

## **Impact of health and maternal investment on survival of endangered Steller sea lion pups**

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### **Abstract**

There is increasing interest in assessing the health of individuals and populations of pinnipeds found in the North Pacific, primarily due to population declines leading to conservation concerns. The western population of Steller sea lions (SSL; *Eumetopias jubatus*) has declined by more than 80% since the 1970's (Loughlin, 1998) and is listed as endangered under the Endangered Species Act (ESA). During three breeding seasons (2005, 2007, and 2008) 76 pups (32 female, 43 male, 1 unknown) ranging in age from 5 to 38 days old were measured, weighed, blood collected and permanently marked with hot-iron branding. This study assessed the "health" of SSL pups by quantifying hormones associated with fat mass (leptin), energy and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine) as well as circulating total and differential leukocyte counts and *in vitro* proliferation of peripheral blood mononuclear cells (PBMC). We hypothesized that age or maternal investment, as quantified by body mass or condition, would influence leukocyte counts, PBMC proliferation, and hormone concentrations in SSL pups. Further, given the inherent requirements of disturbance and animal handling necessary for sampling free ranging pinnipeds, the impact of these activities and the short term effect of hot-iron branding on endocrine and immune profiles were also assessed.

Total white blood cell (WBC) counts, neutrophil counts and T cell proliferation decreased with increasing age in SSL pups, and no relationship between body condition index and circulating concentration of hormones quantified was detected. Circulating monocytes counts and concentrations of cortisol, thyroxine, and triiodothyronine were influenced by the rookery disturbance. However, the variation attributed to the disturbance was low and did not alter total or differential WBC counts or *in vitro* proliferation of PBMC. These findings are presented in three studies outlined in the present report. The aims of these studies were to 1) assess the postnatal changes in circulating immune components and *in vitro* PBMC proliferation; 2) quantify circulating concentrations of cortisol, aldosterone, total and free thyroxine ( $T_4$ ), total triiodothyronine ( $T_3$ ), and leptin, and to determine if these hormones were associated with body condition or age; 3) given the inherent requirements of disturbance and animal handling necessary for sampling pinnipeds, the impact of these activities on endocrine and immune profiles was assessed, and 4) to determine if these hormones and immune parameters were affected by hot-iron branding.

## Introduction

The western population of Steller sea lions (SSL; *Eumetopias jubatus*) has declined by more than 80% since the 1970's (Loughlin, 1998) and is listed as endangered under the ESA. While the mechanistic cause of the decline is a matter of continuing debate, studies have suggested a reduction in juvenile survival or natality (York, 1994; Holmes and York, 2003). As a result, many studies have focused on SSL pups and juveniles. Blood-borne components, including immunological and endocrine parameters have previously been used to assess the health of individuals and populations of SSL. In free ranging pups, cortisol concentrations varied between geographical region and sex (Myers *et al.*, 2010) and thyroid hormones differed between season and age group (Myers *et al.*, 2006). However, previous studies on hormones in SSL pups did not explore the effect of body mass or condition on these parameters. Red Blood cell count, mean corpuscular hemoglobin concentration, mean corpuscular volume, hematocrit, and hemoglobin decreased following birth, the same parameters increased to adult levels between 5 and 9 months in association with the development of diving ability and oxygen storage in SSL pups and juveniles (Richmond *et al.*, 2005). In addition to hematology, peripheral blood mononuclear cell (PBMC) proliferation assays are increasingly used to assess immuno-competence in pinnipeds (Ross *et al.*, 1994; Levin *et al.*, 2005).

Body condition indices (BCI) have been applied to livestock for the purpose of assessment of health and performance measures (Caldeira *et al.*, 2007), and to free ranging populations of terrestrial mammals (Cattet *et al.*, 2002). Increasingly there has been an interest in quantifying body condition in pinnipeds. BCI for SSL have been derived from a combination of mass, length and blubber thickness (Pitcher *et al.*, 2000; Trites and Jonker, 2000; Rea, 2002). BCI previously developed for SSL pups based on mass and length relationship were able to distinguish between dead pups with and without fat stores (Trites and Jonker, 2000); however, how the BCI relate to hormone concentrations has not been explored in SSL pups. Changes in body condition or mass have been associated with changes in circulating cortisol, thyroid hormones and leptin in several species of terrestrial mammals (Concannon *et al.*, 2001; Barboza *et al.*, 2004) and SSL (Jeanniard Du Dot *et al.*, 2009). These previous studies were based on longitudinal sampling during periods of experimental food restriction or fasting in juvenile and sub-adult SSL. Given the high energy demand and rapid growth (0.23–0.48 kg/day) during the postnatal period of SSL pups (Brandon *et al.*, 2005), it is reasonable to expect an association between body condition and circulating concentrations of hormones associated with nutritional status and fat mass (leptin), energy and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine).

The present study sampled pups from a SSL rookery located on the northwestern shore of Chiswell Island in the eastern Gulf of Alaska. Prior to the recent population decline, Chiswell Island supported a dramatically larger population of approximately 2,000 SSL (Mathisen and Lopp, 1963); currently about 90 breeding animals use the rookery producing up to 80 pups annually (Maniscalco *et al.*, 2006). Blood samples were collected from SSL pups of known ages, sex and body condition providing an opportunity to measure circulating hormones during the early postnatal period, and to determine if these hormones could provide predictable markers for evaluating the body condition of pups to better assess the health of the population.

Hot-iron branding enables individual animals to be identified through unique marks that remain present throughout an animals' life. These identifiable individuals allow for the determination of vital rates such as age-specific survival, mortality rates, pup production, and potentially the specifics of mortality events (Maniscalco *et al.*, 2008) which is needed for the

conservation and management of populations. Hot-iron branding has been shown to provide accurate and highly readable marks in pinnipeds (Merrick *et al.*, 1996; McMahon *et al.*, 2006) without negatively affecting the survival of individual pinnipeds (McMahon *et al.*, 2006; Hastings *et al.*, 2007). Research disturbance and/or hot-iron branding have been associated with short-term and temporary changes in behavior of juvenile and adult SSL (Walker *et al.*, 2010; Wilson *et al.*, 2011) and increases in white blood cell counts, platelet levels, globin and haptoglobin concentrations in juvenile SSL (Mellish *et al.*, 2007). However, the effect of hot-iron branding on endocrine and immune profiles immediately (<180 min) following hot-branding has not been previously explored in SSL pups (9-28 days old).

## **Background**

The immune system protects the body from potential infections and is critical for health and survival of an individual. At birth, the mammalian immune system is not fully developed requiring the temporary reliance on maternal antibodies received by consuming colostrum until their immune system matures. A female under nutritional stress may produce a smaller pup or have reduced maternal investment due to more frequent or longer foraging trips (Harding *et al.*, 2005). Low birth weight in other mammals has been associated with impaired cell-mediated immune response, lasting months to years, including a reduced number of T cells and reduction in their response to mitogens (Chandra, 1991). T cells are an important component of the cell-mediated immune response whose function is driven by the presence of circulating antibodies. A compromised cell-mediated immune system may lead to an increase in susceptibility to disease and mortality and may be the result of low birth weight and/or inadequate transfer of immune protection from the mother.

There is a well-established bi-directional communication between the mammalian immune and endocrine systems (Haddad *et al.*, 2002). The endocrine system is largely responsible for maintaining homeostasis and communicates its effectiveness in maintaining homeostasis to the immune system. The immune system in turn responds to the endocrine system by preparing for changes that are potentially detrimental to the animal. These finely tuned interactions are required for the survival and health of an individual and are regulated by circulating hormones and cytokines, which are in turn influenced by body condition, reproductive status, and stress. The potential impact of stress on an animal's ability to survive would be greatest during times of high energy demands such as pregnancy, lactation, and periods of development and rapid growth. Therefore, females and their pups have the highest potential within a population to be affected by stress or any form of metabolic impairment. Given that NMFS has not permitted any research on adult females for a 10 year period (NMFS, 2007), assessing the health of pups may enable us to identify physiological indicators of food limitation and be a proxy for the health and fitness of the mother. Further, the health of pups has the potential to be used as an indication of the overall health of the population.

## **Objective/hypothesis**

### *Objectives*

The overall goal of this project is to determine if different levels of maternal investment as assessed by pup body condition or mass, results in measurable changes in the immune and endocrine systems of SSL pups. Specifically, the objectives of this proposal are to:

1. Assess endocrine function by quantifying hormones previously shown to indicate body condition and/or stress including cortisol, aldosterone, leptin, ACTH, ghrelin, and thyroid hormones (T3 & T4).
2. Assess immune function by complete blood cell count, serum chemistries, lymphocyte proliferation assay and circulating IL-6 levels.
3. Combine measures of maternal investment, pup body condition and endocrine parameters to predict pup health and survival.

### *Hypotheses*

Given the interactions of the endocrine and immune systems and the reported effects of stress, body condition, and maternal investment, we hypothesize:

1. Cell-mediated immunity will change with pup age.
2. Smaller pups will have reduced cell-mediated immunity.
3. Smaller pups will have higher circulating stress hormone levels, specifically cortisol and aldosterone.

### **Study area/field work**

Chiswell Island is home to a small SSL rookery, used by about 90 breeding animals producing up to 80 pups per year (Maniscalco *et al.*, 2006) and is located 35 nautical miles south of Seward within the Alaska Maritime National Wildlife Refuge System. As part of a long-term monitoring and research effort, researchers at the Alaska SeaLife Center (ASLC; Seward, AK) have observed the Chiswell Island rookery via remotely-operated video monitoring equipment since 1998 (Maniscalco *et al.*, 2006). All SSL pups were continuously observed during daylight hours by the remote video monitoring program at the ASLC throughout the entire breeding season.

Table 1. Mean ( $\pm$  SD) for age, body mass (BM), standard length (SL), and axillary girth (AG) for 76 Steller sea lion pups by project. Note: some pups were including in more than one study.

		n	Age (day)	BM (kg)	SL (cm)	AG (cm)
BCI	Male	37	18.3 $\pm$ 7.2	32.2 $\pm$ 4.6	108.0 $\pm$ 4.9	75.6 $\pm$ 6.1
	Female	22	14.7 $\pm$ 4.9	26.7 $\pm$ 4.1	101.6 $\pm$ 4.7	70.3 $\pm$ 5.4
IMMUNE	Male	31	18.9 $\pm$ 7.1	32.6 $\pm$ 4.7	107.9 $\pm$ 5.0	76.1 $\pm$ 6.8
	Female	15	13.8 $\pm$ 4.5	26.8 $\pm$ 4.4	102.1 $\pm$ 4.3	70.7 $\pm$ 5.7
BRANDING	Male	8	20.1 $\pm$ 6.6	32.5 $\pm$ 2.1	108.1 $\pm$ 2.4	74.2 $\pm$ 3.8
	Female	10	16.5 $\pm$ 3.7	27.0 $\pm$ 2.3	101.6 $\pm$ 2.9	71.3 $\pm$ 3.8

### **Materials and Methods**

A total of 76 pups (Table 1) were sampled on June 30, 2005; July 3, 2007 and July 1, 2008 (Permit 881-1890-02; ASLC IACUC 07-001) for the three studies outlined in the present report and supported in part through the Pollock Conservation Cooperative Research Center grant (G00005466). Each pup was measured, weighed, and one blood sample was collected. Body mass (BM) was measured to the nearest tenth of a kilogram using a hanging electronic scale (FWC series 7, FlexWeigh, Santa Rosa, Ca). Standard length (SL) was measured as a straight line from tip-of-nose to tip-of-tail while the pup was lying on a straight board. Axillary

girth (AG) was measured using a tape measure after the pup had exhaled. A body condition index was calculated for 61 pups (male=37, female=24) by applying the stoutness-index [body mass/ (-63.88 + 0.8966\*standard length)] proposed by Trites and Jonker (2000). Pups (n=67) were permanently marked by hot iron branding (Merrick *et al.*, 1995) under isoflurane (USP; Halocarbon Industries, River Edge, NJ) anesthesia (Heath *et al.*, 1997) with the remaining 9 pups having flipper tags attached in the axillary area of both fore-flippers. Branding pups allowed for the identification of mother-pup pairs and determination of pup ages (to within  $\pm 4$  hr) by association with naturally marked females that were tracked from the time they gave birth. Pups ranged in age from 5 to 38 days old at the time of blood collection. In 2008, 11 pups had blood collected prior to branding while 15 pups were branded and held in a temporary corral for 30, 60, 120 or 180 mins after which blood was collected.

### *Blood Collection*

Blood samples ( $\leq 1$  ml/kg) were collected using standard aseptic techniques from the caudal gluteal vein with an 18 gauge needle directly into Vacuette® blood collection tubes. In 2005 and 2008 pups were anesthetized with isoflurane prior to blood collection and in 2007, pups were manually restrained during blood collection. Blood tubes included EDTA-treated tubes for total and differential leukocyte counts and Z serum clot activator tubes for serum chemistries and hormone analysis. Sodium heparin-treated tubes were collected in 2007 and 2008 for isolation of PBMC from 21 of the pups (10 female, 11 male). The serum separator tubes that were collected in 2007 and 2008 were spun on the rookery within 2 hours of blood draw while samples collected in 2005 were separated upon return to the lab. All blood tubes were kept upright and chilled until further processing in the lab ( $< 12$  hrs).

### *Hormone Radioimmunoassays*

Serum was assayed for hormone concentrations with commercially available radioimmunoassay kits (RIA) previously validated for SSL in our lab including cortisol, aldosterone, leptin, and thyroid hormones (Myers *et al.*, 2006; Mashburn and Atkinson, 2008) were used to assess endocrine function.

### *Total and differential leukocyte counts*

CBC counts were determined using the Heska® CBC-Diff Veterinary Hematological System (Heska ® Corporation, Loveland, CO). Total WBC, Hct, RBC, Hb, and Plt were measured and used to calculate the MCV, MCH, and MCHC. Blood smears were made from whole EDTA blood and stained with Wright-Giemsa (Dip Quick Stain, Jorgensen Laboratories, Loveland CO). WBC differentials, including neutrophils, lymphocytes, monocytes and eosinophils were counted manually and reported as percentages and absolute values of differentials (cells/ $\mu$ l).

### *PBMC Isolation and in vitro Proliferation*

PBMC were plated ( $2.0 \times 10^5$  cells/well) and proliferation assessed following exposure to one of two mitogens (ConA or LPS 055:B5; Sigma Aldrich, St. Louis, MO). PBMC were cultured at 37°C with 5% CO<sub>2</sub> in triplicate for each animal in each of the following treatment groups; 1) unexposed control, 2) ConA suboptimal (0.1  $\mu$ g/ml), 3) ConA optimal (1.0  $\mu$ g/ml), 4) LPS suboptimal (1.0  $\mu$ g/ml) and 5) LPS optimal (100  $\mu$ g/ml). These conditions are similar to those previously used for pinnipeds (Levin *et al.*, 2005; Mori *et al.*, 2006).

All PBMC were assessed in two assays with PBMC collected from a captive adult male SSL as an inter-assay control. PBMC were incubated for a total incubation period of 66 h and proliferation was assessed with the Cell Proliferation Biotrack ELISA System version 2 (GE Healthcare, Piscataway, NJ) per manufacturer's instructions using Dynatech MRX Revelation microplate reader (Dynatech Laboratories, Inc, Chantilly, VA) at 450 nm with a reference wavelength of 650 nm. Data are presented as mean of the triplicates for optical density (OD) with standard deviation and as a stimulation index (SI; mean OD of cells exposed to mitogen/mean OD of cells in media only).

### *Statistical Analysis*

Data were analyzed with Systat 10 (Systat Software, Inc, Point Richmond, CA). The best model for each parameter was selected using a stepwise general linear model with an iterative process of comparing the full mixed effects model, which included the categorical variables of sex and anesthesia, age as a continuous variable, and all interaction terms. The full model was compared to reduced models, which included only statistically significant grouping variables and interactions. Three of the 13 hematological parameters (MCV, MCH, and MCHC) showed differences between PR and ISO; however, differences were less than 5% and therefore the hematological parameters were not separated for further analysis. To evaluate the effect of pup handling, serum cortisol concentrations were regressed against either the time elapsed from the initial arrival on the rookery to the time when the individual pup was removed from the corral and taken for blood collection or the immune components measured in the current study. Values were considered statistically significant if  $p \leq 0.05$ .

To evaluate the potential effect of the disturbance at the rookery associated with separation of mother and pup, and corralling the pups, each parameter was regressed against the time elapsed from the initial arrival on the rookery to the time when the individual pup was removed from the corral and taken for blood collection. If anesthesia or sex was identified as a significant factor, then each treatment group was analyzed separately. Values were considered significant if  $p \leq 0.05$ .

### **Summary of results by study**

#### *Study 1:*

There was no effect of sex ( $p=0.338$ ), anesthesia ( $p=0.171$ ) or age ( $p=0.854$ ) on circulating cortisol levels. Cortisol concentrations decreased when regressed against the elapsed time between arrival on the rookery and removal of pup from the holding corral for blood collection ( $p=0.009$ ), indicating that the pups were affected by the initial disturbance of the rookery. However, no significant effects of sex, anesthesia or serum cortisol concentrations ( $p>0.082$ ) were detected in unstimulated or mitogen stimulated PBMC. Proliferation of unstimulated PBMC and PBMC exposed to LPS and the suboptimal ConA did not change with age ( $p\geq 0.137$ ). Further, T cell proliferation decreased with age when exposed to the optimal ConA ( $p=0.038$ ).

There was not a significant effect of sex or anesthesia on total or differential WBC counts, while monocyte counts ( $p= 0.020$ ) decreased in association with increasing cortisol concentrations (Figure 11). However, cortisol concentrations ( $p>0.059$ ) did not affect total WBC, neutrophil, lymphocyte, eosinophil counts or CBC count parameters including Hct, RBC, Hb, or PLT, suggesting handling of pups did not alter circulating immune components. Total WBC and

neutrophil counts decreased while there was no change in lymphocyte, monocyte, or eosinophil counts with age ( $p \geq 0.288$ ). The neutrophil to lymphocyte ratio also decreased with age.

## Study 2

### Age and Body Mass

Body mass ( $p < 0.001$ ), standard length ( $p < 0.001$ ), and axillary girth ( $p = 0.015$ ) were greater in male than female pups. Further, body mass ( $p < 0.001$ ), standard length ( $p < 0.001$ ), and axillary girth ( $p < 0.001$ ) increased with age. BCI was not related to pup age ( $p = 0.216$ ) nor was there an effect of sex ( $p = 0.647$ ).

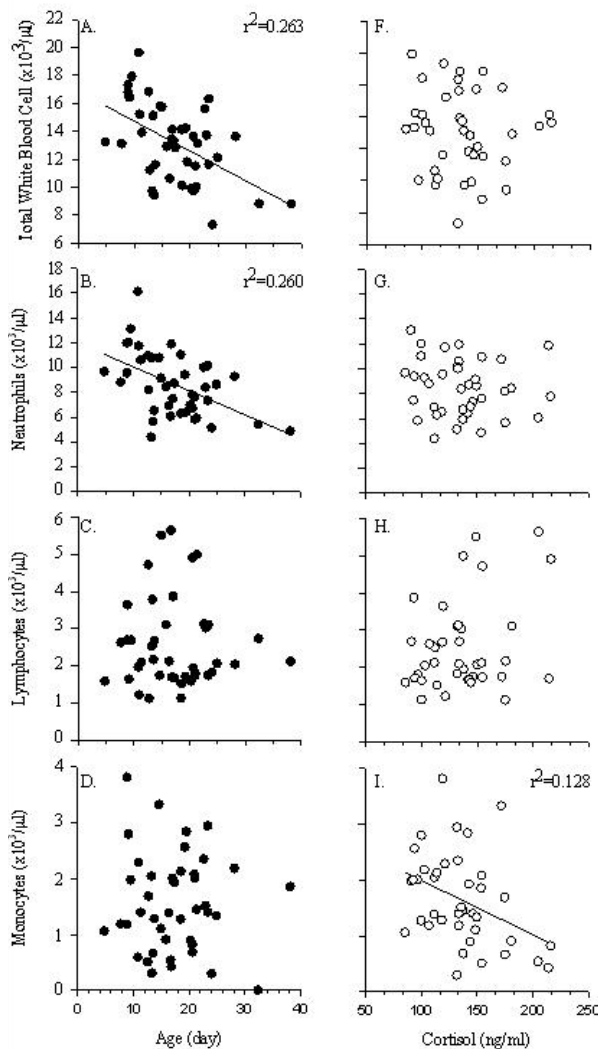


Figure 1 (A) Total WBC counts from 42 pups during the 38 days following birth and differential counts for (B) neutrophils; (C) lymphocytes; (D) monocytes; and (E) eosinophils. (F) Total WBC counts (G) neutrophils; (H) lymphocytes; (I) monocytes; and (J) eosinophils are also presented against level of circulating cortisol (ng/ml). Significant trend lines are presented.

### Hormonal Profiles and Relationships

Aldosterone concentrations were not related to BCI ( $p = 0.055$ ). However, male pups ( $369 \pm 253$  pg/ml;  $5.76 \pm 0.51$  log (pg/ml)) had higher aldosterone concentrations ( $p = 0.015$ ) than females ( $357 \pm 301$  pg/ml;  $5.68 \pm 0.59$  log (pg/ml)) and there was a significant interaction between sex and BCI ( $p = 0.013$ ) suggesting that the relationship between BCI and aldosterone concentration differs between male and female pups (Figure 2). Female pups had an inverse relationship between aldosterone concentration and BCI; however, this relationship was not observed in male pups (Figure 3a). The difference between male ( $323.5 \pm 143.5$  pg/ml;  $5.69 \pm 0.42$  log (pg/ml)) and female ( $276.5 \pm 111.9$  pg/ml;  $5.55 \pm 0.40$  log (pg/ml)) pups remained significant after the outliers (Figure 3b) were removed ( $p = 0.011$ ). No significant effect of age or anesthesia /physical restraint was detected. Nor were aldosterone concentrations effected by the elapsed time between arrival on the rookery and blood collection. Circulating cortisol concentrations were higher in female ( $151 \pm 45$  ng/ml) than male ( $130 \pm 34$  ng/ml) pups ( $p = 0.037$ ) but were not affected by an anesthesia /physical restraint, BCI or age. Cortisol concentrations decreased when regressed against the elapsed time between researchers arrival on the rookery and removal of pup from the holding corral for blood collection ( $p < 0.001$ ).

Leptin (ng/ml HE) serum concentrations were significantly higher ( $p = 0.009$ ) in PR ( $2.0 \pm 0.4$  ng/ml HE) than pups under ISO ( $1.7 \pm 0.4$  ng/ml HE) during blood collection.

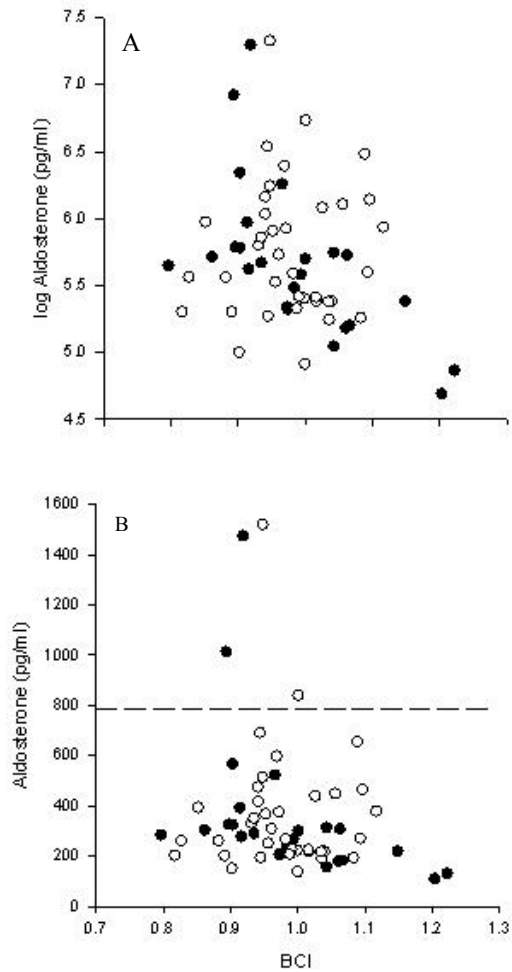


Figure 2. Concentration of Aldosterone (A) (pg/ml) and (B) log(pg/ml) from female (●) and male (○) SSL pups. Dashed line separates the pups with greater than 2 SD of the mean for aldosterone concentration (pg/ml).

When pups under anesthesia or physical restraint were analyzed separately, there was no effect of BCI, sex or age on circulating leptin concentrations. There was no detectable effect of BCI, sex or ISO/PR on circulating total T<sub>4</sub> or free T<sub>4</sub> in SSL pups (Figure 3). Circulating total T<sub>4</sub> ( $p=0.015$ ) and free T<sub>4</sub> ( $p=0.031$ ) decreased with pup age (Figure 3b, d). Pups under anesthesia ( $0.61 \pm 0.15$  pg/ml) had higher ( $p=0.001$ ) total T<sub>3</sub> compared to pups physical restraint ( $0.48 \pm 0.10$  pg/ml) during blood collection. When compared separately there was no significant effect of sex, BCI or age in total T<sub>3</sub>. Thyroid hormones significantly decreased when

regressed against the elapsed time from the initial arrival of researchers on the rookery to the time when the individual pup was removed from the corral and taken for blood collection including total T<sub>4</sub> ( $p=0.008$ ), total T<sub>3</sub> ( $p=0.012$ ), and free T<sub>4</sub> ( $p<0.001$ ) (Figure 3b, c, d).

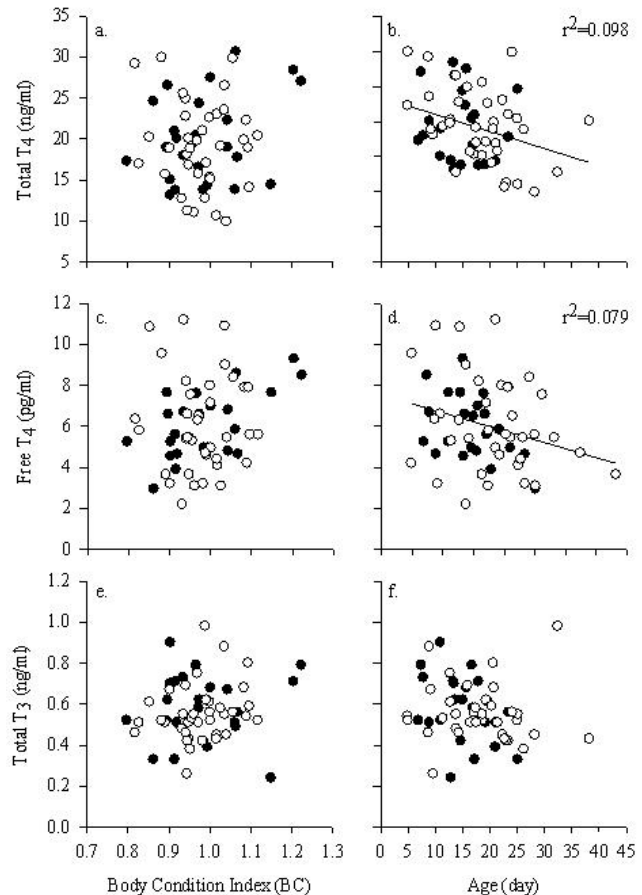


Figure 3. Circulating thyroid hormone concentrations from SSL pups. (A, B) total T<sub>4</sub> (ng/ml); (C, D) free T<sub>4</sub> (pg/ml); (E, F) total T<sub>3</sub> (ng/ml) presented against the body condition index (BCI) or age. Female pups are represented by filled circles, males are by hollow circles. Only significant ( $p \leq 0.05$ ) trend lines are presented.

### Study 3

Thyroid hormone concentrations were not affected by hot-iron branding ( $p \geq 0.197$ ). Cortisol concentrations ( $p=0.036$ ) significantly decreased following branding (Figure 4a). While concentrations of aldosterone appeared to decrease following

branding (Figure 4b) but was not significant ( $p=0.069$ ).

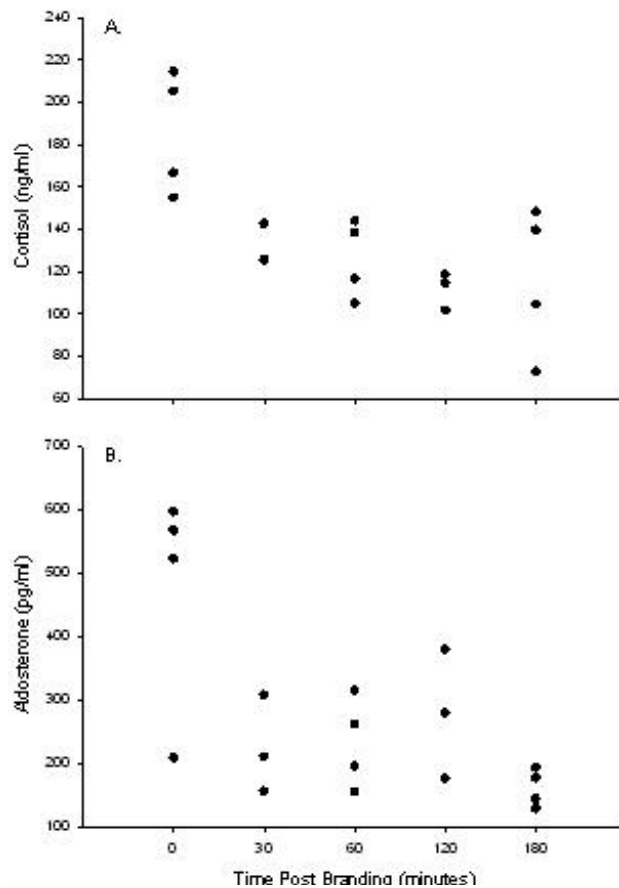


Figure 4. Concentration of (A) Cortisol (ng/ml) and (B) Aldosterone (pg/ml) before and after hot-iron branding in SSL pups.

## Discussion

The ability to acquire blood samples from free ranging SSL pups of known age is a unique opportunity, which enabled us to assess the influence of age and body mass or condition on circulating immune components, *in vitro* PBMC proliferation, and concentrations of a suite of hormones. SSL pups were in a period of rapid growth as evident by the increase in body mass, standard length and axillary girth with age. Males between 5 and 38 days of age were larger than females in body mass, standard length and axillary girth. Brandon *et al.* (2005) found that during the first six weeks following birth, male and female SSL pups grew at the same rate in body mass, standard length and axillary

girth and suggested that males were larger than females at birth by approximately 15%. Estimated mass at birth (female 18.7 kg, male 22.4 kg) did not vary between rookeries while growth rates in body mass ranged from 0.23 kg/day to 0.48 kg/day with higher rates found in the Aleutian Islands (Brandon *et al.*, 2005). Pups from the Chiswell Island rookery were not included in that study and Chiswell Island is geographically closer to rookeries with the lower growth rates. Yet, if we apply the growth rates calculated by Brandon *et al.* (2005) to SSL pups of known ages in the present study, then SSL pups from Chiswell Island rookery are estimated to be between 4.1 and 17.9% (females) and 3.0 and 17.2% (males) larger than predicted by the lowest and maximum estimated growth rates, respectively. These findings are likely an indication that pups sampled in the present studies are from a healthy population of SSL. Further, there were no abandoned or starving pups observed on Chiswell Island at the time of blood collection.

While we detected a significant difference in size between male and female pups, suggesting either a difference in sex-related physiology or maternal investment during gestation as Brandon *et al.* (2005) proposed, only cortisol and aldosterone showed differences between male and female pups. Aldosterone is integral to the regulation of water and sodium balance in mammals. Within phocids, aldosterone concentrations increased during extended periods of fasting in pups (Englehardt and Ferguson, 1980; Ortiz *et al.*, 2000). Unlike phocids, SSL pups have short periods of fasting associated with the maternal foraging trips, which tend to increase in length as the pup ages (Maniscalco *et al.*, 2006). The large variation in aldosterone concentrations observed in the present study was not associated with the rookery disturbance, pup age or BCI and the difference between male and female pups while significant, was small and further studies are needed to determine if

the small difference in aldosterone concentration observed between male and female SSL pups in the present study are biologically relevant. However, the significant interaction between sex and BCI suggests that the relationship between aldosterone concentration and BCI is different for female and male pups. Female pups had an inverse relationship between aldosterone concentrations and BCI while this relationship was not observed in male pups in the present study. Cortisol is well known as a stress hormone and plays a role in gluconeogenesis and promoting the mobilization of fatty acids from peripheral adipose tissues. Cortisol concentrations in the present study increased as a result of the initial disturbance of the rookery and continually decreased as the time elapsed between the initial disturbance and collection of blood. Cortisol concentrations at the time of initial capture in free ranging juvenile SSL (Mellish *et al.*, 2007) were comparable to pups in the present and previous studies (Myers *et al.*, 2010). Similar to the present study, reported cortisol concentrations in free ranging pinnipeds were higher in female pups than male pups in both SSL (Myers *et al.*, 2010) and southern elephant seals (Engelhard *et al.*, 2002). Higher glucocorticoid or metabolite concentrations have also been reported in adult female terrestrial mammals (Garnier *et al.*, 1990; Franceschini *et al.*, 2007) and females exposed to an acute stressor exhibited higher peak cortisol concentrations than males (Guimont and Wynne-Edwards, 2006). While the large sample size in the present study may have allowed for the detection of significant differences between male and female pups, the biological relevance of the small difference in cortisol and aldosterone concentrations between male and female pups is unclear but these sex differences were not related to age or the BCI.

Thyroid hormones including total and free T<sub>4</sub> and total T<sub>3</sub> are integral to maintaining thermoregulation, metabolism and the growth and development of neonatal and juvenile mammals. All forms of thyroid hormones quantified decreased when regressed against elapsed time since arrival on rookery, although the overall variation attributed to the elapsed time was low ( $r^2 < 0.293$ ). The decrease in circulating total and free T<sub>4</sub> and total T<sub>3</sub> could be the result of the initial rookery disturbance and associated increased activity of pups leading to elevated concentrations of thyroid hormones followed by a period of decreasing concentrations as circulating hormones return to basal concentrations. Alternatively, adrenocorticotrophic hormone and glucocorticoids have been shown to alter the secretion of thyroid-stimulating hormone from the pituitary (Wilber and Utiger, 1969) and the conversion of T<sub>4</sub> to T<sub>3</sub> in tissues (Heyma and Larkins, 1982) leading to lower concentrations of thyroid hormones compared to baseline. Further, the elapsed time is by necessity later in the day and given the role of thyroid hormones in regulation of metabolism and thermoregulation, the decrease in thyroid hormones may not solely be related to the rookery disturbance but rather the result of increasing temperatures as the day progressed. Given the experimental design of the present study we are unable to determine the direct cause of the observed decrease in thyroid hormone concentrations over the elapsed time between arrival on rookery and sampling.

Mean thyroid hormone concentrations (total and free T<sub>4</sub>, total T<sub>3</sub>) were similar to those previously reported for SSL pups (Myers *et al.*, 2006). Serum total T<sub>3</sub> concentrations were slightly lower in SSL pups but within the range reported in juvenile and sub-adult SSL (Rosen and Kumagai, 2008; Jeanniard Du Dot *et al.*, 2009). SSL pups had total T<sub>4</sub> concentrations more than two times greater than baseline concentration during summer in juvenile and sub-adult SSL (Rosen and Kumagai, 2008; Jeanniard Du Dot *et al.*, 2009) while free T<sub>4</sub> concentrations in SSL pups were half the circulating concentration found in juvenile and sub-adult SSL (Jeanniard Du Dot *et al.*, 2009). Both total and free T<sub>4</sub> concentrations decreased with pup age, while significant, the relationship was extremely weak ( $r^2 = 0.098$ ) likely due to the short developmental period (5-

38 days) covered in the present study. While male pups were larger than female pups at the time of sampling, no difference in circulating concentrations of any forms of thyroid hormones quantified was observed between male and female pups. These findings may be due to the previously observed growth rates in SSL pups. Brandon *et al.* (2005) determined male and female pups have the same postnatal growth rate (body mass, standard length, and axillary girth) and the difference in size between male and female pups was the result of differences in maternal investment during gestation.

Leptin is secreted by adipose tissue and circulating concentrations have been correlated with fat stores and respond to changes in energy balance in terrestrial mammals (Friedman and Halaas, 1998). We found higher leptin concentrations in pups physically restrained during blood collection compared to those under isoflurane. This finding was unexpected but fluctuating leptin concentrations not associated with changes in fat or body mass have been previously reported in pinnipeds (Arnould *et al.*, 2002; Mashburn and Atkinson, 2008). In addition, the leptin concentrations in our study were near the lower detection limit of the kit (0.5 ng/ml) and similar to previous studies in phocids with similar methods (Hammond *et al.*, 2005). Difficulty in quantifying leptin in SSL (Rosen and Kumagai, 2008) and other pinnipeds (Arnould *et al.*, 2002; Ortiz *et al.*, 2003) has been previously reported with low concentrations or no correlation to body mass or lipid stores being reported (Ortiz *et al.*, 2001; Arnould *et al.*, 2002)

Changes in body condition and mass and associated changes in circulating cortisol, thyroid hormones and leptin have been well documented in several species of terrestrial mammals (Concannon *et al.*, 2001; Caldeira *et al.*, 2007) and pinnipeds (Jeanniard Du Dot *et al.*, 2009). However, we found no relationship between BCI and any of the circulating concentrations of the hormones quantified in the present study. Further, body mass and a density index calculated as  $[\text{body mass} / (\text{standard length} \times \text{axillary girth}^2)] \times 10^6$  [10, 45] (Castellini *et al.*, 1993; Rea, 2002) were similarly not related to circulating concentrations of any hormones assessed in the present study (data not shown).

In addition to accessing the relationship between BCI and circulating concentrations of hormones, we were able to assess the changes in circulating immune components and PBMC proliferation for a subset of SSL pups during the 38 days following birth. In piglets (Hoskinson *et al.*, 1990) and dogs (Toman *et al.*, 2002) high spontaneous proliferation of PBMC were observed during the first week after birth and subsequently decreased within four to six weeks. The cause of the high spontaneous proliferation was unclear but may be due to PBMC being immature or in an activated state (Hoskinson *et al.*, 1990). In the present study, PBMC proliferated when exposed *in vitro* to ConA and LPS compared to unstimulated PBMC, suggesting that peripheral T and B cells are capable of responding to an antigenic challenge as early as 5 days following birth. Similar decreases in T cell proliferation during the first month of life have been observed in dogs followed by a gradual increase after 6 weeks (Gerber and Brown, 1974; Toman *et al.*, 2002). Beckmen (1999) reported a decrease in T cell proliferation exposed to ConA in NFS sampled as neonates (<7 days) and again at an estimated 29-51 days old. Decrease in T cell proliferation following birth observed in the current study and in northern fur seals (NFS) suggests that age at time of vaccination is another factor contributing to the low humoral immune response observed by Beckmen *et al.* (2003). These findings are consistent with observations in New Zealand sea lion following a *K. pneumonia* epizootic event (Castinel *et al.*, 2008). In our study, SSL pups had a decrease in T cell proliferation comparable to changes observed in NFS (Beckmen, 1999) suggesting that SSL pups would have a similarly low or

delayed humoral immune response as observed in other otariid pups (Beckmen *et al.*, 2003; Castinel *et al.*, 2008).

Cortisol levels decreased when regressed against elapsed time from our arrival on the rookery and the time pups were handled indicating that the pups were affected by the initial disturbance of the rookery. In the current study, only monocyte counts were associated with changes in serum cortisol levels suggesting sampling and handling of pups did not negatively affect immune components or *in vitro* PBMC proliferation. WBC counts of SSL pups decreased during the 38 days following birth as a result of decreasing neutrophil counts, while the remaining leukocyte populations were consistent. While neutrophil counts in harbor seals decreased similar to the SSL pups, no change was observed in total WBC counts due to a concurrent increase in circulating lymphocytes and monocytes (Ross *et al.*, 1994). Differential WBC counts in the current study were within ranges reported for healthy adult California sea lions (*Zalophus californianus*) (Roletto, 1993) and SSL pups (Bishop and Morado, 1995). Specifically, Bishop and Morado (1995) found similar percentage of neutrophil, lymphocyte, monocyte and eosinophils though the mean total WBC was lower than in our study. However, the difference in total WBC count is likely explained by methodological differences between studies, since the total WBC counts were based upon estimated WBC density in the blood smear slides in the Bishop and Morado (1995) study.

### **Preliminary conclusions/conclusions**

Circulating concentrations of cortisol, total and free T<sub>4</sub>, and total T<sub>3</sub> decreased when regressed against the elapsed time between researchers' arrival on the rookery and removal of pup from the holding corral for blood collection. While the overall variation attributed to the rookery disturbance was low it may be of significance for future studies on free ranging pinnipeds. While we did not find BCI to be related to circulating hormone concentrations future studies should include pups with a wider range of body conditions including abandoned or starving pups and ideally would employ a longitudinal sampling scheme to address some of the large individual variation in hormone concentrations observed in the present study.

The decreases in total WBC, neutrophil counts and T cell proliferation indicates that SSL undergo a period of postnatal development in cell-mediated immune function, which is comparatively longer than in phocid pups and consistent with other otariids. The postnatal changes in SSL pups may be associated with differences in disease susceptibility between pups and adults. Future studies are needed to assess when pups or juveniles reach immuno-competence and if the development of immune function is associated with disease susceptibility contributing to the reduction in juvenile survival or natality suggested in some SSL populations (York, 1994; Holmes and York, 2003).

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