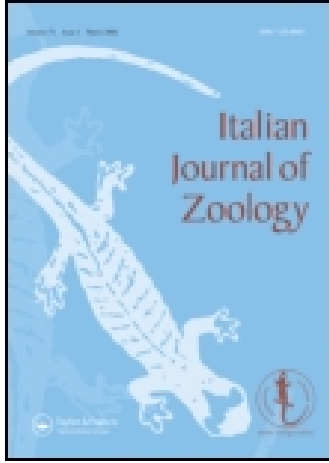


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Morphological and biochemical changes in the thyroid gland of the lizard *Podarcis sicula* (Reptilia, Squamata) induced by VIP treatment

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Morphological and biochemical changes in the thyroid gland of the lizard *Podarcis sicula* (Reptilia, Squamata) induced by VIP treatment

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ABSTRACT

The effects of exogenous vasoactive intestinal polypeptide (VIP) involvement in the regulation of thyroid function were detected in the lizard *Podarcis sicula*. Variations in thyroid gland morphology, thyroid-stimulating hormone (TSH), 3,5,3'-triiodo-L-thyronine (T3) and L-thyroxine (T4) plasma levels, were studied in this species after treatment with VIP, during winter and summer. The polypeptide had a stimulatory effect on thyroid hormone secretion throughout the year. Plasma T4 and T3 levels significantly increased; maximal values were reached in specimens exposed longer to treatment. No difference in plasma TSH levels was observed between control and treated specimens, indicating that, in *P. sicula*, the site of VIP action might be better identified at thyroidal rather than hypophyseal level. Consistently with these results, the morphology of the thyroid gland also appeared stimulated after treatment with VIP, showing an increase in thyroid-cell height, numerous intracellular colloid droplets PAS-positive, and enlarged blood vessels around the follicles.

KEY WORDS: VIP - Thyroid gland - *Podarcis sicula* - Reptiles.

INTRODUCTION

The vasoactive intestinal polypeptide (VIP) is an octacosapeptide with a highly conserved amino acid sequence among vertebrates (Mutt & Said, 1974; Nilsson, 1975). First identified in the gastrointestinal tract of mammals, where it is widely distributed (Said & Mutt, 1970, 1972), it has also been found in the pancreas (Sundler *et al.*, 1978), the genitourinary tract (Larsson *et al.*, 1977) and the nervous system (Larsson *et al.*, 1976). In the thyroid gland, as in many other organs, parasympathetic nerve fibres contain VIP (Ahrén *et al.*, 1980; Grunditz *et al.*, 1988). Immunohistochemical and immunochemical studies have demonstrated that, in some vertebrate species, the thyroid gland shows VIP-containing nerve fibres surrounding the blood vessels and running between and along the follicles. In rat, VIP participates in the regulation of thyroid-hormone release through a cAMP-mediated mechanism (Ahrén *et al.*, 1980). Based on these results, it has been demonstrated that exogenous VIP in rat, besides increasing tissue cAMP levels, increases the number of colloid droplets in follicle cells and, *in vivo*, thyroid-hormone release. Thus, VIP-containing nerve fibres might control thyroid activity through a still undemonstrated functional innervation, which is neither cholinergic nor adrenergic (Green *et al.*, 1982). Other investigators have hypothesized the existence of VIPergic nerve fibres which, together with sympathetic adrenergic and parasympathetic cholinergic nerve fibres, might participate in the autonomous nervous control of thyroid function through a dose-dependent activation of human thyroid follicle cells (Toccafondi *et al.*, 1984). Studies conducted on the plasma membranes of the bovine thyroid gland (Calvo *et al.*, 1990) have elucidated the mechanism responsible for VIP binding to its receptors, showing that it is regulated by guanine nucleotides. Moreover, the role of endogenous VIP in the regulation of thyroid-hormone release and thyroidal blood flow has been studied in normal rats. The results have demonstrated that VIP is not directly involved in the regulation of thyroid-hormone release, whereas, when administered exogenously, it increases thyroidal blood flow, having a tonic vasodilator effect. Therefore, endogenous VIP might be involved in the basal maintenance of thyroidal microcirculation (Michalckiewicz *et al.*, 1994).

The relationships between VIP and the thyroid gland are well documented in homeotherms, whereas little is known about lower vertebrates, particularly reptiles. However in these vertebrates and, of course, in the lizard *Podarcis sicula*, the thyroid gland undergoes a marked annual cycle (Cavagnuolo *et al.*, 1982), characterized by a functional stasis, starting in autumn to become full in December-January. In this period, the follicular epithelium is low, while the colloid is compact and devoid of reabsorption vacuoles. In spring there is an activity resumption that reaches its maximum in May-June; the follicular epithelium is very high and col-

loid is retracted with clear signs of reabsorption. The thyroid gland activity decreases again afterwards. These annual variations in thyroid gland morphology are paralleled by variations in plasma levels of thyroid hormones. In fact, plasma concentrations of 3,5,3'-triiodo-L-thyronine (T3) and L-thyroxine (T4) increased rapidly at the beginning of spring, reaching peak levels in June; thereafter, they gradually decreased, reaching the lowest values in December (Sciarrillo *et al.*, 2000).

Therefore, to have information on the cytophysiological response of *P. sicula* thyroid gland to VIP, the seasonal cycles and the related functional state of the thyroid of these animals throughout the year must necessarily be taken into account. This paper reports the effects of VIP on thyroid gland morphology and physiology of *P. sicula* in winter and in summer.

MATERIALS AND METHODS

Animals and experimental design

Adult male lizards of *P. sicula* were live-captured in the neighbourhood of Naples during winter and summer and housed in environmentally enriched pens. The animals were fed on fly larvae daily, and fresh water was available *ad libitum*.

The specimens were divided into five groups, of 20 animals each:

Group A: the animals were treated with a single intraperitoneal (ip) injection of 5 µg VIP (Sigma Chemical Co., St. Louis, MO) and sacrificed 2 h later;

Group B: the animals were subjected to the same treatment as group A but sacrificed 24 h after injection;

Group C: the animals were treated with a single ip injection of 5 µg VIP/day for three consecutive days and sacrificed 2 h after the last injection;

Group D: the animals were subjected to the same treatment as group C but sacrificed 24 h after the last injection.

A control group for each VIP treatment group was kept under the same conditions as the treated groups, but it was intraperitoneally injected with physiological solution (0.75% NaCl).

Blood samples were collected by intracardiac puncture and put into heparinized capillaries. They were then centrifuged at 1,500 rpm for 10 min to obtain serum.

Tissue preparation

Immediately after collection of blood samples, the animals were decapitated, and the thyroid glands were removed and fixed in Bouin's fixative for 24 h. The tissues were then washed in 75% ethanol overnight, dehydrated through graded ethanols and embedded in Paraplast. Serial cross sections were cut at 7 µm, separated into parallel series, mounted on slides, and stained by Galgano I stain and the periodic acid Schiff (PAS) technique. This technique stains colloid in the follicle lumen as well as colloid droplets in the follicle cells.

The height of the follicular cells was measured in 30 cells every three slides, always on the second section of normal and treated specimens using a micrometric eye-piece (Oknor).

Plasma hormone analysis

Plasma levels of T3 and T4 were determined by radioimmunoassay (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 labelled T3 [¹²⁵I] 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) to release T3 from serum binding proteins. Sensitivity was 0.1 ng/ml with an accuracy of about 97%. The range of intraassay variance in 20 assays was 1.0-2.6%, while the interassay variance ranged between 3.9-5.7% 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 labelled T4 [¹²⁵I] T4, 165kBq (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) to release T4 from serum binding proteins. Sensitivity was 0.45 ng/ml with an accuracy close to 100%; the mean intra- and interassay coefficients of variation were 4.6% and 4.3%, respectively. A logit-log curve fit using a % B/Bo calculation was used. T4 and T3 concentrations were determined by computing the % B/Bo for each sample and then finding the results on the standard curve. 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 thyroid-stimulating hormone (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-specific antibody and ¹²⁵I labelled (10 mCi) 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 (MDD) was 0.01 mIU/ml, with an accuracy close to 100%, and mean intra- and interassay coefficients of variation were 5.0% and 7.5%, respectively.

Statistical Analysis

All data are presented as means ± SE. Statistical analyses were performed by one-way analysis of variance (ANOVA) with repeated measures followed by Duncan's multiple range test for pairwise comparisons. Differences were considered significant if *P* < 0.05.

RESULTS

Winter treatment

Podarcis sicula thyroid gland has low functional activity in winter

During winter, *P. sicula* thyroid gland shows poor functional activity. The follicular epithelium is medium-low (5.91 ± 0.01 µm; Fig. 1A), the thyrocyte nuclei are small, elongated with dense chromatin and a greatly reduced cytoplasm. The colloid shows rare reabsorption vacuoles, without formation of colloid droplets PAS-positive.

In this season, *P. sicula* shows a reduced metabolism, demonstrated by the low TSH blood levels and consequently of thyroid hormones; T3 level is 0.81 ± 0.03 ng/ml and T4 level is 0.99 ± 0.01 ng/ml (Fig. 1B), plasma TSH level is 1.32 ± 0.01 µIU/ml (Fig. 1C).

VIP treatment affects thyroid gland morphology (Fig. 1A)

We analysed four experimental groups, depending on amount of VIP administered and on duration of treatment. In the animals of group A, we observed stimulation of thyroidal activity. In fact, the follicular epithelium was thicker than that of the control specimens (12.5 ± 0.05 µm), nuclei of the thyroid cells were roundish, and cytoplasm was abundant; the colloid showed very

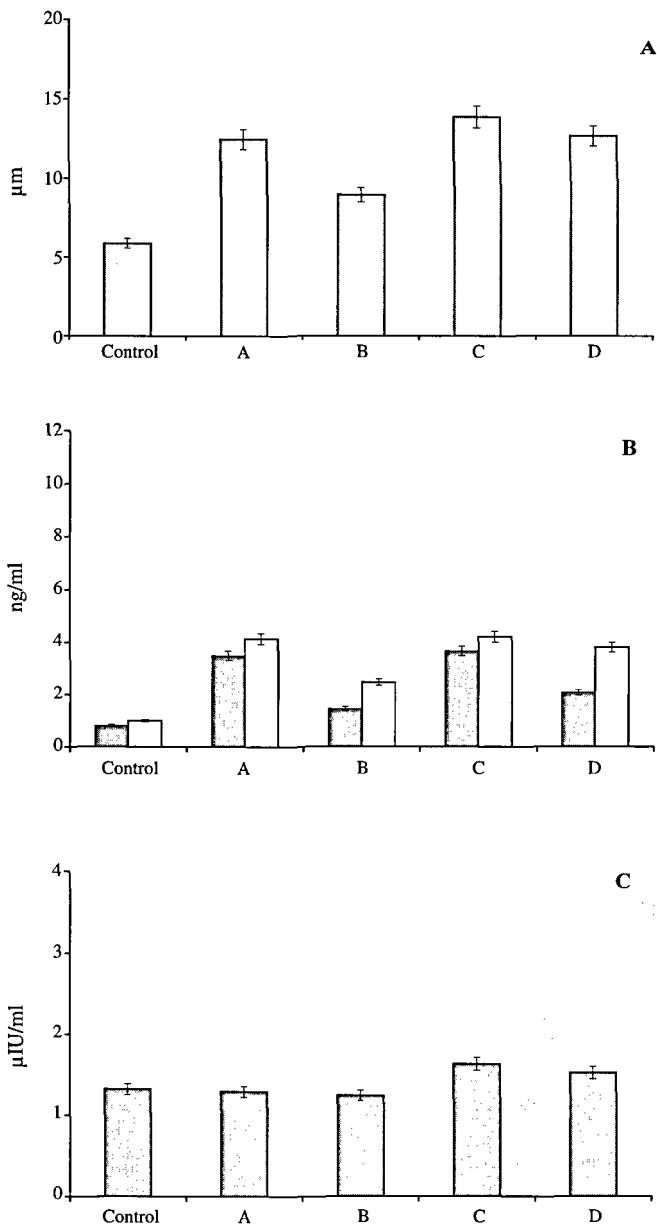


Fig. 1 - Effects of VIP treatment in *Podarcis sicula* in winter (mean \pm SE). **A**, variations in the height of thyroid cells. **B**, variations in T3 (filled bars) and T4 (open bars) plasma levels. **C**, variations in TSH plasma levels.

wide reabsorption phenomena, associated with formation of some intracellular colloid droplets PAS-positive. In group B, the thyroid tended to re-acquire the typical morphological characteristics of the control specimens. In fact, the follicular epithelium appeared medium-high ($8.96 \pm 0.02 \mu\text{m}$) and rare colloid droplets PAS-positive were present. In group C there was an intense stimulation of the follicular epithelium ($13.9 \pm 0.05 \mu\text{m}$), the colloid still revealed wide reabsorption phenomena, associated with the formation of numerous colloid droplets PAS-positive, and enlarged blood vessels were present around the follicles. In the animals of group D the follicular cells were quite high ($12.7 \pm 0.05 \mu\text{m}$),

there were some colloid droplets PAS-positive, and the colloid was a little less vacuolized than the previous group.

VIP treatment has an early effect on T3 and T4 plasma levels increase

Plasma T3 and T4 level varied during VIP treatment (Fig. 1B). Plasma T3 level was increased to $3.49 \pm 0.01 \text{ ng/ml}$ in group A and reached its maximum value ($3.66 \pm 0.05 \text{ ng/ml}$) in group C. Plasma T4 was $4.13 \pm 0.03 \text{ ng/ml}$ in group A; the maximal value was reached in group C ($4.20 \pm 0.05 \text{ ng/ml}$). On the contrary, plasma TSH level did not display great variations in any of the treated specimens, compared to the controls (Fig. 1C).

Summer treatment

Podarcis sicula thyroid gland has high activity in summer

In summer, the thyroid gland of *P. sicula* control specimens shows good functional activity (Fig. 2A). In fact, the thyrocyte nuclei are roundish and in the middle of the cells, the cytoplasm is abundant and the colloid is compact and with marked signs of reabsorption, there are scarce intracellular colloid droplets formations PAS-positive (Fig. 3A), and the follicular epithelium (Fig. 4A) is medium-high ($8.98 \pm 0.02 \mu\text{m}$).

During summer, the metabolism of this lizard is considerably increased in comparison with winter, so much so that T3 and T4 levels were $3.92 \pm 0.02 \text{ ng/ml}$ and $4.25 \pm 0.01 \text{ ng/ml}$ (Fig. 4B), TSH plasmatic levels reached $3.42 \pm 0.02 \mu\text{IU/ml}$ (Fig. 4C).

VIP administration highly stimulates thyroid gland

In group A, there was intense stimulation of thyroid functional activity, which was revealed by the increase in height of the follicular epithelium (Fig. 4A; $14.1 \pm 0.01 \mu\text{m}$). In group B, there was also an increase in height of the follicular epithelium (Fig. 4A; $10.1 \pm 0.01 \mu\text{m}$) compared to controls, the colloid was abundant and compact, and intracellular colloid droplets formation PAS-positive was evident. In group C specimens (Fig. 2B), the follicles were irregular in shape and an increase of blood vessels lumen diameter was evident. The follicular epithelium was very high (Fig. 4A; $16.1 \pm 0.03 \mu\text{m}$), the colloid showed clear signs of reabsorption, documented by numerous colloid droplets formation PAS-positive (Fig. 3B, C). Finally, group D animals showed thyrocytes slightly lower than those of group C (Fig. 4A; $12 \pm 0.04 \mu\text{m}$), and some intracellular colloid droplets PAS-positive.

VIP enhances T3 and T4 plasma level (Fig. 4B)

Plasma T3 increased in the specimens sacrificed 2 h after injection (group A: $4.88 \pm 0.04 \text{ ng/ml}$), reaching the maximal value in group C ($8.28 \pm 0.01 \text{ ng/ml}$). Plasma T4 increased in group A ($5.52 \pm 0.01 \text{ ng/ml}$), and in group C specimens there was a marked increase (9.28

± 0.03 ng/ml). On the contrary, as already observed in winter, TSH level did not show significant variation (Fig. 4C).

DISCUSSION

The presented results show that, at the doses administered, VIP has clear stimulatory effects on thyroid gland activity in *P. sicula*. In fact, morphologically, all the treated animals showed intense stimulation, accompanied by a strong increase in thyroid-hormone release and by enlarged blood vessels, both in winter and in summer.

In this species, a direct influence of VIP on the hypophysis can be ruled out, since TSH plasma levels did not vary significantly. Conversely, the abrupt increase in thyroid-hormone plasma levels suggests a direct action of VIP on the thyroid gland. In our previous studies, it was demonstrated that thyroid gland stimulation in *P. sicula* may be independent of interference at pituitary level (Laforgia *et al.*, 1996; Sciarrillo *et al.*, 1999). These results are in agreement with the hypothesis described in a recent paper (Sciarrillo *et al.*, 2000) about regulation of the pituitary-thyroid axis. In fact, in that paper,

the Authors, as did Eales & Brown (1993), proposed that there is a fundamental distinction between non-mammals and mammals in the functional control of thyroid activity. Therefore, it can be concluded that in *P. sicula* there are two controls: 1) the 'peripheral system', where the hypothalamus-pituitary system serves primarily to optimize thyroid output to meet the prevailing T3 requirements of target tissues; 2) the 'central system', that 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.

In the present paper, in both periods of the year, response to treatment was immediate in the treated specimens receiving only one VIP injection and sacrificed 2 h after injection, whereas, in the specimens sacrificed after 24 h, there was a tendency for the initial value to be restored. Furthermore, a major stimulatory effect was observed in the specimens that received a greater total amount of VIP. The present findings indicate that in *P. sicula* lizards the functional role of VIP in thyroid gland regulation is determined by the ability of this neuropeptide to induce colloid droplet formation PAS-positive and to increase T3 and T4 release. These results suggest that VIP plays a role in thyroid hormone secretion. Ear-



Fig. 2 - Morphological changes in control and VIP-treated *Podarcis sicula* specimens in summer (scale bar, 20 μ m). **A**, thyroïdal section of a normal specimen; note the medium-high follicular epithelium. **B**, thyroïdal section of a specimen treated with three VIP injections and sacrificed after 2 h from the last injection; note the very high follicular epithelium with respect to the controls and the numerous re-absorption vacuoles in the colloid; enlarged blood vessels (arrows) around the follicles are also present.

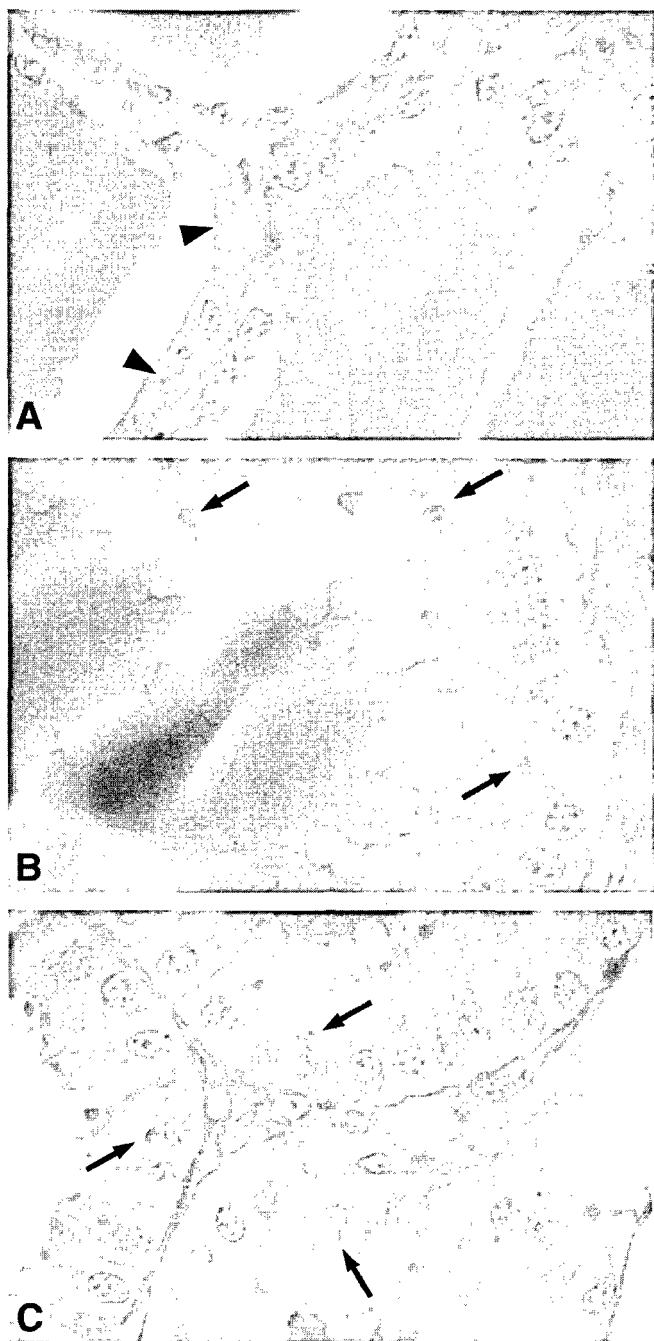


Fig. 3 - Increase of PAS-positive colloid droplets in the thyroid of VIP-treated *Podarcis sicula* specimens in summer ($\times 480$). **A**, thyroidal section of control specimen; note the scarce presence of PAS-positive colloid droplets (head arrows). **B**, **C**, thyroidal sections of a specimen treated with VIP; note the numerous intracellular PAS-positive colloid droplets (arrows).

lier studies have revealed that both adrenergic and cholinergic nerves are involved in the regulation of thyroid activity (Melander & Sundler, 1979).

The interaction between the various nervous mechanisms in the control of thyroid function remains to be elucidated. A third type of apparently autonomic neuronal control was recently demonstrated in thyroid tis-

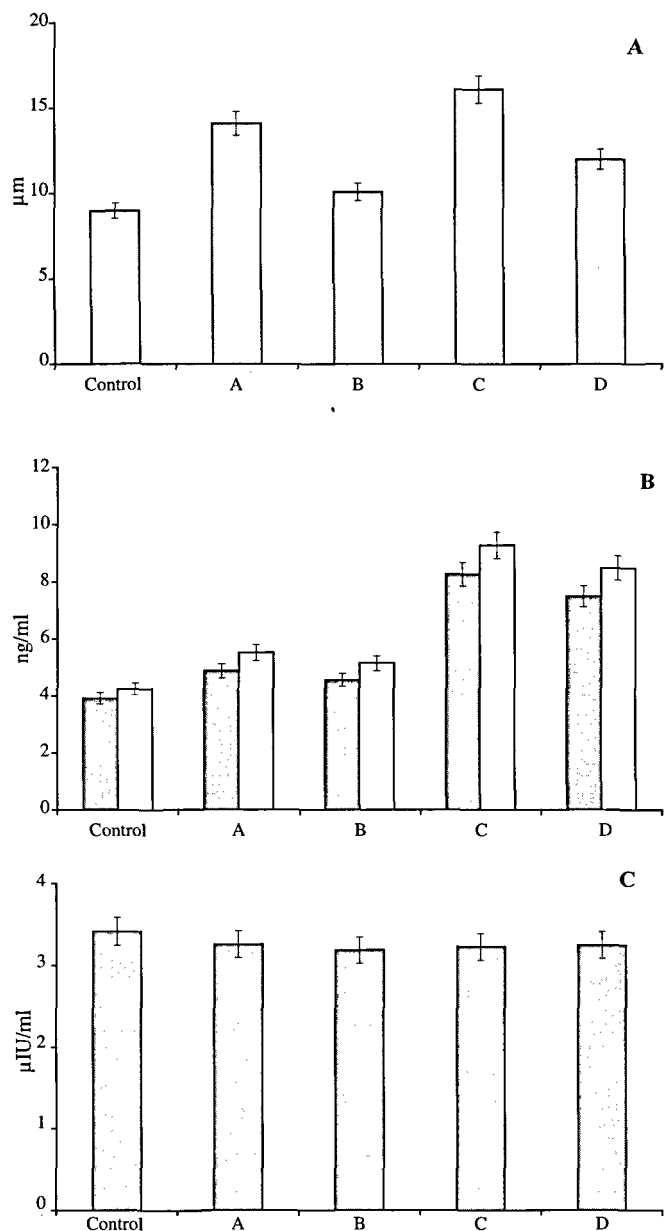


Fig 4 - Effects of VIP treatment in *Podarcis sicula* in summer (mean \pm SE). **A**, variations in the height of thyroid cells. **B**, variations in T3 (filled bars) and T4 (open bars) plasma levels. **C**, variations in TSH plasma levels.

sue; in several species, neurones containing VIP were found to reach thyroid cells, and exogenous VIP was found to induce secretion of thyroid hormone (Ahrén *et al.*, 1980). In the lizard *P. sicula* thyroid, the presence of VIP-terminals was demonstrated by Virgilio *et al.*, (1999, *Abstract* in E.J.B.A.H., 43: 63). VIP nerve fibres were thin, beaded, surrounding blood vessels, and running between and along thyroid follicles.

Our results support the idea that in addition to sympathetic-adrenergic and parasympathetic-cholinergic nerves, VIP-ergic nerves may participate in the autonomic nervous control of thyroid function in lizard.

To conclude, in *P. sicula* lizards experimental treatment with VIP has effects similar to those reported in higher vertebrates. In fact, the observed stimulatory effect agrees with the results reported in literature for mammals, where VIP causes an increase in iodine uptake (Pietrzyk *et al.*, 1992) and colloid droplets (Ahrén *et al.*, 1980, 1982). VIP causes also an increase of thyroidal blood flow (Michalkiewicz *et al.*, 1989, 1994), as noted from the augmentation of blood vessels lumen surrounding the gland, favouring both synthesis and secretion of thyroid hormones (Ahrén, 1991).

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