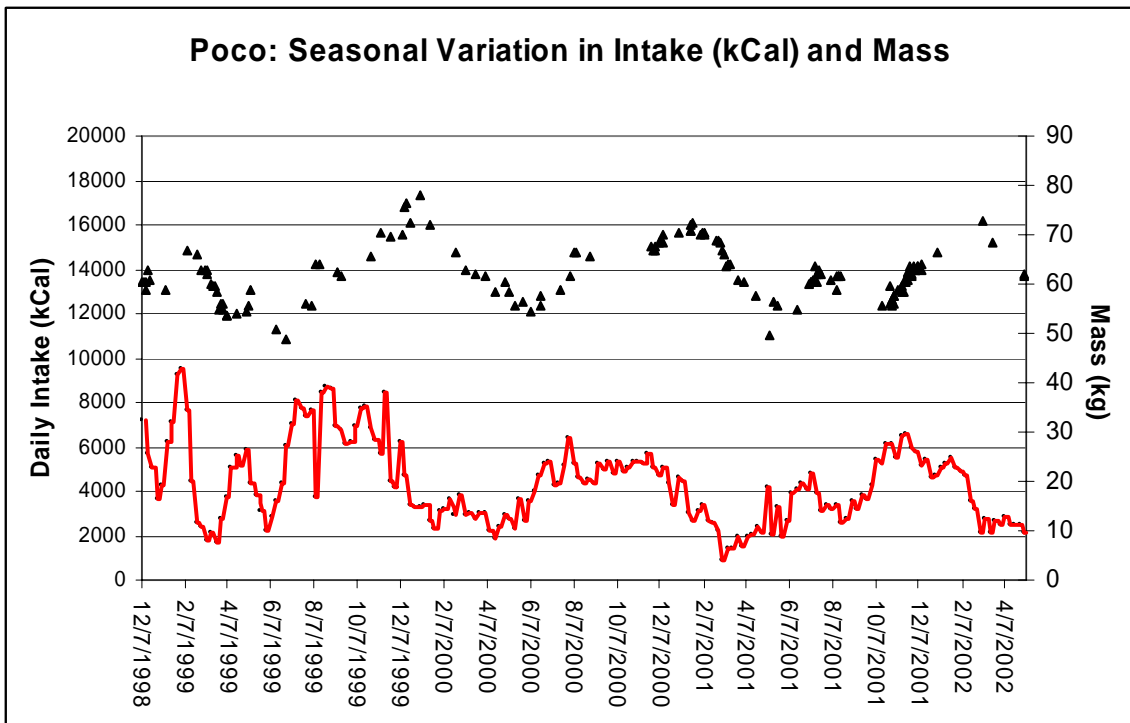


Figure 13 Body mass and caloric intake of permanently captive harbor seal (Skeezix) over time.

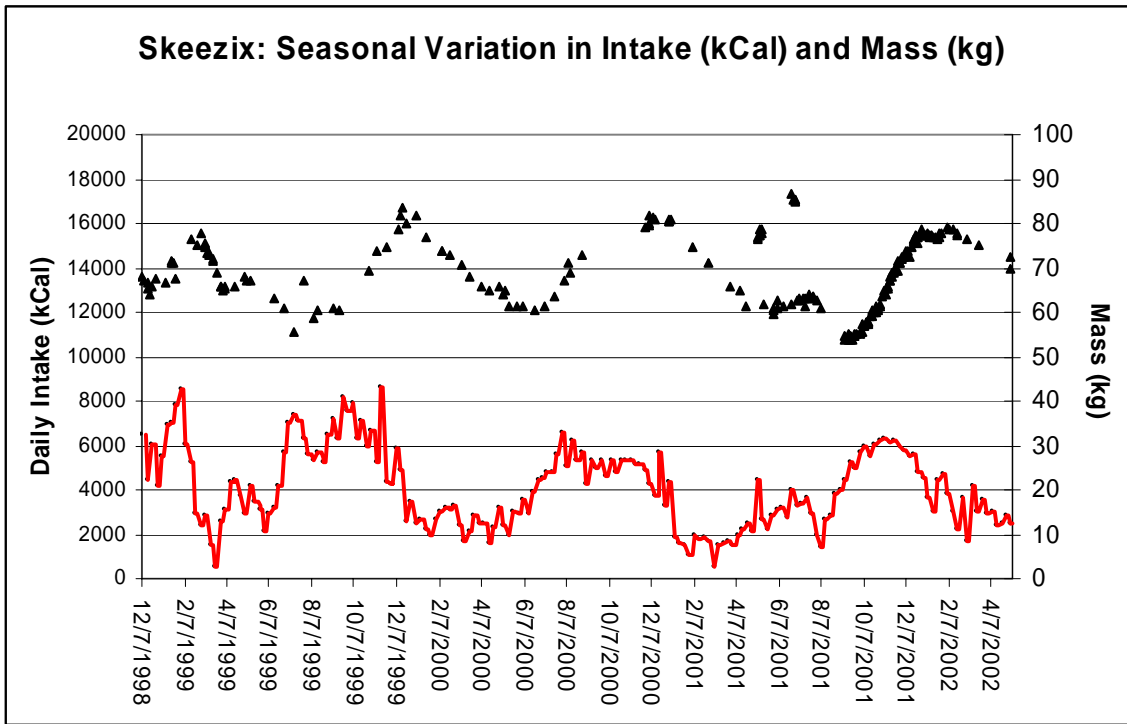


Figure 14 Body mass and caloric intake of permanently captive harbor seal (Snapper) over time.

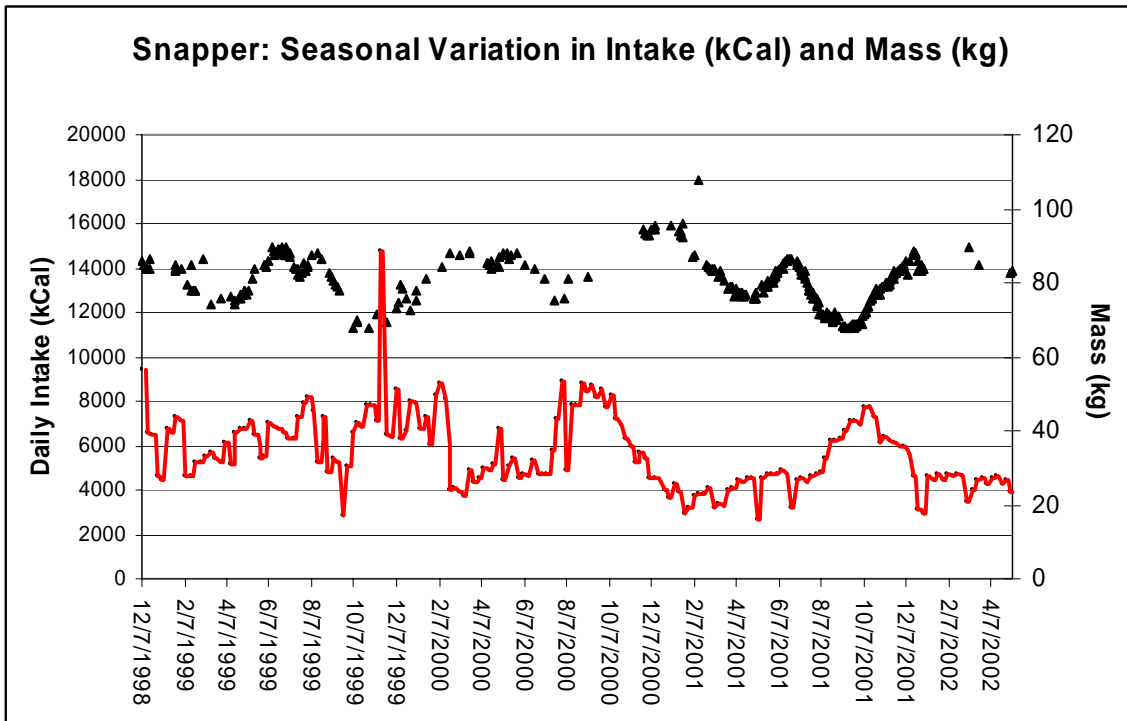


Figure 15 Body mass and caloric intake of permanently captive harbor seal (Sydney) over time.

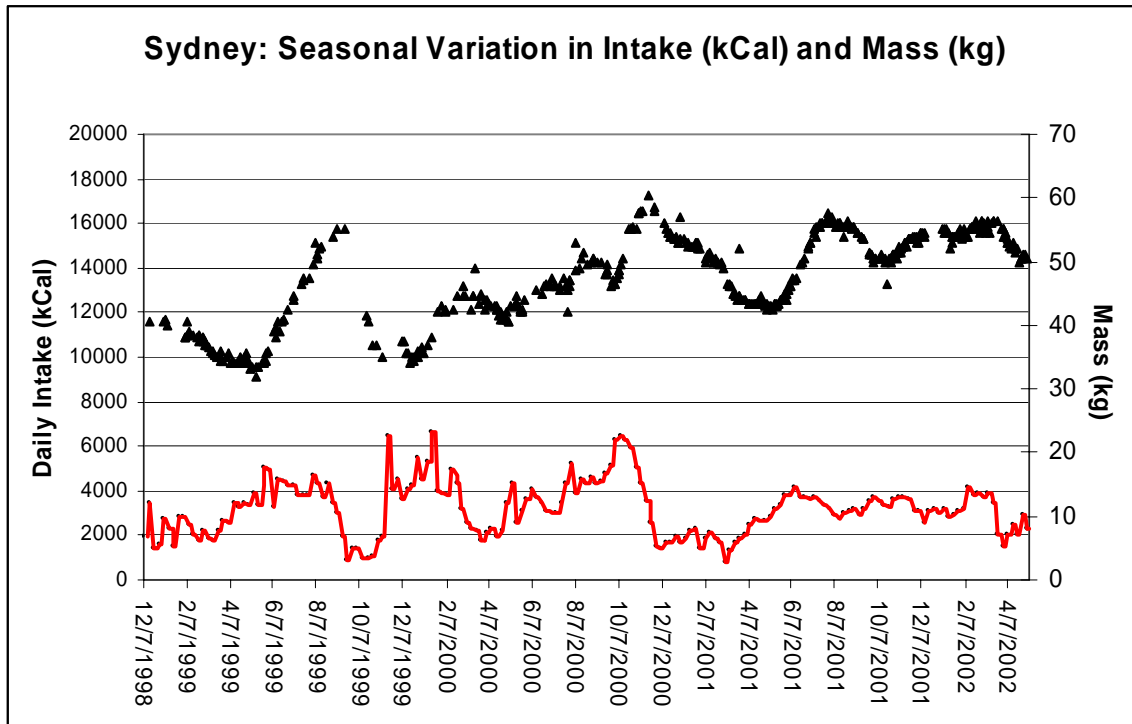


Figure 16 Body mass and caloric intake of permanently captive harbor seal (Tina) over time.

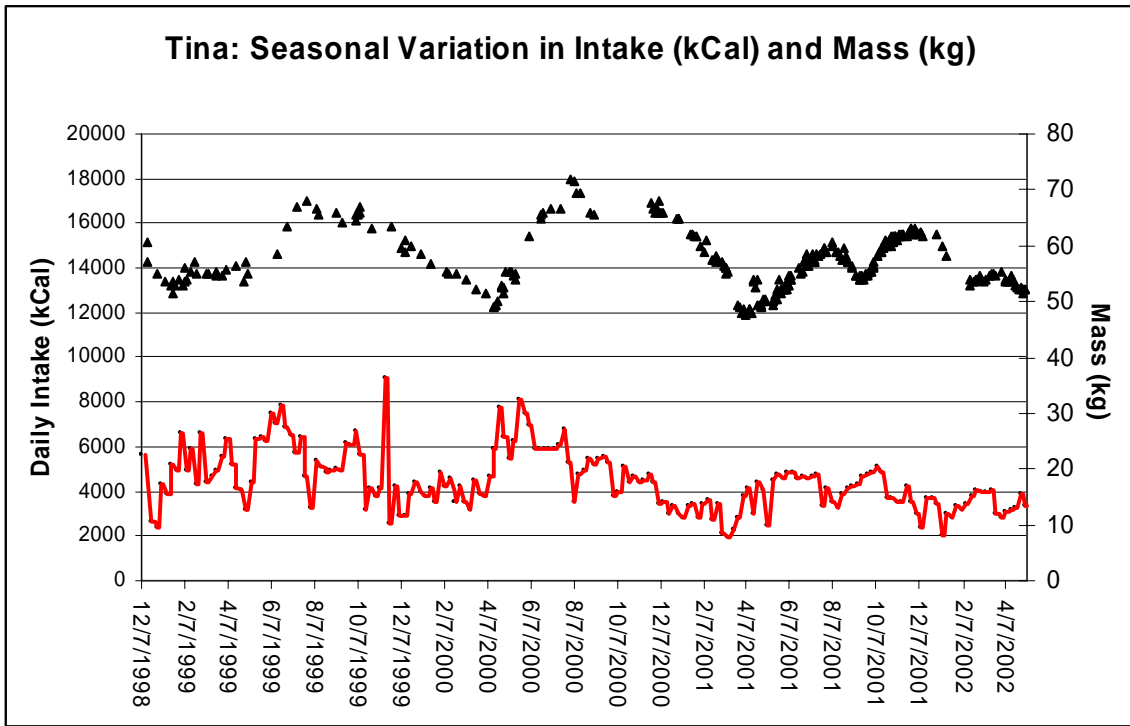


Figure 17 Body mass and caloric intake of permanently captive harbor seal (Travis) over time.

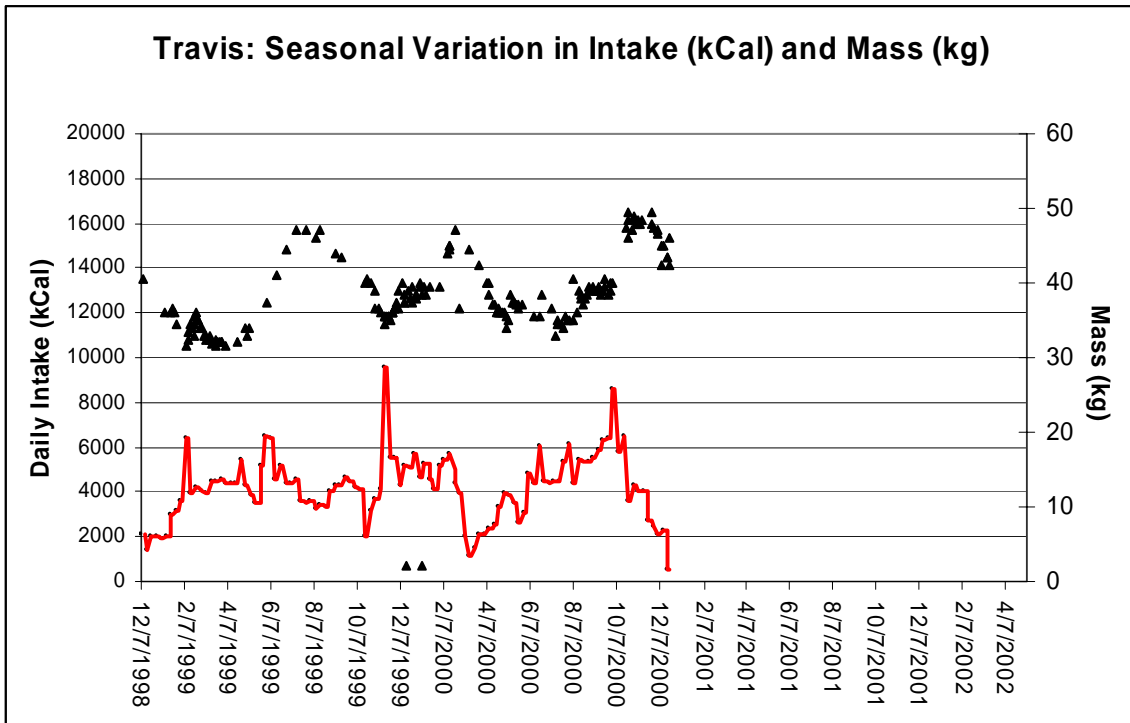


Figure 18 IgA concentrations (mg/ml) in permanently captive harbor seal (Skeexix).

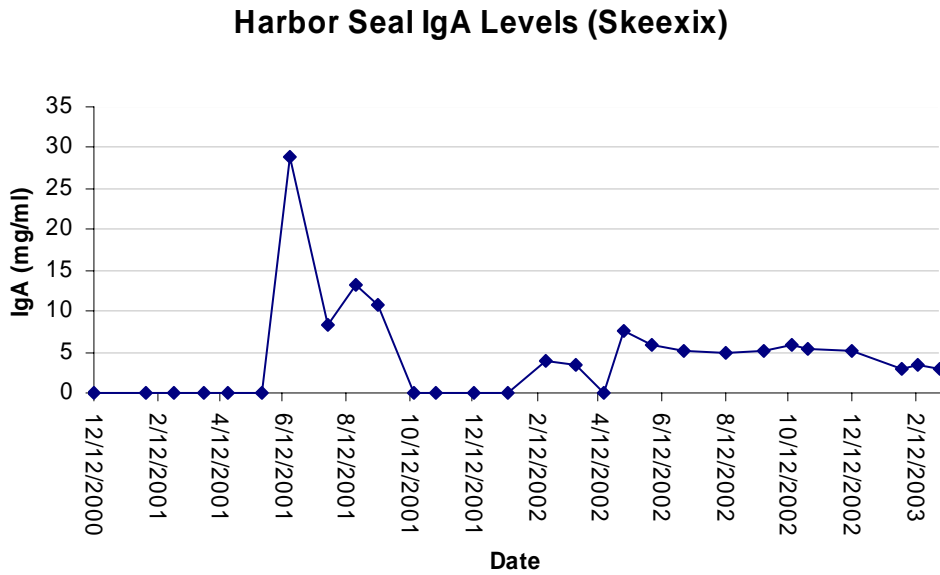


Figure 19 IgA concentrations (mg/ml) in permanently captive harbor seal (Sydney).

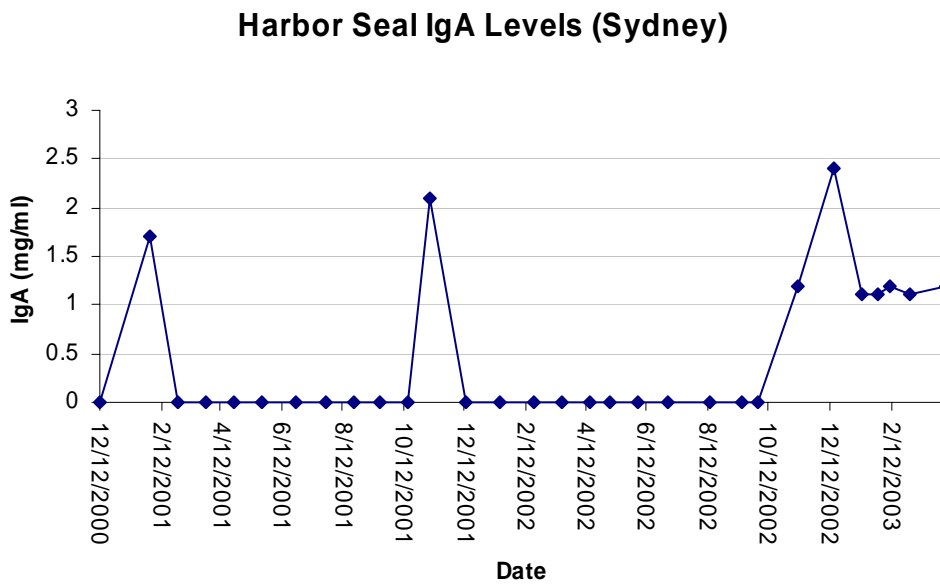


Figure 20 IgA concentrations (mg/ml) in permanently captive harbor seal (Poco).

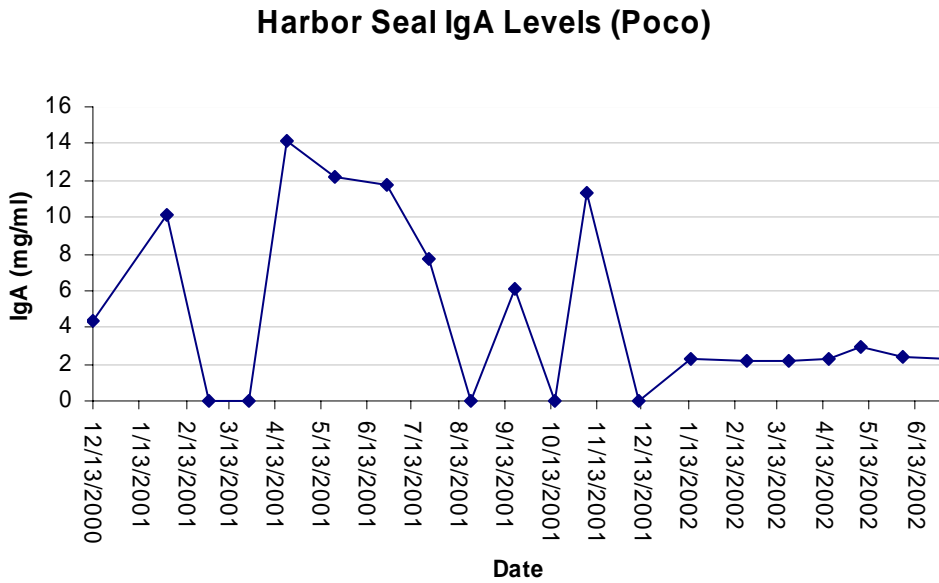


Figure 21 IgA concentrations (mg/ml) in permanently captive harbor seal (Tina).

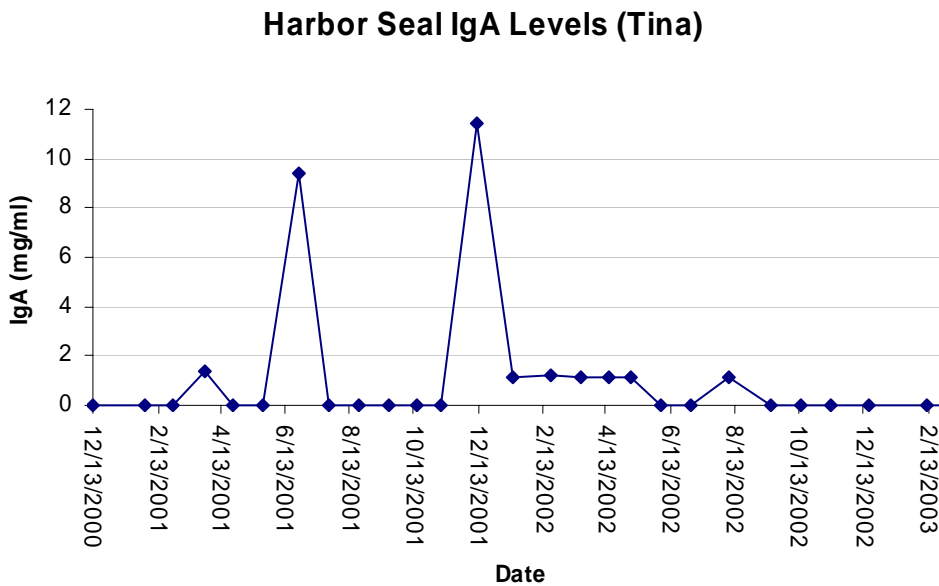


Figure 22 IgA concentrations (mg/ml) in permanently captive harbor seal (Pender).

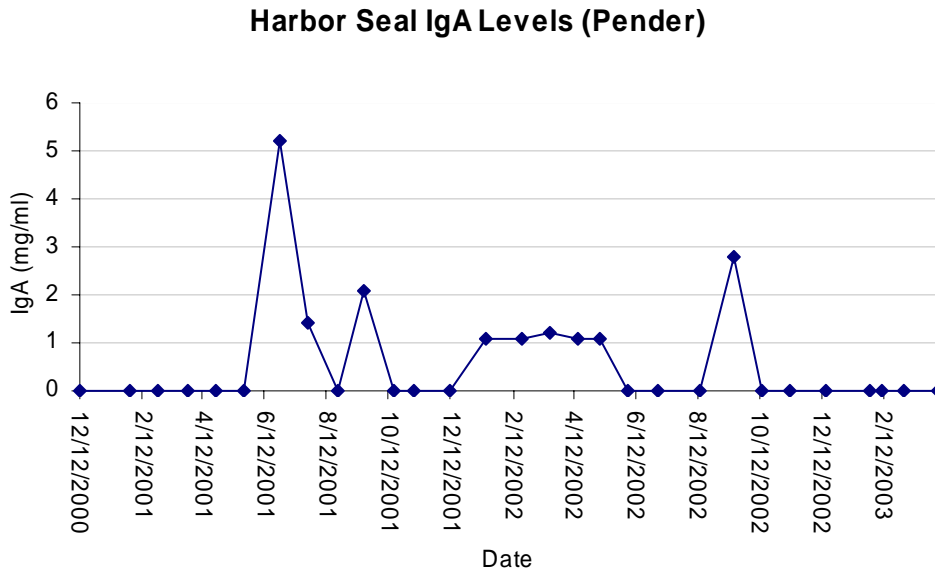


Figure 23 IgA concentrations (mg/ml) in permanently captive harbor seal (Snapper).

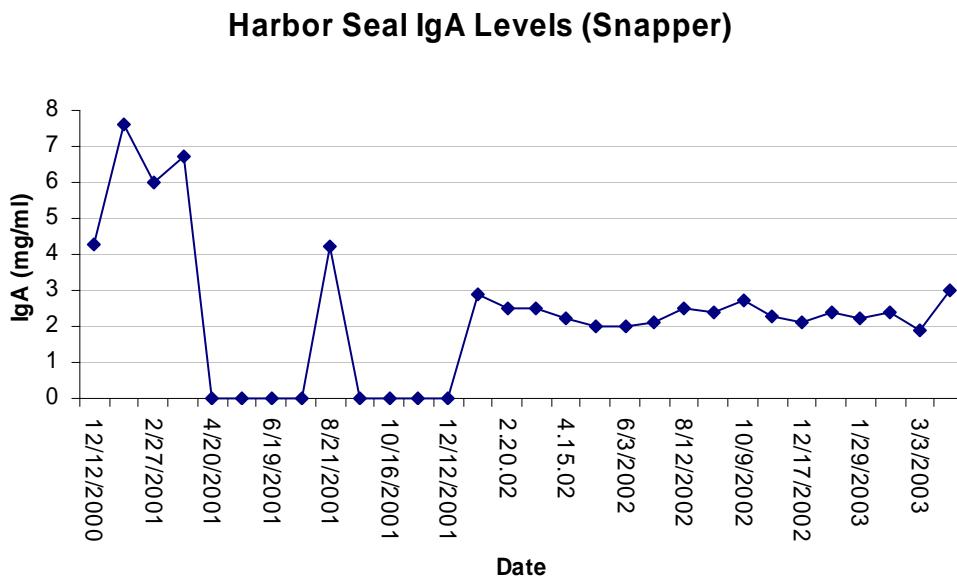


Figure 24 IgA concentrations (mg/ml) in permanently captive harbor seal (Cecil).

