THYROID HORMONE, CORTISOL AND LEPTIN ACTIVITY IN STELLER SEA LIONS
(*Eumetopias jubatus*) IN RESPONSE TO THYROID STIMULATING HORMONE
ADMINISTERED IN DIFFERENT SEASONS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF
HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF:

MASTER OF SCIENCE

IN

ANIMAL SCIENCES

DECEMBER 2014

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Acknowledgements

An extraordinary number of people, agencies and animals came together, on the Steller sea lion's behalf, to make this project possible. My committee members Dr. Shannon Atkinson, Dr. Douglas Vincent and Dr. James Carpenter contributed enormously with their knowledge, advice and an innate quality that allows them to question dogmatic physiological principles – allowing me to do the same. How else could one research marine mammals? Thank you for the freedom to question what has gone before.

Dr. Pam Tuomi and Millie Gray and the rest of the Alaska SeaLife Center mammal staff have shown the highest degree of professionalism and passion for their charges, working long hours with difficult animals (and humans) in the pursuit of understanding how Steller sea lions tick. Your trust in me and your belief in this project is truly the basis for its success.

I could not have finished this without the help of Dr. Tetsuzan Benny Ron. Simply put, he is the endocrinologist's endocrinologist and pushed me to color outside the lines. Dr. Halina Zaleski, thank you, thank you, thank you – for listening and for pushing me to make the right choices.

National Marine Fisheries Service: Thank you for forcing me to be concise and accurately defend my experimental design for permitting purposes. Days of tearing my hair out in exasperation during the permit application process translated into clear and beautifully defined physiological data from the most interesting marine mammal in the world.

This project would never have happened without the support of my family. Period. To my sons, Tristan and Graydon: sorry for the missed dinners – hot dogs can be a food group! To my husband, Jeffrey: you held down the fort. You listened. You believed. I thank you.

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LITERATURE REVIEW

1.1 Steller sea lions (*Eumetopias jubatus*)

Steller sea lions (*Eumetopias jubatus*) (SSL) are members of the Order Carnivora, Suborder Pinnipedia, Family Otariidae, Subfamily Otariinae and are the only existing representative of its genus (Rice, 1998, NMFS, 2008). The geographic range of the SSL extends from Southern California, across Alaska and the Aleutian chain to Japan. In response to genetic and biogeographic data, the population of Steller sea lions have been further divided into two distinct population segments (DPS, Eastern and Western) recognized under the Endangered Species Act (62 U.S Federal Register 24345) in 1997 at 144^0W (Cape Suckling, Alaska) (Bickham, et al., 1996; Loughlin, 1997). Following an observed population decline of approximately 82% from 1960's numbers, National Marine Fisheries Service (NMFS) published a final rule in 1990 listing the SSL as threatened under the Endangered Species Act (55 U.S Federal Register 49204) and the US Fish and Wildlife Service (FWS) published a rule adding SSL to the List of Endangered and Threatened Wildlife (55 U.S Federal Register 50005). In response to a continued decrease in population numbers, NMFS reclassified the Western DPS as endangered while retaining the increasing Eastern DPS as threatened in 1997 (62 U.S Federal Register 30772). Cause(s) of the decline are currently unconfirmed however, recovery of the Eastern DPS population is such that delisting was proposed in 2012 (NMFS, 2012) and completed in 2014. The Western DPS appears to have stabilized in some areas, but not across its entire range and maintains its endangered status as of 2014.

Steller sea lions exhibit pronounced sexual dimorphism, polygyny and are highly seasonal. Males may mate with as many as 30 females (Reidman, 1990) and can outweigh females by as much as 3-fold at the height of the breeding season (Loughlin, 2002) which occurs May through July. Females typically give birth to a single pup shortly following their arrival at rookeries in late May and estrus and mating occur approximately 10 days postpartum (Maniscalco, et al., 2006). Implantation is delayed and is believed to occur in late October/early November, after the embryonic diapause (Pitcher and Calkins, 1981). Approximately one week

following the birth of the pup, the adult female begins to forage and lactation continues most commonly throughout the first year of the pup's life, although cases have been reported in which lactation may last up to 4 years (Mamaev and Burkanov, 2004). The Steller sea lion's reproductive period is coincident with most periods of sample collection for the species in the wild, making the collection of samples that are not confounded by reproductive status in adults difficult, as well as limiting knowledge to a relatively brief period of time over the course of the year. The inaccessibility of free-ranging animals during the winter months of the subarctic precludes the ability to easily define the changing physiology of this highly seasonal species.

1.2 The Thyroid Hormones

Thyroid hormones have a broad range of profound effects on physiological processes. Systems influenced by thyroid hormones include: skeletal (bone growth and development), cardiovascular (cardiac output and protein synthesis), adipose tissues (development and function of both brown and white), fetal brain development, thermogenic function and O2 consumption, pituitary acivity (both synthesis and secretion of hormones), as well as numerous effects on the liver (see Hulbert, 2000, Yen, 2001, Cooper and Landenson, 2011 for extensive reviews). Thyroid hormones function both through direct action on gene transcription and through non-genomic interactions with specific enzymes (Oppenheimer, 1999). In pinnipeds, increased thyroid size and/or hormone concentration increases are closely associated with important life history events, such as neonatal growth (Stokken, et al., 1995, Myers, et al., 2006, Atkinson, et al., 2011), lactation (Harrison, et al., 1962), and molt (Boily, 1996, Routti, et al., 2010), as well as changes in thermoregulatory needs associated with season (Little, 1991, Oki and Atkinson, 2004, Verrier, et al., 2012). Conversely, reduced thyroid hormones are believed to be associated with diving-related hypothyroid states (Weingartner, et al., 2012), as well as general energy conservation via a reduced metabolic rate (Atkinson, et al., in review).

Thyroid hormones are synthesized by the thyroid gland, located in the pharynx and are found on either side of the trachea distal to the larynx in SSL. The cellular organization of the the pinniped thyroid gland is similar to most mammals (Harrison, et al., 1962), in that it is composed of two primary cell types, follicular and parafollicular cells. The follicular cells are

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epithelial cells that express receptors for thyroid stimulating hormone (TSH) and functions include the synthesis of thyroglobulin and concentration of iodide (Kacsoh, 2000, Hulbert, 2000). The thyroid is comprised of epithelial cells surrounded by a basement membrane, throughout which calcitonin-secreting parafollicular cells are located. The follicular cells enclose a lumen which contains proteinaceous fluid mainly consisting of thyroglobulin, which is the stored form of and precursor for thyroid hormones (Larsen, et al., 1998; Hulbert, 2000, Cooper and Ladenson, 2011, Norman and Henry, 2014). Components of the thyroglobulin molecule include up to 140 tyrosyl residues, four to eight of which are oriented sterically for iodination with oxidized iodide (Kacsoh, 2000, Squires, 2010, Cooper and Landenson, 2011). The oxidation of iodide to form iodine as well as the iodination of tyrosine residues is catalyzed by the membrane-bound enzyme thyroid peroxidase (TPO). TPO also catalyzes the linking of pairs of the iodinated tyrosines within the thyroglobulin molecule produce either thyroxine (T_4) or triiodothyronine (T_3) (Hulbert, 2000, Cooper and Landenson, 2011, Norman and Henry, 2014). Thyroid hormones bear several distinctions that make them unique among hormones. Among these, thyroid hormones are the only hormones that are stored in complete form outside the thyroid cells in a follicle by the body as well as being the only hormones that incorporate iodine in their structure (Dohan et al., 2003). In addition, the thyroid gland is believed to be the most ancient of the endocrine glands (Bently, 1998).

There are several forms of thyroid hormone (T_4: thyroxine and T_3; triiodothyronine and rT_3: reverse triiodothyronine, T_2: 3,3'-diiodothyronine,). T_3 is the most biologically active form of thyroid hormone (Tomasi,1991, Hulbert, 2000) and is primarily derived from deiodinated T_4. T_2 and rT_3 represent iodine-conserving, intermediate forms of thyroid hormone metabolism and are generally biologically inactive, although rT_3 has been shown to compete with T_3 for receptors under certain conditions (Hulbert, 2000, Dentice and Salvatore, 2011, Cooper and Ladenson, 2011). As thyroid hormones are hydrophobic in nature (Hulbert, 2000), once they are produced by the thyroid and released into circulation, the majority of T_3 and T_4 are quickly associated with carrier proteins with a small proportion remaining in the free form for direct biological activity. Among the transport proteins, there are 3 major carriers of thyroid hormones: thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin. (Cooper and Ladenson, 2011,

Norman and Henry, 2014). TBG has a single binding site for either T_3 or T_4 and has a high affinity for both hormones and, due to this high affinity, carries as much as 70% of the circulating thyroid hormones in humans (Larsen et al., 1998). TTR has an affinity of T_4 that is as much as ten times higher than T_3 and carries approximately 10% of circulating thyroid hormones (Norman and Henry, 2014). Albumin has a much lower affinity for T_4 and T_3. However, because the concentration of albumin in plasma is much greater than that of TTR and TBG, albumin accounts for approximately 15% of bound circulating thyroid hormones (Cooper and Landenson, 2011). It is thought that binding proteins also contribute to the conservation of iodine by preventing loss through renal clearance (Lechan et al, 2009; Hulbert, 2000; Squires, 2010).

Primary metabolism of thyroid hormones is through deiodination. Approximately 80% of T_4 is deiodinated to either T_3 or rT_3 by deiodinases, while T_3 acts directly with receptors in target tissues (Larsen et al., 1998; Hulbert, 2000; Cooper and Landenson, 2011). The remaining hormones are either sulfated or glucoronidated in the liver or kidneys or undergo deamination, decarboxylation or other cleavages that result in either inactive or compounds of very low biological activity (Hulbert, 2000). Deiodination is carried out by three, transmembrane, 5'-deiodinase enzymes that vary in their primary locations and specificity for substrates, as well as their physiological outcomes. The most abundant form, type 1 5'-deiodinase (D1), is found in the liver, kidneys, skeletal muscle and in the thyroid and its principal function is to provide T_3 in circulation. D1 also has a strong affinity for rT_3, as well as a lesser affinity for T_3, and catalyzes the conversion of both to T_2 (Norris, 1997, Larsen, et al., 1998, Norman and Henry, 2014). Type 2 5'-deiodinase (D2) is found in the brain and in the pituitary and is the primary source of T_3 for the central nervous system. D2 ensures that there is a constant local neuronal concentration of T_3 and is highly sensitive to T_4 concentration, decreasing D2 in response to elevated T_4 and conversely, increasing D2 concentrations in response to decreased T_4 (Hulbert, 2000; Cooper and Landenson, 2011, Norman and Henry, 2014). D2 is also found in brown adipose tissue, where its expression is increased during cold adaptation and the resultant increase of T_3 within the cells, in turn, increase heat production (Kacsoh, 2000). Type 3 5'-deiodinase (D3) is distributed throughout the brain, the placenta and the fetal tissues and is primarily responsible for

inactivation of T_4 and T_3 (Dentice and Salvatore, 2011, Norman and Henry, 2014). Through the conversion of T_3 to T_2 and the conversion of T_4 to rT_3, D3 protects both the brain and the fetus from overexposure to thyroid hormones (Norris 1997; Squires, 2010; Dentice and Salvatore, 2011). In contrast to fetal D3 concentrations, post-natal decreases in D3 allow for neonatal development (Dentice and Salvatore, 2011).

Thyroid hormones may act either through direct genomic pathways or through nongenomic interactions. Thyroid hormone actions taking place in the nucleus or at the mitochondria are mediated by thyroid receptors (TRs) which, upon binding to the hormone, then bind to thyroid responsive elements (TREs) on T_3 target genes (Tsai and O'Malley, 1994, Zhang and Lazar, 2000, Cheng, et al., 2010). TRs belong to the nuclear receptor superfamily and are found in two main forms, TRα and TRβ, that originate from genes on two different chromosomes (Cheng, et al., 2010). Each TR (α and β) gene encodes multiple isoforms and while all isoforms of TRβ (β1, β2) bind T_3, only the TRα1 variant of the TRα isoforms (the others: TRα2 and TRα3) actively bind T_3 in humans (Zhang and Lazar, 2000, Yen, 2001). Generally, TRs are similar in structure; a single chain that contains three distinct domains. The central domain binds with TREs, while the N-terminus varies between the isoforms and the carboxyl-terminus not only binds T3 (ligand-binding) but also interacts with an array of receptor coregulators (Lonard and O'Malley, 2007). TRs can bind to TREs in a number of forms: monomers, homodimers, or heterodimers. The most common form of heterodimer is TR bound to the TRE with retinoid X receptors (RXR) (Zhang and Lazar, 2000). While TR bound with ligand produces a conformational change that initiate transcription in target genes, TRs can bind to TREs in the absence of ligand, effectively repressing transcription. (Zhang and Lazar, 2000, Cheng et al., 2010).

Nongenomic effects of thyroid hormones are typically initiated within the cytoplasm or at the plasma membrane. Extensive reviews by Falkenstein, et al. (2000) and Cheng, et al. (2010) detail specific mechanisms that entail interactions with T_3 and, in a few instances T_4. At the plasma membrane, thyroid hormones modulate the calcium pump (Ca^{2+} - ATPase activity) (Gallo, et al., 1981), the sodium pump (Na, K-ATPase activity) (Bhagava, et al., 2007) and sodium current (Na+ current)(Huang, et al., 1994); 2-deoxyglucose uptake in thymocytes,

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diaphragm, heart and fat cells (Segal, 1989), endocytosis of type 2 and type 3 deiodinases (Davis, et al., 2008, Cheng, et al., 2010), as well as modulation of the epidermal growth factor receptor (Shih, et al., 2004). Thyroid hromones, via interactions with the mitochondria, modulate thermogenesis and ATP generation in addition to influencing mitochondria numbers within cells (Cheng, et al., 2010).

A receptor, integrin αvβ3, of the plasma membrane that contains both T3-specific binding sites and sites where T_3 and T_4 can act has recently been characterized (see Cheng et al., 2010). Thyroid hormone/integrin interactions are involved with movement of proteins in the cell and include the moving TRs to the cell nucleus (Cao, et al., 2009). Through the interactions of integrin and actin (Wiesner, et al., 2005), migration of neuronal and glial cells has been shown to be dependent on thyroid hormones (Farwell, et al., 2006). In addition, both angiogenesis and platelet aggregation, through the integrin membrane receptor, are influenced by thyroid hormones (Cheng, et al., 2010).

1.3 The Adrenal Glucocorticoid Hormone, Cortisol

Cortisol is a steroid glucocorticoid hormone synthesized primarily by the adrenal glands, located cranio-medial to the kidneys, under stimulation by adrenocorticotropin (ACTH) secreted by the anterior pituitary, although there are several lesser extra-adrenal sources (Kostadinova, et al., 2012). The adrenal glands, composed of an outer cortex and an interior medulla, are supplied by three arterial sources: the superior suprarenal artery, the middle suprarenal artery and the inferior suprarenal artery. Arterial supply is through both a plexus in the capsule of the adrenal that perfuses through the cortex into the medulla, in addition to direct arterial supply, and drained through a single adrenal vein from the adrenal medulla. Negative regulation of glucocorticoids is in the form of feedback inhibition on the pituitary and the hypothalamus in addition to the adrenal cortex by glucocorticoids in circulation (Kacsoh, 2000, Briassoulis, et al., 2011).

Lipoproteins in the plasma provide the majority of cholesterol needed for glucocorticoid synthesis in the adrenal cortex, although acetate and a small concentration of free cholesterol in the adrenal also serve as rapidly available substrates (Orth and Kovacs, 1998, Carroll et al., 2011). The primary site of ACTH activity within the adrenal cortex is the conversion of

cholesterol to pregnenolone, the initial step of cortisol synthesis. The site of this conversion is mitochondrial and is catalyzed by the enzyme CYP11A. Further metabolism of cholesterol at microsomal P450 occurs through P450 reductase and the product, pregnenolone is transported out of the mitochondria to the smooth endoplasmic reticulum. In the smooth endoplasmic reticulum, pregnenolone proceeds through a series of enzymatic steps to 17α—hydroxypregnenolone, then to 17α—hydroxyprogesterone, followed by hydroxylation to form 11-deoxycortisol and a final hydroxylation to cortisol (Norman and Litwack, 1997b, Orth and Kovacs, 1998, Kacsoh, 2000, Carroll, et al., 2011). While cortisol is secreted into circulation in unbound form, it becomes associated with transcortin (cortisol binding globulin, CBG). In bound form, cortisol is not biologically active and the CBG-cortisol complex can serve as a readily available source of hormone under stimulation (Orth and Kovacs, 1998, Carroll, et al., 2011). Metabolism of cortisol is carried out primarily in the liver through either conversion to inactive metabolites or conjugation to glucoronides or sulfates. It is thought that over 95% of cortisol metabolites are conjugated and cleared through urinary excretion (Orth and Kovacs, 1998, Kacsoh, 2000).

Cortisol has long been associated with the "fight or flight" response which implies the mobilization of physiological processes in response to extraordinary sources of stress. However, stressors can be acute, simple, daily events such as chasing prey, circadian rhythms or life history events such as adjusting to season or reproductive changes (McEwen and Seeman, 1999). Physiologically harmful effects typically arise due to exposure to cortisol released in response to chronic stressors (Sapolsky et al., 2000). As research into cortisol function in different species over different life history stages becomes more readily available, the definition of stress continues to evolve from contributions of Cannon on homeostasis (1932) and Selye on stress (1936) to attempts to construct concrete and broadly applicable definitions of stress and the introduction of concepts such as homeorhesis (Waddington, 1957) and allostasis (McEwen and Wingfield, 2007).

Cortisol actions affect numerous systems throughout the body. Functions range from what is considered the primary action: the facilitation of gluconeogenesis to actions on the central nervous system (see Raff and Findling, 2003, Carroll, et al., 2011 for extensive reviews)

In the SSL, cortisol responds to ACTH stimulation similarly to terrestrial mammal species (Mashburn and Atkinson, 2004, 2007) although adult females' peak responses appear to occur later in summer than in winter. Both sexes also show a decreased peak response to ACTH in summer when compared to winter. ACTH challenges in juvenile SSLs indicate that juveniles exhibit a cortisol response much more quickly than adults in both seasons (Mashburn and Atkinson, 2008). Of importance to the present study, cortisol has been shown to affect thyroid function (Perrone, et al., 1980, Carroll, et al., 2011, Walter, et al., 2012) yet little is known regarding effects of thyroid stimulation on the adrenal gland., particularly with regards to marine mammals in general or SSLs specifically.

1.4 Leptin Hormone

Leptin, a peptide hormone first decribed in 1994, is produced primarily by adipocytes and is involved primarily in energy balance, although continuing research indicates roles in other physiological functions such as reproduction and puberty (Houseknecht, et al., 1998, Casanueva and Dieguez, 1999, Chehab, 2014). Leptin acts at the level of the hypothalamus to regulate both anorexogenic (appetite suppressor) and orexogenic (appetite stimulant) compounds that ultimately serve to maintain energy balance (Kelesidis, et al., 2010). Leptin released from adipocytes also acts as a lipolytic compound yet suppresses enzymes involved in free fatty acid synthesis while maintaining glycerol release, supporting hydration, and providing a non-carbohydrate source for gluconeogenesis (Wang, et al., 1999, Frubeck and Gomez-Ambrosi, 2001; Chikani, 2004), an important function in species with very little access to dietary carbohydrate. Adipose tissue is the largest reservoir of metabolic water, dramatically illustrated by adipose tissue function in migratory birds or desert dwelling mammals (See Klaassen, 1996, Frank, 1998, Weber, 2011). SSLs rarely drink fresh water (Pilson, 1970, Ortiz, 2001 for extensive review). In fact, the desire to drink fresh water is a clinical symptom of leptospirosis in California sea lions (Dierauf, et al., 1985; Dunn, et al., 2001). Thus, water intake is primarily incidental to prey ingestion and digestion. This may indicate leptin serves an extremely important role in SSL water balance. Relative to the present study, TSH receptors have been identified in adipocytes and promote leptin secretion in humans (Santini, et al, 2010). SSLs have a lipid-based metabolism that is balanced with a need to conserve those lipids for other functions,

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such as thermoregulation, (Winship, et al., 2001, Pendleton, et al., 2006, du Dot, et al., 2009). There may be a seasonally-driven component that ensures appropriate leptin secretion based on current environmental conditions.

Receptors for leptin occur throughout the body, thus leptin exerts effects on numerous physiological systems. Although leptin research has focused primarily on its role in hunger and satiety (Tang-Christensen, et al., 1999; Morrison, et al., 2001, Mustonen, et al., 2005), the functions of leptin in reproduction, such as activation of the hypothalamic-pituitary-gonadal axis and effects on ovarian steroidogenesis (Mantzoros, 2000, Hausman, et al., 2012) are also of significant interest, particularly in puberty and sexual maturation (See Clarke and Henry, 1999, Suter, et al., 2000, Plant and Barker-Gibb, 2004, Cervero et al., 2006 for reviews). Leptin has been found in association with gonad structures in pigs (Rago, et al., 2009), placenta in baboons (Green, et al., 2000), and follicular fluid in humans (Fedorcsak, et al., 2000). Of particular interest to the present study, adrenal hormones and leptin occur in complex association (Bornstein, et al., 1997, Elimam, et al., 1998, Pralong and Gaillard, 2001, Leal-Cerro, et. al., 2001, Koutkia et al, 2003). SSLs utilize lipids for numerous and significant life history strategies, thus the role of leptin, and its relationship with glucocorticoids, have the potential to be magnified or different than that of terrestrial mammals.

1.5 Study Objectives

The roles that the hormones reviewed here play in maintaining homeostasis are significant and must operate in a complimentary fashion to achieve balance. Complex reproductive physiology, challenging environment, and strong seasonal components of the SSL life history underscores the value of understanding how leptin, thyroid hormones and cortisol interact under optimal conditions in healthy animals. This will allow for the construction of seasonal benchmarks of animal condition. Because researchers are often limited to a single sample from a free-ranging individual, it is likely that a suite of hormones as a bioindicator of physiological status will provide a more in-depth profile of that individual. This information, in turn, can provide accurate information for the population at large.

Because growth, survival, and reproductive success all hinge upon functional metabolism, the present project proposes to use bioindicators of metabolic processes to address issues such as metabolic function and the dynamics of the role that the hypothalamic–pituitary-thyroid axis may play in survival strategies across seasons in SSLs. The objectives of this study are (1) to determine whether or not a TSH challenge will elicit different thyroid responses according to season, and (2) to understand the influence of enhanced thyroid activity on other hormones involved in homeostatic balance; primarily cortisol and leptin.

Chapter 2

THYROID HORMONE, CORTISOL AND LEPTIN ACTIVITY IN STELLER SEA LIONS (*Eumetopias jubatus*) IN RESPONSE TO THYROID STIMULATING HORMONE ADMINISTERED IN DIFFERENT SEASONS

Abstract

To assess seasonal influence on thyroid function and its relationship with adrenal and leptin activity in Steller sea lions (*Eumetopias jubatus*; SSL), three captive reproductively intact SSLs (1 male, 2 female) were physiologically challenged using thyroid stimulating hormone (TSH) in each of two seasons, winter and summer. A sham challenge, following an identical protocol, was conducted on the same animals to use as reference. Serial blood samples, obtained over the course of both the sham and the TSH challenges, were assayed for four forms of thyroid hormone: total and free triiodothyronine (TT3 and FT3, respectively) and total and free thyroxine (TT4 and FT4, respectively), as well as cortisol and leptin. In winter, mean percent change from time 0 (T0) to peak concentrations in thyroid hormones were significantly different from the sham procedure (TT3: $P = 0.01$, TT4: $P = 0.007$, FT3: $P = 0.05$, FT4: $P = 0.001$). In contrast, only percent changes in TT4 concentrations of the thyroid suite were significantly different from the sham in summer (TT3: $P = 0.13$, TT4: $P = 0.002$, FT3: $P = 0.16$, FT4: $P = 0.10$). Cortisol mean percent change at peak was significant in both winter ($P = 0.02$) and in summer ($P = 0.004$). However, change from T0 in summer was negative, below baseline, for 150 of the 180 minute sampling period and reached a mean nadir of -48.9% at 105 minutes. Percent change in leptin response concentrations from T0 in winter to peak proved to be significant ($P = 0.002$) and although not significant, summer concentrations of leptin exhibited an overall negative trend during the course of the sampling period in response to TSH administration. The results indicate that exogenously administered TSH had a significant impact on thyroid, adrenal and leptin physiology in winter in SSLs. Most notably, exogenously administered TSH significantly lowered cortisol below baseline concentrations in summer, effectively down-regulating cortisol activity. This indicates that cortisol increases or decreases in response to elevated TSH based on season and suggests that there is a regulatory cascade acting apart from the HPA axis and

stimulated by the HPT axis that serves to actively and rapidly remove cortisol from circulation in summer that is not influenced or suppressed by the HPT in summer. This may indicate that the summer months represent a time of physiological conservation despite being the height of the breeding season, while winter appears to be a season of permissive energy expenditure. Both mechanisms indicate a linking of two or more endocrine systems that control energy balance and metabolism which are intricately controlled by external cues, rather than a reliance on diet or body condition to respond to TSH stimulation.

2.1 Introduction

Steller sea lions (*Eumetopias jubatus*) (SSL) are members of the Order Carnivora, Suborder Pinnipedia, Family Otariidae, Subfamily Otariinae and are the only existing representative of its genus (Rice, 1998, NMFS, 2008). The geographic range of the SSL extends from Southern California, across the Aleutian chain to Japan. In response to genetic and biogeographic data, the population of Steller sea lions have been further divided into two distinct population segments (DPS, Eastern and Western) recognized under the Endangered Species Act (62 U.S Federal Register 24345) in 1997 at 144^0W (Cape Suckling, Alaska) (Bickham, et al., 1996, Loughlin, 1997). Following an observed population decline of approximately 82% from 1960's numbers, National Marine Fisheries Service (NMFS) published a final rule in 1990 listing the SSL as threatened under the Endangered Species Act (55 U.S Federal Register 49204) and the US Fish and Wildlife Service (FWS) published a rule adding SSL to the List of Endangered and Threatened Wildlife (55 U.S Federal Register 50005).   In response to a continued decrease in population numbers, NMFS reclassified the Western DPS as endangered while retaining the increasing Eastern DPS as threatened in 1997 (62 U.S Federal Register 30772). Cause(s) of the decline are currently unconfirmed however, recovery of the Eastern DPS population is such that delisting was proposed in 2012 (NMFS, 2012) and completed in 2014. The Western DPS appears to have stabilized in some areas, but not across its entire range and maintains its endangered status as of 2014. The height of the SSL reproductive period is coincident with most periods of sample collection for the species in the wild, making the collection of physiological samples that are not confounded by reproductive state difficult. The inaccessibility of free-ranging animals during the winter months of the subarctic precludes the capability to completely define the changing physiology of this highly seasonal species.

2.2 Pinniped Thyroid Hormones, Cortisol, and Leptin

Thyroid hormones have a broad range of profound effects on physiological processes. These include effects on fetal development, neurological, neuromuscular and skeletal, cardiovascular effects; O_2 consumption and heat production; effects on the sympathetic nervous system, pulmonary and hematopoetic effects, as well as numerous effects on the liver (see

Hulbert, 2000, Yen, 2001, Cooper and Landenson, 2011 for extensive reviews). Thyroid hormone effects on metabolism and other endocrine systems are of primary concern to the present study (Hulbert, 2000, Cooper and Landenson, 2011). Thyroid hormones function through direct action on gene transcription and through non-genomic interactions with specific enzymes (Oppenheimer, 1999). In pinnipeds, increased thyroid size and/or hormone concentration increases are closely associated with important life history events, such as neonatal growth (Stokken, et al, 1995, Atkinson, et al., 2011), lactation (Harrison, et al., 1962) and molt (Boily, 1996, Myers, et al., 2006, Routti, et al., 2010), as well as changes in thermoregulatory needs associated with season (Little, 1991, Oki and Atkinson, 2004, Verrier, et al., 2012). Conversely, reduced thyroid hormones are believed to be associated with diving hypothyroid states (Weingartner, et al., 2012), as well as general energy conservation via a reduced metabolic rate (Atkinson, et al., in review). Thyroid hormones in numerous marine mammals, including pinniped species, are extensively reviewed and summarized by St. Aubin (2001).

Cortisol actions affect numerous systems throughout the body. Effects range from what is considered the primary function: the facilitation of gluconeogenesis to impacts on the central nervous system (see Raff and Findling, 2003, Carroll et al., 2011 for extensive reviews). In the SSL, cortisol responds to ACTH stimulation similarly to terrestrial mammal species (Mashburn and Atkinson, 2004, 2007) although adult females' peak responses appear to occur later in summer than in winter. Both sexes also show a decreased peak response to ACTH in summer when compared to winter. ACTH challenges in juvenile SSLs indicate that juveniles exhibit a cortisol response much more quickly than adults in both seasons (Mashburn and Atkinson, 2008). Of importance to the present study, cortisol has been shown to affect thyroid function (Carroll et al., 2011) yet little is known regarding effects of thyroid stimulation on the adrenal gland, particularly with regards to marine mammals in general or SSL specifically.

Leptin, a peptide hormone first described in 1994, is produced primarily by adipocytes and functions principally in energy balance, although continuing research indicates roles in other physiological functions such as reproduction and puberty (Houseknecht, et al., 1998, Casanueva and Dieguez, 1999, Chehab, 2014). Leptin acts at the level of the hypothalamus to regulate both anorexogenic (appetite suppressor) and orexogenic (appetite stimulant) compounds that

ultimately serve to maintain energy balance (Kelesidis, et al., 2010). Leptin released from adipocytes also acts as a lipolytic compound yet suppresses enzymes involved in free fatty acid synthesis while maintaining glycerol release, supporting hydration and providing a non-carbohydrate source for gluconeogenesis (Wang, et al., 1999, Frubeck and Gomez-Ambrosi, 2001, Chikani, 2004), an important function in species with very little access to dietary carbohydrate. Adipose tissue is the largest reservoir of metabolic water (Mellanby, 1942), highlighted by adipose tissue function in migratory birds or desert dwelling mammals (See Frank, 1988, Klaassen, 1996, Weber, 2011) SSL rarely drink fresh water and water intake is primarily incidental to prey ingestion and digestion (Pilson, 1970, Ortiz, 2001 for extensive review). In fact, the desire to drink fresh water is a clinical symptom of leptospirosis in California sea lions (Dierauf, et al., 1985, Dunn, et al., 2001). This may indicate leptin from blubber adipocytes serves an extremely important role in healthy SSL water balance.

Relative to the present study, TSH receptors have been identified in adipocytes and promote leptin secretion in humans (Santini, et al., 2010). In addition, adrenal hormones and leptin occur in complex association (Bornstein, et al., 1997, Elimam, et al., 1998, Pralong and Gaillard, 2001, Leal-Cerro, et. al., 2001, Koutkia et al., 2003). Very few SSL studies have included leptin and they have primarily investigated the relationship of leptin to body condition (Rea and Nagy, 2000, Kumagi, 2004) or response to adrenal stimulation in juvenile animals (Mashburn and Atkinson, 2008). Interestingly, Ortiz et al. (2000) found that there was no correlation between fat mass and leptin concentrations in northern elephant seals, as was also the case in Antarctic fur seals (Arnould, et al., 2002). SSL have a lipid-based metabolism that is balanced with a need to conserve those lipids for other functions (Pabst, et al. 1999, Mellish, et al., 2007), there may be a seasonally-driven component that ensures appropriate leptin secretion based on current environmental conditions as is seen in seasonal, terrestrial mammals (Rousseau, et al., 2002*a*, Li and Wang, 2007). The role of leptin, and its relationship with glucocorticoids and thyroid hormones in energy balance, has the potential to be magnified or different in SSL than that of non-seasonal, terrestrial mammals (Ortiz, et al., Arnould, et al., 2002, Rousseau, et al.,2002*b*).

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2.3 Study Objectives

Because growth, survival and reproductive success all hinge upon functional metabolism, the overall goal of this study was to use bioindicators of metabolic processes to address issues such as metabolic function and the dynamics of the role that the hypothalamic – pituitary-thyroid axis may play in survival strategies across seasons in SSL. The objectives of this study were (1) to determine whether or not a TSH challenge would elicit different thyroid responses according to season, and (2) to understand the influence of thyroid activity on other hormones involved in homeostatic balance; primarily cortisol and leptin.

2.4 Methods and Materials

2.4.1 Animals and Samples

Blood samples for TSH challenge trials were obtained from three adult (1 male, 2 females), reproductively intact, permanently captive SSL housed under ambient conditions at the Alaska SeaLife Center (ASLC, Seward, Alaska; N $60^{°}12'$ latitude, W $149^{°}42'$ longitude). Care and feeding of the animals was identical to studies previously reported for SSL by Mashburn and Atkinson (2004, 2007, 2008). Genders were housed separately, although were not cut off from visual, olfactory and auditory contacts. Environmental conditions, such as photoperiod, ambient air and sea temperatures and animal weights and feed intakes are summarized in Figure 2.1. The study was conducted under Federal permits # 782-1532 and 881-1668 and the Alaska SeaLife Center Institutional Animal Care and Use Committee Protocol Number 07-003 (Appendix A.).

All sera used for this study, whether derived from whole blood collected via caudal plexus veinipuncture or through hindflipper vein catheterization, was refrigerated prior to serum harvest and 1 ml aliquots of harvested sera were stored frozen at -80°C until radioimmunoassay (RIA).

2.4.2. TSH Challenge

Animals were treated with a single IV injection (300 μg [2.73 ml] TSH) of a highly purified recombinant form of TSH (Thyrogen; manufactured by Genzyme Corp. Cambridge,

MA 02142) in January and May of 2008. The sham challenge took place in February of 2008 during which the same animals received an equivalent volume (2.73 ml) of sterile saline. Animals were sedated using isoflurane anesthesia, previously shown to not affect concentrations of the hormones being measured (Mashburn and Atkinson, 2004), and a blood sample was drawn from the caudal plexus immediately prior to TSH injection (Time 0, T0) which allowed animals to serve as their own controls.   All methods for sampling and collection were identical to those used in Mashburn and Atkinson (2004). Briefly, animals remained under anesthesia for 3 hours during which blood samples (20 ml) were drawn every 15 minutes via an in-dwelling catheter (18 gauge, 3 in) placed in a hind-flipper vein. The catheter was flushed with approximately 3 ml sterile heparinized saline (concentration: 25 IU/ml) after each blood draw. At each new blood draw, the initial saline portion was discarded. The male was anesthetized pre-TSH injection, during which blood samples were collected as described above. After 3 hours, the animals were removed from anesthesia and, once they had fully recovered, were allowed to return to their enclosures. Twenty four hours post TSH injection, and additional, voluntary blood sample was taken to ensure that all animals had returned to their normal baseline hormonal concentrations.

2.4.3 Radioimmunoassays and Quality Control

Four forms of thyroid hormones, total T_4, total T_3, free T_4 and free T_3, as well as cortisol, were measured in serum, in duplicate, by direct assay using solid phase RIA (Diagnostic Products Corporation, Los Angeles, USA) previously validated in SSL (Mashburn and Atkinson, 2004, Myers et al., 2006). Radioactivity of the bound portion for all assays was counted using a gamma counter (Gamma C12, Diagnostic Products Corporation, Los Angeles CA). All samples were initially run neat and samples were analyzed in duplicate.

Cross-reactivities for all thyroid hormone assays, as listed by the manufacturer, were as follows : Total T_4, L-Thyroxine, 100%; D-Thyroxine, 64%; Tetraiodothyroacetic acid, 104%; and 2% or less for all other compounds tested. Total T_3, Triiodo-L-thyronine (T_3), 100%; Triiodo-L-thyronine (reverse T_3), 0.014%; Triiodo-D-thyronine 100%, and 1% or less for all other compounds tested. Free T_4 100% and 0.03 % or less for all compounds listed. Free T_3 100% and 0.001% or less for all other compounds tested.

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Cortisol concentrations were determined by direct assay using a solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, USA) previously validated in Steller sea lions (Mashburn and Atkinson, 2004, Myers et al., 2006). Leptin was measured using a double-antibody RIA kit for leptin (Linco Research, St. Charles, MO) previously validated for use in SSL by Mashburn and Atkinson (2008).

Manufacturer cross-reactivities for the assays were: Cortisol: Prednisolone, 76%; Methylprednisolone, 12%; 11-Deoxycortisol, 11.4%, and 2% or less for all other compounds tested; Leptin: human leptin (100%), mouse leptin (73%), porcine leptin (67%), rat leptin (61%), canine leptin (3%) and not detectable for all other hormones tested.

Those samples binding above 80% and below 20% or below the assay level of sensitivity were re-assayed following dilution or increased volume to obtain a more accurate measurement of concentration. Intraassay coefficients of variation were $< 5\%$. Inter-assay coefficients of variation for two separate internal controls, in the case of multiple assays for a single hormone, and assay sensitivities are presented in Table 2.1.

2.4.4 Data Analysis

Hormone concentrations were determined using a log-logit transformation of the standard curve for each hormone assayed (Rodbard, 1974). Peak concentrations were defined as those concentrations that exhibited the highest value over the time course of the trial. Absolute concentrations of hormone at peak, corrected for dilution where necessary, were statistically compared to T0 using T-tests for all individuals for each hormone. Time 0 values allow animals to serve as their own controls. Absolute concentrations were also converted to percent change in their concentration relative to T0 for each animal to more precisely reflect individual responses to TSH administration. These values were then compared to percent changes for the same time for each individual's sham trial and significance determined using T-tests. Area under the curve were performed using the trapezoidal rule ($Area = \Sigma_i[y_i(x_{i+1} - x_i) + 0.5(y_{i+1} - y_i)(x_{i+1} - x_i)]$). Statistical analyses and area under the curve calculations were performed using SigmaStat version 12.0 (Systat Software Inc., San Jose, California. USA.).

2.5 Results

Sham trials resulted in no significant percent changes in concentrations from those at T0 (Figure 2.2). However, thyroid hormones TT_3, TT_4, FT_3, and FT_4 exhibited elevated concentrations in response to TSH administration (Table 2.2). Changes in concentration for all four thyroid hormones were significant in winter, while only TT_4 was significantly different from T0 in summer (Table 2.2) and seasonal peak concentration differences of thyroid hormones were significant in TT_4 and FT_4 (Table 2.2). The percent change from T0 in TT_3, TT_4, FT_3, and FT_4 concentrations differed significantly from the sham values in the winter TSH trial (Table 2.3, Fig. 2.3). Among the thyroid hormones, only percent change in TT_4 from sham values was significant in summer (Table 2.3). There were no differences in time to reach peak concentrations between winter and summer trials in any of the thyroid hormones (Table 2.4). A summary of the results for the percent change in thyroid hormone concentrations from T0, statistically compared to sham concentrations for identical sampling times is shown in Figure 2.3.

Cortisol also exhibited a response to TSH administration and proved to be significantly different from T0 concentrations in both winter and summer trials (Table 2.2). However, the direction of response, notably, differed according to season (Fig. 2.4). Cortisol concentrations in winter increased 288.7% while summer concentrations decreased 48.9% relative to T0 (Table 2.3, Fig 2.4). As a consequence of the differences in summer and winter adrenal response, there was a significant seasonal difference between peak concentrations of cortisol (Table 2.2) and percent change (Table 2.3) although there was no seasonal difference in time to peak cortisol response (Table 2.4).

TSH administration also resulted in a response in leptin concentrations which proved to be significantly higher than T0 concentrations in winter only (Table 2.2, Fig. 2.4). Further, mean percent change in in leptin concentrations from T0, was significantly different from sham concentrations in the winter TSH trials but not in summer (Table 2.3). However, leptin percent change from T0, as in cortisol, differed in the direction of response with winter concentrations increasing and summer concentrations decreasing in an overall negative (although not

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significant) response as is shown in (Fig. 2.4). As a result in the opposing directions of response, leptin exhibited a significant seasonal difference in concentrations at peak (Table 2.4).

The response to TSH administration, particularly in thyroid hormones, tended to vary between individuals and is emphasized by area under curves (Appendix B, 1-6). This variability was not related to gender nor season.

2.6. Discussion

All the SSLs in the study exhibited a response to TSH administration in both seasons. However, increases in the complete suite of thyroid hormones proved to be significant only in the winter. In the summer, only TT_4 changes in concentration exhibited statistical significance (i.e. $P < 0.05$). This is consistent with significantly higher thyroid hormone concentrations in winter than in summer found in harbor seals (Oki and Atkinson, 2004). The results suggest that there is an increase in thyroid sensitivity to stimulation by TSH in the winter months relative to summer, as well as a subsequent increase in D1 and D2 iodinase activity in response to heightened circulating T_4 that led to increased T_3 concentrations (Gerenben, et al., 2008, Bianco and Kim, 2006). As the scope of the study did not include T_2 nor rT_3 measurement, it is unknown if D3-driven thyroid hormone metabolism also increased in winter. TT_4 increased significantly without a commensurate increase in FT_3 or FT_4, an indication of a decrease in D2 iodinases in summer in comparison to winter and may be a means to deter local neuronal overexposures to T_3 and overproduction of heat in brown adipose tissue (Silva, 2003, Norman and Henry, 2014). Additionally, reduced free T_3 in circulation provides a means to conserve energy and resources (Silva, 2003, Onur, et al., 2005). That TSH stimulated a significant increase in TT_4 in summer without an increase in TT_3, FT_4 or FT_3 may indicate that other physiological factors are necessary for the initiation of T_3-mediated processes above those required for basic metabolism and homeostasis during the summer in SSL.

In humans, thyroid hormone concentrations vary by individual (Andersen et al, 2003, van der Deure et al, 2010) and is not attributable to one single factor (Johnstone, et al, 2005, Lema, 2014). Thus it was not surprising that SSLs also exhibited variability between individuals in their baseline concentrations (Appendix B, 1-6). Both the timing of peak responses to TSH

administration and the concentrations of thyroid hormones representing those peaks have also been shown to be variable by individual (Benker, et al., 1985, Fail et al, 1999). Therefore, that the SSL in the study were variable, though not significantly, in the time to reach peak response was also not unexpected (Appendix B, 1-6). Differences between individual SSL thyroid responses to TSH administration in the study were not attributable to gender nor to season, as measured by thyroid hormone concentrations, although the gender evaluation is somewhat subjective with only one male SSL.

Additionally, results indicated that under the influence of TSH both cortisol and leptin increased significantly in the winter in SSL. This suggests either a stimulatory effect of increased thyroid hormones upon the adrenal glands and adipoctyes or a permissive milieu that exists under TSH stimulation in the winter in the SSL. Winter conditions in the subarctic are physically demanding, thus it is not surprising that increased metabolic activity in response to several factors, ie. cold, chasing prey or growth-rate demands in juveniles is mirrored by an increase in cortisol-mediated gluconeogenesis or increases in leptin. In essence, life-history functions appear to be recognized as a stressor in winter. The increase in leptin in winter may be an index of enhanced demand for energy in the form of free fatty acids as well as an increased need for metabolic water for physiological processes, rather than a signal of satiety (Margetic, et al., 2002, Carlton, et al., 2012, Zhang, et al., 2013).

For the SSL, allocation of resources is a critical process in winter (Rosen and Kumagai, 2008). Reproductive events, such as implantation of embryos and lactation, occur in breeding females over the winter. While it is likely that the annual cycle of sperm production in breeding males does not begin until roughly two months prior to the breeding season, winter represents a time during which they must recover from breeding losses as well as begin to produce sperm in late winter and early spring. Yearlings and juveniles must maintain a normal rate of growth while learning to hunt at sea over the winter (Winship, et al., 2001), as many of their mothers will produce offspring and breed the following summer (see Calkins and Pitcher, 1982, Pendleton, et al., 2006 for SSL life history demands). Each of these processes incur their own energetic costs and the demand is increased by the need to thermoregulate in winter subarctic waters. Thus, SSL must be able to quickly respond to metabolic demands and the results of this

study indicate that there is a seasonal sensitivity to TSH that allows the SSL to meet these demands in winter and tends toward conservation of resources in summer (du Dot, et al., 2009).

In the summer, in addition to a negative trend in leptin concentrations, increased TSH resulted in a negative effect on cortisol opposite to the enhanced activity in the winter. Cortisol decreased below baseline within 15 minutes post-TSH administration. As the normal half-life of cortisol in human circulation is ~ 66 minutes (McKay and Cidlowski, 2003), this suggests that there is a yet-unreported and rapidly-acting mechanism that removes cortisol from SSL circulation in the summer under the influence of increased TSH. The results of this study indicate that the effect endures for well over 2 hours, with cortisol returning briefly to baseline concentrations at 165 minutes post TSH, before returning to below-baseline concentrations at 3 hours.

At present, it is unclear as to the precise cascade that leads to the removal of cortisol from circulation. It does suggest, however, that there are physiological processes in place in the SSL to prevent TSH-mediated increases in cortisol during the summer while the opposite is true in the winter. The summer is an abbreviated season in the subarctic, a time when females must give birth, breed and maintain lactation in addition to feeding (Calkins and Pitcher, 1982, Pendleton, et al., 2006). Males must maintain a seasonal increase in body weight (Pitcher and Calkins, 1981, Loughlin, 2002) while breeding with numerous females and defending a territory (Reidman, 1990) over the summer. Juveniles must compete for food with a larger congregation of adult animals as well as attain adult size (York, 1994, Thompton, et al., 2008) during the brief summer months. It may be that the SSL physiology distinguishes life-history processes differently according to season. In winter, it may be that energy demands are identified as acute stressors, while in the summer the emphasis is on the reduction of energy expenditure and may be essential to the increase of a blubber layer sufficient to withstand the rigors of the winter months (Winship, et al., 2001, Pendleton, et al., 2006, du Dot, et al., 2009).

It is important to note that, while all animals used in the study were reproductively intact adults, they were not an example of what would be "typical" in the wild. That is, in addition to a small sample size, the animals were not reproductively active and may or may not have exhibited

the endocrine patterns of their wild counterparts. During the summer, adult SSL females typically give birth, experience lactational estrus and embryonic diapause; while males "bulk up", reduce feeding bouts at sea and breed (Pitcher and Calkins, 1981 Mamaev and Burkanov, 2004, Maniscalco, et al., 2006). With such a limited number of animals in the study, it is difficult to draw hard conclusions. However, the results point to a unique, seasonally-driven regulation of metabolism in this species that allows SSLs to thrive under extreme subarctic winters that is outside of the HPT axis. This, in agreement with Rea, et al. (2009), indicates that biomarkers of metabolic function derived from a single sample, such as the thyroid hormones, may be insufficient to determine physiological health or well-being in SSL. Future directions for continued research include an increase in sample size, longitudinal sample collections and inclusion of animals in different reproductive states. This will allow for a more accurate identification of a suite of factors which act to seasonally regulate metabolism in a species from which, typically, only a single field sample can be collected.

Table 2.1. Quality control data for thyroid hormone, cortisol and leptin radioimmunoassays (RIA) including number of RIAs, coefficient of variation for high and low concentration internal assay controls and assay sensitivities for samples collected following TSH administration and sham procedures in Steller sea lions over winter and summer seasons.

Hormone	Internal Control High %CV	Internal Control Low %CV	Assay Sensitivity	n
TT_3	3.81	4.11	18.55 ng/ml	3
TT_4	2.60	0.16	5.60 ng/ml	2
FT_3	3.81	4.11	0.56 pg/ml	4
FT_4	3.01	1.36	0.30 pg/ml	2
Cortisol	4.03	2.47	5.77 ng/ml	2
Leptin	3.04	2.47	0.82 ng/ml	2

Table 2.2. Mean concentrations at Time 0; mean concentrations at peak, differences between Time 0 and peak concentrations ($P < 0.05$); and seasonal differences ($P < 0.05$) in concentrations at peak following TSH administration in Steller sea lion in both winter and summer.

Hormone	Mean T0 ± (SE) Concentration Winter	Mean Peak ± (SE) Concentration Winter	P Winter	Mean T0 ± (SE) Concentration Summer	Mean T0 ± (SE) Concentration Summer	P Summer	Mean Peak Concentration Winter vs. Summer P
TT3 ng/ml	342.0 ± 54.5	810.2 ± 181.9	0.035	318.6 ± 17.4	1005.8 ± 341.1	0.060	0.64
TT4 ng/ml	12.7 ± 3.0	33.1 ± 6.1	0.019	7.4 ± 0.8	14.1 ± 50.9	0.003	0.04
FT3 pg/ml	0.6 ± 0.2	1.1 ± 0.1	0.015	0.2 ± 0.01	1.9 ± 1.1	0.093	0.48
FT4 pg/ml	10.3 ± 0.9	66.9 ± 21.4	0.028	8.7 ± 0.5	15.9 ± 5.3	0.123	0.08
Cortisol ng/ml	46.5 ± 4.7	140.3 ± 29.1	0.017	91.0 ± 20.8	43.5 ± 4.7	0.045	0.03
Leptin ng/ml	3.4 ± 0.2	5.8 ± 0.3	0.001	2.3 ± 0.12	3.5 ± 0.7	0.080	0.03

Table 2.3 Percent change in concentration from Time 0 at time of peak for winter and summer trials and for the same times in sham trial, differences in percent change between sham and TSH concentrations (0.05<P), and seasonal differences between % change at peak in winter and summer (0.05<P).

Hormone	Mean % Change Winter Sham	Mean % Change Peak Winter TSH	Sham vs. TSH Winter P	Mean % Change Summer Sham	Mean % Change Peak Summer TSH	Sham vs. TSH Summer P	Mean % Peak Difference Winter vs Summer TSH P
TT$_3$	-1.2 ± 0.26	113.6 ± 29.1	0.010	-18.0 ± 8.4	228.2 ± 129.2	0.130	0.506
TT$_4$	-12.1 ± 22.9	130.4 ± 16.4	0.007	-1.1 ± 12.2	092.6 ± 007.1	0.002	0.102
FT$_3$	-6.3 ± 6.3	82.7 ± 31.5	0.050	-3.3 ± 8.3	998.0 ± 589.4	0.160	0.196
FT$_4$	-16.3 ± 7.4	533.6 ± 155.2	0.001	-19.7 ± 11.8	106.0 ± 058.2	0.100	0.061
Cortisol	14.5 ± 10.6	288.0 ± 70.3.6	0.020	-12.5 ± 6.4	-48.9 ± 26.9	0.004	0.009
Leptin	9.7 ± 6.45	60.6 ± 7.4	0.002	-6.3 ± 03.4	50.0 ± 27.0	0.112	0.698

Table 2.4. Mean times to peak in winter and summer and seasonal differences ($P < 0.05$) in time to peak following TSH administration in Steller sea lions in both winter and summer seasons.

Hormone	Mean Time to Peak Winter (Min)	Mean Time to Peak Summer (Min)	Time to Peak Difference Winter vs Summer P
TT_3	95.0 ± 13.2	115.0 ± 27.8	0.552
TT_4	130.0 ± 27.8	145.0 ± 21.8	0.693
FT_3	130.0 ± 27.8	95.0 ± 5.0	0.759
FT_4	95.0 ± 21.8	115.0 ± 25.0	0.290
Cortisol	120.0 ± 15.0	80.0 ± 25.0	0.242
Leptin	145.0 ± 18.0	140.0 ± 18.0	0.854
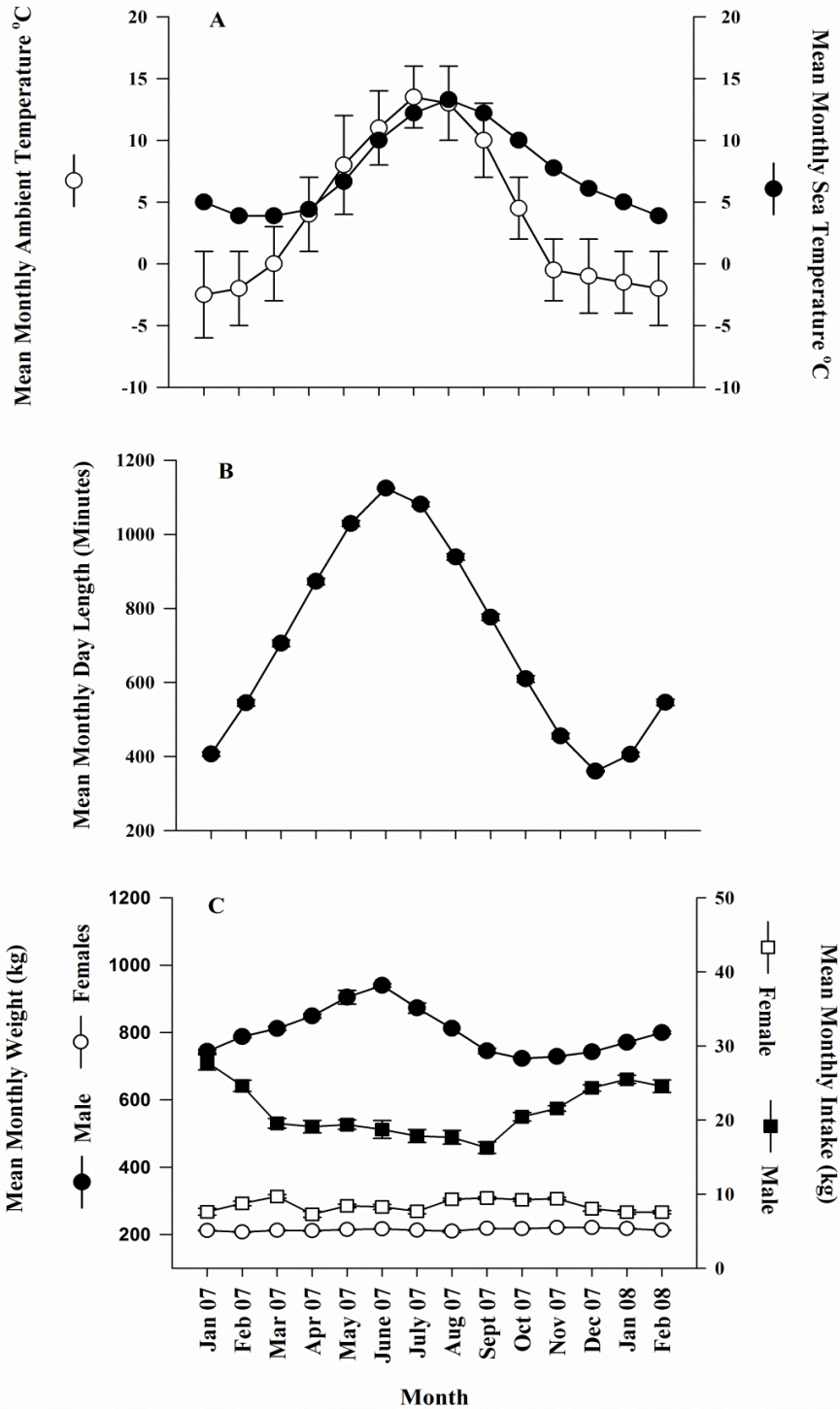
Figure 2.1. A) Mean monthly ambient air and sea temperatures, B) mean monthly day length, and C) mean monthly weights and feed intakes for Steller sea lions housed at the Alaska SeaLife Center, Seward, Alaska from January 2007 to February 2008.
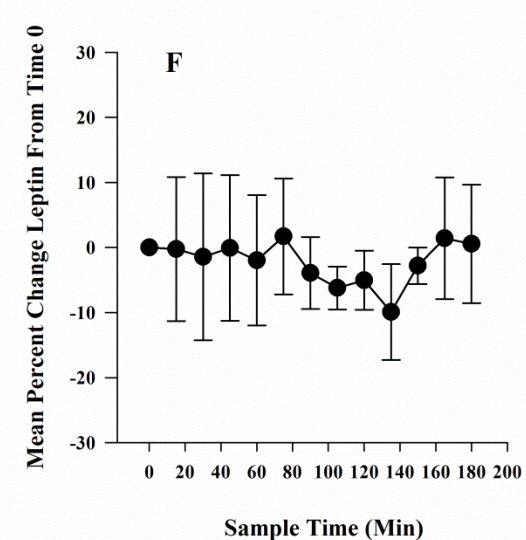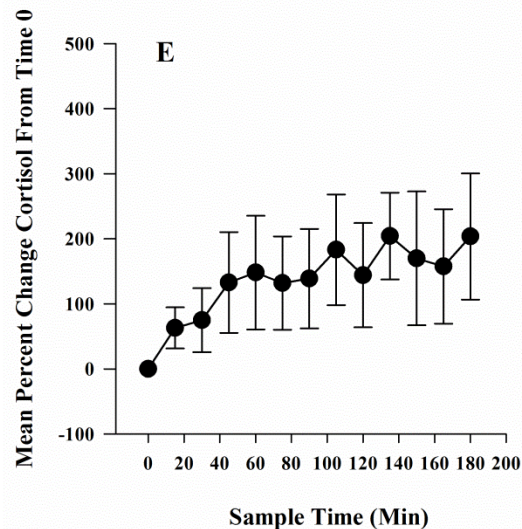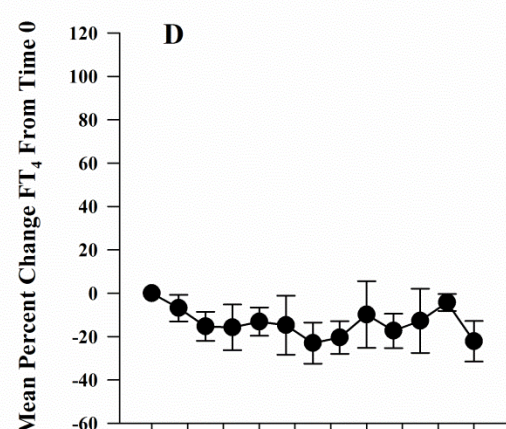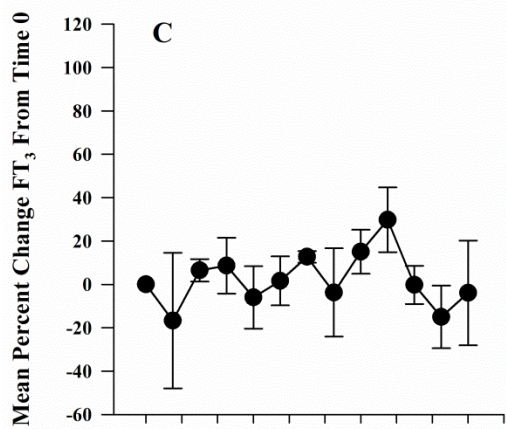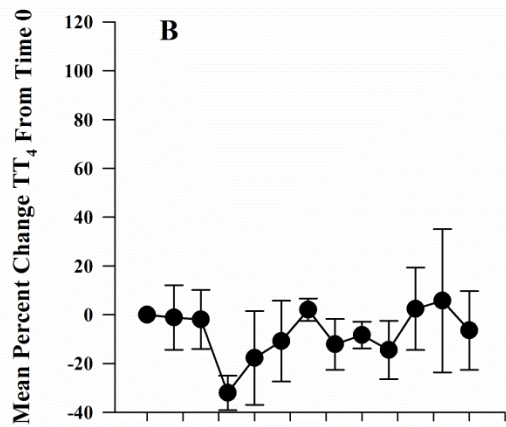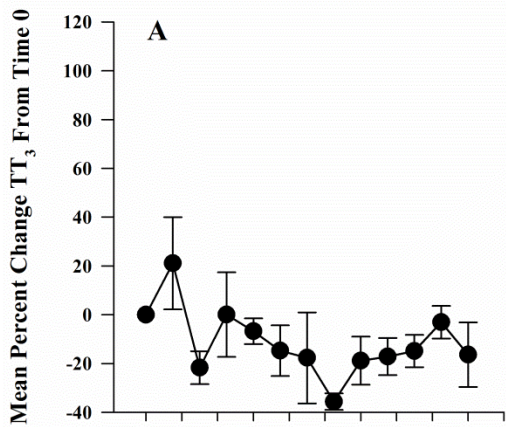
Figure 2.2. Mean percent change from Time 0 concentrations in sham challenges for A) TT_3, B) TT_4, C) FT_3, D) FT_4, E) cortisol and F) leptin in Steller sea lions.
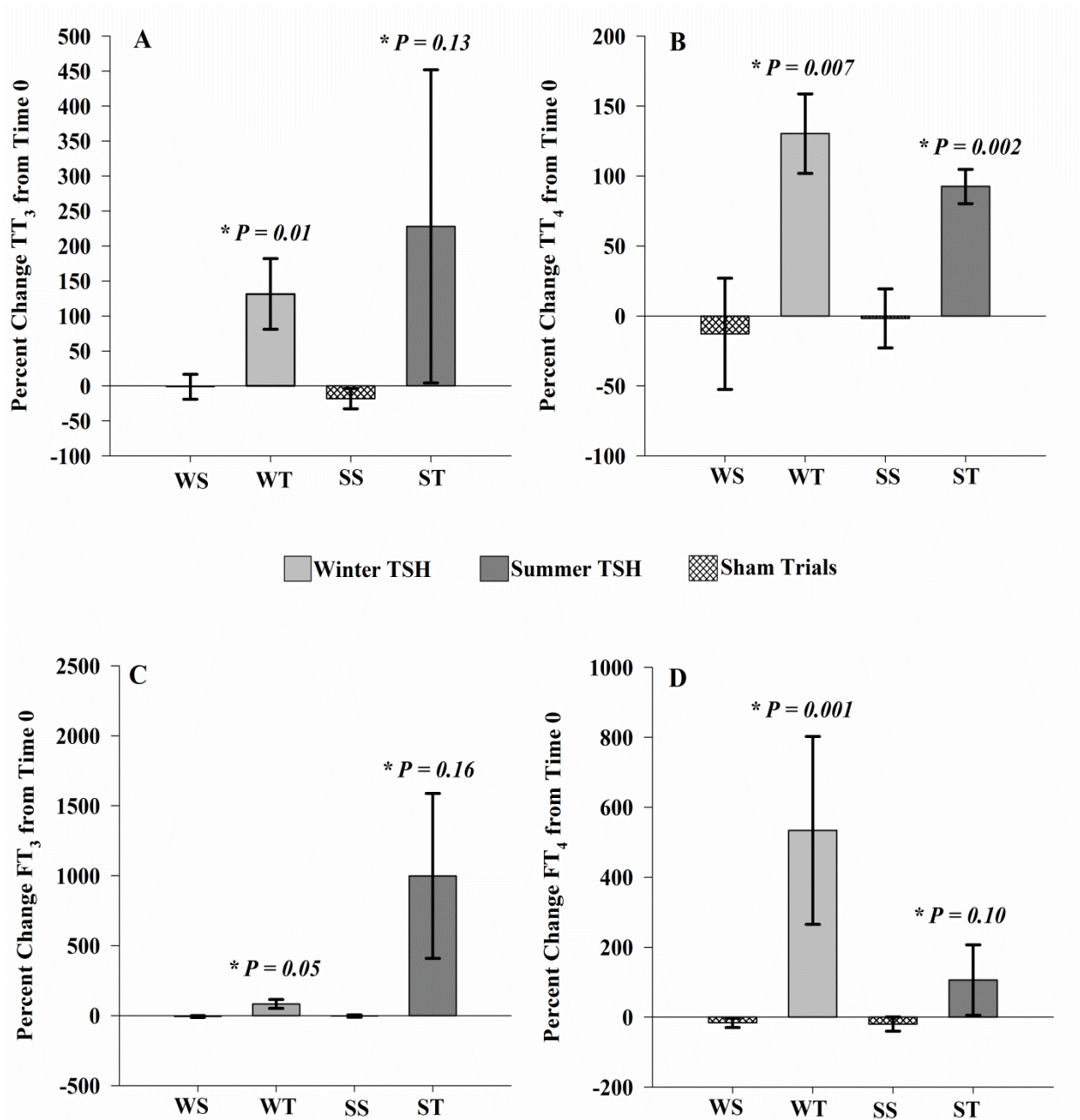
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Figure 2.3. Percent change from Time 0 to concentration peaks at the same times for A) TT$_3$, B) TT$_4$, C) FT$_3$, D) FT$_4$ during winter sham (WS), winter TSH trial (WT), summer sham (SS) and summer TSH trial (ST) in Steller sea lions ($P < 0.05$).
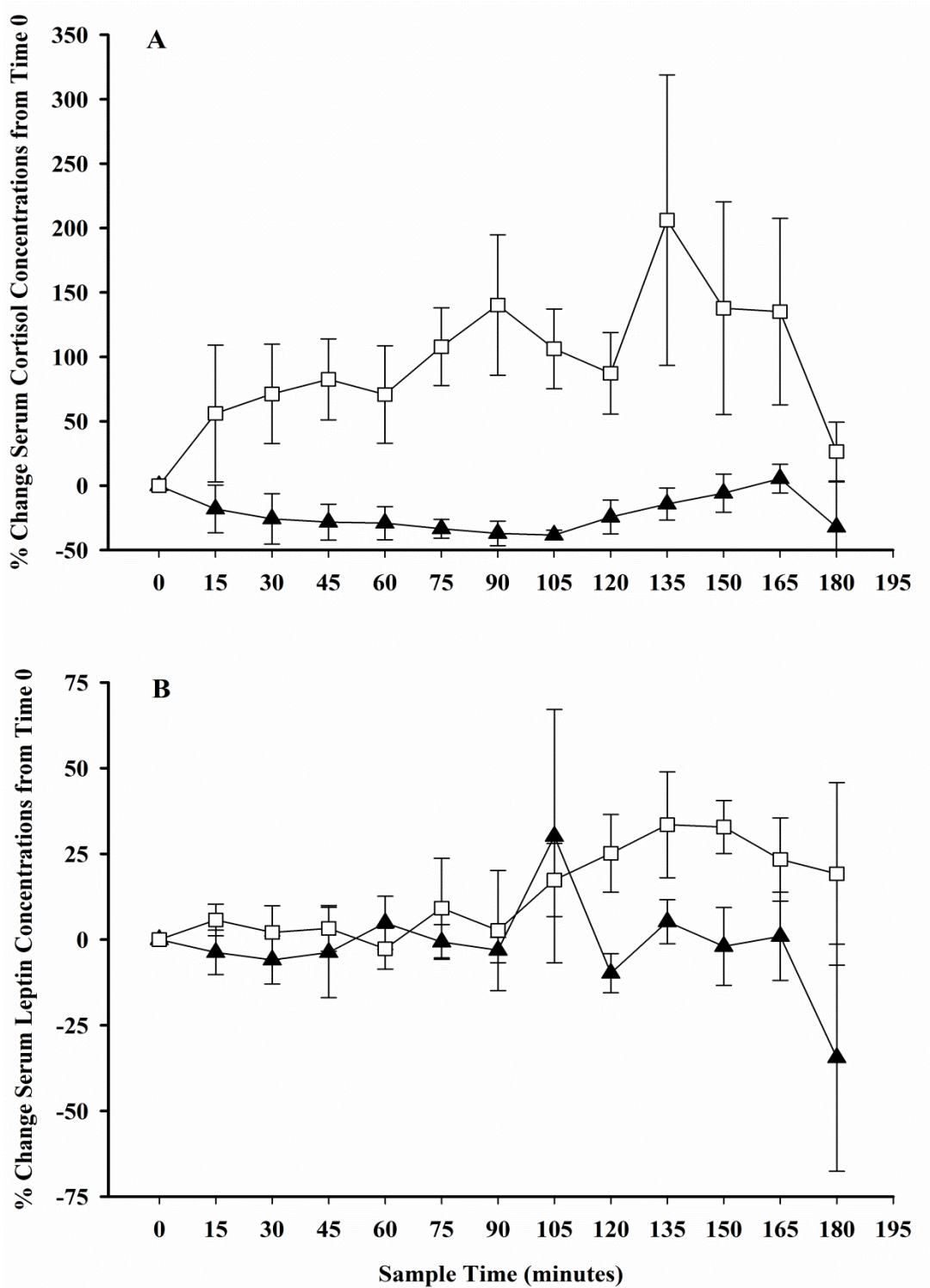
30

Figure 2.4. Percent change in A) cortisol and B) leptin concentrations from Time 0 in Steller sea lions in winter (□) and summer (▲) TSH challenge trials.

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Chapter 3

OVERALL SUMMARY

3.1 Conclusions

In summary, a small sample size ($n = 3$) of captive adult Steller sea lions exhibited a physiological response to exogenously administered TSH that was strongly conservative in the summer as described by thyroid, cortisol and leptin responses. Though there were increases in concentration, with the exception of negative cortisol concentrations in response to TSH, only one hormone (TT_4) was significantly different than T0 concentrations. In the winter, with significant concentration increases in all 6 hormones tested, the reverse was true. This leads, cautiously, to a number of conclusions. First, cortisol increases or decreases in response to elevated TSH based on season. This indicates that there is a regulatory cascade acting apart from the HPA axis and stimulated by the HPT axis that serves to actively and rapidly remove cortisol from circulation in summer that is not influenced or suppressed by the HPT in summer. Second, the role of leptin in SSL becomes much more intriguing. The slow increase to significant concentrations over baseline indicate that adipocytes are undergoing metabolism in response to metabolic requirements. It may be that rather than a primary role as a signal of satiety, leptin is more important in acting as a mediator of water and glycerol production from adipocytes. Third, in sharp contrast to the summer hormonal response, TSH elicits activity in all four of the primary thyroid hormones as well as in cortisol and leptin, suggesting an emphasis on energy mobilization. In this light, the adaptive significance of embryonic diapause and foraging bouts, leaving newborn pups on the rookery, by lactating SSL females becomes clear. As the most important function in summer for Steller sea lions is preparation for winter, yet breeding, birth, and growth must also occur, metabolic activity must be tightly regulated and highly selective in functional priorities.

3.2 Implications

The results of this study indicate that monitoring a single biomarker in defining health of SSL in different seasons is inadequate and has the potential to be misleading, particularly in free-

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ranging animals and most certainly in investigating stress. It also implies that Steller sea lions may be physiologically well-suited to remain healthy under food-limited conditions of short duration over the summer, as all metabolic functions appear to be devoted to accruing energy resources.

3.3 Future Directions

Because this is a small sample size and atypical in that none of the animals were breeding during the summer challenge as their free-ranging counterparts would be, it is of primary importance to obtain longitudinal samples from breeding and pregnant animals. Not only will these samples illustrate the influence of breeding condition on the interactions of thyroid hormones, glucocorticoids and leptin, it will indicate, if these hormones do in fact follow a similar pattern, when the physiology of the SSL converts from energy conservation to energy expenditure. This may also allow researchers to understand the trigger(s) that initiate this conversion.

The role of leptin in SSLs (and other marine mammals) may prove to be important in an accurate definition of water balance in a non-freshwater drinking mammal. Because leptin has proved not to be correlated with body condition, as with terrestrial mammals (including man), and the response to TSH administration changes according to season, the association with glycerol and metabolic water production may be key to SSL survival. Larger sample sizes, different life history stages, and longitudinal sampling are necessary for further investigation.

Appendix A.

Alaska SeaLife Center
w i n d o w s   t o   t h e   s e a

May 4, 2007

Dr. Shannon Atkinson
Alaska SeaLife Center
P.O. Box 1329
Seward, AK 99664

Dear Shannon:

The following protocol using vertebrate animals was reviewed and approved by the Alaska SeaLife Center Institutional Animal Care and Use Committee (IACUC). The "Assurance of Animal Care" form will be kept on file in the office of the IACUC Recording Secretary.

This Assurance is valid for twelve (12) months after approval and must be kept current with respect to new methods or techniques as they evolve. The Assurance will be returned to you each year for review and, if desired, renewal. It may be renewed for a maximum of two (2) times. Thereafter, a new Assurance must be filed with the IACUC.

All students (undergraduates or graduate) and staff must complete a formal training program in animal care and use. All individuals performing manipulations on vertebrate animals (handling, capture, blood collection, surgery, etc.) must demonstrate proper training, experience, and capability.

If a Letter of Assurance is required by a granting agency, you must notify the IACUC Recording Secretary. It is your responsibility to provide the required contact and agency information prior to the grant deadline.

All vertebrate animal mortality events or events that result in unexpected serious injury to study animals associated with this protocol will be reported to the IACUC within one (1) week of occurrence. Failure to report the event may result in suspension of the protocol.

All vertebrate animals under this Assurance must be identified with the assigned IACUC Protocol number.

IACUC Protocol Number:	07-003
Investigator/Instructor:	Dr. Shannon Atkinson
Title of Project/Course:	ASLC captive Steller sea lion research program.
Date Received:	April 23, 2007
Date Approved:	May 4, 2007
Annual renewal:	May 2008
	May 2009

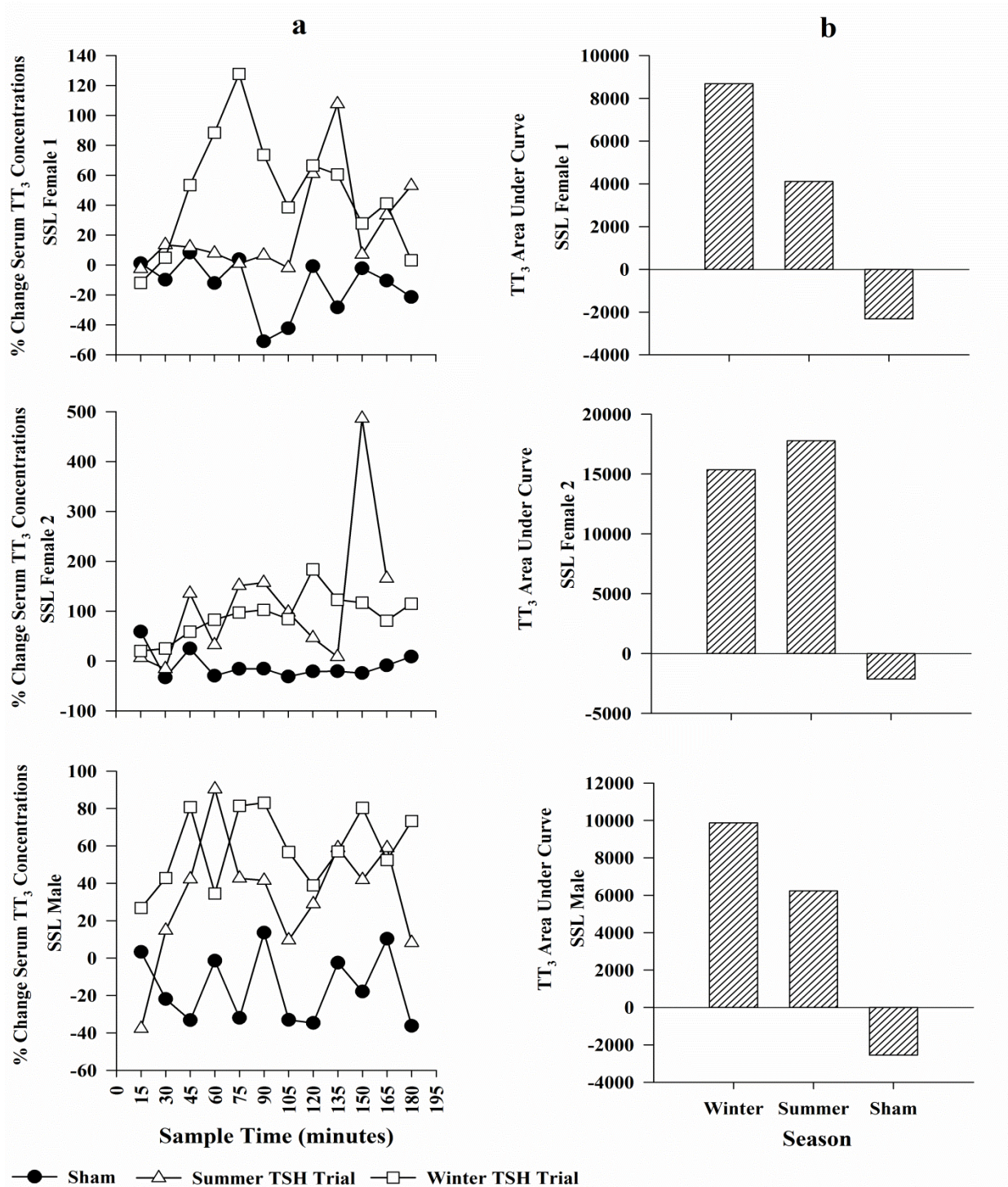APPROVED, as submitted / modified.

Sincerely,

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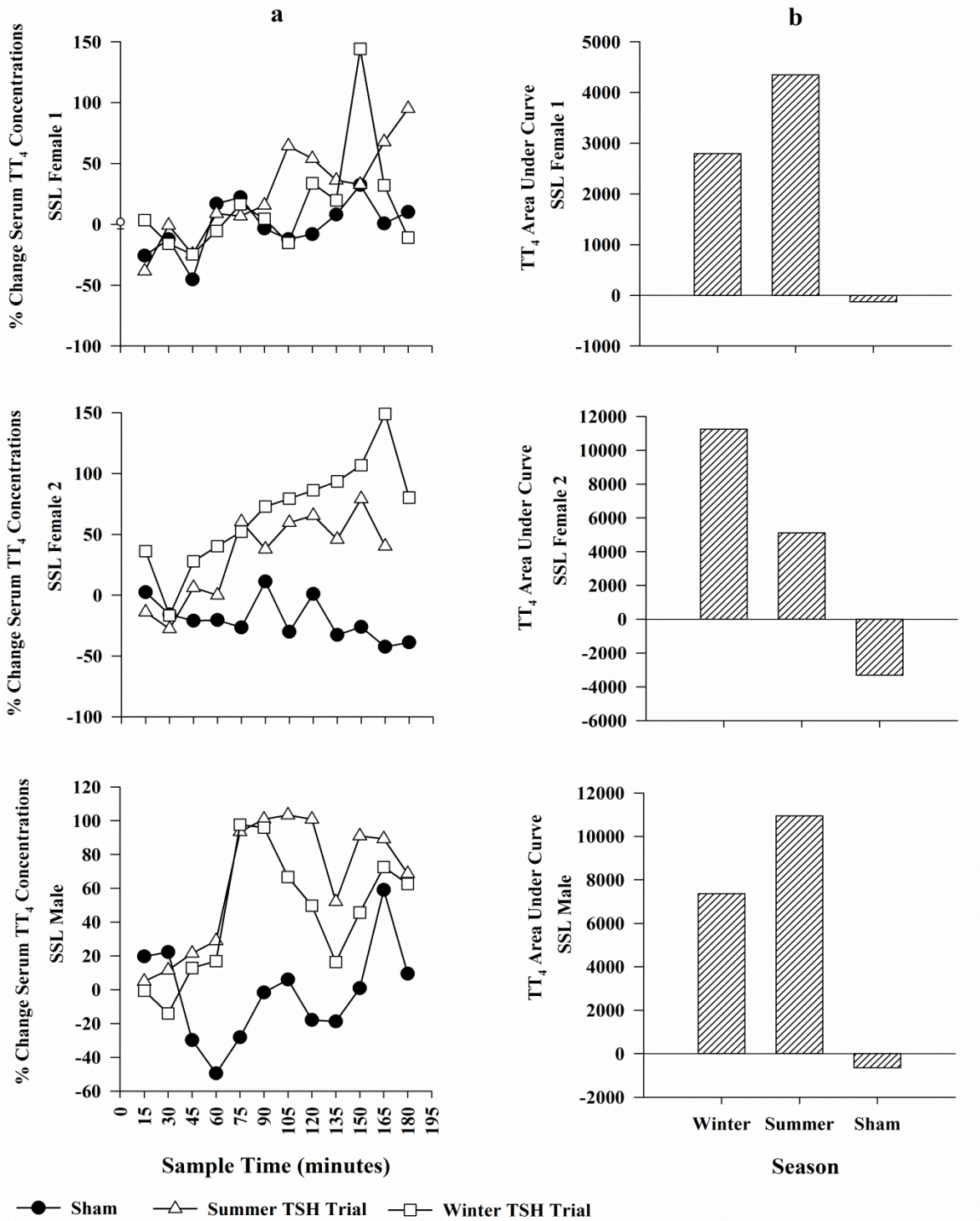
Appendix A. Letter of Alaska SeaLife Center IACUC approval for the presented reseach.

Appendix B1. Percent change for individual animals from Time 0 in TT_3 concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.

Appendix B2. Percent change for individual animals from Time 0 in TT_4 concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.

Appendix B3.

Appendix B3. Percent change for individual animals from Time 0 in FT_3 concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.
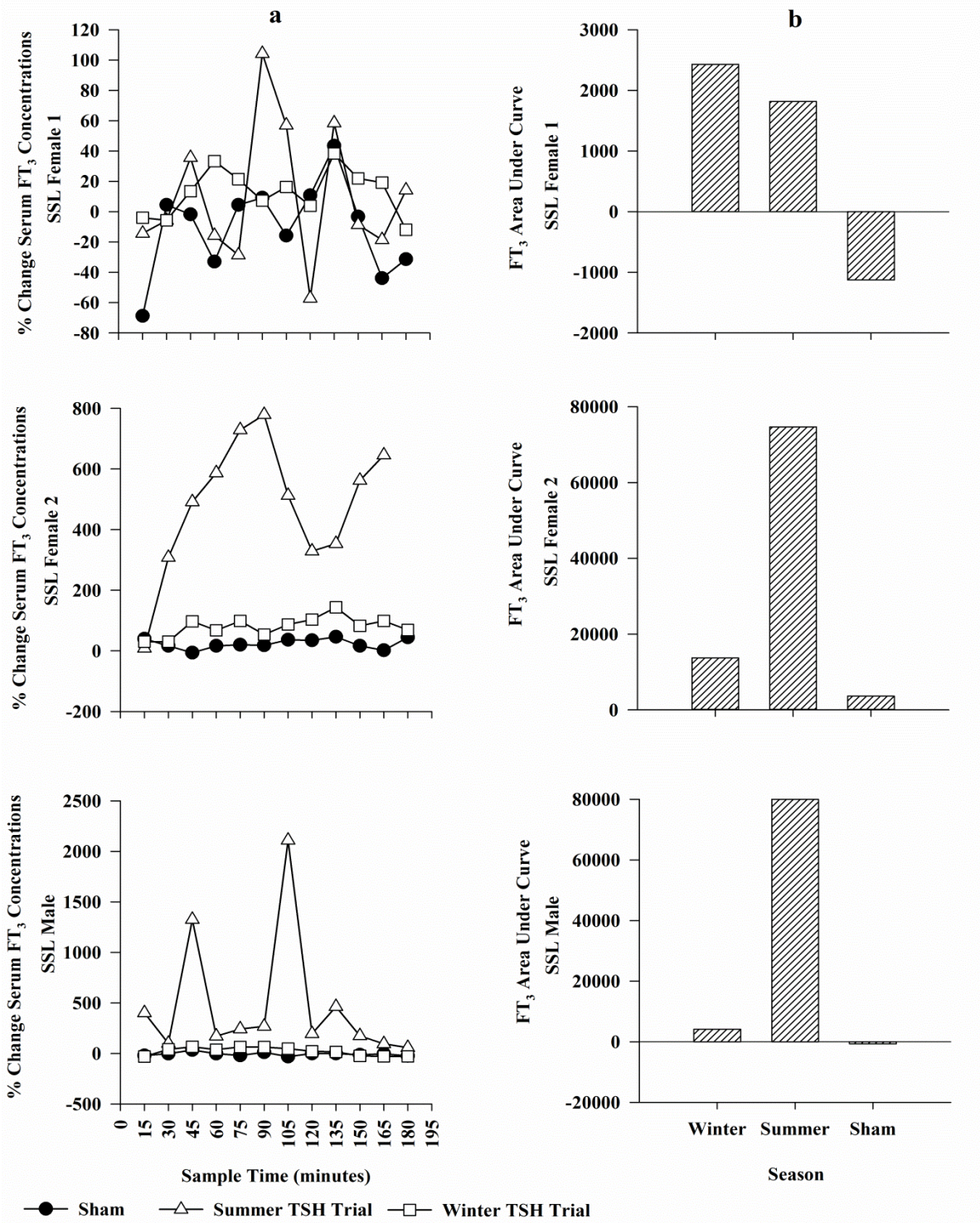
37

Appendix B4.

Appendix B4. Percent change for individual animals from Time 0 in FT_4 concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.

Appendix B5.

Appendix B5. Percent change for individual animals from Time 0 in cortisol concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.
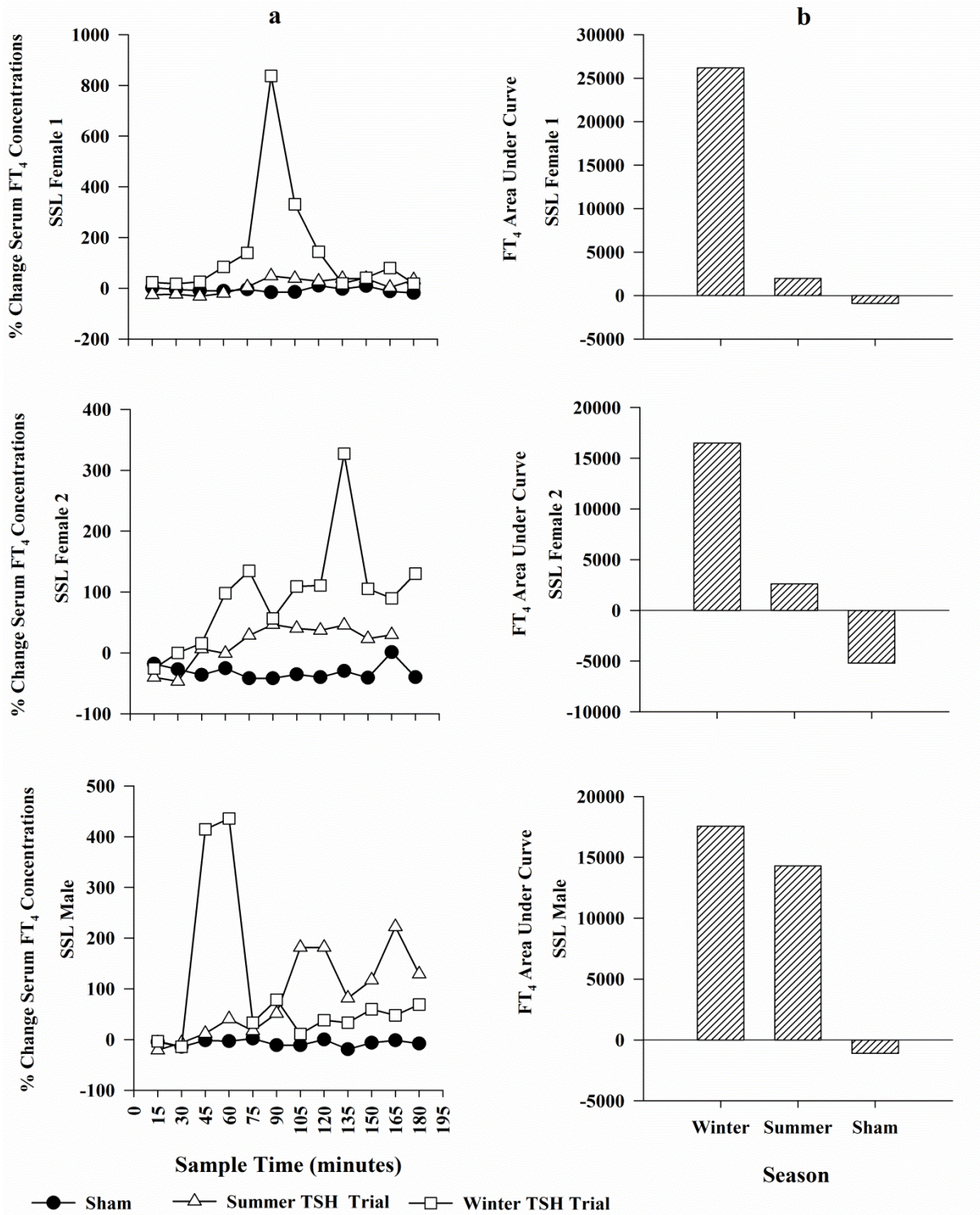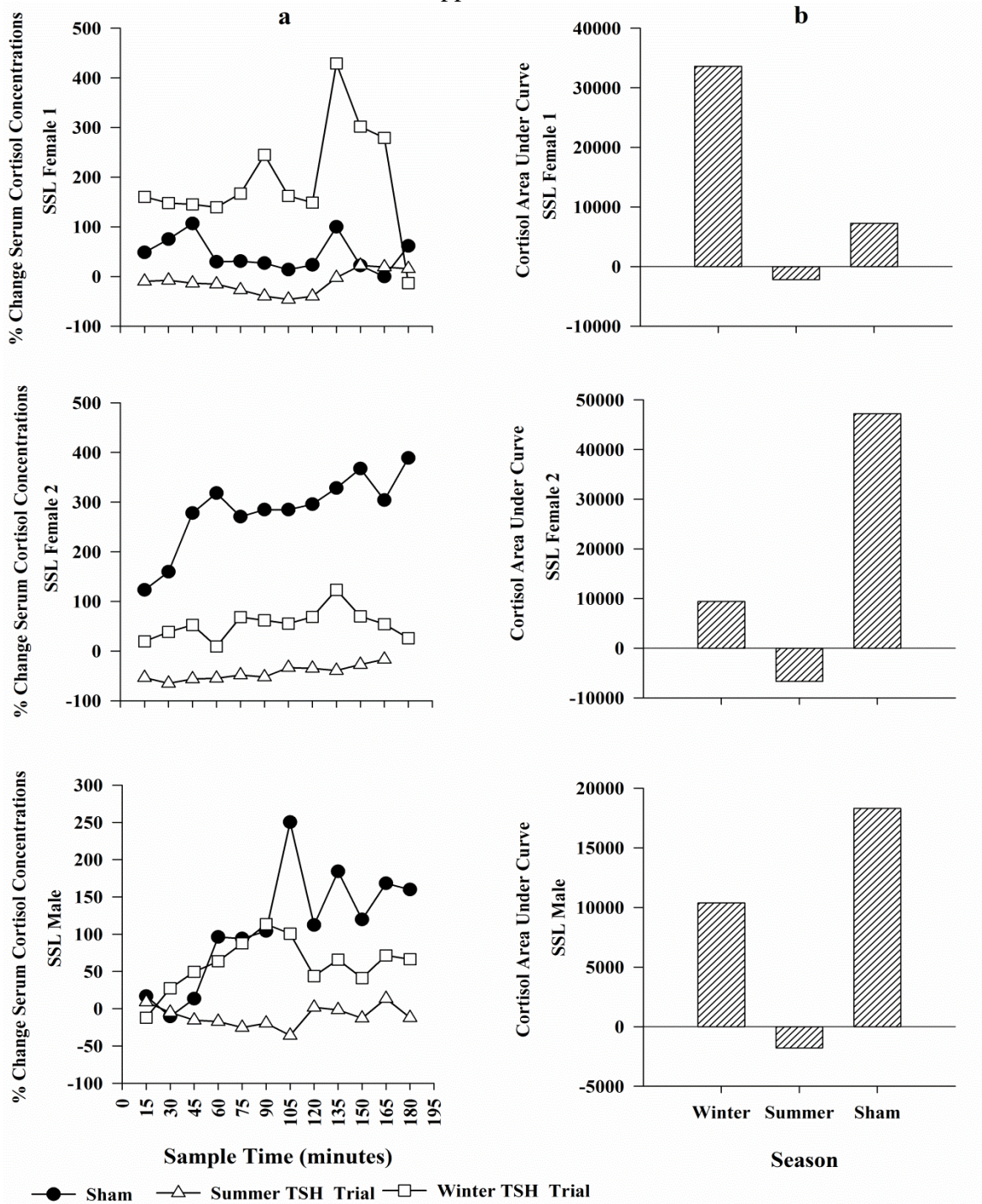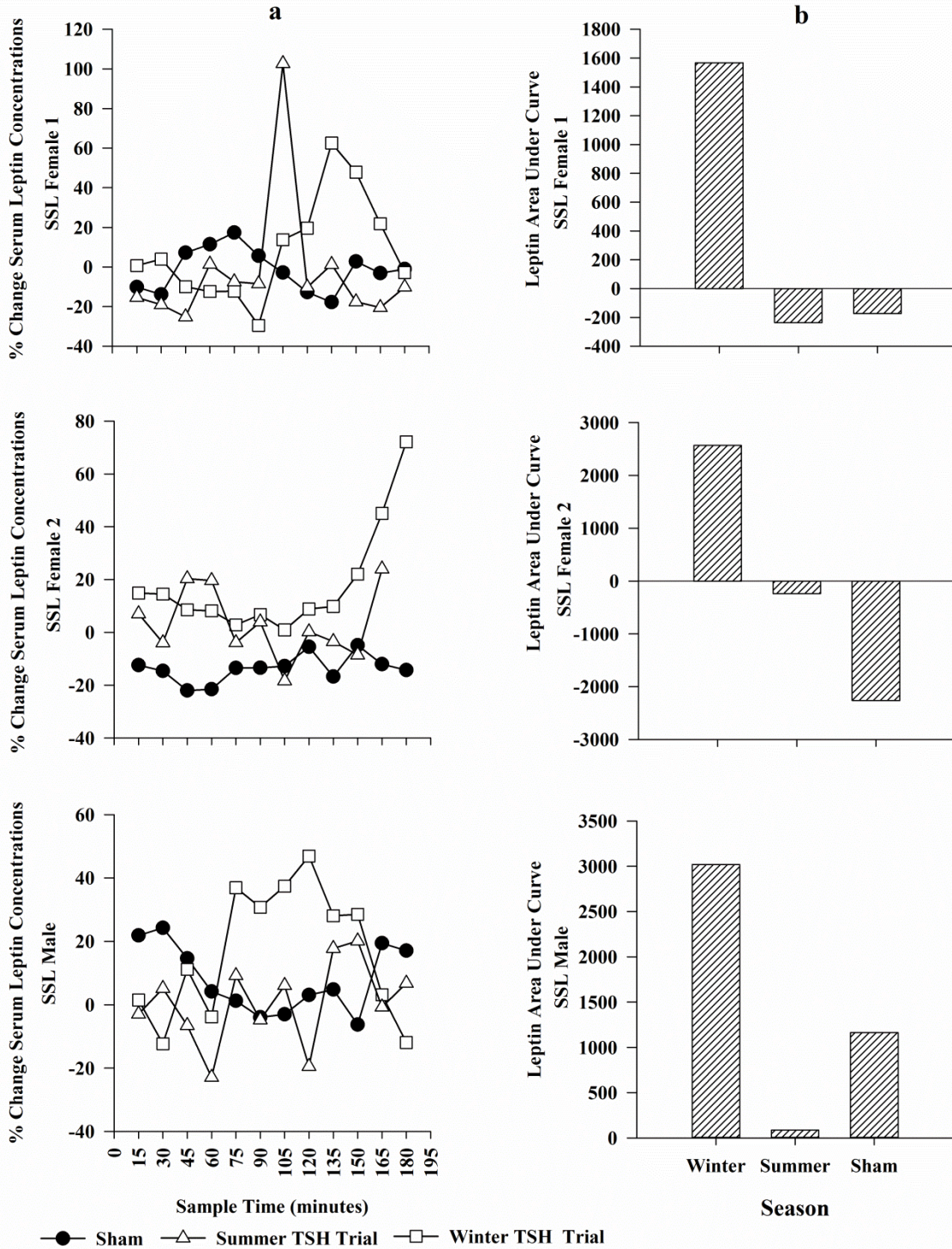
Appendix B6. Percent change for individual animals from Time 0 in leptin concentrations (a) and area under the curve (b) for sham, winter and summer TSH challenge trials in Steller sea lions.

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