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SHORT COMMUNICATION

Direct calorimetry of free-moving eels with manipulated thyroid status

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Abstract In birds and mammals, the thyroid gland secretes the iodothyronine hormones of which tetraiodothyronine (T4) is less active than triiodothyronine (T3). The action of T3 and T4 is calorigenic and is involved in the control of metabolic rate. Across all vertebrates, thyroid hormones also play a major role in differentiation, development and growth. Although the fish thyroidal system has been researched extensively, its role in thermogenesis is unclear. In this study, we measured overall heat production to an accuracy of 0.1 mW by direct calorimetry in a free-moving European eel (*Anguilla anguilla* L.) with different thyroid status. Hyperthyroidism was induced by injection of T3 and T4, and hypothyroidism was induced with phenylthiourea. The results show for the first time at the organismal level, using direct calorimetry, that neither overall heat production nor overall oxygen consumption in eels is affected by hyperthyroidism. Therefore, we conclude that the thermogenic metabolism-stimulating effect of thyroid hormones (TH) is not present with a cold-blooded fish species like the European eel. This supports the concept that TH does not stimulate thermogenesis in poikilothermic species.

Keywords Calorimetry · European eels · Thyroid gland

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Introduction

In endothermic vertebrates (warm-blooded), such as birds and mammals, the thyroid hormones tetraiodothyronine (T4) and triiodothyronine (T3) raise the metabolic rate, especially in organs like muscle and to a minor extent in liver, by activating the Na/K ATPase on the cell membrane. In these two groups, the homoeothermic response of the thyroid is regulated via a temperature-sensitive region in the hypothalamus, which further acts on the pituitary followed by activation of the thyroid. This system is called the hypothalamic–pituitary–thyroid axis (HPT)-axis. An HPT-axis has been described in several fish species (Leatherland 1988).

The main role of thyroid hormones in fish is regulation of growth, development and reproduction (Cyr and Eales 1996). Further, a role in metamorphosis has been suggested for salmon during the parr–smolt transformation (Dickhoff et al. 1978). The universal presence of the thyroid gland and of the thyroid hormones T3 and T4 in vertebrates suggests a continuing and common role in ecto- and endotherms.

Previous studies have shown that thyroid hormones do not stimulate metabolic rate in poikilothermic species as they do in homeotherms. Rossier et al. (1979) failed to demonstrate an increase in oxygen consumption, Na^+ transport, and Na^+–K^+–ATPase activity following T4 injections in amphibians. For Teleostei, limited data are available at present for the role of thyroid hormones in thermoregulation and regulation and control of metabolic rate (Etkin 1978; Plisetskaya et al. 1983). One study looked

at plasma and hepatic nuclear T3 in two lower vertebrates: the lake trout (*Salvelinus namacycush*) and the sea lamprey (*Petromyzon marinus*, Weirich et al. 1987). They found that T3 failed to stimulate hepatic oxygen consumption, mitochondrial glycerophosphate dehydrogenase or malic enymze activity in liver trout whereas it did so in rats.

In homeothermic species, thyroid hormones increase thermogenesis, while the data in poikilothermic species suggested they do not. Direct calorimetry in whole, unrestrained poikilothermic animals, such as the eel, would add support to, or challenge this concept.

The aim of this study was therefore to assess, with direct calorimetry, the extent to which the thyroid is involved in the control of metabolic rate in a cold-blooded fish species, the European eel.

Materials and methods

Animals and hormone treatment

One-year-old male eels (*Anguilla anguilla* L.), with total body weights of 80–120 g, were obtained from a commercial hatchery (Royaal BV., Helmond, The Netherlands). The animals were acclimatized to 20°C and kept under normal laboratory conditions (14 h light, 10 h darkness) and normoxic oxygen saturation values of 80%. The animals were fed daily with Trouvit pelleted food (Trouw, Putten, The Netherlands). There were four groups: control, goitrogenic, T4-treated and T3-treated animals. The goitrogenic group was created by adding phenylthiourea to the water (0.20%) for a period of 6 weeks. Phenylthiourea and other thiocarbimides are known goitrogens (antithyroid substances) inhibiting the iodination of tyrosine residues essential for the production of thyroid hormones (Mountcastel 1980).

The control group (n=6) and goitrogenic group (n=6) consisted of six animals each. One hormone-treated group (n=10) was treated with T3 while a second group (n=9) was treated with T4. In these two groups, T3 or T4 (500 μg/ml per 100 g body weight) were dissolved in 0.1 mM NaOH, dissolved in coconut oil (Pradet-Balade et al. 1997) and injected weekly [intraperitoneally (IP)] for a period of 1 month. Control and goitrogenic eels were also injected weekly with the vehicle (coconut oil) used for T3 and T4 injections.

Calorimeter and oxygen registration system

The calorimetric system and oxygen registration system have been described elsewhere (Addink et al. 1991; van Ginneken et al. 1994). Briefly, it is a differential flow-through calorimeter (Sétaram GF 108, Lyon, France) that measures continuously the rate of heat production of the fish in a vessel with a volume of 1 l. Calibration was performed with a known electrical current and voltage (Sétaram EJ2 joule calibrator) by a current of 3.15 mA with a voltage of 31.64 V, which is applied to a resistor of 1,000 Ω mounted in the measurement vessel. This resulted in a power output signal of 99.7 mW.

The calibration of every sample point (1/min) was performed via a ‘sensitivity coefficient’ using special developed software (van Ginneken et al. 1994).

Oxygen consumption was calculated from the difference in oxygen tension between reference and measurement vessel multiplied by the flow through the system (van Ginneken et al. 1994). We determined the standard metabolic rate (SMR) after 24 h as the minimal metabolic rate over a time interval of 1 h (van Ginneken et al. 1997).

Thyroid measurements

After taking each eel out of the calorimeter at 70 h after the start of the calorimetry experiment, the fish was rapidly anesthetized with 200 ppm MS222 (3-aminobenzoic-acid-ethyl-ester methanesulfonate salt, Sigma, St. Louis, USA). Blood was collected from the caudal vein using a heparinized syringe and stored at −80°C pending further analysis. Total thyroxine (T4), and total triiodothyronine (T3) were determined by fluorescence polarization immunoassay (FPIA) on an Axsym analyzer of Abbott Diagnostics (Hoofddorp, The Netherlands).

Statistics

Normality of the data distribution was tested with a Kolmogorov–Smirnov test. For comparison between groups, one-tailed unpaired t tests with equal (control–goitrogenic) or unequal (control/goitrogenic-T3-T4) sample-size were performed. $P<0.05$ was considered as statistically significant.

Results

In Fig. 1, a registration of four typical calorimetric experiments (control, goitrogenic, T4-treated, and T3-treated) during a period of 3.5 days with one eel in the calorimetric vessel are depicted. The experiment starts with a calibration procedure for the heat signal. This results in a heat production of approximately 100 mW. Thereafter, the fish is introduced into the vessel. The oxygen consumption and heat production are measured for approximately 48 h. The oxygen tension of the in-flowing water is indicated by the top of the spikes in the pO_2 signal, which switches every 55 min to the reference position.

The lowest heat production and, concomitantly, the lowest oxygen consumption, the standard metabolic rate

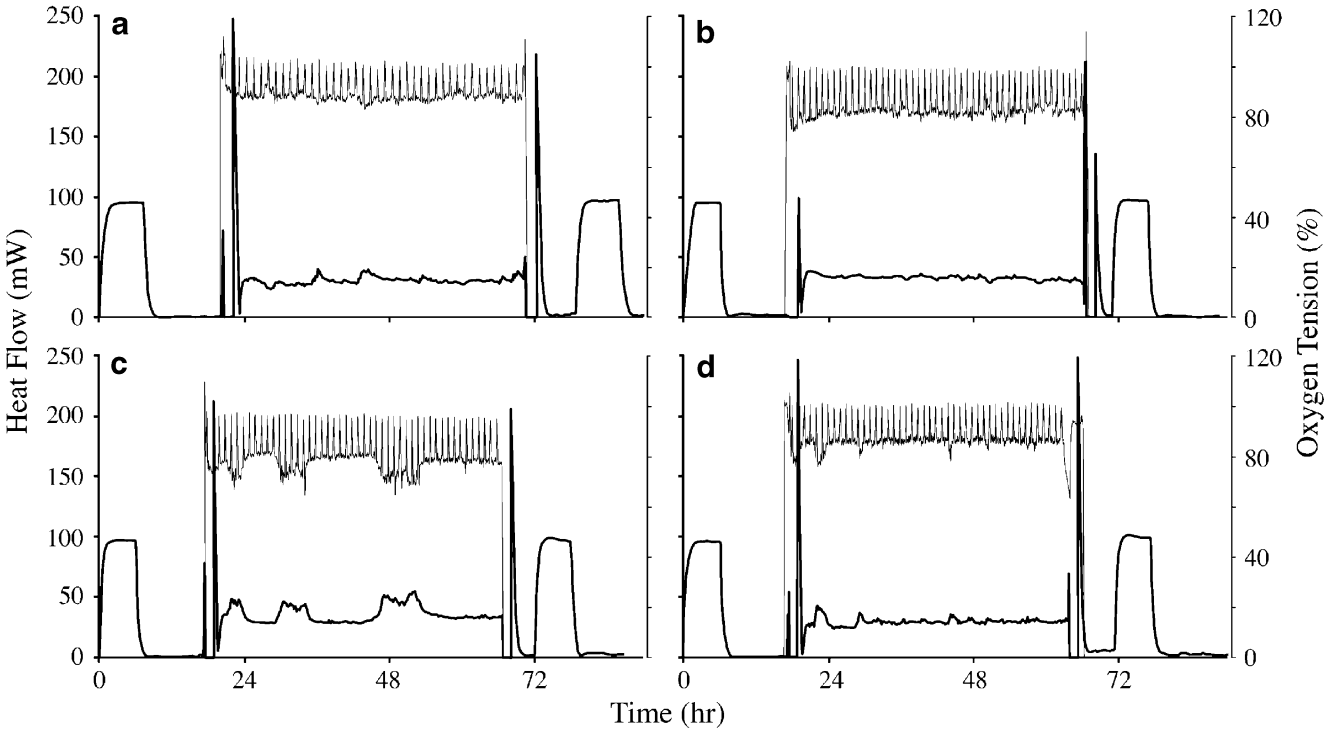

Fig. 1 Registration of four typical experiments during a period of 3.5 days to determine the SMR. Each experiment is performed with one eel. **a** Control animal (102.9 g), **b** goitrogenic animal (105.9 g), **c** T4-treated animal (96.3 g), **d** T3-treated animal (93.4 g). The top signal, alternating between reference and measurement vessel of the calorimetric system, is the oxygen tension signal (right y-axis). The difference in oxygen tension multiplied with the flow through the system gives the oxygen consumption. The lower line is the heat production signal of the fish (left y-axis). The experiment starts the first 20 h with an electrical calibration until 100 mW and finishes at 72 h after removing the fish from the vessel with an electrical calibration

(SMR) was determined over an interval of 1 h for each eel within the four groups (see Fig. 2). Interestingly, despite the goitrogenic animals having low activity levels, which can be concluded from the fluctuations in heat and oxygen signal (see Fig. 1b), they had a significantly higher SMR (Table 1, heat production: *t* test; $n=6$; $P=0.005$; oxygen consumption: *t* test; $n=6$; $P=0.002$).

In Table 1, the Mean±SD for T3 and T4 measured in blood plasma, overall heat production (SMR), overall oxygen consumption (SMR) and oxycaloric coefficient for a control, goitrogenic, T4-treated, and T3-treated European eel (*A. anguilla* L.) group are given. In the control group, T4 levels were 4.4 times higher in comparison to T3 levels. Heat production was around 86 J h^{-1} 100^{-1} g oxygen consumption around 0.2 mmol h^{-1} 100^{-1} g, while the oxycaloric value was in the range of 433 kJ mol^{-1}, which corresponds to a mixed substrate (van Ginneken et al. 1994). Our treatment of the eels with phenylthiourea for a period of 6 weeks resulted in a goitrogenic group with significantly lower T3 levels (*t* test; $n=6$; $P=0.008$) in comparison to the control group. The T4 levels were even zero in comparison to the control group elevating T3 and T4 levels by dissolving these compounds in coconut oil followed by weekly IP injection during a period of 1 month was successful. T3 levels increased 6.9-fold over control, while the T4 levels increased 1.4-fold over control. T4 levels increased 53-fold in comparison to the T4 levels in the control group. In the T4-treated group, the T3 levels increased 15.9-fold in comparison to the T3 level found in the control group.

The most important observation from this study was that heat production and oxygen consumption were not significantly elevated in the T4- (heat production: *t* test; $n=9$; $P=0.504$; oxygen consumption: *t* test; $n=9$; $P=0.466$) and T3- (heat production: *t* test; $n=10$; $P=0.701$; oxygen consumption: *t* test; $n=10$; $P=0.539$) treated groups in comparison with the control group. Only in the goitrogenic group was the metabolic rate significantly increased (heat production: *t* test; $n=6$; $P=0.005$; oxygen consumption: *t* test; $n=6$; $P=0.002$). The oxycaloric value was in the range of 426–433 kJ mol^{-1} for all four groups, which corresponds to mixed substrate (van Ginneken et al. 1994).

Discussion

The procedure to elevate T3 and T4 levels by using a vehicle of coconut oil was successful. T3 levels increased 6.9-fold vs controls while even the T4 levels increased 1.4-fold after T3

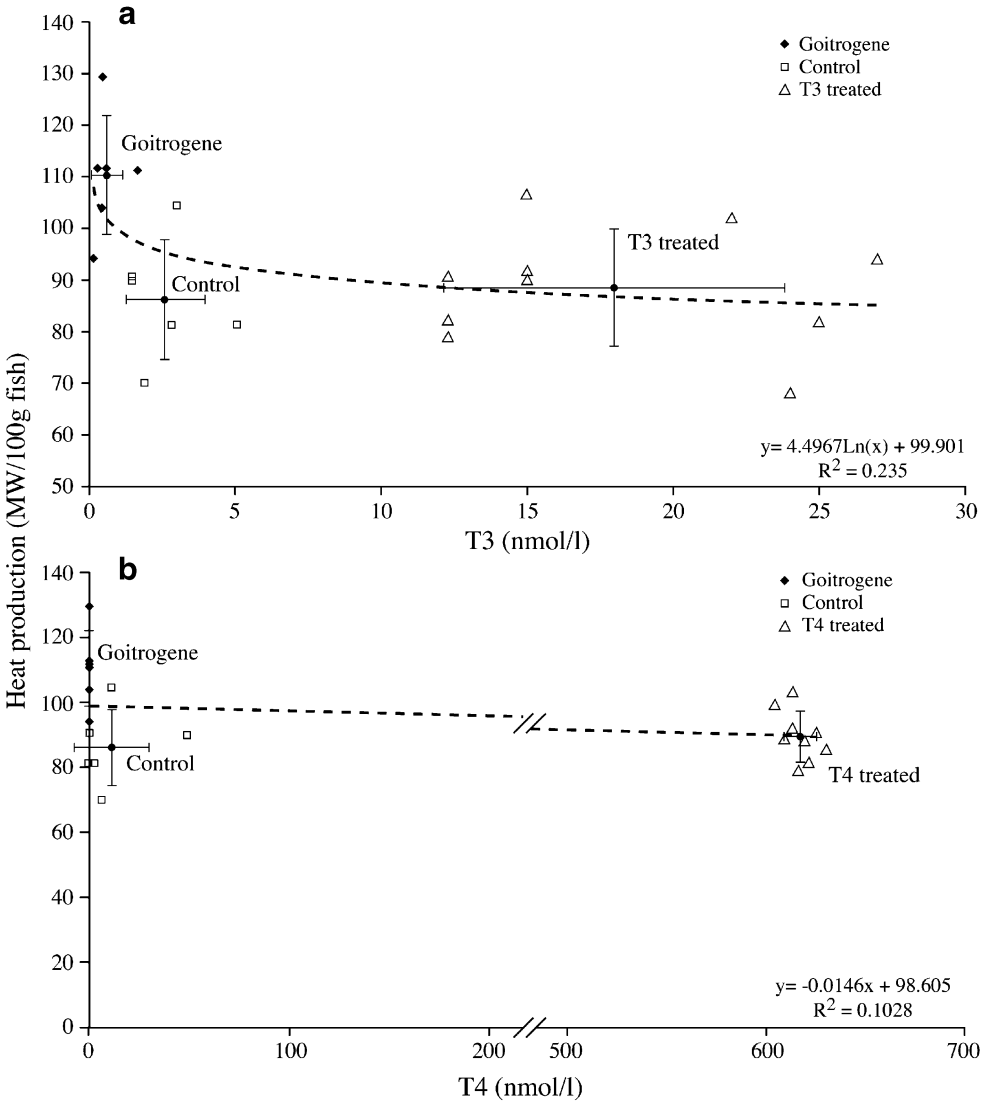

Fig. 2 Heat production (mean± SD) of a control, goitrogenic, **a** T3-, and **b** T4-treated individual European eels (*A. anguilla* L.) measured by direct calorimetry. Heat production was measured via direct calorimetry with a Setaram, 1 l, flow-through twin detection microcalorimeter (van Ginneken et al. 1994)

Table 1 Mean±SD for T3 and T4 measured in blood plasma, overall heat production (SMR), overall oxygen consumption (SMR) and oxycaloric coefficient for a control, goitrogenic, T4-treated, and T3-treated European eel (*A. anguilla* L.) group

	Control (n=6)	Goitrogenic (n=6)	T4 (n=9)	T3 (n=10)	Co–Goi	Co–T4	Co–T3	Goi–T4	Goi–T3
T3 (nmol l^{-1})	2.62 (1.37)	0.61 (0.54)	16.51 (6.67)	17.99 (5.84)	P=0.008**	P<0.001**	P<0.001**	P<0.001**	P<0.001**
T4 (nmol l^{-1})	11.64 (19.08)	0 (0)	617.85 (7.72)	41.56 (27.06)	P=0.166	P<0.001**	P=0.033*	P<0.001**	P=0.002**
Heat (J h^{-1} 100 g^{-1})	86.32 (11.68)	110.52 (11.55)	89.75 (7.81)	88.64 (11.35)	P=0.005**	P=0.504	P=0.701	P<0.001**	P=0.002**
Oxygen (mmol h^{-1} 100^{-1} g)	0.20 (0.03)	0.27 (0.02)	0.21 (0.02)	0.21 (0.03)	P=0.002**	P=0.466	P=0.539	P<0.001**	P<0.001**
Oxycaloric value (kJ/mol O_2)	433.36 (11.02)	430.06 (14.86)	431.35 (16.79)	426.22 (15.95)	P=0.671	P=0.801	P=0.353	P=0.881	P=0.641

Left side table columns 2–5: mean±SD for 3,5,3′-triiodo-L-thyronine (T3) and L-thyroxine (T4), heat production, oxygen consumption and oxycaloric coefficient for a control, goitrogenic, T4-treated, and T3-treated European eel (*A. anguilla* L.) group. Right side table columns 6–10: data were tested between pairs of groups with unpaired student t tests with one-tailed probabilities.

*$P \leq 0.01$

**$P \leq 0.001$

treatment in comparison to control T4 levels. The interaction between the thyroid hormones (T3 & T4), thyroid stimulating hormone (TSH), and the direct effect of this on the pituitary level and its indirect effect via modulation of hypothalamic mediators, such as thyrotropin-releasing hormone, remains a complicated matter (Pradet-Balade et al. 1997). T4 levels increased 53-fold in the T4-treated group compared to controls while there was a 15.9-fold increase of T3 plasma levels. The latter observation can possibly be explained by peripheral T4 deiodination. This observation was also made by Pradet-Balade et al. (1997) for European eel treated with T4.

The increased T4 levels in T3-treated eels is not the consequence of the cross-reactivity of the assay. The T4 assay has less than 10% cross reactivity with T3, and the other way around, the T3 assay has less than 0.05% cross reactivity with T4 (Pers. Comm. Dr. Bart Ballieux). It is possible that the conversion of T4 into T3 is inhibited by deiodinases as a consequence of the high T3 concentrations resulting in T4 accumulation. In humans, the plasma half-life of T4 is seven times higher than the half-life of T3 and is mainly determined by deiodinase activity. The existence of deiodination pathways in fish is demonstrated in the study of Sweeting and Eales (1992).

With conventional techniques like oxygen consumption measurements (indirect calorimetry), some researchers concluded that the energy consumption of cold-blooded animals is not influenced by thyroxine (Bern and Nandi 1964; Gorbman 1964). On the other hand, some reports show a stimulatory effect of thyroid hormones on oxygen consumption in fishes (Ruhland 1969; Pandey and Munshi 1976) while anti-thyroid treatment rapidly decreased oxygen consumption (Ruhland 1969). Differences between the results of different researchers can possibly be ascribed to methodological flaws in the experimental set up including stress of the animals. Therefore, direct calorimetry in whole, unrestrained poikilothermic animals, such as the eel, would add support to, or challenge this concept.

Measuring heat production via direct calorimetry under aerobic conditions has two major advantages: 1) The outcome is independent on the type of metabolism (anaerobic processes or synthesis. 2) The secondary advantage is that direct heat measurements can possibly elucidate if there is a central regulator (heat-center) in the regulation of body temperature even in a cold-blooded fish like the European eel. We thus measured, with a flow-through twin detection calorimeter, the heat-flow and oxygen consumption of eels with low vs high levels of T3 (Fig. 2a, Table 1) and T4 (Fig. 2b, Table 1).

Our results demonstrate no significant difference in heat production or oxygen consumption over a wide range of T4 or T3 levels. Only in the goitrogenic (=low thyroxine) group was the metabolic rate significantly increased (Fig. 2a and b, Table 1). This is an observation not directly related to the most important conclusion from this study, namely that heat production and oxygen consumption are not significantly elevated in the T4- and T3-treated groups in comparison with the control group. The increased metabolic rate in the goitrogenic group can possibly be explained by the toxicity of phenylthiourea.

In contrast to the data presented in this manuscript, published literature data from eu-, hypo- and hyperthyroidic human subjects (Hortling and Hiisi-Brumer 1959) clearly demonstrate that the basal metabolic rate is dependent on the concentration of thyroid hormones.

Our results demonstrate that, despite the universal presence of the thyroid gland and of T3 and T4 in all vertebrates, their calorigenic function is not universal. The study of Hortling and Hiisi-Brumer (1959) supports the view that the ability of thyroid hormones to stimulate thermogenesis is solely restricted to the homeothermic species (birds and mammals), which originated more than 200 million years ago (Freake and Oppenheimer 1995). Our observation, reported here, that thyroxine is not calorigenic in eels, suggests that they lack a mechanism to produce additional heat.

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