A Study of the Testis Tubules, Interstitial Tissue, and Sex Characters (Thumb-pads and Wolffian Ducts) of Normal and Hypophysectomized Frogs (*Rana esculenta*)

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SUMMARY

1. After hypophysectomy in October the testes and some sex characters (Wolffian ducts and thumb-pads) of *Rana esculenta* were investigated during 9 subsequent months.

2. The so-called prespermatogenesis (Champy, 1913) is completed normally after hypophysectomy, but the primary spermatogonia lose their capacity for division. Therefore 8 months after the operation the testis tubules contain only primary spermatogonia.

3. The spermatogonia of the testes of control frogs, kept under laboratory conditions from October onwards, show a precocious activity during the winter, but in July new spermatozoa have not yet been formed.

4. In both control and experimental frogs the spermatozoa, formed during late summer and autumn, almost entirely disappear in the spring, even in the absence of copulations.

5. During the winter and spring months the interstitial testis cells of the control frogs undergo a reduction in both number and function, and this coincides with the increased activity in the testis tubules which occurs under laboratory conditions.

6. Conversely the decreased spermatogenetic activity, caused by hypophysectomy, coincides with an increase in the number of interstitial cells. The cytoplasm of these cells, however, is greatly reduced, although the nuclei maintain their typical form and size till at least 9 months following the operation.

7. Thumb-pads and Wolffian ducts are strongly affected by hypophysectomy.

8. The cytological changes brought about in the interstitial cells of *R. esculenta* after hypophysectomy do not quite coincide with changes in the development of the thumb-pads and Wolffian ducts. Therefore no new evidence for the endocrine function of these cells can be given.

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INTRODUCTION

THE present paper is one of a series in which the endocrine function of
the interstitial tissue of the testis is being examined. It also forms part
of a second series, in which it is intended to deal with the regulation of
spermatogenesis, and in which the phenomenon of spermiation in Rana
and Salamandra (van Oordt, Creutzberg, and Spronk, 1949) has already
been described.

In the papers on the interstitial tissue of mammals (Sluiter, 1945) and of
birds (Sluiter and van Oordt, 1947, 1949) conclusions were reached regarding
its endocrine function and regarding the cells in which the hormone is formed.
A comparison was made between the cytology of normal interstitial cells and
those from animals treated with gonadotropins, the state of the sex accessories
serving as an indicator for the hormone secretion.

In the present paper the reverse experiment, i.e. hypophysectomy, has been
used to obtain further information.

So far a detailed cytological description of the interstitial cells of the frog's
testis, following hypophysectomy, has not been published. However, the
changes in the gonads of hypophysectomized frogs have already been investigat-
ged by Gallien (1940) in Rana temporaria, but as the sexual cycles in the
testes of both Rana-species are very different (Champy, 1913) a careful study
of the testes of R. esculenta after hypophysectomy seems important.

Moreover, the well-known fact (Harms, 1926) must be taken into account
that confinement during the winter months has a great influence on frogs.
When they are protected against extremely low outdoor temperatures and fed
regularly, frogs acquire a sexual cycle which differs completely from that of
frogs hibernating in nature. Consequently, in the present paper we shall com-
pare three different groups of frogs:

(1) animals recently caught in the wild ('normal animals'),

(2) animals kept for some time in the laboratory at room temperature and
fed regularly ('controls'), and

(3) hypophysectomized animals kept in the laboratory under the same con-
ditions ('experimentals').

MATERIAL AND METHOD

On 11 and 13 October 1948, some days after they were caught, the experi-
mental frogs were hypophysectomized, using the method of Mighorst (Sluiter,
Mighorst, and van Oordt, 1949). They were then kept, together with the con-
trols, in a large vivarium at room temperature and fed with mealworms, which
were taken actively. The mortality was rather high only during the first few
days following the operation, and several experimentals lived till July of the
next year.

Both experimentals and controls were killed regularly during the period
from October till July, the intervals varying from 2 or 3 weeks to 2 months.
Of each animal one testis, both thumb-pads, and both Wolffian ducts were fixed in Bouin's solution, sectioned (7 or 10μ), and stained with haemalum-eosin. The other testis was used for detailed cytological investigations and was fixed in Kolster's fluid, sectioned at 3μ, and stained with Altmann's acid-fuchsin, combined with brilliant-cresyl-blue.

**RESULTS**

**Testes**

**Testis Tubules**

*Spermatogenesis.* According to Champy (1913) a so-called prespermatogenesis occurs in *R. temporaria* as well as in *R. esculenta*. It was described as the process whereby the primary spermatogonia do not develop further than into primary spermatocytes, which soon degenerate. As no spermatozoa are formed during this process we think it better to call it prespermatogenetic activity.

In *R. temporaria* it was found (Champy, 1913) that prespermatogenetic activity occurs only during May and June. The formation of spermatozoa takes place in July, August, and September. During autumn and winter very many bundles of spermatozoa are present in the testis tubules, but no spermatogenetic activity occurs. On the other hand, Champy (1913) found that in *R. esculenta* the prespermatogenetic activity continues all the year round, with the exception of the period from July till October, when spermatozoa are formed. Consequently, during the whole year the testis tubules of *R. esculenta* contain cell nests which are derived from spermatogonia by subsequent simultaneous divisions.

In order to determine the prespermatogenetic activity quantitatively, Champy (1913) counted the number of degenerating spermatocytes present. This complicated method was not used by the present authors. Instead, the testis tubules were divided into six different stages after estimations had been made of the number of spermatogenetic cells present in each cross-section. These tubes have been classified as follows (cf. Text-fig. 1):

Stage-number 0: testis tubule with a few primary spermatogonia present.

" " 1: testis tubule with primary spermatogonia and one small cell nest.

" " 2: testis tubule with primary spermatogonia and several small cell nests.

" " 3: testis tubule with primary spermatogonia, several small and at least one large cell nest.

" " 4: testis tubule with a closed row of cell nests at its periphery.

" " 5: testis tubule almost totally filled with cell nests.

In order to determine the spermatogenetic activity of a testis, the various stages present in twenty cross-sectioned testis tubules were estimated. Then
the average stage-number of these twenty tubules was taken as representing the spermatogenetic activity of the gonad as a whole. As a precaution several testis sections were used for each estimation, as in every testis section between three and six different tubule stages are generally to be found.

Text-fig. 1 shows the spermatogenetic activity of controls and experimentals. Controls. During the period October–January a gradual decrease in prespermatogenetic activity takes place, as in normal animals (cf. Champy, 1913, graph I, p. 46) Thus confinement, a relative high temperature, and regular feeding have no influence on the prespermatogenetic activity during this period.

From January onwards the reaction of the controls is not so uniform. At the beginning of January the spermatogenetic activity of one of the controls was very strong, but that of another, representing the normal state, was weak.

In control specimens, killed at the end of March, the end of May, and the beginning of July, the spermatogenetic activity was much stronger than normal. Apparently the laboratory conditions cause a precocious onset of spermatogenesis. In the laboratory spermatogenesis begins as early as February, but in the wild it does not do so before the beginning of July. The process itself is, however, much delayed, because in July spermatozoa were not yet formed in the testis tubules of control frogs.

Experimental. In the period from October to January the spermatogenetic activity of the experimentals shows the same decrease as that of the controls. Three months after hypophysectomy, however, this decrease still continues so that by May stage 0 is reached.
Therefore we may conclude that the increasing activity which was found in the controls from January onwards is at least partly due to the influence of the pituitary. As the spermatogenetic activity of the controls and experimental is almost identical from October till January, it is probable that during this period the pituitary has no gonadotropic function.

*Spermatozoa.* Under natural conditions the testis tubules of *R. esculenta,* like those of other *Rana*-species, contain large quantities of spermatozoa during the winter months. These spermatozoa have been produced in summer and early autumn. In the control specimens, however, the number of spermatozoa varies widely. Whether this variability was due to the laboratory conditions or not could not be ascertained.

In spite of the fact that copulation did not take place, the number of spermatozoa present in the testes of control frogs decreased considerably in May.

As far as could be determined, the quantity of spermatozoa until May was much larger in the experimental than in the controls. In this month a marked decrease in the number of spermatozoa also occurred in the testes of the experimental. This suggests that the decrease is not caused by a hypophyseal influence, but may be due to the limited duration of life of the spermatozoa stored in the testis tubules.

**Interstitial Tissue**

According to Champy (1913) the periodic changes in the interstitial tissue are different in *R. temporaria* and *R. esculenta.* Unlike *R. temporaria,* the interstitial tissue of *R. esculenta* is well developed during the greater part of the year. Only from July till the beginning of October, the spermatogenetic period, is it poorly developed.

Several times already we have pointed out (Sluiter, 1945; Sluiter and van Oordt, 1947, 1949) that in studying the endocrine function of the testis it is not sufficient to pay attention only to the volume of the total intertubular tissue. It is generally known that in most of the vertebrates interstitial cells can be distinguished, which by their structure and contents suggest a secretory or a storage function. To judge the activity of the intertubular tissue at a particular moment it is therefore necessary to pay attention to the number and to the physiological state of these cells.

During the long period in which the intertubular tissue of normal specimens of *R. esculenta* is well developed it is, according to Champy (1913), composed mainly of large cells, containing lipoid droplets and mitochondria. As spermatogenesis increases during July, the lipoid progressively disappears from the interstitial cells and moves into the testis tubules. At the same time a regression of the interstitial cells occurs, with the resulting formation of connective tissue-like cells. At the end of the spermatogenetic period these cells are again transformed into the active cells, described above.

The results of our quantitative investigations into the interstitial cells with regard to their total surface in sections may be seen in Text-figs. 2-5 and 10-
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12; the cytological details of these cells are shown in Text-figs. 6–9 and 13–15.

**Control-specimens** (Text-figs. 2–9). In Text-figs. 2 and 3, showing testis sections of control frogs killed during the autumn and winter months, the

![Text-fig. 2](image1)

![Text-fig. 3](image2)

![Text-fig. 4](image3)

![Text-fig. 5](image4)

**Text-figs. 2–5. Rana esculenta.** Testis sections of control frogs, autopsied respectively 12 Oct. 1948, 17 Nov. 1948, 4 March 1949, and 7 July 1949. The diameter of each circle is proportional to the average lengths of the long and short axis of both testes. Black: total surface of the interstitial cells; dotted: spermatogenerative cell nests. Primary spermatogonia and spermatozoa are not drawn. Champy-fixation; Altmann-staining. (All figs. ×120.)

interstitial cells cover a relatively large area of the testis section. In this period they possess a large round nucleus and a cell body filled with many lipoid droplets and mitochondria. The size and contents of these cells vary during this period between the limits drawn in Text-figs. 6 and 7, respectively. This description is in agreement with that of Champy (1913) for normal green frogs from October to July.

At the end of March, however, the total section area occupied by the interstitial cells of the control frogs is somewhat smaller (Text-fig. 4). This is due
to the fact that most of the interstitial cells have become smaller and more elongated (Text-fig. 8), but they still contain some large lipoid droplets and scattered mitochondria.

In the period from the end of March until July the total section area of the interstitial cells decreases gradually (Text-fig. 5) and the majority of them now take the form of an intertubular connective tissue cell (Text-fig. 9). As stated above, this same regression takes place in the interstitial cells of normal animals, but in these it does not begin until July. Therefore we can conclude that under laboratory conditions the regression of the interstitial cells occurs precociously, and that in laboratory animals it is also accompanied by an increase of the spermatogenetic activity.

Experimentals (Text-figs. 10–15). During the autumn and winter months the total section area of the interstitial cells in the testes of control and experimental specimens is about the same (Text-fig. 10). In spring, however, the section area increases slightly (Text-fig. 11), contrary to what has been described in the controls. In July, about 9 months after hypophysectomy, many interstitial cells are still identifiable as such (Text-fig. 12), but the large cell-groups have divided into small ones (Text-figs. 11 and 12).
In cytological preparations it is obvious as early as 2 weeks after the operation that a varying number of interstitial cells have decreased in size. This decrease affects the cell size, but not the size of the nuclei. Consequently the nuclei lie closer together, the mitochondria are concentrated, and there is no room for lipoid droplets. Moreover, the number of nuclei has markedly increased. Five months after hypophysectomy the condition of Text-fig. 14 is reached. Nine months after the operation the number of cells may have decreased, but the cytological structure is still the same (Text-fig. 15). The cells are intact, but there is very little physiological activity. Obviously the regression stops at this stage.

As regards some of the experimental animals, autopsied during the first 5 months after the operation, the cytology of the interstitial cells differs markedly from that described above. Excessive numbers of vacuoles develop, while most mitochondria disappear (Text-fig. 13). Occasionally there are so many vacuoles that the nucleus is indented, suggesting a fatty degeneration of the cell.

It can be concluded that the regression of the interstitial cells in the testes of hypophysectomized frogs is distinctly different from that of normal and control animals. The cytology of the interstitial cells of the experiments suggests that possibly the secreting and certainly the storage capacity of the cells decreases. But it is important to notice that in hypophysectomized frogs the number of interstitial testis cells increases strongly during their functional regression. This takes place at a time when the spermatogenetic activity is decreasing.
At the end of the autumn the normal animals also show an increase in the number of interstitial cells and a decrease of spermatogenetic activity, but, unlike the experimentals, the function of these cells is resumed at the same time.

**Secondary and Accessory Sex Characters**

Experimentally induced changes in the sex characters, viz. in the thumb-pads and Wolffian ducts, may be of special interest, as they can be used as indicators of a changed hormone-production in the testis.

**Thumb-pads**

As is generally known, the histology of the frog's thumb-pads shows a distinct yearly cycle. After maximum development in the copulation period a marked regression occurs, but during the summer a new development starts, which gradually leads to the maximum condition.

*Controls.* In October and November a thick corneous cuticle with rather large papillae covers the epidermis, beneath which large glands with high secretory cells are visible (Text-fig. 16). Further development is slow, so that during the copulation period the thumb-pads of the controls do not reach their maximum development, this being probably due to the laboratory conditions.

As a regression of the thumb-pads begins after castration (Harms, 1926) it seems possible that the development of these organs is regulated by the inter-
As a matter of fact, in autumn the quick development of the thumb-pads coincides with the presence of interstitial cells, the cytology of which suggests an active secretory function. However, in our control frogs the thumb-pads may maintain their full development till July, whereas the activity of the interstitial cells disappears in spring. Therefore it cannot be demonstrated from the controls alone that the interstitial cells regulate the development of the thumb-pads.

**Experimentals.** According to Gallien (1940) in *R. temporaria* a regression of the thumb-pads begins very soon after hypophysectomy. Two weeks after the operation, in October 1948, the cuticle of the thumb-pads of our hypophysectomized green frogs was already thin, too; its papillae were lacking and the epidermal glands were small, with low cells and a large lumen (Text-fig. 17). This condition was maintained till July 1949.

Now it might be possible that the regression of the thumb-pads is caused by a decreased function of the interstitial cells, which no longer secrete their androgen after the loss of the gonadotropic potency of the pituitary. However, we have seen (p. 138) that in the experimental frogs, 2 weeks after hypophysectomy, the cytology of the interstitial cells suggests only a small decrease in secretory activity, which is not sufficient to explain the quick and active regression of the thumb-pads. It is therefore impossible to decide with certainty whether this regression is caused by the decreased secretion of androgens in the interstitial cells or not.

**Wolffian Ducts**

In the male green frog the Wolffian ducts do not possess seminal vesicles as they do in the grass frog. In the male *R. esculenta* the Wolffian ducts have larger diameters than in the female. Moreover, their interior epithelium shows several folds, which may act as sperm-reservoirs.
In the normal *R. esculenta* periodical changes in the Wolffian ducts similar to those in the seminal vesicle of *R. temporaria* (Harms, 1926) are not known. In control specimens from October to July the epithelium of the Wolffian ducts was always high and folded (Text-fig. 18). Periodical changes could not be demonstrated.

In the experimentals, however, the Wolffian ducts show distinct changes after hypophysectomy; their diameters decrease, while their epithelium becomes low and loses the folds of the normal duct (Text-fig. 19). The regression is visible as early as 2 weeks after the operation and it continues until the end of April. From then on the situation remains stationary.

Again the question arises whether the pituitary acts on the Wolffian ducts directly or indirectly via the testes. As the Wolffian ducts in *R. temporaria*
regress after castration, it is possible that in green frogs the influence of the pituitary acts via the testes. But here, too, the cytological investigation of the interstitial cells does not give any indication. For in our control animals the interstitial cells undergo a strong regression from March till May, whereas the Wolfian ducts do not regress either during or after this period. That both interstitial tissue and the epithelium of the Wolfian ducts show regression after hypophysectomy might, therefore, depend on no causal relation between these two tissues.

**DISCUSSION**

As we have seen, the prespermatogenetic activity, which in *R. esculenta* occurs in normal animals from October to January, is not interfered with by hypophysectomy (Text-fig. 1). The impression is gained that after ablation of the pituitary the mitotic activity of the primary spermatogonia is arrested and that only their descendants, present at the time of operation, develop further till they have all passed over into primary spermatocytes. They then degenerate rapidly. Consequently, only the primary spermatogonia survive, and 8 months after hypophysectomy all other spermatogenetic cell stages have disappeared from the testis tubules. The latter phenomenon has also been mentioned by Gallien (1940) in hypophysectomized grass frogs.

It is therefore not improbable that only one link in the process of spermatogenesis, viz. the division of the primary spermatogonia, is conditioned by the pituitary.

According to Champy (1913) the number of spermatogonia that divide under natural conditions during the winter months is very small. Whether this phenomenon depends on a shortage of pituitary-hormone or on a non-susceptibility of the spermatogonia for the gonadotropic hormone is not easy to decide. On the other hand, spermatogonial divisions can easily be effected during the winter, provided the pituitary is present. Frogs kept under laboratory conditions (room temperature and plenty of food), possess testis tubules showing strong mitotic activity. In these many cell nests arise (Text-fig. 1), although these processes do not lead to the formation of spermatozoa. Under natural conditions, however, spermatogenesis does not begin before June, but spermatozoa are already present in July (Champy, 1913). Consequently, in the laboratory the gonadotropic influence of the pituitary does not seem to be sufficient to stimulate complete spermatogenesis.

The disappearance of the winter spermatozoa from the testis occurs in the laboratory at the same time that copulations are performed in the field, although in the laboratory copulations cannot take place. After hypophysectomy the spermatozoa also disappear, and it appears probable that this phenomenon is due to the limited duration of life of the spermatozoa, which are absorbed 7-10 months after having been formed.

In the foregoing pages (pp. 137 and 138) it has been shown that during a decrease in spermatogenesis, under normal (Champy, 1913) and under labora-
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In tissue and sex characters, there is an increase in the mitotic activity of the interstitial cells, and vice versa. Moreover, the absence of the pituitary does not inhibit the multiplication of the interstitial cells. Therefore the impression is gained that the spermatogenetic and interstitial tissues compete with each other for room and for the food substances supplied by the blood. This explains also the cyclic alternation in the development of both tissues under natural conditions.

The function of the interstitial cells depends on the pituitary. Under normal as well as under laboratory conditions an increase in number of these cells is accompanied by an increase in function (Text-figs. 6 and 7). But following hypophysectomy (cf. Text-figs. 14 and 15) the number of cells increases, whereas their volume, and therefore also their function, decreases. This decrease, however, does not fall below a certain threshold in R. esculenta, for the interstitial cells retain their large typical nuclei at least 9 months after hypophysectomy.

Therefore it is concluded that in R. esculenta the pituitary promotes and maintains only the functional activity and not the mitotic activity of the interstitial cells.

It is probable that in Rana, as in the higher vertebrates, the interstitial cells produce a hormone by which, for example, oestrus, the sex characters, and partly also spermatogenesis are influenced.

According to Champy (1913), however, the activity of the interstitial cells in R. esculenta is not increased either before or during the copulation period, and it alternates with the spermatogenetic activity. The same alternation was stated in R. esculenta under laboratory conditions (p. 137). In R. temporaria the short period during which the interstitial cells are active actually occurs shortly after the copulation period. Therefore in both Rana-species it is not possible from the data of the normal cycle to establish an endocrine activity of the interstitial tissue on oestrus or spermatogenesis.

As to the sex characters, the thumb-pads, and the Wolffian ducts, it is likely that these are conditioned by the interstitial cells. The thumb-pads of R. esculenta resume their development in the period between copulation and spermatogenesis, that is, when the interstitial cells are still very active (Champy, 1913; Harms, 1926). Moreover, an immediate reduction of the thumb-pads and seminal vesicles occurs after castration (Harms, 1926).

We have seen that the same phenomenon takes place after hypophysectomy. Therefore an indirect influence of the pituitary on the sex characters, acting via the interstitial cells, is not impossible. But as we have argued above (p. 140) the cytological changes experimentally induced in the interstitial cells do not point to this action.

De Allende (1939) has demonstrated that in toads, Bufo arenarum, the pituitary has a direct influence on the development of the oviduct. Therefore it might be possible that in Rana sex characters like thumb-pads and Wolffian ducts are also directly dependent on the pituitary. If this were true, it would present another example of double insurance by which the development of these sex characters is directly influenced by both pituitary and gonads.
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