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Inhibin in the testis and adrenal gland of the male lacertid, *Podarcis sicula* Raf.: a light immunocytochemical study

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SUMMARY

Inhibin is a glycoproteic hormone mostly produced by the gonads. Through a feedback at the pituitary level, it selectively inhibits the release of follicle-stimulating hormone. In mammals, inhibin has been found also in some extragonadal tissues such as placenta, pituitary, adrenal, spleen, kidney, brain and spinal cord. At present, no information is available about the existence of inhibin in reptiles. The aim of the present work is to localise, through immunocytochemical methods, the sites of inhibin production in male lizards during the main phases of the reproductive cycle: the *culmination phase* (April-June), the *early regressive phase* (early July), the *maximal regressive phase* (August) and the *winter stasis* (January). In the testis, immunostaining is mainly localised in the Leydig cells during the *early regressive phase*, while it is observed in the Sertoli cells during the *maximal regressive phase*. In the epididymis, the immunostaining is present only during the reproductive period at the level of secreting cells and inside its ducts. In the adrenal gland, after immunostaining, both chromaffin and steroidogenetic tissues are inhibin-positive during the whole spermatogenetic cycle, though with variable intensity throughout the year: cross-reaction appears more evident from January to April (*winter stasis* and *culmination phase*) and weaker in June. However, in captive animals, the reaction persists in chromaffin cells, but disappears in steroidogenetic cells.

The functional meaning of the presence of inhibin as a factor in the local regulation of spermatogenesis is discussed.

INTRODUCTION

Inhibin, a water-soluble non-steroidal glycoprotein hormone inhibiting FSH secretion, is produced by the gonads and several extra-gonadal tissues (Meunier *et al*., 1988; Mather *et al*., 1997). It is composed of two partially homologous subunits, α and β, linked through disulphide bridges. Two forms of the β-subunit have been isolated, β_A and β_B. Inhibin dimers exist as inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$); both forms consist of identical α subunits and different β subunits (Mason *et al*., 1985; Ling *et al*., 1985; Forage *et al*., 1986). In addition, dimerisation of the β-subunit results in the formation of activin, which has been shown to stimulate FSH synthesis and secretion (Vale *et al*.,

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1986; Ling *et al*., 1986a). The mechanism of actionof inhibin has been studied extensively in mammals, where three target organs have been identified: pituitary, hypothalamus and gonads. Inhibin might act also at the local level in different ways, from FSH-specific receptor block to the inhibition of other enzymatic mechanisms (Baird and Smith, 1993). In the female, inhibin produced by the granulosa cells inhibits locally aromatase activity (Norris, 1996). In the male, inhibin interferes with differentiating spermatogonia mitoses, modulating the number of germ cells available for meiosis; moreover, it acts on the Leydig cells, limiting androgen release (van Dissel-Emiliani *et al*., 1989). Inhibin receptors have been found on germ and somatic cells (Leydig and Sertoli) (Woodruff *et al*., 1992) and therefore a paracrine action in the regulation of reproduction is hypothized (Moore *et al*., 1994). Locally, inhibin should link to the R II-activin subunit receptor, preventing its action (Leburn and Vale, 1997). In secreting epididymal cells and in the lumen of mammals, by immunocytochemical methods, intense inhibin reactivity was noticed during the spermiation and mating periods (Hurkadly *et al*., 1991).

In male mammals, the testis is the main source of inhibin, Sertoli cells and, to a lesser extent, Leydig cells being involved (Jègou, 1993). However, inhibin and its mRNAs hare also been detected in such extragonadal sites as adrenal gland, brain, pituitary, kidney and placenta, though the meaning of their presence has not been completely elucidated (Meunier *et al*., 1988; Sawchenko *et al*., 1988; Roberts *et al*., 1989; Merchenthaler *et al*., 1987; Jaffe *et al*., 1993; Voutilainen, 1995; Schwall, 1999).

As in mammals (Skinner, 1991), different substances produced at the gonadal level, display, in reptiles, a paracrine action on the modulation of spermatogenesis and other structures correlated to reproduction; among such substances, inhibin might be of particular importance.

Data on the presence and the meaning of inhibin in the lizard *Podarcis sicula* are lacking. Therefore, the aim of the present work was to localize the sites of inhibin production in the testis and associated tissues (epididymis and adrenal gland) of this species during the different phases of the reproductive cycle: A) *culmination phase*; B) *early regressive phase*; C) *maximal regressive phase*; D) *winter stasis*.

The lizard, *Podarcis sicula,* living in southern Italy, shows an annual spermatogenetic cycle with two periods of activity (spring-summer and autumn); only the former (*culmination phase*) is useful for reproduction, while the latter is considered abortive. In early July, after reproduction, there is a block of spermatogonial mitoses in the testis (*early regressive phase*), whereas the other germ cells continue developing up to the stage of sperm. At the height of summer (mid-August), the spermatogenetic cycle is over, and the testis appears completely regressed (*maximal regressive phase*); only Sertoli cells and spermatogonia being present in its seminiferous tubules. In the fall, a slight spermatogenetical recrudescence is present but it is not useful for reproductive purposes. During the winter months, when the animals are in a state of semihibernation, spermatogenesis is blocked (*winter stasis*), but it is ready to start again as soon as the environmental temperature becomes favourable. Leydig cells also show an annual cycle: they are active during the reproductive period, and progressively inactive during the *early regressive phase*, when they are very large but- completely vacuolised and full of lipid cholesterol-positive droplets (Della Corte *et al*, 1969; Varano *et al*., 1973). During *maximal regressive phase* the Leydig cells appear completely regressed and indistinguishable from fibroblasts. The adrenal gland of *Podarcis* is juxtaposed to the epididymal head and is formed by a parenchyma of steroidogenetic cells and chromaffin tissue (Varano *et al*., 1969a,b; Varano and Laforgia, 1976; Laforgia and Varano, 1978; Varano and Laforgia, 1991; Laforgia and Muoio, 1997). Chromaffin tissue does not show evident changes at the light microscope during the annual reproductive cycle. Steroid-producing cell activity, instead, is low in winter (January-February), and highest in the spring-summer-autumn. Experimental treatments stimulating spermatogenesis and androgen-dependent structures (i.e. epididymis) during the resting period (Angelini e Botte, 1992), stimulate also the adrenal gland (D'Uva *et al*., 1985). Moreover, Leydig cell activity in androgen synthesis is strictly related to the plasma corticoid levels secreted by the adrenal gland, particularly under stress (Manzo *et al*., 1994). Therefore, the gonad-epidydimis-adrenal gland complex can be considered as an integrated system in the regulation of

reproductive activity in these vertebrates. The possible presence of inhibin in these three organs can be useful for understanding the regulation of reproductive mechanisms.

MATERIALS AND METHODS

Animals

Five adult males of *Podarcis sicula* were captured in the vicinity of Naples during different phases of the reproductive period: A) *culmination phase*; B) *early regressive phase*; C) *maximal regressive phase*; D) *winter stasis*. After capture, the animals were kept in a terrarium up to 3 days, exposed to natural temperature and photoperiods, and fed *ad libitum*. The specimens were killed (in the periods indicated above) by decapitation after ether anaesthesia. Five animals of the *culmination phase* period were mantained in a terrarium for twenty days in the same conditions of the preceeding groups and then killed as above.

Tissue preparation

Gonads, epididymis with the adrenal gland were immediately removed, placed in a cryoembedding compound and rapidly frozen. Sections of 8 μm in thickness were cut by a Leica Frigocut cryostat, fixed in methanol-acetone (70:30 v:v) and stored a -80°C until use.

Antisera

The polyclonal antibody used was raised in rabbit against the 1-32 N-terminal sequence of the α-chain of human inhibin, obtained from Peninsula Laboratory Belmont, California (U.S.A.). Normal goat serum, biotinylated affinity-purified goat anti-rabbit IgG and ABC staining reagent were obtained by Pearce Rockford, Illinois (U.S.A.).

Immunocytochemistry

For immunocytochemical localisation of inhibin, the indirect immunoperoxidase ABC staining procedure was used (Hsu *et al*., 1981) with some modifications. Endogenous peroxidase activity was destroyed by incubation with methanol containing 0.3% hydrogen peroxide at room temperature for 30 min. Sections were washed with phosphate-buffered saline (PBS) containing 2% normal goat serum, then incubated with primary antiserum (1:500 dilution) at 4°C for 24 h. in a muist chamber. Nuclear contrast was carried out with Harris haematoxylin. PBS instead of Ab-inhibin or Ab-inhibin after preabsorption with synthetic inhibin (Inhibin α-subunit, Sigma Chemical Co., U.S.A.) were used as controls (Polak and Van Nordeen, 1983).

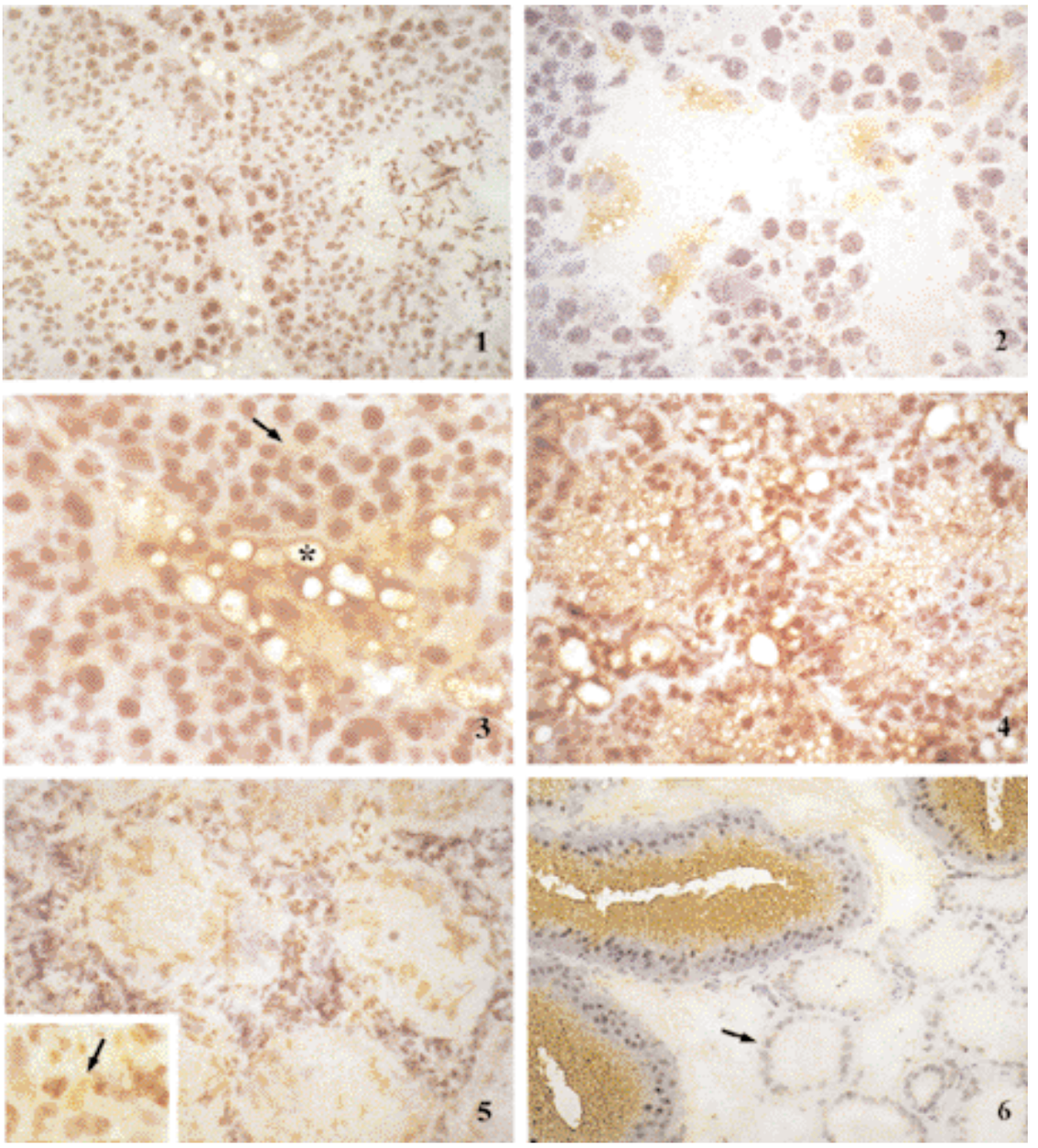
Negative controls were obtained: a) by replacing the primary antisera with non immunised rabbit sera, b) by preabsorbing primary antisera with the corresponding synthetic peptide prior to use.

RESULTS

Testis

During the *culmination phase* of spermatogenetic activity (April-June) no immunopositivity was observed either in Leydig or in Sertoli cells (Fig. 1). The reaction was negative also in twenty days captive animals (data not shown). When, following the slowing down of spermatogonial mitoses, the *early regressive phase* (early July) started, immunopositivity was strong in large Leydig cells, whereas no reaction was observed in the seminiferous tubules (Fig. 2). Afterwards, at the end of the early regressive phase (the end of July), when, after the resting of spermatogonial mitoses, only few stages of the spermatogenetic wave were present in the seminiferous tubules, slight immunopositivity inside the tubules was detected between germinal cells, at the level of Sertoli cell cytoplasm. An evident positivity was also observed in Leydig cells, which appeared completely vacuolised (inactive and predegenerative appearance) (Fig. 3). This situation increased in the first days of August when a strong cross-reactivity in the vacuolised Leydig cells and inside the involuted seminiferous tubules were observed (Fig. 4). During the *maximal regressive phase* (half August), seminiferous tubules were completely regressed and contained only spermatogonia and Sertoli cells; Leydig cells were indistinguishable from connectival fibroblasts. In this condition, the cross-reaction was detected only inside the tubules and in Sertoli cell cytoplasm (Fig. 5). During the *winter stasis*, no testicular immunopositivity was observed (data not shown).

The reaction results always negative in all the specimens obtained after preabsorbing the antisera with the corresponding synthetic peptide.



Figs. 1/6 - 1) Cryostatic section of testis during the culmination phase (x 320). The tubules are in full spermatogenetic activity; note the complete absence of cross-reaction to inhibin. 2) Cryostatic section of testis at the beginning of the *early regressive phase* (x 650). Note the intense inhibin cross-reactivity only at the Leydig cell's level. **3**) Cryostatic section of testis near the end of the *early regressive phase* (x 490). Note the cross-reactivity in the vacuolised Leydig cells (asterisk) and some immunopositivity inside the tubules, between germ cells (arrows). **4**) Cryostatic section of testis during early August (x 320). The tubules are regressed and without lumen. Inhibin cross-reaction is strongly present in degenerating Leydig cells and inside the seminiferous tubules. **5**) Cryostatic section of testis during the *maximal regressive phase* (x 225). Note the immunopositivity only inside the completely regressed tubules at the Sertoli cell's level. Insert: detail of a sector of a tubular wall showing an inhibin cross-reaction (arrow) in the cytoplasm of a Sertoli cell (x 590). **6**) Cryostatic section of the cranial portion of the epididymis during the *culmination phase* (x 200). Note the cross-reactivity only at the level of secreting cells of the channel; the ductules are instead negative (arrow).

Epididymis

The reaction was strongly positive in normal and captive animals during the *culmination phase*, and also in the *early regressive phase* of spermatogenesis (from May to early July) in the secreting cells of epididymal channel and inside its ducts. Conversely, ciliary ducts were always negative. (Fig. 6 and 7).

During the other periods (from the end of August to March), when the epididymis is regressed, the channel cells were negative (Fig. 8).

Adrenal gland

The chromaffin tissue was always positive throughout the year, though with variable intensity. From January to June (*winter stasis* and *culmination phase*), immunolabelling was very evident in all chromaffin cells, no difference was evident between adrenaline and noradrenalin cells at level of optical microscopy (Fig. 9), while in other periods the reaction was slightly weaker (data not shown).

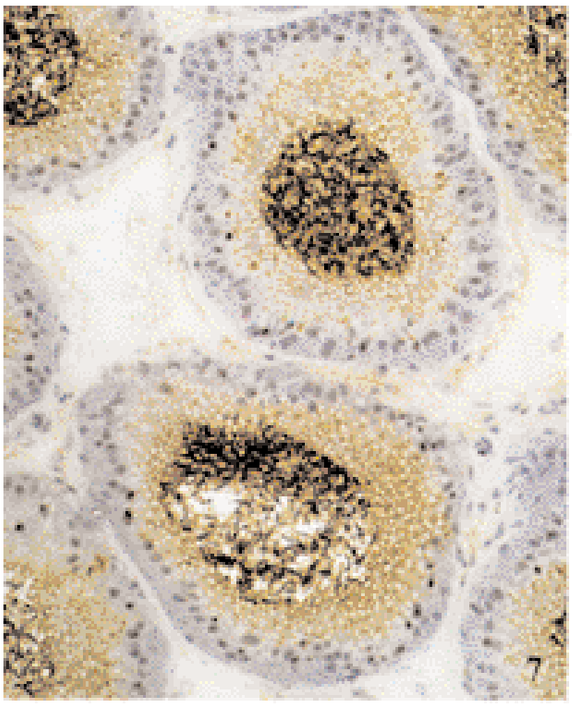
Immunopositivity in steroidogenetic tissue was comparable to that of chromaffin cells, more intense from January to June and weaker in other periods (Fig. 9).

In twenty days captive animals, the reaction was evident in chromaffin cells, but completely absent in steroidogenetic ones (Fig. 10).

DISCUSSION

The present study is concerned with the first immunocytochemical localisation of inhibin in a reptilian species, *Podarcis sicula*. The absence of immunopositivity in control specimens obtained after preabsorbing primary antisera with the corresponding synthetic peptide, is a reliable confirmation, in the absence of biochemical evidence, of the specificity of the reaction in these reptiles. The finding of α-inhibin chain-like immunoreactivity in the testis and adrenal gland cells provides evidence that they are one of the possible sources of inhibin, suggesting a new explanation for the mechanisms that regulate seasonal spermatogenesis in this lizard living in temperate zones.

In the lizard testis showing full spermatogenetic activity (from April to June), no cellular type was immunopositive to inhibin both in normal and captive animals. In early July, when spermatogenesis starts its involution, Leydig cells, instead, resulted positive to it. This suggests, that, in this period, Leydig cells might also secrete inhibin; this hormone, through a feed-back at the hypothalamic and/or the pituitary level, would give rise to a strong decrease in gonadotropins output and, through a local action, might act on the same Leydig cells, limiting androgen release as in Mammals (Moore *et al*., 1994). Gonadotropin decrease in July is followed by a total block of spermatogonial mitoses, a decrease in androgen plasma levels, a regression of the secondary sexual characters and degeneration of the last phases of spermatogenesis (Angelini and Botte, 1992). These effects could be also linked with factors different from gonadotropin decrease. In mammals, inhibin might have a paracrine action on the testis (Moore *et al*., 1994) by limiting testosterone release from Leydig cells and decreasing the number of spermatogonial divisions (van Dissel-Emiliani *et al*., 1989). This effect is of the direct type and not FSH-mediated (Woodruff *et al*., 1992). In *P. sicula*, during the early regressive phase of July, spermatogonial mitoses are scarse and the seminiferous tubules reduce their spermatogenetic activity. Leydig cells are present but largely vacuolised, immediately before their functional involution (Varano *et al*., 1973). In this phase of the cycle Leydig cells are immunopositive to inhibin, and that is in accordance with the loss of their ability to convert precursors into androgens, as is indicated by the collapse in androgen plasma levels (Angelini and Botte, 1992). The inhibin cross-reaction in the Leydig cells in this phase of the spermatogenetic cycle can be considered as a local modulating factor both of steroidogenetic activity and of the resting of spermatogonial mitoses, as proposed for mammals (Moore *et al*., 1994). In August, during the *maximal regressive phase*, the spermatogenetic cycle is completely over; and the seminiferous tubules, reduced and without lumen, contain only spermatogonia and Sertoli cells. Within the interstitium, Leydig cells are morphologically indistinguishable from fibroblasts and no reaction is present in the interstitium. Immunopositivity to inhibin can be, instead, detected only in the Sertoli cells. In this period, both testis and SSC are completely refractory to gonadotropin stimulation (Angelini and Botte, 1992). It could be hypothysed that, through a paracrine mechanism, inhibin would contribute to keeping the gonads refractory, either blocking gonadotropin receptors,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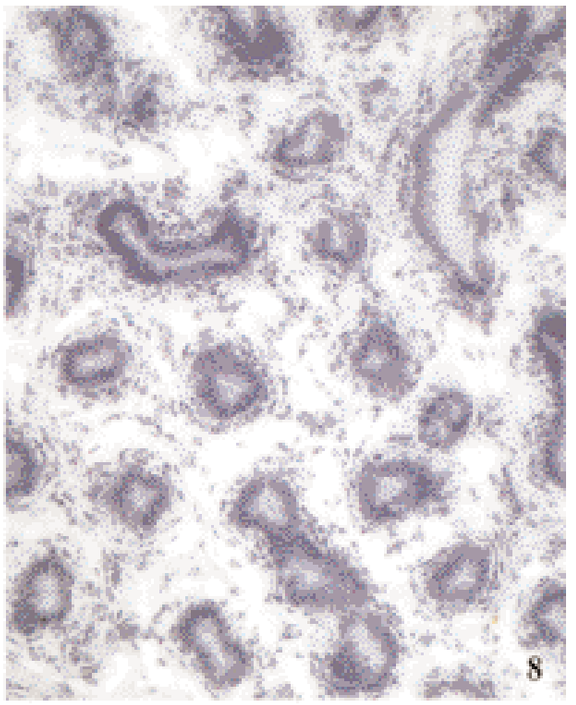
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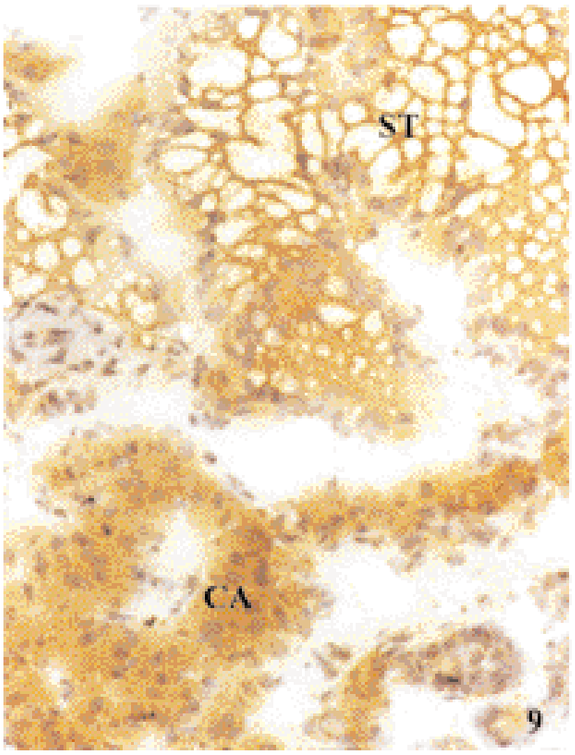
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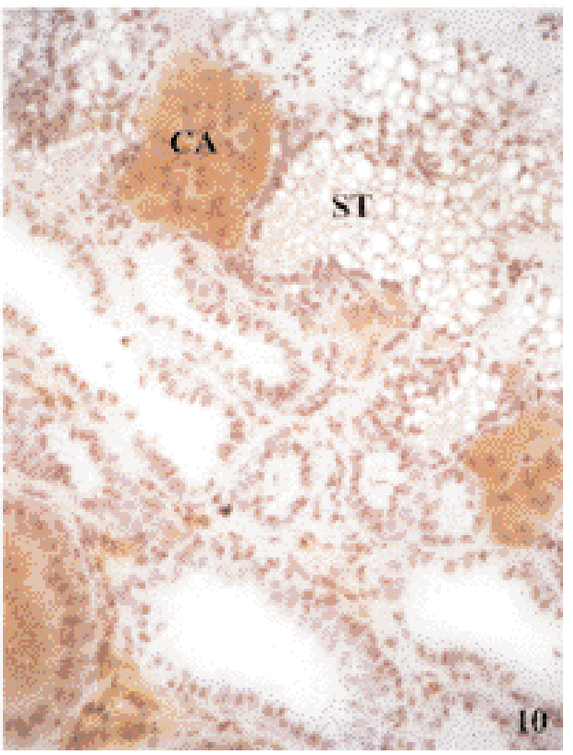
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as has already been hypothesised for mammals (Baird and Smith, 1993), or stimulating sertolian aromatase from the inside, which, in this phase, would lead to the production of a large amount of estrogens (Angelini and Botte, 1992). Thyroid hormones might also be involved in this regulation. In the rat, through a direct action on the testis, high T_3 levels reduce proliferation of Sertoli cells and induce inhibin production in these cells, causing a decrease in FSH levels (van Haaster *et al*., 1993). A similar mechanism might be hypothesised also in *Podarcis*, where the T_3 plasma concentration is very high in the periods considered (Sciarrillo *et al*., in press). After this situation, at the end of August, there is an intense resumption of spermatogonial mitoses, which are known to be stimulated by estrogens in this species (Angelini and Botte, 1992), as well as in mammals (Moger, 1980; van Der Molen and Rommerts, 1981). In August-September, following the resumption of spermatogonial mitoses, the spermatogenetic block is over and germ cells start differentiating by meiosis, giving rise to the autumn spermatogenetic wave (Angelini and Botte, 1992). In this period, Sertoli cells appear to be completely negative to inhibin.

In other vertebrates, especially mammals, inhibin α-subunits have also been identified in both Leydig and Sertoli cells (de Winter *et al*., 1992; Vannelli *et al*., 1992; Vliegen *et al*., 1993; Nagata *et al*., 1998). By a local action, inhibin is believed to modulate both steroidogenesis and gametogenesis (Halvorson and DeCherney, 1996), in particular decreasing androgen production (Hsueh *et al*., 1987). Risbridger *et al*. (1989) and Maddocks and Sharpe (1989) demonstrated that male rat Leydig cells express mRNA for the α-subunit of inhibin and produce biologically and immunologically active inhibin. On the other hand, patients suffering from Klinefelter's syndrome, whose testes are practically devoid of Sertoli cells, show high levels of LH and inhibin (de Kretser *et al*., 1989), which is supposed to be produced by Leydig cells under LH stimulation.

As in several mammalian species by *in vitro* experiments using intact and castrated specimens (Hurkadli *et al*., 1991), also in the lizard, immunopositivity to inhibin has been detected in the epididymis at the level of the channel secreting cells and in the lumen, while ciliary ducts are always negative. In our lizard, inhibin is probably secreted in the lumen and positivity is present only from April up to July, during the mating period, while from August to the following spring, when the epididymis is regressed and empty, all its components are completely negative. At present this aspect has not yet been elucidated; however, it can be hypothesised that, at the epididymal level, inhibin might either take part in the maturation of sperm or protect them from the damage occurring from oxygen radicals, by decreasing the lipid peroxidation, as in mammals (Hurkadli *et al*., 1991).

In mammals, the presence of inhibin α-subunits in the adrenal gland is probably due to the production of inhibin, since the gene for the inhibin α-subunit is known to be expressed in the ovine adrenal cortex (Crawford *et al*., 1987), and immunopositive cells containing inhibin have been observed in the sheep adrenal cortex (Veeramachaneni *et al*., 1989). These data suggest that inhibin may be involved in some stress-related responses, as it is known to have inhibitory effects on the reproductive system through an increase in corticosteroids (Lance, 1984). In the lizard, too, capture-related stress causes a strong increase in corticosteroids, which results in a decrease in androgens, and this effect is reversed after acclimatation in terrarium for some time (Manzo *et al*., 1994). In our captive animals after twenty days of acclimatation, when the stress capture effect is over, steroid producing cells are completely negative. This result could be interpreted as a confirmation that inhibin production from the adrenal gland is related to the stress effects. In the lizard studied, the chromaffin cells, instead, are always positive with a variable intensity in normal and captive animals; but at present, data are lacking to explain these results.

Figs. 7/10 - 7) Cryostatic section of the middle portion of the epididymis during the *culmination phase* (x 280). Note the inhibin cross-reaction in the cytoplasm of the secretory cells and in the lumina filled with sperm and secretion. **8**) Cryostatic section of an involuted epididymis at the end of August (x 390). Cross-reaction appears absent. **9**) Cryostatic section of adrenal gland during the *culmination phase* (x 320). The inhibin cross-reaction is detected in catecholamine (CA) and steroidogenetic cells (ST). **10**) Cryostatic section of adrenal gland during the *culmination phase* of twenty days captive animals (x 295). The inhibin cross-reaction disappears from the steroidogenetic cells but persists in the catecholamine ones. In the lower left corner an inhibin cross-reaction is still present in the secretory cells of the epididymis.

It may be concluded that inhibin production during the different phases of the lizard spermatogenetic cycle can be considered as a systemic hormone acting on gonadotropin release and as a spermatogenesis local inhibitor factor, acting on germ cell proliferation and limiting steroidogenesis.

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