FECAL TRIIODOTHYRONINE ASSAY VALIDATION USING CAPTIVE STELLER SEA LIONS (*EUMETOPIAS JUBATUS*) AND SUBSEQUENT APPLICATION TO FREE-RANGING POPULATIONS TO EXAMINE NUTRITIONAL STRESS

by

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Abstract

Reduced availability of high energy-content prey (nutritional stress) is a predominant hypothesis to explain the decline of Steller sea lion (*Eumetopias jubatus*) populations in western Alaska from the late 1970's to the late 1990's. Animals may respond to eating insufficient prey by increasing stress levels and decreasing metabolic rates. It may thus be possible to identify nutritional stress by measuring concentrations of GC metabolites (stress) and thyroid hormones (metabolism) shed in the feces of Steller sea lions. However, techniques to measure thyroid hormone concentrations from sea lion feces have not been developed.

We quantified variation of triiodothyronine (T3) and thyroxine (T4) concentrations in Steller sea lion feces following two injections of thyrotropin (TSH) at 24 h intervals into four captive animals. Glucocorticoid (GC) metabolites were also assayed to examine any relationship to stimulated thyroid hormone secretion. We found that fecal T3 peaked 48 h post-injection and increased 25-57% in three sea lions (all animals, $p=0.03$). Pre-injection GC increases indicated stress from isolation for baseline fecal collections, but post-injection increases could not be confirmed as a response to TSH injections or as a product of the study design. The results demonstrated that pre- and post-injection changes in fecal GC and T3 concentrations were consistent with predictions of an increased stress response and metabolic rate within the animals.

We then measured T3 and GC concentrations in 834 Steller sea lion fecal samples collected in 2005 and 2006 from 15 resting (haulout) and breeding (rookery) sites between British Columbia and the Central Aleutian Islands. Overall, GC concentrations did not differ between haulout populations (western 2006 pre-pupping and eastern 2005 post-pupping). Fecal hard-part analyses revealed a lower energy-content diet in the western population, suggesting that diet quality is a relevant hypothesis to explain slightly higher GC concentrations found in the western population, specifically the Aleutian Islands region. However, the nutritional stress hypothesis could not be substantiated through T3

concentrations. The rookeries possessed the highest energy-content diets, but also exhibited a nutritional stress response with a significantly higher GC and lower T3 concentration than either haulout population (possibly related to lactation or decreased leptin levels), but T3 comparisons performed at scales of site and region were inconclusive.

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Chapter 1: Introduction

1.1 The Steller sea lion population decline

Steller sea lions (*Eumetopias jubatus*) along the west coast of North America have experienced unexplained declines and increases in abundance since the 1970s. The western sea lion population, occupying the Gulf of Alaska and Aleutian Islands, experienced a dramatic decline from 282,000 animals in the late 1970's to 76,000 by 1992 (Trites and Larkin 1996, Fadely et al. 2006). In contrast, the eastern population occupying southeast Alaska was estimated to be 2,500 in 1957 and increased to 19,000 by 1992 (Trites and Larkin 1996, Calkins et al. 1999). More recent estimates indicated that 45,000 animals occupied the western population, as of 2004-2005, and 46,000-58,000 animals occupied the eastern population, as of 2002 (NMFS 2008).

A number of factors have been proposed as potential contributors to the decline in the western population of Steller sea lions, including ocean climate regime shifts (Benson and Trites 2002, Grebmeier et al. 2006, Trites et al. 2007b, Mueter and Litzow 2008), pollock trawling and other fisheries, killer whale and shark predation (Heise et al. 2003, Williams et al. 2004), and contaminants (Barron et al. 2003, Myers et al. 2008). A final hypothesis to explain the reduced number of pinnipeds is nutritional stress induced by a decrease in the quality or quantity of prey as a result of regime shifts or fisheries (Trites and Donnelly 2003). It is this hypothesis that my thesis addresses.

Of a total 5% annual decline in the western Steller sea lion population through the 1990s, direct anthropogenic conflict and killer whale predation could account for a 2.3% annual decline with the remaining 2.7% to be attributed to regime shifts and competition with fisheries (Loughlin and York 2000). Environmental cycles, intrinsic population cycles, and historical pup harvests have been examined, but models found that only a long-term or catastrophic change in the environment could be responsible for the magnitude of the Steller sea lion decline (Pascual and Adkison 1994). However, this study was unable to discriminate between long-term climatic causes and anthropogenic causes, such

as competition with fisheries. Competition with fisheries is a possibility given that a greater than 51% overlap was found in the sizes of walleye pollock and Atka mackerel taken by the western population of Steller sea lions and the commercial trawl fishery (Zeppelin et al. 2004). Furthermore, the percent overlap between walleye pollock taken by nocturnally feeding northern fur seals and the commercial trawl fishery at St. George and St. Paul of the Pribilof Islands was respectively 15.5% and 4.1% in feces, and 94.6% and 89.6% in spews (Gudmundson et al. 2006). However, the abundance of pollock appears to be great enough that the removal of all Alaskan trawl fishing effort was estimated to affect the rate of population change by only 0.56% (Dillingham et al. 2006). Thus, neither commercial fishing effort nor prey abundance appear to explain the decline.

Regardless of whether oceanic or anthropogenic changes occurred, the result has been an alteration to the quality or quantity of prey available to marine mammals and pelagic seabirds. Ocean climate regime shifts have occurred within the Pacific Ocean multiple times in the last century, causing increases or collapses of fish populations (Anderson and Piatt 1999). For instance, a shift in walleye pollock abundance occurred in 1977 in the western Gulf of Alaska (Alton et al. 1987) and nearly concurrently, the last shift to a warm regime began approximately 1976-1977 (Hare and Mantua 2000).

1.2 Glucocorticoids

One of the greatest challenges in identifying an inadequate diet as a cause of the Steller sea lion decline is documenting the degree to which changes in diet affect morbidity and mortality. The application of physiological techniques have allowed researchers to link the body condition of individuals' to environmental factors, with glucocorticoid hormones often serving as an indicator of stress within the individual. Glucocorticoids are an end product of the stress response mediated through the hypothalamic-pituitary-adrenal (HPA) axis. A real or perceived stressor triggers the hypothalamus to release corticotropin-releasing hormone (CRH), which signals the anterior lobe of the pituitary gland to

release adrenocorticotropic hormone (ACTH). ACTH in turn signals the adrenal cortex of the pancreas to produce and release glucocorticoids (GC). The two primary GCs are corticosterone and cortisol, which is the dominant form in Steller sea lions. Changes to the morphology of the HPA axis have also been documented as a result of chronic stress. The adrenal gland mass and the adrenal cortex to medulla ratio increased in chronically stressed bottlenose dolphins (*Tursiops truncatus*) compared to those acutely stressed (Clark et al. 2006).

Glucocorticoids increase feeding behavior and act to mobilize energy through gluconeogenesis, the conversion of protein and lipid into glucose for immediate use. The stress response is therefore beneficial under acute circumstances, such as when fleeing predators. However, the stress response also comes at the cost of digestion, growth and tissue repair, and can impair the immune system, and reproductive physiology and behavior (Sapolsky 2002), which notably are the same effects that occur from decreases in the hormone leptin (Flier et al. 2000). Decreases in leptin can occur from the metabolization of adipose tissue in lieu of a sufficient diet and can also increase the stress response. Despite this, GC metabolites are a better measure of the general stress response. For instance, fecal GC metabolite concentrations correlated positively with social factors such as group size and dominance rank in African elephants (Foley et al. 2001, Gobush et al. 2008). Peak fecal GC metabolites were also found to reflect responses to acute stressors in the rehabilitation of a Steller sea lion pup (Petrauskas et al. 2006).

Despite the acute benefits, chronically increased GC concentrations could affect the rate of survival for a species. For example, the process of gluconeogenesis requires 30% of the energy available in the endogenous tissues being metabolized (Sapolsky 2002). Increased GC concentrations decrease allocation towards growth rate in younger animals, and towards embryonic growth and lactation in older animals (Sapolsky 2002). Glucocorticoids also inhibit the release and conversion of hormones necessary for reproduction in both sexes, primarily through decreases in sensitivity to luteinizing hormone (LH),

which in turn decreases the secretion of testosterone, estrogen, and progesterone (Sapolsky 2002).

1.3 Thyroid hormones

Thyroid processes are essential to the control of metabolism and mediated through the hypothalamic-pituitary-thyroid (HPT) axis. The hypothalamus releases thyrotropin-releasing hormone (TRH), which signals the pituitary gland to release thyroid-stimulating hormone (TSH). TSH in turn signals the thyroid to produce, store, and release the hormones thyroxine and triiodothyronine (Flier et al. 2000). The hormones thyroxine (T4) and triiodothyronine (T3), act as moderators of metabolism. T4 has a moderate influence on metabolism and is the more abundant of the two hormones, but may be converted via deiodination to highly active T3 or inactive reverse triiodothyronine (rT3).

T3 concentrations affect the rate of glucose oxidation and, as a result, determine the conservation of endogenous energy reserves. Decreases in T3 concentrations are associated with acute and chronic reductions in energy intake in endothermic mammals and birds, as well as ectothermic teleost fish (Eales 1988). As with the relationship between energy consumption, adipose metabolization, and an increased stress response; decreases in leptin concentrations also play a role in decreasing metabolism (Harris 2000). The lowest T3 concentrations were associated with the highest T4 utilization rates in the serum of fasted pigs and the highest T3 concentrations with the lowest T4 utilization (Ingram and Evans 1980). T4 and T3 concentrations and metabolic clearance did not change in the serum of physically deconditioned humans (Balsam and Leppo 1975a). Furthermore, plasma T3 concentrations and fecal T3 metabolic clearance did not change in physically trained humans, although urinary clearance increased by 8.5% (Balsam and Leppo 1975b).

T3 concentrations are commonly increased in non-hibernating animals of colder environments to assist in thermoregulation. T3 utilization rates increase with cold exposure and decrease with fasting in a variety of mammals (Tomasi

1991). In Steller sea lions, increased T3 concentrations could assist in thermoregulation of the western population, whereas decreased T3 concentrations could assist rookeries while fasting or areas of decreased prey quality. Similar to temperature, photoperiod was also found to have an inverse relationship with T3 concentrations in the serum of cotton rats (Tomasi and Mitchell 1996).

1.4 Nutritional stress

The detrimental effects of chronically increased GC concentrations and the rate of gluconeogenesis could be energetically mitigated through concurrently decreased T3 concentrations and the rate of glucose oxidation. The combined measurement of the hormones could theoretically identify populations experiencing chronic nutritional stress. Energy utilization rates could thus be optimized under conditions of chronic nutritional stress, but still at the cost of reproduction and other effects of increased GC concentrations. For example, cortisol concentrations increased and T3 concentrations decreased in human plasma following a 72 hour fast (Beer et al. 1989). Sources of stress unrelated to diet, such as killer whale predation, assume no need for long-term metabolic adjustment. However, other factors such as contaminants and disease cannot be dismissed as contributors to changes in hormone concentrations.

Diet is a factor commonly investigated in stress research and is often examined relative to glucocorticoid concentrations. Plasma corticosterone concentrations decreased following foraging trips of wandering albatross (*Diomedea exulans*) and correlated negatively with foraging success as defined by total mass gain (Angelier et al. 2007a). The effect of adult foraging success is not lost on chicks. Nutritional restriction of red-legged kittiwake chicks (*Rissa brevirostris*) not only increased corticosterone concentrations, but restriction using low-lipid prey resulted in even greater concentrations and impaired cognitive abilities associated with foraging (Kitaysky et al. 2006b). Furthermore, the mass of the low-lipid diet consumed by chicks equaled or exceeded the maximum capacity of adult provisioning and, as a result, the impacts of a low-

lipid diet could not be offset through further provisioning in the wild (Kitaysky et al. 2006b).

Male Steller sea lions may wean later on average than females, prolonging the consumption of a high-calorie intake without foraging investment (Marcotte 2006). Weaning at a later age may provide a mass advantage in holding territories as adults, but could also be hypothesized as necessary for males to increase gastric capacity in order to subsist on lower energy-content prey. The depths and durations of dives were found to be similar between juvenile and subadult Steller sea lions, and were consistent with seasonal and diurnal changes in prey (Rehberg and Burns 2008). However, pup dives were shallower, shorter, and did not reflect prey activity, suggesting that pups were dependent on nursing during the winter (Rehberg and Burns 2008).

Steller sea lions provided a squid diet of similar mass to a control maintenance herring diet exhibited a 20% mass-corrected decrease in resting metabolic rate (RMR) independent of satiation (Rosen and Trites 1999). T3 concentrations were also positively correlated with food intake in reindeer (*Rangifer tarandus tarandus*) serum and greater T3 decreases occurred during summer than fall (Ryg 1984). Additionally, metabolic depression was observed in pregnant humans and to greater degrees in thinner pregnant females (Prentice et al. 1989). Collectively, these studies suggest metabolism may be associated with nutritional status, season, and breeding status.

Decreased serum T3 concentrations as a result of fasting caused a reduction in the rate of endogenous protein metabolization in humans (Gardner et al. 1979). Phase II fasting metabolizes endogenous lipids in order to conserve proteins, but chronically may enter Phase III fasting or terminal starvation, marked by depleted endogenous lipids and a shift to protein metabolization as the primary energy source. For Steller sea lions, a reduction in endogenous protein metabolization implies dependence on endogenous lipid metabolization, leading to decreases in buoyancy and the ability to thermoregulate.

1.5 Fecal sampling

Glucocorticoids are steroid hormones that travel via blood as free GCs readily available for target cells, but also as bound GCs attached to corticosteroid-binding globulins and unavailable for biological activity. Determining the general status of an animal from blood requires taking multiple samples, because physiological indicators in blood change almost instantaneously in response to stimuli and are influenced by circadian rhythms. The time associated with fecal GC metabolite excretion diminishes these complications resulting from blood-sampling techniques. Radioimmunoassay has proven a successful method of fecal GC metabolite measurement in various species (Wasser et al. 2000) and has been validated specifically for use with Steller sea lions (Hunt et al. 2004, Mashburn and Atkinson 2004).

The measurement of fecal GC metabolites may be of use in assessing the health of wild populations, but factors such as the life history of the species and the handling of samples must be considered in study design (Millspaugh and Washburn 2004, Keay et al. 2006). Once free GCs are metabolized, mainly in the liver but also in the intestine, GC metabolites are excreted via feces and urine, with the proportion of the two routes differing between sexes and species (Palme et al. 2005). In the case of Steller sea lions, population composition (male, female, or mixed population) is recorded prior to feces collection to serve as a basis for the interpretation of hormone concentrations.

1.6 Thesis organization

This thesis consists of two major chapters, each written as a manuscript intended for publication in a scientific journal. The first (Chapter 2) is a validation of the radioimmunoassay of T3 from the feces of Steller sea lions through the provision of TSH to captive animals. The second (Chapter 3) is an application of the radioimmunoassay of T3 and GC metabolites to the feces of free-ranging Steller sea lion populations, which when combined with diet and population trends may provide insights into the nutritional stress hypothesis.

Chapter 2 presents the first validation of a fecal T3 radioimmunoassay for any species. As a result, little literature is available to determine methodologies specific for our purposes of detecting physiological changes in Steller sea lions via feces. We therefore collected feces before and after applying a method to stimulate the thyroid that was similar to that used to test thyroid activity via blood in humans. However, we were cautious about over-stimulating the thyroid of the sea lions, due to concerns about the safety of the animals. Thus, we anticipate that our attempts at validation may not elicit as strong a response as might occur in free-ranging populations, although remain confident that significant increases in thyroid hormone concentrations will occur in the feces of the animals.

Chapter 3 applies the fecal T3 radioimmunoassay technique to free-ranging populations to investigate whether correlations could be detected among physiology, diet, and population status at scales of site, region, and population, with attention to breeding and resting status within the decreasing western and increasing eastern populations. We predict that resting sites in the western population will have a lower quality diet, a higher GC metabolite concentration, and a lower T3 concentration relative to resting sites in the eastern population. Adult females dominate breeding sites (as a result of the polygamous breeding strategy used by Steller sea lions) and likely consume a diet with a higher energy content than males due to sex-specific differences in foraging patterns (Trites and Calkins 2008). Despite a higher quality diet, the influences of lactation and limited foraging opportunities may bring into question predictions of the relationship between diet quality and physiology.

Collectively, this thesis demonstrates the value of non-invasive techniques, and provides a new approach for monitoring free-ranging pinniped populations.

Chapter 2: Fecal triiodothyronine and thyroxine assay validation for the Steller sea lion with concurrent glucocorticoid responses

2.1 Introduction

Chronic nutritional stress — induced by a decrease in the quality and availability of prey — is a leading hypothesis to explain the decline of Steller sea lion (*Eumetopias jubatus*) populations (Trites and Donnelly 2003). A shift to nutritionally poor diets reduces breeding success in seabird colonies, which often feed on similar prey bases as marine mammals (Wanless et al. 2005, Osterblom et al. 2008). A decrease in prey quality may reduce the ability of female Steller sea lions to bring a pregnancy to term and nurse a pup (Pitcher et al. 1998). A shortage of high quality prey could also affect juvenile survival due to a lower foraging efficiency and a restricted winter diet (Merrick et al. 1997).

Free-ranging populations may produce elevated levels of glucocorticoids in response to negative changes in diet, as could other sources of stress. Thyroid hormones represent an alternate physiological parameter that may be particularly useful for identifying inadequate nutrition as a specific stressor. Alterations of thyroid hormone concentrations are a common adaptive response to decreased energy intake (van der Heyden et al. 1986, Hennemann et al. 1988, Blake et al. 1991, Douyon and Schteingart 2002). A decrease in free-circulating thyroid hormones reduces the rate of energy expenditure and hence limits energy deficits, partly through decreases in metabolic and growth rates. Together, an increase in glucocorticoid concentrations accompanied by a concurrent reduction in thyroid hormones may indicate that an individual is experiencing nutritional stress.

To date, the predicted response of circulating GC and T3 to diet quality has been successfully observed in mice using food deprivation with other stressors (Cremaschi et al. 2000, Silberman et al. 2002), but results for Steller sea lions have been mixed (Jeanniard du Dot 2007, Rosen and Kumagai 2008), possibly due to the use of invasive serum sampling techniques which can increase GC concentrations independently of diet or other factors. Noninvasive

measures of fecal steroid hormones have proven increasingly valuable for monitoring stress and reproductive function in free-ranging animals (Wasser 1996, Wasser et al. 1997, Foley et al. 2001, Mostl and Palme 2002). Fecal thyroid quantification may further provide biologically meaningful measures of thyroid function.

Artificial, predictable changes in circulating hormone concentrations need to be induced in an animal to test the efficacy of using fecal thyroid hormone levels to monitor circulating levels. For diagnostic purposes, thyroid hormone production can be increased and measured in serum by injecting an animal with thyroid-stimulating hormone (TSH), Injecting northern elephant seals (*Mirounga angustirostris*) for example with TSH significantly increased serum T4 concentrations in healthy elephant seals, as well as those that had northern elephant seal skin disease (Yochem et al. 2008). However, the measurement of thyroid hormone concentrations in feces has never been validated for any species.

Increased TSH up-regulates the hypothalamic pituitary thyroid axis, resulting in a peak in circulating concentrations of thyroxine (T4) and triiodothyronine (T3). While T4 is the primary thyroid-produced metabolic hormone, it is relatively inactive until deiodinated to T3 (Tomasi 1991). If significant increases in fecal T4 or T3 concentrations occur after TSH injections (accounting for secretion/excretion lag-times), then it reasons that fecal thyroid hormone measures also reflect thyroid function. Similar validations have been previously completed for the radioimmunoassay of fecal glucocorticoid (GC) metabolites of Steller sea lions (Hunt et al. 2004, Mashburn and Atkinson 2004). This study marks the first fecal validation of thyroid-produced hormones in the Steller sea lion.

TSH serum equilibrium and peak thyroid activity occur approximately 1.5 h (canine) to 2 h (human) after administrating a TSH bolus followed by an infusion (Ridgway et al. 1974a, Ridgway et al. 1974b). In northern elephant seals, serum T3 peaked at 1.5 h and T4 at 3 h following a direct 5 IU TSH injection (Yochem et al. 2008). Human subjects retain approximately 10% of radiolabeled T3

injections in the serum 24 h post injection (Chopra 1976), while T4 retention is approximately 50% at nine days post injection. An approximate 24 h minimum T3 clearance rate has also been documented in beluga whales (*Delphinapterus leucas*) injected with TSH multiple times (St. Aubin and Geraci 1992). TSH stimulation was also found to increase rates of cholesterol synthesis in the thyroid cells of rats and return to basal rates after 48 hours (Grieco et al. 1990). Preliminary tests using high-pressure liquid chromatography (HPLC) of feces from canids fed ^{131}I demonstrated that T3 is the predominant metabolic hormone excreted in canine feces (Wasser Unpublished-b).

Our objective was to determine whether increased thyroid hormone production could be measured under controlled conditions (by injecting animals with TSH to alter their physiology). We also sought to examine the relationship between thyroid stimulation and adrenal activation in Steller sea lions. A peak in fecal T3 concentrations following the injections would show that thyroid hormone concentrations can be measured in feces in combination with glucocorticoid metabolites as possible indices of chronic nutrition-specific stress in free-ranging Steller sea lion populations.

2.2 Methods

2.2.1 Subjects, facilities, and dosage

Four female Steller sea lions housed at the Vancouver Aquarium (Vancouver, B.C., Canada) participated in the study – SSL1 (134 kg) and SSL2 (151 kg), aged four years, and SSL3 (191 kg) and SSL4 (192 kg), aged seven years. All animals were captured as pups and raised within the facility using positive reinforcement methods to provide daily contact with staff and equipment.

The thyroid gland is unique, because it secretes and stores thyroid hormones (Dierauf and Gulland 2001). To stimulate the thyroid, we injected the Steller sea lions with two 10 IU TSH injections, 24 h apart, to assure that increases in thyroid hormones would enter circulation rather than simply increase thyroid hormone stores in the gland. A review of published protocols for TSH injections for canines suggested a maximum injection of 10 IU (Feldman and

Nelson 1996). Other sources recommended 0.1 ug TSH / Kg to a maximum injection of 5 IU TSH for canines, and a maximum injection of 10 IU TSH for horses (Plumb 2005). The volume of TSH required relative to the mass of each captive Steller sea lion would have exceeded 10 IU, thus we used a 10 IU TSH dosage as a standard injection for each animal on the recommendation of the Staff Veterinarian of the Vancouver Aquarium (Vancouver, BC) (Haulena 2007).

The two TSH injections were expected to promote sustained circulating hormone concentrations over a 48 h period and allow for prolonged metabolite accumulation in feces. The lag time from stimulating GC metabolite secretion through adrenocorticotropic hormone (ACTH) injections to the subsequent peak excretion in the feces of Steller sea lions was 5 and 28 h for females (Hunt et al. 2004) and 32 h for both sexes combined (Mashburn and Atkinson 2004). We predicted a similar lag time in the excretion of T3 and T4, reflected by peak concentrations in feces approximately 24 h after the second TSH injection. We were also interested in whether the TSH injections would influence glucocorticoid metabolite concentrations since the metabolic effects associated with the TSH injections might in turn affect leptin, which also appears to influence the expression of thyrotropin-releasing hormone and the activation of the stress axis (Ahima et al. 1996).

All injections were given intramuscularly, under isoflurane gas anesthesia, in 2 cc saline solution using TSH sourced from bovine pituitary (Sigma-Aldrich, Oakville, Ontario, Canada). Each animal served as its own control by being housed in separate holding pens for approximately two weeks prior to TSH injections to establish baseline (pre-injection) fecal hormone concentrations. We injected SSL1 and SSL3 with TSH on two consecutive mornings starting 26 June 2007, while SSL2 and SSL4 were injected on two consecutive mornings starting 16 July 2007.

The second injection was designated hour zero for the interpretation of hormone concentrations between baseline and post-injection samples, because no notable peak occurred within 24 h of the first TSH injection. Steller sea lion initial defecation time has ranged from 2-56 h for hard remains (Tollit et al. 2003).

The hard remains and ACTH infusion defecation times suggest a lag period greater than 24 h may exist in Steller sea lions, although mean peak fecal glucocorticoid concentrations have been determined to occur 24 h after radiolabeled glucocorticoid injection in canines (Schatz and Palme 2001). Passage rates predicted that fecal T3 concentrations would decrease to baseline approximately 3 days after the second injection. Fecal collections continued for two weeks post-injection to ensure all affected hormone metabolites passed through the digestive tract. Fecal checks were performed five times daily (08:00 and 16:00 PST, and at each of three feeding sessions), recording date and time, location (pool or dry haulout), and animal. All samples were immediately frozen at -20^{o}C for subsequent analysis.

Outside of the TSH stimulus, care was taken to keep environmental conditions consistent by controlling activity and diet. The Steller sea lions were fed a standard maintenance diet (Pacific herring – *Clupea pallasi*) with daily vitamin supplements at intake quantities determined by animal-trainer interactions. No notable changes in environment or animal behavior were observed during the study. All procedures were in accordance with animal care guidelines established by or binding to the University of British Columbia and the Vancouver Aquarium.

2.2.2 Sample preparation and assay

The frozen fecal samples were thawed overnight for hormone extraction and manually homogenized the following morning by massaging the storage bags. A 2-5 g subsample was taken by passing a portion of homogenized feces through 0.5 mm mesh to remove hard remains. Subsamples were returned to -20^{o}C and lyophilized to halt biological activity and remove variation among subsample mass due to water content (Wasser et al. 1993). Hormone extractions were completed using an ethanol vortex method for radioimmunoassay, modified from Wasser et al. (Wasser et al. 2000, Wasser Unpublished-a).

We performed the glucocorticoid (GC) metabolite assay using a double-antibody ^{125}I radioimmunoassay kit (MP Biomedicals, LLC, Irvine, CA; catalog # 07-120103). Manufacturer's protocols were followed except for the halving of kit volumes. The immunoreactivity of the GC radioimmunoassay kit was 100% for corticosterone. Specificity of the antiserum to cross-reactions with common steroids was less than 1%. Those equal to or greater than 0.01% included; 5ALPHA-Dihydrotestosterone 0.01%, Androstenedione 0.01%, Progesterone 0.02%, Aldosterone 0.03%, Cortisol 0.05%, Testosterone 0.10%, and Desoxycorticosterone 0.34%.

The T3 assay was performed using a coated tube ^{125}I radioimmunoassay kit (Diagnostic Systems Laboratories, Inc., Webster, TX, catalog # DSL-3100), with modifications to the manufacturer's protocol. Modifications included the use of a phospho-saline-BSA buffer and standards made from crystalline T3 to replace the serum-based standards and diluent. The specific cross-reactivity of the T3 antibody to other compounds was: Triiodo-L-thyronine (reverse) 0.004%, L-thyroxine 0.003%, 3,5-diiodo-L-thyronine 0.002%, 3-monoido-L-tyrosine 0.001%, 3,5-diiodo-L-tyrosine 0.001%, and Triiodothyroacetic acid 2.76%. The T4 assay was performed using a coated tube ^{125}I radioimmunoassay kit (Diagnostic Products Corporation/Siemens, Los Angeles, CA, catalog # TKT45) with protocol modifications identical to T3. The specific cross-reactivity of the T4 antibody to other compounds was: Triiodo-L-thyronine 2%, and Triiodothyroacetic acid 2%.

A pool from the extractions of five samples were serially diluted and examined for parallelism of triiodothyronine, thyroxine, and glucocorticoid metabolites. Serial dilutions paralleled respective standard curves and fifty percent binding occurred at a 1:30 dilution in each assay. T3, T4, and GC metabolite assays were also tested for accuracy, respectively producing slopes of 0.95, 1.1, and 0.94, illustrating that fecal extracts were not interfering with measurement precision (Wasser Unpublished-a). All hormones were measured as nanograms hormone per gram dry feces (ng g^{-1}) and samples that resulted in a percent bound outside 15-85% on the standard curve or had a coefficient of

variation between duplicate pairs greater than 10% were re-assayed at the appropriate concentrations.

2.2.3 Statistical analyses

Accuracies of the GC, T3, and T4 assays were tested at The Center for Conservation Biology at the University of Washington where the noninvasive techniques were developed (Wasser Unpublished-a, b). All hormone concentrations were log-transformed for all statistical analyses. We compared 95% confidence intervals to determine whether there was a significant difference in hormone concentrations between the age groups for the baseline concentration of each hormone — and used paired *t*-tests (α=0.05) to compare the T3 baseline and time 0 concentrations of all animals to those of the peak samples that followed the TSH injections.

2.3 Results

2.3.1 Response to TSH injections

Hormone concentrations over the course of the study ranged from 450-898 ng g^{-1} T3, 1489-4276 ng g^{-1} T4, and 65-164 ng g^{-1} GC metabolites (Table 2.1). The 95% confidence intervals (data not shown) of pre-injection fecal hormone concentrations suggest there were no differences between trial groups and no seasonal effect. Baseline concentrations significantly differed between age classes for T3, but not for T4 or GC metabolites (Table 2.1). Mean (SE; 95%CI) baseline T3 exhibited by the older females SSL3 and SSL4 of 514 ng g^{-1} (14; [486,542]) was lower than the younger age group of SSL1 and SSL2 of 652 ng g^{-1} (43; [567,737]), which was influenced by the high baseline T3 concentration of SSL1.

All four Steller sea lions experienced a fecal T3 spike following TSH injections (Figure 2.1). Three animals (SSLs 1-3) displayed a fecal T3 peak 48 h after the first injection with each peak occurring in the first scat collected after the second injection. SSL4 did not peak until 71 h following the first injection (898 ng g^{-1}) and the sample contained the highest T3 concentration in the study (Figure

2.1). The physiological response to the TSH injections varied among animals (Table 2.1).

T3 concentrations differed significantly between the time 0 samples and the peak T3 concentrations ($p=0.03$). Three of the four animals (SSLs 2-4) showed increases of 25-57% above the time 0 sample. Alternatively, no difference was found between the mean baseline T3 concentrations and the peak T3 concentrations ($p=0.12$). Three of the four animals (SSLs 2-4) showed increase hormone concentrations of 25-75% above baseline. The remaining animal (SSL1) showed an 18% increase above the time 0 sample and only a 1% increase above mean baseline. However, the baseline for SSL1 was 37% higher than the mean of SSLs 2-4.

A return to fecal T3 concentrations similar to baseline was detected for SSL1 at 95 h after the first TSH injection, SSL2 at 101 h, SSL3 at 76 h, and SSL4 at 119 h, resulting in a mean (SE) of 97.75 h (8.86). Mean fecal T3 peak and return occurred at 54 h and 98 h, respectively. Post-peak T3 concentrations in SSLs 1-3 declined below baseline and then began to increase. Comparable data were unavailable for SSL4 owing to the delayed response to the TSH injections. T4 production, while showing no pre-injection differences between ages or trials, did show a post-injection peak that appeared to parallel T3 production in SSL2 and SSL4 in scale and time.

2.3.2 Glucocorticoid metabolite response

Three of the four animals (SSLs 1-3) displayed a pre-injection peak in glucocorticoid metabolites. The peaks were detected in the second sample collected post isolation, which represented the first complete digestive cycle while separated. A fecal sample was collected from two animals (SSLs 1 and 2) during the period between the first and second TSH injection, but both samples occurred less than three hours after the first injection and had to be considered baseline samples. All animals displayed a GC metabolite increase following the second TSH injection. SSLs 1 and 3-4 peaked in the first sample collected after the second injection (Figures 2.2 and 2.3).

2.4 Discussion

Fecal T3 concentrations revealed a biologically significant response to the dual injections of TSH in the Steller sea lion, as demonstrated by spikes in fecal T3 concentrations 48-71 h after the first TSH injection. Although T4 paralleled T3 post-injection in two animals of different age classes, T4 did not appear to provide biologically meaningful results overall. A correlation was found between log10 TSH and T3 concentrations, both decreasing in the plasma of fasted humans, but not T4 (Beer et al. 1989). A large portion of serum T3 is derived by the monodeiodination of T4 (Surks et al. 1973), which may have been completely monodeiodized prior to entering the digestive tract of the two animals that did not display a T4 increase. This parallels results found by Wasser (Wasser Unpublished-b) following ^{131}I ingestion studies in domestic dogs. Despite the lack of definitive T4 peaks across subjects in response to the TSH injections, fecal T4 concentrations were higher than T3 concentrations across all samples, as is common across most mammals.

T3 in SSL4 peaked 71 h after the first injection (23 h later than SSLs 1-3), but also returned the highest T3 concentration of the study. Individual digestive passage rates, gut flora, and diet can also affect both serum and fecal hormone concentrations (Goldin et al. 1982, Wasser et al. 1993, Lewis et al. 1997), which may have influenced the results of SSL4. The peaks of all animals, whether triggered solely by the first injection or a product of both injections, coincided with the expected period of elevated serum concentrations and passage rates as all spikes rose and fell over approximately three days.

The pre-injection peaks in fecal glucocorticoid metabolite concentrations in three of the animals were likely the result of stress caused by separation and acclimatization. Cortisol concentrations decreased in the serum of captive harbour porpoises (*Phocoena phocoena*) habituated to invasive sampling procedures, but effects of the procedures could not be removed (Desportes et al. 2007). Initial peaks occurred in the second sample from each sea lion, which also represented the first complete digestive cycle since being moved to

separate holding pens (Figure 2.2). Overall, stress responses were similar across animals immediately following the TSH injections.

The continued increase of GC metabolite concentrations following the peak T3 concentration of all four animals (Figure 2.3) may be the result of leptin influencing the thyroid and stress axes. The increases in metabolism associated with increased T3 concentrations could have resulted in decreased leptin concentrations, which suppresses the thyroid axis and activates the stress axis. The immediate decrease of T3 concentrations occurring after the peak T3 response and continued decrease to concentrations below the time 0 samples of all four animals is consistent with this hypothesis (Figures 2.2 and 2.3). However, we cannot exclude the possibility that the increased GC metabolite concentrations are simply a response to stress associated with the study design. Together, the changes in fecal concentrations of T3 and GC metabolites reflected a non-invasive physiological response representative of events occurring a day or two prior to excretion and collection.

These results support the use of this non-invasive fecal technique as a biologically valid measurement to examine thyroid activity and physiological stress among free-ranging populations. Physiological differences between individuals comprising a population may indeed be capable of capturing differences between populations when used with an appropriate experimental design. Serum-measures of thyroid hormone have been shown to differ in Steller sea lions based on region, season, and age groups of pups and juveniles from those 4 years and older (Myers et al. 2006). While variable, our higher baseline fecal T3 concentrations in the younger age group also suggest that Steller sea lions may experience a decrease in basal metabolic activity as animals age and mature. This suggests the importance of sampling representative individuals from representative sites when collecting feces.

The ability of this technique to non-invasively detect changes in fecal hormone concentrations within individual animals and between groups has the potential to determine the physiological status of wild populations and isolate sources of stress. The results establish the ability of fecal hormone analysis to

non-invasively capture physiological alterations to Steller sea lion thyroid hormone production and metabolization, although we did not examine relationships between serum and fecal concentrations. Our results indicate that fecal thyroid hormone measures can be combined with other fecal measures of stress and diet quality to examine nutrition-specific stress in free-ranging Steller sea lions.

2.5 Summary

Thyroid hormones shed in feces may help to identify nutritional stress, but techniques to measure concentrations need to be developed and validated. We therefore determined whether a known physiological alteration to thyroid hormone production could be measured in the feces of Steller sea lions. We quantified variation of triiodothyronine (T3) and thyroxine (T4) concentrations in feces following two intramuscular injections of thyrotropin (TSH) at 24 h intervals into four captive females of two age classes. Glucocorticoid (GC) metabolites were also assayed to examine the relationship to stimulated thyroid hormone secretion.

The mean (SE; 95%CI) baseline T3 concentration of older animals at 514 ng g^{-1} (14; [486,542]) was significantly lower than that of the younger age class at 652 ng g^{-1} (43; [567,737]). Fecal T3 concentrations in three of the four animals increased 25-57% over time 0 samples (all animals, p=0.03) and 25-75% over mean baseline concentrations (all animals, p=0.12). The peak T3 response occurred 48 h post-injection in three animals and 71 h in the fourth. Post-injection changes in fecal T4 concentrations paralleled T3 in two animals.

Fecal GC metabolite concentrations increased twice in all animals. The pre-injection increases indicated stress in the animals due to being isolated for baseline fecal collections. The post-injection increases could not be confidently designated as a response to TSH injections or as a product of the study design. These results demonstrate that pre- and post-injection changes in fecal GC and T3 concentrations were consistent with predictions of an increased stress

response and metabolic rate within captive Steller sea lions, although the utility of fecal T4 measures remains unclear.

Table 2.1: Individual and mean fecal hormone concentrations (ng g^{-1}) showing baseline values and increases following TSH injections, percent increases in T3, and time of T3 events in four Steller sea lions.

	Steller sea lion					
Hormone measurement	SSL1	SSL2	SSL3	SSL4	Mean	SEM
GC metabolite baseline (ng g^{-1})	103	106	98	76	95.75	6.79
GC peak pre-injections (ng g^{-1})	164	128	115	79	121.50	17.55
GC peak post-injections (ng g^{-1})	146	146	121	156	142.25	7.47
T4 baseline (ng g^{-1})	2636	1529	1897	1987	2012.25	230.31
T4 peak (ng g^{-1})	2414	2623	2637	4276	2987.50	432.52
T3 baseline (ng g^{-1})	729	575	514	514	583.00	50.75
T3 time 0 sample (ng g^{-1})	626	524	509	572	557.75	26.42
T3 peak (ng g^{-1})	739	733	636	898	751.50	54.24
T3 increase from mean baseline (ng g^{-1})	10	158	122	384	168.50	78.44
T3 increase from mean baseline (%)	1	27	24	75	31.75	15.54
T3 increase from time 0 sample (%)	18	40	25	57	35.00	8.65
T3 peak (h)	48	48	48	71	53.75	5.75
T3 return to baseline (h)	95	101	76	119	97.75	8.86

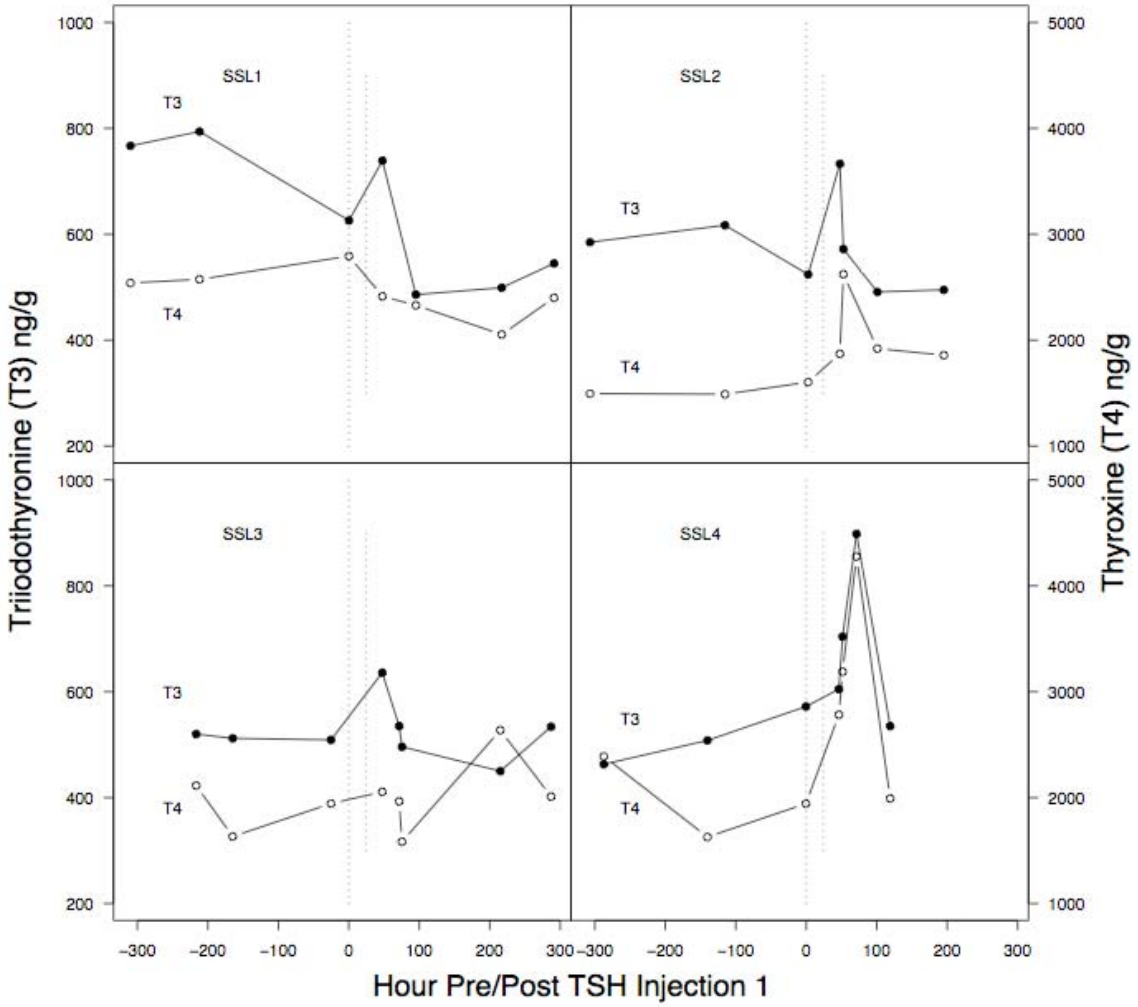

Figure 2.1: The effects of intramuscular TSH injections in four Steller sea lions on fecal concentrations of the thyroid-produced metabolic hormones triiodothyronine (T3 -●-) and thyroxine (T4 -○-) measured by radioimmunoassay from an ethanol vortex extraction. The first TSH injection occurred at 0 h, but passage rates predicted physiologically altered fecal samples approximately 24 h later and the second injection served as hour zero for statistical comparisons.

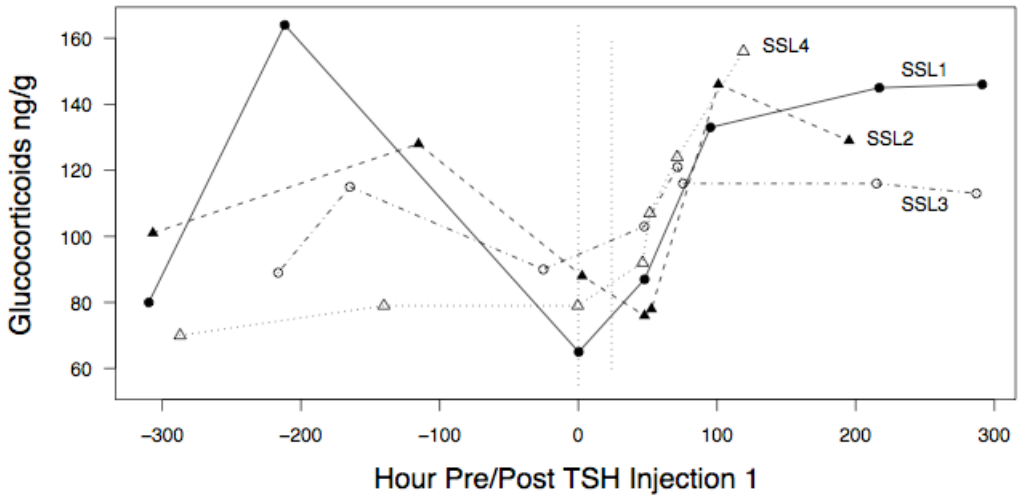

Figure 2.2: Fecal glucocorticoid metabolite concentrations of four Steller sea lions over the course of a TSH stimulation study. Concentrations increased following initial separation from the group (approximately -300 h) and again after intramuscular injections were completed (0 h and 24 h).

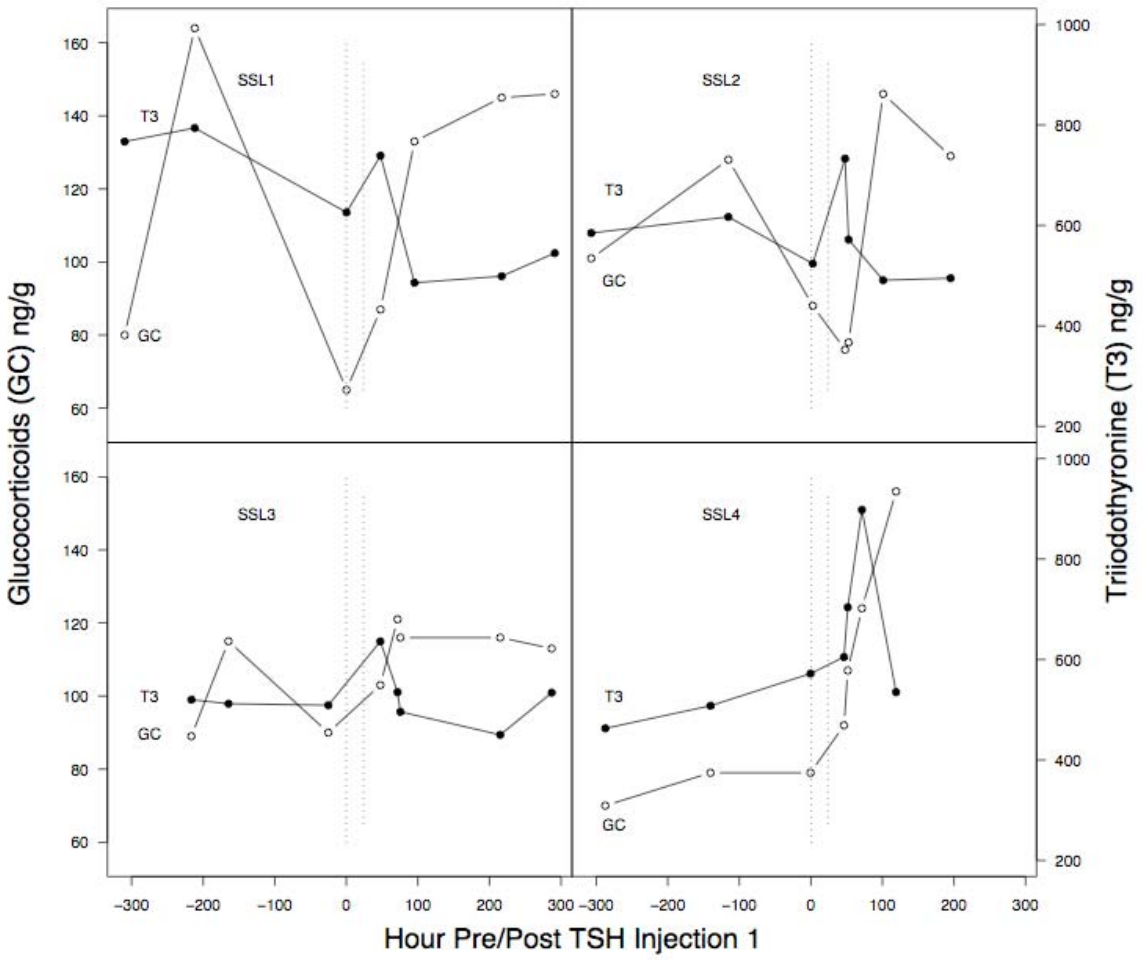

Figure 2.3: The effects of intramuscular TSH injections in four Steller sea lions on fecal concentrations of glucocorticoid metabolites (GC -○-) and triiodothyronine (T3 -●-) measured by radioimmunoassay from an ethanol vortex extraction. The first TSH injection occurred at 0 h, but passage rates predicted physiologically altered fecal samples approximately 24 h later and the second injection served as hour zero for statistical comparisons.

Chapter 3: Fecal triiodothyronine and glucocorticoid metabolite concentrations in relation to the diets and population dynamics of Steller sea lions in Alaska and British Columbia

3.1 Introduction

The western population of Steller sea lions (*Eumetopias jubatus*) occupying the Gulf of Alaska and Aleutian Islands experienced a dramatic decline between the late 1970's and the late 1990's (Merrick et al. 1987, Trites and Larkin 1996). This decline was in sharp contrast to increases that occurred in the eastern sea lion population, which ranges from southeast Alaska to California (Pitcher et al. 2007). Chronic nutritional stress, induced by a decrease in the quality or quantity of prey, is a leading hypothesis to explain the reduced number of Steller sea lions in the western population (Trites and Donnelly 2003). A decrease in prey quality may reduce the ability of female sea lions to bring a pregnancy to term and successfully nurse a pup (Pitcher et al. 1998). A deficiency of high quality prey also has the potential to delay weaning age by one or more years (Rosen and Trites 2005) and reduce juvenile survival (Merrick et al. 1997).

Diet quality appears to be critical to the reproductive success of a number of marine predators (Osterblom et al. 2008). For example, growth of captive marine bird chicks was affected by differences in the energy density of fish they consumed, and likely influenced pre- and post-fledging survival (Romano et al. 2006). Furthermore, consuming lower quality prey reduced the cognitive ability (Kitaysky et al. 2006a) and breeding success (Wanless et al. 2005) of seabirds, which have a similar diet as marine mammals. Diet quality thus has the potential to influence all phases of life history and impact the physiology of the consumer. However, it is difficult to identify individuals and populations that may be unable to meet their daily energy requirements while satiated on sub-quality prey that is insufficient to sustain reproduction and survival.

A promising technique that may identify individuals and populations subsisting on sub-quality prey is measurement of hormone concentrations

contained in blood or feces. The measurement of steroid hormone concentrations in feces has been shown to provide insight into the stress and reproductive status of free-ranging species (Wasser 1996, Wasser et al. 1997, Foley et al. 2001, Mostl and Palme 2002). Among seabirds, for example, a significant portion of the variation in glucocorticoid (GC) hormone concentrations — a physiological indicator of stress — was explained by diet and foraging success (Angelier et al. 2007b), even across seasons and years (Shultz and Kitaysky 2008, Benowitz-Fredericks et al. in press). Acute stressors, such as natural predation or direct anthropogenic conflict may also induce temporary increases in the GC concentrations of individuals, but may not be detectable on a population scale, because it is unlikely that all individuals experience the same conditions. Conversely, potentially chronic stressors, such as reductions in diet quality resulting from fisheries or ocean climate regime shifts, may affect an entire location. However, even chronic increases in GC concentrations are not conclusive evidence of nutritional stress, as correlations do not necessarily mean causation.

Thyroid hormones, specifically the metabolically active triiodothyronine (T3), may also reflect the nutritional status of an individual. Free-circulating thyroid hormones determine energy deficits or surpluses by controlling the rate of energy utilization. A reduction in thyroid hormones conserves endogenous energy reserves through decreases in metabolic rate and body growth. Alterations to thyroid hormone concentrations are therefore a common adaptive response to reductions in diet quality (van der Heyden et al. 1986, Hennemann et al. 1988, Blake et al. 1991, Douyon and Schteingart 2002). Thus, measuring variation in GC and T3 concentrations together has the potential to identify individuals that are experiencing chronic nutritional deficits.

Physiologically, Steller sea lions that eat low quality prey to the point of satiation or who are unable to find sufficient amounts of prey regardless of quality might respond by increasing GC concentrations and concurrently decreasing T3 concentrations.

Among marine mammals, the metabolism of adult Steller sea lions has been shown to change in response to diet quality. For example, sea lions that were provided a diet of squid in equal mass as a control maintenance diet of herring exhibited a 20% reduction in resting metabolic rate that was independent of satiation (Rosen and Trites 1999), suggesting reductions in metabolic rate occur to compensate for reductions in prey quality. A separate captive study found that the metabolism of juvenile sea lions did not change when fasted (Rea et al. 2007), suggesting a limited ability of this age group to cope with prey shortages, because metabolic rates were not being adjusted in relation to changes in consumption.

In mammals, concentrations of GC metabolites and T3 found within feces are representative of events approximately 24-48 h prior to defecation. Serum GC concentrations peaked approximately 30 minutes after adrenocorticotropic hormone (ACTH) infusion in pinnipeds (St. Aubin and Geraci 1986) and serum T3 concentrations peaked 1.5 h after thyrotropin (TSH) infusions in both canines (Ridgway et al. 1974a) and elephant seals (Yochem et al. 2008). Stress response times were similar regardless of sex or age in canines (Feldman et al. 1978) and mean initial defecation time of marked prey was reported to be 4.2 h in California sea lions (Helm 1984). Mean fecal GC peak concentrations following stimulation infusions of captive male and female Steller sea lions occurred at 32 h (Mashburn and Atkinson 2004) and 50.5 h (Hunt et al. 2004). Similarly, mean fecal T3 peak concentrations occurred at 53.75 h in female Steller sea lions (Chapter 2).

Our study was designed to use noninvasive fecal techniques to provide insight into the nutritional status of Steller sea lions at rookeries (breeding sites) and pre- and post-pupping haulouts (resting sites) from increasing eastern and endangered western populations. More specifically, we wanted to determine whether fecal GC and T3 concentrations correlated with each other, but also sought to examine relationships between these hormones and population status (increasing or decreasing), diet quality (determined from fecal hard remains), use type (rookery and haulout), and populations (west and east).

3.2 Methods

3.2.1 Fecal sample collection

We collected 834 Steller sea lion fecal samples from rookeries (n=5) and pre- and post-pupping haulouts (n=7 and n=4, respectively) ranging from British Columbia to the Central Aleutian Islands to examine hormone concentrations, diets, and population trends at scales of individual site, geographic region, and genetic population (Figure 3.1). Fecal samples were collected from eastern rookery populations (samples from western rookeries were unavailable), as well as from eastern and western haulout populations. We attempted to select rookeries in close proximity to haulouts to compare site-types (i.e. rookeries vs. haulouts) in similar oceanographic areas.

Rookery fecal samples were collected post-pupping, June-July of 2005, from British Columbia (Triangle and Sartine Islands of the Scott Islands complex and Cape St. James) to southeast Alaska (Hazy Island, White Sisters, and Graves Rocks) (Table 3.1). Graves Rocks is a former haulout, now classified as a rookery, which provided a unique comparison to the nearby rookery, White Sisters. The remainder of the rookeries corresponded to the haulouts at Ashby Point, Gosling Rocks, and Timbered Island. A final eastern haulout, McInnes Island, was sampled pre-pupping April 2004 and post-pupping July 2005, providing a comparison between seasons and years at one location. The western population haulout samples were collected pre-pupping, May 2006, from the Gulf of Alaska (Sea Otter Island, Cape Shaw, and Takli Island) to the Aleutian Islands (Aiktak Island, Rootok Island, and Cape Izigan). Two western haulouts, Sea Otter and Aiktak, were classified as male-dominated at the time of collection.

The male-dominated and 2004 McInnes collections were excluded from analyses at the region and population scales. Aiktak was male-dominated, whereas Sea Otter had no animals present aside from a lone male in the water. Regardless of an undefined population structure, the sample size collected at Sea Otter (n=10) was too small to be used for diet analyses using prey species combined into groups (Trites and Joy 2005).

Four geographic regions were delineated: British Columbia, southeast Alaska, the Gulf of Alaska, and the Aleutian Islands. These regions reflect distinct geographic shifts in population sizes and trends that occurred over the 1990's (York et al. 1996, Trites et al. 2007b). The eastern population was comprised of British Columbia and southeast Alaska (the southeast Alaskan border to the 144° W longitude demarcation), while the western population was comprised of the Gulf of Alaska (144° W longitude demarcation to the western Alaskan peninsula) and the Aleutian Islands (ending at Samalga Pass). The eastern and western populations were divided at 144° W longitude in reference to established genetic boundaries (Baker et al. 2005). Further genetic differentiation (O'Corry-Crowe et al. 2006), as well as differences in population trends (Trites et al. 2007b), occur roughly at Samalga Pass of the Aleutian Islands, dividing the western population into two distinct subpopulations. Within the western population, fecal samples from across the eastern subpopulation (the Gulf of Alaska to Samalga Pass) were available for this study.

3.2.2 Hormone analyses

The frozen fecal samples were thawed overnight for hormone extraction and manually homogenized the following morning to reduce variation from uneven hormone distribution (Wasser et al. 1988, Wasser et al. 1996). A 2-5 g subsample was taken by passing a portion of homogenized feces through 0.5 mm mesh to remove hard remains. Subsamples were returned to -20^{o}C and lyophilized to halt biological activity and remove variation among subsample mass due to water content (Wasser et al. 1993), a method which often serves as a control when determining the reliability of other storage methods (Terio et al. 2002, Hunt and Wasser 2003). Hormone extractions were completed using an ethanol vortex method for radioimmunoassay, modified from Wasser et al. (Wasser et al. 2000, Wasser Unpublished-a).

We performed the glucocorticoid metabolite (GC) metabolite assay using a double-antibody ^{125}I radioimmunoassay kit (MP Biomedicals, LLC, Irvine, CA; catalog # 07-120103). Manufacturers protocols were followed except for the

halving of kit volumes. The immunoreactivity of the GC radioimmunoassay kit was 100% for corticosterone. Specificity of the antiserum to cross-reactions with common steroids was less than 1%. Those equal to or greater than 0.01% included; 5ALPHA-Dihydrotestosterone 0.01%, Androstenedione 0.01%, Progesterone 0.02%, Aldosterone 0.03%, Cortisol 0.05%, Testosterone 0.10%, and Desoxycorticosterone 0.34%.

The triiodothyronine (T3) assay was performed using a coated tube ^{125}I radioimmunoassay kit (Diagnostic Systems Laboratories, Inc., Webster, TX, catalog # DSL-3100), with modifications to the manufacturers protocol. Modifications include the use of a phospho-saline-BSA buffer and standards made from crystalline T3 to replace the serum-based standards and diluent. The specific cross-reactivity of the T3 antibody to other compounds was: Triiodo-L-thyronine (reverse) 0.004%, L-thyroxine 0.003%, 3,5-diiodo-L-thyronine 0.002%, 3-monoido-L-tyrosine 0.001%, 3,5-diiodo-L-tyrosine 0.001%, and Triiodothyroacetic acid 2.76%.

A pool from the extractions of five samples were serially diluted and examined for parallelism of triiodothyronine and glucocorticoid metabolite metabolites. Serial dilutions paralleled respective standard curves and fifty percent binding occurred at a 1:30 dilution in each assay. T3 and GC metabolite assays were also tested for accuracy, respectively producing slopes of 0.95 and 0.94, illustrating that fecal extracts were not interfering with measurement precision (Wasser Unpublished-a). All hormones were measured as nanograms hormone per gram dry feces (ng g^{-1}) and samples that resulted in a percent bound outside 15-85% on the standard curve or had a coefficient of variation between duplicate pairs greater than 10% were re-assayed at the appropriate concentrations.

We assigned each sample a designation of “dry” or “wet” (estimates of old and new fecal samples). We then compared whether hormone concentrations differed by physical state by pooling all samples, as well as within individual sites. Dry samples were to be excluded from further analyses, if hormone concentrations were clearly different from moist samples. Once the hormone

concentrations were found to be unaffected by these physical conditions, we proceeded to examine differences in each hormone across the scales of site, region, and population.

3.2.3 Diet analyses and indices

Each fecal sample was rinsed of soft tissues and the hard remains used to determine the presence and absence of prey species (Pacific IDentifications, Inc., Victoria, BC). Diets were reconstructed from 849 fecal samples, of which 93 were removed from further analyses as a result of being "empty" or containing only "unknown" hard remains. Prey species were categorized into one of eight groups based on similarities in taxonomy, energy content, behavior, and historical prevalence in the diet of Steller sea lions (Merrick et al. 1997, Trites et al. 2007b): (1) forage fish: Pacific herring (*Clupea pallasii*), sandlance (*Ammodytes hexapterus*), sardine (*Sardinops sagax*), eulachon (*Thaleichthys pacificus*), and capelin (*Mallotus villosus*); (2) salmon (*Oncorhynchus spp.*); (3) rockfish (*Sebastes spp.*); (4) cephalopods: squid and octopus; (5) other prey: Irish lord (*Hemilepidotus spp.*), skate (*Raja spp.*), dogfish (*Squalus acanthias*), threespine stickleback (*Gasterosteus aculeatus aculeatus*), sandfish (*Trichodon trichodon*), unidentified polychaetes, lamprey (*Lampetra spp.*), and snailfish (*Liparidae spp.*); (6) hexagrammids: Atka mackerel (*Pleurogrammus monopterygius*) and greenling (*Hexagrammos spp.*); (7) flatfish: arrowtooth flounder (*Atheresthes stommias*), rock sole (*Lepidopsetta bilineatus*), starry flounder (*Platichthys stellatus*), halibut (*Hippoglossus stenolepis*); and (8) gadids: Pacific hake (*Merluccius productus*), pollock (*Theragra chalcogramma*), Pacific cod (*Gadus macrocephalus*), and Pacific tomcod (*Microgadus proximus*).

Diet comparisons were completed using the prey group raw frequencies at each geographic scale and for each use-type (rookeries or haulouts). The prey group raw frequencies were also used to calculate the split-sample frequency of occurrence (SSFO, frequency of occurrence), which estimates prey group proportions as a unit of one (Merrick et al. 1997). Frequency of occurrence proportions were then used to calculate two indices of diet quality; a diet diversity

index (DDI) (Merrick et al. 1997) and an energy content index (ECI). Potential DDI values ranged from 1.0 to 8.0, indicating a single prey group present to all eight groups being equally represented. The respective equations used to calculate the DDI of individual sites and higher scale combinations were:

$$DDI_{site} = e^{-\sum_{i=1}^{8} SSFO_i \ln SSFO_i} \qquad DDI_{area} = e^{\sum_{area=1}^{\#sites} [DDI_{site}(count_{site} / count_{area})]}$$

ECI was calculated as the summation of prey group split-sample frequency of occurrence multiplied by the corresponding prey group mean energy content wet weight (J mg^{-1} WW). Mean energy content of the prey groups were taken from (Cauffope and Heymans 2005): forage (7.5); salmon (7.0); rockfish (6.0); cephalopods (5.0); hexagrammids (4.5); flatfish (4.0); and gadids (4.0). The energy content of the prey group "other" (4.7) was determined by (Joy 2008). Potential ECI values therefore ranged from 4 to 7.5 J mg^{-1} WW.

$$ECI = \sum_{i=1}^{8} SSFO_i J\left(mg^{-1} WW\right)$$

3.2.4 Population trends

Count data was restricted to the largest annual occurrence of non-pups within the breeding season (June-July) from 2000-2007 in order to determine Steller sea lion population trends following the 1998-99 ocean climate regime shift (McFarlane et al. 2000, Peterson and Schwing 2003). Aerial photography counts were used for sites in U.S. waters to assure quality, but aerial counts in Canadian waters were of limited availability. Boat counts were therefore used exclusively within Canadian waters to retain observational consistency within the region. Counts were conducted at sites in U.S. waters by the National Marine Mammal Laboratory and in Canadian waters by the Alaska Department of Fish and Game and the Marine Mammal Research Unit.

The restricted count data were used to calculate rates of population change and percent change (2005-2006) at each geographic scale and additionally subdivided as rookeries and haulouts. Ashby Point (a rate of

population decrease of 26 animals per year, but then an increase of 103 after outlier removal) was excluded from analyses at the region and population scales because of high variation in counts. A study of hourly Steller sea lion counts found a coefficient of variation of 9% at a rookery and an average of 32% across multiple haulouts (Kucey 2006). White Sisters (a rate of population decrease of 59 animals per year) resulted in population trends inconsistent with the history of the rookery. As a result, pup-counts (a rate of population increase of 40 animals per year) were used as a proxy for the non-pup population.

An additional regression was performed using site counts recorded prior to fecal collection against site counts estimated from regressions of the 2000-2007 counts. The correlation between the field and estimated site counts was significant ($p<0.001$, $r^2=96\%$). Field counts were retained for analyses, but estimates were used for sites where field counts were not conducted prior to fecal collection.

3.2.5 Statistical analyses

Tests for the accuracy of the GC and T3 assays were completed by The Center for Conservation Biology at the University of Washington where the noninvasive techniques were developed (Wasser Unpublished-a). All hormone concentrations were log-transformed for all statistical analyses. *t*-tests were used to compare the physical state of samples (i.e. “dry” or “moist”) and were also used to determine whether mean hormone concentrations differed between the eastern and western population. The Tukey HSD test with cluster analyses was used to compare hormone concentrations across the geographic scales of site, region, and population. All statistical tests were considered significant at $p<0.05$, except for 120 diet comparisons that were completed using the Pearson chi-square test with a Bonferroni correction ($p=0.00042$), 28 region comparisons ($p=0.00179$), and 6 population comparisons ($p=0.00833$). Percent population change, rates of population change, and population counts were estimated from linear regressions.

Analyses began with random-effects models using the geographic scale

as the random factor and either GC or T3 against all factors that resulted in $p \leq 0.2$ when independently analyzed under the same model design. Factors examined included GC and T3 concentrations, Julian collection day (1-365), season (spring or summer), year (2005 or 2006), use-type (haulout or rookery), proportions of the eight prey groups, DDI, ECI, percent population increase (2005-2006), rate of population increase, site count, and estimated site count. Correlations were examined as linear regressions if the models failed to uncover any significance greater than the correlations found between GC and T3 concentrations. The remainder of comparisons between factors relied upon relative values.

3.3 Results

3.3.1 Hormone analyses

The physical state of all samples when pooled as "dry" or "moist" did not affect GC concentrations ($p=0.8024$), but T3 values were lower when dry ($p<0.0001$). Conversely, the physical state of "dry" or "moist" samples from individual sites had similar T3 concentrations, but four sites developed differences in GC metabolites. Two sites (Rootok, $p=0.0080$ and Timbered, $p=0.0132$) exhibited lower T3 concentrations in dry feces and two (Cape Izigan, $p=0.0436$ and Cape Shaw, $p=0.0039$) exhibited higher T3 concentrations in dry feces. As a result, no samples were removed for factors related to physical state.

Mean GC concentrations of the 15 sites ranged from 81.67 ng g^{-1} (Takli) to 254.96 ng g^{-1} (Aiktak) (Table 3.2, Figure 3.2). A cluster analysis comparison of GC metabolite concentrations determined the highest cluster order to include Sea Otter (184.21 ng g^{-1}) and the male-dominate site at Aiktak. McInnes was also included in the highest GC order as the 2004 pre-pupping collection (188.17 ng g^{-1}), but as the 2005 post-pupping collection exhibited the second lowest GC concentration (86.30 ng g^{-1}). Aside from the notably increased GC concentration of Graves Rocks (157.39 ng g^{-1}), the remaining sites were relatively similar regardless of being rookeries or haulouts.

Mean GC metabolite concentrations of the four haulout regions did not differ (Figure 3.2 and Table 3.3). The haulout GC concentration was lowest at the Gulf of Alaska (93.54 ng g^{-1}) and increased outward in both directions, with the highest haulout concentration at the Aleutian Islands. Mean GC concentrations of the two rookery regions differed significantly (p=0.0004). Southeast Alaska rookeries (138.42 ng g^{-1}) occupied a higher cluster with Aleutian haulouts (127.49 ng g^{-1}), but British Columbia rookeries (107.58 ng g^{-1}) exhibited a GC concentration similar to all haulouts.

The mean GC metabolite concentration of the western and eastern haulout populations did not differ (p=0.2148), but the pre-pupping 2006 western population (115.24 ng g^{-1}) exhibited a higher concentration than the post-pupping 2005 eastern population (98.57 ng g^{-1}). The mean GC concentration of all rookeries was significantly higher than that of all haulouts combined (p=0.0013). The rookery population also exhibited a mean GC concentration (129.05 ng g^{-1}) similar to that of the western haulout population (Figure 3.2 and Table 3.3).

T3 concentrations ranged from 1576.56 ng g^{-1} (Cape St. James, rookery) to 4120.56 ng g^{-1} (Ashby Point, haulout). T3 cluster analysis comparisons revealed no immediate ecologically significant results at the site scale. Region-scale T3 comparisons revealed a significant difference between rookery concentrations ($p \leq 0.0001$). Southeast Alaska rookeries (3511.82 ng g^{-1}) exhibited a T3 concentration similar to the majority of haulout regions, whereas British Columbia rookeries (1846.99 ng g^{-1}, lowest region concentration) were similar to southeast Alaska haulouts. In contrast to the British Columbia rookery region, British Columbia haulouts (3718.81 ng g^{-1}) exhibited the highest T3 concentration. The haulout T3 concentration was lowest at southeast Alaska (2075.39 ng g^{-1}) and increased outward in both directions, concluding with the highest haulout concentration at British Columbia. T3 concentrations to the west shared a similarity between regions not observed in eastern haulouts, ending at the Aleutian Islands (3644.22 ng g^{-1}), which was similar to British Columbia haulouts.

The rookery population exhibited the lowest T3 concentration (3006.27 ng g^{-1}) and was significantly lower than all haulouts ($p<0.0001$), as well as the western ($p<0.0001$) and eastern ($p=0.0034$) haulout populations. The T3 concentration of the western (3438.00 ng/g) and eastern (3248.12 ng g^{-1}) haulout populations were significantly different ($p=0.0248$), but were not directly comparable due to the high variation that exists across years (Myers et al. 2006).

GC and T3 concentrations were positively correlated when examined using all rookery samples ($p<0.0001$, $F=89.63$, $r^2=0.19$), but were not correlated in the 2006 western pre-pupping or 2005 eastern post-pupping haulout populations. When examined at a finer scale though, only five sites exhibited correlations between GC and T3 concentrations. A negative correlation was present at the haulout Takli ($p=0.0366$, $r^2=0.11$), but positive correlations were present at four sites: Graves Rocks ($p<0.0001$, $r^2=0.32$), White Sisters ($p<0.0001$, $r^2=0.46$), Hazy ($p=0.00107$, $r^2=0.11$), and Timbered ($p=0.0022$, $r^2=0.14$). The four positively correlated sites occupied the southeast Alaska region ($p<0.0001$, $r^2=0.26$) and three were rookeries ($p<0.0001$, $r^2=0.25$).

3.3.2 Diet analyses

Comparisons of raw prey group data revealed similarities among sites, but not within scales of region or population (frequency of occurrence data, Table 3.4). Diets at the haulouts Cape Izigan and Rootok were similar ($\chi^2=3.104$, $p=0.8752$), supporting the use of a single Aleutian Island region. The diet at Cape Shaw was similar to region partner Takli ($\chi^2=3.315$, $p=0.6516$), but neither was similar to sites at the Aleutian Islands, suggesting correct region design to the west. Cape Shaw was also similar to the southeast Alaska haulout, Timbered ($\chi^2=23.121$, $p=0.0008$). The three haulouts at British Columbia shared similarities, but not to southeast Alaska haulouts, suggesting a correct region design was also applied to the east. Moreover, the two McInnes collections were found to be similar ($\chi^2=18.694$, $p=0.0092$), while the 2004 pre-pupping collection differed from all other pre- and post-pupping haulout sites. Furthermore, of the two McInnes collections, only the 2004 pre-pupping collection was similar to the

Scott Islands complex (χ^2=18.557, p=0.0097), the only haulout similar to a neighboring rookery. Among rookeries, Graves Rocks was the only rookery dissimilar to the Scott Islands complex, but was similar to partner White Sisters. Rookeries and haulouts in close proximity exhibited no similarities; nor was the male-dominated site, Aiktak, similar to other collections.

Site-scale examinations of prey groups accounting for 50% or more frequency of occurrence revealed that Graves Rocks possessed the highest salmon content (52%) of all sites (Table 3.4). Rookeries exhibited a nearly continuous increase in salmon content from British Columbia westward. The male-dominated haulout, Aiktak, consumed the highest hexagrammid content (87%), which was 55% more than the highest mixed-population haulout and 86% more than the highest rookery. Finally, two British Columbia haulouts, McInnes and Gosling Rocks, consumed the highest gadid contents (65% and 63%, respectively), which were more than 30% higher than other mixed-population haulouts and more than 40% higher than the highest rookery. Comparisons of individual prey group frequency of occurrence values to site GC and T3 concentrations revealed no significant correlations.

Region-scale examinations of prey groups accounting for 25% or more frequency of occurrence revealed further differentiation between diets at rookeries and haulouts. The southeast Alaska rookery region was associated with high proportions of salmon and forage fish (37% and 25%) and British Columbia with salmon and rockfish (27% and 25%). Haulout regions in contrast revealed the Aleutian Islands to be the only region containing hexagrammids (25%), the Gulf of Alaska and southeast Alaska (which exhibited site-scale similarities) contained large quantities of flatfish (46% and 36%, respectively), and British Columbia contained a high proportion of gadids (45%).

When all rookery samples were combined, 77% of rookery diets were comprised of the four highest energy-content prey groups. The proportion of the four highest energy-content prey groups in the combined diet of eastern haulout populations was 42%, while that of western haulout populations was 31% (which also had the lowest individual proportions of these prey groups). Furthermore,

the western haulout populations combined consumed four times the proportion of the prey group “Other” than the eastern haulout or rookery population and was the only population to consume hexagrammids (Table 3.4 and Figure 3.3).

3.3.3 Diet indices

Diet Diversity Indices (DDI) values did not separate rookeries from haulouts at any scale (Table 3.4). The most westerly haulout, Cape Izigan (6.06), exhibited the highest DDI of all sites. The male-dominated haulout, Aiktak (1.84), exhibited the lowest DDI, yet the potentially male-dominated Sea Otter exhibited the second highest diversity. The next two lowest DDI values occurred at the post-pupping McInnes and Gosling Rocks haulouts (3.32 and 3.48, respectively), within British Columbia. Region-scale DDI revealed the Aleutian region (5.93) exhibited the highest diet diversity and the Gulf of Alaska (3.82) the lowest diversity. Population-scale DDI revealed eastern rookeries (5.56) exhibited higher diet diversity than eastern haulouts (5.20), but the highest diversity occurred at western haulouts (6.41).

Rookeries exhibited consistently higher Energy Content Indices (ECI) values (ECIs>6.0) than haulouts (ECIs<6.0) (Table 3.4, Figure 3.4). The pre-pupping McInnes collection (5.97) was the haulout exhibiting the highest ECI and Aiktak (4.63) the lowest energy content. Region-scale ECI confirmed the site-scale trend of rookery energy content values greater than 6.0: southeast Alaska (6.22) and British Columbia (6.18). The Gulf of Alaska possessed the lowest ECI (5.04) of all regions, with haulout region energy content increasing outward in both directions, concluding with the highest haulout energy content at British Columbia (5.31). The ECI of the rookery population (6.20) was higher than both the western (5.16) and eastern (5.30) haulout populations.

3.3.4 Population trends

Population percent change over the two years of fecal collection increased for all locations and at all scales (Table 3.5). Individual sites grouped by population and use-type resulted in similar ranges of percent change increases:

western haulouts (1%-14%), eastern haulouts (2%-18%, excluding Ashby Point), and eastern rookeries (5%-12%). One rookery and three haulouts (excluding Ashby Point) exhibited percent change increases of 10% or greater (Graves Rocks, Cape Izigan, Takli, and McInnes) and each was located in a different region.

The percent change increases of the two rookery regions were lower than those of haulout regions, aside from southeast Alaska. The British Columbia rookery and haulout region each exhibited the greatest percent change increase among respective regions of similar use-types. Percent change by region decreased east to west for both haulouts and rookeries, aside from southeast Alaska, which possessed the lowest value. The percent change of all rookeries combined was lower than that of haulouts combined in either population and the western haulout population possessed the greatest percent change.

3.4 Discussion

Our study marks the first fecal T3 investigation of any free-ranging species. It is also the first to compare T3 and GC metabolite concentrations to diet measures and population status to identify nutritional stress in Steller sea lions. We set out to determine whether hormone concentrations differed between rookeries and haulouts at multiple scales and whether any differences could be attributed to diet quality. What we ultimately found was twofold. First, the predicted hormone profile indicative of fasting was present in the rookery population when all rookeries were pooled. This result does not appear to be associated with diet quality, although diet quantity may be a factor in this response. Second, the western haulout population contained indicators of nutritional stress, evidenced by a relatively higher GC metabolite concentration and lower diet quality, but conclusive evidence was limited by the inability to compare T3 concentrations across years (Myers et al. 2006).

The ranges of fecal hormone concentrations found in the free-ranging Steller sea lion populations were vastly different from those of a previous study involving injections of TSH into captive female sea lions (Chapter 2). The free-

ranging populations in our study had a GC metabolite range of 27-1065 ng g^{-1}, whereas the captive range was only 65-164 ng g^{-1}. In addition, T3 concentrations were 550-12,315 ng g^{-1} in the free-ranging populations, but only 450-898 ng g^{-1} for captive females (Chapter 2). The amount of TSH provided the captive animals may not have been enough to maximally stimulate the thyroid gland and the range of conditions in the wild are more variable and more severe than in captivity.

3.4.1 Biases and limitations

The western and eastern population was sampled over different months, seasons, and years, due to a legal decision that revoked all Steller sea lion research permits in 2006. The multiple categories we used to qualify fecal samples attempted to account for the potential confounding effects of the time and location of fecal sampling, as well as sex, in order to compare the diets and hormone concentrations of Steller sea lions (Table 3.6). Despite the complexity inherent in the interpretation of hormone concentrations, other studies have confirmed the validity of drawing comparisons across seasonal diets (Dellinger and Trillmich 1999), seasonal GC concentrations (Oki and Atkinson 2004), and comparisons of T3 concentrations between the sexes (Myers et al. 2006). Despite these similarities, a number of other factors can still confound the simple interpretation of hormone concentrations and diets.

The metabolism of pinnipeds, which is influenced by air and water temperatures, can vary between years (Myers et al. 2006), seasons (Oki and Atkinson 2004), and genetic populations (Hoopes et al. 2004). Even lactation in pinnipeds can influence metabolism (Hall et al. 1998, Trillmich and Kooyman 2001) and seasonal energy requirements (Winship et al. 2002). Total GC concentrations increase during pregnancy in the serum of many species, but breakdown by the liver and metabolic clearance rates are also reduced (von der Ohe and Servheen 2002). Thus, it is questionable whether our fecal GC measurements could be influenced by pregnancy.

Some of the fecal samples we used were collected in May when sexually mature males were departing from haulouts and arriving at rookeries, approximately one month prior to females. Such sex-biased movement patterns to and from rookeries could seasonally alter the sex ratio of Steller sea lions at haulouts. Other fecal samples were collected in June and July during the breeding season when haulouts are dominated by immature animals and nonterritorial males, and rookeries are dominated by territorial males who have ceased fasting, and lactating females that fast intermittently while tending pups. Fasting also occurs among lactating females for approximately a week following parturition while attending to pups, prior to initiating regular foraging bouts (Milette and Trites 2003). Such variations in population composition and breeding status could have important implications for interpreting differences in hormone concentrations and diet quality.

To account for the potential effects that the time and location of fecal sampling and sex may have on the interpretation of Steller sea lion hormone concentrations, we grouped the samples by scale (site, region, and population), use type (haulout or rookery), population composition (male or mixed population), season (pre- or post-pupping), and year. The use of these categories provided the potential to disentangle influences other than diet on T3 and GC metabolite concentrations, such as seasonal physiological trends that may be associated with each sex. Despite taking precautions to make appropriate comparisons, we were not confident that differences between western and eastern populations were meaningful. T3 concentrations are known to vary across years (Myers et al. 2006). It would also be unreasonable to not expect GC metabolite concentrations to vary across years, although a significant portion of the variation in GC concentrations of seabirds was explained by diet and foraging success across seasons and years (Shultz and Kitaysky 2008, Benowitz-Fredericks et al. in press). As a result, comparisons between the western and eastern populations were conducted for purposes of examining the potential of the noninvasive technique, but without statistical significance, and whether the relative hormone concentrations and diet quality held biological implications.

3.4.2 Male-dominated haulouts

Of all the sites, the male-dominated haulout had the lowest quality diet (by all measures) and the highest GC metabolite concentration. Steller sea lions are known to exhibit this pattern of sex-specific differences in diet (Trites and Calkins 2008) and GC metabolite concentrations (Mashburn and Atkinson 2007). These qualities associated with the male-dominated haulout suggest that male Steller sea lions possess a unique foraging behavior and physiology, with an increased gastric capacity and the ability to subsist on prey of lower diversity and energy content. The natural foraging behavior and physiology of males influences the interpretation of data. Thus comparisons of diets and hormone concentrations must account for the different proportions of males and females present within the various breeding, resting, and seasonal population compositions.

3.4.3 Pre- vs. post-pupping haulouts: A site case study

McInnes Island was the only site sampled twice, which provided insight into how seasonal changes in physiology at haulouts might have affected hormone concentrations. Furthermore, McInnes Island had the greatest percent population increase of all the sites sampled (aside from sites with outliers removed) and had a similar overall diet between seasons and years, thus indicating an ideal control population to examine seasonal physiology. The first collection of fecal samples occurred in April 2004, one month prior to any other collection, and exhibited a high GC metabolite and low T3 concentration relative to all the sites sampled. In contrast, the July 2005 collection exhibited the lowest GC metabolite and moderate T3 concentrations. Regardless of a similarity in diets, the April collection also contained higher proportions of high energy-content prey, while the June collection was dominated by the lowest energy-content prey group. Such results suggest that these hormonal indicators do not always correlate strongly with diet quality and that season has a considerable influence on the physiology of Steller sea lions. Season may affect the proportion of males and females at haulouts, as well as their respective breeding physiology, which can in turn influence measures of diet quality and hormone

concentrations. The similar trends found at the April 2004 McInnes collection and in the general rookery population suggest that a greater proportion of females may have been present at the time of fecal collection, possibly due to the early male dispersion to rookeries for purposes of establishing breeding territories.

3.4.4 Rookeries vs. haulouts: Characteristics among sites

Diets consumed by Steller sea lions using rookeries and haulouts in close proximity of one another differed, as did the diet of the male-dominated haulout from all other sites. Conversely, similarities in diet were found between haulouts within the regions of the study. These findings suggest different foraging behaviors were detected at rookeries and haulouts. Rookery diets were of the highest energy content at all scales examined, despite also having greater population densities (i.e. greater competition). In contrast, the British Columbia haulout region had a diet with the highest proportion of gadids, yet also had the greatest percent population increase. These results suggest that post-pupping period summer diets dominated by gadids were not singularly reflective of nutritive state and may be a product of a higher proportion of male sea lions in a post-breeding or nonterritorial physiological state at haulouts.

Although the nutritive state of Steller sea lions cannot be directly stated through ECI values, the ECI of the sites sampled filtered out according to the proportions of females present. From highest to lowest energy content and female presence: rookeries, mixed population haulouts, and the male-dominated haulout. Collectively, these findings suggest that the proportions of females and males at sites may have a considerable influence on diet composition and thus energy content, reinforcing previous findings that female Steller sea lions consume higher quality diets than males.

In general, we found relatively similar GC metabolite concentrations across all the sites sampled, with a few notable exceptions—the male-dominated haulout, and the significant difference between pre- and post-pupping periods at the McInnes Island haulout. It is also noteworthy that Graves Rocks in southeast

Alaska had the highest rookery GC concentration (although it did not differ significantly from other rookeries). Graves Rocks is a former haulout that developed into a rookery as increasing numbers of pups were born at the site. The site also had the greatest percent population increase of any rookery and a diet that contained the highest proportion of salmon. The higher than average GC metabolite concentration measured at Graves Rocks may thus reflect an incomplete transition from haulout to rookery with associated social stress in an otherwise ideal population.

3.4.5 Regional differences in diet and physiology: The Aleutian Islands

Previous studies had found a positive correlation between diet diversity and population trends in the 1990s (i.e., areas with the greatest rate of decline had the lowest diet diversity) (Merrick et al. 1997, Trites et al. 2007a). However, the region we found to have the highest DDI value, the Aleutian Islands region, had experienced only a moderate percent population increase and had a moderate ECI value. Furthermore, previous studies found the Aleutian Island region had a lower probability for extinction relative to other regions of the western population (Winship and Trites 2006). Our finding of a higher DDI value at the Aleutian Islands region was similar to previous research examining only western population rookeries from 1990-1998 (Call and Loughlin 2005), but not research examining rookeries and haulouts from all Alaskan regions from 1993-1999, which found southeast Alaska was associated with the highest diet diversity and energy content (Trites et al. 2007a). Together, these studies suggest that the geographical range examined may have an influence on the interpretation of DDI values.

DDI was not associated with GC or T3 concentrations. To the contrary, the Aleutian Islands region had an increased GC metabolite concentration most similar to those of the two eastern rookery regions, thus signaling potential concern for Steller sea lion populations beyond the Bering Sea shelf.

3.4.6 Western vs. eastern haulout population

The western haulout population exhibited a higher, although insignificant, concentration of GC metabolites than the eastern haulout population and had a diet of lower energy-content. The influence of diet cannot be excluded as a potential contributor to the higher GC concentration of the western population. Furthermore, if males are departing haulouts in May for rookeries one month prior to females, then a greater proportion of scats at the haulouts will be from females. Since females also consume higher energy-content diets than males, it follows that the May western haulout population diet (which is already lower in energy content compared to the June-July eastern haulout population) may be of a higher quality than if we had also sampled the western population during the summer. Therefore we could predict a further decreased diet quality at the western population during the summer and that the difference in diet quality between the western and eastern haulout population may be greater than our findings. Thus, had fecal samples from these populations been available from the same period, greater differences in diet quality may have been detected.

We expected higher GC metabolite concentrations in the western population as a result of diet quality, but the reproductive status of females and males pre- and post-pupping may have also contributed to the minor increase. For example, the serum cortisol concentrations in male Weddell seals decreased as estrus and the breeding season ended, as well as cortisol concentrations being higher in territorial than nonterritorial males and unsuccessful territorial males decreasing to nonterritorial concentrations by estrus (Bartsh et al. 1992). Seasonal reproductive status may influence the GC metabolite concentrations of Steller sea lions and may have contributed to the higher concentration found in the western haulout population, as well as been the cause of the pre- and post-pupping difference found at the McInnes Island haulout.

The western haulout population also exhibited a higher, although insignificant, T3 concentration than the eastern haulout population. T3 concentrations may be affected by temperature and pregnancy, however the relationship between metabolism and pregnancy in pinnipeds is unclear (Renouf

and Gales 1994, Sparling et al. 2006). On the other hand, T3 utilization rates are known to increase with cold exposure and decrease with fasting in various mammals (Tomasi 1991). The higher T3 concentration of the western haulout population suggests such an influence of decreased temperatures at higher latitudes, but cannot be confirmed under the present study design.

3.4.7 Rookeries vs. haulouts: Insights into physiology between sexes

Combined, the rookeries had a significantly higher GC metabolite concentration and a significantly lower T3 concentration than the eastern and western haulout populations and indicate a fasting response within the general rookery population. Long-term fasting associated with the breeding and perinatal periods at rookeries should have ceased by the time our fecal samples were collected. However, intermittent fasting should have continued for females while dividing time between foraging and nursing. This presents the possibilities that hormone concentrations found in the rookery population were related to seasonal physiological changes or nutritional restrictions in females. Lactation and the influence of decreasing leptin concentrations are two potential explanations, and not necessarily mutually exclusive.

Free and total GC concentrations are known to increase in mammals as parturition nears and lactation begins (von der Ohe and Servheen 2002). This has been shown in harp seals that had greater GC concentrations during lactation than post-lactation (Engelhardt and Ferguson 1980). Thyroid hormones have also been shown to be associated with lactation. For example, the serum T3 concentrations of phocid seals increased from lower post-partum concentrations to higher concentrations over the lactation period, albeit insignificantly ($p=0.05$), while total and free T4 concentrations increased significantly (Haulena et al. 1998). T3 concentrations decreased in the plasma of food-restricted, non-lactating rats and not individuals provided a regular diet, but, once lactating, T3 concentrations decreased in both groups (Oberkotter and Rasmussen 1992). These results ultimately suggest that lactation may mask any influence of diet consumption on T3 concentrations in females.

The intermittent fasting experienced by females while dividing time between foraging and nursing suggests that nutritional restriction may determine hormone concentrations of the general rookery population. Not only are potential foraging times halved due to nursing, but the limited foraging opportunities must also meet the energetic demands of more than one animal. Under these circumstances, nutritional stress would predict decreasing body conditions, which for marine mammals is often a measure of lipid mass, and brings into question the influence of leptin on GC and T3 concentrations.

Leptin concentrations are decreased when adipose tissue is metabolized as a result of consuming a diet inadequate to meet maintenance requirements. Reductions of leptin suppress growth, reproduction, and thyroid activity, while concurrently increasing the stress response (Flier et al. 2000). Leptin is often considered an indicator of lipid reserves because the rate at which leptin is secreted and the concentration of leptin in plasma correlate with total lipid mass (Reidy and Weber 2000). However, research with pinnipeds has failed to expose such a relationship. The plasma leptin concentrations of female Antarctic fur seals (*Arctocephalus gazella*) during fasting periods of normal duration did not positively correlate with body mass or condition (Arnould et al. 2002). A study using captive Steller sea lions fed isocaloric restricted diets of herring or pollock for 8-9 day periods, four times a year, found no seasonal changes in leptin and no relationship to body mass or body lipids (Kumagai 2004). Another captive study, this time using fasted Steller sea lions, again found no correlation between leptin concentrations and body lipid mass, but also found decreasing leptin in three males and, counter to expectations, increasing leptin in a single female (Rea and Nagy 2000).

A final captive study of particular interest using female Steller sea lions fed restricted isocaloric amounts of herring or pollock found that total body lipid loss of the herring group was -90.8% (summer) and -73.4% (winter), while the pollock group lost only -47.6% (summer) and -13.1% (winter) (Jeanniard du Dot et al. 2008). The author suggests that the animals fed herring did not reach satiation as a result of the lower prey volume, the effects of which could be the

metabolization of adipose tissue and decreased leptin secretion. These findings complement our results for the general rookery population in terms of sex, possible intake restriction, diet quality, and the potential effects of decreased leptin on GC and T3 concentrations.

The overall variation we found in the T3 concentrations of Steller sea lions at the scales examined prevents any certainty in the interpretation of our results. This may have resulted from our inability to control for aspects of use-type, population composition, season, and year. Nevertheless, the hormone concentrations found in fecal samples from the general rookery population fit the physiological response predicted for females nursing during the post-pupping period.

3.4.8 Fecal hormone radioimmunoassay and study design

Our study could discriminate between diets consumed by males and females, as well as between diets consumed at haulouts and rookeries. However, we could not identify nutrition-specific stress, despite suggestive trends in fecal hormone concentrations. Overall, our findings revealed that diets differed between rookeries and haulouts, as well as by sex. Hormone concentrations also differed by sex, breeding status, and even seasonally at non-breeding sites.

The western haulout population was sampled before the breeding season and had higher GC and T3 concentrations than the eastern haulout population sampled following the breeding season. However, the difference was not statistically significant. Despite exhibiting a higher GC metabolite concentration and lower diet quality, T3 concentrations could not confirm nutritional stress in the western population. The general rookery population had a significantly higher GC and lower T3 concentration than the western and eastern haulout populations. However, rookeries also had the highest quality diets at all scales. Despite this, our findings were not necessarily counter to predictions of nutritional stress. The demands of nursing compounded by limited foraging opportunities, may not have allowed the females to reach satiation or meet maintenance energy requirements even while consuming higher quality diets, thus inducing a

fasting response.

Our study design could not confirm nutritional stress in these populations, but our results suggest that once the degrees to which sex and annual physiological cycles influence hormone concentrations are determined, conditions specific to the feeding physiology of Steller sea lions may become clear. Means of achieving this are to radioimmunoassay reproductive hormones to determine the sex of the animal that defecated, as well as to collect fecal samples over four seasons at select rookeries and haulouts to determine annual hormone cycles. Further analyses of fecal GC and T3 concentrations combined with these approaches will help to better determine the influence of diet on changes in the population abundance of Steller sea lions.

3.5 Summary

Reduced availability of high energy-content prey remains the predominant hypothesis to explain the dramatic decline of Steller sea lion populations in western Alaska from the late 1970's to the late 1990's. We measured hormone concentrations (glucocorticoid metabolites - GC - and the thyroid hormone triiodothyronine - T3) in 834 fecal samples collected in 2005 and 2006 from 15 sites between British Columbia and the Central Aleutian Islands. We sought to determine whether hormone profiles at breeding (rookery) and resting (haulout) sites were consistent with physiological fasting responses (GC increasing to mobilize energy reserves and T3 decreasing to conserve rates of energy utilization) in relation to reduced diet quality. We also compared hormone concentrations and diet between the western population (Gulf of Alaska and Aleutian Islands) and the eastern population (southeast Alaska and British Columbia).

Regionally, Steller sea lions using rookeries exhibited higher GC metabolite concentrations than did animals at haulouts in coastal areas. Haulouts located in open waters of the Aleutian Islands, however, exhibited a regional GC metabolite concentration that was similar to rookeries. Overall, GC metabolite concentrations did not differ between the western 2006 pre-pupping

haulout population and the eastern 2005 post-pupping haulout population. Steller sea lions using haulouts in the western population consumed the lowest energy-content diet, suggesting diet quality remains a relevant hypothesis to explain a slightly higher GC concentration in the western population and previous population declines. However, the hypothesis could not be substantiated through the measurement of fecal T3 concentrations.

The rookery population exhibited a higher GC metabolite and lower T3 concentration than either haulout population, but T3 comparisons at site and regional scales were inconclusive. The physiological fasting response potentially found at rookeries does not appear to be related to diet quality, as the sea lions at rookeries also consumed the highest energy-content diets. However, as a result of limited foraging opportunities while nursing pups, reduced diet quantities may have been consumed by the females at rookeries. The fasting response may thus be a product of lactation or decreased leptin concentrations, which are not necessarily mutually exclusive.

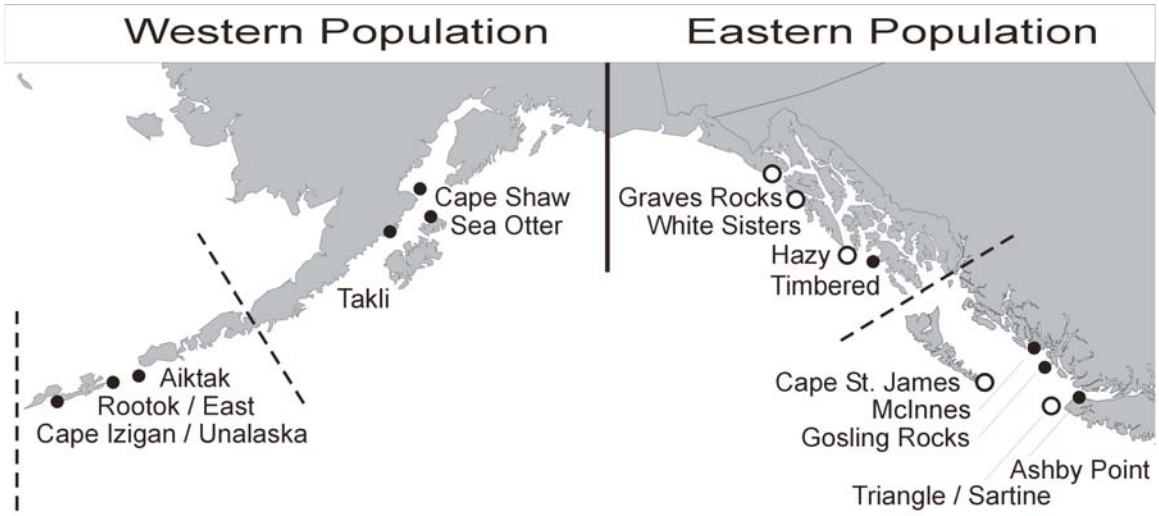

Figure 3.1: Map designating locations where Steller sea lion fecal samples were collected. Solid and dashed lines represent divisions of populations and regions, respectively. Left to right: Samalga Pass, western Alaskan peninsula, 144° W longitude population division, and southeast Alaska / British Columbia.

Table 3.1: Steller sea lion fecal collection locations categorized by site type (H=haulout or R=rookery) and scale (region and population), as well as collection periods and coordinates. Regions are denoted as: A=Aleutian Islands, GOA=Gulf of Alaska, SEAK=southeast Alaska, and BC=British Columbia. Populations are denoted as: W=Western and E=Eastern.

Site	Type	Region	Pop.	Season	Date	Julian	Lat (N)	Long (W)
Cape Izigan	H	A	W	Spring	21-05-06	141	53.23	167.66
Rootok / East	H	A	W	Spring	12-05-06	132	54.05	165.49
Aiktak	H	A	W	Spring	18-05-06	138	54.18	164.85
Takli	H	GOA	W	Spring	15-05-06	135	58.01	154.31
Cape Shaw	H	GOA	W	Spring	18-05-06	138	59.00	153.22
Sea Otter	H	GOA	W	Spring	23-05-06	143	58.31	152.13
Graves Rocks	R	SEAK	E	Summer	30-06-05	181	58.24	136.76
White Sisters	R	SEAK	E	Summer	27-06-05	178	57.64	136.26
Hazy	R	SEAK	E	Summer	24-06-05	175	55.87	134.57
Timbered	H	SEAK	E	Summer	08-07-05	189	55.70	133.80
Cape St. James	R	BC	E	Summer	22-07-05	203	51.91	130.97
McInnes	H	BC	E	Summer	12-07-05	193	52.26	128.72
				Spring	14-04-04	105		
Gosling Rocks	H	BC	E	Summer	12-07-05	193	51.88	128.45
Ashby Point	H	BC	E	Summer	11-07-05	192	50.94	127.92
Triangle	R	BC	E	Summer	25-07-05	206	50.86	129.07
Sartine	R	BC	E	Summer	25-07-05	206	50.82	128.90

Table 3.2: Site glucocorticoid metabolite (GC, top) and triiodothyronine (T3, bottom) concentrations ($ng \cdot g^{-1}$) (mean raw, mean natural log, and standard error of natural log values) measured from (n) Steller sea lion fecal samples collected at haulouts (H) and rookeries (R) at the western and eastern populations. Also shown are similarities in natural log hormone concentrations across sites determined by use of Tukey HSD with cluster analysis.

Site	Type	n	GC	lnGC	lnGC SE	Cluster					
Cape Izigan	H	63	130.19	4.61	0.08			C	D	E	
Rootok / East	H	38	123.02	4.62	0.09			C	D	E	
Aiktak	H	33	254.96	5.22	0.13	A					
Takli	H	31	81.67	4.31	0.08				D	E	
Cape Shaw	H	26	107.68	4.56	0.10			C	D	E	
Sea Otter	H	10	184.21	4.95	0.24	A	B	C	D	E	
Graves Rocks	R	79	157.39	4.76	0.08		B	C			
White Sisters	R	97	136.27	4.70	0.06			C	D		
Hazy	R	90	124.08	4.68	0.05			C	D	E	
Timbered	H	59	93.83	4.46	0.05			C	D	E	
Cape St. James	R	70	112.97	4.49	0.08			C	D	E	
McInnes: Pre-pup	H	45	188.17	5.10	0.08	A	B				
McInnes: Post-pup	H	29	86.30	4.28	0.10					E	
Gosling Rocks	H	68	104.43	4.52	0.06			C	D	E	
Ashby Point	H	50	103.31	4.55	0.06			C	D	E	
Scott Is. complex	R	46	99.38	4.43	0.08			C	D	E	

Site	Type	n	T3	lnT3	lnT3 SE	Cluster						
Cape Izigan	H	63	3776.78	8.19	0.04	A	B					
Rootok / East	H	38	3424.46	8.08	0.05	A	B	C	D			
Aiktak	H	33	3775.30	8.22	0.03	A	B					
Takli	H	31	2727.53	7.82	0.08			C	D	E	F	
Cape Shaw	H	26	3483.98	8.14	0.03	A	B	C				
Sea Otter	H	10	2260.34	7.67	0.11				D	E	F	G
Graves Rocks	R	79	2921.63	7.84	0.06				D	E		
White Sisters	R	97	3727.61	8.13	0.04	A	B					
Hazy	R	90	3797.31	8.14	0.05	A	B					
Timbered	H	59	2075.39	7.59	0.04						F	
Cape St. James	R	70	1576.56	7.31	0.04							G
McInnes: Pre-pup	H	45	1905.69	7.53	0.03						F	G
McInnes: Post-pup	H	29	2994.79	7.92	0.08		B	C	D	E		
Gosling Rocks	H	68	3732.18	8.17	0.04	A	B					
Ashby Point	H	50	4120.56	8.26	0.05	A						
Scott Is. complex	R	46	2258.52	7.70	0.03					E	F	

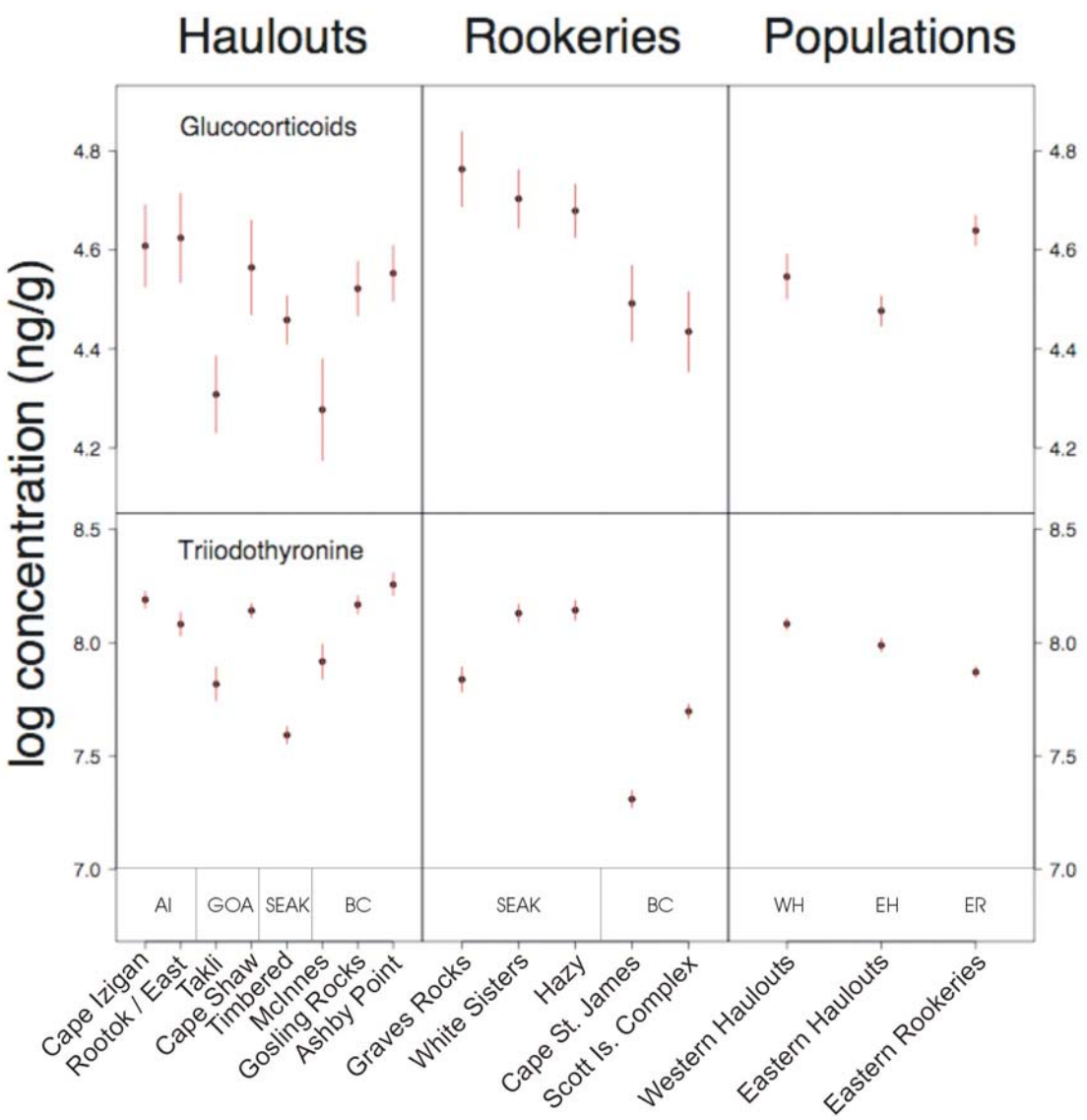

Figure 3.2: Steller sea lion glucocorticoid metabolite and triiodothyronine concentrations ($ng \cdot g^{-1}$) (mean natural log and standard error of natural log values) measured from fecal samples. Sites are categorized west to east by region (A=Aleutian Islands, GOA=Gulf of Alaska, SEAK=southeast Alaska, BC=British Columbia) and population (WH=western haulout, EH=eastern haulout, and ER=eastern rookery).

Table 3.3: Region and population glucocorticoid metabolite (GC, top) and triiodothyronine (T3, bottom) concentrations ($ng \cdot g^{-1}$) (mean raw, mean natural log, and standard error of natural log values) measured from (n) Steller sea lion fecal samples collected at haulouts (H) and rookeries (R). Also shown are similarities in natural log hormone concentrations across regions determined by use of Tukey HSD with cluster analysis and across populations by use of t-test.

Region	Type	n	GC	lnGC	lnGC SE	Cluster		
Aleutians	H	101	127.49	4.61	0.06	A	B	
Gulf of Alaska	H	57	93.54	4.43	0.06		B	
Southeast Alaska	H	59	93.83	4.46	0.05		B	
Southeast Alaska	R	266	138.42	4.71	0.04	A		
British Columbia	H	147	100.47	4.48	0.04		B	
British Columbia	R	116	107.58	4.47	0.06		B	

Population	Type	n	GC	lnGC	lnGC SE	Cluster	
Western	H	158	115.24	4.55	0.05	A	B
Eastern	H	206	98.57	4.48	0.03		B
Eastern	R	382	129.05	4.64	0.03	A	

Region	Type	n	T3	lnT3	lnT3 SE	Cluster		
Aleutians	H	101	3644.22	8.15	0.03	A	B	
Gulf of Alaska	H	57	3072.58	7.97	0.05		B	
Southeast Alaska	H	59	2075.39	7.59	0.04			C
Southeast Alaska	R	266	3511.82	8.05	0.03	A	B	
British Columbia	H	147	3718.81	8.15	0.03	A		
British Columbia	R	116	1846.99	7.46	0.03			C

Population	Type	n	T3	lnT3	lnT3 SE	Cluster	
Western	H	158	3438.00	8.08	0.03	A	
Eastern	H	206	3248.12	7.99	0.03	A	
Eastern	R	382	3006.27	7.87	0.03		B

Table 3.4: Steller sea lion diet identified from (n) fecal samples compiled at the site, region, and population scale. Prey groups are ordered from highest to lowest energy content (J mg^{-1} wet weight): F=forage fish; S=salmon; R=rockfish; C=cephalopods; O=other; H=hexagrammids; Fl=flatfish; and G=gadids. Diet is expressed as split-sample frequency of occurrence (%), Diet Diversity Index (DDI, 1-8 prey groups), and Energy Content Index (ECI, 4-7.5 J mg^{-1} wet weight). Fecal samples were collected from the western population during the spring season and from the eastern population during the summer season, aside from McInnes Island: Pre-pupping samples.

Site	n	F	S	R	C	O	H	Fl	G	DDI	ECI
Cape Izigan	59	4%	23%	8%	0%	23%	23%	11%	8%	6.06	5.28
Rootok / East	37	2%	19%	6%	0%	26%	32%	5%	10%	5.31	5.12
Aiktak	32	3%	0%	2%	2%	4%	87%	1%	1%	1.84	4.63
Takli	30	22%	2%	0%	1%	19%	0%	43%	13%	4.04	4.97
Cape Shaw	26	27%	2%	0%	0%	16%	0%	49%	7%	3.52	5.11
Sea Otter	10	20%	6%	2%	10%	10%	1%	18%	32%	5.85	5.11
Graves Rocks	68	17%	52%	3%	1%	8%	1%	5%	15%	4.23	6.27
White Sisters	97	37%	34%	12%	1%	4%	0%	4%	9%	4.59	6.57
Hazy	62	23%	29%	14%	6%	1%	0%	6%	21%	5.35	6.03
Timbered	39	12%	14%	17%	1%	7%	0%	36%	13%	5.40	5.24
Cape St. James	62	17%	30%	33%	5%	6%	0%	0%	9%	4.77	6.24
McInnes: Pre	44	31%	5%	30%	6%	11%	0%	3%	14%	5.42	5.97
McInnes: Post	28	11%	2%	9%	4%	8%	0%	1%	65%	3.32	4.72
Gosling Rocks	62	13%	9%	3%	1%	3%	0%	9%	63%	3.48	4.81
Ashby Point	50	22%	29%	6%	0%	3%	0%	11%	30%	4.74	5.77
Scott complex	49	27%	25%	20%	3%	5%	1%	12%	9%	5.81	6.13

Region	n	F	S	R	C	O	H	Fl	G	DDI	ECI
AI: H	96	4%	22%	7%	0%	23%	25%	9%	9%	5.93	5.24
GOA: H	56	24%	2%	0%	1%	18%	0%	46%	10%	3.82	5.04
SEAK: H	39	12%	14%	17%	1%	7%	0%	36%	13%	5.40	5.24
SEAK: R	227	25%	37%	10%	4%	4%	0%	5%	16%	5.20	6.22
BC: H	140	18%	19%	5%	1%	4%	0%	9%	45%	4.52	5.31
BC: R	111	23%	27%	25%	3%	5%	0%	7%	9%	5.68	6.18

Population	n	F	S	R	C	O	H	Fl	G	DDI	ECI
Western: H	152	13%	14%	4%	0%	21%	14%	25%	9%	6.41	5.16
Eastern: H	179	16%	17%	8%	1%	5%	0%	16%	37%	5.20	5.30
Eastern: R	338	24%	32%	17%	4%	4%	0%	6%	13%	5.56	6.20

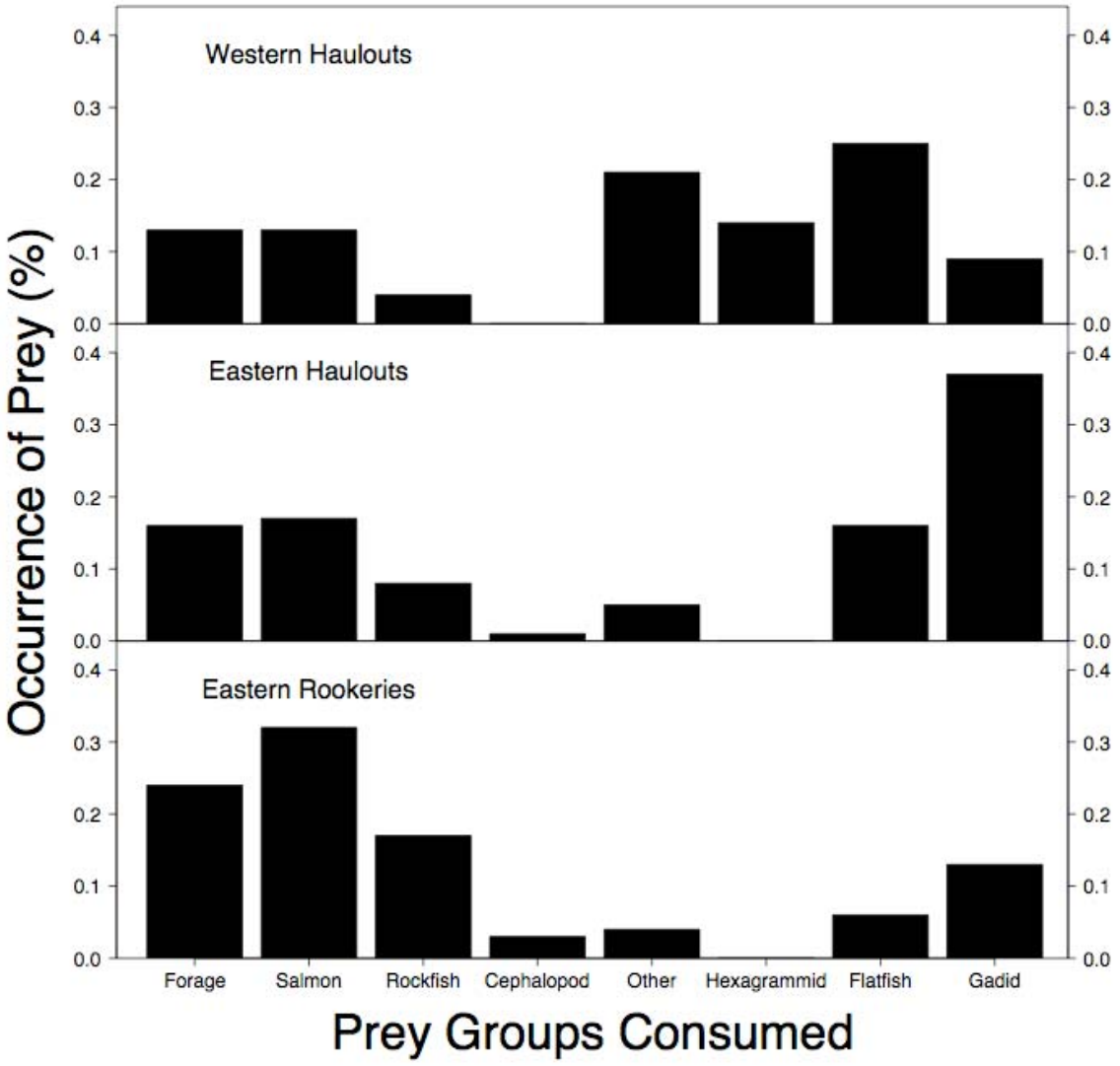

Figure 3.3: Steller sea lion diets identified from fecal samples for populations and use types expressed as prey group split-sample frequency of occurrence (%) ordered from highest to lowest energy content (J mg^{-1} wet weight). Fecal samples were collected from the western population during the spring season and from the eastern population during the summer season.

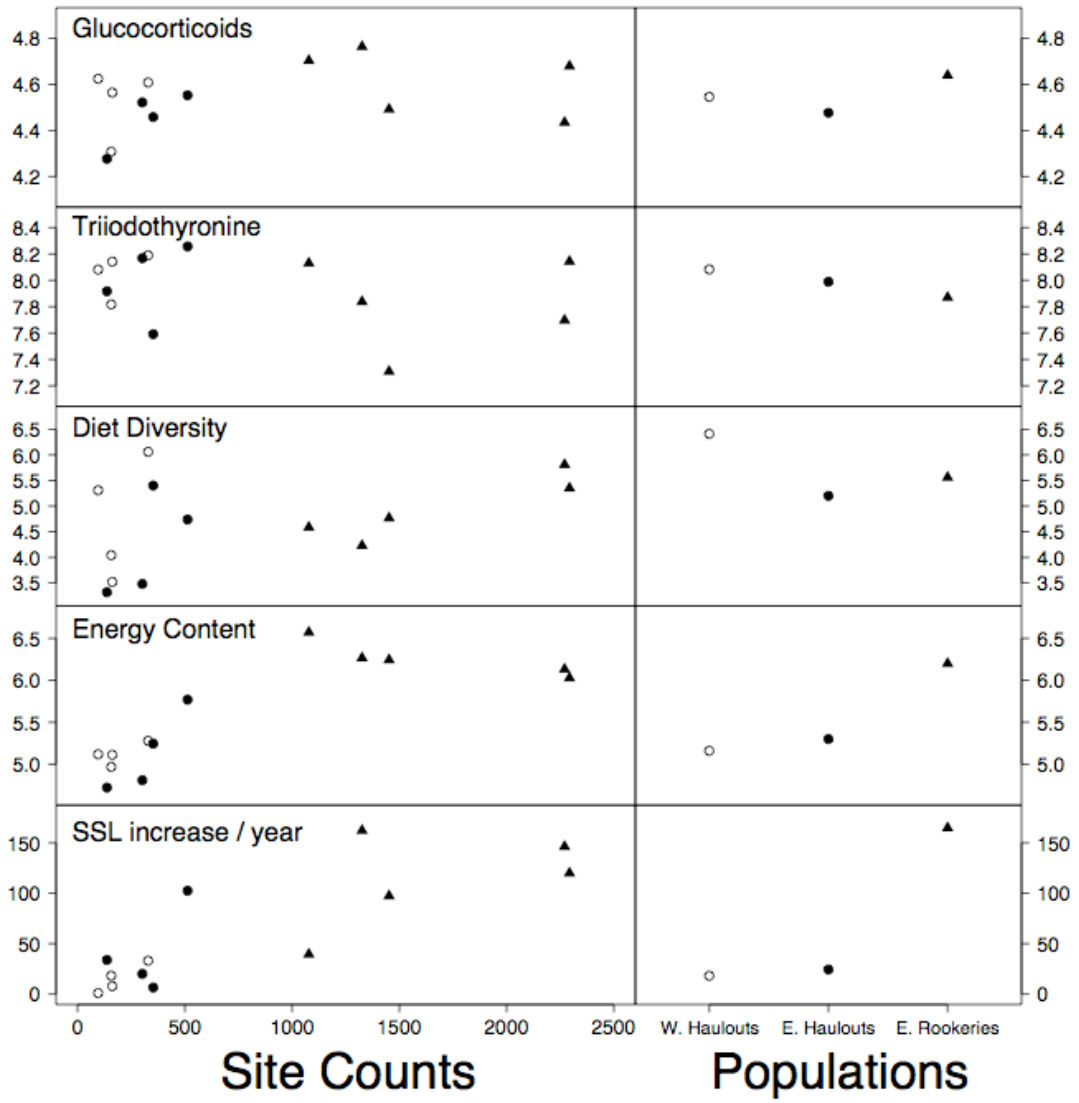

Figure 3.4: Hormone concentrations, diet indices, and the rate of population change per year compared to Steller sea lions site counts (the number of animals present at the time of fecal collection) and populations (western haulouts ○, eastern haulouts ●, and eastern rookeries ▲). Glucocorticoid and triiodothyronine hormone concentrations are expressed as the mean log value ($ng \cdot g^{-1}$). Indices of diet diversity (1-8 prey groups) and energy content (4-7.5 J mg^{-1} wet weight) were calculated using frequency of occurrence from fecal hard remains. Population growth is expressed as the increase in animals between the years of fecal collection (2005 and 2006), calculated using counts conducted over 2000-2007.

Table 3.5: Steller sea lion population trends for sites, regions, and populations. Number (slope, m) and percent change per year were calculated from regressions of counts from 2000-2007 between two years of fecal collection (2005-2006). Trends for regions and populations were calculated by pooling site counts for a given area. Counts are of non-pups (unless noted) from the year fecal samples were collected (western population=2006, eastern population=2005, and McInnes: Pre-pup=2004), unless estimated from regressions (*).

Site	Type	Count	# change	% change
Cape Izigan	H	329	33	11%
Rootok / East	H	96	1	1%
Aiktak	H	111	4	4%
Takli	H	157	18	14%
Cape Shaw	H	162	8	7%
Sea Otter	H	*113	2	2%
Graves Rocks	R	1326	162	12%
White Sisters	R	1078		
White Sisters	R	(Pup) 520	40	8%
Hazy	R	2293	120	5%
Timbered	H	*353	7	2%
Cape St. James	R	1452	97	9%
McInnes: Pre-pup	H	85	34	18%
McInnes: Post-pup	H	137	34	18%
Gosling Rocks	H	302	20	9%
Ashby Point	H	513	103	21%
Scott Is. complex	R	2270	146	5%

Region		# change	% change
Aleutian Islands	H	21.23	10%
Gulf of Alaska	H	13.08	11%
Southeast Alaska	H	6.50	2%
Southeast Alaska	R	169.80	8%
British Columbia	H	36.43	14%
British Columbia	R	170.59	9%

Population		# change	% change
Western	H	18.17	10%
Eastern	H	24.39	9%
Eastern	R	164.76	8%

Table 3.6: Summary of all previous variables categorized by use type, region, and population.

	Haulout						Rookery		
	AI	**GOA**	**SEAK**	**BC**	**West**	**East**	**SEAK**	**BC**	**East**
Month	May	May	July	July	May	July	June	July	Jun-Jul
Year	2006	2006	2005	2005	2006	2005	2005	2005	2005
Rate	21.23	13.08	6.5	36.43	18.17	24.39	169.80	170.59	164.76
Percent	10%	11%	2%	14%	10%	9%	8%	9%	8%
DDI	5.93	3.82	5.40	4.52	6.41	5.20	5.20	5.68	5.56
ECI	5.24	5.04	5.24	5.31	5.16	5.30	6.22	6.18	6.20
GC	127	94	94	100	115	99	138	108	129
T3	3644	3073	2075	3719	3438	3248	3512	1847	3006

Chapter 4: Conclusion

The overall objective of our study was to characterize the nutritional status of Steller sea lions in the western and eastern populations through the application of noninvasive fecal techniques. These techniques included estimating diet composition by identifying fecal hard parts and assessing physiological status by measuring fecal T3 and GC metabolite concentrations. We first validated the measurement of fecal T3 concentrations in captive Steller sea lions (Chapter 2), and then applied the technique to free-ranging populations, along with fecal measures of diet quality and GC metabolite concentrations (Chapter 3).

We predicted that populations consuming diets of low energy-content (specifically the endangered western Steller sea lion population) would exhibit hormone profiles consistent with a fasting response, as a means of coping with chronic nutritional stress. We defined a fasting response as increased GC metabolite concentrations (indicating stress and the mobilization of endogenous energy reserves) and decreased T3 concentrations (indicating metabolism and a decreased rate of endogenous energy utilization) relative to the other sites sampled. Fecal hard remains revealed that the western population had a lower energy-content diet, as predicted, and a slightly higher GC metabolite concentration, but without concrete implications to population status. Comparisons of T3 concentrations between the western and eastern populations could not be interpreted with confidence because fecal samples were collected from the two populations over two different years.

Despite the limitations of our comparison between the western and eastern haulout populations, we found that the hormone profile of all the rookery populations combined was consistent with a fasting response. The rookery population had a higher GC metabolite concentration and a lower T3 concentration than those of the eastern and western haulout populations. Males at rookeries should have ceased fasting by the time the fecal samples were collected, however lactating females fasting intermittently while tending pups

should have still been present. The female-biased population composition and higher energy-content diets at rookeries suggested that the fasting response we found may be a physiological response to lactation or limited foraging opportunities.

Lactation has been associated with a fasting response independent of fasting in rats (Oberkotter and Rasmussen 1992), which suggests that the energetic demands of lactation may be so great as to induce a hard-wired physiological response. Decreased leptin concentrations combined with limited foraging opportunities while meeting the energetic demands of pups (in addition to self-maintenance) may also have contributed to the fasting response. The combination of limited foraging opportunities for females during the nursing period and the high energy-content prey consumption at rookeries highlights the need to apply measures of both quality and quantity in future studies of nutritional stress.

4.1 Current research, general conclusions, and limitations of this thesis

My thesis presents the first validation and application of fecal T3 measurements. Further research using the measurement of fecal T3 is currently underway at the Marine Mammal Research Unit of the University of British Columbia on northern fur seals (*Callorhinus ursinus*) and the Center for Conservation Biology at the University of Washington (various species). To our knowledge, the Alaska SeaLife Center (Seward, AK) is also pursuing the validation of fecal T3 measurement for Steller sea lions. Until these studies are completed, the value of these non-invasive techniques in addressing nutrition-specific stress or even in describing the general physiology of other species cannot be approved or discredited.

Overall, our captive and free-ranging studies yielded mixed results. T3 measurement in the feces of captive Steller sea lions (Chapter 2) was validated when comparing the scats collected before injections occurred (time 0 samples) to the samples of peak concentration. Furthermore, trends observed in post-injection GC and T3 concentrations fit the prediction that leptin decreases as a

result of metabolic stimulation would decrease T3 concentrations and increase GC concentrations. These findings justified our application of the technique to free-ranging populations as a means of assessing metabolic activity and possibly nutritional status. Our findings of increased population growth at the free-ranging populations (Chapter 3) suggest that at the time of this study, nutritional stress may not have been present at the populations sampled.

Further complicating matters of interpretation are that vastly different ranges of hormone concentrations were found in the captive and free-ranging populations for GC metabolites (captive, 65-164 ng g^{-1} and free-ranging, 27-1065 ng g^{-1}) and T3 concentrations (captive, 450-898 ng g^{-1} and free-ranging, 550-12,315 ng g^{-1}). Since the thyroid serves as both a hormone producing and storage organ, it could be that the amount of TSH provided the captive females failed to produce hormone responses similar to ranges experienced outside of a controlled setting, such as changes due to greater variation in ages, diets, and temperature.

It must be noted that our inability to identify nutrition-specific stress in Steller sea lions may lie in the fact that our original study design was rendered impossible by the 2006 Humane Society litigation against the U.S. National Marine Fisheries Service (NMFS) over the issuance of Steller sea lion research permits. This case resulted in permits being revoked by the NMFS Office of Protected Resources until the following year. We were therefore unable to collect fecal samples from sites across the entire range in a single year, which necessitated the use of samples available from different years and seasons. Despite this, our measures of diets and hormone concentrations suggested, but could not confirm, the presence of nutrition-specific stress in the western and rookery population. Ultimately, the free-ranging study was valuable as a means of determining the foraging behavior and physiology specific to each sex at rookeries and haulouts.

4.2 Future research

The different diets and hormone concentrations that occurred at rookeries and haulouts, pre- and post-pupping haulouts, and the male-dominated haulout present various avenues for more concise study designs using fecal techniques with Steller sea lions. First and foremost, captive studies of baseline, nutritionally restricted, and fasted animals must be conducted that combine hematological and fecal measurements to understand the differences between hematological responses and what evidence of nutritive status may be excreted. In essence, the problem is that we validated the ability to measure the fecal hormones of Steller sea lions, but did not determine what the range of hormone concentrations meant in relation to the actual physiological status of the animals.

We used the presence and absence of prey in our diet analyses and recognize that this excludes estimates of the quantity and mass of each prey species consumed to more accurately determine energy intake. Until these additional measures are made possible, it will remain difficult to determine the degree at which decreased energy-content or limited prey consumption must be accounted for by detectable physiological changes.

It has also been suggested that predators may assimilate the hormones within consumed prey. Hormones present within consumed prey can be absorbed, metabolized, and excreted by the carnivorous consumer (Chanoine and Junien 1984, Heazelwood et al. 1984, von der Ohe and Servheen 2002), and may be differentially excreted by species and sex into urine or feces (Cooper et al. 1996). Studies have shown that peak fecal glucocorticoid concentrations in bears occur during salmon spawning months (von der Ohe et al. 2004), a period of peak cortisol concentration within the salmon and a time of high food security for the bears.

We conducted a pilot study extracting blended samples of pollock (n=10) and herring (n=10) as classic representatives of low and high lipid content prey used within captive studies. Comparisons between the prey type concentrations were significant for GC metabolites ($p=0.00$, $t=4.32$), but not T3 ($p=0.20$, $t=-1.36$). Under the most simple of diet assessments using pollock and herring as

indicators of a consumer's nutritional state, our results suggest that the assimilation of the increased GC load within pollock could potentially cause a deceptive addition to a consumer's actual physiological response to the lower quality prey. We found gadid consumption in the eastern haulout population was 28% higher than the western population. As the western haulout population GC concentration was higher than the eastern haulout population, many possibilities exist. For example, it may be that consumed GC does not significantly affect consumer fecal concentrations, or that the GC concentration of the western haulout population was greater due to prey quality or female pre-pupping physiology. It may also be that the GC concentration of the eastern population was inflated due to the proportion of pollock consumed, masking a greater difference between the populations.

An additional means of improving the interpretation of hormone changes is to sex individual animals through the use of fecal samples, rather than using the observed population composition at the time of fecal collection. Knowing the sex of the defecator will allow for the foraging strategies and hormone profiles specific to each sex to be differentiated. Fecal sexing can be completed by radioimmunoassay of the reproductive hormones; androgens, estrogens, and progestins (Rolland et al. 2005, Wasser and Hunt 2005), although the ability to further differentiate between pregnant and pseudopregnant pinnipeds is seasonally dependent (Browne et al. 2006, Greig et al. 2007). If fecal sexing is not possible, then fecal samples should be collected soon after pupping when the population composition of rookeries and haulouts would best function as a proxy for sex, as 55% of the adult Steller sea lion population returns to breed at rookeries in the summer (Trites and Larkin 1996). Haulouts could also be sampled in September, when males are returning from open water foraging and prior to blastocyst implantation, to best remove any influence of pregnancy, lactation, and breeding behavior from optimally mixed populations.

A final variation in study design that might be considered is to sample select rookeries and haulouts multiple times throughout the year to determine annual variations of T3 and GC metabolite concentrations, as well as diet, in the

different Steller sea lion populations. Times of the year other than the breeding season may present circumstances under which nutritional stress may be either more prevalent or difficult to discern due to other physiological demands on the animals. For instance, bull Steller sea lions proceed to haulouts for the molt in September, where they mix with females and pups. In harbor seals, molt induces a physiological response in serum similar to our predicted fasting response, with increased plasma GC and decreased T4 concentrations (Ashwell-Erikson et al. 1986). Additionally, mating takes place shortly after pupping, but implantation of the blastocyst does not occur until October (Pitcher and Calkins 1981), which, along with winter climate, may change the energetic demands of Steller sea lions. Collecting fecal samples throughout a year could resolve the degrees to which such influences as sex, population composition, and breeding status have on the interpretation of hormones associated with nutritional stress. Seasonal collections would also help to identify the time of year when diet quality is lowest, and hence the time of year when more concise research should be conducted on nutritional stress in each population.

4.3 Implications

The study design we were forced to use as a result of the litigation was not specific enough in design to address the needs of identifying nutrition-specific stress, despite our attempts to control for confounding factors. Although an animal's physiology may change in response to stimuli such as diet, it also serves to maintain homeostasis, which makes nutrition-specific stress a difficult theory to address. Despite this, our study was valuable in validating a non-invasive measure of metabolism, which until now was performed using various invasive techniques. Furthermore, our findings suggest an ability to detect nutrition-specific stress, while highlighting the need to account for annual cycles of the sexes in terms of physiology and diet before conclusive work on nutrition-specific stress can be initiated. Our work also shows that male and female Steller sea lions are different in both physiology and diet, necessitating that they be considered independently of each other. Ultimately though, until a study can

compare the western and eastern populations, as well as rookeries and haulouts, over an identical or continuous time period, the western population must remain an area of continued concern due, by current calculations, to small population numbers and low energy-content diets.

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