Evidence of the Presence of Thyroid Hormones in *Achatina fulica* Snails

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ABSTRACT

The objective of this study was to identify thyroid hormones and to examine their putative site of synthesis in *Achatina fulica* snails. For this purpose, radioimmunoassays were performed for T_3 and T_4 before and after long starvation with or without hemolymph deproteinization. Sodium/iodide symporter activity *in vivo* was analyzed through ^{125}I administration with and without $KClO_4$ pretreatment. Only T_4 was detected, and its concentration decreased due to starvation or deproteinization. However, high-performance liquid chromatography analysis also showed the presence of T_2 and T_3 apart from T_4, but rT_3 was not detected in the *A. fulica* hemolymph. The sodium/iodide symporter activity was greater in cerebral ganglia than digestive gland, but $KClO_4$ treatment did not inhibit iodide uptake in any of the tissues analyzed. Altogether, our data confirm for the first time the presence of thyroid hormones in *A. fulica* snails and suggest their participation in the metabolism control in this species, although the putative site of hormone biosynthesis remains to be elucidated.

Key words: *Achatina fulica*, Sodium/iodine symport, Thyroid hormones, Thyroglobulin.

INTRODUCTION

In mammals, the thyroid gland is responsible for the biosynthesis and secretion of 3,3′,5,5′-tetra-iodo-l-thyronine l-thyroxine (l-T_4) and

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3,3′,5-triiodo-l-thyronine (l-T_3), so-called thyroid hormones (THs). The biosynthesis of T_3 and T_4 occurs through several steps that are dependent on thyroglobulin (TG) synthesis, iodide transport across basal membrane (by Na^+/I^- symporter, NIS), iodide oxidation, tyrosyl iodination sites in TG and coupling of the iodotyrosines, a process catalyzed by the enzyme thyroperoxidase (TPO) (Larsen et al. 1998). So, TG protein is endocyted by the apical

membrane of the follicular cell, and once inside the phagolysosome it is degraded to release T_3 and T_4 into the bloodstream. In the circulation, THs play an important role in the regulation of energy homeostasis by stimulating oxygen consumption and heat generation. They are also important to the normal growth and development of the organism (Haber et al. 1988, Gupta and Chakrabarty 1990).

THs and their receptors have been well studied in vertebrates, but less is known about the mechanisms by which they regulate the physiology and behavior in mollusks. Tensen et al. (1994) showed the presence of thyroid-stimulating hormone (TSH) receptors in the freshwater gastropod *Lymnaea stagnalis* and Heyland et al. (2006) showed evidence of the endogenous synthesis of THs in the sea hare *Aplysia californica*, and of the gene that encodes a peroxidase (AcaTPO) that is similar to the TPO found in mammals. More recently, Huang et al. (2015) demonstrated that THs are produced in the Pacific oyster, *Crassostrea gigas*. However, to our knowledge the presence of THs and the mechanisms of regulation and synthesis/metabolism of THs in land snails are still poorly unknown.

The terrestrial snail *Achatina fulica*, known as the giant African snail, is considered an agricultural pest and intermediate host of different parasites (Thiengo et al. 2007). These characteristics along with its rapid dissemination on the American continents have drawn attention to developing strategies for control of this invasive snail. Since many behavioral and physiological processes in mollusks are regulated by hormones, including growth and reproduction (Gomot et al. 1992, Gonzalez et al. 1995, Gooding and LeBlanc 2004), knowledge about endocrine aspects in *A. fulica* can support strategies for its control. Thus, the aim of the present study was to identify the presence of THs in *A fulica* snails (under basal and starvation conditions) using radioimmunoassay and high-performance liquid chromatography (HPLC)

analysis. Furthermore, NIS activity was evaluated to try to identify the production site of these hormones.

MATERIALS AND METHODS

Achatina fulica COLLECTION AND MAINTENANCE

Specimens of *A. fulica* were collected manually early on summer mornings from residential gardens located in Seropédica, Rio de Janeiro state (22° 46' 59" S and 43° 40' 45" W, 33m height). The snails were maintained under laboratory conditions in transparent plastic boxes (50 × 30 × 15cm) with a 3cm layer of moistened earth at the bottom. The animals were fed with lettuce leaves *ad libitum* and the food was renewed on alternate days. The mollusks were submitted to an acclimation period of three weeks to the laboratory conditions before starting the experiments (26 ± 2°C).

To identify the presence of T_4 and T_3 hormones in hemolymph of *A. fulica* snails, two groups were formed: Control group (n=10) that was fed as described above throughout experimental period and Starvation (n=10), a group of snails that were left without food for four weeks (Rudolph and Bailey 1985). So, the specimens from each group were randomly chosen, dissected by removing their shell and then, the hemolymph (~2mL) was collected with the aid of a syringe by heart puncture and stored in microtubes at -10°C until utilization. Samples were maintained in an ice bath during the dissections. To assess whether THs could be bound to hemolymph proteins, the same hemolymph samples were submitted to deproteinization protocol (Nelson 1944), thus two new subgroups were formed: No changes (n=10), with normal hemolymph and Deproteinized (n=10), in which the hemolymph was deproteinized.

Furthermore, to correlate TH concentrations with the biomass of animals, the new group of snails (n=7) under basal condition was used. For that, the snails were individually weighed using

a digital analytical balance and body size was measured by using a metric ruler when the snails were resting and so, hemolymph was collected as described above.

The protocol studies followed the international guidelines and standards for the ethical use of animals in research and agree with Ethical Principles in Animal Research adopted in Brazil.

RADIOIMMUNOASSAY (RIA) FOR TOTAL T_4 AND T_3

Circulating T_3 and T_4 levels were determined by specific coated-tube RIA kits: T_3, 3100 Active; and T_4, 3200 Active (Diagnostic System Laboratory, Webster, TX). Hormone-stripped rat serum was used for the standard curves of total T_4, and T_3. T_3 assay sensitivity was of 0.1nmol/L, and inter- and intra-assay coefficients of variation varied from 8.3 to 8.6% and from 2.9 to 3.3%, respectively. T_4 assay sensitivity was 13nmol/L and inter- and intra-assay coefficients of variation varied from 5.6 to 8.6% and 4 to 5.1%, respectively. All procedures were performed following the recommendations of the kit.

HPLC ANALYSIS

For this assay, we adapted the technique from Alexander and Nishimoto (1979), de la Vieja et al. (1997), Gika et al. (2005). Briefly, the reagents 3,3′,5,5′-tetra-iodo-l-thyronine l-thyroxine (l-T_4), 3,3′,5-triiodo-l-thyronine (l-T_3), reverse 3,3',5'-triiodo-L-thyronine (rT_3) and 3,5-diiodo-l-thyronine (l-T_2) were used, obtained from Sigma–Aldrich (Steinheim, Germany). Standard solutions of the compounds were prepared at a concentration of 2mg/mL in a mixture of MeOH and 0.01M NaOH (1:1v/v). These were stored protected from light at 4ºC, since under these storage conditions they were stable. All HPLC experiments were carried out with a Shimadzu LC-20AD liquid chromatograph system (Kyoto, Japan) associated with an SPD-M20A photodiode array detector,

with a column oven (CTO-20A). The system was controlled by the LC-Solution Single software. The analyses were performed with a Shim-Pack CLC-ODS C18 column (250 mm x 4.6 mm i.d., 5µm particle size, Shimadzu). A Rheodyne 7125i sample injector valve with 20 µL filling loop (Berkeley, CA, USA) was used. The mobile phase consisted of a mixture of solvent A (water, 1% acetic acid, pH 3.9) and solvent B (90% MeOH, 10% acetonitrile), starting from B-A, 65 to 35%. The column oven temperature was set at 25°C and the mobile phase was pumped at a flow rate of 1mL/min, and the eluent was monitored at 254 nm.

Aliquots of the same hemolymph samples from the first experiment were vortexed and centrifuged for 10 min at 3500rpm. The supernatant was removed and the undissolved particles were removed by filtration using 45µm membrane filters. Aliquots of 20µL were used for the chromatographic analysis.

SHORT-TERM RADIOIODIDE UPTAKE: *In vivo* NIS FUNCTION

It was previously shown that the measurement of radioiodide uptake 15 min after ^{125}I–NaI administration (short-term iodide uptake) reflects iodide transport through the sodium–iodide symporter (NIS) without influence of *in vivo* thyroid iodine organification activity (Ferreira et al. 2005). Thus, to evaluate the *in vivo* NIS function in snails under basal conditions (n=6) using digestive gland (DG) and cerebral ganglia (CG) material and measure the radioiodine uptake, Na–^{125}I (3700 Bq, Amersham, Buckinghamshire, England) was administered in the medial region of the cephalopodal mass with a hypodermic syringe 15 min before decapitation. In the thyroid, iodine organification is catalyzed by TPO and consists of binding of iodine to tyrosil residues in thyroglobulin, an essential step of TH biosynthesis. The radioactivity of the tissues was measured using a gamma counter (LKB) and expressed as percentage of total ^{125}I injected per mg of DG

or CG. After this, six different snails received a solution of potassium perchlorate (0.40mg/100μL), an inhibitor of NIS activity, two minutes before Na-^{125}I administration, after which the radioactivity was measured as above. As there is no published information about pharmacological approaches to thyroid function in snails, the Na–[125]I and $KClO_4$ administrations were based in values used for rats (Ferreira et al. 2005).

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error. The unpaired Student t-test was used to compare two groups. Nonlinear regression was applied to analyze the relation between T4 concentration and body weight and body size of the snails. The statistical analyses were performed using GraphPad Prism® 5 (Graphpad Software, Inc., San Diego, USA). For all comparisons, the differences between means were considered statistically significant for P < 0.05.

RESULTS

As observed in Figure 1, only T_4 was found in the *A. fulica* hemolymph by RIA analysis (3.49 ± 0.55μg/dL). After four weeks of starvation, there was a significant decrease of T_4 concentrations (2.27 ± 0.16 vs. 3.49 ± 0.55μg/dL, P<0.05) in *A. fulica* hemolymph in relation to the control. Similarly, the T_4 concentrations decreased after hemolymph deproteinization both in non-starved (0.63 ± 0.08 vs. 3.49 ± 0.55μg/dL, P<0.0001) and starved groups (0.69 ± 0.11 vs. 2.27 ± 0.16μg/dL, P<0.0001) when compared to their respective controls. Also, there was a weak relation between T_4 levels and body size and body weight (r²= 0.53 and 0.26, respectively) (Fig. 2a and b).

Nevertheless, qualitative HPLC analysis showed that T_2, T_3 and T_4 were present in *A. fulica* hemolymph but not reverse T_3 (Fig. 3). This analysis was confirmed by the similar retention

time between the standards and samples, as well as the ultraviolet absorbance curve designed for the hormones (Fig. 4). The profiles obtained here suggest that THs have low concentrations in this snail's hemolymph.

In an attempt to identify the putative production site of these hormones, NIS activity was measured. For this assay, CG and DG were collected because earlier studies have shown that these tissues are able to produce several hormones (Davidson et al. 1971, Geraerts 1976, Roubos et al. 1980, Jong-Brink et al. 1981). The results showed that CG seems to take up more ^{125}I than DG (194.11 ± 40.99 and 68.11 ± 16.17 ^{125}I uptake/mg tissue, P<0.05, respectively). However, these results may be deceiving because: i) these animals have an open circulatory system; and ii) the protocol was conducted *in vivo*. So, $KClO_4$, an inhibitor of NIS activity, was used before ^{125}I-NaI administration. Curiously, $KClO_4$ did not impair iodide uptake (Fig. 5).

DISCUSSION

Only T_4 was found in *A. fulica* hemolymph by RIA analysis, and its levels declined after both starvation and hemolymph deproteinization. The decrease of T_4 concentrations is a well-established condition observed during starvation that is secondary to decrease of the thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH) secretion (Krotkiewski 2000). Also, since THs are known to increase energy metabolism and thermogenesis (Ribeiro et al. 2001), it is reasonable to expect the animals subjected to fasting to present a lower level of THs, in line with the smaller energy input during fasting (adaptive phenomenon). On the other hand, it was previously demonstrated that echinoids can also obtain THs from food (algae) (see Heyland and Moroz 2005), suggesting that the decrease of THs after starvation could also be associated with the lower support of exogenous THs. However, to our knowledge there
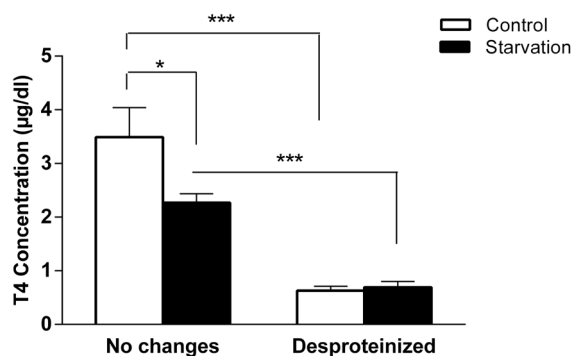
Figure 1 - Thyroxin (T$_4$) concentration in hemolymph of *Achatina fulica*, expressed in µg/dL, under basal and starvation conditions as well as hemolymph desproteinization. The hemolymph samples were obtained from 10 snails per group, but when necessary the samples were pooled. * Indicates significant difference between groups (P < 0.05). *** Indicates significant difference between groups (P < 0.0001).
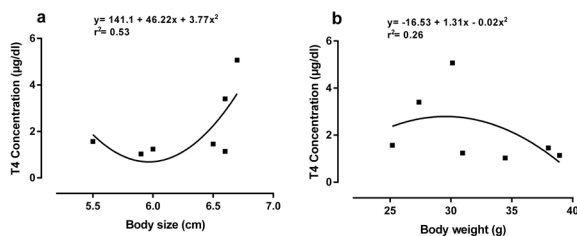

Figure 2 - Relation between the thyroxin (T$_4$) concentration, expressed in µg/dL, in hemolymph of *Achatina fulica* (n= 7) and its body size (**a**), expressed in centimeters (cm) and body weight (**b**), expressed in grams (g).

is no information regarding TH synthesis in lettuce plants (used as food in our experiments). Besides this, the presence/synthesis of THs in different species of mollusks and of TSH hormones in the pulmonate gastropod, *L. stagnalis* (Tensen et al. 1994), indicate that *A. fulica* snails are also able to produce endogenous THs.

In the present study, the T$_4$ concentrations varied greatly among animals. Since we had no data on age, we tried to correlate the biomass of the mollusks with the T$_4$ concentrations. However, no significant correlation was detected.

The decrease of T$_4$ concentrations after hemolymph deproteinization is consistent with the fact that THs bind to plasmatic proteins, mainly
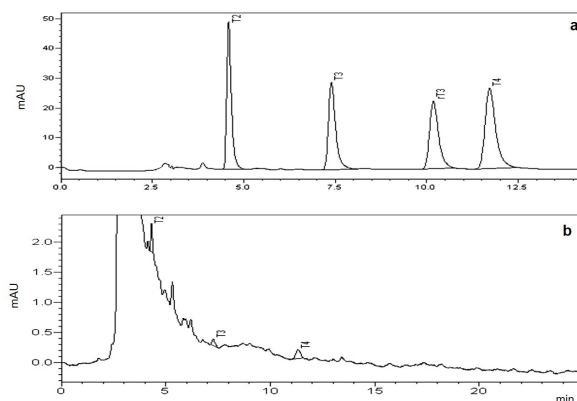

Figure 3 - Indicative chromatograms of spiked biological samples: (**a**) standard iodoamino acids and (**b**) hemolymph samples from *Achatina fulica* snails on a Shim-Pack CLC-ODS C18 column (250mm x 4.6mm i.d., 5µm particle size, Shimadzu). The mobile phase consisted of a mixture of solvent A (water, 1% acetic acid, pH 3.9) and solvent B (90% MeOH, 10% acetonitrile), starting from B - A, 65 to 35%. The column oven temperature was set at 25°C and the mobile phase was pumped at a flow-rate of 1mL/min, and the eluent was monitored at 254nm. Peaks: (T$_2$) 3,5-diiodo-l-thyronine (4.58 vs. 4.30min, respectively), (T$_3$) 3,3',5-triiodo-l-thyronine (7.38 vs. 7.24min), (rT$_3$) reverse 3,3',5'-triiodo-L-thyronine (10.17 min), and (T$_4$) 3,3',5,5'-Tetra-iodo-l-thyronine l-thyroxine (11.71 vs. 11.30min). On the y-axis, the peak area is expressed as absorbance units (mAU).

thyroxin-binding globulin (TBG), in vertebrates (Yen 2001), although more elaborate experiments are needed to elucidate this issue. So, the deproteinization process must have depleted the hormone binding proteins that probably exist in the hemolymph of *A. fulica* snails. Moreover, hormone-binding proteins have previously been detected in mollusks (D'Aniello et al. 1996, Bogoeva and Russev 2008).

In addition, HPLC analysis showed the presence of T$_2$, T$_3$ and T$_4$, but not rT$_3$. These results confirm the presence of THs in *A. fulica* snails. However, the evaluation of the chromatographic profile obtained suggests these hormones have low concentration in the *A. fulica* hemolymph. A study by Tensen et al. (1994) detected TSH receptors in the freshwater gastropod *L. stagnalis*, which supports our idea of a functional activity of THs
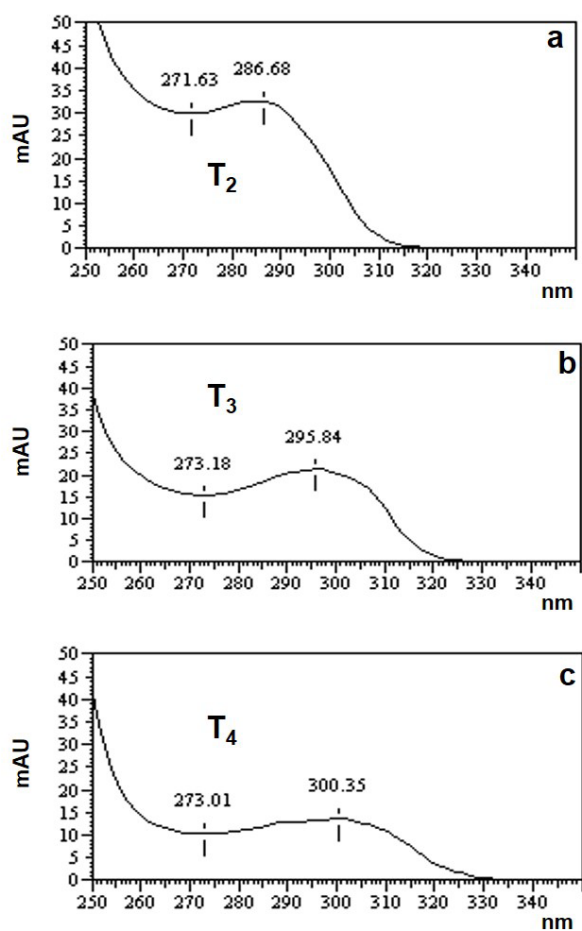
Figure 4 - Comparison of standard iodoamino acids spectra, wavelength of 250 to 350nm, obtained using HPLC- PDA. (**a**) T$_2$, (**b**) T$_3$, and (**c**) T$_4$. These data are similar to those reported by de la Vieja et al. (1997). On the y-axis, the ultraviolet spectrum is expressed as absorbance units (mAU).

Figure 5 - Short-term radioiodide uptake study performed in *Achatina fulica* snails with (n= 6) and without KClO$_4$ treatment (n= 6). The tissues of both cerebral ganglia (CG) and digestive gland (DG) were removed, weighed and the radioactivity was measured using a gamma counter and expressed as 15min ^{125}I uptake/mg tissue. Data are expressed as mean ± SEM. * Indicates significant difference between groups (P < 0.05). ** Indicates significant difference between groups (P < 0.001).

in mollusks. Due to the phylogenetic proximity of these two species, it is possible suggest that *A. fulica* snails have a functional thyroid system. The decrease of T$_4$ levels after starvation strengthens this idea.

To date we have no explanation for the fact that iodide uptake activity was not blocked after KClO$_4$ treatment. Similar results were obtained by Heyland et al. (2006). These authors demonstrated that KClO$_4$ does not change the uptake of iodide in echinoderms, suggesting that the results found were due to differences in iodine transport between the mammalian thyroid gland and
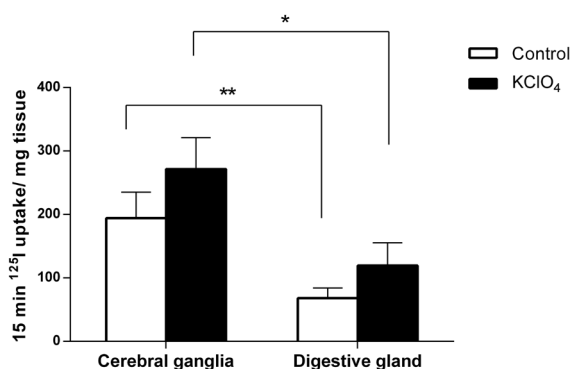
echinoderm cells. Moreover, Miller and Heyland (2013) demonstrated that iodine accumulation in the sea urchin *Strongylocentrotus purpuratus* is independent of NIS transport and seems to be mediated by peroxide-dependent diffusion. In turn, it is possible that in *A. fulica* the iodine uptake occurs by epithelial diffusion. Considering that *A. fulica* does not have follicular thyroid tissue or its homologous endostyle, our results suggest that CG could be able of synthetize THs in *A. fulica* since this neural tissue showed greater uptake of iodine than DG, which is consistent with the results of previous studies showing that the CG has the ability to secrete various hormones (Geraerts 1976, Roubos et al. 1980).

The identification of THs in snails is extremely important because it suggests the presence of a new pathway that can regulate the metabolism of this invertebrate group. This sheds more light on the spread, survival and reproduction of this species of snail. Moreover, recently Wu et al. (2007) demonstrated the presence of TH receptors in the genome of *Schistosoma mansoni,* a parasite distributed worldwide that infests snails and

vertebrates during its complex life cycle. Saule et al. (2002), studying the effects of murine infection with *S. mansoni*, found that rats treated with thyroxine had an increase in the number and development of giant worms, whereas a diet deficient in iodine reduced the maturation of the parasite and number of eggs laid, and caused liver disease. The idea that neurohumoral systems of definitive or even intermediate hosts play a key role in infection processes of parasites has grown enormously. However, in relation to THs, the importance of the humoral loop is poorly understood in these animals. Taken together, these results provide strong evidence that THs are highly conserved during phylogenetic evolution and may have an important role in species conservation.

In conclusion, the present study demonstrates for the first time the presence of THs in the snail *A. fulica,* a terrestrial gastropod. We observed the presence of T_4, T_3 and T_2 in the hemolymph of these animals through HPLC analysis, although these were present in low concentrations. The decrease in T_4 levels after fasting and hemolymph deproteinization indicates similarities with vertebrates, and suggests its participation in the metabolic control of this snail, but the physiological role of this hormone remains elusive.

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