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Fecal triiodothyronine and thyroxine concentrations change in response to thyroid stimulation in Steller sea lions (*Eumetopias jubatus*)

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ABSTRACT

Variation in concentrations of thyroid hormones shed in feces may help to identify physiological states of animals, but the efficacy of the technique needs to be validated for each species. We determined whether a known physiological alteration to thyroid hormone production was reflected in hormone concentrations in the feces of Steller sea lions (*Eumetopias jubatus*). We quantified variation of triiodothyronine (T3) and thyroxine (T4) concentrations in feces following two intramuscular injections of thyrotropin (thyroid-stimulating hormone, TSH) at 24 h intervals in four captive female sea lions. We found fecal T3 concentrations increased 18–57% over concentrations measured in the baseline sample collected closest to the time of the first TSH injection ($p = 0.03$) and 1–75% over the mean baseline concentration ($p = 0.12$) for each animal of all samples collected prior to injections. Peak T3 concentrations were greater than the upper bound of the baseline 95% confidence interval for three animals. The peak T3 response occurred 48 h post-injection in three animals and 71 h in the fourth. Post-injection T4 concentrations did not differ between the baseline sample collected closest to the time of the first TSH injection ($p = 0.29$) or the mean baseline concentration ($p = 0.23$) for each animal. These results indicate that induced physiological alterations to circulating thyroid hormone concentrations can be adequately detected through analyses of fecal T3 concentrations and that the technique may provide a means of non-invasively detecting metabolic changes in Steller sea lions.

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 was found to reduce breeding success in colonies of seabirds, 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).

Nutritional stress may be detected in free-ranging populations of Steller sea lions through characteristic changes in hormone concentrations. However, obtaining blood samples from large marine mammals is logistically difficult and invasive sample collection may produce biased results. Non-invasive 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, 2000; Foley et al., 2001; Mostl and Palme, 2002).

Free-ranging populations may produce elevated concentrations of glucocorticoids in response to negative changes in diet, but such concentrations could also result from other sources of physiological stress. 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. Fecal thyroid hormone quantification may provide diagnostically relevant measures of thyroid function and represents an additional physiological parameter that may be useful for identifying inadequate nutrition as a specific stressor.

To date, the predicted response of circulating glucocorticoids and the thyroid hormone triiodothyronine to food deprivation combined with other stressors has been successfully observed in mice (Cremaschi et al., 2000; Silberman et al., 2002). Results for Steller sea lions subjected to alterations in diet quality have been

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mixed (Jeanniard du Dot et al., 2008; Rosen and Kumagai, 2008), possibly due to the use of invasive serum sampling techniques which can increase glucocorticoid concentrations independently of diet or other factors.

The efficacy of using fecal hormone levels to monitor circulating levels can be tested by studies that induce predictable changes in circulating hormone concentrations. Thyroid hormone production can be measurably increased in serum by injecting an animal with thyrotropin (thyroid-stimulating hormone, TSH). For example, injecting northern elephant seals (*Mirounga angustirostris*) with TSH significantly increased serum concentrations of the thyroid hormone thyroxine in healthy seals, as well as those that had northern elephant seal skin disease (Yochem et al., 2008). We tested whether TSH injections significantly increase concentrations of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) in the feces of Steller sea lions.

TSH stimulates the hypothalamic–pituitary–thyroid axis, increasing circulating concentrations of T3 and T4 (Flier et al., 2000). While T4 is the primary thyroid-produced metabolic hormone, it is relatively inactive until deiodinated to T3 (Tomasi, 1991). Significant increases in fecal T3 or T4 concentrations following TSH injections (accounting for secretion/excretion lag-times) would suggest that fecal thyroid hormone measures adequately reflect gross thyroid function. Similar validations have been previously completed for the radioimmunoassay of fecal glucocorticoids with 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.

Our objective was to determine whether increased T3 and T4 production could be detected in the feces of Steller sea lions under controlled conditions in response to the administration of TSH. A peak in fecal T3 or T4 concentrations following the injections would indicate that diagnostically relevant variations in thyroid hormone concentrations can be measured in feces, providing potential indices of metabolism and chronic nutrition-specific stress in free-ranging Steller sea lion populations.

2. Methods

2.1. Subjects, facilities, and dosage

Four female Steller sea lions housed at the Vancouver Aquarium (Vancouver, BC, Canada) participated in the study – SSL1 (134 kg) and SSL2 (151 kg), aged 4 years, and SSL3 (191 kg) and SSL4 (192 kg), aged 7 years. The animals were captured as pups and raised within the facility using positive reinforcement methods to provide daily contact with staff and equipment.

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*) supplemented with vitamins. The quantity of herring consumed was determined by animal-trainer interactions, not to exceed the maintenance allowance for each animal. No notable changes in environment or animal behavior were observed during the study. All procedures were in accordance with University of British Columbia and the Vancouver Aquarium animal care guidelines.

TSH sourced from bovine pituitary (Sigma–Aldrich, Oakville, Ontario, Canada) was administered intramuscularly in 2 cc saline solution, while the animals were under isoflurane gas anesthesia. The sea lions were injected with TSH on two consecutive mornings, either starting 26 June 2007 (SSL1 and SSL3) or 16 July 2007 (SSL2 and SSL4). Each animal was housed in a separate holding pen for approximately 2 weeks prior to TSH injections to establish baseline fecal hormone concentrations (the mean fecal hormone concentration of all samples collected prior to injections for each animal) and continued for 2 weeks post-injection to ensure all affected hormone metabolites passed through the digestive tract.

Checks for fecal samples were performed five times daily (08:00 and 16:00 PST, and at each of three intervening feeding sessions), recording the date and time, location (dry haulout or pool), and animal. All available fecal samples were collected, although some could have dissipated completely before recovery within the holding pen pool. All samples were immediately frozen at −20 °C for subsequent analyses.

The thyroid gland is unique, because it both secretes and stores thyroid hormones (Dierauf and Gulland, 2001). The captive Steller sea lions in our study were administered two 10 IU TSH injections, 24 h apart, to ensure that thyroid stimulation would result in increases in circulating thyroid hormones rather than simply increase thyroid hormone stores in the gland. A review of published TSH doses suggested a maximum injection of 10 IU for canines (Feldman and Nelson, 1996), whereas other sources recommended 0.1 μg TSH/kg to a maximum injection of 5 IU TSH for canines, and a maximum injection of 10 IU TSH for horses (Plumb, 2005). Under these guidelines, the volume of TSH required relative to the mass of each sea lion would have exceeded 10 IU, thus a 10 IU TSH dosage was used as a standard for each injection.

TSH serum equilibrium and peak thyroid activity occur approximately 1.5 h (canine) to 2 h (human) after administrating TSH (Ridgway et al., 1974a,b). 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 9 days post-injection. An approximate 24 h minimum T3 clearance rate has also been documented in beluga whales (*Delphinapterus leucas*) administered multiple TSH injections (St. Aubin and Geraci, 1992). We administered two 10 IU TSH injections to promote sustained circulating thyroid hormone concentrations over a 48 h period and allow for prolonged metabolite accumulation in feces. We did not examine relationships between serum and fecal concentrations, because we wanted to limit the use of invasive procedures with the potential to alter the animals' physiological state.

Steller sea lion initial defecation time for hard prey remains has ranged from 2 to 56 h (Tollit et al., 2003). The lag-time from stimulating glucocorticoid secretion through adrenocorticotropic hormone 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. Together, serum clearance and digestive passage rates suggest that fecal T3 concentrations should decrease to baseline approximately 3 days after the second injection.

Two animals (SSLs 1 and 2) defecated less than three hours after the first TSH injection. Despite being collected after the first injection, these two samples were categorized as baseline based on serum clearance and digestive passage rates previously reported for Steller sea lions. The baseline sample collected closest (before or after) to the time of the first TSH injection for each animal (the previously mentioned samples for SSLs 1 and 2) was also termed the time 0 sample. The time 0 sample for each animal was used both to determine mean baseline concentrations and to represent the status of each animal immediately prior to any changes induced by the TSH injections.

2.2. Sample preparation and assay

The frozen fecal samples were thawed overnight and manually homogenized for hormone extraction the following morning by

Table 1
Individual and mean fecal T3 and T4 concentrations (ng g^{-1}) following TSH injections in four Steller sea lions, showing baseline and peak values, percent increases, and time of events.

	Steller sea lion				Mean	SE
	SSL1	SSL2	SSL3	SSL4		
T3 mean baseline (ng g^{-1})	729	575	514	514	583	30.09
T3 baseline 95%CI (ng g^{-1})	627–831	522–629	507–520	452–576	524–642	
T3 time 0 sample (ng g^{-1})	626	524	509	572	558	26.42
T3 peak (ng g^{-1})	739	733	636	898	752	54.24
T3 peak (h)	48	48	48	71	54	5.91
T3 increase from baseline (ng g^{-1})	10	158	122	384	168	78.35
T3 increase from baseline (%)	1	27	24	75	32	15.38
T3 increase from time 0 (ng g^{-1})	113	209	127	326	194	48.90
T3 increase from time 0 (%)	18	40	25	57	35	8.64
T3 rebound from depletion (h)	95	101	215	n/a	137	39.01
T4 mean baseline (ng g^{-1})	2636	1529	1897	1987	2012	133.89
T4 baseline 95%CI (ng g^{-1})	2480–2792	1456–1603	1621–2173	1554–2419	1750–2275	
T4 time 0 sample (ng g^{-1})	2794	1604	1944	1943	2071	253.86
T4 peak (ng g^{-1})	2414	2623	2055	4276	2842	492.18
T4 peak (h)	48	53	48	71	55	5.62
T4 increase from baseline (ng g^{-1})	−222	1094	158	2289	830	559.57
T4 increase from baseline (%)	−8	72	8	115	47	28.61
T4 increase from time 0 (ng g^{-1})	−380	1019	111	2333	771	595.94
T4 increase from time 0 (%)	−14	64	6	120	44	30.21

massaging the storage bags. A 2–5 g subsample was removed by passing a portion of homogenized feces through 0.5 mm mesh to exclude hard prey remains. Subsamples were returned to −20 °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 the ethanol vortex method described by Wasser et al. (submitted for publication), with the exception that subsamples were extracted once and the supernatant was not dried prior to dilution in assay buffer.

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 using a phospho-saline-BSA buffer and standards made from crystalline T3 to replace the serum-based diluent and standards, as described by Wasser et al. (submitted for publication). The specific cross-reactivity supplied by the manufacturer 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 those of T3. The specific cross-reactivity supplied by the manufacturer of the T4 antibody to other compounds was: triiodo-L-thyronine 2%, and triiodothyroacetic acid 2%.

Parallelism and accuracies of the T3 and T4 radioimmunoassays were tested at The Center for Conservation Biology at the University of Washington where the non-invasive techniques were developed (Wasser et al., submitted for publication). Serially diluted samples did not differ from their respective standard curves and accuracy studies produced slopes of 0.95 and 1.1 for T3 and T4, respectively. All hormones were measured as nanograms hormone per gram dry feces (ng g^{-1}). Samples with a percent-bound outside 15–85% on the standard curve were re-run, as were those with a coefficient of variation greater than 10% between duplicate pairs.

2.3. Statistical analyses

All hormone concentrations were log-transformed prior to statistical analyses. We compared 95% confidence intervals to determine whether baseline thyroid hormone concentrations differed significantly between trial groups or age classes — and used paired *t*-tests ($\alpha = 0.05$) to compare the baseline and time 0 concentrations of T3 and T4 from each animal to the peak values exhibited following the TSH injections.

3. Results

3.1. Response to TSH injections

Thyroid hormone concentrations over the course of the study ranged from 450 to 898 ng g^{-1} T3 and 1489 to 4276 ng g^{-1} T4 (Table 1). Baseline T3 concentrations, expressed as mean (ng g^{-1}) with standard error and 95% confidence interval (SE; [95%CI]) were: SSL1 729 ng g^{-1} (52; [627, 831]), SSL2 575 ng g^{-1} (27; [522, 629]), SSL3 514 ng g^{-1} (3; [507, 520]), SSL4 514 ng g^{-1} (32; [452, 576]), and all animals 583 ng g^{-1} (30; [524, 642]). Baseline T4 concentrations were: SSL1 2636 ng g^{-1} (80; [2480, 2792]), SSL2 1529 ng g^{-1} (37; [1456, 1603]), SSL3 1897 ng g^{-1} (141; [1621, 2173]), SSL4 1987 ng g^{-1} (221; [1554, 2419]), and all animals 2012 ng g^{-1} (134; [1750, 2275]). The 95% confidence intervals of baseline T3 and T4 concentrations suggest there were no differences between trial groups (data not shown). The 95% confidence intervals of baseline concentrations differed significantly between age classes for T3, but not for T4. Baseline T3 concentrations exhibited by the older females SSL3 and SSL4 of 514 ng g^{-1} (14; [486, 542]) was lower than the younger age class of SSL1 and SSL2 of 652 ng g^{-1} (43; [567, 737]), although this was largely due to the high baseline T3 concentration of SSL1.

The physiological response to the TSH injections varied among animals (Table 1). All four sea lions experienced a fecal T3 spike following TSH injections (Fig. 1). Three animals (SSLs 1–3) displayed a fecal T3 peak 48 h after the first injection with the peaks occurring in the first scat collected after the second injection. Concentrations of T3 for SSL4 did not peak until 71 h following the first injection, such that the mean peak fecal T3 concentration of all animals occurred at 54 h following the first TSH injections. T4 production showed a post-injection peak that appeared to parallel T3 production in scale and time for SSL2 (53 h) and SSL4 (71 h). The results for SSL3 were less clear; the highest recorded T4 concentration occurred at 215 h, which would suggest an abnormally delayed response. Alternately, the actual peak physiological response may have occurred in samples collected at 48 h and

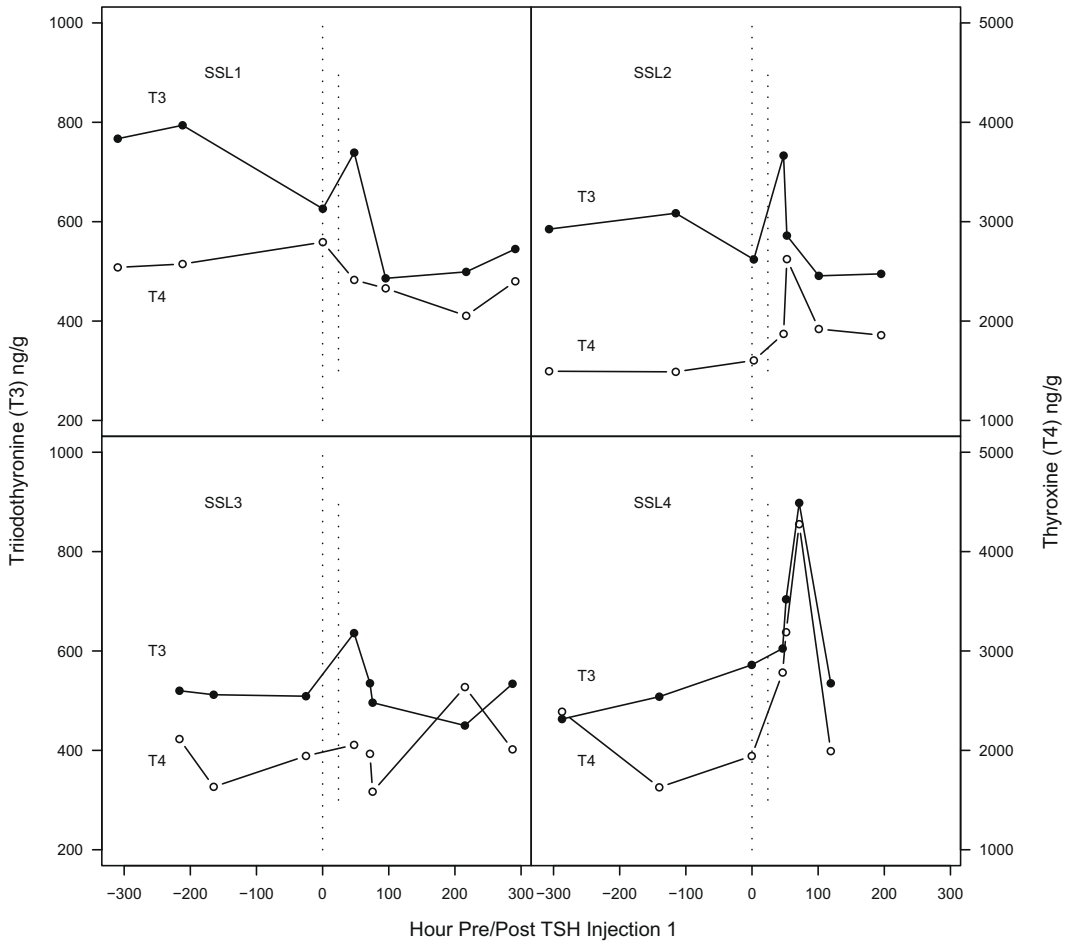

Fig. 1. The effects of intramuscular TSH injections in four Steller sea lions on fecal concentrations of the thyroid-produced metabolic hormones triiodothyronine (T3 –●–) and thyroxine (T4 –○–) measured by radioimmunoassay from an ethanol vortex extraction. Dashed vertical lines represent TSH injection times. The first TSH injection occurred at 0 h, but passage rates predicted physiologically altered fecal samples approximately 24 h later.

72 h (Fig. 1). The absolute T4 concentrations from these two samples were not as high as that seen from the 215 h sample. However, the timing of the two samples fit with the generally known endocrine and digestive physiology of this species, and correspond with the timing of the T3 peak in that animal, as was also seen in SSL2 and SSL4. Rather than estimating a mean value between the 48 and 72 h samples, the 48 h concentration was used for subsequent average timing and peak calculations. SSL1 did not exhibit post-injection T4 concentrations greater than baseline or time 0. Despite not exhibiting an increased T4 concentration, the post-injection maximum for SSL1 was retained for mean calculations using T4 peak and time (Table 1).

Peak T3 concentrations (defined as the post-injection sample of highest concentration) for the sea lions were: SSL1 (739 ng g^{-1}), SSL2 (733 ng g^{-1}), SSL3 (636 ng g^{-1}), and SSL4 (898 ng g^{-1}). The peak concentrations were greater than the upper bound of the baseline 95% confidence interval for SSLs 2–4 (Table 1). T3 concentrations differed significantly between the time 0 samples and peak values ($p = 0.03$). A T3 increase of 25–57% above time 0 samples was exhibited by SSLs 2–4, while SSL1 exhibited an 18% increase. SSLs 2–4 exhibited T3 concentrations 24–75% greater than baseline, while SSL1 only exhibited a 1% increase mainly due to having a baseline concentration 37% greater than the mean baseline of SSLs 2–4. As a result, no difference was found overall between baseline and peak T3 concentrations ($p = 0.12$).

Peak T4 concentrations for the sea lions were: SSL1 (2414 ng g^{-1}), SSL2 (2623 ng g^{-1}), SSL3 (2055 ng g^{-1}), and SSL4 (4276 ng g^{-1}). The peak concentrations were greater than the upper bound of the baseline 95% confidence interval for SSLs 2 and 4 (Table 1). T4 concentrations did not differ between peak values and time 0 samples ($p = 0.29$) or baseline ($p = 0.23$). The T4 concentrations of SSLs 2–4 increased 6–120% above time 0 samples, whereas SSL1 exhibited a decrease of 14%. The T4 concentrations of SSLs 2–4 increased 8–115% above baseline samples, whereas SSL1 exhibited a decrease of 8%.

The period over which post-injection fecal samples were collected was not long enough to identify the time at which T3 concentrations returned to baseline values, but did reveal a point following the T3 peak where concentrations depleted to below baseline and then began to rebound. The time at which these lowest T3 concentrations occurred (potentially signaling the start of a return to baseline values) was at 95 h for SSL1, 101 h for SSL2, and at 215 h for SSL3 for a mean (SE) of 137.10 h (39.01) post-injections. Comparable T3 data were unavailable for SSL4 owing to the delayed peak response to the TSH injections.

4. Discussion

Fecal T3 concentrations were found to reflect physiological activity in Steller sea lions, exemplified by the significant differences found between the upper bound of the baseline 95% confidence intervals and time 0 samples to the T3 peaks that occurred 48–71 h later. By contrast, peak T4 concentrations were only

greater than the upper bound of the baseline 95% confidence intervals of two animals of different age classes. Although SSL3 demonstrated its greatest post-injection T4 concentration at 215 h, it is likely that the animal exhibited a T4 peak response at 48 h of a lesser concentration (possibly because it was split between two samples) than the variation that occurred in the samples thereafter.

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 et al., submitted for publication). 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 in serum across most mammals. In fasted humans, a correlation was found between log 10 TSH and T3 plasma concentrations, 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 converted prior to entering the digestive tract of the two animals that did not display a T4 increase.

Individual differences in the timing of T3 peaks (48 h after the first injection in SSLs 1–3 and 71 h in SSL4) may reflect individual differences known to occur in gut flora and digestive passage rates (Goldin et al., 1982; Wasser et al., 1993; Lewis et al., 1997). The peaks for 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 increases from and returns towards baseline values occurred over approximately 3 days. Since undertaking our study, synthetic TSH has become more commonly used than homogenized pituitary TSH, which has a greater potential for interference with other trophic hormones. Despite any potential interference, our findings support the use of this non-invasive technique to measure thyroid hormones in feces.

Overall, the results of our study indicate that induced physiological alterations to circulating thyroid hormone concentrations can be adequately detected through analyses of fecal hormone concentrations and may provide a means of detecting metabolic changes in captive Steller sea lions under controlled conditions. Although we cannot confirm the utility of applying fecal T3 analyses to free-ranging populations, the physiological differences we detected between individuals within our captive population suggest that this non-invasive technique should also capture differences between free-ranging populations when used with an appropriate experimental design. However, the variability found across samples in our study highlights the need to explore issues pertaining to longitudinal sampling. Nevertheless, our results indicate that induced physiological alterations to circulating thyroid hormone concentrations can be adequately detected through analyses of fecal T3 concentrations and that the technique may provide a means of non-invasively detecting metabolic changes in Steller sea lions.

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