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Role of thyroidal and testicular hormones in regulation of tissue respiration in male air-breathing fish, *Clarias batrachus* (Linn.)

D S Lynshiang & B B P Gupta*

Environmental Endocrinology Laboratory, Department of Zoology, North-Eastern Hill University, Shillong 793 022, India

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In vivo and *in vitro* effects of thyroidal hormones (MIT, DIT, T_3, T_4), propyl thiouracil (PTU), testosterone and cyproterone acetate were studied on the rate of tissue (liver, muscle, kidney and brain) respiration of adult male *C. batrachus* during winter and summer/rainy seasons. Monoiodotyrosine (MIT) and diiodothyrosine (DIT) increased the respiratory rate in a dose-dependent and temperature-independent manner. Triiodothyronine (T_3) and thyroxine (T_4) stimulated tissue respiration during summer/rainy months but not during winter. PTU decreased tissue respiration during summer/rainy season and also at simulated low temperature. Testosterone invariably stimulated the rate of respiration of the tissues, while *in vivo* treatment with cyproterone acetate significantly decreased the metabolic rate of all the tissues. The findings suggest that in *C. batrachus* MIT and DIT may be more important than T_3 and T_4 at low temperature, endogenous thyroid hormones are involved indirectly in energy metabolism even during winter/at low temperature and testicular hormones are actively involved in the respiration.

The oxidative metabolism in vertebrates is regulated by a complex mechanism involving both external (climatic) and internal (endocrine) factors. Thyroid hormones are known to play a major role in the regulation of energy metabolism of homeotherms irrespective of natural fluctuation in the environmental temperature[1-3]. However, the role of thyroid hormones in the oxidative metabolism of poikilotherms seems to be temperature-dependent[4-7]. A careful analysis of the available information reveals that the thyroid hormones stimulate the rate of respiration in poikilothermic vertebrates only at or above the preferred (ecretic)-temperature and do not produce calorigenic effects at low temperature (below 20°C)[8]. Comprehensive studies have suggested that testicular hormones regulate the energy metabolism of amphibians and reptiles during winter with low temperature where thyroid hormones are calorigenically ineffective[6,8,9]. Further, it has been reported that during winter endogenous thyroid hormones are indirectly involved in the energy metabolism of two amphibian species[6]. Unlike in amphibians and reptiles, no comprehensive study has been conducted to investigate the calorigenic role of thyroid hormones in the oxidative metabolism of any fish species[8]. Earlier reports regarding the metabolic effects of thyroid hormones are found to be confusing and contradictory[4,8]. These reports did not consider the environmental temperature and/or the time of the year which might be the prime reason for stimulatory, inhibitory and no effect of thyroid hormones on fish respiration[8]. There is also practically no information on a possible role of testicular hormones in the regulation of fish oxidative metabolism at low temperature of the winter season. There are indications that monoiodotyrosine (MIT) and diiodotyrosine (DIT) are present in blood of vertebrates[10-13]. But so far no attempt has been made to examine the calorigenic role of candidate hormones MIT and DIT in any vertebrate species, particularly in poikilotherms where the calorigenic action of thyroxine (T_4) and triiodothyronine (T_3) is doubtful. Therefore, it was thought worthwhile to undertake a comprehensive study to investigate the role of thyroid hormones (including MIT and DIT) and testicular steroids in the male air-breathing fish, *Clarias batrachus* maintained under natural climatic conditions during winter and summer seasons. The findings of the present study suggest that the rate of tissue respiration of the fish is regulated jointly by thyroid and testicular hormones, and the calorigenic effects of MIT, DIT and testosterone seem to be temperature-independent.

Materials and Methods

All *in vivo* and *in vitro* experiments were conducted on male *Clarias batrachus* which lives in

*Correspondent author.

shallow rivers, ponds and muddy places, and survives even in water with low O_2 content in nature. *Clarias batrachus* breeds during monsoon[14]. The gonadal activity undergoes a cyclic change (both in morphology and histology) with the change in season so that spawning takes place in the most propitious time of the year. The gonadal activity slowly increases during the months of January and February. It then becomes fully active from March-April and spawning occurs from June to October. Then gonads become inactive again in November-December. The hormones used in this study were purchased from Sigma Chemical Company, USA. General chemicals were purchased from BDH. Cyproterone acetate was gifted by Prof. Dr. M.F. ElEtreby, Berlin.

Adult male *Clarias batrachus* (weighing : 70-80 g; body length: 18-22 cm) were purchased locally during the first week of the months in which the experiments were performed. Fishes were maintained in the earthen pots and acclimatized at least for 15 days in the laboratory under natural climatic conditions at Shillong (Latitude 25° 30′N, Longitude 91° 52′E; Altitude 1450 ASL; minimum water temperature 4°C and maximum water temperature 22°C). During the acclimatization, the fishes were fed daily with minced earthworms *ad libitum*. Water was changed frequently to avoid infections.

In vivo experiments—The *in vivo* experiments were conducted during both winter and summer/rainy seasons. After acclimatization, fishes were divided into different groups (four in each group) for treatments with different hormones. The desired dose of hormone was injected intra-muscularly on the lateral sides of the dorsal fin at an interval of 24 hr between 1000 and 1100 for 4 consecutive days. The details of doses of hormones, duration of treatments, water temperature and the months are mentioned in the following experimental protocol. Water temperature was recorded daily at 1030 hrs. After 24 hr of the last injection, fishes were decapitated, the tissues (liver, muscle, kidney and brain) rapidly removed, rinsed in ice-cold fish buffer saline and stored in the ice-chamber (−10°C) of a refrigerator. Though no significant alteration was found in the rate of tissue respiration up to one month, the rate of tissue respiration was measured within 15 days of storage. For the measurement of tissue respiration, the tissues were first blotted, weighed and homogenized in a loose fitting all-glass homogenizer (Remi Homogenizer, Remi equipments, Bombay) in ice-cold fish buffer saline (9:1) solution (*p*H 7.4). Homogenate (1 ml) was added to 4 ml of fish buffer saline solution and placed into the incubation chamber of the oxygen electrode for measuring the rate of tissue respiration.

In vitro experiments—*In vitro* effects of selected hormones on the rate of tissue respiration were also conducted during both winter and summer/rainy seasons. Adult male fishes (4) were first weighed, and then decapitated. The tissues (liver, muscle, kidney and brain) were quickly removed separately, rinsed in ice-cold fish buffer saline and stored in a freezer as mentioned earlier. The tissues were used to study the *in vitro* effects of hormones within 15 days. For *in vitro* treatments, the tissues were blotted, weighed and homogenized in a loose fitting all glass homogenizer in ice-cold fish buffer saline solution (*p*H 7.4). Homogenate (1 ml) was added to 3.9 ml of fish buffer saline and incubated with 0.1 ml of hormone solutions having the desired concentration (for details, see experimental protocol). The tissue homogenates treated with monoiodotyrosine (MIT), diiodotyrosine (DIT), L-T_3, L-T_4 and testosterone were pre-incubated at 4°C for 1 hr prior to measurement of the rate of oxygen consumption. This incubation was necessary to allow the binding of these hormones to the tissues. Then the rate of tissue respiration was measured with the help of an oxygen electrode.

Measurement of tissue respiration—The rate of oxygen consumption of each tissue (liver, muscle, kidney and brain) was measured with the help of an oxygen electrode (Digital Oxygen System, Model 10; Rank Brothers Ltd., England). For measuring the rate of respiration, polarizing voltage was kept at 0.6 V. Fish buffer saline (*p*H 7.4) was used as the polarizing medium. The rate of oxygen consumption of tissue homogenates was measured at 25°C by circulating water at 25°C in water jacket of the incubation chamber using the thermostatic water circulator. The homogenate was incubated in the chamber for 20 min before recording the readings. Readings were recorded at an interval of 5 minutes for 30 min in the linear range of oxygen consumption. The rate of tissue respiration was expressed as µl O_2/mg wet weight tissue/hr.

Statistical analysis—The data were analysed statistically with the help of Student's t-test[15]. In order to find out statistical correlation between dose and the rate of tissue respiration in terms of correlation coefficient (r), the data of Table 3 were also analysed with the help of 'Regression analysis'. A $P < 0.05$ was considered as significant.

Experimental protocol

Exp no.	Treatment	*In vivo/ In vitro*	Month (Temp.)	Dose	Duration of Treatment
(A)	Saline	*In vitro*	Jan (9.6°C) & July (20°C)		
	MIT			1 μ*M*	
	DIT			1 μ*M*	
	L-T_3			1 μ*M*	
	L-T_4			1 μ*M*	
	Testosterone			1 μ*M*	
(B)	Saline	*In vivo*	Jan (9.6°C) Sept (20°C)		4 days
	MIT			2 μg/fish/day	
	DIT			2 μg/fish/day	
	L-T_3			2 μg/fish/day	
	L-T_4			2 μg/fish/day	
	Testosterone			2 μg/fish/day	
	Cyproterone acetate			1 μg/fish/day	
(C)	Saline	*In vivo*	May (22°C)		4 days
	PTU			1 μg/gm/day	
	L-T_3			2 μg/fish/day	
	L-T_4			2 μg/fish/day	
	PTU + L-T_3			1 μg/g+2 μg/fish/day	
	PTU + L-T_4			1 μg/g+2 μg/fish/day	
(D)	Saline	*In vivo*	Jan (9.6°C)		4 days
	MIT			2 μg/fish/day	
				4 μg/fish/day	
				8 μg/fish/day	
	DIT			2 μg/fish/day	
				4 μg/fish/day	
				8 μg/fish/day	

Results and Discussion

The results are presented in Tables 1-4.

In vivo and *in vitro* administration of L-T_3 and L-T_4 during winter (water temperature 9.6°±1°C) did not stimulate liver, muscle and kidney tissue respiration. These hormones, when administered *in vitro*, stimulated brain tissue oxygen uptake. During summer/rainy months (water temperature 20°C ± 1°C), both L-T_3 and L-T_4 significantly stimulated the rate of tissue respiration both *in vivo* and *in vitro* experiments (Tables 1 & 4). These findings seem to suggest that the oxidative machinery of the fish tissues becomes insensitive to thyroid hormones at low temperature during winter. It may be due to the lack/insufficiency or adverse modification of receptors of the thyroid hormones. The energy-generating pathway regains its sensitivity to thyroid hormones (L-T_3 and L-T_4) during summer/rainy months. Thus, the tissues of *C. batrachus* seem to exhibit a rhythm of sensitivity and insensitivity to thyroid hormones over an annual time scale. The changes in the sensitivity of the metabolic machinery/pathway of the tissues may be an adaptation to conserve energy during winter and increased energy output/metabolic rate during summer/rainy months to meet the increased energy requirements for higher degree of physical and metabolic activity associated with reproduction. In amphibians[6,16] and reptiles[17,18] also,tissues become insensitive to L-T_3 and L-T_4 during winter and sensitive to the hormones during summer.

In vivo treatment with MIT during winter stimulated muscle and brain oxygen uptake, while it increased only muscle tissue during summer/rainy months (Table 1). Further, *in vitro* treatment with MIT stimulated the respiratory rate of all the tissues during winter and only of liver and muscle tissues during summer/rainy months (Table 4). Unlike MIT, *in vivo* and *in vitro*

administration of DIT invariably stimulated the respiratory rate of all the tissues irrespective of water temperature and seasons (Tables 1 and 4). These findings strongly suggest that both MIT and DIT are involved in the regulation of the tissue respiration of *C. batrachus*. The dose-dependent stimulatory effects of MIT and DIT strongly support this suggestion (Table 3). The metabolic action of MIT seems to be dependent on tissues, mode of treatment, and ambient temperature/seasons. However, the calorigenic effect of DIT seems to be unequivocal and independent of temperature and seasons. It, thus, seems that DIT may be more important than MIT for the oxidative metabolism of *C. batrachus*. It has been reported recently that administration of diiodothyronine (T_2) in the gold fish, *Carassius auratus* and the lake-char, *Slavelinus namaycush* increased the state-3 oxidation of pyruvate in liver mitochondria and state-4 oxidation in red muscle mitochondria[19,20]. T_2 has also been reported to increase the activity of the enzyme cytochrome oxidase (COX) in mitochondria of rat liver and bovine heart[21-26]. Therefore, there is a possibility that MIT and DIT also stimulate tissue respiration in fish by stimulating mitochondrial enzymes as reported

Table 1—*In vivo* effects of thyroid hormones, testosterone and cyproterone acetate on the rate of tissue respiration of male *C.batrachus* during (a) winter and (b) summer/rainy seasons
[Values are mean ± SE from 4 animals]

Treatments	Rate of tissue oxygen consumption (µl oxygen/mg /hr)			
	Liver	Muscle	Kidney	Brain
Saline (Control)	a 2.88±0.07	1.12±0.05	2.69±0.08	5.26±0.09
	b 4.71±0.08	2.72±0.05	4.10±0.05	4.30±0.07
Monoiodotyrosine	a 3.17±0.11	1.47±0.11[a]	2.98±0.10	5.55±0.05[a]
	b 4.91±0.02	2.98±0.03[b]	4.20±0.03	4.49±0.04
Diiodotyrosine	a 3.27±0.03[b]	1.54±0.05[c]	3.08±0.04[b]	5.65±0.04[b]
	b 5.26±0.05[b]	3.08±0.08[b]	4.46±0.09[a]	4.59±0.06[a]
Triiodothyronine	a 2.82±0.05	1.18±0.09	2.72±0.05	5.10±0.11
	b 5.61±0.06[c]	3.27±0.07[c]	4.65±0.05[c]	4.75±0.08[b]
Thyroxine	a 2.85±0.07	0.96±0.07	2.66±0.06	5.04±0.07
	b 5.45±0.12[b]	3.49±0.09[c]	4.84±0.07[c]	4.97±0.09[c]
Testosterone	a 3.88±0.03[c]	1.66±0.14[a]	3.65±0.12[b]	5.68±0.05[b]
	b 5.26±0.04[c]	3.52±0.08[c]	4.47±0.07[b]	4.59±0.09[a]
CA	a 2.53±0.08[a]	0.80±0.09[a]	2.40±0.05[a]	4.94±0.07[a]
	b 4.46±0.02[a]	2.37±0.07[a]	3.85±0.04[b]	3.98±0.06[a]
CA + testosterone	a 3.24±0.09[a,f]	1.31±0.05[a,e]	3.08±0.04[b,e]	5.48±0.02[a,d]
	b 4.97±0.05[a,e]	3.30±0.05[e,d]	4.26±0.03[a,d]	4.36±0.04[d]

[a, b, c] Differ from saline treated controls : $P < 0.05$, 0.01 and 0.001, respectively.
[d, e, f] Differ from the group treated with testosterone : $P < 0.05$, 0.01 and 0.001, respectively.

Table 2—*In vivo* effects of thyroid hormones and propyl thiouracil (PTU) on rate of tissue respiration of male *C.batrachus* during summer
[Values are mean ± SE from 4 animals]

Treatments	Rate of tissue oxygen consumption (µl oxygen/mg /hr)			
	Liver	Muscle	Kidney	Brain
Saline (control)	4.87±0.04	3.17±0.05	3.98±0.05	5.29±0.05
L-T_3	5.45±0.03[c]	3.66±0.07[b]	4.59±0.05[c]	5.71±0.07[a]
L-T_4	5.55±0.10[c]	3.56±0.03[c]	4.87±0.04[c]	5.65±0.09[a]
PTU	4.43±0.07[b]	2.82±0.08[a]	3.62±0.10[a]	4.94±0.10[a]
PTU + T_3	5.20±0.03[c,f]	3.30±0.02[a,e]	4.23±0.05[a,e]	5.45±0.03[a,d]
PTU + T_4	5.23±0.05[a,g]	3.33±0.04[a,h]	4.46±0.09[b,h]	5.42±0.02[a,g]

[a, b, c] Differ from saline treated controls : $P < 0.05$, 0.01 and 0.001, respectively.
[d, e, f] Differ from the group treated with L-T_3 : $P < 0.05$, 0.01 and 0.001, respectively.
[g, h] Differ from the group treated with L-T_4 : $P < 0.05$ and 0.01, respectively.

for L-T_2, L-T_3 and L-T_4. It is important to mention that MIT and DIT were the first iodinated molecules associated with proteins in protochordates[27-29]. Later on, during the course of evolution iodothyronines (e.g., T_2, T_3 and T_4) appeared in the blood of vertebrates. Therefore, it seems that MIT and DIT were first to be evolved to serve as regulatory molecules (hormones) in lower vertebrates before the evolution of thyroid gland followed by synthesis of more effective hormones like T_3 and T_4 in vertebrates. Recent reports regarding

Table 3—Dose-dependent *in vivo* effects of monoiodotyrosine (MIT) and diiodotyrosine (DIT)on the rate of tissue respiration of male *Clarias batrachus* during winter
[Values are mean ± SE from 4 animals]

Treatments	Rate of tissue oxygen consumption (µl oxygen/mg /hr)			
	Liver	Muscle	Kidney	Brain
		MIT		
Saline (control)	2.92±0.09	1.15±0.05	2.66±0.06	5.29±0.03
2 µg MIT	3.14±0.05	1.50±0.10[a]	2.95±0.12	5.52±0.06[a]
4µg MIT	3.46±0.10[b,d]	1.92±0.08[c,d]	3.43±0.11[c,d]	5.84±0.03[c,e]
8µg MIT	3.78±0.16[b,e]	2.37±0.07[c,f,h]	3.88±0.14[c,e,g]	6.09±0.07[c,f,g]
Correlation coefficient (r)	+0.98	+0.98	+0.97	+0.96
		DIT		
Saline (Control)	2.92±0.09	1.15±0.05	2.66±0.06	5.29±0.03
2 µg DIT	3.40±0.11[a]	1.79±0.10[b]	3.11±0.05[b]	5.77±0.08[b]
4µg DIT	3.78±0.07[c,d]	2.24±0.09[c,d]	3.53±0.12[c,d]	6.09±0.03[c,d]
8µg DIT	4.10±0.05[c,e,h]	2.56±0.10[c,e]	3.98±0.09[c,f,g]	6.32±0.05[c,e,h]
Correlation coefficient (r)	+0.97	+0.95	+0.98	+0.95

[a, b, c] Differ from saline treated controls : $P < 0.05$, 0.01 and 0.001, respectively.
[d, e, f] Differ from the group treated with 2 µg MIT/DIT : $P < 0.05$, 0.01 and 0.001, respectively.
[g, h] Differ from the group treated with 4 µg MIT/DIT : $P < 0.05$ and 0.01, respectively.

Table 4—*In vitro* effects of thyroid hormones and testosterone on the rate of tissue respiration ofmale *Clarias batrachus* during (a) winter and (b)summer/ rainy seasons
[Values are mean ± SE from 4 animals]

Treatments	Rate of tissue oxygen consumption (µl oxygen/mg /hr)			
	Liver	Muscle	Kidney	Brain
Control	(a) 2.69±0.06	0.89±0.08	2.53±0.05	5.01±0.04
	(b) 4.81±0.12	2.85±0.02	4.01±0.03	5.07±0.07
Monoiodotyrosine	(a) 2.95±0.05[a]	1.34±0.03[c]	2.79±0.08[a]	5.26±0.08[a]
	(b) 5.16±0.05[a]	3.11±0.06[b]	4.10±0.09	5.36±0.11
Diiodotyrosine	(a) 3.05±0.07[b]	1.28±0.05[b]	2.92±0.11[a]	5.45±0.07[b]
	(b) 5.36±0.07[b]	3.37±0.09[b]	4.43±0.07[b]	5.48±0.05[b]
Triiodothyronine	(a) 2.72±0.09	0.79±0.02	2.56±0.05	5.65±0.06[c]
	(b) 5.52±0.04[b]	3.53±0.07[c]	4.68±0.12[b]	5.61±0.06[c]
Thyroxine	(a) 2.63±0.12	0.75±0.07	2.60±0.09	5.87±0.05[c]
	(b) 5.45±0.03[b]	3.40±0.16[a]	4.81±0.07[c]	5.71±0.07[c]
Testosterone	(a) 4.75±0.04[c]	1.63±0.05[c]	3.59±0.09[c]	4.81±0.03
	(b) 5.32±0.03[b]	3.75±0.05[c]	5.01±0.04[c]	5.58±0.12[a]

[a, b, c] Differ from saline treated controls : $P < 0.05$, 0.01 and 0.001, respectively.

calorigenic function of T_2 strongly suggest a gradual evolution of iodinated molecules for regulation of the oxidative metabolism to meet the increasing requirement of energy with the evolution of complex organs/systems in higher vertebrates. Considering the phylogenic position of fish, it is suggested that the iodinated tyrosine molecules, particularly DIT, may be more important than T_3 and T_4 in calorigenesis especially at low temperature of winter months when both T_3 and T_4 are calorigenically ineffective[6,8,17].

In vivo administration of propyl thiouracil (PTU) significantly suppressed the respiratory rate of liver, muscle and kidney tissues during the summer/rainy season (Table 2) as well as at simulated low temperature (10°C) (unpublished data). PTU inhibited brain tissue respiration only during summer/rainy months (Table 2) but not at the simulated low temperature (unpublished data). Administration of L-T_3 reversed the inhibitory effect of PTU. These findings seem to indicate that the metabolic rate of brain is influenced by thyroid hormones only during summer/rainy months, while during winter it may be regulated by hormones other than that of thyroid. The decreased respiratory rate following PTU treatment during summer/rainy months may be due to inhibitory effect of PTU on iodine uptake by the thyroid tissue as well as inhibition of peripheral conversion of T_4 to T_3 leading to decreased synthesis of thyroid hormones and of low availability of T_3[12,30]. Thus, inhibition of thyroid hormones' production and conversion of T_4 to T_3 may be responsible for the decline in the rate of tissue oxygen uptake of the fish following PTU treatment. There are reports that administration of PTU decreased the whole body oxygen consumption in *Mugil auratus*[31] and carps[32]. Further, PTU has been reported to inhibit the activity of Na^+-K^+-ATPase and mitochondrial enzymes in fish, while administration of thyroid hormones reversed the inhibitory effect of PTU[33-35]. As reported in amphibians[36] and reptiles[8], indigenous thyroid hormones may be involved indirectly in the energy metabolism of *C. batrachus* by potentiating the calorigenic action of catecholamines at low temperature of winter.

In vivo and *in vitro* administration of testosterone significantly increased the respiratory rate of all the tissues during both winter and summer/rainy months except brain (Tables 1 and 4). Brain tissue respiration was stimulated by *in vitro* treatment with testosterone only during summer/rainy seasons, but not during winter (Table 4). These findings clearly suggest that testosterone is calorigenic in fish, and its stimulatory effect on the rate of tissue respiration seems to be independent of ambient temperature. Further, *in vitro* stimulation of tissue respiration by testosterone strongly suggests that the hormone has a direct calorigenic effect on the tissues. Brain respiration during winter, however, seems to be indirectly regulated by testosterone. *In vivo* administration of cyproterone acetate always inhibited the respiratory rate of all the tissues during both winter and summer/rainy months (Table 1). Cyproterone acetate blocks androgen receptors and does not allow binding of testosterone and other androgens to their receptors. A significant decrease in the rate of tissue respiration clearly indicates that indigenous testicular androgens are involved in the regulation of the metabolic rate of the tissues in the fish, *C. batrachus*. These findings seem to suggest that testicular hormones play an important role in the regulation of the oxidative metabolism of the fish. The temperature-independent and direct calorigenic action of testosterone in fish may be of great adaptational importance. Further, significant decrease in the rate of tissue respiration following cyproterone acetate treatment, especially during winter months, seems to suggest that indigenous testicular androgens may be actively involved in the regulation of metabolic rate to ensure minimum physiological activity and survival of the fish at low temperature.The increased basal metabolic rate in some mammals has been reported to be related to the concentration of testicular hormones[37]. There are several reports on the stimulatory effect of testosterone and inhibitory effect of castration on the respiratory rate of reptiles and amphibians[6,8]. It is, thus, obvious that testosterone (the major androgen), due to its direct and temperature-independent calorigenic action, plays a major role in the regulation of the oxidative metabolism in fish and other poikilothermic vertebrates, particularly at low temperature.

On the basis of these findings it is concluded that exogenous L-T_3 and L-T_4 are calorigenic in fish only at higher water temperature (above 20°C). However, the indigenous thyroid hormones seem to be involved in calorigenesis even at low temperature of winter months. The stimulatory effect of MIT varies with tissues, mode of treatment and season, while L-DIT stimulates tissue respiration irrespective of temperature and seasons. Besides T_3 and T_4, MIT and DIT also seem to be actively involved in the regulation of the oxidative metabolism of *C. batrachus*. Further,

testicular hormones seem to play an important role in the regulation of tissue respiration of the fish especially at low temperature where T_3 and T_4 are not calorigenic. These findings strongly support the view that testicular hormones may also be regulating the energy metabolism of the poikilothermic vertebrates to ensure their survival at extremely low temperature of winter months[8].

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