NMR-Based Metabolomics and Breath Studies Show Lipid and Protein Catabolism During Low Dose Chronic T1AM Treatment

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Objective: 3-Iodothyronamine (T1AM), an analog of thyroid hormone, is a recently discovered fast-acting endogenous metabolite. Single high-dose treatments of T1AM have produced rapid short-term effects, including a reduction of body temperature, bradycardia, and hyperglycemia in mice.

Design and Methods: The effect of daily low doses of T1AM (10 mg/kg) for 8 days on weight loss and metabolism in spontaneously overweight mice was monitored. The experiments were repeated twice (n = 4). Nuclear magnetic resonance (NMR) spectroscopy of plasma and real-time analysis of exhaled 13CO2 in breath by cavity ring down spectroscopy (CRDS) were used to detect T1AM-induced lipolysis.

Results: CRDS detected increased lipolysis in breath shortly after T1AM administration that was associated with a significant weight loss but independent of food consumption. NMR spectroscopy revealed alterations in key metabolites in serum: valine, glycine, and 3-hydroxybutyrate, suggesting that the subchronic effects of T1AM include both lipolysis and protein breakdown. After discontinuation of T1AM treatment, mice regained only 1.8% of the lost weight in the following 2 weeks, indicating lasting effects of T1AM on weight maintenance.

Conclusions: CRDS in combination with NMR and 13C-metabolic tracing constitute a powerful method of investigation in obesity studies for identifying in vivo biochemical pathway shifts and unanticipated debilitating side effects.

Introduction

3-Iodothyronamine (T1AM) is thought to be an endogenous derivative of thyroid hormone discovered in 2004 (1). To date, the physiological effects of endogenous T1AM remain elusive, although there is increasing interest in its physiological function and pharmaceutical potential due to the role it plays in lipid and glucose metabolism (2–4). Research shows that T1AM action is mediated through nongenomic signaling (5), binding to G-protein coupled receptors such as trace amines associated receptors Type 1 (6), and Alpha-2A adrenergic receptors (2). Time-course studies indicate that T1AM has a fast action and causes significant changes in body temperature, activity level, and glucose metabolism (1,4,7). On the basis of these findings, we hypothesize that T1AM is involved in rapid regulation of lipid metabolism that is central to its metabolic functions.

Reports on measurements of endogenous T1AM concentrations remain conflicting (8–10), which is likely due to the differences in measuring unbound versus total (bound and unbound) T1AM (11). Pharmacological studies of T1AM administration (on the order of nmol/kg body weight) show that T1AM causes symptoms consistent with a hypermetabolic phase, such as increased activity and food consumption (12). However, the majority of T1AM research has used a high dose (50 mg/kg body weight) that induces a severe hypometabolic state, suggesting that T1AM works in opposition to
High doses of T1AM (i.e., 50 mg/kg body weight) could have potential applications in emergency medicine because it protects against ischemic injury following stroke (15) or in space travel because it induces profound hypothermia and torpor-like symptoms (7). However, the pharmacological potential of multiple lower doses of T1AM to regulate metabolism has not yet been explored. Previous studies have noted no observable adverse effects up to 2 months following a single administration of 50 mg/kg body weight T1AM (1), indicating that long-lasting adverse side effects following the discontinuation of treatment are unlikely.

In this study, we focused on investigating the effects of chronic treatment of T1AM at a lower pharmaceutical dose on lipid metabolism in a spontaneously obese mouse model by multianalytical techniques. T1AM was administered to mice daily at a dose of 10 mg/kg body weight for 8 days, whereas breath stable isotope ratios were monitored continuously by cavity ring down spectroscopy (CRDS) and plasma samples were collected and analyzed by nuclear magnetic resonance (NMR)-based metabolomics to determine the efficacy of the treatment to induce weight loss and screen for possible unexpected side effects. Untargeted NMR-based metabolomics is a powerful tool to allow general screening of potential metabolic effects of T1AM treatment, whereas a targeted method uses 13C-metabolic labeling to specifically trace endogenous versus exogenous metabolites contributing to the treatment. Our results show that using CRDS in combination with NMR and 13C-metabolic labeling and tracing can provide a powerful analytical tool for identifying in vivo T1AM associated changes in energy substrate utilization.

In the first study, animals had access to food during breath collection and blood samples were collected in nonfasting condition. In the second study, animals were fasted 4 h before blood collection.

Preparation of T1AM
A stock solution of T1AM (56 mg in 112 μL of dimethyl sulfoxide (DMSO) carrier) was dissolved in 33.6 mL of medical grade 0.9% saline, which was then aliquoted into volumes for single injections at final 10 mg/kg body weight. A vehicle solution was made of 112 μL DMSO carrier in 33.6 mL 0.9% saline solution for injecting sham animals. All T1AM and vehicle solutions were stored at –80°C until use.

Preparation of glucose solution
Two grams of [U-13C]-glucose in 6 mL medical grade 0.9% saline and individually aliquoted and were stored at –80°C until injection time.

Breath sample collection
Single animals were placed in a flow through custom-made 1230 cm^3 metabolic chamber as previously described (16,17). The chamber contained Aspen bedding and free access to food and water. Animals were acclimated to the chamber 1 week before the study to reduce stress and associated weight loss. Furthermore, animals were placed in the chamber for 2 h before injection to capture a steady baseline δ13C value before injection with T1AM or saline. All animals were then injected at 12:00 h daily from day 0-7 intraperitoneally with either 10 mg/kg T1AM or vehicle (total injection volume in mL was 0.06 times body weight in grams). Immediately after injection, the animals were returned to the chamber, and breath delta values measured for four more hours. Then, they were returned to their home cage. This process was repeated for each animal separately and the treatment was done each day at the same time for a period of 1 week per animal. All data were collected at the same time of day to account for diurnal variation that is known to affect δ13C values in mice (16). Isotopic CO2 was measured from exhaust air using CRDS (Picarro, Sunnyvale, CA) (16,18,19).

Plasma sample collection
Baseline reference plasma was collected 7 days before the start of injections (day 7) and then on day 0, day 7, and day 21 (followed 2 weeks after last day of T1AM treatment). All plasma samples were collected at 16:00 h (2-h after injection with T1AM). For all blood collections, animals were anesthetized with 2.5% isoflurane, and blood was collected from the retro orbital venous plexus with heparinized capillary tubes. Plasma was then separated by refrigerated centrifugation at 1,000g for 10 min at 4°C and stored at –80°C until preparation for NMR data collection.

Analysis of long-term effects of T1AM on energy substrate utilization via [U-13C]-glucose
On day 21 of the experiment, mice were fasted 2 h before intraperitoneal injection of 20 g/kg of [U-13C]-glucose. Blood was collected 2-h later at maximum metabolic response (17). Plasma samples were prepared and stored as described above.

Preparation of plasma samples for NMR
Frozen plasma samples were allowed to thaw on ice. Ice-cold methanol (2:1, v/v) precipitation was performed to remove
proteins as previously described (17). The supernatant was then dried in a speed vacuum overnight. The dried supernatant was redissolved in 20 mM phosphate buffer containing 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid and 0.1 mM sodium fluoride and the pH was adjusted to 7.4 ± 0.05.

Collection of NMR spectra
All one-dimensional (1D) $^1$H-NMR spectra were collected at 25°C on a 600 MHz Varian VNMRS spectrometer equipped with a cryogenic probe (17). Each 1D spectrum was accumulated for 1,028 scans, with an acquisition time of ~400 ms (4,096 complex points) and a 3 s repetition delay for a total collection time of ~2 h (17). 1D data were then analyzed using Chenomx NMR suite (Chenomx, Edmonton, Alberta, Canada) and Mestranova (Mestrelab Research, Santiago de Compostela, Spain) software to identify relative concentrations of metabolites of interest. To obtain 1D spectra that contain only signals from $^{13}$C-bound protons, two different 1D spectra were collected as interleaved scans: one specific to $^{12}$C and the other isotopically nonspecific ($^{12}$C + $^{13}$C) as described previously (17). Each 1D spectrum, $^{12}$C and $^{12}$C + $^{13}$C, was accumulated for 1,028 scans, with an acquisition time of ~400 ms (4,096 complex points) and a 3 s repetition delay for a total collection time of ~2 h. 1D subtracted spectra were then analyzed as described above.

Statistical analysis
Despite the small sample size ($n = 4$), normality was confirmed by 95% of the data fitting within ± two standard deviations of the mean. Two-tailed student’s $t$-test was performed on breath delta values from 0 to 80 min after T1AM injection each day for the T1AM- and the sham-treated groups. Body weight, feed consumption, and NMR metabolite data were analyzed with two-tailed student’s $t$-tests. Differences were considered significant with $P < 0.05$ unless otherwise stated.

Results

Body weight
Exogenous T1AM administration was associated with a body weight loss of 8.2% of initial body weight by day 9 ($P = 0.038$) (Figure 1). On the other hand, sham mice lost only 0.1% of their initial body weight by day 9. After T1AM withdrawal, mice regained only 1.8% of initial weight in the following 2 weeks. The chronic treatment with 10 mg/kg T1AM administration was repeated for a second time, which resulted a similar trend with a total weight loss of 8.6% of initial body weight by day 7 (data not shown).

Food intake
Weight loss was not associated with any change in food intake. No difference in food intake was observed at any time during the study between T1AM- and sham-treated animals. There were
insignificant fluctuations in the amount of food consumed at different points in the study, which was consistent for both T1AM- and sham-treated animals. Preinjection food consumption averaged 3.91 g/day ± 0.17 for sham and 3.30 g/day ± 0.45 for T1AM (P = 0.32). Food consumption during treatment decreased slightly (sham = 3.36 g/day ± 0.11; T1AM = 3.12 g/day ± 0.47; P = 0.47). Post-treatment food consumption increased slightly for both groups (sham = 4.13 g/day ± 0.02; T1AM = 4.18 g/day ± 0.14; P = 0.76).

Real-time breath analysis
Upon switching to stored fat as a fuel source, the effect on breath δ13C values become more negative (16,19,20). Thus, to follow the effect of T1AM on lipid breakdown, we measured real-time stable isotope fractionation in breath using CRDS reported as δ13C values (19). We determined the baseline real time breath δ13C values for 120 min before any treatment. The first 30 min allowed animals to become acclimated to the metabolic chamber. However, the breath delta values showed a steady decline shortly after T1AM injection with a minimum δ13C value at about 80 min post injection as compared with the control group that did not show a decline. Figure 2 shows the average daily difference between T1AM and sham injected mice for the δ13C value 80 min postinjection. The δ13C value was significantly lower (~3.6%) in T1AM-treated mice from day 0 to 4 with (P < 0.05) (Figure 2). However, later in the treatment (day 5–7), the T1AM treatment effect on lipolysis was diminished (δ13C value ~2%).

NMR plasma analysis
Blood samples taken on day 7 (pre-T1AM injection) and day 0 (the first 2 h post-T1AM injection) revealed no differences in plasma metabolites between T1AM (n = 7) and sham (n = 8) animals either in the fasted or the nonfasted conditions (data not shown). However, after 8 days of T1AM treatment, plasma 3-hydroxybutyrate, a lipid breakdown intermediate, was significantly higher in nonfasted T1AM-treated animals compared with sham animals (n = 4) (P = 0.031) (Figure 3A), indicating increased lipolysis in the nonfasting regime. Similarly, under fasted condition, 3-hydroxybutyrate was still significantly higher in the T1AM-treated group as compared with the sham group (n = 4) (P = 0.03). Blood samples collected on day 21, after an uniformly labeled 13C-glucose challenge, revealed increases in unlabeled (12C) glycine (1.35× controls, P = 0.05) and valine (1.70× controls, P = 0.001) in T1AM-treated mice (Figure 3B). Interestingly, 13C labeled acetate was 2.95 times higher in the T1AM animals compared with the sham group (Figure 3C) under the nonfasting condition and still remained higher in the T1AM-treated mice even in the fasted condition (P = 0.286). The increased level in 13C-acetate is consistent with the lower weight gain at day 21 seen in the T1AM-treated animals (1.8% weight regained by day 21).

Discussion
This study investigated subchronic effects of T1AM treatment at a low pharmaceutical dose on lipid metabolism using three complementary technologies (CRDS, NMR-based metabolomics, and
We administered 10 mg/kg/day of T1AM for 8 days as a potential method for inducing weight loss. We followed the effect of T1AM for two additional weeks to evaluate long-lasting consequences of T1AM on weight gain and to assess the effectiveness of T1AM in maintaining a new reduced weight level and its side effects. Based on the combination of these analytical methods, we were able to show that chronic T1AM exposure induced a rapid increase in lipid mobilization, followed by a shift in macronutrient substrate oxidation from lipids to proteins in the last days of treatment.

T1AM-treated mice showed continued reduction in body weight compared with sham animals, without reduction in food intake. Although daily reduction in body weight in T1AM-treated mice did not reach statistical significance during the 8 days of treatment, weight loss was significantly greater than in sham mice at day 9 (−8.2%; \(P = 0.038\) for the first study, and −8.6%; \(P < 0.05\) in the second study). Importantly, after T1AM withdrawal, the mice that were allowed ad libitum access to food, regained only a small part of the lost weight (1.8% of initial weight regained) in the following 2 weeks versus sham mice.

Dhillo et al. reported decreased levels of activity in mice at a high dose of T1AM, and no change in activity at a low dose of T1AM (12), consistent with our observations that the weight loss seen in our study is not due to increased activity. The results from this study suggest that T1AM alone is capable of promoting weight loss and may show increased efficacy for weight maintenance at a pharmaceutical level, if proteolysis is controlled. A recent study showed that changes in food consumption post-T1AM administration follow a biphasic dose-dependent response (21). The biphasic responses associated with T1AM may explain no changes on food consumption in our study, whereas other studies have shown various effects depending on the dose and route of administration (12,21).

A single high-dose injection of T1AM was shown to play a role in regulating glucose and lipid metabolism (2–4,21). Specifically, T1AM induced a shift in energy metabolism from carbohydrates to lipids, which resulted in decreased fat mass (3). Our study provides the first evidence that T1AM alone at a subchronic, lower pharmacological dose administration, plays a role in increased lipid oxidation. We measured T1AM associated changes in energy substrate utilization by monitoring carbon stable isotope ratio (\(^{13}\)CO\(_2\)/\(^{12}\)CO\(_2\), or \(\delta^{13}\)C value) in exhaled breath. CRDS can be used continuously for assessing lipid oxidation in real-time and noninvasively (16,20). The results from breath analysis corroborate existing studies linking T1AM induced weight loss to increased lipolysis.

Our results showed that 2 h before treatment, the baseline \(\delta^{13}\)C values were similar in both sham and T1AM treatment groups. Therefore, the depression in \(\delta^{13}\)C values following T1AM administration indicates that the differences in observed \(\delta^{13}\)C values between the two groups are attributed to the effect of T1AM shifting substrate utilization to lipids and increasing rate of expired \(^{12}\)CO\(_2\) release in breath. Because of the isotope effect of pyruvate dehydrogenase during synthesis, lipids were enriched in the lighter isotope \(^{12}\)C; thus, during lipolysis, more \(^{13}\)CO\(_2\) is generated.

\[ \text{FIGURE 3} \]
Analysis of plasma by NMR-based metabolomics reveals an increase in the lipid oxidation and protein break down in nonfasting condition. (A) A significant increase in 3-hydroxybutyrate at day 7 of T1AM treatment. The concentration of 3-hydroxybutyrate is shown as light gray bars for T1AM-treated mice (\(n = 3\)) and dark gray bars for control mice (\(n = 4\)) (\(P = 0.03\)). (B) Increases in the plasma amino acids glycine (glucogenic) and valine (branched and ketogenic/glucogenic) in post-T1AM treatment regime. Plasma samples were collected in the following 2 weeks after discontinuation of T1AM treatment and were 2 h post \([\text{U-}\text{13C}]\)-glucose injection. The concentration of glycine (\(P = 0.05\)) and valine (\(P = 0.001\)) is shown as light gray bars for T1AM-treated mice and dark gray bars for sham mice. T1AM associated increases in both of these amino acids indicate a shift in energy metabolism following glucose administration. (C) Increase in the plasma acetate concentration (lipid intermediate) at day 7 of T1AM post-treatment. Light gray bars are for T1AM-treated mice and dark gray bars represent control mice, respectively. Error bars represent the SEM. Statistical significance (\(P < 0.05\)) compared with the sham-treated group is indicated by asterisks.
resulting in a more negative $\delta^{13}\text{C}$ value (18–20). The day 0 breath data indicated a rapid drop in $\delta^{13}\text{C}$ value that reached the minimum within the first 80 min after T1AM injection (Figure 2). This time frame is consistent with other rapid effects of T1AM reported in the literature (1,3) and is indicative of its rapid lipid oxidation. Thus, breath $\delta^{13}\text{C}$ value can be used as a sensitive marker for early detection of lipolysis.

During the early days of T1AM treatment (day 0-4; day 0 being the first day of treatment), there is a significant decrease (–3.6 to –4.1) in the $\delta^{13}\text{C}_{80\text{min}}$ value in the T1AM-treated animals (Figure 2) consistent with results from microarray studies showing increased lipase gene expression in chronic T1AM treatment in rats (M. Righi, personal communication).

Later in the treatment (day 5-7), the change in $\delta^{13}\text{C}_{80\text{min}}$ value only decreased by 1-2 units in T1AM treated versus sham animals (Figure 2). These results suggest a prolonged action of T1AM on lipolysis. The continued weight loss through day 9 in the T1AM-treated animals (Figure 1) is consistent with the breath data.

Nontargeted NMR metabolomics data confirmed the increase in lipid utilization. On the last day of treatment, T1AM-treated mice showed a significant elevation in the plasma level of the ketone body, 3-hydroxybutyrate (Figure 3A), providing further evidence that T1AM is directly affecting lipid utilization through day 7 of treatment. Even though, Braulke et al. (3) demonstrated development of ketonuria in rodents treated with a single injection of T1AM at 50 mg/kg, our study is the first to report that low dose chronic T1AM treatment causes a significant increase in plasma ketone bodies.

We followed the long-term effect T1AM, after 2 weeks discontinuation, by $^{13}\text{C}$-isotope tracing and NMR spectroscopy. Mice were challenged with [U-$^{13}\text{C}$]-glucose to evaluate lipid synthesis versus lipolysis in the post weight loss regime. Two hours after $^{13}\text{C}$-U-labeled glucose administration, both plasma $^{12}\text{C}$-glycine and $^{12}\text{C}$-valine were found to be elevated (Figure 3B), whereas the rate of $^{13}\text{C}$-glucose consumption was similar in T1AM-treated versus sham animals. The $^{13}\text{C}$-U-glycine injection resulted in no statistically significant labeled ($^{13}\text{C}$) metabolites, indicating that the elevated levels of $^{13}\text{C}$-valine and $^{13}\text{C}$-glycine found are indeed a product of endogenous protein catabolism. The increase in these amino acids further supports the breath data that showed a lesser decreased $\delta^{13}\text{C}$ values indicating a shift in macronutrient substrate oxidation from lipids to carbohydrates or proteins in day 5-7 of treatment. The rise in unlabeled amino acids thus may reflect a temporary metabolic shift resulting from the need of the body to use amino acids as precursors for energy production (pyruvate in the case of glycine and Krebs cycle intermediates, such as succinyl-CoA, in the case of valine). These long-term changes in metabolites levels in exogenously T1AM-treated animals suggest that T1AM may have substantially long-term effects on energy metabolism than previously thought, with increased endogenous protein catabolism when administered subchronically. The plasma samples following the $^{13}\text{C}$-U-glycine injection also showed an increase in $^{13}\text{C}$-acetate, which was 6–7 times higher than in sham-treated animals. Even though the $^{13}\text{C}$-acetate levels did not reach statistical significance the results are consistent with a decrease in lipid synthesis in T1AM-treated animals that still persists 2 weeks post-termination of T1AM treatment. While this study begins to decode long-term effects of T1AM on metabolism, additional studies with mildly feed restricted mice are needed to examine the effects of T1AM treatment for periods longer than 8 days. The breath $\delta^{13}\text{C}_{80\text{min}}$ value indicates that the biological response to exogenous T1AM over an 8-day period is dynamic based on the fluctuations in energy substrate utilization. Our finding that prolonged use of T1AM affects protein catabolism is consistent with prior studies showing reduced inotropism and diminished muscle contraction strength with high doses of T1AM (1). However, to date, protein catabolism has not been reported following an acute administration of T1AM. The discovery that protein catabolism induction can occur after chronic application of T1AM at low concentration is important and demonstrates the power of combined analyses for anti-obesity drug evaluations to identify unexpected side effects.

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References

1. Scanlan TS, Suchland KL, Hart ME, et al. 3-Iodothyronamine is an endogenous and rapid-acting derivative of thyroid hormone. Nat Med 2004;10:638–642.
2. Regard JB, Kataoka H, Cano DA, et al. Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. J Clin Invest 2007;117:4034–4043.
3. Braulke LJ, Klingenspor M, DeBarber A, et al. 3-Iodothyronamine: a novel hormone controlling the balance between glucose and lipid utilization. J Comp Physiol B 2008;178:167–177.
4. Klieverik LP, Poppen E, Ackermans MT, et al. Central effects of thyronamines on glucose metabolism in rats. J Endocrinol 2009;201:377–386.
5. Axelband F, Dias J, Ferrao FM, Einicker-Lamas M. Nongenomic signaling pathways triggered by thyroid hormones and their metabolite 3-iodothyronamine on the cardiovascular system. J Cell Physiol 2011;226:21–28.
6. Chiellini G, Frascarelli S, Ghielardoni S, et al. Cardiac effects of 3-iodothyronamine: a new aminergic system modulating cardiac function. FASEB J 2007;21:1597–1608.
7. Ju H, So H, Ha K, et al. Sustained torpidity following multi-dose administration of 3-iodothyronamine in mice. J Cell Physiol 2010;226:853–858.
8. Saba A, Chiellini G, Frascarelli S, et al. Tissue distribution and cardiac metabolism of 3-iodothyronamine. Endocrinology 2010;151:5063–5073.
9. Ackermans MT, Klieverik LP, Ringeling P, Endert E, Kalsbeek A, Fliers E. An online solid-phase extraction-liquid chromatography-tandem mass spectrometry method to study the presence of thyronamines in plasma and tissue and their putative conversion from $^{13}\text{C}$-thyroxine. J Endocrinol 2010;206:327–334.
10. Hoefig CS, Kohrle J, Brabant G, et al. Evidence for extrathyroidal formation of 3-iodothyronamine in humans as provided by a novel monoclonal antibody-based chemiluminescent serum immunoassay. J Clin Endocrinol Metab 2011;96:1864–1872.
11. Scanlan TS. Endogenous 3-iodothyronamine (T1AM): more than we bargained for. J Clin Endocrinol Metab 2011;96:1674–1676.
12. Dhillo WS, Bewick GA, White NE, et al. The thyroid hormone derivative 3-iodothyronamine increases food intake in rodents. Diabetes Obes Metab 2009;11:251–260.
13. Zaccari R, Chiellini G, Scanlan TS, Grandy DK. Trace amine-associated receptors and their ligands. Br J Pharmacol 2006;149:967–978.
14. Frascarelli S, Ghielardoni S, Chiellini G, et al. Cardiac effects of trace amines: pharmacological characterization of trace amine-associated receptors. Eur J Pharmacol 2008;587:231–236.
15. Doyle KP, Suchland KL, Ciesielski TM, et al. Novel thyronine derivatives, thyronine and 3-iodothyronamine, induce transient hypothermia and marked neuroprotection against stroke injury. Stroke 2007;38:2569–2576.
16. Butz DE, Cook ME, Eghbalnia HR, Assadi-Porter F, Porter WP. Changes in the natural abundance of $^{13}\text{C}2\text{O}3/12\text{CO}2$ in breath due to lipopolysacchride-induced acute phase response. Rapid Commun Mass Spectrom 2009;23:3729–3735.
17. Haviland JA, Tonelli M, Haughey DT, Porter WP, Assadi-Porter FM. Novel diagnostics of metabolic dysfunction in breath and plasma by selective isotope assisted labeling (SIAL). *Metabolism* 2012;8:1162–1170.
18. DeNiro MJ, Epstein S. Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* 1977;197:261–263.
19. Schoeller DA, Brown C, Nakamura K, et al. Influence of metabolic fuel on the $^{13}$C/$^{12}$C ratio of breath CO$_2$. *Biomed Mass Spectrom* 1984;11:557–561.
20. Hatch KA, Pinshow B, Speakman JR. The analysis of $^{13}$C/$^{12}$C ratios in exhaled CO$_2$: its advantages and potential application to field research to infer diet, changes in diet over time, and substrate metabolism in birds. *Integr Comp Biol* 2002;42:21–33.
21. Manni ME, De Siena G, Saba A, et al. 3-Iodothyronamine: a modulator of the hypothalamus-pancreas-thyroid axes in mouse. *Br J Pharmacol* 2012;166:650–658.