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Increased Sensitivity of Thyroid Hormone-mediated Signaling Despite Prolonged Fasting

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Abstract

Thyroid hormones (TH) can increase cellular metabolism. Food deprivation in mammals is typically associated with reduced thyroid gland responsiveness, in an effort to suppress cellular metabolism and abate starvation. However, in prolonged-fasted, elephant seal pups, cellular TH-mediated proteins are up-regulated and TH levels are maintained with fasting duration. The function and contribution of the thyroid gland to this apparent paradox is unknown and physiologically perplexing. Here we show that the thyroid gland remains responsive during prolonged food deprivation, and that its function and production of TH increase with fasting duration in elephant seals. We discovered that our modeled plasma TH data in response to exogenous thyroid stimulating hormone predicted cellular signaling, which was corroborated independently by the enzyme expression data. The data suggest that the regulation and function of the thyroid gland in the northern elephant seal is atypical for a fasted animal, and can be better described as, "adaptive fasting". Furthermore, the modeling data help substantiate the *in vivo* responses measured, providing unique insight on hormone clearance, production rates, and thyroid gland responsiveness. Because these unique endocrine responses occur simultaneously with a nearly strict reliance on the oxidation of lipid, these findings provide an intriguing model to better understand the TH-mediated reliance on lipid metabolism that is not otherwise present in morbidly

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obese humans. When coupled with cellular, tissue-specific responses, these data provide a more integrated assessment of thyroidal status that can be extrapolated for many fasting/food deprived mammals.

Keywords

Thyroid hormones; obesity; fasting; mathematical modeling; deiodinase; thyroxine; thyroid hormone receptor; thyroid stimulating hormone

Introduction

Hypothalamic-pituitary-thyroid axis (HPT) regulates circulating levels of thyroid hormones (TH), which in turn regulate cellular metabolism (Joseph-Bravo et al., 2015). The pituitary gland secretes thyroid stimulating hormones (TSH), which stimulates the release of thyroxine (T4) and triiodothyronine (T3) in a ratio of approximately 17:1 (Dayan and Panicker, 2009; Segerson et al., 1987). Because T3 is the most biologically active form of the hormone, T4 must be deiodinated by either deiodinase type 1 (DI1) or deiodinase type 2 (DI2) before it can exert genomic physiological effects (Bernal and Refetoff, 1977; Bianco and Kim, 2006; Koenig, 2003; St. Germain and Galton, 1997). DI1 catalyzes the deiodination of both inner and outer rings of primarily T4, and DI2 catalyzes only outer ring deiodination (converting T4 into biologically active T3), with its activity providing an indicator of increased cellular TH availability (Bianco and Kim, 2006; St. Germain and Galton, 1997). DI3 exclusively catalyzes inner ring deiodination converting T4 into reverse T3 (rT3) and rendering the hormone inactive (Bianco and Kim, 2006; St. Germain and Galton, 1997). Once DI1 or DI2 deiodinate T4, T3 enters nucleus and binds to its tissue-specific nuclear receptor to increase the transcription of specific metabolism-driving genes (Brunelle et al., 2011; Handschin and Spiegelman, 2006; Pascual and Aranda, 2013; Vatner et al., 2013). Thyroid hormone receptor beta 1 (THrβ1) is the principal receptor in peripheral tissues such as muscle and adipose and is localized on specific DNA promoter sequences for TH target genes. T3-bound THrβ1 increases the transcription of genes, and eventually, the translation of proteins associated with substrate turnover (Bernal and Refetoff, 1977; Dimitriadis et al., 1985; Samuels and Tsai, 1973).

Fasting is typically characterized by a decrease in cellular metabolism to abate substrate depletion and further consequences of starvation (Azizi, 1978; Haymond et al., 1982; Herlihy et al., 1990; Janan et al., 1995; LoPresti et al., 1991). As such, this suppression of cellular metabolism is an essential adaptive response for survival, and regulated by the hypothalamus (Laurberg et al., 2012). The response is also characterized by a suppression of circulating levels of TH coupled with a decrease in DI1, DI2, and THrβ1 transcription, and an increase in DI3 expression and rT3 levels (Azizi, 1978; Herlihy et al., 1990; Janan et al., 1995; Kmiec et al., 1996; LoPresti et al., 1991; Tveit and Almlid, 1980; van Haasteren et al., 1995). However, in prolonged fasting northern elephant seal (*Mirounga angustirostris*) (NES) pups, the mRNA expressions of DI1, DI2, and THrβ1 are increased with fasting duration (Martinez et al., 2013; Martinez et al., 2016). Moreover, this fasting bout is coupled with increased or maintained circulating levels of TH (Ortiz et al., 2003a). This unique and

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paradoxical, physiological response to prolonged fasting is further perplexing, given that these mammals are non-hibernators, remain physically active, and remain normothermic throughout their fast (Bryden, 1972; Kirby and Ortiz, 1994; Ortiz et al., 2003a). Collectively, these data suggest that NES have evolved unique endocrine responses to prolonged food deprivation, namely, that cellular TH-mediated events increase with fasting duration. However, it remains unclear whether these observed changes are functional or the consequence of altered clearance from circulation or both, or simply driven by enzymatic activity of the deiodinases and availability of the receptor.

Here, we use a mathematical model to better assess the dynamics of cellular, TH-mediated events, and production and clearance rates after exogenous TSH infusion, comparing changes between early- and late-fasted pups. Aside from producing a mathematical model that describes hormonal dynamics *in vivo* in a mammal naturally adapted to prolonged fasting, we also show that the increase in endogenous T4 levels resulting from reduced clearance, which has been observed in hibernating squirrels (Magnus and Henderson, 1988a, b), is a function of active secretion. Ascertaining the functional contributions of TH on metabolism based solely on changes in circulating levels is problematic especially when changes in hormone clearance and cellular signaling cannot be measured and coupled simultaneously. Thus, a strength of this study is the construction of a mathematical model that predicts the dynamic changes in TH observed, which are influenced by production rates, and to a lesser extent, changes in clearance rates. The model, coupled with complimentary gene expression data demonstrate that the **F**asting-**A**ssociated **M**etabolism **I**n **N**orthern **E**lephant-seals, or perceived FAMINE is a robust *adaptive* fast.

Material and methods

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee’s of both the University of California Merced and Sonoma State University. All work was conducted under the National Marine Fisheries Service marine mammal permit #87–1743.

Animals

Northern elephant seal pups were studied at the Año Nuevo State Reserve (30 km north of Santa Cruz, CA, USA) during their natural post-weaning fast while they are still on land. Ten pups were sampled during the early (1–2 weeks post-weaning) and ten were sampled during late fasting period (6–8 week post-weaning) as independent cohorts. The pups were initially sedated with ~1 mg/kg Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft Dodge, IA, USA) administrated intramuscularly and once immobilized, an 18 gauge, 3.5-inch spinal needle was inserted into the extradural spinal vein to facilitate blood sampling and the infusion of TSH or saline (Ortiz et al., 2003a; Ortiz et al., 2003c). The body mass was measured using a hangingload cell suspended from a tripod.

Intravenous Thyroid Stimulating Hormone Infusion

Pre-infusion (time 0) blood samples were collected in chilled, EDTA-treated vacutainer sample tubes and kept on ice until they could be centrifuged. Pre-infusion (time 0) adipose

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and muscle tissue biopsies were collected by first cleaning a small region in the flank of the animal near the hind flipper with alternating wipes of isopropyl alcohol and betadine. A small (<1.5 cm) incision was made using a sterile scalpel, and a blubber and muscle biopsy (ca. 50–200 mg) was collected with a sterile biopsy punch needle (Henry Schein). The biopsy samples were rinsed with cold, sterile saline, placed in cryogenic vials, immediately frozen by immersion in liquid nitrogen, and stored at −80°C until later analyses. Following initial sample collection, animals (n=10 early, n=10 late) were infused with 8 IU of bovine TSH (Sigma) (n=6) or with saline (n=4). Following the infusion, blood samples were collected at 15, 30, 60, 120, and 1440 mins. Additionally, adipose and muscle biopsies were collected at 60, 120, and 1440 mins. Blood samples were centrifuged for 15 min at 3000***g***, and the plasma was transferred to cryo-vials, immediately frozen by immersion in liquid nitrogen, and stored at −80°C (Viscarra et al., 2011; Viscarra et al., 2013; Viscarra et al., 2010; Viscarra et al., 2012).

Quantification of mRNA Expressions

Quantification of DI1, DI2, DI3, and THrβ1 mRNA expressions was performed as previously detailed (Martinez et al., 2013; Martinez et al., 2016). RNA was extracted using the standard Trizol RNA isolation protocol and the RNA quality after extraction was determined by measuring the absorbance at 260/280 nm and 260/230 nm using a NanoDrop (Maestrogen, Inc. MN913, USA) and by assessing the RNA integrity number (RIN) using a bioanalyzer (Agilent Technologies, USA). RIN values for all samples were above seven, on a scale of 1–10. RNA samples underwent two treatments of genomic DNA wipeout before being subsequently reversed transcribed. Contamination of genomic DNA in total RNA was eliminated by digestion with DNase I (Roche, Indianapolis, IN) as specified by the manufacturer. Isolated cDNAs from muscle and adipose were synthesized from total DNA-free RNA (1 μg) using oligo-dT and the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA), which also included a genomic DNA wipeout step. Gene expression data was calculated using the standard curve method, dilutions 5e-3 to 5e-8 were used. The expression of beta-actin (β-actin) was used as an internal standard to normalize the expression of each target gene; forward primer sequence 5-CAGGATGCAGAAGGAGATCAC-3' and reverse primer sequence 5'-GCATTTGCGGTGGACGATGG-3' were used. β-actin expression did not change with fasting duration or in response to the exogenous infusions, confirming its utility for normalizing as a reference gene.

Plasma Analyses

The plasma concentrations of TSH, total thyroxine (tT4), free thyroxine (fT4) and total triiodothyronine (tT3) were measured by radioimmunoassay as previously validated and detailed (Martinez et al., 2016; Ortiz et al., 2003a; Ortiz et al., 2003b; Ortiz et al., 2003c; Ortiz et al., 2001). All samples were analyzed in duplicate and run in a single assay with intra-assay, percent coefficients of variability of <10% for all assays.

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Statistics

Repeated measures ANOVA was used to determine changes in all analyses following the infusions. Changes were considered significantly different at $P < 0.05$. Statistical analyses were performed using R Software.

Results

TSH Infusions are Equivalent at Both Early and Late Fasting Periods

To confirm that our infusion of exogenous TSH increased circulating levels (Figures 1 and 2), plasma TSH was measured. Furthermore, the disappearance of TSH from circulation provides an indication of the clearance of TSH in elephant seals. The measurable amount of exogenous TSH infused was the same in both early and late fasting, which allows us to predict the responsiveness of the thyroid gland and its rate of production as a function of only fasting duration, and not variations in TSH fluctuations (Figure 3A). During both the early and late fast, concentrations of tT4, fT4 and tT3 increased in response to TSH, with levels peaking after 120 minutes. While, concentrations return to baseline after 24 hours in the early fast, in the late fast circulating levels of tT4 and fT4 remain elevated after 24 hours. The area under the curve (AUC) for TSH, $AUC_{TSH} = i_0 \tau_{tg}$; early: 362 minutes*ng/ml vs. late: 486 minutes*ng/ml. AUC for tT4 curve under the constant clearance model, $AUC_{tT4} = i_0 s_{fT4} \tau_{fT4,d}$: 31682 minutes*ng/ml early vs. 69524 minutes*ng/ml late. AUC for fT4 curve under the constant clearance model, $AUC_{fT4} = i_0 s_{fT4,d}$: 671 minutes*pg/ml early vs. 1475 minutes*pg/ml late. Lastly, AUC for tT3 curve under the constant clearance model, $AUC_{tT3} = (\tau_{tT3,d}\ i_0(s_{43} \tau_{tT4,d} \tau_{T3,g} + s_{tT4,g} \tau_{43}))/(\tau_{tT3,g} \tau_{43})$: 815 minutes*ng/ml early vs. 815 minutes*ng/ml late (Table 1).

Modeling of TSH-induced Thyroid Hormones

In order to estimate the changes in TH levels during the period in which measurements were not taken, we mathematically modeled the measured levels of T4 and T3 as a function of time after TSH infusion (Figure 3, Figure 4A). This model considered the following conditions and processes, variables for which have also been summarized (Table 1):

1. Before TSH infusion, endogenous TSH is present at a constant level, n_{eTSH}. The level is considered to remain constant over the course of the experiment.
2. Prior to TSH infusion, the levels of total T3 (n_{T3}), free T4 (n_{fT4}), and total T4 (n_{TT4}) are constant in time.
3. The endogenous TSH disperses into the body from where it is generated at a rate n_{eTSH}/τ_e.
4. The infused TSH, n_{iTSH}, disperses into the body of the animal at a rate, $n_{iTSH}\tau_i$.
5. The total TSH is depleted from the body at a rate, $\frac{n_{\mathrm{eTSH}} - n_{\mathrm{iTSH}}}{\tau_{\mathrm{TG}}}$
6. The thyroid gland produces free T4 (n_{fT4}), total T4 (n_{tT4}), and total T3 (n_{tT3}) due to TSH stimulation and releases it into the body at a rate, $n_{tT4}/\tau_{tT4,g}$, $n_{fT4}/\tau_{fT4,g}$ and $n_{T3}/\tau_{T3,g}$ respectively.

7. Free T4, total T4, and total T3 are metabolized at a rate, $n_{tT4}/\tau_{tT4,d}$, $nfT4/\tau fT4,d$ and $nT3/\tau T3,d$, respectively.

8. Total T3 may also be generated from T4 at a rate, τ_{43}. However, we assume that T4 is not being affected by T3 production as the amounts of T3 generated from T4 are typically much smaller than the amounts of T4 available, as is the case in this study.

Mathematically we describe this model by the following set of rate-equations:

$$\frac{\mathrm{dn_{eTSH}}}{\mathrm{dt}}=0 \quad (1)$$

$$\frac{\mathrm{dn_{iTSH}}}{\mathrm{dt}}=-\frac{n_{\mathrm{iTSH}}}{\tau_i} \quad (2)$$

$$\frac{\mathrm{dn_{bTSH}}}{\mathrm{dt}}=\frac{n_{\mathrm{eTSH}}}{\tau_e}+\frac{n_{\mathrm{iTSH}}}{\tau_i}-\frac{n_{\mathrm{bTSH}}}{\tau_{\mathrm{TG}}} \quad (3)$$

$$\frac{\mathrm{dn_{TG}}}{\mathrm{dt}}=\frac{n_{\mathrm{bTSH}}}{\tau_{\mathrm{TG}}}-\frac{n_{\mathrm{TG}}}{\tau_{x,g}} \quad (4)$$

$$\frac{\mathrm{dn_{tT4}}}{\mathrm{dt}}=s_4\frac{n_{\mathrm{TG}}}{\tau_{\mathrm{tT4},g}}-\frac{n_{\mathrm{tT4}}}{\tau_{\mathrm{tT4},d}} \quad (5)$$

$$\frac{\mathrm{dn_{tT3}}}{\mathrm{dt}}=s_3\frac{n_{\mathrm{TG}}}{\tau_{\mathrm{tT3},g}}-\frac{n_{\mathrm{tT3}}}{\tau_{\mathrm{tT3},d}}+s_{43}\frac{n_{\mathrm{tT4}}}{\tau_{43}} \quad (6)$$

Here the scaling factors s_x (with $x = fT4$ or $x = tT4$), s_3 and s_{43}, are introduced to account for the fact that, for example, TSH does not stimulate total T4 on a one-to-one basis, and that only a fraction of T4 is converted to T3. In this model we treat the total T3 semi-independently from the total T4, and free T4 equivalently to total T4. That is,

1. the dynamics of total T4 is not influenced by the dynamics of total T3,

2. the dynamics of free T4 is considered identical to that of total T4, and

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3. the dynamics of total T3 may be influenced (however weakly) by the dynamics of total T4 through the mechanism that generates total T3 from T4, or deiodination.

We justify these three assumptions as follows:

1. the levels of total T4 being much larger than the levels of total T3,
2. the ratio between the endogenous total T4 and free T4 levels remains the same between the early and late fasting seasons,
3. the gene expression data indicates that the T4 to T3 conversion is increased during the late fasting season (Figure 5A–F).

Endogenous Levels: Steady-state Considerations

In this analysis we treat the endogenous levels as constant over the time scale of the experiment. Mathematical expressions for the endogenous levels are obtained by setting each of the previous equations 1–6 equal to zero. The resulting initial conditions presume that all endogenous levels are constant prior to TSH infusion:

$$n_{\mathrm{eTSH}}(0)=e_0 \quad (7)$$

$$n_{\mathrm{bTSH}}(0)=\frac{e_0\tau_{\mathrm{TG}}}{\tau_e} \quad (8)$$

$$n_{\mathrm{TG}}(0)=\frac{e_0\tau_{x,g}}{\tau_e} \quad (9)$$

$$n_x(0)=s_x\frac{n_{\mathrm{TG}}(0)t_{x,d}}{\tau_{x,g}}=\cdots=s_x\frac{e_0\tau_{x,d}}{\tau_e} \quad (10)$$

$$n_{\mathrm{tT}3}(0)=s_3\frac{n_{\mathrm{TG}}(0)\tau_{\mathrm{tT}3,d}}{\tau_{\mathrm{tT}3,g}}+s_{43}s_4\frac{n_{\mathrm{tT}4}(0)\tau_{\mathrm{T}3,d}}{\tau_{43}}=\cdots=\frac{e_0\tau_{\mathrm{tT}3,d}}{\tau_e}\left(s_3\frac{\tau_{\mathrm{tT}4,g}}{\tau_{\mathrm{tT}3,g}}+s_{43}s_4\frac{\tau_{\mathrm{tT}4,d}}{\tau_{43}}\right) \quad (11)$$

Together with the measured endogenous levels from Table 2, equations 7–11 offer some initial insights on the metabolic dynamics during early and late fasting. Table 2 reveals that the endogenous levels between early and late fasting change for free and total T4, while the endogenous levels of TSH and total T3 remain unchanged within the measurement error (Table 2). Consequently, we can assume that the dynamics associated with endogenous TSH remains unchanged between the early and the late fasting seasons. That is, $n_{\mathrm{bTSH}}^{\mathrm{Early}}=n_{\mathrm{bTSH}}^{\mathrm{Late}}$,

which suggests $e_0^{\mathrm{Early}}=e_0^{\mathrm{Late}}$, $\tau_e^{\mathrm{Early}}=\tau_e^{\mathrm{Late}}$, and $\tau_{\mathrm{TG}}^{\mathrm{Early}}=\tau_{\mathrm{TG}}^{\mathrm{Late}}$. The observation that $n_{T3}^{\mathrm{Early}}=n_{T3}^{\mathrm{Late}}$, but that $n_{T4}^{\mathrm{Early}} \neq n_{T4}^{\mathrm{Late}}$ suggests that not just the T4-related dynamics change, but also the T3-related dynamics, yet in a way that keeps the endogenous T3 level unchanged.

TSH Infusion Dynamics

Further information on the metabolic dynamics is obtained from the response to TSH infusion. At time $t = 0$, an amount estimated by $n_{iTSH}(t = 0) = i_0$ is being infused. To assess only the effect of TSH infusion on circulating levels of TH, we subtract the endogenous levels at time $t = 0$ from all measured values, which is equivalent to setting $e_0 = 0$ in the above equations. Fitting the resulting TSH levels according to

$$n_{\mathrm{TSH}}(t)-n_{\mathrm{TSH}}(0)=\frac{i_0\tau_{\mathrm{TG}}}{\tau_{\mathrm{TG}}-\tau_i}\left(e^{-\frac{t}{\tau_{\mathrm{TG}}}}-e^{-\frac{t}{\tau_i}}\right) \quad (12)$$

yields the values summarized in Table 3. The fitted values reveal that the dynamics of the infused TSH is nearly identical during the early and late fasting seasons (Table 3).

Thyroid Hormone Dynamics: Constant Generation Model

Before fitting the T4 and T3 data according to the above described model it is necessary to evaluate the data at time points $t = 60$mins and $t = 120$mins (Table 4). These data suggest that the generation of T4 and T3 induced by the TSH infusion follows the same dynamics at early and late fasting periods. That is, for the purpose of fitting the data we may assume, that changes in the dynamics between early and late fasting results from a change in clearance. Here it is important to note, that the generation rates involved in the endogenous dynamics may change between early and late fasting. Whether that is the case or not can be addressed by assuming that the underlying dynamics are not affected by the TSH infusion, and that the changes in the clearance dynamics are solely responsible for the change in the endogenous levels. If so, then the fitted values for the clearance effect can be directly related to the measured endogenous levels (Table 2) with equations 7–11 as such:

$$\frac{n_{\mathrm{tT4}}^{\mathrm{Early}}(0)}{n_{\mathrm{tT4}}^{\mathrm{Late}}(0)}=\frac{\tau_{\mathrm{tT4}}^{\mathrm{Early}}}{\tau_{\mathrm{tT4}}^{\mathrm{Late}}} \quad (13)$$

A discrepancy between the calculated values and what is observed endogenously using this model, will indicate that the endogenous generation of hormone levels changes between early and late fasting. To fit the total T4, free T4, and total T3 data according to the above model, we proceed as such:

1. We fitted the TSH data for both early and late fasting by solving for $n_{bTSH}(t)$, which yielded values for i_0, τ_i and τ_{TG}.

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2. We used the (average) values obtained for the TSH dynamics (Table 3) as fixed parameters for fitting the total T4 data by solving for $n_{tT4}(t)$, to obtain values for s_x, $\tau_{x,g}$, and $\tau_{x,d}$, with $x = \text{tT4}$.
3. We repeated the previous step for the free T4 data. (Note, the same equations as for total T4 are used, just with the subscripts tT4 replaced by fT4)
4. We used the values obtained from fitting the TSH and total T4 data as fixed parameters for fitting the total T3 data by solving for $n_{tT3}(t)$, which yields values for s_3, $\frac{s_{43}}{\tau_{43}}$, $\tau_{3,g}$ and $\tau_{3,d}$.
5. Fitting the data for late-fasted animals was performed as described above (steps 1–4), while keeping all parameters pertaining to the generation fixed to their values from the early fasting season. That is, only the clearance times remained as fit parameters. These data are summarized summarized, (Table 5).

We find for total T4:

$$\begin{array}{ccc} \frac{n_{tT4}^{\text{Late}}(0)}{n_{tT4}^{\text{Early}}(0)} & = \cdots = & \frac{\tau_{tT4,d}^{\text{Late}}}{\tau_{tT4,d}^{\text{Early}}} \\ \| & & \| \\ 1.24 & \neq & 2.14, \end{array} \quad (14)\ (15)$$

and for free T4:

$$\begin{array}{ccc} \frac{n_{fT4}^{\text{Late}}(0)}{n_{fT4}^{\text{Early}}(0)} & = \cdots = & \frac{\tau_{fT4,d}^{\text{Late}}}{\tau_{fT4,d}^{\text{Early}}} \\ \| & & \| \\ 1.24 & \neq & 2.24, \end{array} \quad (16)\ (17)$$

which indicates that the assumption that the changes in clearance are solely responsible for the changes in endogenous levels is false.

Thyroid Hormone Dynamics: Constant Clearance Model

The data was analyzed under the assumptions of a constant clearance model (Table 6). Here, we assumed that the clearance remained unchanged between early and late fasting and that changes in endogenous TH levels were caused solely by changes in generation. In order to fit the data according to this model, we proceeded as before using the TSH values obtained by fitting the early fasting T4 data. To fit the late T4 data, we kept the T4 depletion time constant, $\tau_{tT4,d}^{\text{Early}} = \tau_{tT4,d}^{\text{Late}}$, and left the generation time, $\tau_{tT4,g}^{\text{Late}}$, and the scaling factor, s_{tT4}, as free fitting parameters. Then, we used the obtained values to fit the T3 data (Table 6). We then compared the fitted values to their matched endogenous ratios.

For total T4 we solved with Equation 10 as follows:

$$\begin{array}{ccc} \frac{n_{tT4}^{Late}(0)}{n_{tT4}^{Early}(0)} & =\cdots= & \frac{s_{tT4}^{Late}\tau_{tT4,g}^{Early}}{s_{tT4}^{Early}\tau_{tT4,g}^{Late}} \\ \| & & \| \\ 1.24 & \approx & 1.19, \end{array} \quad (18)\ (19)$$

and for free T4:

$$\begin{array}{ccc} \frac{n_{fT4}^{Late}(0)}{n_{fT4}^{Early}(0)} & =\cdots= & \frac{s_{fT4}^{Late}\tau_{fT4,g}^{Early}}{s_{fT4}^{Early}\tau_{fT4,g}^{Late}} \\ \| & & \| \\ 1.24 & \approx & 0.95, \end{array} \quad (20)\ (21)$$

The obtained ratios using the constant clearance assumption matched the endogenous ratios much better than those obtained under the constant generation assumption. An example of how the two fitted models differ is provided in Figure 4B. This analysis indicates that the assumption that the changes in the endogenous levels between early and late fasting are solely caused by changes in clearance is highly unlikely, suggesting instead that the changes in the endogenous T4 levels are the result of active T4 secretion. Collectively, these thorough analyses provide robust evidence for a functional and active thyroid gland throughout the elephant seal's fast. It is interesting to note that the fitted models yielded quasi infinite τ_{43}/s_{43} ratios for early fasting and finite values for late fasting. This observation is consistent with the up-regulation of the T4 to T3 conversion between early and late fasting that is independently corroborated by the gene expression data (Figure 5A–F).

TSH increases circulating TH levels

Measured plasma hormone levels show that endogenous levels of TSH are increased to the same capacity, rendering the response of the thyroid gland to hormonal stimulation a consequence of sensitivity (Table 7). To address our hypothesis that the thyroid gland remains responsive to TSH, with sensitivity increasing with fasting duration, we measured circulating thyroid hormones levels in response to TSH during the early and late fast. During the early fast, concentrations of tT4, fT4 and tT3 increased throughout the infusion period in response to TSH with levels peaking at 120 minutes and returning to baseline after 24 hours. However, although concentrations of tT4, fT4 and tT3 also increased in response to TSH during the late fast, with levels peaking at 120 minutes, circulating levels of tT4 and fT4 did not return to baseline after 24 hours (Figures 1 and 2).

TSH Increases mRNA Expressions of DI1 and THrβ1

DI1 and THrβ1 mRNA expression increased in response to TSH; however, the increases peaked at 120 min in adipose, and at 60 min post-infusion in muscle (Figure 5). Moreover, the increase in DI2 mRNA expression observed with fasting duration alone, are not changed with THS infusion (Figure 5).

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Discussion

A review of the literature clearly demonstrates that thyroidal status during times of food deprivation, fasting, and/or starvation are variable and dynamic among mammals. But typically for most mammals, the response to food deprivation includes suppression of TH secretion, circulating levels, and TH-mediated cellular signaling (Azizi, 1978; Haymond et al., 1982; Herlihy et al., 1990; Janan et al., 1995; LoPresti et al., 1991). Furthermore, the differences between responses help to assess the ecological and/or evolutionary pressures on that particular species' metabolism to conform and adapt for the survival of that species. Therefore, the motivation for this study was to better assess the responsiveness of the thyroid gland and the concomitant cellular events to define the physiological adaptations evolved in seals which enable them tolerate and thrive during prolonged bouts of absolute food deprivation. The study of the thyroid gland and cellular TH-mediated events in such a model of unique physiology characterized by physical activity and normothermia, is intriguing and offers a number of intellectual challenges. We hypothesized that the previously reported increases in TH between the early and late fasting were not solely a result of reduced clearance, but rather a result of increased secretion, influenced by: **(1)** production at the level of the thyroid gland, **(2)** cellular metabolism by deiodinases, and **(3)** receptor availability.

The infusion of TSH has long been used as a tool for assessing thyroidal regulation and its contribution to disease. For example, the potential of infused TSH to alter deiodination rate has been examined by Nicoloff (Nicoloff, 1970). However, deiodination was assessed by examining changes or alterations in labeled T4 via urinary excretion and not surprisingly, no changes were measured (Nicoloff, 1970). Similarly, a number of studies have assessed thyroidal secretion and sensitivity using pre-labeled radioactive iodine, which are only proxy measurements at best. Moreover, in a study where T4 and T3 concentrations were measured following TSH infusion, levels remaining unchanged acutely, but increased with time and remained elevated after TSH stimulation was removed (Laurberg, 1976). In this same study, deiodination was not assessed, although the author suggested that TSH may have also directly affect deiodination based on T3 being preferentially secreted during TSH stimulation (Laurberg, 1976). Our results are similar in that TH levels were not immediately nor acutely increased in response to TSH, but levels are predicted to increase substantially and are maintained elevated after TSH levels have diminished. These responses suggest that the thyroid gland does not have a pool of free hormone that can be released immediately. Moreover, the prolonged secretion of TH even after TSH stimulation is removed suggests that TSH remains bound to its receptor sufficiently long enough to perpetuate stimulation of the thyroid gland despite the disappearance of the acutely elevated TSH levels, this physiological maintenance of TSH stimulation due to prolonged receptor binding has previously been reported (Manley et al., 1974). Moreover, TSH has been shown to directly increase mono-deiodination in rat kidney, although the mechanism is not clear (Ikeda et al., 1985). Additionally, TSH stimulates both inner and outer ring deiodination (Ishii et al., 1983). This effect is at the very least suggestive of ubiquitous genomic regulation, whose inhibition would likely come from epigenetic regulation, or methylation. However, it should be noted that the number of TSH infusion studies in an effort to better assess thyroidal status, are starkly overshadowed by the number of thyrotropin releasing hormone (TRH)

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studies (Dizerega et al., 1978; Extein et al., 1980; Ikeda and Greer, 1993; Mongioí et al., 1983; Noel et al., 1974; Pavasuthipaisit et al., 1983). Furthermore, of the few TSH infusion studies (Ahrén et al., 1978; Laurberg, 1981), to the best of our knowledge, none have simultaneously examined deiodinases and receptor availability in different tissues, highlighting the significance of the present study.

An additional intriguing discovery includes the differential response in the timing of observed peak gene expressions between adipose and muscle following TSH infusion. For example, TSH increased both DI1 and THrβ1 in the late fast at 120 mins in adipose, but were increased at 60 mins in muscle. The tissue-specific increases in receptor availability and deiodinase transcript elucidate an additional level of regulation, where there also exists a change in tissue-specific sensitivity, with muscle deiodination and clearance increasing faster when compared to adipose. Furthermore, the timing of these cellular responses are closer to the peaks in TSH than to the peaks in THs suggesting that TSH may directly stimulate cellular events. TSH receptors have been found in a variety of peripheral tissues including, adipose, liver, and the pituitary gland (Balzan et al., 2007; Burgos et al., 2016; Prummel et al., 2000). TSH receptors are G-coupled protein receptors, which are integral cellular components that produce enzyme modification and gene expression (Schöneberg et al., 2016). Thus, the differential, tissue-specific responses suggest that peripheral TSH receptors regulate the sensitivity of that tissue as a function of fasting duration.

While adipose and muscle are both targets of TH, they may contribute differentially to tissue-specific cellular metabolism. For example, lipid and glucose metabolism may be regulated differentially within the two tissues examined (Obregon, 2014; Salvatore et al., 2014). The differences in the timing of the cellular events suggest that, while TH are likely contributing to the maintenance of the lipid metabolism, the sensitivity to TSH is increased in muscle- which is additionally intriguing given that earlier studies show that lean mass is the primary determinant of resting metabolic rate (RMR), which decreases in fasting northern elephant seal pups (Rea and Costa, 1992; Tift et al., 2013). Moreover, in mice and human muscle, T3-liganded THrβ1 increased the expression of mitochondrial uncoupling proteins as well as glucose transporter 4 (GLUT4) among others, resulting in TH-induced shifts in muscle function ending with high oxidative capacity and glycolytic potential (Solanes et al., 2005; Zorzano et al., 2005). Thus, the stimulation of TH-mediated events within muscle especially in late-fasted pups may be important to support muscle function and development to help prepare the animals for their initial diving experiences, which immediately follow their fasting period.

Given that RMR decreases across the fast in NES pups, it is plausible that the observed increases in response to thyroidal stimulation with fasting duration, may be contributing in part, to fulfill other physiological purposes. To that end, we know that TH contribute to a multitude of physiological functions in mammals, including erythropoiesis; a potent effect which can be observed in human subjects with hypothyroidism, often exhibiting concomitant symptoms of associated forms of anemia (Das et al., 2012). Also underlining the possible contribution of TH to erythropoiesis during this developmental stage in NES pups, are studies assessing the established contribution and requirement of TH receptors to drive erythropoiesis (Angelin-Duclos et al., 2005; Gauthier et al., 1999). These studies

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suggest that receptor availability is primarily only necessary during periods of early development. In adult mice for examples, THrβ and/or its isoform, thyroid hormone receptor alpha (THrβ) knock-outs have no detrimental consequence on steady state erythropoiesis (Angelin-Duclos et al., 2005; Gauthier et al., 1999). However, the extent of the thyroidal contribution to erythropoiesis is debatable given that previous studies show a lack of relationship between TH and hematological parameters, including the crucial red cell mass-maintaining hormone, which is erythropoietin (Somo et al., 2015). This differential response of tissue sensitivity may highlight the intricate relationship between TH regulation and insulin signaling, given that adipose develops insulin resistance with fasting duration in NES pups (Viscarra et al., 2011; Viscarra et al., 2013; Viscarra et al., 2010; Viscarra et al., 2012).

An additional point of notable consideration is that the regulation of the expression of DI2 is subject to endoplasmic reticulum (ER) stress (Arrojo e Drigo et al., 2011). Specifically, ER stress acts as a highly effective disruptor of D12 activity (Arrojo e Drigo et al., 2011). It is well established that ER disruption, which is caused by metabolic disorders such as diabetes, insulin resistance, and obesity, increase ER stress (Özcan et al., 2004). Although DI2 expression is relatively lower in adipose, which is insulin resistant compared to muscle (Viscarra et al., 2011; Viscarra et al., 2013; Viscarra et al., 2010; Viscarra et al., 2012), adipose DI2 expression increases with fasting duration suggesting that insufficient stress is generated to disrupt DI2 expression. Oxidative stress is also abrogated in these tissues as a function of fasting duration further corroborating the contention that cellular stress is not likely contributing to the regulation of DI2, and likely the other deiodinases, in fasting elephant seals (Vázquez-Medina et al., 2010). The mathematical model employed here compliments and offers further insight into our unique dataset. Given federal regulations, the need to keep stress levels of the animals minimal and sampling periods low, hinders continuous data collection from the NES pups. Instead, the experiment was designed to obtain data (tissue sample) in closely spaced time intervals right after the introduction of a TSH bolus, with an additional measurement after 24 hours. Based on prior data and observations, we anticipated that an acute response to a TSH bolus would occur in the first few hours, with cessation occurring well before 24 hours. Much to our surprise and despite the rapid clearance of TSH from the system the NES pups actually demonstrate a maintained and elevated metabolic response even after the end of the sampling period. In order to quantitatively analyze the response despite the lack of measurements between hour 2 and hour 24 we setup a flow diagram, which diagrammatically describes the processes of T3/4 generation and clearance. This flow diagram was then translated to a mathematical rate equation system. The solution of the rate equation system is used here to fit the experimentally obtained data. The parameters extracted (generation and clearance rates) from the fit allow us to visualize the most probable temporal evolution of the T3/4 levels at and between the measured time steps, based on the available measured data and the flow diagram. In other words, if the assumed flow diagram is correct, the corresponding mathematical rate equation model and the measured data points yield the transient T3/4 levels as indicated by the solid lines in Figures 3 and 4. The fitting, according to the modeled plasma data yielded insights which suggested that T4 to T3 conversion was increased during the late fast given the time parameters generated in Table 5, which complimented our empirical gene analysis given the increases in DI gene expression. Collectively and

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complimentarily, both the modeled TH data and the empirically generated gene expression results provide a more comprehensive and novel picture of thyroidal regulation during fasting duration in a large mammal, naturally adapted to prolonged fasting. From the modeled data we observed and predicted the following: **(1)** T4 clearance decreased in the late fast compared to the early fast, resulting in the maintenance of circulating levels of TH with fasting duration, **(2)** the secretion of T4 in response to TSH was greater in late fasting compared to early suggesting that the sensitivity of the gland increased with fasting and that this increased production provides an additional means to help maintain circulating levels, **(3)** T3 was cleared faster than T4 regardless of fasting duration, which would help ensure the availability of T4 for cellular deiodination, and thus, cellular activity, and **(4)** the T4:T3 ratio decreased after TSH infusion suggesting that T3 and T4 are generated at a different rate than prior to TSH infusion. The model independently predicted that during the late fast, the conversion of T4 to T3 is upregulated. This would suggest that deiodination and receptor clearance are increased, which is what the empirical gene expression data demonstrated.

Conclusions

This study revealed three significant contributions to the disciplines of cell and evolutionary biology, endocrinology, comparative and ecological physiology, and biomedicine. Among these important findings were: **(1)** given the potential issues with assessing thyroid status from a single plasma sample, which only accounts for a static measure of circulating TH levels, the modelling of the endocrine response to a TSH challenge provides unique insight to clearance rates, thyroid gland responsiveness, and production rates that when coupled with cellular, tissue-specific events, provides a more robust assessment of the dynamic changes associated with the thyroid gland and its related cellular signaling, **(2)** evidence that elephant seal pups undergo a unique fasting period that is driven by paradoxical, fasting-induced changes in thyroid gland production that can be better characterized as “adaptive fasting”. Additionally, this study coupled with additional data now published, further suggests that the reliance on lipid oxidation to support the metabolism of fasting northern elephant seals makes it likely that TH-mediated signaling contributes to lipid metabolism, which may provide unique insights into the complex interactions between TH, impaired lipid metabolism, and human obesity (Martinez et al., 2016b). Unraveling the biology behind this truly exceptional fasting response, perhaps better known as, “adaptive fasting”, may provide alternative insight to addressing the multitude of metabolic disorders in humans. For example, identifying mechanisms that would allow humans to preferentially metabolize lipid, all the while not eating and maintaining the integrity of their cellular capacity, could provide beneficial changes on many scales.

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- description of a novel adaptation to fasting, highlighting a unique contribution from the thyroid gland, which appears paradoxical and is physiologically perplexing
- demonstration that the thyroid gland remains responsive during prolonged food deprivation, and more importantly that its function and production of TH change with fasting duration in elephant seals
- employment of mathematical models that confirmed the cellular, tissue-specific responses that combined provide unique insight on hormone clearance, production rates, and thyroid gland responsiveness
- an approach that offers a more integrated assessment of thyroidal status that can be extrapolated for any fasting/food deprived mammal.

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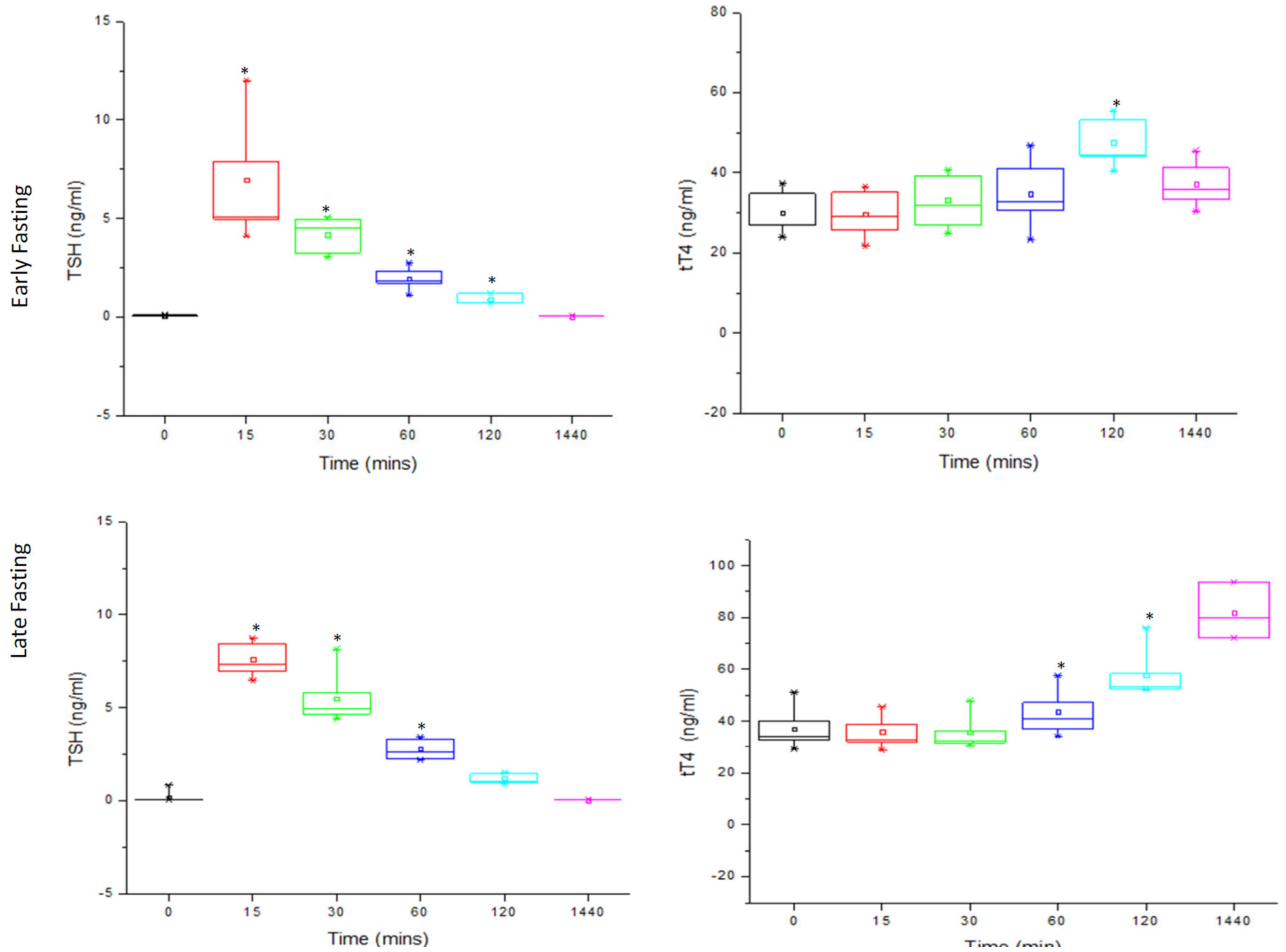

Figure 1.

Raw plasma (± s.e.m.) concentrations of plasma **(A)** thyroid stimulating hormone, **(B)** total thyroxine (tT4) from northern elephant seal pups before (0) and 15, 30, 60, 120, and 1440 mins post-TSH infusion in the early (n=6; 2–3-weeks post-weaning) and late (n=6; 6–8 weeks post-weaning)-fasted elephant seal pups. * denotes significant ($P<0.05$) difference from time 0.

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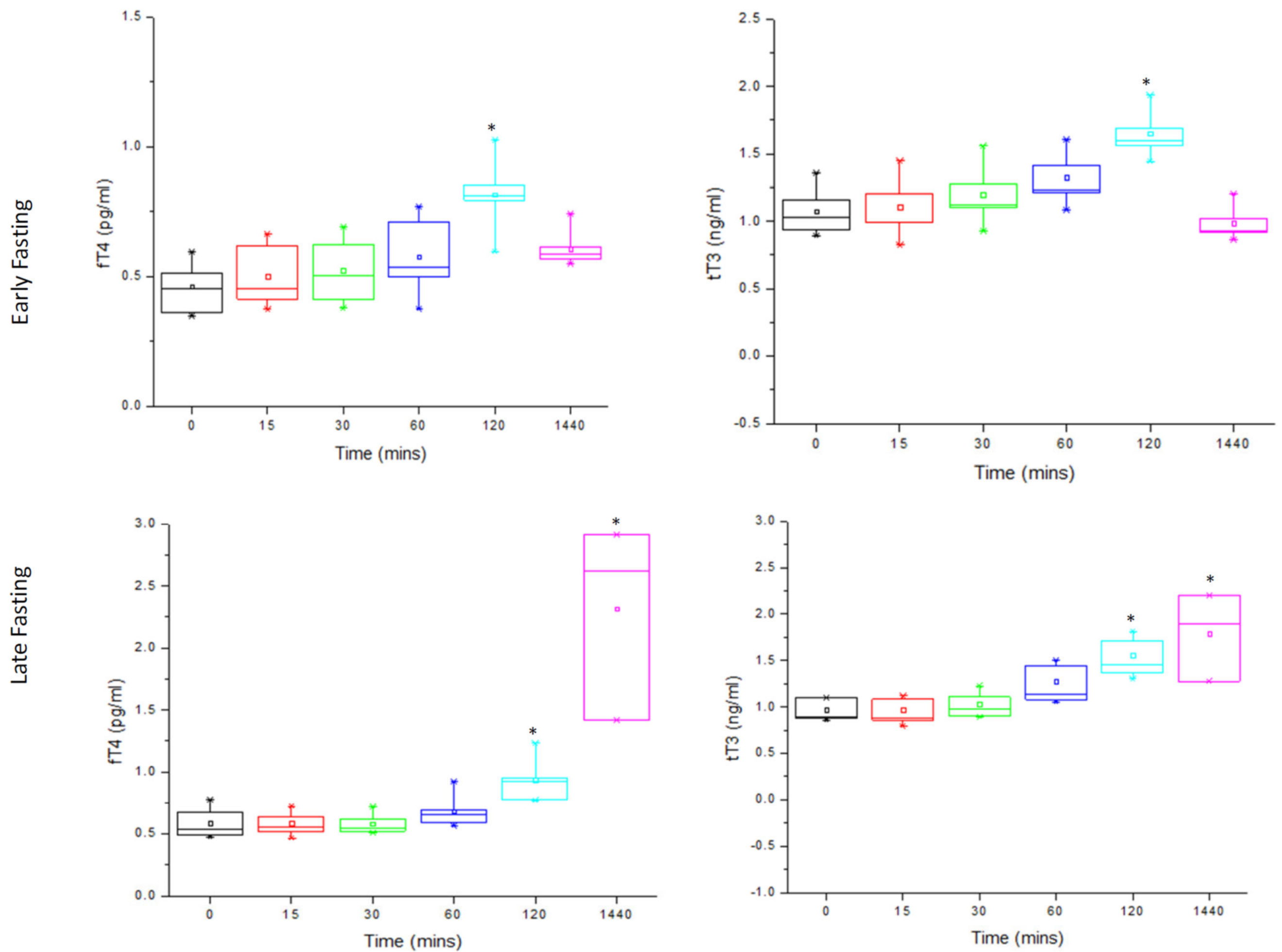

Figure 2.

Raw plasma (± s.e.m.) concentrations of plasma **(A)** free thyroxine (fT4), and **(B)** total 3,5,3′-triiodothyronine (tT3) from northern elephant seal pups before (0) and 15, 30, 60, 120, and 1440 mins post- TSH infusion in the early (n=6; 2–3-weeks post-weaning) and late (n=6; 6–8 weeks post-weaning)-fasted elephant seal pups. * denotes significant (P<0.05) difference from time 0.

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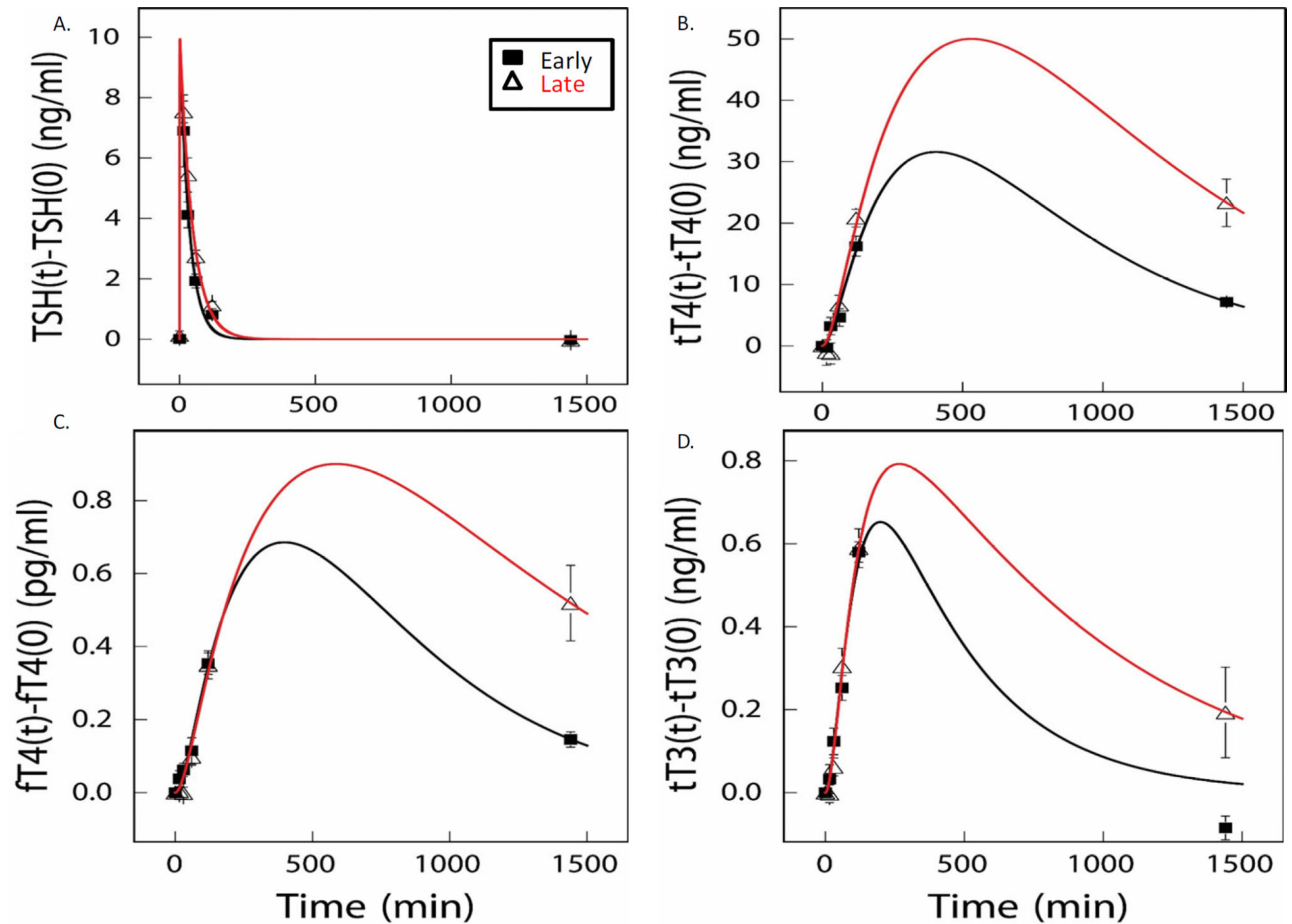

Figure 3.

Modeled mean (± s.e.m.) concentrations of plasma **(A)** thyroid stimulating hormone (TSH), **(B)** total thyroxine (tT4), **(C)** free thyroxine (fT4), and **(D)** total 3,5,3′-triiodothyronine (tT3) from northern elephant seal pups before (0) and 15, 30, 60, 120, and 1440 mins post-TSH in the early (n=6; 2–3-weeks post-weaning) and late (n=6; 6–8 weeks post-weaning)-fasted elephant seal pups. The black line is the fit to the early fasting data (solid squares). The red line is the fit to the late fasting data (open triangles) according to the constant clearance model.

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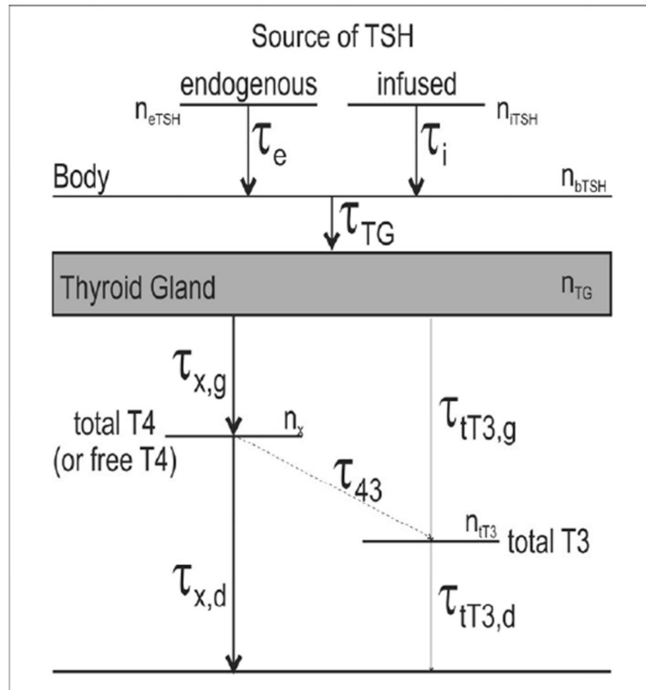

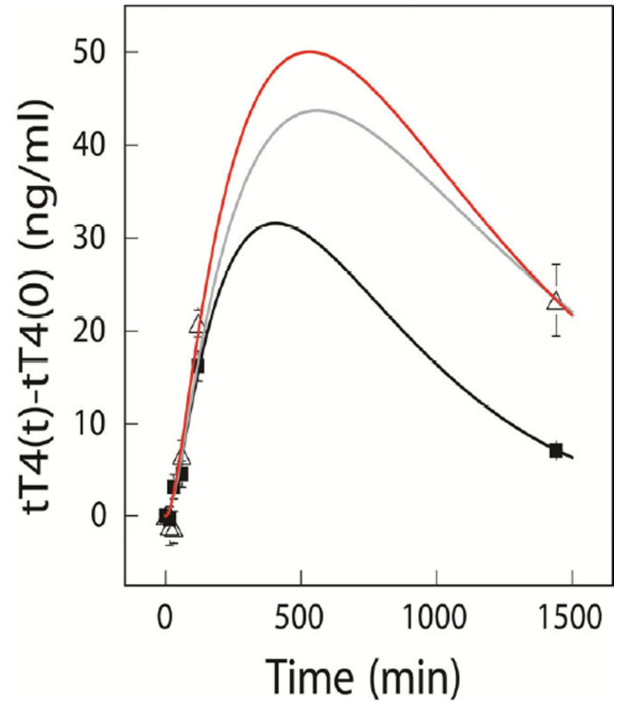

Figure 4.

(**A**) A diagrammatic model depicting the developed mathematically model assessing the measured levels of T4 and T3 as a function of time after TSH infusion in fasting northern elephant seal pups, (**B**) Fitted total T4 dynamics using mathematical model. The black line is the fit to the early fasting data (solid squares). The grey line is the fit to the late fasting data (open triangles) according to the constant generation model. The red line is the fit to the late fasting data according to the constant clearance model.

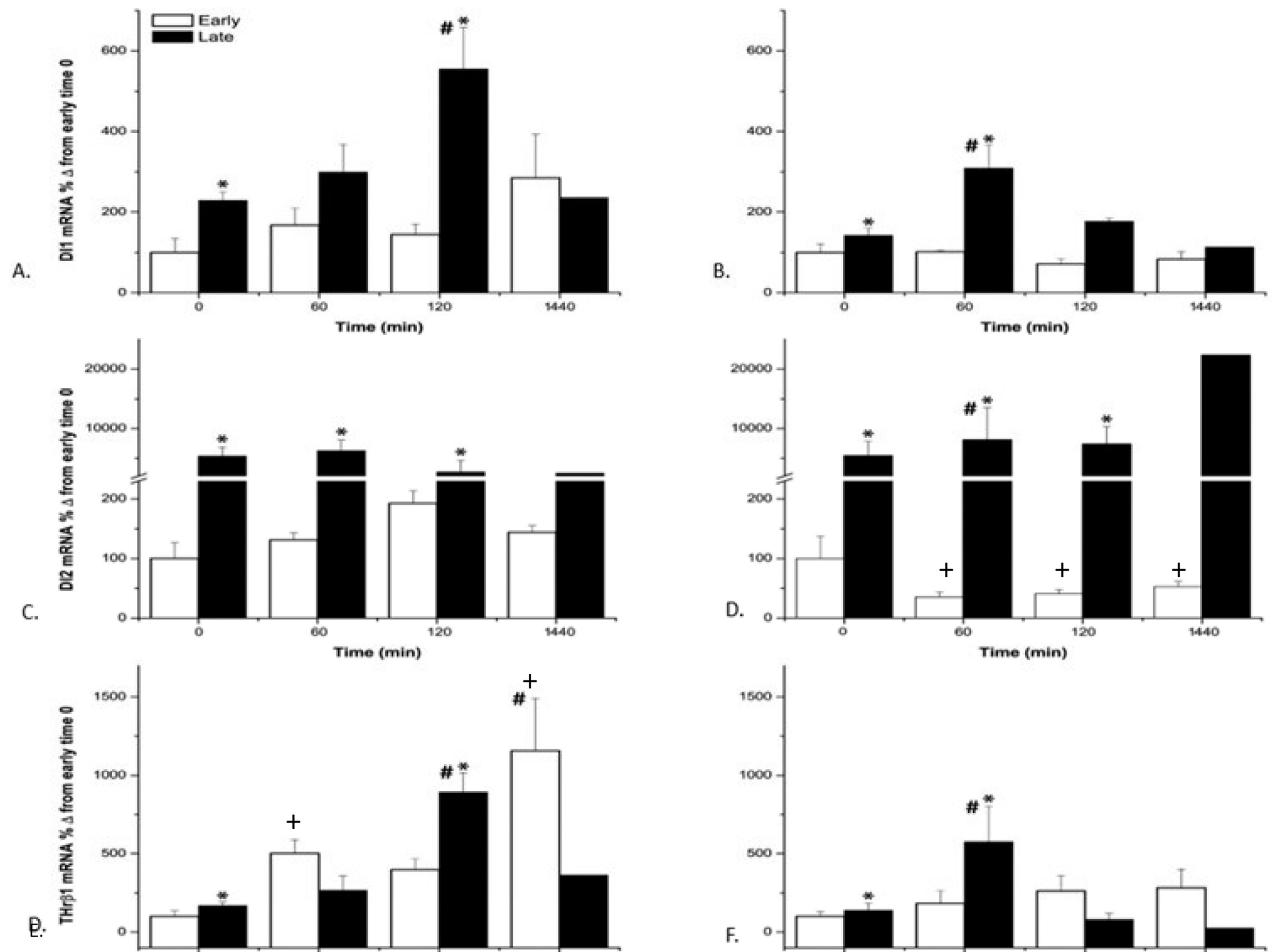

Figure 5.

Mean (± s.e.m) mRNA expressions of **(A)** adipose deiodinase type I (DI1), **(B)** muscle deiodinase type I (DI1) **(C)** adipose deiodinase type II (DI2), **(D)** muscle deiodinase type II (DI2) **(E)** adipose thyroid hormone receptor beta-1 (THrβ1) and **(F)** muscle thyroid hormone receptor beta-1 (THrβ1) from northern elephant seal pups before (0) and 60, 120, and 1440 mins postthyroid stimulating hormone infusion in the early (n=6; 2–3-week post-weaning) and late (n=6; 6–8-week post-weaning)-fasted elephant seal pups. * denotes significant ($P<0.05$) difference from 2–3 weeks post-weaning; + denotes significant ($P<0.05$) difference from T0 within the early fasting group; # denotes significant ($P<0.05$) difference from T0 within the late fasting group.

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Table 1

Summary of variables used in mathematical model.

Symbol	Dimension	Description
n_{eTSH}	ng/ml	Endogenous TSH concentration where generated
n_{iTSH}	ng/ml	Concentration of the injected TSH
n_{bTSH}	ng/ml	Concentration of TSH at location of tissue extraction
n_{TG}	ng/ml	Concentration of TSH at location of thyroid gland
n_x with x=tT3, fT4, tT4	ng/ml	Concentration of tT3, fT4, tT4
τ_e	s	Time constant of dispersion of the endogenous TSH
τ_i	s	Time constant of dispersion of the injected TSH
τ_{TG}	s	Time constant of TSH depletion
$\tau_{tT4,g}$	s	Time constant of tT4 generation (& dispersion)
$\tau_{tT3,g}$	s	Time constant of tT3 generation (& dispersion)
$\tau_{tT4,d}$	s	Time constant of tT4 depletion
$\tau_{tT3,d}$	s	Time constant of tT3 generation
τ_{43}	s	Time constant of T3 generation from T4
s_x with x=fT4, tT4, 3, 43		scaling factors

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Table 2

Measured endogenous levels at time zero (before TSH or saline infusion).

Season	endogenous levels of			
	TSH (ng/ml)	**total T4 (ng/ml)**	**free T4 (pg/ml)**	**total T3 (ng/ml)**
Early	0.053 ± 0.010	31 ± 2	0.45 ± 0.02	1.06 ± 0.04
Late	0.058 ± 0.003 [†]	38 ± 3	0.56 ± 0.04	1.08 ± 0.07

Values represent the averages from 10 animals each during the early and the late fasting seasons.

(†) The value for the late TSH was calculated from the average of nine animals, as the tenth value exceeded the average by seven standard deviations.

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Table 3

Fitted values (including standard errors) describing the dynamics of the infused TSH during the early and late fasting seasons.

Season	Fitted values for		
	i_0 (ng/ml)	τ_i (min)	τ_{TG} (min)
Early	10.2 ± 0.9	0.24 ± 0.27	35 ± 5
Late	10.2 ± 0.5	0.20 ± 0.11	48 ± 4

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Table 4

Comparison of the $t = 60$ mins and $t = 120$ mins data points for early and late fasting.

Season	Data values for		
	n_{tT4} (ng/ml)	n_{fT4} (min)	n_{tT3} (min)
t=60 minutes			
Early	4.62 ± 1.42	0.11 ± 0.04	0.25 ± 0.03
Late	6.65 ± 1.58	0.10 ± 0.02	0.30 ± 0.04
t=120 minutes			
Early	16.25 ± 1.63	0.35 ± 0.03	0.58 ± 0.02
Late	20.81 ± 1.44	0.35 ± 0.04	0.59 ± 0.05

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Table 5

Constant Generation Model Fitting: Fitted values describing the total T4, free T4 and total T3 dynamics after TSH infusion during the early and late fasting seasons.

Parameter	Fasting Season	
	Early	**Late**
Total T4		
$\tau_{tT4,g}$	350 min.†	
s_{tT4}	9.64±1.09††	
$\tau_{tT4,d}$	(384±39)min.	(824±77)min.
Free T4		
$\tau_{fT4,g}$	360 min.†	
s_{fT4}	0.180±0.011††	
$\tau_{fT4,d}$	(366±30)min.	(819±46)min.
Total T3		
$\tau_{tT3,g}$	60 min.†	
s_{tT3}	0.077±0.032††	
$\tau_{tT3,d}$	(80±73)min.	(73±9)min.
$\frac{\tau_{43}}{s_{43}}$	$(6 \cdot 10^{15} \pm 0)$min.	(10900±2900)min.

† The values for the generation times were determined through an initial fitting step in which they were left as fitting parameters. In a second step they were fixed at values near the fitting value obtained initially. The displayed value represents a value for which the standard error of the other fitting parameters is approximately minimized.

†† The values for the scaling parameters were obtained from fitting the data from the early fasting season. Those values were then fixed when fitting the data from the late fasting season.

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Table 6

Constant Clearance Model Fitting: Fitted values describing the total T4, free T4 and total T3 dynamics after TSH infusion during the early and late fasting seasons assuming a constant clearance.

Parameter	Fasting Season	
	Early	**Late**
Total T4		
$\tau_{tT4,g}$	(350±37)min.	(647±74)min.
s_{tT4}	(8.09±0.74)	(17.75±1.37)
$\tau_{tT4,d}$	384 min.†	
Free T4		
$\tau_{fT4,g}$	(350±30)min.	(873±87)min.
s_{fT4}	(0.180±0.013)	(0.395±0.021)
$\tau_{fT4,d}$	366 min.†	
Total T3		
$\tau_{tT3,g}$	(70±0)min.	(565±0)min.
s_{tT3}	(0.089±0.045)	(0.680±0.039)
$\tau_{tT3,d}$	80 min.†	
$\frac{\tau_{43}}{s_{43}}$	$(7 \cdot 10^{11} \pm 0)$min.	(30200±14100)min.

†The values for the depletion times were taken from the fitting of early fasting data performed under the under constant generation assumption.

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Table 7

Mean (± s.e.m.) concentrations of plasma thyroid stimulating hormone (TSH), total (t) and free (f) thyroxine (T4), and total (t) triiodothyronine (T3) from northern elephant seal pups before (0) and 15, 30, 60, and 120 post- saline infusion during the early and late fasting period.

	0	**15**	**30**	**60**	**120**
			Early Saline Infusion		
TSH (ng/ml)	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.09 ± 0.07
tT4 (ng/ml)	31 ± 2	30 ± 2	32 ± 2	27 ± 3	34 ± 1
fT4 (pg/ml)	0.44 ± 0.01	0.44 ± 0.02	0.45 ± 0.03	0.43 ± 0.01	0.47 ± 0.02
tT3 (ng/ml)	1.04 ± 0.02	1.04 ± 0.03	1.07± 0.05	1.0 ± 0.05	1.0 ± 0.03
			Late Saline Infusion		
TSH (ng/ml)	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.07 ± 0.03
tT4 (ng/ml)	39 ± 5	35 ± 5	36 ± 4	34 ± 4	36 ± 4
fT4 (pg/ml)	0.52 ± 0.08	0.52 ± 0.08	0.52 ± 0.07	0.47 ± 0.07	0.54 ± 0.09
tT3 (ng/ml)	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1