Role of thyroidal and testicular hormones in regulation of tissue respiration in hibernating and non-hibernating species of frogs

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***In vivo* and *in vitro* effects of L-triiodothyronine ($L–T_3$), L-tetraiodothyronine ($L–T_4$), propyl thiouracil (PTU), testosterone (T) and cyproterone acetate (CA) were studied on the rate of tissue respiration in Indian streaked frog, *Rana limnocharis* (a hibernating species) and Indian skipper frog *Rana cyanophlyctis* (a non-hibernating species) maintained under natural climatic conditions during winter and summer months. The rate of tissue respiration was measured with the help of an oxygen electrode. Hormones were used in physiological doses. PTU invariably decreased respiratory rate in both the species. However, tissues of *R. cyanophlyctis* were comparatively more sensitive to thyroid hormones. Both *in vivo* and *in vitro* administration of testosterone significantly increased and *in vivo* administration of CA significantly decreased the rate of tissue respiration in both the species irrespective of seasons and temperature. These findings suggest that while the oxidative mechanisms of the two species differ in their sensitivity to thyroid hormones, endogenous thyroid hormones seem to play an important role in energy metabolism of frogs. Further, testicular hormones seem to be directly involved in the regulation of the oxidative metabolism and might be responsible for maintaining normal activity of *R. cyanophlyctis* during winter months.**

HORMONES play a major role in the regulation of the oxidative metabolism in vertebrates and ensure energy production for normal functioning of a large number of organs. Investigations over the last four decades have revealed that the energy metabolism in homeotherms is controlled primarily by thyroid hormones[1–3]. However, these hormones stimulate the metabolic rate of poikilotherms only at ecritic/high temperature (25°C or above)[4,5]. There are only a few reports regarding the role of thyroid hormones in the regulation of metabolic rate of amphibians in relation to ambient temperature[6,7]. Moreover, these studies were conducted at simulated temperatures on temperate zone amphibians maintained under laboratory conditions[8–12], and so far no attempt has been made to investigate the role of thyroid hormones in the calorigenesis of amphibians maintained under natural climatic conditions[5]. Recent studies suggest that gonadal hormones might be actively involved in the regulation of respiration in reptiles exposed to low temperatures where thyroid hormones generally do not influence the oxidative metabolism[5,13]. Notwithstanding these reports, however, there is scarcity of information on the role of gonadal hormones in the amphibian oxidative metabolism, particularly at low temperature[14,15]. Further, there are anurans which do not experience cold torpor (hibernation) even during severe cold winter months. But so far no attempt has been made to study the differences between hormonal regulation of the oxidative metabolism in hibernating and non-hibernating species of frogs with special reference to natural ambient temperature and gonadal activity[5]. Therefore, keeping in view the critical phylogenetic position of amphibians and lack of information, it was thought worthwhile to undertake a study of the role of thyroidal and gonadal hormones in the regulation of the oxidative metabolism of a hibernating and a non-hibernating species of frogs maintained under natural climatic conditions during winter and summer months.

Materials and methods

All experiments were conducted on male Indian streaked frog, *Rana limnocharis* (a hibernating species; body weight: 8–10 g; snout to vent length: 31–33 cm) and Indian skipper frog, *Rana cyanophlyctis* (a non-hibernating species; body weight: 10–12 g; snout to vent length: 35–39 mm) which are found in and around Shillong (Lat., 25°30′N; Long., 91°52′E; altitude: 1450 m ASL; minimum temperature: 0–4°C, maximum temperature: 26°C). *R. limnocharis* develops anorexia and becomes lethargic during winter months (November to January) and breeds during rainy/summer season (April to August). *R. cyanophlyctis* does not exhibit cold torpor/hibernation and remains physically and reproductively active throughout the year[16,17].

Chemicals

All the hormones and the chemicals were purchased from Sigma Chemicals, USA.

In vitro experiments

Male *R. limnocharis* and *R. cyanophlyctis* were collected from nature during December (winter, temperature: 6–14°C) and July (summer/rainy season: temperature: 14–21°C). Four frogs of each species were killed immediately after collection, and tissues (liver and skeletal muscle) were rapidly removed, rinsed in ice-cold frog Ringer's solution and stored in a freezer (– 10°C to – 15°C) for *in vitro* hormonal treatments. Storage in freezer for up to 15 days has no effect on the rate of tissue respiration. The stored tissues were used within 10 days from the date of collection for *in vitro* treatments. For treating the tissues with hormones, tissues were blotted, weighed and homogenized in ice-cold frog Ringer-phosphate buffer solution (pH 7.4) using a loose-fitting glass homogenizer. One ml of tissue homogenate (16–20 mg tissue/ml), 3.9 ml of Ringer-phosphate buffer and 0.1 ml of hormone solution (having desired concentration of hormones) were used for measuring the rate of tissue respiration. The homogenates treated with L-triiodothyronine (L–T_3) (0.6 µM), L-tetraiodothyronine (L–T_4) (0.5 µM) and testosterone (1.38 µM) were pre-incubated at 4°C prior to the measurement of the rate of tissue respiration using an oxygen electrode.

In vivo experiments

Male *R. limnocharis* and *R. cyanophlyctis* were collected from the field during December (temperature: 7–15°C) and July (temperature: 12–21°C) and maintained in plastic cages with mud and water. The cages were fully exposed to the natural climatic conditions. The frogs were acclimatized for 10 days. After acclimatization, the frogs of both species were separately divided into 6 groups of 4 each during winter. The first group of each species was injected only frog saline (0.1 ml) and served as control. Groups 2, 3, 4, 5 and 6 were administered L–T_4 (2 µg/frog), L–T_3 (2 µg/frog), propyl thiouracil (PTU) (1 µg/g body weight), testosterone (2 µg/frog) and cyproterone acetate (CA) (1 µg/g body weight) respectively. All the injections were given intra-muscularly at an interval of 24 h between 9 am and 10 am for four days. The frogs were provided with live earthworms as food *ad libitum*. Twentyfour hours after the last injections, frogs of each group were decapitated, tissues (liver, skeletal muscle and kidney) were rapidly removed, rinsed in ice-cold frog Ringer's solution, and stored in a freezer (– 8°C) for measuring the rate of tissue respiration with the help of an oxygen electrode. During July, after acclimatization, frogs of each species were divided into ten groups of four each. Group 1 of both the species received only saline (0.1 ml) and served as control. Groups 2, 3, 4, 5 and 6 were administered L–T_3, L–T_4, PTU, testosterone and CA respectively. The doses, mode of administration and the duration of administration of the hormones were the same as those during the winter months. Frogs of groups 7, 8, 9 and 10 were intra-muscularly injected epinephrine (2 µg/frog), norepinephrine (2 µg/frog), PTU + epinephrine (1 µg/g + 2 µg/frog) and PTU + norepinephrine (1 µg/g + 2 µg/frog) respectively at an interval of 24 h between 9 am and 10 am for four days. Twentyfour hours after the last injections, frogs were decapitated and tissues were procured, processed and stored for the measurement of respiratory rate following the procedure mentioned earlier. The stored tissues were processed within 10 days for the measurement of the rate of tissue respiration.

Measurement of the rate of tissue respiration

The rate of tissue oxygen consumption was measured with the help of an oxygen electrode (Digital Oxygen System Model 10, Rank Brothers Ltd, UK). For measuring tissue respiration, tissues were blotted, weighed and homogenized in ice-cold frog Ringer-phosphate buffer solution (pH 7.4) using a loose-fitting glass homogenizer. The polarizing voltage was kept at 0.6 V and the homogenizing medium was used as the polarizing medium. Since the preferred body temperature of most amphibians ranges between 25 and 28°C (ref. 18), temperature of the incubation chamber of the electrode was maintained at 25°C with the help of a thermostat-controlled water circulator. The homogenates were incubated in the incubation chamber of the oxygen electrode for 20 min before recording the readings. Readings for each tissue were taken for 30 min in the linear range of oxygen consumption. The rate of tissue respiration was calculated and expressed as µl O_2/mg wet tissue/hour (ref. 15). The data were tabulated and analysed using Student's *t* test[19]. A probability of less than 5% was considered to be significant.

Results

In vitro experiments

The data are presented in Table 1. *In vitro* treatments with L–T_3 and L–T_4 did not stimulate liver and muscle tissues respiration of both the species during winter and summer (Table 1). However, the respiratory rate of the tissues was significantly increased following *in vitro* treatment with testosterone during both winter and summer (Table 1).

In vivo experiments

The data are presented in Table 2. *In vivo* administration

Table 1. *In vitro* effects of thyroid hormones, propyl thiouracil and testosterone on the rate of tissue respiration of *R. limnocharis* and *R. cyanophlyctis* during winter and summer

	Rate of tissue oxygen consumption (µl oxygen/mg/h)			
	R. limnocharis		*R. cyanophlyctis*	
Treatment	Liver	Muscle	Liver	Muscle
Winter				
Control	0.88 ± 0.07*	0.61 ± 0.03	0.80 ± 0.07	0.40 ± 0.02
L–T_3	0.92 ± 0.09	0.71 ± 0.09	0.90 ± 0.06	0.45 ± 0.04
L–T_4	0.86 ± 0.02	0.60 ± 0.09	0.81 ± 0.05	0.40 ± 0.05
Testosterone	1.26 ± 0.10[a]	0.80 ± 0.03[b]	1.24 ± 0.08[b]	0.81 ± 0.03[a]
Summer				
Control	1.81 ± 0.02*	1.65 ± 0.04	1.69 ± 0.005	1.32 ± 0.04
L–T_3	1.98 ± 0.08	1.76 ± 0.06	1.86 ± 0.09	1.44 ± 0.07
L–T_4	1.82 ± 0.04	1.62 ± 0.06	1.80 ± 0.10	1.34 ± 0.04
Testosterone	1.99 ± 0.05[b]	1.89 ± 0.06[a]	1.86 ± 0.06[a]	1.51 ± 0.04[a]

*Mean ± standard error (SE); $n = 4$.
[a,b] Differ from the respective control value: $P < 0.05$ and $P < 0.01$ respectively.
L–T_3 = L-triiodothyronine; L–T_4 = L-tetraiodothyronine.

Table 2. *In vivo* effects of hormones and inhibitors on the rate of tissue respiration in *R. limnocharis* and *R. cyanophlyctis* during winter and summer

	Rate of tissue oxygen consumption (µl oxygen/mg/h)					
	R. limnocharis			*R. cyanophlyctis*		
Treatment	Liver	Muscle	Kidney	Liver	Muscle	Kidney
Winter						
Saline	1.04 ± 0.05*	0.79 ± 0.03	0.92 ± 0.05	1.44 ± 0.05	1.12 ± 0.02	1.30 ± 0.02
L–T_3	1.08 ± 0.07	0.80 ± 0.01	0.98 ± 0.04	1.46 ± 0.01	1.20 ± 0.01[a]	1.48 ± 0.04[b]
L–T_4	1.05 ± 0.04	0.80 ± 0.05	0.96 ± 0.03	1.44 ± 0.005	1.19 ± 0.03	1.45 ± 0.08
PTU	0.87 ± 0.03[a]	0.65 ± 0.04[a]	0.69 ± 0.05[a]	1.20 ± 0.06[a]	0.76 ± 0.12[a]	0.96 ± 0.09[a]
T	1.31 ± 0.07[a]	0.97 ± 0.06[a]	1.15 ± 0.04[a]	1.71 ± 0.05[b]	1.22 ± 0.03[a]	1.61 ± 0.09[a]
CA	0.74 ± 0.07[a]	0.64 ± 0.05[a]	0.76 ± 0.03[a]	1.18 ± 0.07[a]	0.96 ± 0.03[b]	0.96 ± 0.04[c]
Summer						
Saline	2.05 ± 0.07	1.44 ± 0.09	1.82 ± 0.03	2.01 ± 0.08	1.36 ± 0.06	1.72 ± 0.02
L–T_3	2.10 ± 0.07	1.52 ± 0.03	1.93 ± 0.05	2.17 ± 0.03	1.44 ± 0.06	2.02 ± 0.02[b]
L–T_4	2.04 ± 0.02	1.45 ± 0.05	1.84 ± 0.06	2.12 ± 0.04	1.42 ± 0.08	1.96 ± 0.05[a]
PTU	1.67 ± 0.03[c]	1.20 ± 0.01[a]	1.70 ± 0.03[a]	1.75 ± 0.03[a]	1.16 ± 0.04[a]	1.51 ± 0.05[b]
T	2.24 ± 0.03[a]	1.80 ± 0.02[b]	1.99 ± 0.05[b]	2.39 ± 0.11[a]	1.60 ± 0.07[a]	2.04 ± 0.07[b]
CA	1.70 ± 0.05[b]	1.29 ± 0.07	1.71 ± 0.03[a]	1.72 ± 0.05[a]	1.06 ± 0.09[a]	1.52 ± 0.04[b]
EP	2.40 ± 0.10[a]	1.88 ± 0.07[b]	2.20 ± 0.07[b]	2.50 ± 0.14[a]	1.81 ± 0.03[c]	2.14 ± 0.09[b]
NE	2.60 ± 0.04[c]	1.94 ± 0.06[b]	2.27 ± 0.03[c]	2.54 ± 0.12[a]	1.90 ± 0.07[c]	2.30 ± 0.10[c]
PTU + EP	2.09 ± 0.04[f]	1.58 ± 0.10[d]	1.92 ± 0.09[d]	2.10 ± 0.05[d]	1.42 ± 0.07[e]	1.85 ± 0.06[d]
PTU + NE	2.15 ± 0.07[e]	1.50 ± 0.01[f]	1.99 ± 0.05[e]	2.29 ± 0.09	1.49 ± 0.11[d]	1.93 ± 0.08[d]

*Mean ± standard error (SE), $n = 4$.
[a,b,c] Differ from saline treated control group: $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[d,e,f] Differ from respective EP or NE treated group: $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
CA = cyproterone acetate; EP = epinephrine; NE = norepinephrine; PTU = propyl thiouracil; L–T_3 = L-triiodothyronine L–T_4 = L-tetraiodothyronine, T = testosterone.

of L–T_3 and L–T_4 had no significant effect on the respiratory rate of tissues in *R. limnocharis* during winter and summer (Table 2). However, L–T_3 administration in *R. cyanophlyctis* significantly stimulated the respiratory rate of muscle and kidney during winter and only of kidney during summer. Unlike L–T_3, *in vivo* treatment with L–T_4 had no effect on the respiratory rate of tissues of *R. cyanophlyctis* during winter and summer except kidney respiration which was stimulated during the summer (Table 2). Administration of PTU invariably and significantly decreased the respiratory rate of all tissues in both the species during summer as well as during winter seasons (Table 2).

In vivo administration of testosterone significantly stimulated and CA significantly decreased the respiratory rate of all the tissues (except of muscle in *R. limnocharis* during summer) in both the species during both summer and winter seasons. CA-induced decrease in the respiratory rate of muscle in *R. limnocharis* during summer was found to be marginally insignificant (Table 2).

In vivo administration of epinephrine (EP) and norepinephrine (NE) significantly stimulated the respiratory rate of tissues in both the species during summer (Table 2). However, when EP and NE were administered in combination with PTU during summer, the stimulatory effect of the catecholamines on tissue respiration was significantly reduced compared to the respective groups which received only catecholamines (Table 2).

Discussion

The thyroid hormones did not stimulate the respiratory rate of tissues of *R. limnocharis* which undergoes hibernation/cold torpor during winter months irrespective of seasons, temperature and mode of treatment. However, *in vivo* administrations of L–T_3 and L–T_4 were found to stimulate the respiratory rate of muscle and kidney of *R. cyanophlyctis* (which remains active throughout the year) during winter and summer respectively (Table 2). It seems that tissues of the two species differ in their response to calorigenic action of thyroid hormones. Further, *in vivo* administration of PTU (a goiterogen which blocks synthesis of thyroid hormones) significantly decreased tissue respiration in both the species during both winter and summer months (Table 2). Thus, there is a possibility that the endogenous levels of T_3 and T_4 are actively involved in the regulation of the oxidative metabolism in both the amphibian species during both summer and winter months. There are reports that exogenous thyroid hormones significantly influence intermediary metabolism of poikilotherms exposed to low temperature[20,21]. In mammals exposed to low temperature, catecholamines regulate energy metabolism, and thyroid hormones reportedly support the non-shivering thermogenesis by potentiating the calorigenic action of catecholamine hormones[22–24]. Therefore, there is a possibility that endogenous thyroid hormones might be involved in maintaining the metabolic rate indirectly by potentiating the calorigenic action of catecholamines. This assumption is strongly supported by significant reduction in the stimulatory effect of EP and NE on tissue respiration when the catecholamines are administered with PTU compared to effects of catecholamines alone during summer (Table 2). The observed significant reduction in the calorigenic action of catecholamines in the presence of PTU might be due to decreased production of thyroid hormones and/or due to inhibition of deiodination of T_4 to T_3 (ref. 3). Potentiation of the calorigenic action of catecholamines in frogs by thyroid hormones seems to be of evolutionary significance. In reptiles also, thyroid hormones reportedly potentiate the metabolic effects of catecholamines at low temperature[25,26]. On the basis of these findings it can be suggested that for evaluating the calorigenic role of thyroid hormones in any amphibians/poikilotherms, use of goiterogens should be preferred over the use of exogenous thyroid hormones. In *R. cyanophlyctis*, comparatively higher tissue sensitivity to thyroid hormones might be associated with higher levels of physical and/or physiological/metabolic activity[5]. Thyroid hormones are reported to stimulate activities of several oxidative enzymes in fish and reptiles exposed to preferred body temperature[27–30].

Gonadal hormones have been reported to be directly involved in the regulation of the oxidative metabolism in several species of reptiles[2,5,13,31,32] and birds[33–35]. In the present study the rate of tissue respiration was invariably and significantly stimulated by testosterone in both the species in all the experiments. Further, in both the species *in vivo* administration of CA (a blocker of androgen receptors) significantly decreased the respiratory rate of tissues irrespective of seasons and ambient temperature. It, thus, seems that the observed decrease in tissue respiration following CA administration might be due to inability of endogenous androgens to bind to their specific receptors already occupied by CA. *In vitro* calorigenic action of testosterone strongly suggests that, as in reptiles[5,13], androgens are also directly involved in the regulation of the oxidative metabolism of amphibians. It is important to mention that during winter while spermatogenesis is arrested in *R. limnocharis*, in *R. cyanophlyctis* spermatogenesis has been reported to occur throughout the year with slight depression during winter months[16,17]. Thus, there is a probability that a comparatively higher level of androgens (as indicated by spermatogenesis) might be responsible for maintaining higher levels of metabolic rate of tissues and physiological activity in *R. cyanophlyctis* as compared to *R. limnocharis*. Direct involvement of testicular hormones in the oxidative metabolism of amphibians (present

study), reptiles[5] and birds[1,33–35] suggests that androgens might be playing a dominant role in regulation of the metabolic rate in sub-mammalian vertebrates.

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