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The Effects of Testosterone and Estradiol on Mast Cell Number in the Harderian Gland of the Frog, *Rana esculenta*.

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ABSTRACT—The Harderian gland (HG) of the frog *Rana esculenta* contains mast cells in the interstitial tissue. The mast cell number (MCN) is influenced by sex hormones. Gonadectomy in both sexes provoked a decrease in MCN in January, while no effect was observed in September. Sex hormone-replacement therapy gave different results; estradiol treatment in castrated males and females always increased MCN, while testosterone did not. Acute estradiol treatment provoked an increase in MCN on days 2 and 4 of treatment and the morphology of the glandular compartment appeared normal. On days 8, 10 and 12 of treatment the MCN drastically decreased. The majority of glandular acini appeared strongly disorganized and the interstitial tissue became hypertrophic in concomitance with an increased vascularization. Our results suggest that estradiol acts by stimulating mast cells and acute estradiol treatment provokes proliferation of interstitial connective tissue together with glandular cells damage.

INTRODUCTION

The Harderian gland is a large orbital gland of many tetrapod species possessing the nictitating membrane [13]. Although its main role is to lubricate the eyeball, in the last few years several other functions have been proposed [16, 22, 24].

The Harderian gland is the only orbital gland of the frog *Rana esculenta*, and it contains scattered mast cells of the connective tissue type both in the interstitial tissue and in the connective tissue capsule [2].

In previous studies we have reported that the secretory activity of the frog Harderian gland is cyclic (for review see [16]) and concomitantly, the mast cell number (MCN) shows annual variations, being more numerous during winter [2]. Interestingly, plasma testosterone and estradiol in males show high values during winter months and early spring respectively [8], while in females estradiol reach a peak during autumn [23]. In several vertebrates the MCN seems to be sex hormone dependent in both the Harderian gland ([for review see [22]) and testis [7, 10].

The present study was undertaken to investigate the influence of sex hormones on mast cell number in the frog Harderian gland. Here we report the effects of castration and replacement therapy in two different periods of the secretory cycle (September and January) as well as those following an acute estradiol administration in the frog, *Rana esculenta*, of both sexes.

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MATERIALS AND METHODS

Animals

Experiment 1

Adult males (n=25) and females (n=25) of *Rana esculenta* were collected in the vicinity of Naples in the months of September (when the secretory activity drastically drops and MCN is at lower value) and January (when the secretory activity is resuming and MCN is a high value) [2, 6]. The frogs were maintained in plastic tanks with food (meal worms) and water available *ad libitum*. Fifteen females and 15 males were gonadectomized. Five males and 5 females were sacrified as initial controls; and 5 males and 5 females were sham-operated and used as controls. One day after operation, the castrated (CS) animals were divided into 3 groups and injected on alternate days for 4 weeks, in the dorsal lymph sac with: 1) vehicle alone (100 μl of Krebs-Ringer bicarbonate buffer pH 7.4, KRB) (n=5); 2) 2 μg of estradiol (E_2)/100 μl/injection per animal (n=5); 3) 10 μg testosterone (T)/100 μl/injection per animal (n=5).

Experiment 2

In February, 60 adult frogs of both sexes were injected daily (5 days/week) with E_2 (2 μg/injection) for two weeks. The animals were sacrificed on days 0, 2, 4, 8, 10 and 12 of the treatment.

At the end of each experiment, the animals were decapitated after anaesthesia with MS 222 (Sigma) and the Harderian glands (HG) was quickly dissected from the orbits.

Histology and ultrastructure

For histological studies, the left gland from each animal was fixed in Bouin's fluid and 5 μm paraffin sections were stained with haematoxylin-eosin and/or trichrome Mallory stain.

For electron microscopic study, small pieces of the right HG (1 mm^3) were fixed in Karnovsky's fluid in phosphate buffer (pH 7.4) for 2 hr at 4°C, postfixed for 2 hr at 4°C in 1% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series and embedded in TAAB 812 resin. Ultrathin sections were stained with 4% uranyl

acetate followed by 1% lead citrate and examined with a Philips 301 trasmission electron microscope (EM).

Histochemistry

Mast cells were stained differentially using 0.2% toluidine blue in Walpole buffer at pH 4.2 [9]. Mucosubstances were demonstrated histochemically by the periodic acid-Schiff (PAS), Alcian blue 8GX at pH 2.5 and Alcian-PAS methods.

Numerical and statistical analysis

Five randomly chosen sections from each animal were viewed in a light microscope at a magnification of $\times 400$ using an eyepiece with a square-ruled grid with a total area of 0.062 mm^2. The mast cell number (MCN) within interstitial tissue of the gland was counted in 5 different areas of each section (total area: 0.310 mm^2). This allowed the calculation of MCN/acinus. The groups were compared by one-way analysis of variance followed by Duncan's test for multigroup comparison.

RESULTS

The Harderian gland of the frog *Rana esculenta* is an acinous gland, located at the medial corner of the orbit and is associated with the nictitating membrane. The gland is richly vascularized, particularly at the periphery and is surrounded by a thin connective capsule (for review see [1, 16]). The secretory activity of the HG displays seasonal changes: it reaches a maximum during July-August, drops in September and resumes slowly from October onwards [6].

Experiment 1

Histology and ultrastructure

In September, the secretory activity of the HG is at its lowest level: the glandular cells became shorter and almost devoid of secretory granules. Also the acinar lumina are empty. The round nuclei, centrally located, are euchromatic. Following castration, the morphology of both male and female HG is different from that of control animals. The majority of acini appear disorganized and the acinar lumina are filled with debris. Numerous glandular cells appear normal but with pyknotic nuclei [3, 6].

In January, the HG shows the resumption of the secretory activity; the glandular cells with round nuclei become taller and the cytoplasm is filled with secretory granules. Some basophilic material is usually present in the acinar lumen. The nuclei round in shape, contain a prominent nucleolus. The cytoplasm appears more homogeneous and basophilic.

On the EM, in September the glandular cells show rather distended cisternae of rough endoplasmic reticulum (RER). Mitochondria are small and rare. The nuclei contain large masses of heterochromatin and only few secretory granules can be found in the cytoplasm. Following castration, most of the glandular cells of both sexes show loss of cellular adhesion and display signs of degeneration: partial rupture of membranes, dilated cisternae of RER and degenerated mitochondria in the vacuolated cytoplasm. The nuclei of some acinar cells tend to be pyknotic. Testosterone but not estradiol therapy prevents these effect.

In January, the RER and the mitochondria become more abundant. The nuclei possess a large nucleolus associated with a large mass of chromatin, and secretory granules can be found throughout the cytoplasm. No histological or ultrastructural differences have been evidenced in the HG of the animals castrated in January.

We have already published some of these histological and ultrastructural data [3, 6].

Histochemistry

The results of the histochemical tests are summarized in Tables 1a and 1b. In September no PAS positive material is present in either epithelium or lumina of the HG of the control frogs, while in the CS+E_2- and CS+T-treated animals a paint staining is observed in the epithelium (Table 1a). Both acinar cells and luminar content of all the experimental groups are Alcian blue positive (Table 1a). After Alcian blue/PAS sequence a positive reaction is present in both epithelium and lumina of all groups of frogs exept in the lumina of CS animals (Table 1a).

In January, PAS positive material is present only in the epithelium of the gland; in those of the CS+E_2-treated and CS+T-treated animals the reaction is stronger and appears also in the lumina of the CS+E_2-treated animals (Table 1b). Alcian blue staining shows orthochromatic material both in the acinar cells and lumina of all groups of frogs (Table 1b). After Alcian blue/PAS sequence the majority of the secretory granules in the cytoplasm stain blue and when the granules coalesce they become PAS positive. In the CS+E_2 -treated animals the reaction becomes stronger in both the acinar cells and the lumina (Table 1b).

Mast cell number

September experiment The HG of the control frogs shows the lowest MCN/acinus. Following castration, no effect is observed on MCN/acinus (Fig. 1). Estradiol treatment induces a significant increase of the MCN in both male and female glands ($P<0.01$), while testosterone treatment has no effect (Fig. 1).

January experiment The HG of the control frogs (males and females) show a high value of MCN/acinus. Following castration, MCN/acinus decreases significantly in both male and female animals ($P<0.01$) (Fig. 2). Estradiol treatment causes a marked increase of MCN/acinus in both male and female frogs ($P<0.01$). After testosterone administration no significant differences between CS+T and Control groups in males became evident ($P<0.01$) (Fig. 2).

Experiment 2

Histology and ultrastructure

Histological examination of control frogs (day 0) shows a typical morphology of February HG. The cytoplasm of the secretory cells filled with secretory granules appears more

TABLE 1. Histochemistry of the Harderian gland in both sexes of *Rana esculenta*.

a) September experiment

	CONTROL		SHAM-OPERATED		CS		CS+E_2		CS+T	
	E	L	E	L	E	L	E	L	E	L
Alcian blue pH. 2.5	±	±	±	±	±	−	+	+	+	+
Alcian-PAS	±	±	±	±	±	±	+	+	+	+
PAS	−	−	−	−	−	−	±	−	±	−

The intensity of the reaction is classified into 4 categories from − to ++
E: epithelium; L: lumina; CS: castrated.

b) January experiment

	CONTROL		SHAM-OPERATED		CS		CS+E_2		CS+T	
	E	L	E	L	E	L	E	L	E	L
Alcian blue pH. 2.5	+	±	+	±	±	±	++	+	+	±
Alcian-PAS	±	+	±	+	±	+	+	++	±	+
PAS	+	−	+	−	±	−	+	+	+	−

The intensity of the reaction is classified into 4 categories from − to ++
E: epithelium; L: lumina; CS: castrated.

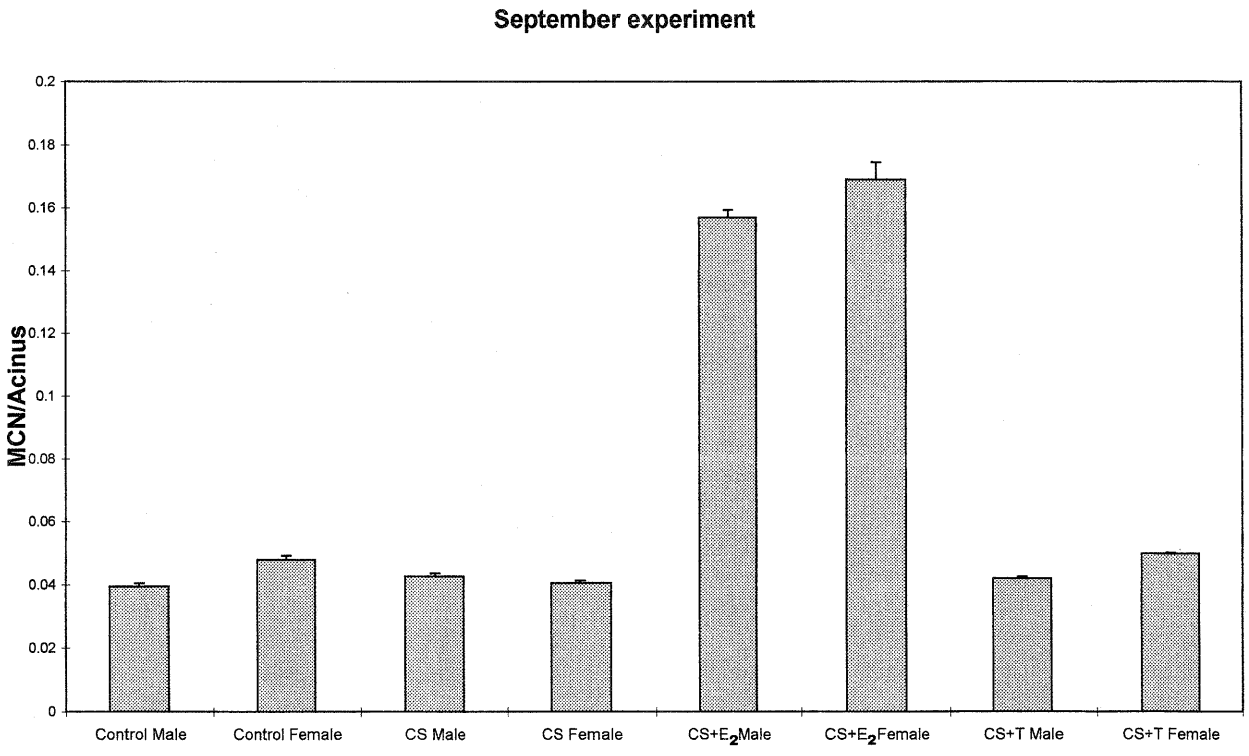


FIG. 1. Influence of sex hormones (estradiol and testosterone) on MCN of male and female castrated frogs in September period. CS: castrated. Vertical bars indicate standard error.

homogeneous and basophilic, and the nuclei are round in shape and contain a prominent nucleolus. The free border of the cells is often marked by a thin basophilic line and in the majority of acini the lumen is very small or absent (Fig. 3 and [6]).

On days 2 and 4 following estradiol administration, many

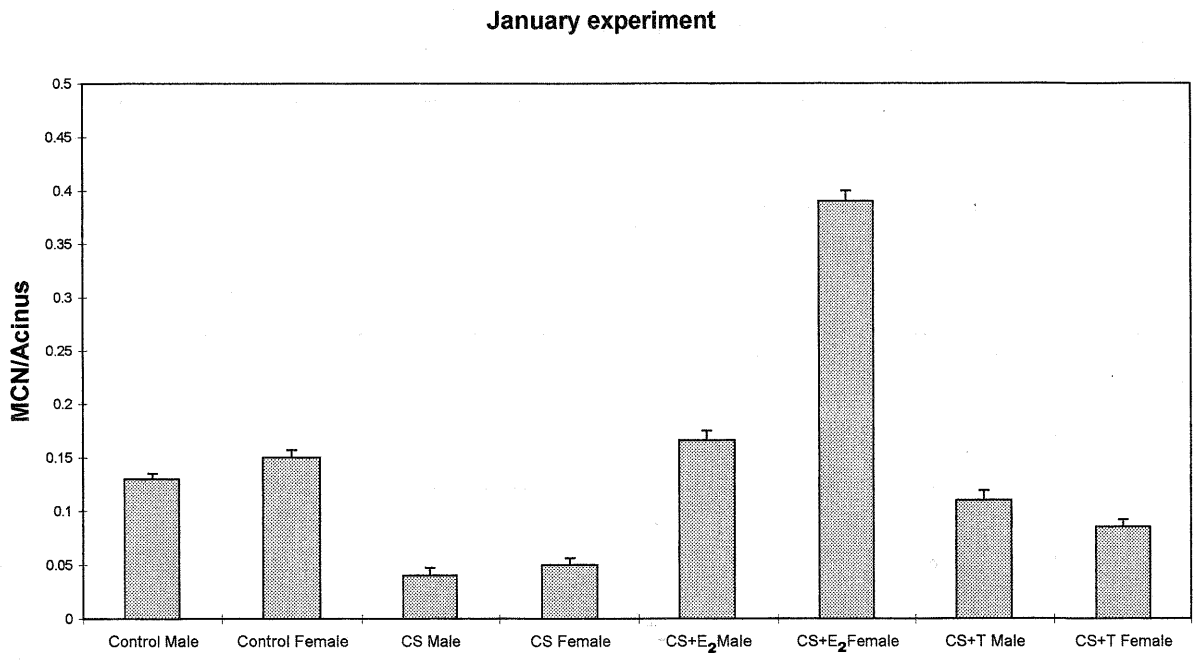


Fig. 2. Influence of sex hormones (estradiol and testosterone) on MCN of male and female castrated frogs in January period. CS: castrated. Vertical bars indicate standard error.

mast cells can be found in the developed connective tissue around the acini (Figs. 4 and 13). Interestingly on day 8 in concomitance with a dramatic decreases in MCN/acinus the gland became richly vascularized. In fact, numerous blood vessels appear among the glandular acini (Figs. 5 and 13). The acinar cells are shorter and the lumina are evident, some of them containing discharged material (Fig. 5). Worthy of note is the strongly hypertrophic connective tissue surrounding the acini (Fig. 5). On days 10 and 12, the hyperaemia of the gland is less evident. On day 10 the interstitial connective tissue around the acini is greatly developed and the majority of the acini show degenerating glandular cells. On day 12 the gland shows clear signs of degeneration. The majority of glandular acini appear disorganized, sometimes they coalesce and glandular cells possess pyknotic nuclei (Fig. 6).

On the EM, the HG of the control animals (Day 0) shows well-organized acini with glandular cells filled with secretory granules. The basally located euchromatic nuclei contain one or more nucleoli (Fig. 7). The secretory cells are columnar or pyramidal in shape and contain a nonhomogeneous type of secretory granule; the RER is well developed and is located around the nuclear envelope, and many mitochondria with transverse cristae in light matrix are distributed in the cytoplasm (Fig. 7).

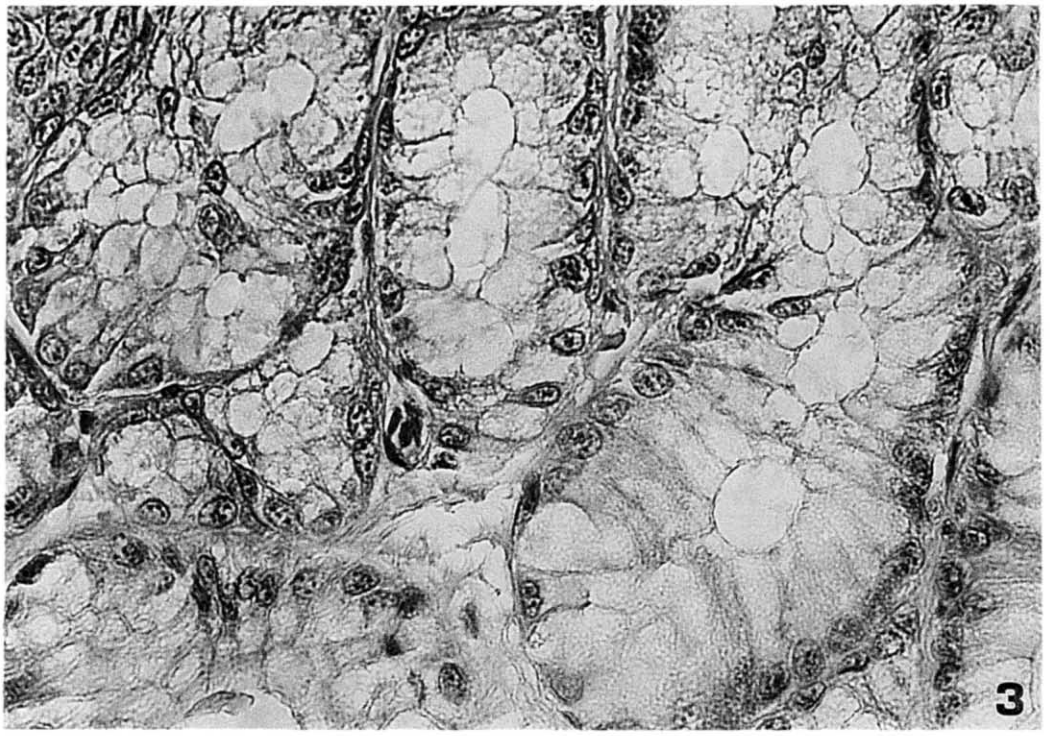
On day 4 the only ultrastructural difference compared with the control is due to the presence of well-developed interstitial connective tissue around the acini (Fig. 8). Mast cells possess heterochromatic nuclei and their cytoplasm is filled with mature secretory granules containing "scroll-like" components and very few mitochondria [2, 7, 16](Fig. 9).

On day 8 the majority of the acini appear disorganized. Within the same acini, glandular cells devoid of secretory granules but with dilated cisternae of RER are present together with other secretory cells containing some granules and normal RER (Fig. 10). The nuclei tend to be heterochromatic; sometimes they assume an irregular shape. The interstitial connective tissue among the acini is hypertrophic (Fig. 10).

On day 12 the majority of the glandular acini appear disorganized, and the secretory cells are damaged and present clear signs of degeneration. Debris and membrane fragments are seen in the acinar lumina. Secretory granules coalesce at the apex of the majority of the glandular cells while the basally located nuclei tend to be pyknotic (Fig. 11). Sometimes the glands are particularly damaged: the glandular acini appear completely destroyed with most of the glandular cells collapsed and it is very difficult to distinguish cellular components among the debris (Fig. 12), however, few cells are still present in the disorganized epithelium of the gland (Fig. 12).

Histochemistry

The results of the histochemical tests are summarized in Table 2. PAS positive material is present mainly on days 4 and 8 of the treatment in both the epithelium and lumina. On days 0 and 2 PAS positive material is found only in the epithelium, while on days 10 and 12 PAS positive material is found only in the lumina (Table 2). Alcian blue staining shows ortochromatic material in both the acinar cells and lumina of all groups of frogs: on day 4 the reaction is more positive in the epithelium than in the lumina while on days 10 and 12 it becomes more positive in the lumina than in the acinar cells (Table 2). After Alcian blue/PAS sequence the



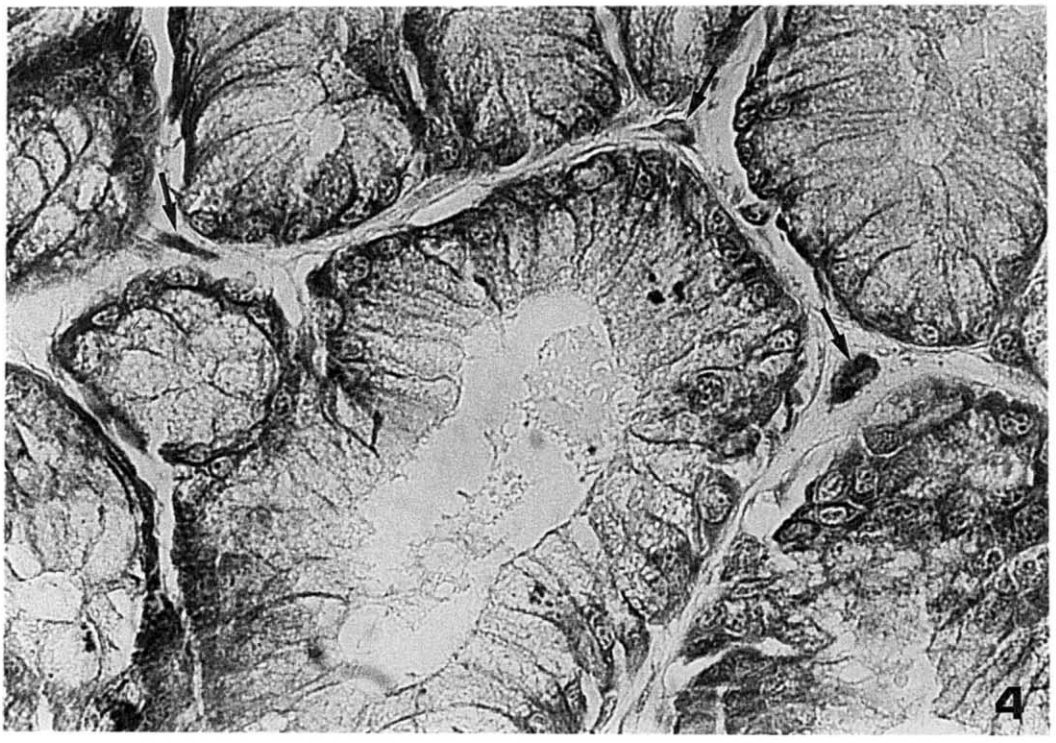


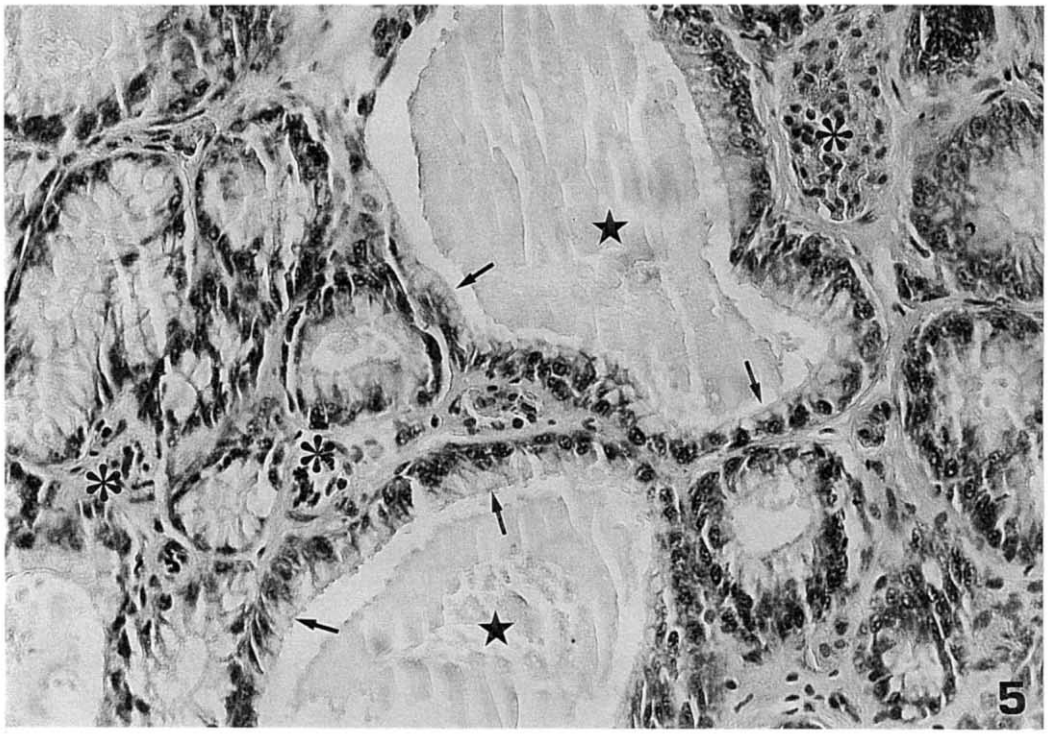
FIG. 3. Harderian gland (HG) of February frogs on Day 0, showing a typical morphology of the acinar cells characterized by round nuclei with a prominent nucleolus; the acinar lumen is very small or absent. ×500.

FIG. 4. HG of a frog on Day 4 of acute estrogen-treatment, showing several mast cells in the interstitium between the acini (arrows). ×500.

majority of the secretory granules stain blue; however when the granules coalesce they become PAS positive. In the E_2-treated animals the Alcian blue/PAS reaction becomes more positive in the lumina (Table 2).

Mast cell number

On days 2 and 4 MCN/acinus increases significantly ($P<0.01$), while a dramatic decrease occurs on days 8, 10 and 12 of treatment ($P<0.01$) (Fig. 13).



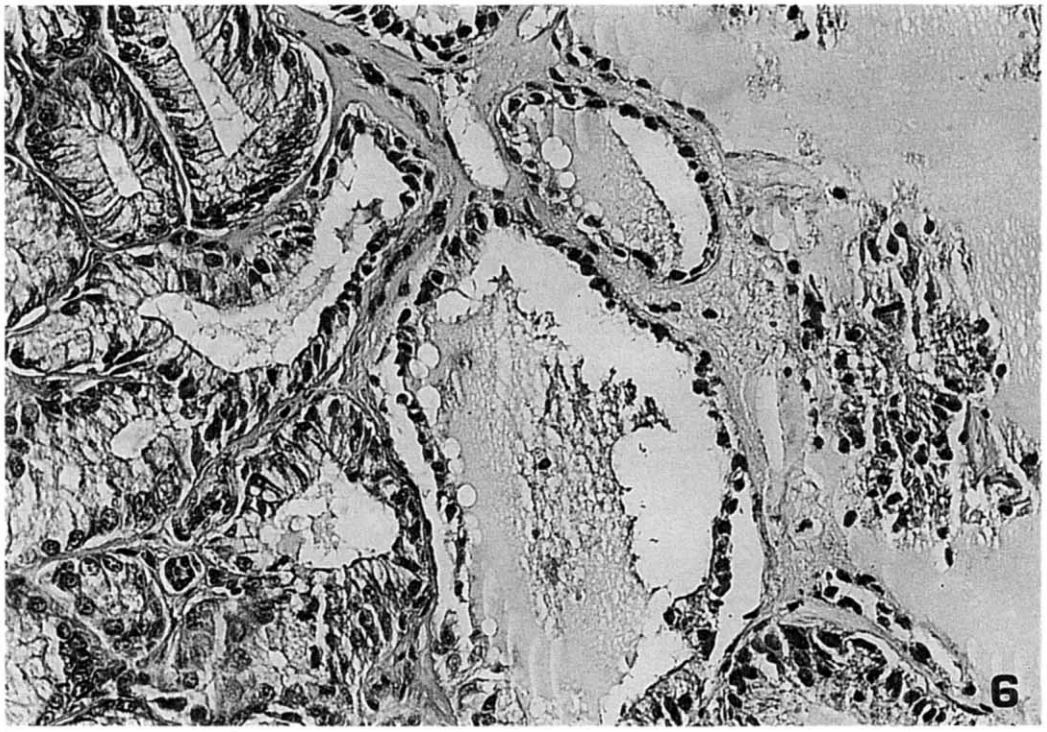
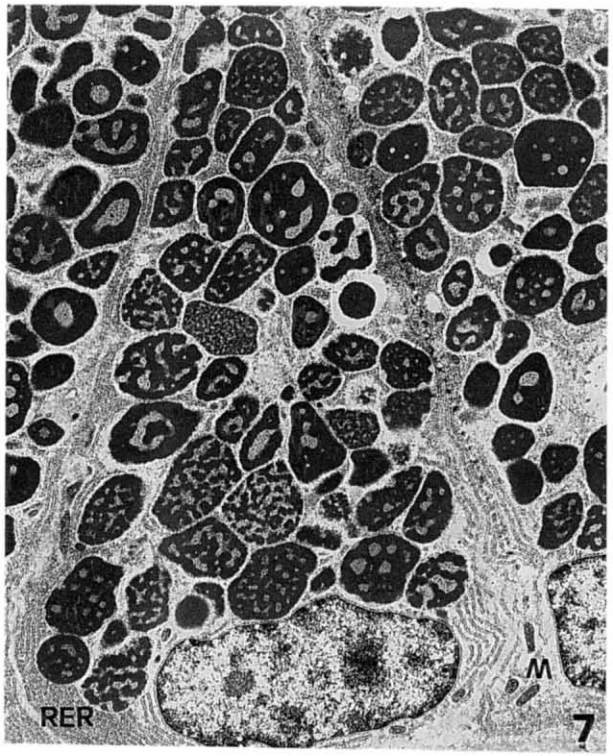


Fig. 5. HG of a frog on Day 8 of acute estradiol-treatment, showing the shortening of the acinar cells (arrows). Note the enlargement of the acinar lumina (stars) and the numerous blood vessels (asterisks) among the acini. ×500.

Fig. 6. HG of a frog on Day 12 of acute estradiol-treatment, showing the disorganized and sometimes confluent glandular acini with pyknotic nuclei (stars). ×500.

DISCUSSION

Humoral control of the HG includes also sex hormones and the implications of this, firstly shown in the golden hamster [4], have been recently demonstrated also in amphibia. In the green frog, *Rana esculenta*, the active sex hormone is testosterone in both sexes. In fact, only androgen receptors have been identified in the HG of the golden



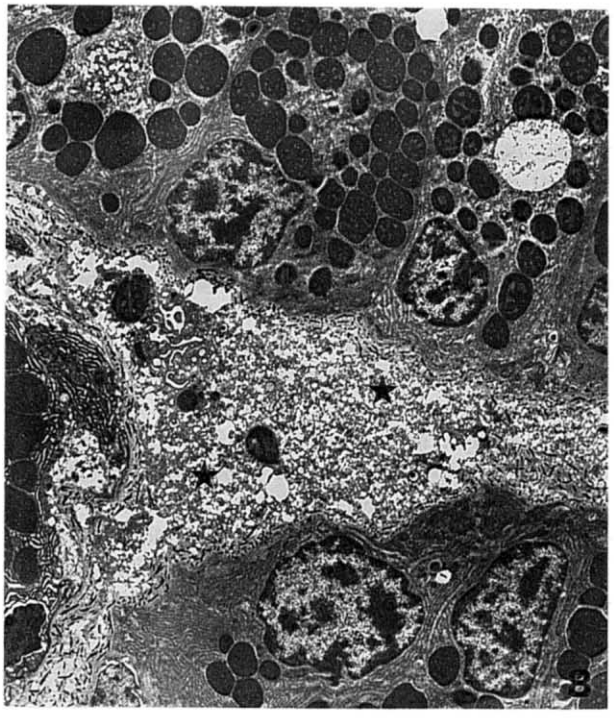
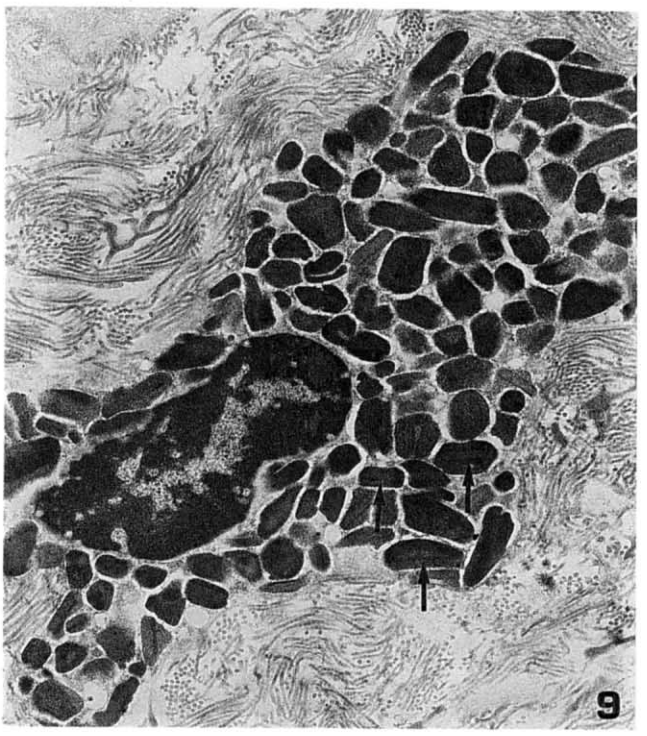


FIG. 7. Electron micrograph of the HG of a control animal (Day 0) showing a pyramidally shaped cell containing non-homogeneous secretory granules. RER: rough endoplasmic reticulum; M: mitochondria. ×500.

FIG. 8. Electron micrograph of the HG of a frog on Day 4 of the acute E_2-treatment: note the hypertrophy of the interstitial tissue around the acini (stars). The glandular cells, with euchromatic nuclei and one or more nucleoli, are filled with secretory granules. ×2600.



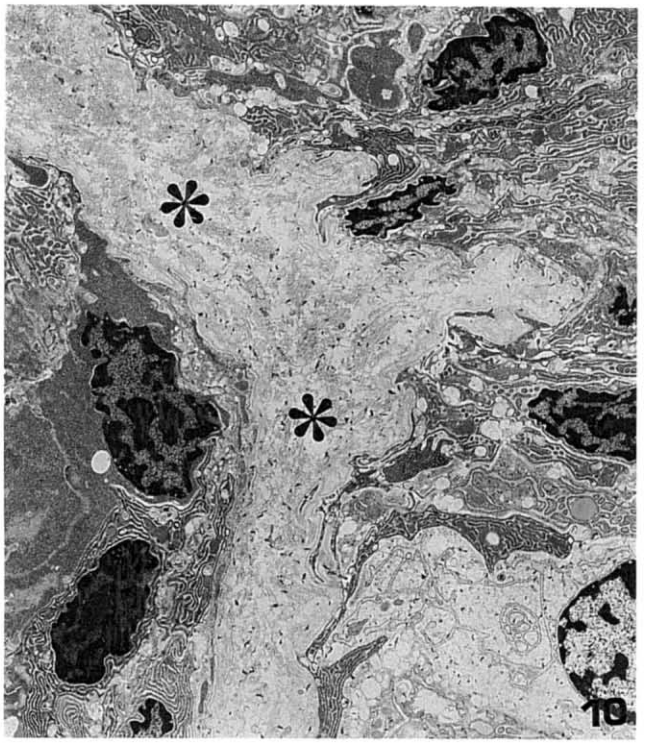
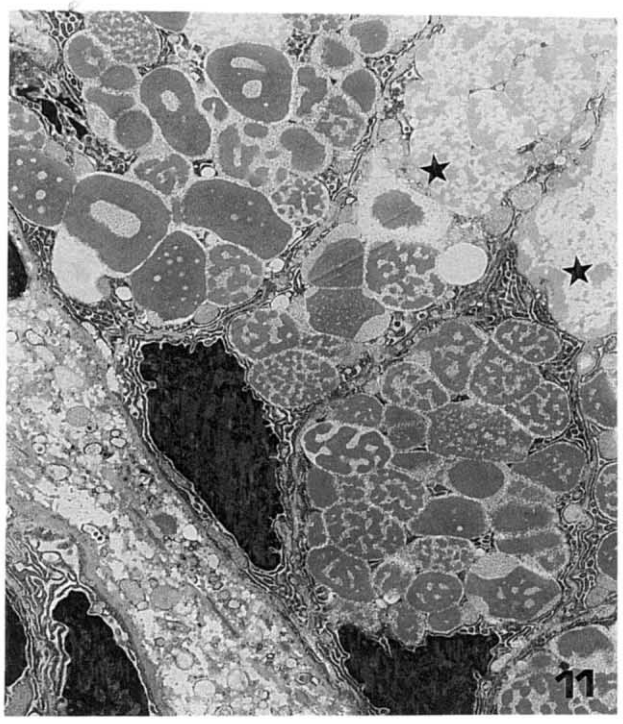


FIG. 9. Electron micrograph of a mast cell in the hypertrophied interstitial tissue of the HG on Day 4 of acute E_2-treatment. Note the heterochromatic nucleus and the cytoplasm filled with mature secretory granules containing "scroll like" components (arrows). ×4600.

FIG. 10. Electron micrograph of the HG of a frog on Day 8 of acute E_2-treatment. The acinar cells appear devoid of the secretory granules with a dilated cisternae of RER. Note the heterochromatic and irregular shaped nuclei. The interstitial tissue among the acini appears hypertrophic (asterisks). ×3800.



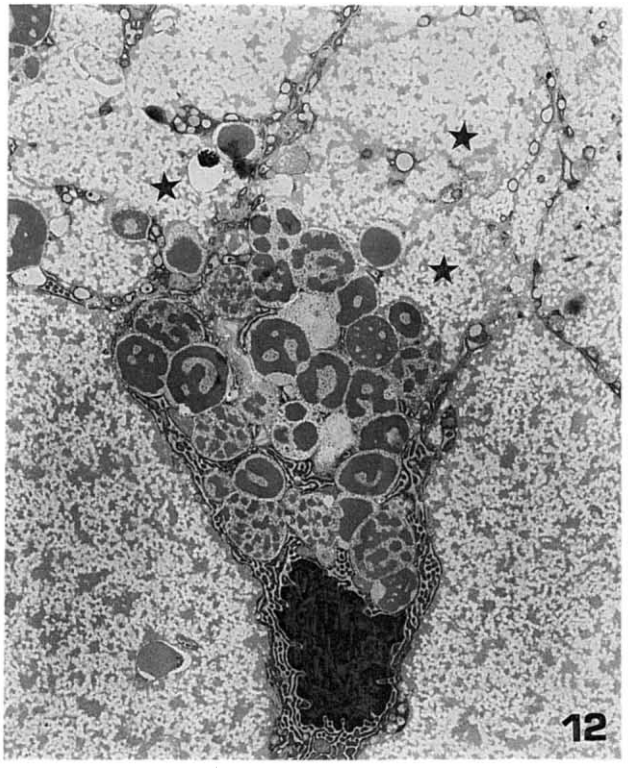


Fig. 11. Electron micrograph of acinar cells of the HG of a frog on Day 12 of acute E_2-treatment, showing damaged secretory cells. At the apex of the acinar cells the secretory granules coalesce (stars); the basally located nuclei tend to be pyknotic. ×2800.

Fig. 12. Electron micrograph of acinar cells of the HG of a frog on Day 12 of acute E_2-treatment, showing collapsed glandular cells (stars). Only one cell is still distinguishable in the disorganized epithelium of the acinus. ×2800.

hamster [27] and green frog [5]. The presence of specific testosterone-binding sites in the HG of both sexes of *Rana esculenta*, suggests that the cyclic activity might be correlated with the plasma levels of androgens [5]. In addition, it has been demonstrated by *in vitro* experiments that testosterone increases the incorporation of ^{3}H-uridine into the poly(A)$^{+}$ -RNA fraction and [^{35}S]methionine incorporation into HG proteins suggesting that the frog HG represents a target for androgens [1, 26].

The present observations have shown, besides the previously described effect of castration on the morphology of the glandular components [3], a hypertrophy and increased vascularization of the interstitial tissue following acute treatment with estradiol. Furthermore, castration provokes a decrease of MCN in January in both sexes, while no effect was observed in September when MCN is at its lowest value.

Estradiol-administration in both males and females prevents the castration-induced effect and increases the MCN both in January and in September. In addition, it causes an increase of the Alcian blue/PAS positive mucosubstances in both epithelium and lumina of the glandular acini; this last effect strongly suggests that estradiol may act as a maturating factor of the HG secretory granules. On the contrary, testosterone-treatment has no effect in rising MCN, while it is able to prevent the CS-effect in January.

Acute estradiol treatment provokes an increase in MCN on days 2 and 4. However, the morphology of the glandular compartment appears normal. MCN increase per cross section also occurs after 7 days of estradiol-treatment in the uterus of the musk shrew [19]. On days 8, 10 and 12 of treatment, when the total dose of estradiol/animal is non-physiological, the MCN drastically decreases and the majority of the glandular acini appear strongly disorganized; the interstitial connective tissue becomes hypertrophic in concomitance with the increased vascularization. Connective tissue proliferation after estradiol-treatment has also been found in different accessory sex organs of the rabbit [20] and rat [11]. Many other studies are derived from acute treatment with high doses of estradiol to help explain the higher prevalence of neuroendocrine disorder in females, the augment of mast cell secretion and the possible therapeutic effects following hormonal administrations (for review see [28]).

Similar results have been obtained in the toad *Bufo viridis*. Estradiol-treatment to ovariectomized animals provokes a marked increase in MCN. In contrast ovariectomy does not affect the MCN while a slight increase in MCN occurs in testosterone-treated toads [17].

Also in mammals sex hormones influence MCN in the HG of the hamster and mouse. The female HG contains more mast cells in the interstitial tissue than does the male gland; castration of males causes an increase of MCN and this change is prevented by androgen administration or subcutaneous implantation [15, 21, 25].

The responsiveness of mast cells to steroid hormones has been well-established in the female rat and hamster uterus in

TABLE 2. Histochemistry of the Harderian gland of the frog, *Rana esculenta* after the acute E_2-treatment

	CONTROL Day 0		Day 2		Day 4		Day 8		Day 10		Day 12	
	E	L	E	L	E	L	E	L	E	L	E	L
Alcian	+	±	+	±	+++	+	+	++	+	++	++	+++
Alcian-PAS	+	+	+	++	±	++	±	++	±	++	±	++
PAS	+	−	+	−	++	±	+	+	±	+	−	+

E: epithelium; L: lumina.
The intensity of the reaction is classified into five categories from- to +++.

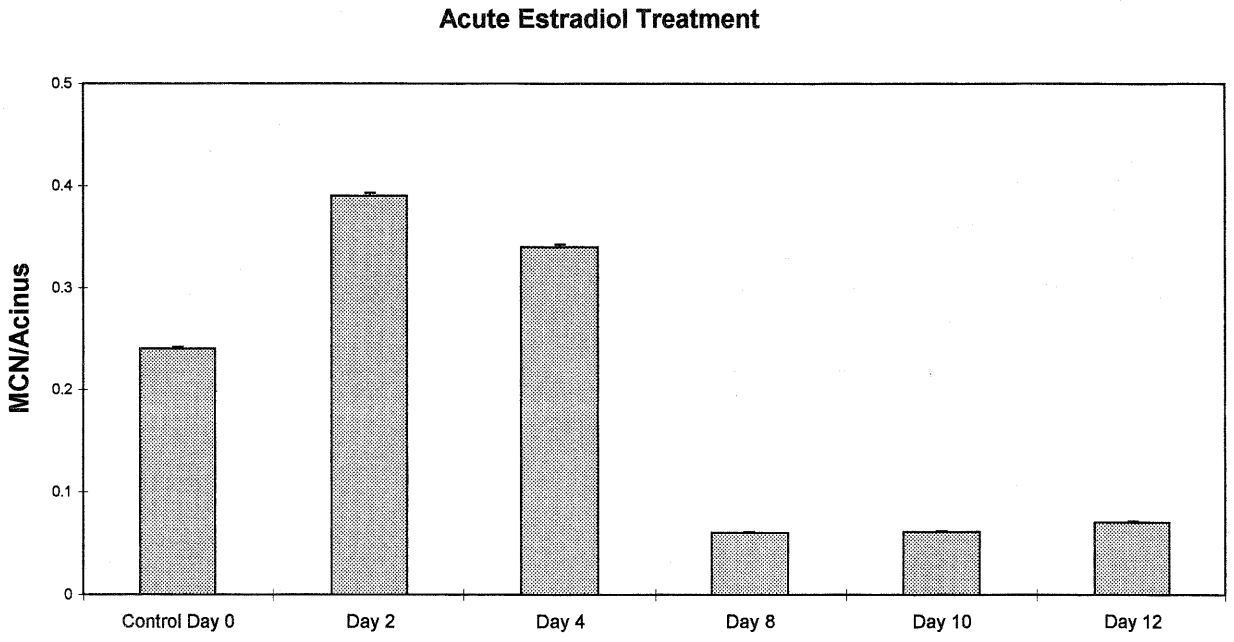


FIG. 13. Variation of MCN in the HG during acute E_2-treatment. Vertical bars indicate standard error.

which changes in MCN have been found during the estrous cycle [12, 14] as well as in other tissues such as the peritoneum [18].

Gaytan *et al.* [11] have found an increase of mast cells in the interstitial tissue of the rat testis after estradiol treatment, and suggested that estradiol treatment alters an inhibitory factor for mast cells which is normally present in the interstitium.

Our data suggest that acute estradiol-treatment is more effective in rapidly promoting proliferation of the interstitial connective tissue, together with a strong acinar cell damage than chronic E_2-treatment. The increase in MCN and subsequent decrease on days 8, 10, and 12 of the treatment could be due to these rapid and dramatic changes of both the connective tissue and glandular acini.

In conclusion, the present results suggest that estradiol-treatment induces changes in MCN and, following acute estradiol-treatment also, proliferation of the interstitial connective tissue together with acinar damage in the HG of the green frog. The mechanism of these changes is still unclear [14, 18].

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