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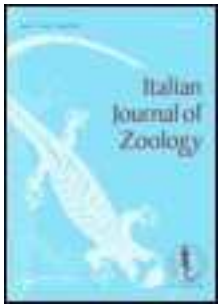

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Reproductive activity and plasma androgen concentrations in the male of Rana dalmatina

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Reproductive activity and plasma androgen concentrations in the male of *Rana dalmatina*

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ABSTRACT

Reproductive activity (spermatogenesis, testis and fat-body weight, thumpad) is related to environmental factors and plasma androgen levels in the male of *Rana dalmatina*. The population studied, living at 1000 m a.s.l., showed a markedly discontinuous spermatogenetic cycle. From November to February, spermatogenesis stopped completely. Spermiation occurred from late February to late March. In May, there was recovery of spermatogenesis. The mating season and ovipositions took place from the end of February to late March or early April, when environmental temperatures increase and the pool where this species reproduces thaws. Significant seasonal variations were observed in the testis and fat-body weight, thumpad epithelium, and plasma testosterone but not in plasma androstenedione. Plasma testosterone peaked in February, two months after the maximum development of nuptial callosity; however, there was a positive correlation between these two characters, which was not observed, instead, between nuptial callosity and plasma androstenedione levels. Finally, an explanation is provided for the occurrence of developed interstitial tissue after the reproductive season, when androgen (both T and A) levels in the plasma are at the minimum, and the nuptial callosity is completely regressed.

KEY WORDS: Testosterone - Androstenedione - Testis and thumpad histology - *Rana dalmatina*.

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INTRODUCTION

In the male of different anuran species, seasonal variations in plasma androgen concentration have been correlated with testis condition, callosity development, and sexual behaviour (D'Istria *et al.*, 1974; Wada *et al.*, 1976; Siboulet, 1981; Licht *et al.*, 1983; Polzonetti *et al.*, 1983; Rastogi *et al.*, 1986; Kuhn *et al.*, 1987; Townsend & Moger, 1987; Delgado *et al.*, 1989; Itoh & Ishii, 1990; Itoh *et al.*, 1990). The investigations so far carried out have shown that species-specific differences exist within Anura, and plasma androgens do not always appear to be closely related to the various characters of reproduction, including gonadal weight and sexual behaviour.

The reproductive biology of *Rana dalmatina* Bonaparte is known only in some aspects. This is probably due to the fact that this species is not abundant in the biotopes in which it is present, although its distribution in Europe is wide (Sofianidou & Kyriakopoulou-Sklavonou, 1983). Investigations were mainly carried out on its reproductive ecology (Geisselmann *et al.*, 1971; Guyetant, 1976; Sofianidou & Kyriakopoulou-Sklavonou, 1983); little interest was devoted to its reproductive cycle (Cei, 1944).

The present study aims at detecting the changes in the testis and secondary sexual characteristics (thumbpad) in relation to variations in plasma testosterone (T) and androstenedione (A) in the male of *Rana dalmatina*.

MATERIALS AND METHODS

The study was conducted from 1988 to 1990 on a *Rana dalmatina* population from Vesalo (Salerno, Italy), located about 1000 m a.s.l. The animals (34 in total and showing an average total length of 53 ± 3.42 mm) were sacrificed within one day from the capture, under anaesthesia in MS-222 (Sigma). They were measured and weighed. Right testes and fat bodies were also weighed, and seasonal variations were expressed as organo-somatic indices (organ weight/body weight in grams). Testes, seminal vesicles and nuptial callosities were fixed in Bouin and prepared for routine light-microscopy techniques. The Leydig cell nuclear area was measured with an ocular micrometer, taking 15 randomly selected nuclei for each testis. Small fragments of testes were sectioned in a cryostat, and subjected to the Schultz test for cholesterol (Mazzi, 1977).

Dosages of T and A were performed on plasma taken by heart puncture, using radioimmunoassay (RIA) kits supplied by Sorin Biomedica (Testo K; Δ_4). Plasma sex steroids had already been assayed by this method successfully in *Rana perezi* (Delgado *et al.*, 1989). For T, cross-reactivity was 100% with T, 7.2% with 5α-dihydrotestosterone, 0.81% with androstenedione, <0.20% with other steroids. For A, cross-reactivity was 100% with A, 0.03% with androsterone, <0.002% with other steroids. The sensitivity of the method was 0.02 ng/ml for T and 0.01 ng/ml for A. The intra- and interassay coefficients of variations were <10% and <15%, respectively.

During sampling, air and water temperatures were measured and are reported in Figure 1.

All numerical data were presented as means ± standard deviation. One-way analyses of variance (ANOVA) combined with Tukey's test or, when variances were not homogeneous, the Kruskal-Wallis test were used to analyze the differences between months. The correlation between variables was analyzed by means of Spearman's correlation test. For statistical tests, $P \leq 0.05$ was considered as significant. Statistical analyses were performed using computer programmes provided by Vannini (1991).

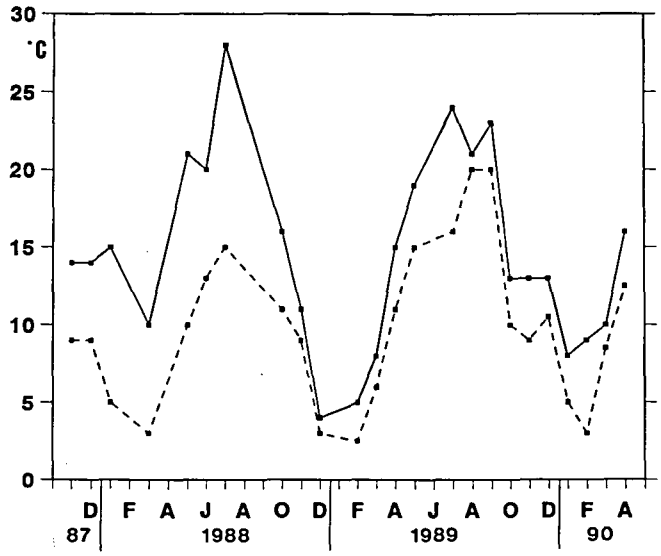

Fig. 1 - Changes in air (continuous line) and water (dotted line) temperatures in the Vesalo area during the study period. Each point represents the value obtained from 11 a.m. to 1 p.m. of the day of sampling.

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RESULTS

In October, spermatogenesis appeared to be almost completed. In fact, mainly primary spermatogonia and numerous sperm bundles were observed in the seminiferous tubules, whereas cysts of the other spermatogenetic stages were not numerous. From November to February, all the animals showed complete spermatogenetic stasis (Fig. 2). In this period, the seminiferous tubules contained only non-mitotic primary spermatogonia and spermatozoa; cysts of degenerating meiocytes were sometimes observed. The interstitial tissue showed prevailingly round Leydig cell nuclei (Leydig cell nuclear area: in December-January, $\bar{X}$ = 23.6 ± 2.4 μm^2, n = 9; in February-early March, $\bar{X}$ = 22.5 ± 7.23 μm^2, n = 6). Spermiation always occurred in February and March, and almost all sperm were usually released at the end of the reproductive season (early March-early April), thus almost exclusively primary spermatogonia were observed in the testis. In May, there was a recovery of spermatogenetic activity characterized by proliferation of primary and secondary spermatogonia. During April-May the interstitial tissue still appeared developed (Fig. 3), with Leydig cells showing either large and round or elliptical nuclei (Leydig cell nuclear area: $\bar{X}$ = 23.3 ± 2.6 μm^2, n = 4). In addition, the Leydig cell cytoplasm was strongly cholesterol-positive.

Figure 6 shows the variations in the gonado-somatic index (GSI) and the fat body somatic index (FBSI) from October to May and Figure 7 the height of the thumbpad epithelium, and plasma A and T levels in the same period. The data related to the period of this investigation were plotted in a single year. Table I summarizes the ANOVA (for the GSI, FBSI, thumbpad and T) and the Kruskal-Wallis test (for A) concerning the parameters of Figures 6 and 7 referring to the months from December to April. GSI and FBSI showed a drastic decrease from October to May, the variation from December to April being significant. The variations of the two indices were positively correlated (Table II). Thumbpad resulted most developed in December and January: at a gross examination it appeared dark grey, whereas in section conspicuous epidermal papillae with keratin storages could be observed (Fig. 4). In April and May the thumpad appeared whitish and completely regressed, and the smooth epidermal surface was observed (Fig. 5). Variations in monthly averages were significant also for the thumbapd. Plasma T and A levels were higher in individuals captured in February. However, variations in plasma T were significant, but those in A were not, often being at the limit of assay sensitivity. The plasma T peak registered in February was significant compared with the values registered during the other months of the year (Tukey's test, $P < 0.01$). Finally, the variations in plasma T, unlike those in plasma A, positively correlated with the variations in thumpad epithelium (Table II). T and A were also combined as a single variable (T + A), showing a significant correlation with the changes in thumpad epithelium height.

DISCUSSION

In the population of *R. dalmatina* studied, testicular activity shows four discrete stages: completion of spermatogenesis (October), spermatogenetic stasis (from November to early February), spermiation (February-March) and recovery of spermatogenetic activity (from May onwards). Therefore, like other anurans from the temperate regions, *R. dalmatina* shows a markedly discontinuous spermatogenetic cycle (Galgano, 1952; Rastogi, 1976; Rastogi & Iela, 1992). The scarcity of animals in the summer months does not allow a detailed histological picture of testicular activity in this period. On the other hand, immediately after reproduction (March), *R. dalmatina* leads a terrestrial life going even very far from the site of reproduction: this is the reason why it is difficult to find it in the wild. This difficulty has been reported also by other investigators (Cei, 1944; Guyetant, 1976; Dolce *et al.*, 1982). In this station, almost the whole population oviposits from late February to the first half of March, when drastic changes in climatic conditions occur, such as increase in water and air temperature, and thawing of the temporary pool in which this species reproduces. In April, few newly-laid egg masses are found. A shorter breeding season observed in the *R. dalmatina*, population we studied, in comparison with Greek populations (Sofianidou & Kyriakopoulou-Sklavounou, 1983), may be due to the colder climate experienced by the former. Males reach the site of reproduction earlier than females, and stay there longer.

Plasma levels of T and A in *R. dalmatina* males undergo characteristic changes throughout the sexual cycle. Although potential effects on plasma hormone levels

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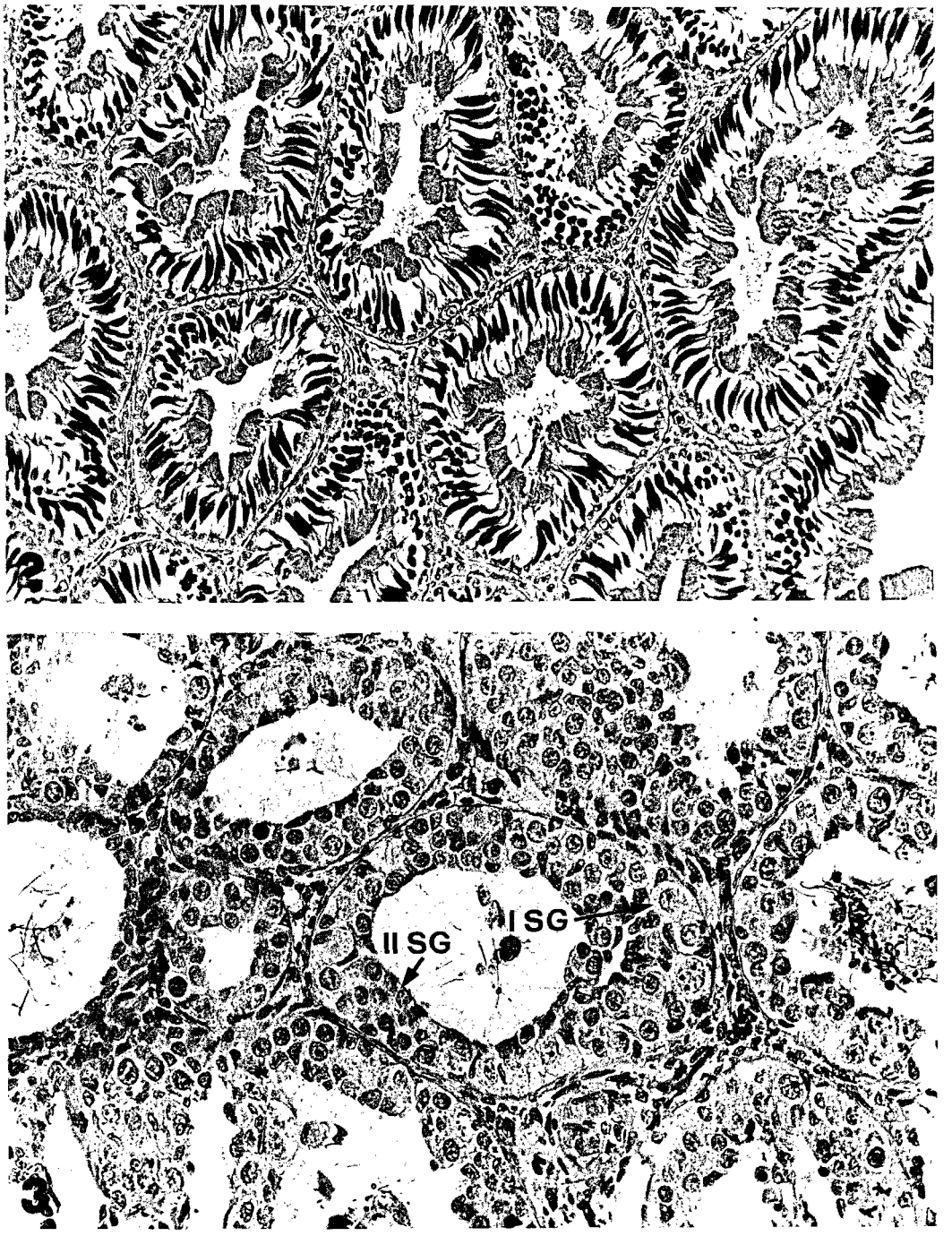

Figs 2-3 - **Fig. 2** - Cross section of the testis of a male sacrificed in late February. The seminiferous tubules are filled only with sperm bundles and primary spermatogonia. ×180. **Fig. 3** - Cross section of the testis of a male sacrificed in May. The seminiferous tubules are filled with primary spermatogonia (I SG) and cysts containing secondary spermatogonia (II SG). ×490).

following capture and short-term confinement have not been investigated, we are confident that the changes in plasma hormone levels detected in this study have given a reliable plasma hormone profile of *R. dalmatina* throughout the sexual cycle. In fact, in *Rana esculenta* the annual pattern of plasma androgens, as well as their correlation with the secondary sexual character cycle, is not significantly different in just captured and short-term captive frogs (Paolucci *et al.*, 1990). In *R. dalmatina*, T peaks during the breeding season. Although this increase has also been recorded in plasma A, no significant differences have been observed between months for this hormone. This might depend on the marked individual variability in the A plasma level found within each group. In addition, only T significantly correlates with thumpad epithelium height. This finding does not agree with what

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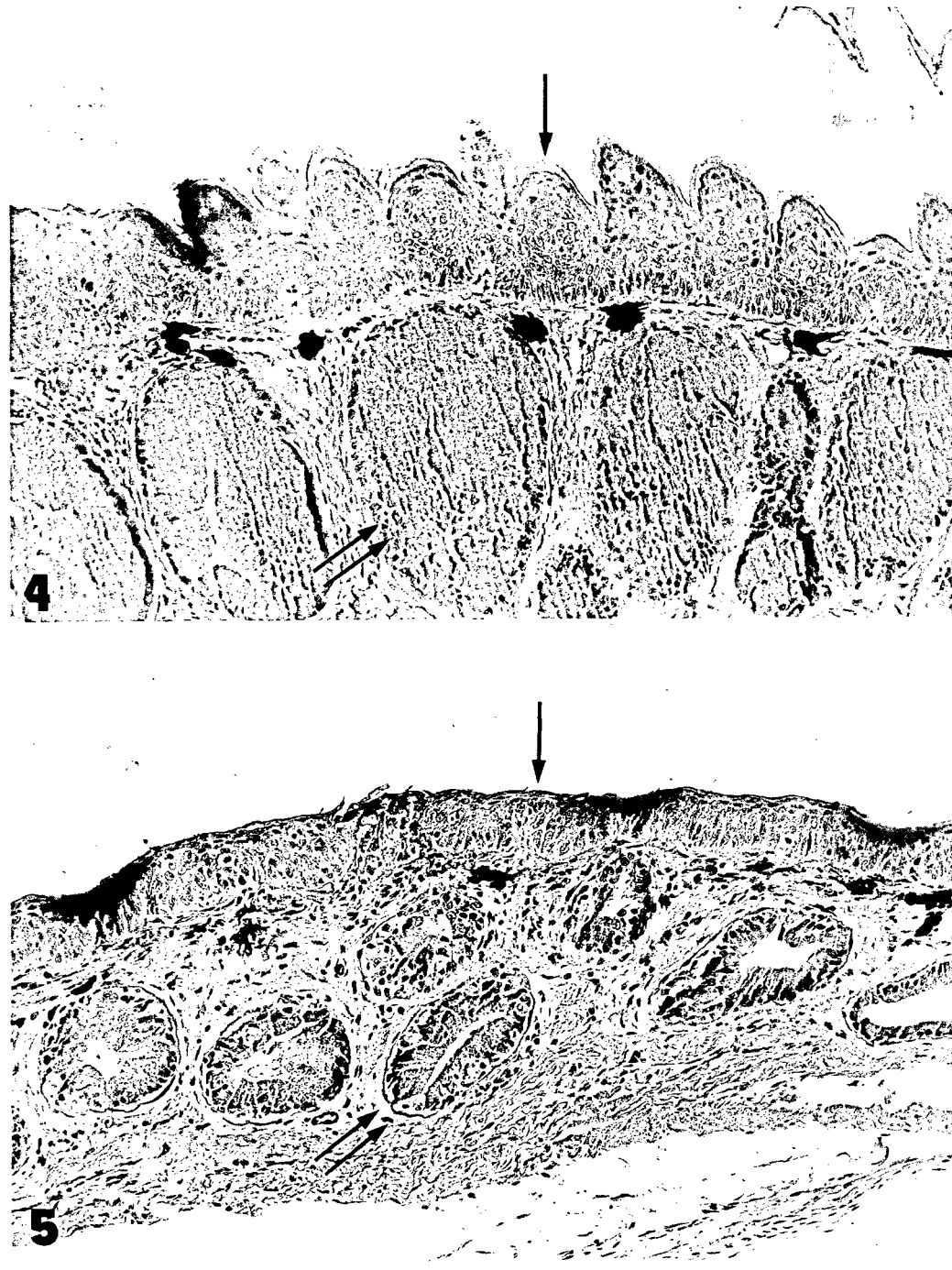

Figs 4-5 - **Fig. 4** - Cross section of the thumpad of the same male as Figure 2, showing epidermal papillae (arrow) and trophic mucous glands (double arrows). ×160. **Fig. 5** - Cross section of the thumpad of the same male as Figure 3, showing smooth epidermal surface (arrow) and regressed mucous glands (double arrows). ×160.

has been reported in *Pachymedusa dacnicolor*, the only anuran in which T and A have been measured separately during the various stages of the reproductive cycle (Rastogi *et al.*, 1986). In fact, in this anuran species, thumpad development and reproductive behaviour better correlate with plasma A than with T.

In *R. dalmatina*, combined T and A values show a correlation coefficient with thumpad epithelium height larger than those obtained with either T or A. Similar results have been reported by Itoh *et al.* (1990) in the simple and multiple correlation analyses between plasma gonadotropin levels (FSH and LH) and plasma androgen titres in *Bufo japonicus*. It could be supposed that in *R. dalmatina*, as in *P. dacnicolor* (Rastogi *et al.*, 1986), the

TABLE I - *ANOVA (F) and Kruskal-Wallis (H) test of the data concerning the parameters reported in Figures 6 and 7.*

Parameters	F or H	P
GSI	$F_{(5,25)} = 10.9$	< 0.01
FBSI	$F_{(5,25)} = 4.5$	< 0.01
Thumb.	$F_{(5,19)} = 19.9$	< 0.01
T	$F_{(5,19)} = 215$	< 0.01
A	H = 7.4 , df = 5	NS

TABLE II - *Spearman's rank correlation (r_s).*

Variables correlate	*n*	r_s	P
GSI-FBSI	33	0.56	<0.001
thumbad-T	18	0.48	<0.05
thumbad-A	18	-0.03	NS
T + A-thumbad	18	0.53	<0.05
T - A	18	-0.06	NS

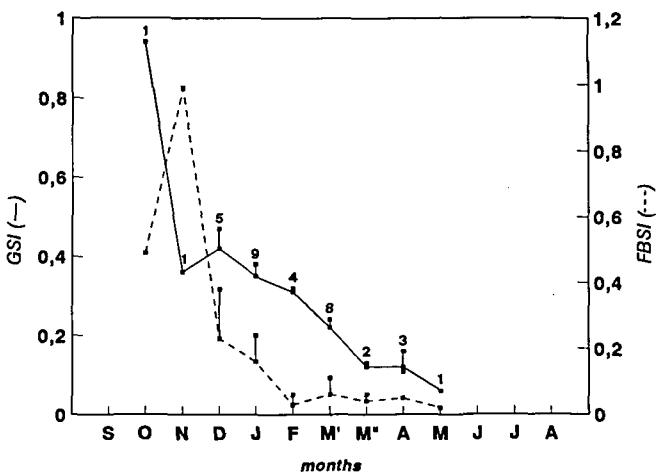

Fig. 6 - Changes in the gonado-somatic indexes (GSI) (solid line) and fat body-somatic indexes (FBSI) (dotted line) during the period October-May. Each point represents the $\bar{X} \pm SD$ (bars). The numbers above the GSI curve indicate the numbers of the individuals sampled. M', first half of March; M", second half of March.

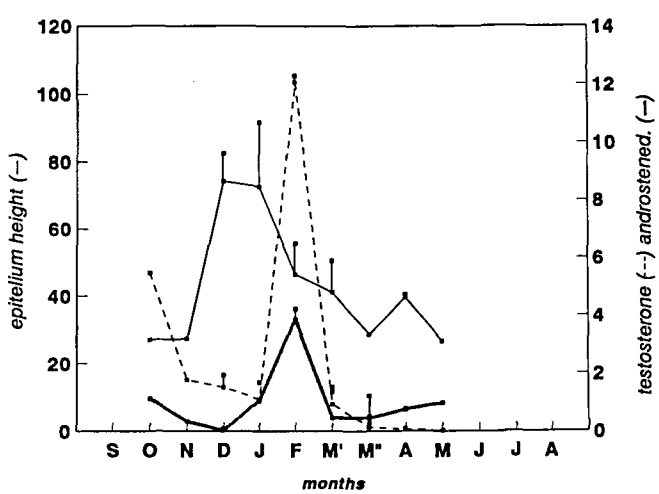

Fig. 7 - Changes in thumpad epithelium height (μm) (thin solid line), plasma testosterone (ng/ml) (dotted line) and androstenedione (ng/ml) (bold solid line) during the period October-May. Each point represents the $\bar{X} \pm SD$ (bars). Androstenedione values have been multiplied by 10. For the number of individuals sampled, see Figure 6.

two androgens cooperate in the control of the calling and amplectant behaviour; however, only experimental treatments might corroborate this hypothesis.

In the male of *R. dalmatina*, the lowest plasma androgen level and the complete regression of the thumbpad occur after the breeding season (late February-late March), even though testicular interstitial tissue appears still developed at histological examination. The low concentrations of plasma T and A during the postreproductive period are probably due to a reduction in LH secretion, as observed in other anurans (Itoh *et al.*, 1990). The persistence of developed interstitial tissue also in the months immediately following the breeding is typical of *R. dalmatina*, being not exhibited by other temperate-region anurans (Rastogi, 1976; Delgado *et al.*, 1989; Guarino, 1992). Moreover, in this period, there is an accumulation of cholesterol-positive material in the Leydig cells, similar to that observed in other amphibians (Lofts, 1974; Jorgensen *et al.*, 1979) and some reptiles (Mahmoud *et al.*, 1985; Angelini *et al.*, 1986; Limatola *et al.*, 1991). These data indicate that, throughout the post-breeding period, Leydig cells accumulate androgen precursors which are not probably converted into androgens. Thus, the morphological aspect of Leydig cells does not always sustain their actual steroidogenetic activity.

Our data on the relation between environmental factors (temperature and photoperiod), spermatogenesis, and steroidogenesis in the testis, show that in *R. dalmatina*, during winter when temperature is low and photoperiod is short, spermatogenesis is stopped and plasma T levels are relatively low. However, these levels remarkably increase when the breeding season starts in late winter (late February). Conversely, in spring when temperature and photoperiod markedly increase, there is a recovery of spermatogenesis, whereas plasma androgen levels are the lowest in the whole study period. These results are consistent with those obtained in other temperate-region anurans (Rastogi, 1976; Delgado *et al.*, 1989, Rastogi & Iela, 1992). It can be suggested that, as demonstrated by Rastogi *et al.* (1978) in *Rana esculenta*, also in *R. dalmatina*, the various stages of the annual spermatogenetic cycle depend on a combination of critical temperature and critical photoperiod.

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