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Annual variations of thyroid activity in the lizard *Podarcis sicula* (Squamata, Lacertidae)

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ABSTRACT

The present paper describes the annual variations in plasma concentrations and thyroid and hepatic contents of thyroid hormones, as well as the activity of hepatic 5'-T4 ORD (type II) monodeiodinase, in the male lizard, *Podarcis sicula*, during two consecutive years. Plasma concentration of T4 was low in winter and increased in March, reaching maximal levels in May-June; plasma levels of T3 varied throughout the year, reaching minimum values in December and February. The thyroid content of T3 was high between March and July; the T4 content followed a similar seasonal pattern and showed a minimum in January and a maximum in July. Low hepatic contents of T3 and T4 were found in winter, increasing rapidly after spring to reach maximal values in July-October. Moreover, the maximal activity of hepatic 5'-T4 ORD (type II) monodeiodinase was observed in May and June-July. Minimal activity was detected in winter and in October-November. Plasma TSH levels were high between March and July and low from November to January.

KEY WORDS: Thyroid - Lizard - *Podarcis sicula*.

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INTRODUCTION

In many vertebrate species, the thyroid gland releases both L-thyroxine (T4) and tri-iodo-L-thyronine (T3) into the bloodstream. In addition, a portion of the T3 present in the blood is produced extrathyroidally, via the monodeiodination of T4 in peripheral tissues. In fact, most T4 is converted enzymatically to active or inactive derivatives in extrathyroidal tissues. Outer-ring monodeiodination (ORD) removes an iodine for the T4 phenylring creating the biologically active T3. Alternatively, inner-ring deiodination (IRD) removes an iodine from the tyrosylring to form the biologically inactive 3,3',5'-triiodo-L-thyronine (reverse T3 = rT3). These ORD and IRD pathways may contribute to the regulation of the thyroidal status by adjusting T3 availability in plasma and tissues.

The mechanisms of cellular uptake of thyroid hormones in non-mammalian species have been studied particularly in fishes (Frith & Eales, 1996; Sefkow *et al.*, 1996; Eales *et al.*, 1997), birds (Darras *et al.*, 1996; Schew *et al.*, 1996), and amphibians (Kuhn *et al.*, 1983, 1985; Gancedo *et al.*, 1995). In reptiles has been demonstrated that T4 and T3 are the major circulating thyroid hormones, and that there is extrathyroidal conversion of T4 and T3 (Bona-Gallo *et al.*, 1980; Chiu, 1982; Sellers *et al.*, 1982; Kar & Chandola-Saklani, 1985a, b). An annual cycle of thyroid hormones has been described in numerous reptilian species (Gabe & Saint-Girons, 1962; Saint-Girons & Duguy, 1966; Lynn, 1970). However, most studies have widely used indirect histological and histochemical criteria, and only few of them have evaluated the thyroid function by direct measurement of the circulating plasma levels of thyroid hormones. Bona-Gallo *et al.* (1980) demonstrated that, in the Chinese cobra *Naja naja*, thyroid weight and morphology may not accurately reflect the blood levels of thyroid hormones; yet, they reported a very pronounced annual cycle of plasma T4 with a peak in summer and a marked depression during the coldest months of the year. In the lizard *Cnemidophorus sexlineatus*, Sellers *et al.* (1982) determined a cycle of plasma T4 with levels ranging from 1.0 to 4.2 ng/ml during 15 months of study. Then, in *Vipera aspis*, Naulleau *et al.* (1987) demonstrated an annual cycle of plasma T4 with a maximum concentration from February to March, a decrease from September to October (two months before the beginning of hibernation) and a minimum in November-December.

These annual variations of T3 and T4 plasma concentrations in reptiles appear to be related to such factors as hibernation or reproduction, and are influenced by climatic variations and temperature. In fact, several data indicate seasonal variations in the activity of the hypothalamo-hypophyseal-thyroid axis of these vertebrates. Chiu (1982) concluded that the thyroid function is influenced by temperature through the hypophysis, and Walker (1973) observed in *Sceloporus cyanogenys* that

continued cold reduces thyroid activity. The low thyroidal activity observed in *Vipera aspis* in winter can be considered as a passive response to the disadvantageous outdoor temperature and the consequent suspension of feeding (Naulleau *et al.*, 1987). Low thyroidal activity in winter (low plasma T4 levels and glandular activity) has also been observed in other reptiles. For example, in *Pseudemys scripta elegans*, Vivien-Roels (1969) showed low glandular activity in winter followed by marked stimulation in spring. This was also shown by Saint-Girons & Duguy (1962, 1966) in *Vipera aspis* and *Natrix maura*. In addition, many Authors have demonstrated a relationship between testicular and thyroid functions; in fact, reactivation of gonadal activity appears to be responsible for the decrease in plasma T4 levels at the end of hibernation (Licht *et al.*, 1984, 1985). In the cobra, Bona-Gallo *et al.* (1980) observed a maximal level of plasma T4 one month after the peak of plasma testosterone, the increase in plasma T4 levels coinciding with the decrease in plasma testosterone. These Authors concluded that T4 acts negatively on plasma testosterone levels by blocking gonadotropins. The same conclusion was reached by Sellers *et al.* (1982). Then Nilson (1982) showed that, in *Vipera berus*, T4 regulates spermatogenesis, while Naulleau *et al.* (1987) observed that, in *Vipera aspis*, plasma testosterone and T4 were in phase in winter and in opposition after emergence from hibernation, with a peak of plasma T4 one month after the peak of plasma testosterone. Thus, on the basis of the literature data, thyroid activity in reptiles seems to vary during the year, also showing variation according to the species and the environmental conditions.

Morphologically, the thyroid of the most common Italian lizard, *Podarcis sicula* Raf, exhibits seasonal variation in activity (Cavagnuolo *et al.*, 1982). Therefore, the following month-to-month study was made in order to obtain complete annual information on plasma concentrations and hepatic contents of T3 and T4. Outering deiodinating activity of T4 in hepatic tissues and annual plasma thyrotropic hormone (TSH) concentrations were also investigated.

MATERIALS AND METHODS

Animals and experimental design

All the experiments were performed on adult male specimens of *P. sicula*, weighing between 12 and 15 g, caught in fields near Naples. In order to study annual variation in the thyroid function, a group of 10 animals were killed two days after capture on the 15th day of each month for two consecutive years. The blood samples were collected into heparinized capillaries, centrifuged 10 min at 1500 rpm, and the plasma was stored at -20° C until radioimmunoassay (RIA) was performed.

Determination of plasma hormones

Plasma T3 and T4 levels were determined by RIA. In the T3 assay, a measured amount of sample serum and standards was added to a tube coated with anti-T3 rabbit antibody, along with a trace amount of radioactively labeled T3 ([^{125}I]-T3, 165 kBq; Byk-Sangtec Diagnostica, Dietzenbach, Germany) and a blocking agent (Tris buffered saline, 4 mM ANS, 6 mM sodium salicylate with 0.2% sodium azide as a preservative; Sigma Chemical Co., St. Louis, USA) to release T3 from serum binding proteins. The sensitivity was 0.1 ng/ml with an accuracy of about 97%. The range of intra-assay variance in 20 assays was 1.0-2.6%, while the inter-assay variance ranged between 3.9 and 5.7% in 12 assays.

For T4, a measured amount of sample serum and standards was added to a tube coated with anti-T4 rabbit antibody, along with a trace amount of radioactively labeled T4 ([^{125}I]-T4, 165 kBq; Byk-Sangtec Diagnostica) and a blocking agent (Tris buffered saline, 4 mM ANS, 6 mM sodium salicylate with 0.2% sodium azide as a preservative; Sigma Chemical Co.) to release T4 from serum binding proteins. The sensitivity was 0.45 ng/ml with an accuracy close to 100%; the mean intra-assay and inter-assay coefficients of variation were 4.6 and 4.3%, respectively. The cross-reactivity for T4 in the T3 RIA (1.3%) was not considered for data calculations, neither was that for T3 in the T4 RIA (0.1%).

Serum TSH was determined by immunoradiometric assay (IRMA). Sample serum and standards were added to anti-ligand coated tubes. The tracer/capture reagent, a blend of ligand-tagged TSH-rabbit antibody and ^{125}I labeled (10 µCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose was 0.01 µIU/ml, with an accuracy close to 100% and a mean intra-assay and inter-assay variance of 5.0% and 7.5%, respectively. Cross-reactivity studies were performed using substances which could theoretically interfere with the performance of the assay. The cross-reactivity for FSH, hCG and LH in TSH IRMA was less than 0.001 and therefore was not considered for data calculations.

Determination of thyroid content of T3 and T4

Thyroid glands were sonicated in 0.01 M sodium-phosphate buffer with a Vibra cell sonicator three times each for 10 s (35 W final power output) and centrifuged at 10000 rpm for 15 min. The supernatant was subjected to enzymatic digestion with pronase (Sigma Chemical Co.) as described by Darras *et al.* (1996) but without using the subsequent LAP-enzyme digestion. Thereafter, T3 and T4 contents were measured by RIA and were expressed as ng/thyroid.

Determination of hepatic thyroid hormones content and 5'-T4 ORD (type II) monodeiodinase activity

The livers were removed and rinsed in a buffer consisting of 50 nM morpholinopropane sulfonic acid (Mops, Sigma Chemical Co.) with 1 mM EDTA at pH 7.4. They were then thawed, minced on a glass plate, and homogenized with 3 volumes of sucrose buffer (0.25 M sucrose, 5 mM Tris, pH 8.0) in a pyrex tissue grinder held on ice. The resulting homogenate was centrifuged at 1000 rpm and 4° C for 10 min. The supernatant was reserved and the pellet was suspended in an equal volume of sucrose buffer and centrifuged again. The pooled supernatant was then centrifuged at 12000 rpm and 4° C for 5 min. The resulting final supernatant (premicrosomal fraction) was centrifuged for 90 min at 78000 rpm and 4° C in an ultracentrifuge (Beckman, Palo Alto, USA). Microsomal pellets were suspended in Mops buffer and stored in aliquots at -80° C. The contents of T3 and T4 in hepatic tissue were determined by RIA and were expressed as ng/mg of tissue (fresh weight).

An aliquot (30 µl) of the homogenate plus 3 volumes of buffer containing 50 mM of 1-4 dithio-DL-threitol (DTT) and 1 nM radiolabeled T4 was incubated 20 min at 12° C. Cold ethanol was added to the samples kept overnight at 4° C and, subsequently, centrifuged 20 min at 1400 rpm. The supernatant was used for the measurement of 5'-T4 ORD type II (ORD II) monodeiodinase activity. The activity of the enzyme is expressed as pM T3/g (of liver)/h.

Statistical analysis

All data were presented as means ± SE. Statistical analysis was performed by means of one-way ANOVA for repeated measures, with least squared means as a post-hoc test, or unpaired Student's t test. A two-tailed probability of less than 5% (i.e., $P < 0.05$) was considered statistically significant.

RESULTS

One-way ANOVA for repeated measures of the plasma T3 and T4 concentrations in all months showed a significant difference between groups ($P < 0.001$); all other specific comparisons were derived from post-hoc tests ($P < 0.05$). Plasma thyroidal levels of both hormones were very low in winter (December-February T3 = 0.08-0.82 ng/ml; T4 = 0.09-0.99 ng/ml), but they increased rapidly in March-April to reach maximal values in May (T3 = 5.20 ± 0.01 ng/ml; T4 = 6.70 ± 0.05 ng/ml). They remained elevated in June (T3 = 4.21 ± 0.03 ng/ml; T4 = 5.53 ± 0.05 ng/ml) and July (T3 = 3.69 ± 0.01 ng/ml; T4 = 4.28 ± 0.03 ng/ml), and, thereafter, slowly decreased (Fig. 1A). The thyroid contents of T3 and T4 fluctuated significantly ($P < 0.001$) during the year. The T3 content remained low from November (1.08 ± 0.03 ng/thyroid) to January (1.01 ± 0.01 ng/thyroid), with higher values in June (2.06 ± 0.03 ng/thyroid) and in July (2.56 ± 0.05 ng/thyroid). The T4 content followed very similar seasonal patterns to that of T3, with a minimum in January (2.01 ± 0.05 ng/thyroid) and a maximum in summer (July = 3.64 ± 0.05 ng/thyroid, Fig. 1B). On the other hand, the thyroid content of T4 is markedly greater than the thyroid T3 content, supporting the well-known general thyroid preference for thyroxine production.

One-way ANOVA for repeated measures of the hepatic T3 and T4 contents and 5'-ORD II monodeiodinase activity in all months showed a significant difference between groups ($P < 0.001$); all other specific comparisons were derived from post-hoc tests ($P < 0.05$). Hepatic contents of T4 were low in December (1.48 ± 0.01 ng/mg) and January (1.03 ± 0.03 ng/mg), and started to increase in spring (March = 2.18 ± 0.05 ng/mg), reaching a maximum in July (3.33 ± 0.04 ng/mg, Fig. 1C). Low hepatic contents of T3 were found in December (1.65 ± 0.02 ng/mg) and January (1.82 ± 0.05 ng/mg). In February-March, these values increased, reaching a maximum in July (3.85 ± 0.07 ng/mg, Fig. 1C). The 5'-ORD II monodeiodinase activity of the liver was low in January-February (0.71-0.64 pM T3/g/h), and began to increase in March (2.20 ± 0.05 pM T3/g/h), reaching peak values in July (2.98 ± 0.01 pM T3/g/h). Thereafter, its level decreased to 1.81 ± 0.03 pM T3/g/h in October, went down to 1.48 ± 0.02 pM T3/g/h in November and reached a low level again in December (Fig. 1D).

One-way ANOVA for repeated measures of the plasma TSH concentrations in all months showed a significant difference between groups ($P < 0.001$); all other specific comparisons were derived from post-hoc tests ($P < 0.05$). As shown in Figure 1E, plasma concentrations of TSH were low in December (0.91 ± 0.05 µIU/ml) and January (1.13 ± 0.04 µIU/ml), and increased in March (3.12 ± 0.01 µIU/ml) and April (3.18 ± 0.02 µIU/ml). They remained elevated in May (3.25 ± 0.08 µIU/ml), showing a peak in July (5.57 ± 0.05 µIU/ml); then, they slowly decreased in November (1.31 ± 0.03 µIU/ml) and December (0.91 ± 0.05 µIU/ml).

DISCUSSION

The present study describes the annual profiles in plasma levels and thyroid content of both T3 and T4 in the male *P. sicula*. Plasma concentrations of T4 and T3 exhibited profound variations in the period studied. In fact, they increased rapidly at the beginning of spring, reaching peak levels in May. Thereafter, these levels gradually decreased, reaching the lowest values in December. In addition, both thyroid T3 and T4 contents are seen to be high in early spring and summer, while low contents were observed in November-January. This annual variation in the plasma levels and in the thyroid content of these hormones is paralleled by variations in the thyroid gland morphology, as demonstrated by histological observations (Cavagnuolo *et al.*, 1982). In fact, in the specimens captured in the field we observed an annual cycle of the thyroid gland morphology with a reduction of activity in November, a complete stop in December-January (low follicular epithelium and compact colloid and devoid of reabsorption vacuoles), a good secretory activity in March-April, and maximal activity in May-June (very high follicular epithelium and retracted colloid, with clear signs of reabsorption). Therefore, annual variation in the plasma concentration of thyroid hormones in *P. sicula* might be the result of a seasonal variation in the ability of the thyroid gland to release hormones. In agreement with previous studies on other species of vertebrates, our results suggest that, in *P. sicula*, the primary hormone produced and secreted by the thyroid gland is T4, while T3 is produced principally by extrathyroidal monodeiodination, responsible for the 5'-T4 ORD II monodeiodinase system. In fact, the plasma T3/T4 ratio is much higher than the thyroid T3/T4 ratio, which indicates that most of the plasma T3 comes from peripheral monodeiodination, as suggested for other vertebrates, even if a portion of the T3 present in the blood is secreted by the thyroid gland. Moreover, our results show a correlation between plasma T3 concentrations and 5'-T4 ORD II monodeiodinase activity, proposing the liver as a major T3 donor to plasma. Therefore, as suggested for other vertebrates, a system of deiodinases might autoregulate T3 levels.

There is less information on the phylogenetic distribution of monodeiodinases (MD). Their activity has been characterised in a few mammals, birds, frog, and fish, but it remains unexplored in reptiles. There are only two reports of MD activity in reptiles, a snake (Wong *et*

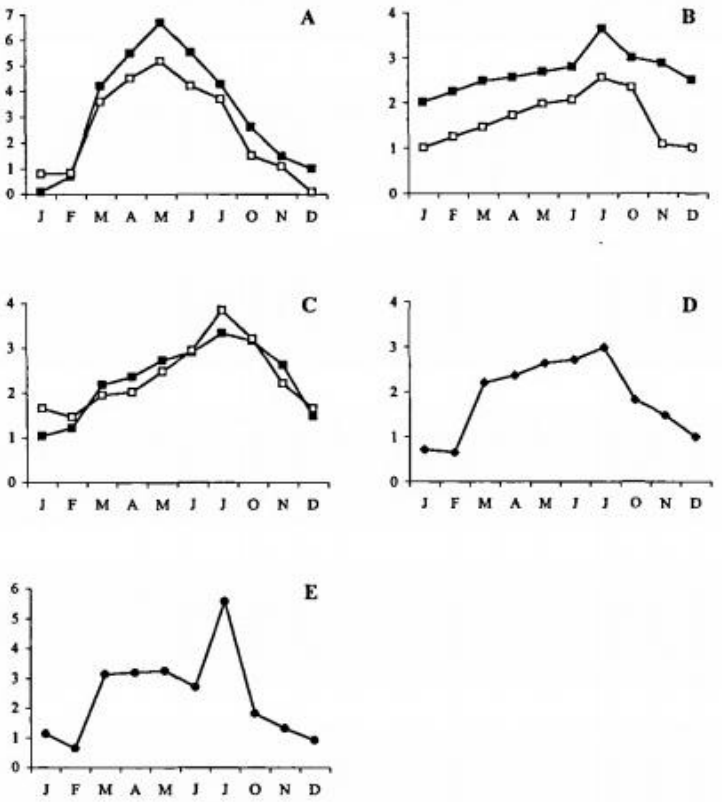

Fig. 1 - Annual variations of thyroid activity in *Podarcis sicula*. **A**, plasma levels (ng/ml), **B**, thyroid contents (ng/thyroid) and **C**, hepatic contents (ng/mg) of T4 (■) and T3 (□). **D**, 5'-T4 ORD II monodeiodinase activity (pM T3/g/h) of the liver. **E**, plasma TSH concentrations (μIU/ml).

al., 1993) and a lizard (Kar & Chandola-Saklani, 1985a, b); both reported the presence of a MD type I (MDI)-like activity, but neither fully characterised the enzyme nor explored the possibility of other forms. Recent study (Hungerberger & Licht, 1999) in the turtle (*Trachemys scripta*) has altered the traditional view of the typical reptilian thyroid system and suggests that turtles possess a mammalian-like type I activity and have colocalised MD forms in the liver. However, the second turtle MD form (MDH) is not comparable to the mammalian or avian MD type II (MDII)-like activity. Besides, turtle MDI produces T3 from T4, while MDH produces rT3 from T4 as does the mammalian type III forms, but MDH has a wider tissue distribution (kidney, liver, pancreas, heart, ovary, and brain) and distinct enzyme kinetics. Moreover, MDH activity in the turtle kidney is 100-fold higher than in the liver, indicating that the kidney may play a critical role in the metabolism of thyroid hormones in the turtle; this high renal activity distinguished the turtle from all other vertebrates studied.

On the basis of the data available it is reasonable to conclude that the *P. sicula*, T4 ORD has a high affinity for T4 (K_n = 1 nM), and a high cofactor requirement (50mM DTT); therefore it resembles more closely type II. Besides, this low K_n, combined with the increased cofactor dependence suggests that the high-affinity enzyme may be more similar in function to the mammalian MDII. Properties of other various reptile deiodinases are incompletely understood, and their homology with mammalian types I, II, and III deiodinase isozymes is still unresolved. The T4 ORD II characteristics for the lizard resemble those described previously for talapia *Oreochromis niloticus* liver (Mol *et al.*, 1993; Darras *et al.*, 1994). A low K_n T4 ORD has also been reported for the liver of salmonids (MacLatchy & Eales, 1992; Sweeting & Eales, 1992 a, b; Morin *et al.*, 1993;) and for the liver of red drum *Sciaenops ocellatus* (Van Putte C. L. M. *et al.*, 1994, *Abstract* in Am. Zool., 34: 26A) and it is likely the primary deiodinase isozyme present in several teleost tissues.

It is noteworthy that plasma T4 concentrations increased with increased levels of plasma TSH, except in July, when a greater TSH release (5.57 μIU/ml) was not paralleled by high plasma T4 concentrations. Therefore, it can be concluded that the thyroid becomes virtually refractory to stimulation with TSH. In our experiments, refractoriness of the thyroid gland in July might be the result of a prolonged exposure of this gland to TSH. In mammals (Verhoeven *et al.*, 1980; Jahnsen *et al.*, 1982) and amphibians (Kuhn *et al.*, 1985), several cases have been described in which endocrine glands evolve in a state of refractoriness or desensitization following previous contacts with physiological or high concentrations of their homologous hormones. These observations, therefore, support the hypothesis that, in *P. sicula*, thyroid desensitization might have occurred in July, when a high concentration of plasma TSH is unable to induce a greater T4 release.

Our results can be explained on the ground of the well-known hypothalamic regulation of the pituitary-thyroid, which has been described for several vertebrate classes. In mammals, it is well established that certain hypothalamic regions are involved in the control of the thyroid function through the regulation of the pituitary secretion of TSH (Aizwa & Greer, 1981; Taylor *et al.*, 1990). This control would mainly occur in the preoptic paraventricular nucleus by means of neurons which secrete the thyrotropin-releasing factor (TRF) to stimulate TSH secretion of both T4 and its biologically active metabolite T3. In fishes, particularly in teleosts, there is evidence that pituitary hormones other than TSH may play a role in the regulation of T4 secretion and its con version to T3. In fact, in a review, Eales & Brown (1993) proposed that there is a fundamental distinction between teleosts and mammals in the functional control of thyroid activity. They argued that the demand of target tissues for T3 is the main determinant of the level of thyroid T4 secretion in teleosts. According to this 'peripheral control' hypothesis, the hypothalamus-pituitary system serves primarily to optimize thyroid output to meet the prevailing T3 requirements of target tissues. In contrast to this hypothesis, the 'central control' model suggests that, in mammals, it might have an inductive role, serving as a master control to influence the functioning of various peripheral tissues through the increased production of T4 and thus T3. On the basis of

these data and of our results, it can be concluded that in *P. sicula* there is a 'central system' determining plasma T4 levels, and a 'peripheral system' regulating T3 availability through T3 production.

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