V. *The Reproductive Cycle of the Three-Spined Stickleback,* Gasterosteus aculeatus, *Linn.*

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PART IV.—THE REGULATORY MECHANISMS OF THE REPRODUCTIVE CYCLE.

Introduction.

The investigations described in this paper were made in the Zoological Laboratory, Cambridge. I am greatly indebted to Professor J. S. GARDINER, F.R.S., for providing the special apparatus and accommodation required for the experimental work. I am also very grateful to Dr. F. H. A. MARSHALL, F.R.S., for his valuable advice and criticism.

Objects of the Research.

A study has been made of the cyclical changes which occur annually in the reproductive organs of a fish living in a temperate climate. The fish chosen has marked seasonal secondary sexual characters in the male, and the relation between the development of these and the changes in the testis has been investigated in detail.

An experimental examination, suggested by these studies, has been made of the possible influences of physical and biological changes in the environment on the reproductive cycle. The migration of the fish which occurs in connection with reproduction was examined to ascertain whether it bears a relation to the reproductive cycle.

Previous Work on the Reproductive Cycle of Fish.

The cycle of changes occurring in the reproductive organs of fishes has been fully investigated in a very limited number of cases.

The nuclear changes accompanying spermatogenesis are known from AGAR'S investigations on *Lepidosiren* (AGAR, 1911). The maturation of the egg of Teleosts has been described by CUNNINGHAM (1890), WALLACE (1904), WHEELER (1924), and others.

The complete history of the germ cells in both the sexes of *Cottus bairdii* is given by HANN (1927). This a fresh-water species of *Cottus* found in Michigan, Canada.

HANN'S paper is primarily concerned with the origin and early phases of the germ cells, but it also gives a clear account of spermatogenesis and oogenesis.

Oogenesis.—Oogenesis in fish may be divided into two stages—(*a*) the division of the oogonia, and (*b*) the transformation of the oocytes into mature ova.

Resting oogonia are found in the ovary of *Cottus* throughout the whole year. Oogonial division and early maturation phases are found in the young female seven weeks old, that is about in the second week of June, and this oogonial division continues until October. A portion of the oocytes which begin development the first year mature when the individual is two years old. In the next and successive years oogonial division commences in the middle of May and continues until October. A reserve supply of oocytes is thus formed. " Thus each year after the first, a portion of the oogonia change to oocytes and increase the reserve supply, and at the same time a portion of the reserve supply enter the secondary growth phase and develop during the year into mature ova." (HANN, 1927, p. 464).

The spawning season of *Cottus bairdii* in the locality from which HANN took his samples was in April. Secondary yolk formation (" maturation " as used by HANN) begins about midsummer and lasts until October.

Other investigators (CUNNINGHAM, etc., *cit.* above) have found the growth period of the egg varies with the species, in a number of cases this occupies only one year. Where known, the period of oogonial division occurs as in *Cottus*, shortly after the breeding season.

Spermatogenesis.—As in oogenesis, there are two phases in spermatogenesis: (*a*) the division of the spermatogonia (" germ cells," see HANN, *loc. cit.*, p. 470), and (*b*) the maturation stages.

In *Cottus* the division of the germ cells of the male occurs when the individual is 13 months old, about a year later than in the female. " There result within the testis groups of spermatogonia within thin-walled cysts. With the beginning of maturation these cysts become very distinct and all cells of one cyst are practically at the same stage, though different cysts show different stages. Maturation continues until the spawning time in April."

TURNER'S account of the seasonal changes in the testis of the Perch, *Perca flavescens* (TURNER, 1919), differs from that given by HANN for *Cottus* in two respects.

According to TURNER, in this species there is " a cord of germ cells outside the body of the testis from which the testis is periodically supplied. . . . An active migration of germ cells occurs from the cord, outside the testis, to the ends of the lobules at the periphery of the testis " (TURNER, *loc. cit.*, p. 703). Secondly, the case of *Perca flavescens* differs from that of *Cottus bairdii*, in that spermatogenesis is completed by November, and breeding begins in March. The translation of the " germ cells " from the cord outside the testis into the latter is in progress from March to August; the proliferation of these germ cells within the testis, which corresponds with the division of the spermatogonia in *Cottus*, lasts from March to November.

GEISER has given an interesting but short account of the spermatogenesis of *Gambusia affinis*, the top-minnow (GEISER, 1922). " The testis does not possess a cord of germ cells for the renewal of the testis after spermatogenesis. The spermatogonia develop from inconspicuous germ cells lying in the stroma of the testis between the cysts.

These cells migrate periphally (outside the zone of the *vas deferens*) and give rise to new spermatogonial cysts. The period of greatest sexual activity (maximum testis volume) in early spermatogenesis is March-July. The onset of this period appears to be conditioned largely by the temperature and light conditions. In November-February the testes attain their maximum size. No spermatogenesis occurs during the cold winter weather" (GEISER, 1922, p. 105). The Cyprinodonts, to which family *Gambusia* belongs, are sub-tropical fishes with many tropical representatives (GUNTER, 1880).

VAN OORDT (1924) described the spermatogenesis of the Nine-spined Stickleback, *Pygosteus* (*Gasterosteus*) *pungitius*. When spawning is over there is a division of the resting spermatogonia within the tubules of the testis. This is followed by the maturation of the spermatocytes which, as in *Cottus*, occur within cysts. According to VAN OORDT, the spermatozoa are usually retained within these cysts until the breeding season, although active division of the spermatocytes may be completed some time before the beginning of the spawning season.

COURRIER (1922) kept Three-spined Sticklebacks at 17° C. for one and a half months and found that spermatogenesis was completed at the end of that time. COURRIER does not state when the experiment was begun, but it seems clear that it was made during the winter months. The result was that, although spermatogenesis was completed, breeding did not take place.

There is a close similarity between the time and relations of the different phases of spermatogenesis in *Cottus*, *Perca* and *Pygosteus*. The division of the "germ cells" (spermatogonia) begins soon after the spawning season. Maturation occurs in autumn and is completed in late winter. Spermatogenesis may be completed prior to the spawning season in all three species.

TURNER (1919) points out the close relation between these times and certain phases of the temperature cycle of the environment of the fish. "The beginning of the period of spermatogenesis is contemporaneous with the beginning of the seasonal reduction of the temperature of the water in which the Perch is found. The expulsion of the spermatozoa occurs at the same time as the seasonal rise in temperature of the water" (TURNER, 1919, p. 704).

Definitions and General Considerations on the Reproductive Cycle of Fish.

The life history of a fish living under temperate climatic conditions is divisible into three clearly marked periods :—

I.—*Embryonic Period*, terminating with hatching.
II.—*Prepubertal Period*, terminated with sexual maturity.
III.—*Sexual Maturity*, which period lasts until death.

(A *Sexually Mature Fish* is one which during the breeding season produces functional gametes.)

At the end of the Prepubertal Period the fish enters upon a recurrent cycle of changes directly associated with reproduction. *The Period of Sexual Maturity* may therefore be divided into the following *phases* :—

Phase.	In the Male.	In the Female.
i.	Growth of the Spermatocytes ..	.. Growth of the Oocytes.
ii.	Maturation (or Spermatogenesis)	.. Secondary growth phase of the Oocyte (yolk formation).
iii.	Discharge of the Spermatozoa ..	.. Discharge of the Ova (frequently accompanied by Maturation).

Phases i and ii are distinct processes, but they follow one another without pause. Although the differences between Phase ii in the male and female are at first sight considerable, these differences are more apparent than real. Both phases result in functionally mature gametes, that is gametes capable of fertilization or of being fertilized. Phase ii in the female is often referred to as Maturation (*e.g.*, HANN, 1927, p. 464). On the other hand, Maturation is frequently restricted to the nuclear changes (meiosis or reduction division) (WILSON, 1925, p. 1136; MARSHALL, 1922, p. 127). The combination of Phases i and ii in both sexes will be termed *Gametogenesis* in this paper.

Phase iii does not necessarily follow on the end of Phase ii in the male (*e.g.*, TURNER, 1919, quoted page 200 herein). It will be shown in this paper, and it may also be deduced from HANN (1927) that Phase iii is not always immediately followed by Phase i.

Four *Critical Points* may be distinguished therefore in the annual cycle of the adult fish :—

Critical Point.

1. The beginning of Phase i.
2. The end of Phase ii.
3. The beginning of Phase iii.
4. The end of Phase iii.

From the standpoint of the reproduction of the fish, the commencement of the period of *Sexual Maturity* (Period III) is a major *Critical Point* in the life of the animal.

It will have been noticed from the account of previous investigations on the reproductive cycle of fish that there is some disagreement as to the term which should be applied to the small resting cells in the testis which give rise by mitotic division to each annual crop of spermatocytes. These are frequently referred to as " resting germ cells " (*vide* HANN, TURNER, GEISER, *loc. cit.*) but VAN OORDT terms them spermatogonia. By definition these cells must be spermatogonia (WILSON, 1925), and in this paper I have used the term spermatogonia for all germ-cells in the testis which are not spermatocytes, spermatids, spermatozoa, or cells of Sertoli.

The cycle of the germ cells corresponding to the *Periods* in the life of the fish described in this paper (*Gasterosteus aculeatus* L.) is as follows :—

Period.	In the Male.	In the Female.
I.—Indifferent period, origin and migration of germ-cells.		
II.—Period of Sex differentiation.		
	Division of the Spermatogonia.	Division of the Oogonia.
III.—i.	Growth of Spermatocytes	Growth of the Oocytes.
ii.	Division of the Spermatocytes ..	Division of the Oocytes.
	Secondary Spermatocytes	Yolk formation.
	Spermatids.	
	Spermatozoa.	
iii.	Spermatozoa	Nuclear maturation of the egg.

Two further definitions may be considered here.

The *Spawning Season* of fish may be defined as that season of special activity of the generative organs when the fish is normally capable of extruding generative products, a definition adapted from that of HEAPE for the Rutting Season of Mammals (HEAPE, 1900).

The *Breeding Season* is " that period during which any male or female (mammal) is concerned in the production of young " (HEAPE, *loc. cit*).

The force of this distinction is apparent when the case of such fish as the Cyprinodonts is considered. Females of many members of this group may bring forth litters at approximately monthly intervals during eight to ten months during the year (VAN OORDT, 1925). A number of fish normally considered as belonging to a temperate climatic fauna show a reproduction of this type (*e.g.*, *Leuresthes*, CLARK, 1925).

A fish during *Periods* I and II of its life may be conveniently termed a *Young Fish*, those in *Period III*, *Adult Fish.*

The Interstitial Tissue of the Testis of Fish.

The evidence in favour of the hypothesis of BOUIN and ANCEL (1903) that the interstitial tissue of the testis is the source of the hormone which causes the development of the secondary sexual characters of the male, in mammals and probably other vertebrates, is considered at length in LIPSCHUTZ's book (LIPSCHUTZ, 1924) and the cytology of the tissue in relation to its possible hormonal function is reviewed by RASMUSSEN (1928). The hypothesis of BOUIN and ANCEL has been attacked by a number of workers, and STEIVE (1921) has proposed an alternative hypothesis that the interstitial tissue has a trophic relation to the germ cells of the testis. Since the reviews of LIPSCHUTZ and RASMUSSEN, COURRIER (1927) has shown that in a number of mammals with periodic spermatogenesis, there is strong circumstantial evidence in favour of the hypothesis of BOUIN and ANCEL.

The interstitial tissue of the testis of fish was first described by STEPHAN (1902) and since then it had been investigated by CHAMPY (1923), COURRIER (1921, *a*, *b*, *c*, 1922, *a*, *b*), KOLMER and SCHEMINZKY (1922), VAN OORDT (1923, 1924, *a*, 1924, *b*, *c*, and 1925), and ESSENBERG (1923).

CHAMPY (1923, *b*), ESSENBERG (1923) and VAN OORDT (1925) show that in the Cyprinodonts there is probably no correlation between the development of the secondary sexual dimorphisms of this group and the development of the interstitial tissue of the testis.

On the other hand, such fish as *Callionymus*, *Cottus*, *Girardinus* and *Gobius*, COURRIER (COURRIER 1921, *a*) found to have the interstitial tissue well developed when the secondary sexual characters were developed, and in the case of *Callionymus* at least, this tissue showed a cyclical development closely correlated to the cyclical changes of the secondary sexual characters. KOLMER and SCHEMINZKY (1922) record a further number of species in which there is interstitial tissue in the testis of fish.

CHAMPY (1923) denied the existence of interstitial tissue in *Spinanchia vulgaris* at the time when the secondary sexual characters were developed, but my own observations on this species do not confirm CHAMPY'S statement. In *Phoxinus lævis*, a species in which KOPEČ has shown that the secondary sexual characters are dependent on the gonad (KOPEČ, 1927), CHAMPY was unable to find any trace of interstitial tissue.

Gasterosteus aculeatus and the closely allied *Pygosteus* (*Gasterosteus*) *pungitius* have been investigated by CHAMPY (1923), COURRIER (1921, 1922, *a*, 1925) and VAN OORDT (1923, 1924, *b*, 1924, *c*). COURRIER observed that during the winter there was little interstitial tissue in the testis, but at the time of the breeding season this was well developed; CHAMPY criticised this conclusion, citing some specimens in which he claimed the secondary sexual characters were developed, but in which there was no interstitial tissue.

VAN OORDT examined a considerable number of *Pygosteus pungitius*, and confirmed COURRIER'S observations in part, but found a number of cases in which the interstitial tissue was well developed (" Ziemlich breit, mit zahlreichen Zwischenzellen "), and the secondary sexual characters absent.

In OSLUND'S review of " *Seasonal Modifications in Testes of Vertebrates* " (1929) this author makes some statements on the reproduction of fish which are so much in conflict with the accounts of the workers he cites that, in order to prevent misunderstandings, they must be considered here.

" Fish testes are made up of lobules containing cysts of germ cells. During the non-spermatogenetic season these lobules are small and contain only a few germ cells. (OSLUND, *loc. cit.*, p. 254–255). In the absence of a definition to the contrary by OSLUND, I presume *the non-spermatogenetic season* is that part of the year during which spermatogenesis does not occur, in the fish which have discontinuous spermatogenesis. OSLUND refers to TURNER'S account of the cyclical changes in the testis of the Perch (TURNER, 1919). From TURNER'S diagram (*loc. cit.*, p. 701) it is clear that during the

three months December to March there is no spermatogenesis, but the testis is full of spermatozoa.

" Though COURRIER, CHAMPY and VAN OORDT made observations on the same species *Gasterosteus aculeatus* " (it may be noted in passing that VAN OORDT made observations on *G. pungitius* not on *G. aculeatus* (VAN OORDT, 1924)), " the data offered are very meagre and do not cover the entire year . . . Chart I, which is based on all available data, makes it quite certain that the interstitial cells do not promote the appearance of nuptial apparel in this species" (OSLUND, *loc. cit.*, p. 257). It is impossible to reconcile this chart with the observations of VAN OORDT, which OSLUND summarises a few lines above and which include the observation that " in winter when the secondary sex characters are not developed, he found some testes in which there existed a broad interstitium with many interstitial cells" (Quoted from OSLUND, p. 256).

OSLUND continues summarising VAN OORDT : " He concludes from cytological findings that the interstitial cells increase when spermatogenesis is suspended because they become charged with nourishment." I have been unable to find any statement by VAN OORDT on the cytology of the interstitial tissue of his material ; on the contrary, he states " Da meine Hodenpräparate in BOUIN's Gemisch fixiert und mit Hämatoxylin-Eosin gefärbt waren, ist vom Zelleib der Zwischenzellem nicht viel zu sehen " (p. 387), and VAN OORDT used material fixed in BOUIN's mixture and stained with hæmatoxylin exclusively (p. 381, VAN OORDT, 1924).

Choice of Material.

For the object of this investigation it was considered that a fish with a single annual spawning season would be the most suitable material. The situation of the laboratory made the choice of a fresh-water species desirable.

A preliminary examination was made of the reproductive organs of three fresh-water species which had considerable differences in the duration of the spawning season and which were known to be suitable for experimental purposes. These fishes were :— The River Trout, *Salmo fario*, the Gold-fish, *Carassius* sp. and the Three-spined Stickleback, *Gasterosteus aculeatus*. A number of the Nine-spined Stickleback, *Pygosteus pungitius**, were also examined.

It was decided from these observations to make a detailed study of the changes occurring in the testis of the male *Gasterosteus aculeatus*.

The male fish was chosen because the typical phases in the maturation of the male (p. 5, *b*) are more readily recognised than the corresponding phases in the female. VAN OORDT's investigations (1922, 1923, 1924, etc.) on the reproductive cycle of the male *Pygosteus pungitius*, in addition to supplying a considerable amount of data, suggested

* *Pygosteus pungitius*, GILL is syn. *Gasterosteus pungitius* L. The nomenclature of TATE REGAN (1910) is followed in this paper.

some experimental investigations. The Three-spined species was chosen in preference to the Nine-spined on account of the nature of its secondary sexual characters (p. 213).

Source of Material.

An examination of specimens from different localities showed that there is a considerable variation in the rate of growth and in the maximum size reached by the fish. It was also found that there was considerable variation in the state of the reproductive organs. It seemed possible that the apparently contradictory conclusions of COURRIER and VAN OORDT as to the time of completion of spermatogenesis might be due to different sources from which they drew their supplies, but it also has to be borne in mind that the species examined by COURRIER and VAN OORDT were different and there is possibly a difference due to this.

Accordingly an examination was made of both fish from different sources, and of both species. Detailed records were made of fish from one source and these were supplemented by observations made on fish from other localities.

Three typical habitats in the neighbourhood of Cambridge were selected, care being taken to choose those where there was least likelihood of the fish mixing with others from a different locality.

A fourth series of samples were taken from Berkhamsted, the fish here being probably a distinct race from any of those found near Cambridge.

The four sources are as follows :—

Source.	Times Collections were made.
Ga. Watercress beds, adjoining the Grand Junction Canal, Berkhamsted	January, June.
Gb. Shallow streams, Coe Fen, River Cam, Cambridge ..	May, 1928.
Gc. River Cam and streams connected with river, below Baitsbite Lock, Cambridge	All year round.
Gd. Brick Pond, Garlic Row, Chesterton, Cambridge ..	May–August.

The pond is isolated and densely populated with both *Gasterosteus* and *Pygosteus*. The series Ga and Gb are from streams connected with the river or canal at all times of the year, and the fish probably pass most of the year in these streams. In the case of the fish from Baitsbite, their natural habitat except at the time of the breeding season is the river.

This stock of fish at Baitsbite, although living in the river for about nine months of the year, is comparatively isolated. The river within the two-mile stretch between locks has only four streams flowing into it, at least two of which are only accessible to Sticklebacks when they are in flood.

At a point on the west bank about 150 yards below Baitsbite lock is the opening of

the Corporation sewage effluent. Around this a large shoal of Gasterostidæ congregate, feeding on the small animals growing in the sewage effluent.

This shoal is present at all times, though much reduced during the breeding season. During November and December there was an influx of *Gasterosteus aculeatus* var. *semiarmatas*. These fish, with the lateral bone plates extending far down the flanks, are considered by BERTIN (1925) to be developed from the common form at Cambridge, *Gasterosteus aculetaus* var. *gymnura*. Since the variety *semiarmata* was not found at other times of the year anywhere within at least twelve miles of Baitsbite, the appearance of this variety in the Baitsbite shoal seems to support BERTIN's theory. This theory of the origin of this variety also supports the contention that one race of fish only is present in the Baitsbite shoal. The belief that there is one local race here is based on its constant appearance at this one place, and that the range of sizes is constant.

This shoal of fish was rather heavily infected with *Glugea anomalum* (identified according to STEMPELL, 1904). *Schizstocephalus*, on the other hand, was rare among these fish. The frequency of occurrence of these parasites was reversed in samples taken from habitats around Cambridge town, a fact which also points to the Baitsbite fish being a pure race.

Methods of Obtaining Samples.

Where possible the fish were caught with a coarse-mesh tow-net. Where the water was too shallow or too overgrown to use this, a hand ring net was used.

The collections made were of two types, those merely to collect male fish and those intended to give a representative sample of the constitution of the race of fish at any locality at one time.

When the tow-net was used at Baitsbite, it was sunk and drawn gently under the shoal of fish feeding at the sewer outflow. After about two minutes it was drawn rapidly to the surface, a distance of about 4 feet. In using the net in this manner only a small area was covered, although about 40 fish could be caught with one haul. On several occasions the fish scattered in the river were sampled. It was found that as far as size and maturity were concerned they were essentially the same as those in the large shoal.

The constant composition of the samples taken in this manner during any one month indicated that this manner of sampling gave a reasonably accurate picture of the fish in the locality.

When the fish dispersed at the time of the spawning season, the only manner in which they could be caught was with the hand-net. At this time the mature adult fish are found either over the shallows in the river or ascending streams joining the river. With the hand-net it is impossible to sample the population with a degree of accuracy comparable with that reached with the tow-net. It was found that only the young fish and immature adults remained at the sewer outflow, and only mature adults were

found over the shallows and ascending the streams. This migration is considered more fully below.

Results of Examination of Samples.

The shoal of fish at Baitsbite (Source Gc) were examined by sampling with the tow-net in the manner described above, on average twice per month. The samples comprised 70–100 fish which were taken alive to the laboratory and examined there. A certain number of these were reserved for experimental purposes, the rest were killed and a histological examination of the reproductive organs made.

These samples threw light on the following points of the life history of the fish :—

i. The Rate of Growth and Maximum Age reached.
ii. The Age at Maturity.
iii. The Duration of the Spawning Season.

Rate of Growth.

The total length of *Gasterosteus* from Baitsbite rarely exceeded 5·5 cm.

The length of the breeding season makes it difficult to determine the rate of growth from the length of the fish in the samples. A number of measurements were made of fish reared in the laboratory (Table V). Both the specimens shown in the table were females, but it was found that the rate of growth of the male was about the same as that shown. It will be seen that there is a difference of 0·5 cm. at the end of five months between these specimens, although the feeding and other conditions were identical.

These examples are cited to illustrate the great variability of rate of growth in length in the early life of the fish, a variability which is readily observed in growing broods.

TABLE V.—Rate of Growth of Young Fish in Laboratory from Time of Hatching. Individual Rates of Two Specimens from the same Brood.

Age in months :	1.	2.	3.	4.	5.
Specimen A ... ... cm.	1·5	1·9	2·5	2·8	3·1
Specimen B ... ... cm.	1·1	1·5	1·9	2·4	2·6

TABLE VI.—Rate of Growth of Adult Fish in the Laboratory. Specimen Gc 117. Age at commencement : At least 12 Months.

Month :	May.	August.	October.	December.	February.
Length in cm.	4·6	4·7	4·7	4·8	4·9

The rate of growth in length of the adult fish, on the other hand, is apparently very much slower. Records of the total length of fish kept in the laboratory at a temperature between 15°–17° C. were made. The greatest length of time over which these records were made was 10 months (Table VI, Specimen Gc 117) and the growth during these 10 months amounted to only 0·3 cm. (viz., from 4·6 to 4·9 cm.). The fish was fed freely and lived in a normal manner, nesting during the breeding season in its aquarium. There is good reason to believe (see p. 241) that the rate of growth in the aquarium is at least equal to that of the fish in its natural habitat at the same temperature.

BERTIN (1925) has deduced the rate of growth of *Gasterosteus* for the length frequencies of a number of samples from different sources, and has come to the conclusion that in general the duration of life of the fish is two years. This opinion is at variance with my conclusions.

If the differences of rate of growth in length shown in Table V are combined with the variation due to the length of the breeding season (the rate of growth of the fish is depressed during winter) there will be a difference of at least $\mp$ 0·4 cm. in fish of the same year group. BERTIN made measurements of dead fish, and I have found that this will introduce a variation of $\pm$ 0·15 mms., owing to the variation of protrusion of the mouth, and notably the variation of extension of the dorsal and pelvic spines. There will therefore be a variation in the measurements of fish of the same year group of at least $\pm$ 0·5 cm. This alone is sufficient to throw doubt on the validity of BERTIN'S conclusions.

There is, however, in addition, a considerable variability in the results of examination of samples from different sources and at different times of the year found by BERTIN. The samples were collected for this author by different people, by methods unspecified. *Gasterosteus* is gregarious during the non-breeding season. I have observed in a number of sources that the fish form themselves into shoals of approximately the same length, a form of shoaling which occurs in the case of a number of fishes (BROWN GOODE, 1878). It appears to me that the variation between samples investigated by BERTIN may be due to some of these having been drawn from only one shoal.

BERTIN also draws evidence of the duration of life of the Stickleback from the observations of WARRINGTON (1885), who found that the adult fish die after nesting, shortly after the young cease to have need of their care. This only occurs if the fish are confined in small aquaria or if kept in badly maintained aquaria (see p. 213 and CROSER, 1928).

If the records of *Gasterosteus* living in aquaria for four years (LACEPEDE (1802), SOLAND (1869)) be taken with the foregoing considerations, it is doubtful if BERTIN'S generalisation that *Gasterosteus* lives only two years can be tenable.

In the case of the race of fish at Baitsbite, it was found impossible to distinguish the age of the fish after its first year on the basis of length.

Age of Maturity.

From an examination of the histology of the testis it is possible to distinguish a fish which has spawned from a maiden fish (p. 225 *et seq.*). For a given locality it appears to be possible to distinguish a length at which the fish reaches sexual maturity. For the fish from Baitsbite this length is 4·5 cm. This makes it possible to divide the samples into two groups, adult fish and immature fish, on the basis of length. Occasionally the fish may exceed 4·5 cm. without reaching maturity. It is clear from the above observations on the rate of growth, that the number of immature fish at the commencement of any breeding season will be approximately the number of 9–10 months' fish.

From a histological examination of the testis, it is also possible to distinguish fish which are mature for the first time from those which are in their second or third spawning season (p. 225 *et seq.*). There is, however, not always a distinction in the total length of these fish.

Duration of the Spawning Season.

From the external appearance of the fish it is possible to deduce the duration of the spawning season by the presence of the secondary sexual characters of the male. The accuracy of this as a criterion for the spawning season is discussed below (p. 261). As has been mentioned, the scattering of the fish at the time of spawning renders accurate sampling difficult. This can be overcome to some extent by the recognition of the early stages in the development of the secondary sexual characters.

It has been found from observation of the development of the secondary sexual coloration of *Gasterosteus* in aquaria, that traces of this may be observed some time before the characteristic red colour appears.

It was found in nature that this trace may appear before the fish begin to migrate to the spawning grounds. Under the natural conditions it has been found that these early traces always lead to the full coloration. Hence it was possible by the recognition of these traces to determine those fish which were going to breed while they were still in the large shoal.

These secondary sexual characters are confined to the male. In the case of the female, those fish which were going to breed could be recognised some time before they moved to the breeding grounds by the distention of the posterior part of the abdomen.

The time of commencement of the breeding season in the years 1928 and 1929 varied, this occurring about two weeks later in 1929, owing no doubt to the delayed spring rise in temperature of the habitat in that year. In the records the samples taken between April, 1928, to April, 1929, are considered, records for other years are less complete.

It will be seen (Table I, p. 235) that the breeding season for the year 1928 extended from the end of April to the middle of August, in the source Gc. In the other sources breeding commenced slightly earlier and the breeding season ended also rather earlier.

The general features of the reproductive cycle, including the extent of breeding season, are shown in Table I.

Evidence of the Migration of Gasterosteus.

There is strong presumptive evidence that the *Gasterosteus* at Baitsbite migrate to definite breeding grounds, for at the time of the breeding season the shoal at the sewer outflow disperses and the fish appear in the shallows. As, however, scattered fish may appear on these shallows at times other than the breeding season it was thought possible that this movement was not a definite migration.

In the spring of 1929, however, very striking evidence of the migration of the fish in the neighbourhood of Baitsbite was obtained. About 20 yards from the sewer outflow there is a tunnel under the towing path, connecting a shallow stream with the river. The tunnel itself, which is lined with bricks, is on a sharp incline, about 1 in 5. At the upper end of this tunnel is a simple weir, to maintain some water in the ditch.

During the dry spring of 1929 the amount of water passing down the tunnel was only sufficient to cover the brickwork bottom by about half an inch.

At the river mouth of the tunnel there is a concrete landing stage, the space between the tunnel and the landing stage forming a shallow backwater, connected with the river under the platform. This backwater had been regularly examined for fish when samples were taken from the shoal at the sewer outflow. At the beginning of May fish were noticed in this backwater for the first time since the preceding breeding season, and some were caught with a hand-net. On examination they were found to be predominately fish in an advanced state of maturity.

On the evening of May 5 a number of fish were noticed endeavouring to ascend the tunnel against the flow of water. The fish could swim only a few inches against the rapid flow of water down the tunnel. Having done this the fish anchored itself by extending all its spines. The force of the current would frequently sweep the fish on its side, where it remained anchored between one pelvic spine and the dorsal spines. After a few minutes' rest the fish would make a further ascent. It is rather interesting to note that at the upper end of the tunnel where the stream joins it through the weir there was a drop of about 15 inches which, I think, would be absolutely impassable to the Stickleback. No large fish were ever found in the stream above the weir.

The backwater at the junction of the tunnel and the river seemed to offer a suitable breeding ground, and it was with some surprise that the fish were found migrating up the tunnel instead of breeding in the backwater. On May 12 many fish were caught trying to ascend the tunnel. On examination they were all found to be in an advanced state of maturity (Stage II, p. 217).

At the same date two males with fully developed colours were found nesting in the backwater. On and after this date males were found nesting in the shallows of the river.

The shoal of fish at the sewer outflow had become much reduced at this time.

In June and July the fish were clearly returning to the shoal at the sewer outflow Further, they had disappeared from the streams in the neighbourhood.

There is thus evidence that the fish return towards the end of the breeding season to the river. It seems probable that this return migration is not executed by the fish hatched that season.

Throughout the autumn and winter months small fish are present in the breeding streams, but never large fish. In the river the proportion of small fish (under one year old) in the autumn and winter months is always far less than if the whole of the Stickleback population was present in the shoal. Assuming that the natural life of the fish does not exceed four years, the proportion of small fish in the whole population should be at least one-third or more. Actually the numbers of small fish are nearer one-seventh to one-tenth.

During September, 1928, there was a very heavy mortality of Sticklebacks in a ditch in the neighbourhood of Baitsbite. The fish were in a ditch which at that time had no connection with the river, but which during the spring of 1928 was in direct connection owing to the extensive flooding which occurred. The fish died apparently from starvation, though it is interesting to notice that only the large fish died. Later in the year a number of small fish were found in this ditch, but not one large fish was seen. This suggests that a possible cause of the return of the large fish to the river is an insufficiency of food in the streams.

It is possible to predict from laboratory experiments the length of time taken by the fish to develop from the stage of maturity, referred to above as Stage II, to the condition of full breeding activity. As will be shown later, the full development of maturity might be expected about seven days after the fish were observed migrating up the tunnel.

This was confirmed by transferring the fish from the river to a stream similar to that at the top of the tunnel. This second stream was known not to contain any fish before the experiment was started, nor was it connected either with the river or another stream. About fifty fish, the majority of which were in Stage II of maturity, were caught in the river and placed in the stream. After a week these fish could still be seen in this stream, two nests were found and a number of males with the full breeding colours seen. The fact that these fish in the streams began breeding earlier than those which finally nested in the shallows of the river, is probably due chiefly to the higher temperature of the water in the stream as compared with that of the river. Experimental evidence of this will be given later.

The migrations of *Gasterosteus* in the neighbourhood of Baitsbite appear to be as follows :—

The fish leave the feeding ground off the sewer outflow and enter the shallows of the river. Some of the fish then endeavour to move into the shallow streams connected with the river. In about ten or twelve days from their first movement away from the feeding ground the fish nest, either in the shallow breeding streams or in the sheltered

parts of the river. While the fish are migrating the secondary sexual coloration of the male is not fully developed. This development occurs shortly after the fish has reached the breeding ground.

Towards the end of the breeding season (June to July) these fish which have bred return to the river, probably on account of the lack of food in the shallow water. The young fish which have just been born do not move to the river at once. They gradually join their parents in the autumn. Some of these young fish may pass the winter in the breeding streams. In this case their growth is slow, and they probably do not reach maturity until the succeeding breeding season.

These migrations appear most accentuated in the case of fish living in a river devoid of suitable quiet shallows, but are probably made by all Sticklebacks. In the case of fish in a pond, for example, the migration consists of a movement from the deep parts of the pond to the shallows where they are found nesting.

Day (1880), Meek (1916) and Bertin (1925) refer to the shoal movements of *Gasterosteus*, particularly to the inshore movements of *G. spinanchia* in the spring. Bertin, whose paper summarises the literature referring to this genus, makes no reference to the specific spawning migrations of *Gasterosteus aculeatus*; rather he attributes to this species a "rheotropism negative," by which he means apparently that the fish dislikes currents.

The migrations of *Gasterosteus* may well have been overlooked in the past; it is only recently that the migrations of a number of fresh-water Teleosts have been recognised.

Breeding Habits of Gasterosteus.

The site chosen for the nest of *Gasterosteus* in the case of the fish I have examined in nature was always in a comparatively sheltered spot, with not more than a foot of water. A sandy or even muddy bottom is chosen in preference to a gravelly bed. It is interesting to notice that *Pygosteus*, which lives in muddy ditches, constructs its nest in the branches of water-weeds, and not on the bottom.

Whether *Gasterosteus* will build a nest in a depth of water exceeding about 18 inches is difficult to decide. The nest is inconspicuous, it being generally noticeable from the presence of the male fish guarding and attending it. No case of a nest at this depth of water has been observed by me.

The fish seem to prefer slow flowing or still water to the main current of the river. Day (1880), however, quotes a description of a nest built on a gravelly bottom with a current of water running over it.

The construction of the nest is difficult to observe in nature. In aquaria the whole occupies less than an hour. It is difficult to determine therefore what is the stimulus leading to nesting under natural conditions. In aquaria the presence of a female with well-developed ovaries, a "ripe" female, acts as a stimulus to the fish. At first the male darts around the female. The colours become heightened by the retraction of

the melanophores (see p. 217). It then builds the nest and induces the female to enter it.

A male with the secondary sexual characters well developed will, however, show desire to nest in the absence of a "ripe" female. It makes a hollow in sand at the bottom of the aquarium with its snout, but it carries the nesting operations no further. Rarely will the male build an entire nest in the absence of a female.

The presence of a "ripe" female does not always act as a stimulus, and the initiative does not always lie with the male. A pair of sexually mature fish were put together to nest, but after a week, the male having shown no signs of nesting, it was removed and replaced by a second male. The female at once sought out the second male, who showed no signs of being stimulated by her presence. The female followed the male about for some hours, occasionally gently dragging it about by its tail in a manner precisely similar to that of a male coaxing a female.

The whole of the mature eggs of a female fish are deposited in the nest at one time and in about five minutes. The male passes the spermatozoa over the eggs in the nest directly after the female has spawned. The male will receive three or four females into one nest within an interval of three days.

The length of time during which the eggs remain in the nest varies from two to three weeks, varying with the temperature (p. 257).

The male breaks open the nest to liberate the young fish which emerge with the yolk sac still unconsumed. The young may emerge before the nest is opened; in this case the male "puffs" them back into the nest.

In aquaria it has been found that after the emergence of the first brood, the male may remake the nest and receive a female. In the two cases in which this occurred the second brood failed to hatch. It is difficult to decide whether this was due to failure to fertilise the eggs or was due to other causes. It is a moderately common occurrence in aquaria according to my experience for the eggs to fail to hatch. An examination of the testis of the male at the time when it might construct a second nest in the aquarium shows that there are comparatively few spermatozoa present. In the case of these fish which built a second nest the breeding season was being artificially prolonged. It seems probable therefore that in nature the fish normally nests only once.

The Secondary Sexual Characters of the Male Gasterosteus aculeatus.

The secondary sexual characters of this species have been described in their fully developed condition by TITSCHACK (1923). In their fully developed state these consist of the following modifications:—

1. A red coloration is developed on the throat and ventral aspect of the body.
2. The iris of the eye and the back of the body appear blue.
3. A large number of the kidney tubules are modified in correlation with the production of the mucous secretion used in building the nest.

4. The musculature of the pectoral fins is enlarged, in relation to the use of these fins in " fanning " water over the nest.

The above characters are developed only during the breeding season. The further points of sexual dimorphism described by TITSCHACK are permanent, viz. :—

5. The urinary bladder of the male is larger than that of the female.
6. The whole of the brain of the male is larger than that of the female, and also shows differences in its fine structure.

The first two of these characters are visible externally, and the third by simple dissection, the volume of the kidney greatly increasing when it is modified.

VAN OORDT (1924) has taken the depth of the epithelium of the kidney tubules as a convenient index of the development of the secondary sexual characters. The situation of the kidney next to the testis makes it simple to fix and section both structures and thus make a permanent record of the state of the secondary sexual characters.

The secondary sexual characters of *Pygosteus* differ from those of *Gasterosteus* only in that the red colour (1) is replaced by a dense black pigment. In the specimens of *Pygosteus* examined by VAN OORDT he was apparently unable to recognise stages in the development of the secondary sexual characters.

In *Gasterosteus*, on the other hand, I have found it possible to recognise definite stages in the development of the secondary sexual characters and therefore chose this species in preference to *Pygosteus* (p. 204). The time taken by the fish in passing through these developmental stages depends on the conditions, as will be shown.

Red Coloration.

The red coloration is due to a pigment which is absent during the non-breeding period of the life of the fish. TITSCHACK came to the conclusion that there were special chromatophores developed for this pigment. He traced the disappearance of the pigment while the chromatophores were still present.

Although the situation of the chromatophores in the thin layer of skin overlying the bone of the lower jaw, the operculum and the ventral pelvic plate, presents certain difficulties, I have found it possible to demonstrate their presence prior to the development of the pigment, by using the silver impregnation method of VERNE (1921). This was possible in a fish which from the degree of the development of the modification of the eye colour and the kidney would be expected to develop the red coloration shortly. Hence the appearance of the red pigment does not mark the commencement of the development of the secondary sexual characters.

The composition of this pigment is unknown. It is distinct from the yellow pigment (xanthophyll) which is present in small chromatophores all over the body of the fish in both sexes. TITSCHACK terms these latter xanthophores and the red pigment containing chromatophores erythrophores (after BALLOWITZ). The pigment of the xantho-

phores is a lipochrome. The erythrophore pigment does not give the characteristic reactions of a lipochrome with iodine, or concentrated sulphuric acid, although it dissolves and is bleached rapidly in alcohol, ether and acetone. It lasts for a few days after the death of the fish if not placed in any solution. It is apparently precipitated by carbon bisulphide, becoming a granular brick red colour. It shows no definite absorption spectrum.

On immersing a fish after death in carbon bisulphide the red becomes very clear and can be seen to extend further over the body than is apparent in the living fish. This observation is useful in demonstrating the presence of the red colour, which is at times difficult to see in the living fish.

During the life of the fish the red colour varies in intensity. This does not appear to be due to any contraction or expansion of the erythrophores, which cannot be made to expand or contract in excised pieces of skin when put in dilute solutions of potassium chloride, magnesium chloride or other solutions which affect the melanophores (SPÄTH, 1916). This heightening of the red colour which occurs when the fish is excited, either when it is nesting or when it is disturbed while tending the nest, appears to be due to the contraction of the melanophores. The whole of the body becomes lighter, and both the red and the blue colour become more pronounced.

The appearance of the red pigment marks a definite phase of the development of the secondary sexual colours. Variations of the intensity of the red colour are transitory and cannot be regarded as different stages in its development.

Blue Coloration.

The blue colour of the iris of the eye TITSCHACK showed to be due to the reduction of the amount of guanin crystals in the guanophores. When these are reduced there is a dispersion effect of the light falling on the iris. It seems probable that the blue appearance on the body is due to the same effect, although this blue on the body was not noticed by TITSCHACK. The reduction of the guanin crystals occurs first in the guanophores situated in the dorsal part of the iris, and the blue colour thus appears first in the upper part of the iris. This blue colour is well marked and the presence of the trace of blue colour in the eye is the first external appearance of the secondary sexual characters in the male. At this stage there is no other trace of the secondary sexual characters to be found. The trace of blue in the eye is not confined exclusively to the male, having been found in four female fish out of about 200 of this sex examined during the year.

This reduction of the guanin crystals over the iris of the eye is not in the nature of an adaptation to different light intensities, since of the four female fish found with a trace of the blue colour, two were living in the brightly lit aquarium, and two from the dimly lit natural habitat. It may also be noticed that the male fish will develop this colour in total darkness or in a bright light.

The trace of blue in the eye expands quite suddenly, in the course of a day or so, all over the eye. This condition has never been found in any female *Gasterosteus*. It is a highly characteristic stage in the development of the secondary sexual characters, for at this time the chromatophores of the red pigment may be found with the pigment not yet developed and also the first traces of the modification of the kidney.

Kidney Modification.

The increase in depth of the epithelium of the kidney tubules precedes the production of the mucous secretion. This preliminary modification of the tubules precedes slightly the development of the red coloration.

The mucous secretion of the kidney tubules may be readily stained in sections with muci-carmine or aniline blue. With MASSON'S Triple Stain, the unmodified tubules stain red, the modified blue. When the secretory phase begins the central parts of the tubule cells stain blue while the rest of the cell remains red.

The transformation of the kidney tubules is not associated with any other changes in the kidney, the amount of blood remaining about the same. When the kidney is re-formed into the normal condition the modified tubules are invaded by blood corpuscles, and their cells appear to be broken down. The modification of the kidney occurs first in the ventral part and it persists longest there.

Stages in the Development and Loss of the Secondary Sexual Characters of Gasterosteus.

The annual cycle of the development and loss of the secondary sexual characters of the male *Gasterosteus* may be divided into five stages, recognisable externally. Regarded as indices of the amount of secondary sexual character development, the intervals between these stages are not of equal value. Stages I and II are developmental stages. Stages III and IV are externally quite distinct, but there is probably no real change in the development of the secondary sexual characters between them. Stage V is the only stage recognisable in the loss of the sexual characters.

The fish prior to the development of the secondary sexual characters is in respect to these characters at least, exactly similar to the female externally. (There is a slight sexual dimorphism most pronounced in large fish. The female is deeper in the body in proportion to its length than the male. The head of the male is rather more angular than that of the female. It is, however, by no means always possible to distinguish a male without any secondary sexual characters from a female). This condition when the secondary sexual characters are not developed may be called Stage 0. An adult male fish is in Stage 0 for eight or nine months every year.

Stage 0.—No trace of the secondary sexual characters, internally or externally.

Stage I.—A small patch of blue colour in the upper part of iris of the eye. No other trace of the secondary sexual characters developed.

Stage II.—Blue all over the iris of the eye. A slight bluish tinge on the dorsal part of the body. The opercular region frequently shows a pinkish colour. This does not appear to be due to any trace of the erythrophore colour. The chromatophores, however, are developing at this stage. The pinkish colour of the opercular region is probably due to the blood in the gills. The reason for the hæmoglobin colour being visible is due no doubt to the reduction of the quantity of guanin crystals in the guanophores on the operculum.

Internally the secondary sexual modifications are developing in the kidney. In the ventral part of the kidney modified tubules appear. The condition of the pectoral musculature has not been examined at this stage.

Stage III.—The red erythrophore colour is developed on the under side of the mouth, throat and body, and over the opercular region. The blue colour on the dorsal aspect of the body is more pronounced than in Stage II. The modification of the kidney is complete, and the mucous secretion is present in the kidney and frequently also in the urinary bladder. The modification of the pectoral musculature is completed, and the fish shows its nesting instincts.

Stage IV.—The red colour appears heightened, the blue most pronounced, and the whole fish is of a light colour. These changes in appearance are caused by the contraction of the melanophores in the skin, these being seen as small dots on the dorsal part of the body. This stage is always associated with active movement on the part of the fish, generally in connection with reproduction, and also occurs when the fish is endeavouring to evade capture.

This condition, Stage IV, rarely lasts more than an hour, after which the fish return to the condition of Stage III.

Stage V.—The red colour has disappeared, the blue colour of the eye and a slight bluish tinge on the body remaining. Externally this stage is almost indistinguishable from Stage II. In the kidney the modified tubules are in the process of being broken down.

Structure and Cyclical Changes of the Testis and Ovary. Histological and Cytological Methods.

The microscopic preparations of the gonads were made with two objects in view, a routine examination of the condition of the gonads of a number of fish monthly throughout the annual cycle, and a cytological examination of the gonads at particular phases of the reproductive cycle.

For the routine examination the whole testis or ovary was fixed with the alcoholic variation of BOUIN'S picro-formol. Material was fixed for 15 to 18 hours, washed with 70 per cent. alcohol, and treated with lithium carbonate in 90 per cent. until the picric coloration was removed from the object. Material was cleared with xylol and

cut in sections 6μ thick. As a rule the anterior half of the testis was sectioned and the remaining part stored in the wax.

For good fixation of the cytoplasm and a study of the intracellular inclusions of the cells of the testis a number of fixatives were used, such as neutral formalin, HELLEY'S Formol Zenker and FLEMMING'S strong mixture without acetic acid. FLEMMING without acetic gave very satisfactory results. The only objection to it as a routine fixative, in addition to the long fixation required, is that material so fixed will not stain satisfactorily with MASSON'S Triple Stain, which stain was of great value in the study of the interstitial tissue.

The fish were killed by section behind the head. Care must be taken to handle the testes only by the *vas deferens*, any pressure on the gonad disturbs the interstitial tissue.

For routine examinations iron hæmatoxylin and MASSON'S Trichromic Stains were used (MASSON, 1912). MASSON'S stain gave very precise differentiation of the collogen connective tissue and the interstitial cells (Plate 19). For a critical examination of the germ cells iron hæmatoxylin alone was used.

External Morphology of the Testes.

The testes of *Gasterosteus* are oblong-ovate bodies situated between the intestine and the kidney in the posterior part of the abdomen.

The volumetric variation of the testis is comparatively small for a Teleost fish with a single annual spawning. No doubt the breeding habits of the fish, which ensure the spermatozoa being discharged directly over the eggs in the nest, results in an economy of the sperm.

At its maximum size, which occurs during January to May, the testis is 1·5 mm. long and 0·9 mm. in diameter. At its minimum, found in July and August, the dimensions are 1·0 mm. by 0·6 mm., the cubic capacity of the larger testis being rather less than four times that of the smaller. In the case of *Perca flavescens*, TURNER (1919) found ten times this volumetric variation.

It is difficult to measure the small variations in size of the testis of *Gasterosteus* without recourse to microscopic methods of measurement or to accurate gravimetric methods. An examination of a large number of cases by eye showed that it was possible to classify the testes into three groups, maximum size, minimum size and intermediate. These estimates were checked at times by measuring with a micrometer eyepiece and low power objective. There are very large individual variations in the size of the testes, independent of the size of the fish. The two testes of a fish were almost without exception of equal size.

It may be pointed out that the maximum size of the testes never cause them to press on other organs in the abdominal cavity. The suggestion of MÖBIUS (1885, 1886) that the secretion of the kidney is of a semi-pathological nature caused by the mechanical pressure of the enlarged testes upon the kidney seems improbable, both on

account of the small variations in size of the testes and because the increase of the kidney occurs posteriorly to that of the testes.

The *vasa deferentia* are very much smaller in diameter than the testes and their point of origin is sharply marked. Their length is about the same as the testes, 1·5 mm. They open at the cloaca between the anus and the opening of the urinary bladder. The *vasa deferentia* are white, never having any of the melanophores which are found abundantly on the peritoneal covering of the kidney and rectum.

The testes are of a white colour with a varying number of melanophores scattered over them. The intensity of pigmentation of the testes of *Gasterosteus* varies considerably. According to VAN OORDT (1924), the testes of *Pygosteus* are darkly pigmented, although this was not always the case in specimens of this species I have examined. Young *Gasterostidæ* show very little pigmentation of the testes. In adult specimens the pigmentation varies with the density of the pigmentation of the peritoneum. After the discharge of the spermatozoa, the testes decrease in size markedly, and the pigmentation appears darker, owing to the contraction of the surface of the testes bringing the existing melanophores close together. During the increase in size of the testes which accompanies spermatogenesis, the pigmentation of the testes is uneven. It has not been found possible to show any correlation between the light and dark parts of the testes and spermatogenetic activity in those regions. During the winter months, when spermatogenesis is nearly completed, the testes of a number of fish show a bluish sheen. The cause of this colour has not been determined.

The testes are supported by a pair of thin double mesenteries from the dorsal wall of the peritoneum. The *vasa deferentia* arise dorsally in the testes and their origin is marked by a groove. The genital artery enters anteriorally in this groove, lying between the mesenteries supporting each testis and arises from the dorsal aorta some way in front of the gonads.

Malformations of the testes are rare. Occasionally one of them may be kinked in association with the intestine. One of the results of unilateral castration may be the hypertrophy of the remaining testis. This occurs at the anterior end of the testis, and is noticeable externally by the hypertrophied portion being very lightly pigmented. Incomplete extirpation of one or both testes also results in the hypertrophy of the remaining portion of the testes. The new portion thus formed is very distinctly lobulated, and is externally unlike the normal structure. Cases of hermaphroditism in Teleost fish are moderately common (DEAN, 1923). One case was found among some 500 fish examined. This fish (Gc 100) was reaching its first maturity; the left gonad was a normal testis with spermatogenesis in active progress, the right a complete ovary with eggs in the process of maturation.

The sex of the fish may be distinguished externally after about four months. The young ovary and testis are both of about the same white colour but the testis is lightly dotted with chromatophores. Further, the sharp division of the testis and *vas deferens* distinguishes the male from the female.

Microscopic Structure of the Testis.

(i) *General Arrangement.*—The germ cells are contained in 150 to 200 tubules radiating outwards from a dorsally situated hilus. The hilus contains, in addition to the main collecting ducts of the tubules which unite to form the *vas deferens*, the main blood vessels of the testis.

The tubules are at their maximum development or greatest distention, about 0·6 mm. long. In transverse section the tubules are 0·1 mm. in diameter, in longitudinal section rather less than this.

The testis of *Gasterosteus* is precisely similar to that of *Pygosteus*, and in general form like that of *Perca* and *Cottus* (TURNER, 1919 ; HANN, 1927), that is, of the type termed *Acanthopterian* by BROCK (1878).

In place of the word *tubule*, the term *lobule* has been used by TURNER (1919) and *canal* by VAN OORDT (1923). The tubules of the testis of fish are not strictly comparable with mammalian seminiferous tubules, there being no permanent germinal epithelium in fish.

(ii) *Spermatogenesis : the Cycle of the Male Germ Cells.*—The germ cells of the testis of *Gasterosteus aculeatus* undergo a cycle of changes agreeing in general features very exactly with the cycle in *Cottus bairdii* described by HANN (1927).

Unlike *Cottus*, the majority of *Gasterosteus aculeatus* reach Period III, Sexual Maturity, in their second year. The testis of a fish in the process of maturation for the first time differs in certain characteristics from that of a fish in its second or subsequent maturation, these differences being in the amount of pigmentation and the amount of the interstitial tissue present (see below.)

(*a*) *Division of the Spermatogonia.*—The early stages of the germ-cells of *Gasterosteus* were determined from specimens reared in the laboratory ; as far as could be determined, the rate of growth of such fish is not very different from that of the young animals in their natural habitat. At the end of the first three months the division of the spermatogonia in the testis appears to stop, and some of these cells by a process of growth become transformed into primary spermatocytes. These spermatocytes are contained within thin-walled cysts and these cysts entirely fill the lumen of the tubules (Plate 21, fig. 11). A very few spermatogonia are found closely pressed against the walls of the tubules of the testis at all times of the year ; they are seen most readily in the testis of an adult fish after the spermatozoa have been discharged (Plate 21, figs. 12 and 14).

The divisions of these spermatogonia within the tubules have not been observed in the adult fish and it is improbable that they are the only source of germ-cells for each spermatogenesis. On the other hand, spermatogonia are found lining the *vas deferens* within the testis and migrating towards the periphery of the tubules during the winter months in adult fish (text-fig. 1). (It may be noted that these cells give a superficial appearance of the existence of a patch of well-developed interstitial tissue in the testis.) These spermatogonia in this position, at the point of origin of the *vas deferens* agrees

with the descriptions of GEISER (1922) and conforms to the account given by TURNER (1919), both of which are quoted above (p. 199).

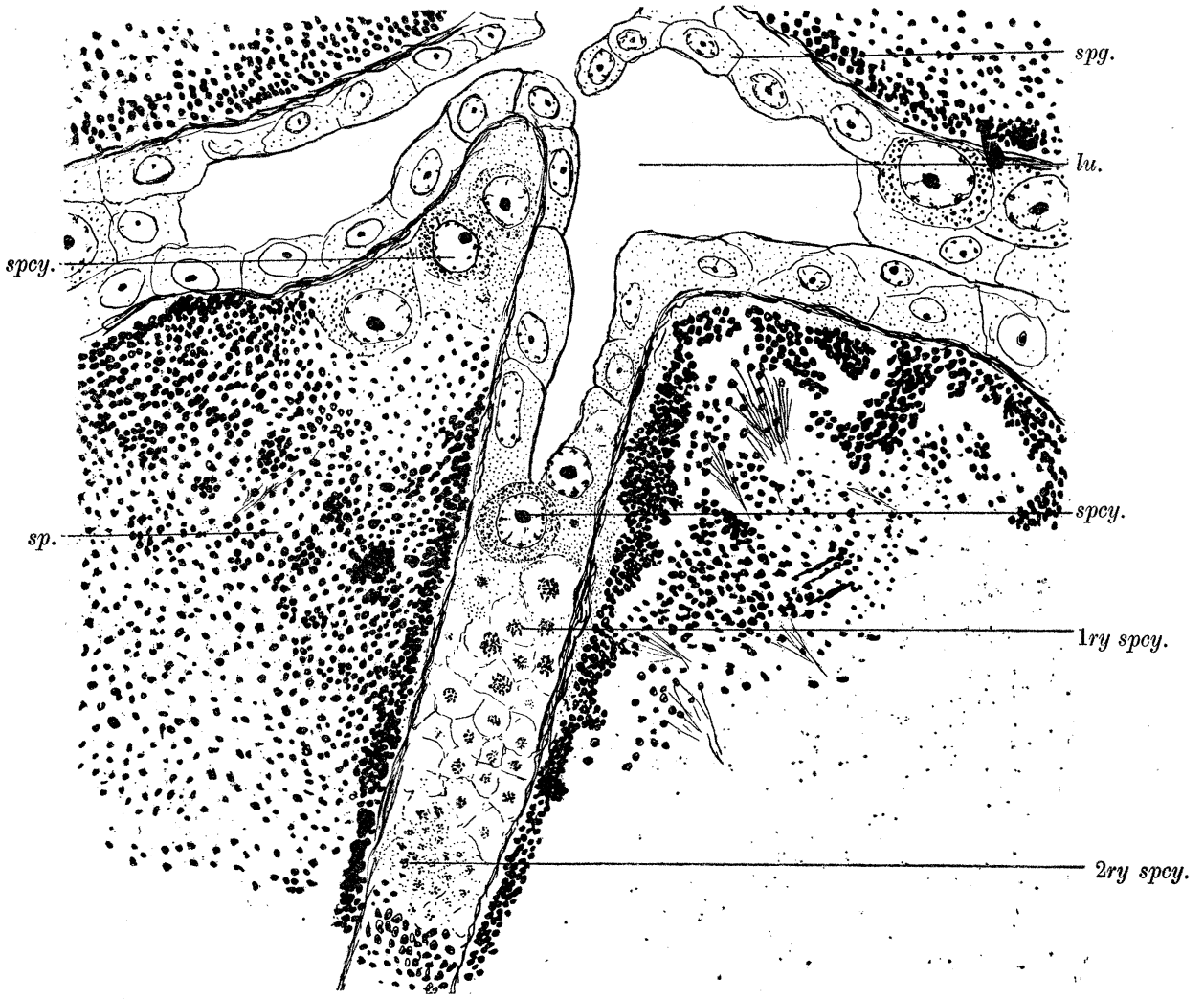


FIG. 1.—Portion of testis near hilus, showing migration of spermatogonia and their transformation into spermatocytes. *lu.* = lumen of tubules meeting *vas deferens*; *sp.* = spermatozoa; *spcy.* = spermatocyte (1*ry* = primary, 2*ry* = secondary); *spg.* = spermatogonium.

(*b*) *Maturation of the Male Germ-cells.*—The commencement of maturation is recognisable by the appearance of the primary spermatocytes in the testis. This phase (see p. 201) occurs during late July, August and the beginning of September, both in the case of the first maturation and the second or subsequent maturations. In a few cases, however, the first maturation may commence as early as May.

The time of completion of spermatogenesis, the end of Phase ii, is variable. In the majority of adult fish it is completed in two or three months, but in some cases it may be prolonged until the breeding season (Tables I and III). Certain fish fail to

complete their first maturation, stopping at the formation of spermatids, Phase i, the growth of the spermatocytes immediately following.

(*c*) *The Primary Spermatocyte.*—The primary spermatocyte is considerably larger than the spermatogonium, being about 12μ in diameter, with the nucleus 7μ in diameter, in contrast to the 6μ spermatogonium with its 4μ nucleus. In the spermatocyte the nucleolus is large, and the cytoplasm granulated. The beginning of the reduction division is accompanied by the disappearance of the nucleolus, and the chromatin collects at one side of the nuclear membrane, the so-called "synzesis." (Plate 21, fig. 15.)

(*e*) *Secondary Spermatocytes.*—The secondary spermatocytes are of short duration, and are smaller than the primary spermatocytes. The maximum size of the testis is reached, however, during this phase of maturation, partly owing to the fact that Phase i, the growth of the spermatocytes, does not occur synchronously, the maximum number being present at the time when the earliest formed have just undergone division, and partly owing to the development of the interstitial elements of the testis. The chromosomes are most clearly seen at this stage, but they are always minute. The diploid number is estimated to be 36 to 38, a number typical of most Teleost fish (Bresslau and Harnisch, 1927).

(*f*) *Spermatids.*—The cysts enclosing the germ-cells during maturation are considerably less distinct in *Gasterosteus aculeatus* than in *Pygosteus pungitius*, but a number of definite cases showed that these cysts disappear at the time of formation of the spermatozoon. The chromatin of the spermatid is dense, but appears occasionally collected as a cup to one side of the nuclear membrane.

(*g*) *Spermatozoa.*—The head of the spermatozoon is slightly oval and about 1·5μ long, the tail is 15μ.

(*h*) *Cells of Sertoli.*—While spermatogenesis is in progress it is impossible to distinguish the cells of Sertoli, unless, as is probable, they are to be recognised as the cells forming the cysts. When spermatogenesis is completed, the spermatozoa at the periphery of the tubules are associated with cells of Sertoli as small dense masses. After the discharge of the spermatozoa the cells of Sertoli can be seen very clearly, but soon become obliterated by the primary spermatocytes which fill the tubules.

(i) *Time Relations of Spermatogenesis.*—The beginning of each spermatogenesis is marked by the development of the spermatocytes. The first specimen showing this condition in 1928 was caught in July, and the last in September. (Phase i, p. 201.)

The completion of spermatogenesis occurs first in the posterior part of the testis. Advanced stages in maturation are found earliest in the central parts of the tubules, this position being due no doubt to the growing primary spermatocytes displacing the older groups inwards.

The transformation of the spermatogonia into primary spermatocytes occurs only during the period from the end of July to the middle of September. (Phase i, p. 201.)

In the samples of fish from Baitsbite (Series Gc) nearly 80 per cent. of the adult

male fish have spermatogenesis in progress in October, but by November the proportion has fallen to about 40 per cent. (Table III). During the winter months the proportion falls slowly to about 20 per cent. in May. In samples from the Grand Junction Canal (Series Ga) 80 per cent. show spermatogenesis in progress in January and 20 per cent. in June.

TABLE III.—Percentage of Adult Fish per Month with Spermatogenesis in Progress.

Samples Series Ga.

Month.	Fraction of Sample.	Percentage.
January	3/12	25
February	0/5	0
March	3/12	25
April	1/7	14
May	5/20	25
June	1/18	5·5
July	1/10	10
August	12/12	100
September	6/6	100
October	11/14	78
November	6/16	36
December	4/10	40

Samples Series Ga.

Month.	Fraction of Sample.	Percentage.
January	8/10	80
June	2/10	20

These differences in the time of completion of spermatogenesis appear to be due to the differences in rates of fall of temperature in the autumn of the two habitats. COURRIER'S (1922) and my own experimental observations described below show that the cell division of spermatogenesis is accelerated by a rise of temperature. It follows therefore that in a habitat in which the fall of temperature in autumn is slow or delayed (as is the case of a large body of water such as the river Cam) spermatogenesis will proceed more rapidly than in a habitat where the fall of temperature is rapid (as in the shallow water-cress beds at Berkhamsted.

VAN OORDT (1924) considers that in *Pygosteus* the spermatozoa remain in cysts until the breeding season, although active spermatogenesis may end some months earlier (Conclusion 5). Direct observation of the testis (p. 228) show that this is not true of *Gasterosteus aculeatus,* and this observation is supported by the experiments on artificial

fertilization (p. 257) which show conclusively that functional spermatozoa may be present in the testis one to two months prior to the beginning of the breeding season.

The special interest in the time of completion of spermatogenesis lies in the question as to what relation this bears to the beginning of the breeding season. It will be seen from Table IV that in the sample of fish from the River Cam at Baitsbite (Series Gc) about 7 per cent. of the fish with the secondary sexual colours developed had spermatogenesis in progress. In the sample from Berkhamsted (Series Ga) 20 per cent. of the fish had the breeding colours developed and spermatogenesis in progress in June.

TABLE IV.—Relation between the Condition of Spermatogenesis and the Presence of the Secondary Sexual Characters of the Adult Male Fish in samples Series Gc.

Condition of Spermatogenesis.	Completed.		In Progress.	
Sexual Characters.	Present.	Absent.	Present.	Absent.
	Per cent.	Per cent.	Per cent.	Per cent.
January	—	75	—	25
February	—	—	—	—
March	—	75	—	25
April	—	86	—	14
May...	70	—	8	2
June	94	—	6	—
July	90	5	—	5
August	—	—	50	50
September	—	—	—	100
October	—	12	—	78
November	—	64	—	36
December	—	60	—	40

It is impossible, therefore, to consider that the beginning of the breeding-season is directly dependent on the completion of spermatogenesis. This is further supported by experimental evidence; it is possible experimentally to bring fish into the breeding condition, as judged by the development of the secondary sexual characters, while the earliest stages of spermatogenesis are present in the testis. (See p. 253.)

VAN OORDT'S conclusions were as follows:—

"5. In einigen Herbst- und Winterhoden (von Tieren also, bei welchen sich noch keine sekundaren Geschlechtsmerkmale entwickelt haben) war die Spermatogenese beendet. Die Spermien liegen jedoch noch meistens in Cysten. Das Interstitium dieser Hoden ist breit und enthalt zahlreiche Zwischenzellen."

"6. Die Hoden der Brunsttiere weisen keine Spermatogenese auf. In den Hodenkanalchen liegen auser vereinzelten Spermatogonies in Ruhestadium grosse Mengen freier Spermien." (VAN OORDT, 1924, p. 396–7.)

The difference between VAN OORDT'S samples and those recorded in this paper appears to be due to a difference in habitat. VAN OORDT'S were taken from a canal near Rotterdam, and probably conditions were such that spermatogenesis was completed rapidly. It has to be noted also that VAN OORDT'S samples taken directly from nature as distinct from fish kept in the laboratory for some time, only number 8 in March and 5 in June, and of these, 5 are less than 4 cm. long and almost certainly immature. These samples do not appear sufficiently numerous to justify his conclusion " 6," that spermatogenesis is not in progress in the testis of fish in the breeding season.

(iii) *The Interstitium.*—In addition to the cycle of changes occurring in the germ cells there is a cycle of changes in the elements of the interstitium of the testis. The interstitial, connective and vascular tissues of the testis undergo an annual development and regression. This annual cycle does not occur synchronously with respect to all the tissues and therefore the composition of the interstitium is not constant.

The histological elements of the testis are seen most clearly in that of an adult fish just prior to the breeding season (Plate 19).

(*a*) *The Connective Tissue.*—The connective tissue of the testis consists of a fibrous tissue which forms the basis of its structure, a thin peritoneal covering of the testis and the melanophores. There is no muscular tissue in the testis, and the discharge of the spermatozoa must be brought about chiefly by the elastic contraction of the connective tissue.

The fibrous connective tissue of the adult fish does not show an obvious cellular structure. Such stains as MASSON'S Trichromic show that it is a collagen tissue. Interstitially it is closely mixed with the interstitial tissue and the nuclei of the latter sometimes appear to belong to the connective tissue. In the peripheral wall of the testis, however, where the interstitial cells are absent, no connective tissue nuclei can be seen.

The connective tissue appears homogeneous, and no part of it stains definitely with elastic tissue stains such as orcein or van Gieson. The collagen stains are, however, most intense in the epithelium lining the tubules.

After the discharge of the spermatozoa the whole testis contracts and the interstitial space and the periphery of the testis become relatively wide (Plate 20, fig. 6). Shortly after this there is a marked reduction in the space occupied by the connective tissue, the cavities of the tubules being separated by a very thin division (Plate 20, fig. 7). It is difficult to determine whether this is accompanied by actual cell destruction or whether it is due merely to a volumetric reduction of the connective tissue only.

The interstitium of young fish is extremely thin, consisting of a thin wall of connective tissue. When spermatogenesis is nearly completed the interstitium increases in volume. A similar increase in the amount of connective tissue in the testis occurs in the case of an adult fish when spermatogenesis is reaching completion. During this increase cell division has not been distinguished. In young fish especially, occasional elongated small nuclei are found in association with the connective tissue, and perhaps belong to cells which give rise to fibrous tissue.

(*b*) *The melanophores.*—The melanophores occur in the peripheral coat of the testis and interstitially. The testis of young fish prior to its first maturity is lightly pigmented externally and there are very few melanophores interstitially situated. After the discharge of the spermatozoa, contraction of the testis results in the superficial melanophores becoming closely placed one to another and also brings about a limited involution of the superficial melanophores. During the next and subsequent enlargements of the testis with the maturation of the spermatozoa, these melanophores remain in the interstitium. At the same time further melanophores appear at the surface of the testis, and also to a less extent in the interstitium. It follows that the older the fish the deeper is the pigmentation of the testis.

(*c*) *The Blood Corpuscles in the Testis.*—The blood corpuscles are found in the testis at all times in a vessel in the hilus and in small capillaries between two tubules at the surface of the testis (Plate 21, fig. 14). Blood corpuscles are found interstitially only when the testis is approaching its maximum size, at the time when the connective tissue of the testis is reaching its maximum development. On the other hand, lymph spaces occur in the interstitium at all times, especially during spermatogenesis (Plate 21, fig. 16). These spaces are always to be found, even when the testis is fixed with agents which cause the minimum of contraction.

In comparison with the testes of other fish, the proportion of blood corpuscles in the interstitium of *Gasterosteus* is extraordinarily small. The reason for this is probably due to the small size of the testis itself. Since there are blood vessels at the surface and at the hilus, no part of the testis is situated further than about 0·3 mm. from a blood vessel. Absence of blood corpuscles interstitially is therefore explicable, the lymph spaces fulfilling the nutritive requirements of the tubules. The size of the blood corpuscles, 8μ by 5μ, would prohibit their free entry into much of the interstitium except when this is at its maximum size.

(*d*) *The Interstitial Cells.*—The interstitial cells are at their maximum development in an adult fish just prior to the breeding season, but are seen most clearly after the discharge of the spermatozoa. At these times they are large cells with granular cytoplasm with a large nucleus and a prominent nucleolus (Plate 19). If fixed with a good cytoplasmic fixative, such as FLEMMING's Strong Mixture without acetic acid, the cytoplasm is rather more extensive, and is carried out into processes; the rounded appearance of the interstitial tissue in Plate 19 is partly an artefact due to the fixation with BOUIN. In a teased preparation of a fresh testis, the interstitial cells have a yellow colour which is lost during fixation.

Neutral osmic fixatives show a number of black granules in the cells which may be distinguished from the smaller mitochondria by extracting the blackening of the latter with turpentine. Plate 22, fig. 20, shows some of these black granules in the interstitial cells after fixation with FLEMMING without acetic, and stained with iron hæmatoxylin. The amount of these granules in the interstitial cells varies considerably, some showing a large number of small granules, others a few larger, and some none at all. With

special cytological fixatives it was found impossible to distinguish satisfactorily all the interstitial cells from the connective tissue cells owing to the impossibility of using critical counterstains after this type of fixation. The close association between the interstitial tissue and the connective tissue is seen in Plate 20, fig. 22. Reasons are given

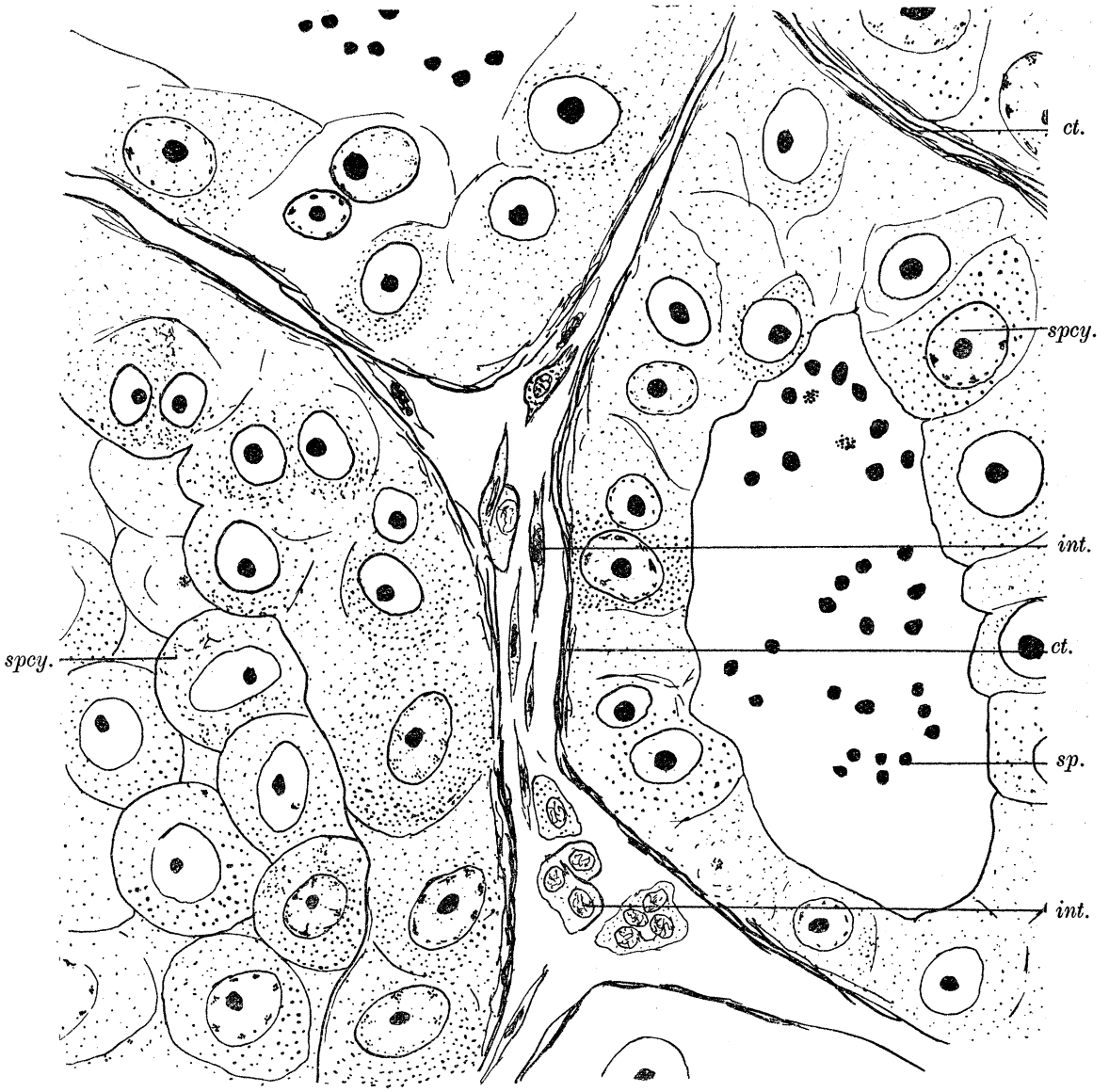


FIG. 2.—Portion of testis at commencement of spermatogenesis, showing interstitial tissue at its minimal development. *ct.* = connective tissue; *sp.* = spermatozoa; *int.* = interstitial tissue; *spcy.* = spermatocyte.

below for the probability that at all times these tissues are perfectly distinct and except for this point, COURRIER'S description of the secretory condition of the interstitial tissue is entirely confirmed (COURRIER, 1921).

" Si l'on a traité l'object par une technique spéciale les éléments intertubulaires révèlent des détails cytologiques intéressants. Les cellules conjonctives ont acquis un aspect glandulaire parfaitement distinct ; autour d'un noyau arrondi à gros nucléole central se trouve un protoplasme de structure différente suivant les éléments considérés. Il est le plus souvent foncé grâce a l'abondance de granulations extrêmement fines qui sont des mitochondries. D'autres cellules possèdent des granulations plus volumineuses et enfin quelques éléments renferment dans leur cytoplasme de gros grains de sécrétion."

This type of evidence as to the functions of this tissue, the presence of granules in the cytoplasm, may of itself not appear to be of much significance. But it must be pointed out that (i) the interstitial cells do not always contain " secretory granules," they only do so prior to and during the beginning of the breeding season, they do *not* show secretory granules during the commencement of spermatogenesis, (ii) that different cells show very clear phases in the formation of large granules, and (iii) *no other cells in the testis show similar granules.*

The original appearance of small granules distributed throughout the cytoplasm and the later stages in which the granules are concentrated into larger masses, are paralleled in the phases of secretion of a number of gland cells (BOWEN, 1929). In connection with Plate 22, fig. 20, which shows the granules in the cells, it may be mentioned that under the microscope there is no possibility whatever of mistaking the brilliant yellow-brown melanin granules for the black inclusions of the interstitial cells. This distinction cannot be seen clearly in the microphotograph.

It is therefore my opinion that COURRIER is fully justified in saying that the interstitial tissue of *Gasterosteus* has the cytological structure that would be expected of a gland of internal secretion (COURRIER, 1921).

(*e*) *Distribution of Interstitial Tissue.*—The distribution of the interstitial tissue is uneven. At the anterior end of the testis when the interstitial tissue is at its maximum development, it occurs in groups, especially where three or four tubules meet. In this region of the testis the hilus is not well developed. In the middle of the testis the interstitial tissue appears both where two or three tubules meet and also in the interstitital space between two adjacent tubules. Posteriorly the interstitial tissue is altogether less well developed.

(*f*) *Cyclical Changes in the Interstitial Tissue.*—After the spawning season both the nuclei and the cytoplasm of the interstitial cells are reduced. When the testis is at its minimum size the interstitial and connective tissues are indistinguishable (Plate 21, fig. 12). While spermatogenesis is in progress the interstitial space is small (Plate 21, fig. 13 ; Plate 21, fig. 14 ; Plate 21, fig. 16), but in the neighbourhood of the hilus of the mature fish where the connective tissue is fairly abundant the interstitial nuclei can be seen. Further, a careful examination of the various small interstitial spaces show the presence of considerably reduced interstitial cells (Fig. 2, p. 31 ; Fig. 3, p. 34). Throughout the winter there is a gradual increase in the volume of the cytoplasm of the interstitial cells, the increase being, however, not quite even. Patches of well-

developed interstitial cells appear first anteriorly in the testis. Reduced interstitial cells between the walls of two adjacent tubules show flattened and elongated nuclei (Fig. 2, p. 227). During the breeding season a number of exactly similar nuclei can be seen closely mixed with the fibrous connective tissue. As has been mentioned above, the lack of connective tissue nuclei at the rather thick wall of the testis makes it probable that these nuclei are really those of interstitial cells.

A number of workers have concluded that the interstitial cells are derived from connective tissue-cells, and that they revert to this type (MAZZETTI, 1911 ; RASMUSSEN, 1917 ; HUMPHREY, 1921). It is interesting to note therefore that BLOUNT'S (BLOUNT, 1929) description of the interstitium of the horned toad, *Phrynosoma solare*, agrees very exactly with that given above for *Gasterosteus*. Having quoted MAZZETTI (*loc. cit.*) BLOUNT states : " In the present investigation there has been no indication of such a reversion of interstitial cells to connective-tissue-cell type. There are flattened nuclei between the tubules, but they are only the interstitial-cell nuclei flattened by pressure. It must be emphasised that there are at all times interstitial cells in the centre of the intersection where the pressure is less and at no time are these flattened cells between the tubules absent " (BLOUNT, 1929, pp. 336–7).

The interstitial cells are present, therefore, in the interstitium of the adult fish at all times during the year. It seems probable that not all the interstitial cells reach full secretory activity annually, a certain number remaining in the partly reduced condition (Fig. 3, p. 230). It has not been possible to recognise the interstitial cells in the testis before the appearance of the late stages of spermatogenesis in a young fish.

(*g*) *Origin of the Interstitial Tissue.*—Regarding the origin of the interstitial tissue in *Gasterosteus*, COURRIER (1921, *a*), in the paper quoted above, refers to the interstitial tissue as modified connective tissue, but in a later paper (1921, *b*) considers that the interstitial cells are formed from leucocytes derived from the kidney. " Il est probable que, chez l'Épinoche, les cellules interstitièlles se forment aux dépens de leucocytes qui viennent directement du rein. On trouve, en effect entre les tubes rénaux des Teleostéens un tissu lymphoide bein developé. Cette origine des cellules est â rapprocher de ce qui BOUIN et ANCEL ont décrit au sujet de l'histogenèse de la glande diastematique chez le Cheval."

VAN OORDT (1924) was unable to distinguish between the connective tissue cells and the interstitial cells while the latter were developing.

Evidence will be given for concluding that the interstitial tissue of *Gasterosteus* is an endocrine gland, and it would seem unlikely on general principles that such a tissue should be formed directly from connective tissue. Two observations of the author support the theory of origin from lymphoid tissue. The lymphoid tissue of the kidney on *Gasterosteus* bears an extraordinarily close resemblance to the interstitial tissue in the testis. Such similarity of histological form does not necessarily imply identity ; as has been mentioned above, the early stages of the spermatogonia have a very close resemblance to the interstitial cells. The second observation is that the interstitial

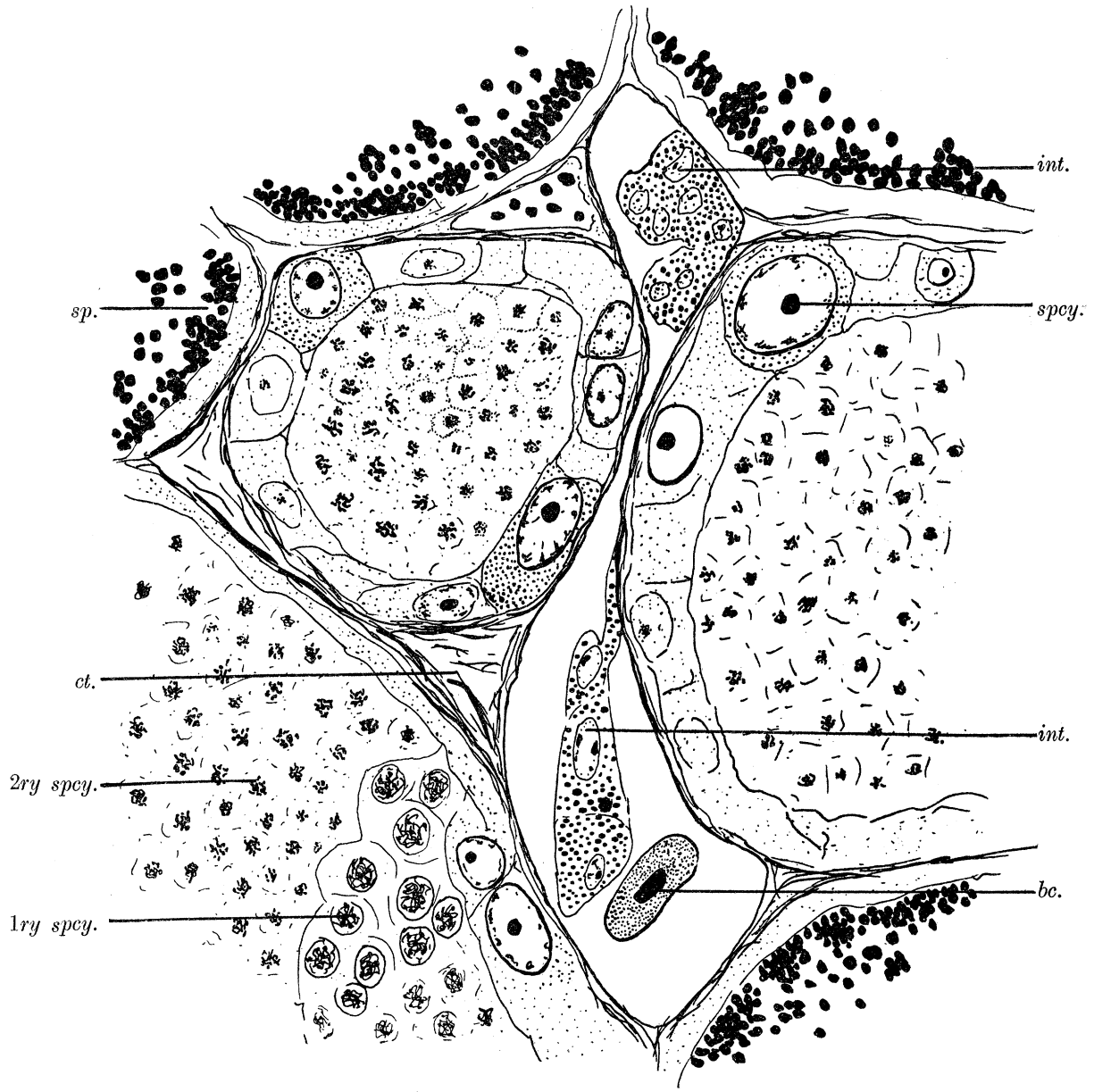


FIG. 3.—Small portion of testis in late stages of spermatogenesis, showing the developing interstitial tissue. *bc.* = blood corpuscle ; *ct.* = connective tissue ; *int.* = interstitial tissue ; *sp.* = spermatozoa ; *spcy.* = spermatocyte.

tissue in young fish makes its appearance suddenly ; it is therefore improbable that it is derived from connective tissue already pre-existing in the testis.

(*h*) *Cycle of Changes in the Interstitium.*—From the fully developed condition which is found at the time of breeding, the regression of the interstitial cells commences slightly before that of the connective tissue. In the developing interstitium in winter it appears at first sight that the development of the connective tissue precedes that of the interstitial tissue. When fixed with BOUIN the testis at this time shows a loose

network of fibres in the interstitium. The collagen tissue, however, is restricted to the walls of the tubules, the periphery of the testis, and around the blood vessels. If fixed with FLEMMING without acetic or HELLEY'S Formol Zenker, the interstitial space is found to be nearly filled with cells with a clear cytoplasm extended into a number of amœboid processes. A few of these cells contain granules of the same type which are found in the fully developed interstitial cells. The development of the interstitial cells therefore precedes that of the connective tissue.

As judged by the results of fixation with BOUIN the interstitial cells become gradually less subject to distortion into a fibrous appearance by plasmolysis as they develop. The secretory granules may appear in the cells before they attain their maximum development.

During this stage the blood corpuscles are confined to the blood vessel in the hilus and the smaller vessels at the surface of the testis. When the connective tissue is reaching its maximum development occasional specimens are found with the testis suffused with blood.

During the winter and while the spermatogenesis is either almost or entirely completed, the interstitium remains about the same width ("Medium wide," see below). In its actual constitution, however, the interstitium is changing; the interstitial tissue which at the first enlargement of the interstitium is in an amœboid condition, becomes firmer, and the connective tissue develops. The result of these two developments is to produce the "wide" interstitium which accompanies the spawning season.

For the purpose of records, the cyclical changes in the interstitium were divided into four stages, which after experience had been gained from the examination of a large number of sections, were readily recognisable:—

Thin.—Interstitial cells greatly reduced or absent (Plate 20, fig. 7) found in the testis of young fish, and in adults at the end of the spawning season.

Medium wide.—Interstitial cells developed to an amœboid condition, some containing osmophil granules. Connective tissue little developed (Plate 22, fig. 18).

Wide.—Interstitial tissue fully developed, often occurring in patches. Connective tissue well developed (Plate 19, fig. 4; Plate 22, fig. 19).

Very wide.—Interstitial elements developed as in *Wide*, above, but owing to the discharge of the spermatozoa, the interstitium testis appears relatively very wide (Plate 20, fig. 6).

(i) *Significance of the Interstitial Tissue.*—The examination of the samples taken directly from the natural habitat affords some presumptive evidence as to the rôle of the interstitial tissue. Below are enumerated the main points of interest in the cyclical changes of the interstitial tissue.

1. At its maximum development the interstitial tissue shows a close general resemblance to mammalian interstitial tissue, and has the cytological structure which would

be expected in a gland of internal secretion. The resemblance to mammalian interstitial tissue is masked to some extent by the great development of fibrous connective tissue. The interstitial tissue has been found to be well developed in every testis of *Gasterosteus* or *Pygosteus* when the secondary sexual characters are developed. (CHAMPY (1923, *a*) records observations which disagree with this generalisation. His statement is as follows: " J'ai eu cet hiver plusieurs Épinoches qui ont eu leur parue de noces avec un tissu interstitiel peu abondant ou nul." This observation is in direct conflict with VAN OORDT'S observations and with mine. VAN OORDT (1923) states that " all testes of *Gasterosteus* possess a more or less large number of interstitial cells." The occurrence of fish in winter with the secondary sexual characters developed has never been observed by me nor recorded elsewhere. On the other hand, if fish are caught in winter and kept for a short time (two or more weeks) in the laboratory, the secondary sexual characters develop, as is described below (p. 242). In this case the interstitial cells develop to their maximum before the connective tissue. The interstitium therefore remains narrow, and the interstitial cells are not obvious. It seems probable to me that this is the explanation of CHAMPY'S statement.)

2. The development of the interstitial tissue precedes the development of the secondary sexual characters, and its reduction is accompanied by the loss of the secondary sexual characters. The development of the interstitial tissue frequently occurs as much as three months before the development of the secondary sexual characters.

3. The development of the interstitial tissue occurs when spermatogenesis is nearly completed; the reduction of the interstitial tissue coincides with the growth phase of the spermatogonia.

The lack of direct connection between the time of development of the secondary sexual characters and the relation between the development of the interstitial tissue and spermatogenesis, supports VAN OORDT'S conclusion that in *Gasterosteus* the interstitial tissue has a trophic relation (" praassimilienende Funktion " of ROUX) to the germ cells. On this theory the interstitial tissue develops when spermatogenesis is nearly completed as a result of the " superfluity " of food material entering the testis, and the reduction of the interstitial tissue is due to the stored food matter being utilised in the growth of the spermatogonia. The apparent lack of blood in the testis was explained in this manner (but see p. 226).

The cyclical development of the fat-bodies of the fish is of interest in connection with this Trophic theory of the interstitial tissue.

Cyclical Changes in the Fat-body of Gasterosteus.

Examination and record of the fat-bodies of *Gasterosteus* were made with two objects in view. The work of ANDRE (1927), BULL (1928), POLIMANTI (1912) and REACH (1912) show that in Elasmobranchs and also in some Teleost fish there is a relation between the development of fat and sexual maturity. I have been unable to trace any reference

to records of the cycle of development and loss of the fat-bodies in Teleost fish. My own observations on *Mugil, Salmo* and *Gasterosteus* have shown that there is a reduction of the fat-body at the time of maturation of the genital products.

The condition of the fat-body also was considered to be interesting as an index of the state of nutrition of the fish. The fat-bodies are developed in *Gasterosteus* in the autumn, rapidly reach their maximum size, and disappear in January and February. The store of fat is therefore laid down at the time when the growth phase of the spermatogonium occurs. It would appear probable that when the fat-body was developing there would be an abundance of food available. If this assumption is correct it seems doubtful whether a special store of food in the interstitium would be necessary at the time of commencement of spermatogenesis. It may also be pointed out that the increase in volume of the testis which accompanies spermatogenesis, and for which presumably food is required, occurs in October, some two to three months after the reduction of the interstitial tissue.

It will be shown that under experimental conditions the fish can be brought into the breeding condition with or without the fat-body being developed. The rôle of the fat-body is probably entirely that of a store of material for use during the winter.

External Morphology of the Ovaries.

The ovaries of *Gasterosteus* are separated throughout their length. The oviduct is at all times short. The annual changes in volume of the ovaries are six to eight times as great as those of the testis, the large size of the ovaries when mature is due to the size of the eggs rather than to their number. The mature or " ripe " ovary measures 10 mm. by 4 mm. and contains 70–150 mature eggs. The fully developed eggs have a diameter of 1–1·5 mm.

After spawning the ovaries decrease in diameter to about 6 mm. by 2 mm. With the discharge of the yellow mature eggs the ovary becomes whitish grey. The ovary during the time of yolk formation (Phase ii, p. 201) increases in length more rapidly than in diameter, and the yellow colour is gradually acquired. The final increase in size of the ovary occurs rather rapidly.

The external appearance of the ovary of the young fish is similar to that of the testis of the young male, but differs, as has been stated above, in that the ovary is less pigmented than the testis.

Microscopic Structure of the Ovary.

(i) *General Arrangement.*—The cavity of the ovary is divided by a number of transverse lamellæ originating from the dorsal wall. The growing oocytes are embedded in these lamellæ, surrounded by a fine follicular epithelium.

(ii) *Oogenesis:* (*a*) *The Cycle of the Female Germ Cells.*—The ovary of a very young fish shows little difference from that of a very young male in respect to the germ cells. In the adult directly after spawning the discharged follicles become filled with cells derived

from the follicular epithelium, analogous to the cells of the mammalian *corpora lutea*, but the structures resulting are histologically much simpler than *corpora lutea* and have only a short duration.

Oogonial division has only been observed in *Gasterosteus* soon after spawning, but it may occur at other times, being masked by the shrinking of the yolk which causes a distortion of the stroma of the ovary.

(*b*) *Primary Oocytes.*—The commencement of maturation is marked by the growth of the oogonia: secondary yolk formation, by the appearance of numerous nuceoli beneath the nuclear membrane and by the presence of a reticular yolk. HANN (1927) found in *Cottus* that only a part of the primary oocytes underwent full maturation each year, the others remaining in a resting state at the end of the primary growth stage (Phase i, p. 201). In *Gasterosteus*, also, this is the case.

HANN'S terms (primary and secondary yolk formation) conveniently express the two phases of growth of the oocytes, and I have used them here for that reason, although, properly speaking, it may be incorrect to speak of two types of yolk in the egg. Not only may these two types of yolk be distinguished by their structure (HANN, 1927, p. 465), but also they may be stained differentially by MASSON'S Trichromic Stain, the primary yolk staining deep red, the secondary purple-blue. Chromatin threads in the nucleus disappear rapidly with the onset of the secondary growth phase of the oocyte.

Phase ii, the onset of secondary yolk formation of the oocytes, takes place in *Gasterosteus* during August to October.

(*c*) *Ova.*—Nuclear maturation of the female germ-cell almost certainly occurs when the ripe oocytes are discharged from the ovary.

(*d*) *Time Relations of the Phases of Maturation in the Female* (p. 201).—The division of the oogonia has been observed in July, but may occur at other times in the year.

Phase i.—The formation of the oocyte, marked by the primary growth phase, occurs in July to September.

Phase ii.—The secondary yolk formation continues from its commencement in October until the time of the breeding season, May.

Phase iii.—Nuclear maturation, and the discharge of the mature eggs, occur simultaneously during the breeding season, viz., from May to July.

(iii) *Interstitial Elements of the Ovary.*—There is a complete absence of melanophores either on the surface of the ovary or in the interstitium. The interstitium of the ovary is always thin, consisting of a simple stroma of connective tissue. There are no interstitial cells in the ovaries of *Gasterosteus*.

Correlations and Coincidences of the Natural Reproductive Cycle of the Adult Male, Gasterosteus aculeatus.

The main features of the reproductive cycle of the adult male are shown in tabular form in Table II. The evidence on which this table is based is seen in Tables I and III.

TABLE I.

Percentage of Adult Male Fish, showing:—

(1) Secondary sexual characters fully developed.

(2) Spermatogenesis in progress.

(3) Interstitial tissue in condition of "Medium wide," "Wide" or "Very wide" (p. 231).

Temperature of natural habitat, River Cam, Baitsbite (p. 238). May, 1928–April, 1929.

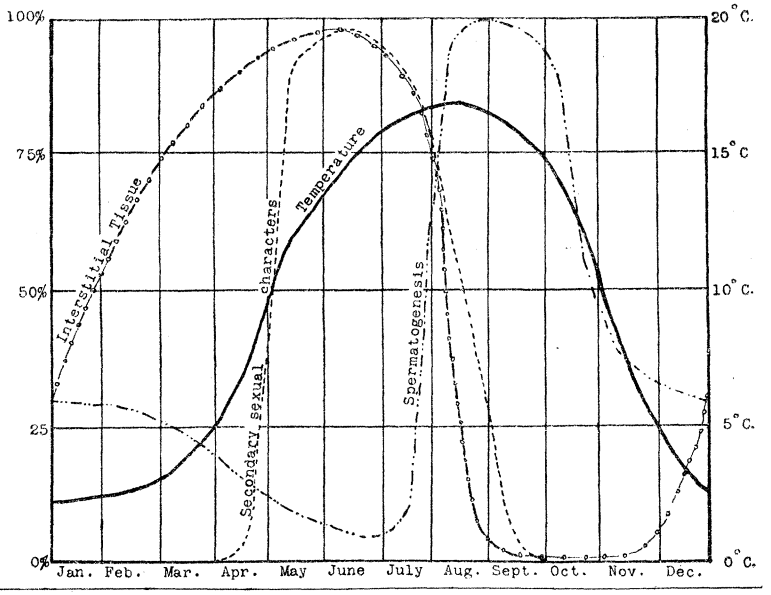


TABLE II.—Synopsis of the Reproductive Cycle of the Adult Male.

	February–April.	May–July.	August–September.	October–January.
Locality of fish	River ...	Breeding streams	River ...	River.
Secondary sexual characters	Absent ...	Fully developed	Absent ...	Absent.
Interstitial tissue	Increasing to maximum	Decreasing ...	Greatly reduced	Minimum.
Spermatogenesis	Frequently completed	Completed ...	Commencing...	In progress.
Fat-bodies	Decreasing ...	Absent ...	Developing ...	Maximum.
Temperature of habitat	Rising slowly	Rising ...	Maximum ...	Falling to minimum.

Taking the spawning season as the central feature of the reproductive cycle, it will be seen that there is a coincidence (i) between the migrations of the fish and the beginning of the spawning season, (ii) between this time and the reduction of the fat-bodies, and (iii) between this time and the completion of spermatogenesis.

Table III (p. 223), however, shows that there is no exact correlation between the completion of spermatogenesis and the beginning of spawning.

The close of the spawning season is coincident with the commencement of spermatogenesis. There is also a coincidence between the reduction of the interstitial tissue and the commencement of maturation (Table I).

There is no correlation between the development of the interstitial tissue and beginning of the breeding season.

The experimental investigations were made with a view to determining which, if any, of these coincidences were obligatory correlations. These experiments, described below, suggested that the temperature of the habitat of the animal plays an important part in the reproductive cycle. Accordingly the temperature cycle of the natural habitat is included in Tables I and II. It will be seen that there is a coincidence between the vernal rise of temperature and the beginning of the breeding season, and between the maximum temperature of the habitat and the commencement of spermatogenesis.

Experimental Investigations.

Introduction.—In the experimental investigations an endeavour was made to reproduce as exactly as possible the conditions of the natural habitat of the fish, while at the same time the main physical and biological conditions were controlled. From this datum the conditions were varied both singly and collectively.

In deciding the conditions which it was desirable to control, the following were selected :—

(i) Temperature.

(ii) Light.

(iii) Food.

(iv) Oxygen concentration.

These were selected for the following reasons.

(i) *Temperature.*—The evidence of the influence of temperature on animals with no internal temperature regulating mechanism need not be enlarged upon.

(ii) *Light.*—The effect of varying light intensities, apart from local reactions (WHITE, 1919) on the life of fish is little known. ROWAN (1926), from his experiments on birds, has made the suggestion that fish are affected by variations of duration of light. In view of this it was considered desirable to control and investigate the influence of light on the reproductive cycle.

(iii) *Food.*—There is no direct evidence of the influence of food on the reproduction of fish apart from the effects of starvation. Intensity of feeding plays an important part in some aspects of the reproductive cycle of mammalia (MARSHALL, 1922).

(iv) *Oxygen concentration.*—HENZE found that in two species of fish that the oxygen consumption is within wide limits practically independent of the oxygen tension of the water (HENZE, 1910). L. ROULE (1916) has advanced the hypothesis that the migration of fish are in co-relation with the oxygen requirements of the animal at the time of maturation of the gonads, this being supported by the measurements of the oxygen concentrations of the habitat at the time of migration. This received support from

the experiments of LOEWY and RICHTER (1899) who found that castration of a male dog produced a decrease in the respiratory exchange.

In the experiments the oxygen tension was kept at the saturation value corresponding to the temperature of the water. The River Cam, the natural habitat of the fish which were chosen for the experiments, is a shallow slow-flowing river, and contains in the immediate neighbourhood from which the samples were taken abundant water weeds. It is very probable therefore that the oxygen tension of the river is at all times very close to the saturation point, if not over.

The following conditions were not closely controlled in the experiments :—

i. The carbon dioxide tension in the water.

ii. The hydrogen-ion concentration in the water.

The experiments of POWERS (1921) indicate that the hydrogen-ion concentration may play a part in the reproduction of fish, at least under some experimental conditions. On the other hand, PRUTHI (1927) has shown that *Gasterosteus aculeatus* lived well within the range of p_H 6·0–8·5. PRUTHI found that this fish also lived well in carbon dioxide concentrations up to 10·0–13·0 c.c. per litre.

The consistency of results which have been secured without rigorously controlling these two factors has in part justified their neglect.

(i) *Arrangement of Aquaria.*—The aquaria used for experimental purposes were glass battery jars, measuring 12 inches high by 14 inches by 8 inches. These were filled to a depth of 1 inch with fine sand and water taken directly from the tap. Five or six adult fish were kept in each aquarium and a pair of aquaria were used in each experiment.

Each aquarium was provided with artificial aeration, some Elodea and a few fresh-water Gastropods. Such aquaria containing plants and snails were found to maintain the fish in better condition than those containing tap-water alone.

The aquaria were situated in a room on the roof of the laboratory with windows on three sides and on the roof. Except where otherwise stated, the lighting conditions remained constant throughout the experiments. The aeration maintained the oxygen concentration at saturation, and it is probable that the carbon dioxide concentrations did not vary greatly.

(ii) *Food.*—The feeding of a large number of *Gasterosteus* in the laboratory is a matter of some difficulty, the fish being voracious and greatly preferring living food. VAN OORDT (1924) used *Chironomus* larvæ as food. As these do not breed continuously, the supply may be quickly exhausted. The fish take small pieces of earthworm, but it is a matter of considerable labour to provide a sufficiency of this daily for a hundred or more fish.

Throughout the experiments the fish were fed daily with *Daphnia magna*. At the time of beginning the experimental work I was fortunate in finding a pond abundantly

stocked with this species. Using a hand net, sufficient *Daphnia* could be caught to supply about a hundred fish for three weeks. This source was almost unfailing.

The fish were fed daily with sufficient *Daphnia* to last them half an hour. With this amount of food they grew slightly faster than in their natural habitat. The amount of food required varied according to the temperature.

A diet of *Daphnia* may be criticised as being different to the natural food of the species. SAUNDERS (1914) and BLEGVAD (1916) record that except under exceptional conditions Crustacea form 80 per cent. of the food of this species. Further, if the temperature of the aquarium be maintained exactly at that of the natural habitat, although the diet be exclusively *Daphnia*, the reproductive cycle of the fish in the aquarium is exactly that of the fish in the natural habitat.

(iii) *Temperature.*—The control of temperature received considerable attention. Throughout the winter, during which time most of the experiments were made, the aquarium room was thermostatically regulated at 12° C. Temperatures below this were maintained by circulating tap-water through pipes in the aquaria. By varying the rate of flow of the tap-water, the temperature could be regulated from that of the room down to about 1 degree above the tap-water.

Constant high temperatures were kept by thermostatically regulated water-baths and variable high temperatures by an electric heating element in conjunction with a variable resistance. A temperature varying by daily increments was brought about by combining the heating element and the cooling-tube.

The temperatures of the experiments were recorded with the daily records. Recording thermographs were used for the aquaria in which the temperature was varying. The maximum daily variation of the constant temperature aquaria was on average 1° C. In the aquaria subjected to a varying temperature the range obtained was from 8° C. to 26° C., at the rate of 5° C. per week with a variation of about 2° C. per day.

Owing to low temperatures being maintained with tap-water, it was not possible to keep fish at temperatures much below that of their natural habitat during the summer months.

Continuous records of the temperature of the principal habitat were not available. From the temperature records obtained at the time of taking samples, the temperature cycle shown in Graph I was constructed. This has been smoothed by reference to the records of the Ministry of Fisheries Station on the River Itchen.

(iv) *Light.*—In the experiments in which the lighting conditions were varied from that of the aquarium-room, continuous light was supplied by a 4 c.p. electric lamp placed in the aquarium, precautions being taken to nullify the heating effect of the lamp. Increasing " daylight " was experimented with several times. In these experiments the duration of daylight was increased by means of a quartz-tube arc which was switched on for increasing periods at sunset daily.

(v) *Survival of Fish in Experimental Aquaria.*—The fish chosen for experimental purposes were selected as being normally healthy adult fish. Owing to the high

proportion of fish infected with *Glugea* (p. 206), it was often impossible to exclude such fish from the experiments. No death occurred which could be directly attributed to this disease. The mortality during the experiments, which usually lasted at least four months, was low. The precaution of dividing the fish into two aquaria during an experiment was useful, as occasionally after two months there was a sudden high death rate in one aquarium, the cause being unknown.

(vi) *Observation of Results of Experiments.*—The determination of the time of development of the stages of development and loss of the secondary sexual characters under different conditions was the objective of most of the experiments. As each experiment included at least ten fish, and since several experiments were in progress at the same time, it was impracticable to keep individual records of the fish. Various means of marking the fish were experimented with, such as cutting the spines. As, however, the fish became very tame after being in the aquaria about a week, they do not extend their spines and this method thus presented difficulties. It is one which would probably be very suitable for marking a large number of fish in their natural environment for the purposes of tracing migratory movements.

To ensure uniformity the observations were made by artificial light, a lamp being placed directly over each aquarium at the time of observation. The secondary sexual colours show very clearly under these conditions. Daily records were kept of the number of fish in each aquarium in each stage of development of the colours. Owing to the number of observations made daily the element of personal error was greatly reduced. The maximum range of individual variation in the time of development of the secondary sexual characters in any one experiment was never greater than one week.

Owing to the difficulties of distinguishing the male fish from the female when the secondary sexual characters are absent, a certain number of females were accidentally included in the experiments. This could only be determined after the conclusion of the experiment when all the fish were killed and examined.

(vii) *Range of Experiments.*—The experiments were of three types :—

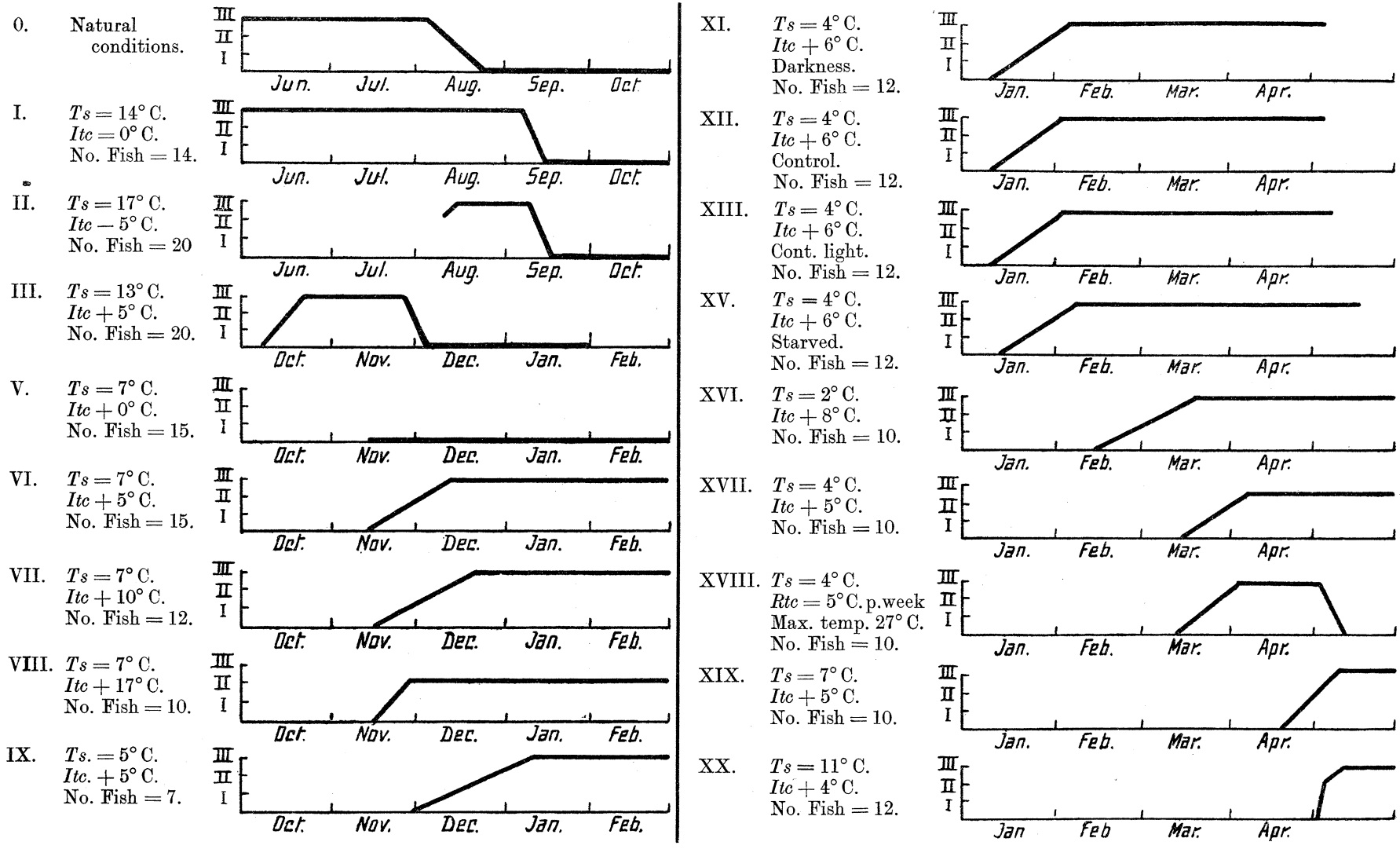
(*a*) Experiments of the effect of keeping the fish at different temperatures, under different conditions of light and under combinations of temperature and light (20 experiments).

(*b*) Experiments on partial castration, total castration, and heterotransplantation of the gonads (33 experiments).

(*c*) Experiments on the effects of sterilty and sub-sterility doses of X-rays (10 experiments).

In order to save labour, particularly that of fixing and embedding and sectioning objects, the experiments were combined. For example, the condition of the testis of a fish after it had been kept at a given temperature for three weeks was determined by removing one testis by an operation. This testis also gave the condition of the gonads at the commencement of a unilateral castration experiment.

GRAPH *a*I.—Rate of Development and Loss of the Secondary Sexual Characters under Experimental Conditions.



Records and Analysis of Experimental Results.

The results of the principal experiments are shown by a graph of the rate of development of the secondary sexual characters. The following experimental data is also given :—The number of adult male fish in each experiment (number of fish) ; the temperature of the habitat of the fish at their time of capture, *i.e.*, at the beginning of the experiment (*Ts.*) ; the initial change of temperature (*Itc*), or the rate of change of temperature (*Rtc*). Except where otherwise stated, the food and lighting conditions of the experiments were constant.

The experiments recorded are those in which at least 70 per cent. of the fish behaved alike in respect to the secondary sexual characters. The occasional non-conformity of fish in other experiments was almost always attributable to the health of the fish suffering in the course of the experiment. The uniformity attained, despite the comparative small numbers (generally 10 to 12 fish in each experiment), I consider to be due to the careful selection of healthy adult fish.

In addition to the experiments here referred to, a number of other experiments were performed. These additional experiments were either preliminary experiments or repetitions of the experiments recorded. Although these additional experiments were made with a smaller number of fish, the results agreed closely with the general plan of the main experiment in each case.

Series A.—Experiments on the effect of variations of food, light and temperature on the rate of development of the secondary sexual characters.

(i) *Food Experiments.*—Experiments on the effect of different diets and of different intensity of feeding showed that the food of the fish played a small part in the reproductive cycle. For example, if the temperature of the fish was maintained constant at that of its habitat during any time of the non-breeding season, then intensive feeding, varying diets or starvation were equally without effect in inducing the development of the secondary sexual characters.

It was found impossible to subject a fish to complete starvation if kept in an aquarium containing water-weeds and detritus at the bottom. If the fish were kept in plain water, they died before any changes had taken place in the gonad.

If the fish are subjected to a change of temperature which induces a development of the secondary sexual characters, the rate of development of these is very little affected by variations of the food of the fish (Experiment XV).

In view of these experiments, the fish in other experiments were fed sufficiently to keep them in good health and growing moderately rapidly, as this was considered to be probably the optimum conditions for the development of the secondary sexual characters, when this has been stimulated by some other factor.

(ii) *Light Experiments.*—Different light intensities, as with variations of food supply

alone, are insufficient to cause a development or inhibition of the secondary sexual characters, when the fish is kept at the temperature of its natural habitat during the non-breeding season. In view of ROWAN'S results on the effect of artificially lengthening exposure to light at a constant temperature of birds (ROWAN, 1926,), several experiments of this type were made with the fish. ROWAN found that under the stimulus of lengthening exposure to light the ovaries of the birds underwent the same changes which accompany the approach of the natural breeding season. Increasing exposure to either artificial light or light from an arc (containing a moderate proportion of ultra-violet light) caused no change in the testis nor the development of the secondary sexual characters. Prolonged exposure (one and a half hours) to the arc caused no observable change to the fish.

If the secondary sexual characters are stimulated to develop by a suitable change in the temperature conditions of the fish, the rate of development of these characters is unaffected by a wide range of light intensities (Experiments XII-XIV, Graph *a*I).

Light intensity has apparently little effect on the general health of these fish when in aquaria. A moderately brightly lit aquarium was in general favourable to the fish. The animals become rapidly acclimatised to bright lights, and feed in a normal manner in darkness. Throughout the experiments on the effect of different temperatures, the fish were in aquaria at the level of and close to north windows. In addition the whole room was well lighted with a top-light.

(iii) *Temperature Experiments.*—In these experiments on the effect of variations of temperature on the secondary sexual characters, two types of temperature change were used, an initial rise of temperature (*Itc*) and a definite rate of change of temperature (*Rtc*). In contrast with the experiments in which the temperature was continuously altered, in all the experiments in which the fish were subjected to an initial rise of temperature, the rate of change of temperature was of the order of not less than 5° C. per hour.

In order to make a logical presentation of the evidence of the effect of changes in temperature, the experiments are arranged in groups irrespective of chronological order.

A.—*The Development of the Secondary Sexual Characters.*

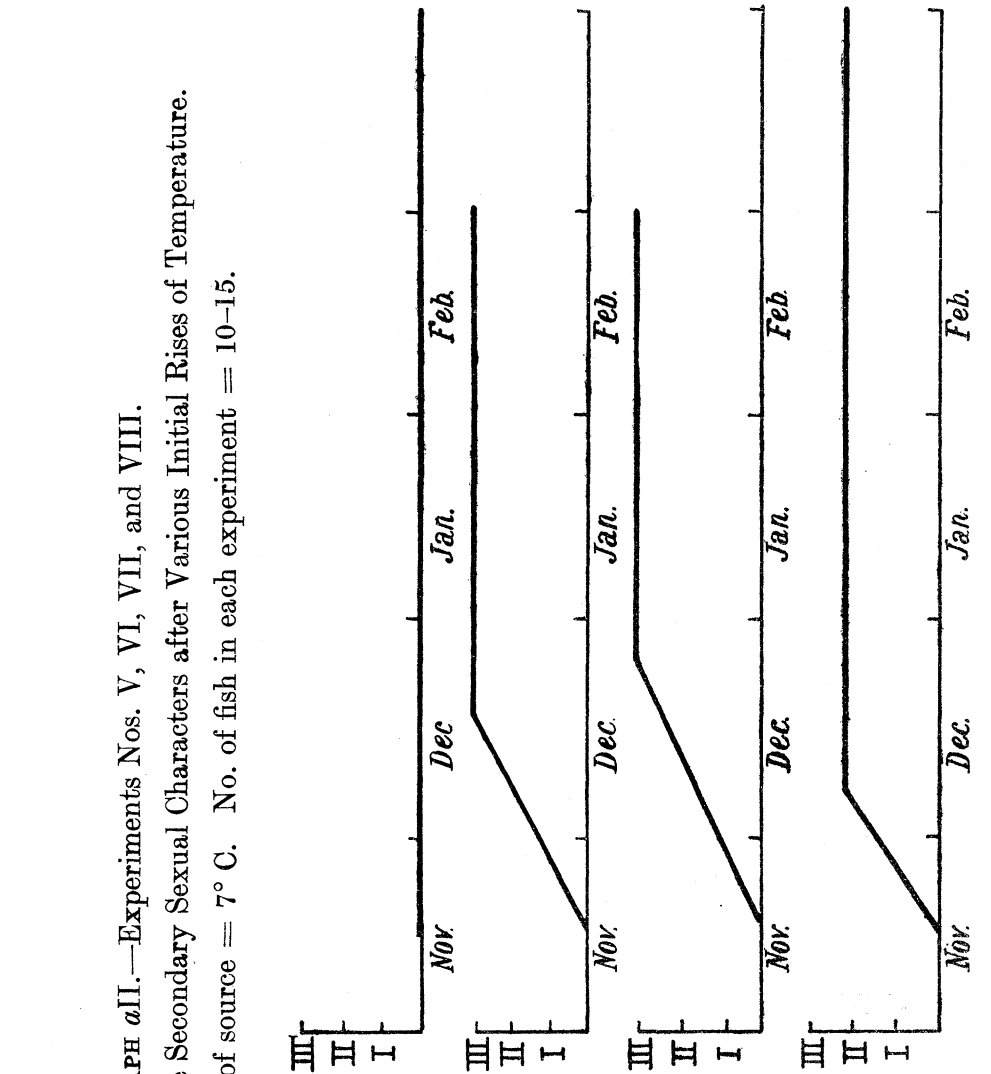
Experiments V–VIII (Graph *a*II) show the rate of development of the secondary sexual characters under the influence of varying initial rises of temperature. It will be noticed that the maximum rate of development occurred when the rise of temperature was 5° C. It was impossible to make a closer analysis of this *optimum initial rise of temperature,* as observational errors would cover two or three days' difference in the time of appearance of the secondary sexual characters.

Experiments VI, X, XVI, XVII, XIX and XX (Graph *a*III) form a series in which the fish were subjected to approximately the same initial rise of temperature (5° C.) but differing in their dates and therefore in their initial temperature. The greatest time

GRAPH *a*II.—Experiments Nos. V, VI, VII, and VIII.

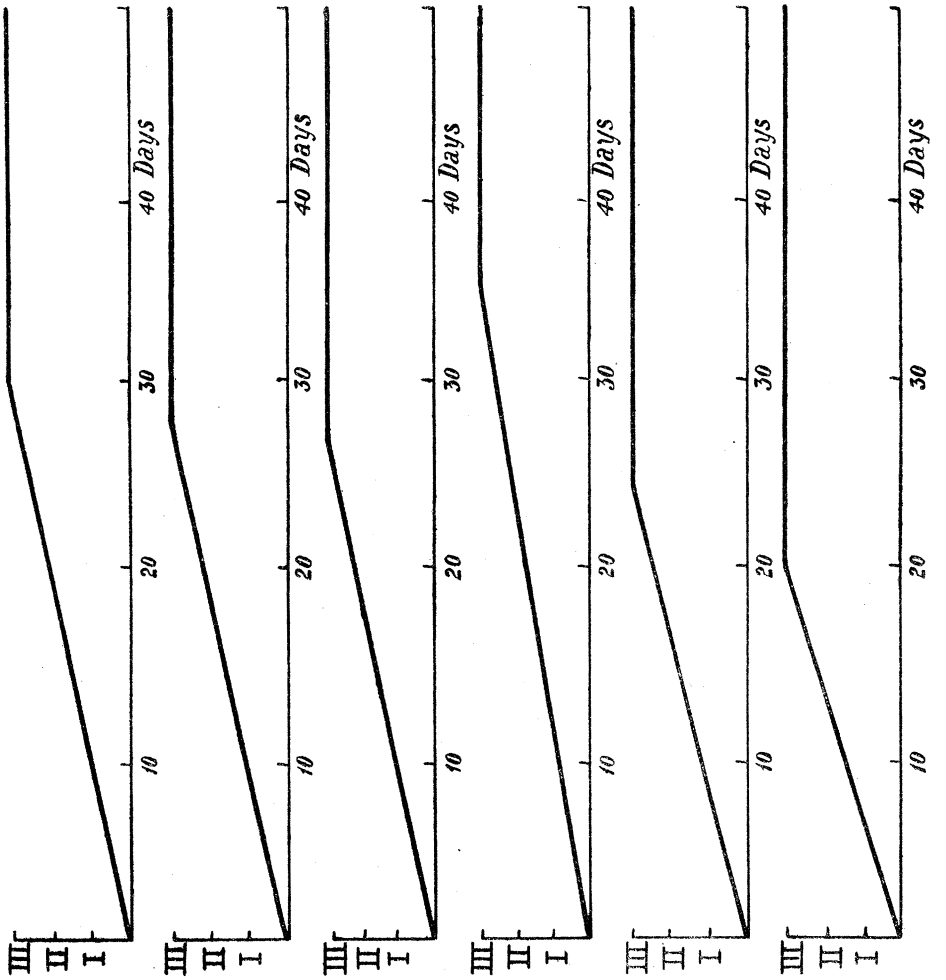
Rate of Development of the Secondary Sexual Characters after Various Initial Rises of Temperature.

Temperature of source = 7° C. No. of fish in each experiment = 10–15.



for the total development of the sexual characters occurred in December (Experiment IX), the total time decreasing as the natural breeding season of the fish approached.

GRAPH αIII.—Rate of Development of the Secondary Sexual Characters at different Times under the same Initial Rise of Temperature.



No. VI. November 15.
Temp. of source = 7° C. *Itc* = 5° C.
No. of fish = 14.

No. X. December 28.
Temp. of source = 4° C. *Itc* = 6° C.
No. of fish = 10.

No. XIV. January 9.
Temp. of source = 4° C. *Itc* = 6° C.
No. of fish = 12.

No. XVI. February 15.
Temp. of source = 2° C. *Itc* = 8° C.
No. of fish = 10.

No. XVII. March 15.
Temp. of source = 6° C. *Itc* = 4° C.
No. of fish = 10.

No. XIX. April.
Temp. of source = 7° C. *Itc* = 5° C.
No. of fish = 8.

The explanation of the decrease in the rate of development which occurs if the initial rise of temperature is increased may be due to the disturbance of the physiological state of the fish which must occur when the temperature of its environment is suddenly changed. It is significant, however, that in Experiment VIII, in which the secondary sexual characters failed to develop fully, the fish lived at the high temperature for three to four months. On the other hand, Experiment XVIII shows that if the rate of change of temperature is moderate, the secondary sexual characters develop at a high temperature. In all the experiments on the development of the sexual characters after exposure to a rise of temperature the only change in the testis, in those cases in which the secondary sexual characters appeared, was a growth of the interstitial elements in the testis. In Experiment VIII, on the other hand, there was no development of the interstitial tissue, and no development of the secondary sexual characters, but in the germ cells there was a marked change, since spermatocytes and early stages in spermatogenesis appeared. It is possible that a very rapid rise of temperature may have either upset the rhythm of the interstitial cells but not that of the germ cells (under natural conditions the development of the interstitial tissue being associated with a rising temperature), or that this rapid rise of temperature has a specific action in inhibiting the development of the interstitial tissue.

A continued high temperature is not essential to the development of the secondary sexual characters. In Experiment IV the fish were exposed to the higher temperature (18° C.) for seven days, and then the temperature was reduced to that of their natural habitat at the beginning of the experiment (11° C.). The secondary sexual characters developed at the same rate as the control (Experiment III) in which the higher temperature was maintained. The development of the secondary sexual characters therefore does not depend on the environment of the fish being above a certain threshold temperature. Further evidence of this fact is given by the development of the sexual characters at a temperature of 8° C. in Experiment XVI. The fish are at this temperature both in their natural habitat in April and in Experiment V, without the secondary sexual characters. It is clear that a rise of temperature is one of the conditions essential to the development of the secondary sexual characters.

B.—*The Duration of the Fully Developed Sexual Characters.*

In Experiment I the breeding season of the fish was extended one month in comparison with the natural breeding season. Actually the extension of the breeding season in the experiment was probably greater than one month. The loss of the secondary sexual characters of the fish in their natural habitat is gradual, and the highest development of the sexual characters is passed by the beginning of August.

The cause of this extended breeding season in Experiment I is the constant moderate temperature (12° C.) at which the fish were kept. In Experiment II, the fish were taken in August at the end of their natural breeding season and kept at a temperature 5° C.

below that of their natural habitat. As a result the secondary sexual characters were redeveloped, and maintained for a month. The total breeding season of these fish therefore was approximately the same as those in Experiment I.

In October (Experiment III) the fish developed the secondary sexual characters moderately rapidly after they had been subjected to an initial rise of temperature at 8° C. The secondary sexual characters resulting persisted for only one month. A comparison of this experiment and those made in November, suggests that in this experiment there was a redevelopment of the previous breeding season, rather than a precocious development of the following season.

The full duration of the secondary sexual characters was also followed in Experiment XVIII (Graph *a*I). The secondary sexual characters were developed at a temperature of 27° C. in this experiment, and persisted for one month.

The durations of the breeding season at different temperatures are therefore as follows :—

Four to four and a half months at a temperature from 12°–18° C. (Natural Breeding Season).

Five months at a temperature of 12° C. (Experiments I and II).

One month at a temperature of 27° C. (Experiment XVIII).

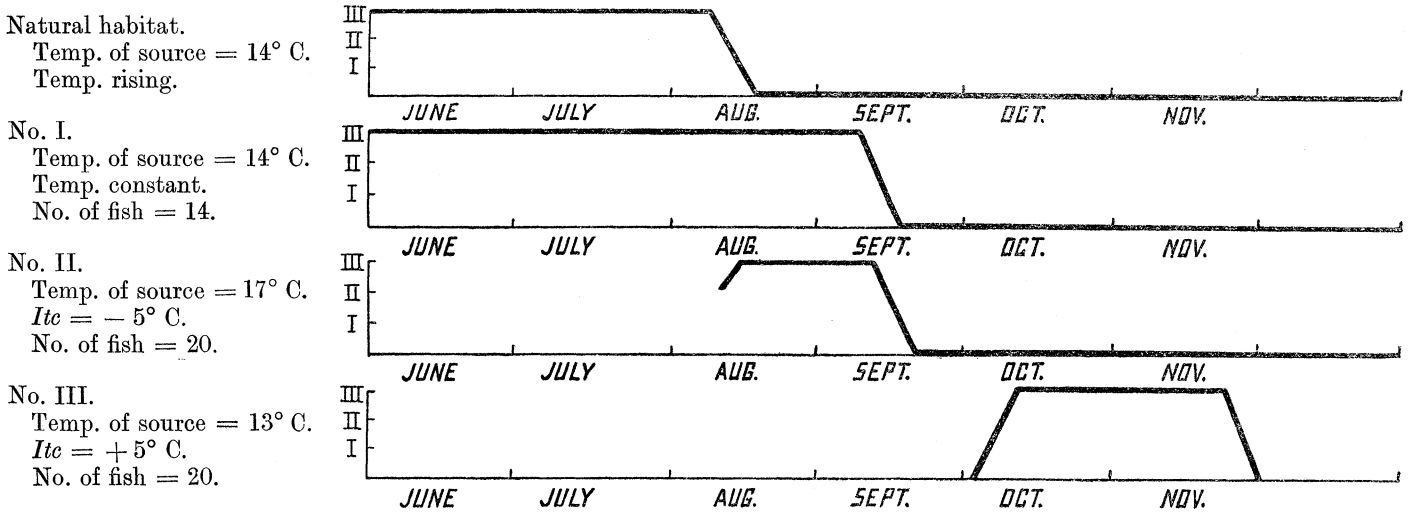
If, as is very probable from the above, the duration of the breeding season is inversely proportional to the temperature, the conditions being kept constant, it will be seen that in no other case was an experiment continued sufficiently long for the completion of the breeding season at the temperature of the experiment. (The duration of the experiments was limited by the mortality of the fish, the depletion of numbers by the removal of samples and by the requirements of space.)

C.—*The Loss of the Secondary Sexual Characters.*

Under experimental conditions the loss of the secondary sexual characters (Graph *a*IV) occurred without any change in the physical conditions of the experiment. The duration and loss of the secondary sexual characters agrees therefore with the hypothesis that the duration of the secondary sexual characters is dependent on the continuous supply of some supporting substance, the total amount of which available is fixed for one annual breeding season. This substance is utilised in a reaction which is accelerated by temperature. Evidence is given below for the hypothesis that the interstitial tissue produces such a substance.

The loss of the secondary sexual characters by the fish in its natural habitat may be due to either a specific inhibition or to the total exhaustion of the interstitial hormone. The reduction of the secondary sexual modifications under experimental conditions cannot be brought about prematurely by alterations of temperature, light or food, nor do such alterations necessarily accompany the reduction of the secondary sexual

GRAPH *a*IV.—Experiments I–IV.
Rate of Loss of the Secondary Sexual Characters under Experimental Conditions.



characters when this occurs under experimental conditions. There is no indication of any external stimulus causing the inhibition of the secondary sexual characters. It is concluded, therefore, that the reduction of the secondary sexual characters which marks the end of the natural breeding season is due to the total exhaustion of the available interstitial hormone. The redevelopment of the secondary sexual characters in Experiment II is presumed to be due to a development of further interstitial hormone.

Conclusions from Experiments of Type A.—The effect of variations of food, light and temperature on the secondary sexual characters and spermatogenesis :—

i. The development of the secondary sexual characters is a function of a rise of temperature.

ii. The duration of the secondary sexual characters varies inversely with the temperature.

iii. A high temperature is associated with the appearance of the commencement of spermatogenesis, *i.e.*, the spermatogonia.

Experiment II is to some extent an exception to Conclusion (i). The redevelopment of the partially reduced sexual characters in this case was not associated with a rise of temperature.

The fact that the secondary sexual characters developed more rapidly in October (Experiment III) than in November (Experiment VI) suggests that in the former experiment the short breeding period produced is to be associated with the prolongation of the previous natural breeding season rather than a precocious development of the succeeding breeding season.

The experiments also show that :—

iv. The secondary sexual characters develop, under the influence of the same stimulus, more rapidly as the natural breeding season is approached, the minimum rate of development occurring in November and December.

Series B.—Experiments on the effects of unilateral and total castration and on the transplantation of the gonads.

(i) *Previous Work on the Castration of Fish.*—The only direct castration experiments on the possible connection between the gonads and the secondary sexual characters are those of KOPEČ in 1918. KOPEČ used the Central European Minnow, *Phoxinus laevis*, in which the male, and to a less extent the female, develops a red nuptial hue during the breeding season.

KOPEČ's first series of experiments (KOPEČ, 1918) were not entirely satisfactory, 7 out of 30 total castrates developing the nuptial hue, although the fish survived only three weeks. KOPEČ suggests that, "At the time of catching, the gonads of these fishes were already so far developed physiologically, that in many individuals hormones from the gonads had already reached the interior of the body and remained there, they caused the nuptial colour notwithstanding the following castration" (KOPEČ, 1918, p. 113).

In 1927 KOPEČ showed that if the gonads were removed sufficiently early the secondary sexual characters did not develop. KOPEČ also made some experiments on the expansion of the erythrophores and concluded as follows :—

"1. The development of the nuptial red in *Phoxinus laevis* Agass, indubitably depends on the presence of the testicles or of the ovaries.

2. The gonads may not be considered as exclusively influencing the expansion of the erythrophores ; besides optical impressions there are several other factors, internal as well as external, which also have a distinct influence on this process.

3. The nuptial expansion of the erythrophores seems to be provoked in the two sexes, perhaps even directly, by the secretion of the pituitary gland stimulated by the sex hormones (*cf.* the experiments of ABOLIN).

4. The nuptial red is a character which is either acquiring a dependence on the gonads or is already becoming independent from the sexual glands."

(The reference to ABOLIN in Conclusion 3 is to this investigator's experimental production of the nuptial hue in both sexes of *Phoxinus* by the injection of infundin (ABOLIN, 1925).

KOPEČ lays stress on the influence of external factors on the development of the secondary characters, but does not appear to have attempted to control these in his experiments. The presence of the nuptial hue in both sexes renders the question of

the cause of development of this in *Phoxinus* a less concise problem than that of the secondary sexual characters of the male *Gasterosteus.* Lastly, although as KOPEČ points out, it affected the controls as much as the experimental fish, the fact that the fish only survived the operation by three weeks cannot be considered altogether satisfactory.

While, therefore, KOPEČ's investigations show that the nuptial hue in fish is dependent on the presence of the gonads, neither has that part of the gonad which brings this about, nor the mechanism of this relation, been determined by him.

KOPEČ'S own observations recorded in his summary given above (2), support the experiments recorded (p. 247) on the dependence of the development of the secondary sexual characters in Teleost fish on the environment.

The experiments of ABOLIN (1925) do not necessarily show that the internal secretion of the gonads act by way of the posterior lobe of the pituitary, since the expansion of the chromatophores can be brought about in other ways than by infundin (SPÄTH, 1916). In considering the results of injections of extracts of the anterior or posterior lobe of the pituitary, it is necessary to take into account the post-mortem diffusion of the hormone of one lobe into the other (SPAUL, 1929). It is known that the extracts of the anterior lobe may have a direct effect on the gonads (see p. 256).

(ii) *Method of Operating.*—After several experiments with different anaesthetics, 0·9 per cent. urethane was found to be the most convenient. The operation was performed under a low-power binocular, the fish lying on damp cotton-wool. The wound was closed with a hot needle and coated with celloidin in acetone.

Several attempts were made to perform hetero-transplantation of the gonad. In one case only, when the gonad of a male in Stage III (p. 217) was planted in the kidney of a totally castrated female, was any positive result obtained. In this case the fish developed to Stage II of the secondary sexual characters.

The survival of fish after these operations was remarkably good. At least 80 per cent. survived six weeks, many considerably longer. After the first month the scar tissue healed completely, leaving no external trace of the operation.

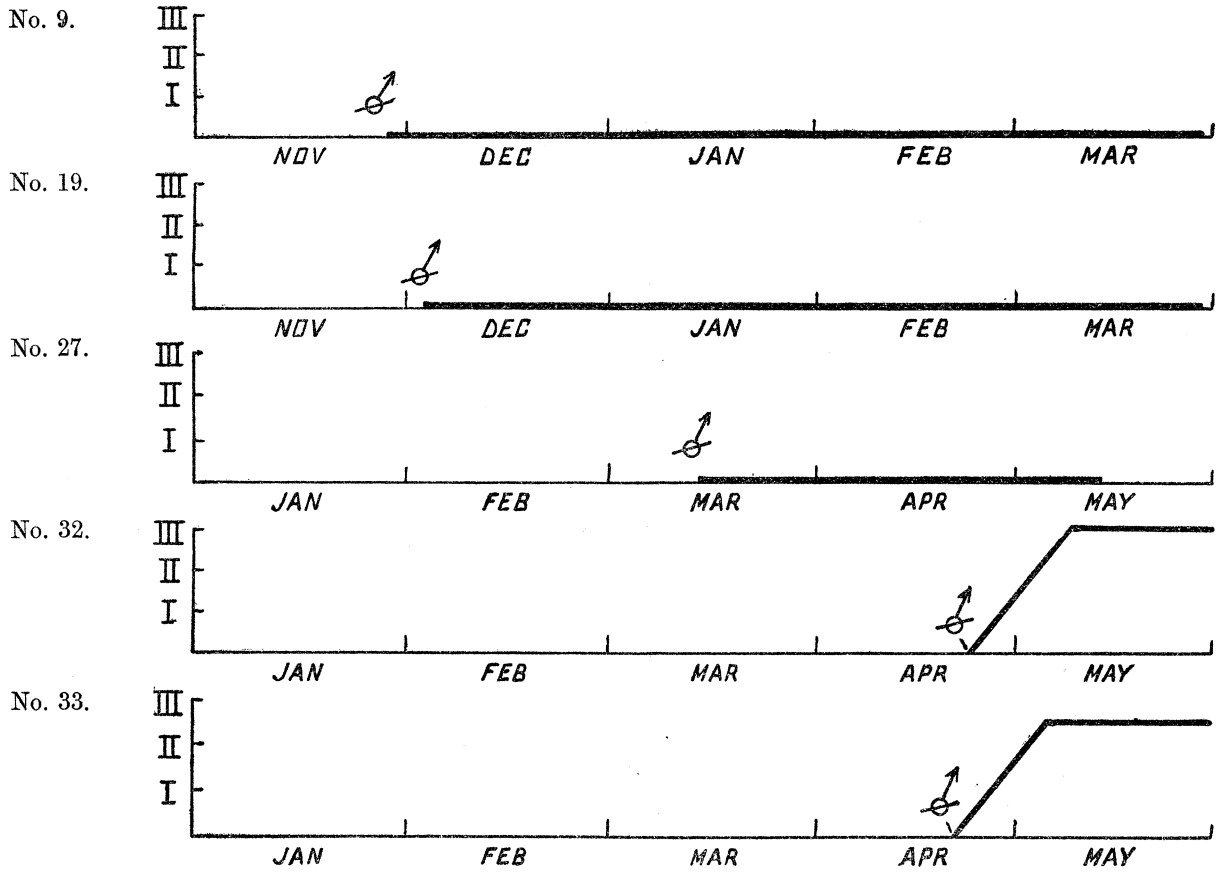
(iii) *Total Castration.*—In a few preliminary experiments total extirpation of the testes was not brought about. Such fish subsequently developed the secondary sexual characters. In these first operations the *vas deferens* was cut close behind the testis. That apparently the whole testis was removed was verified by sectioning. After four months these fish developed the sexual colorations, and on examination it was found that both testes had regenerated, as small much lobulated masses. In subsequent operations both the testes and the anterior halves of the *vas deferens* were removed and no regeneration occurred. These observations bear out the opinion expressed above (p. 220) that the resting spermatogonia of the testis are contained in the anterior part of the *vas deferens.*

If at the time of total castration the testis contains only thin interstitial tissue (p. 231), the totally castrated fish fails to develop the secondary sexual characters even

though it be subjected to a rise of temperature (Operation Nos. 9, 19, 27, etc., Graph *b*I).

GRAPH *b*I.—Effects of Total and Unilateral Castration on the Secondary Sexual Characters.

Total Castration—Secondary Sexual Characters not developed at time of operation.



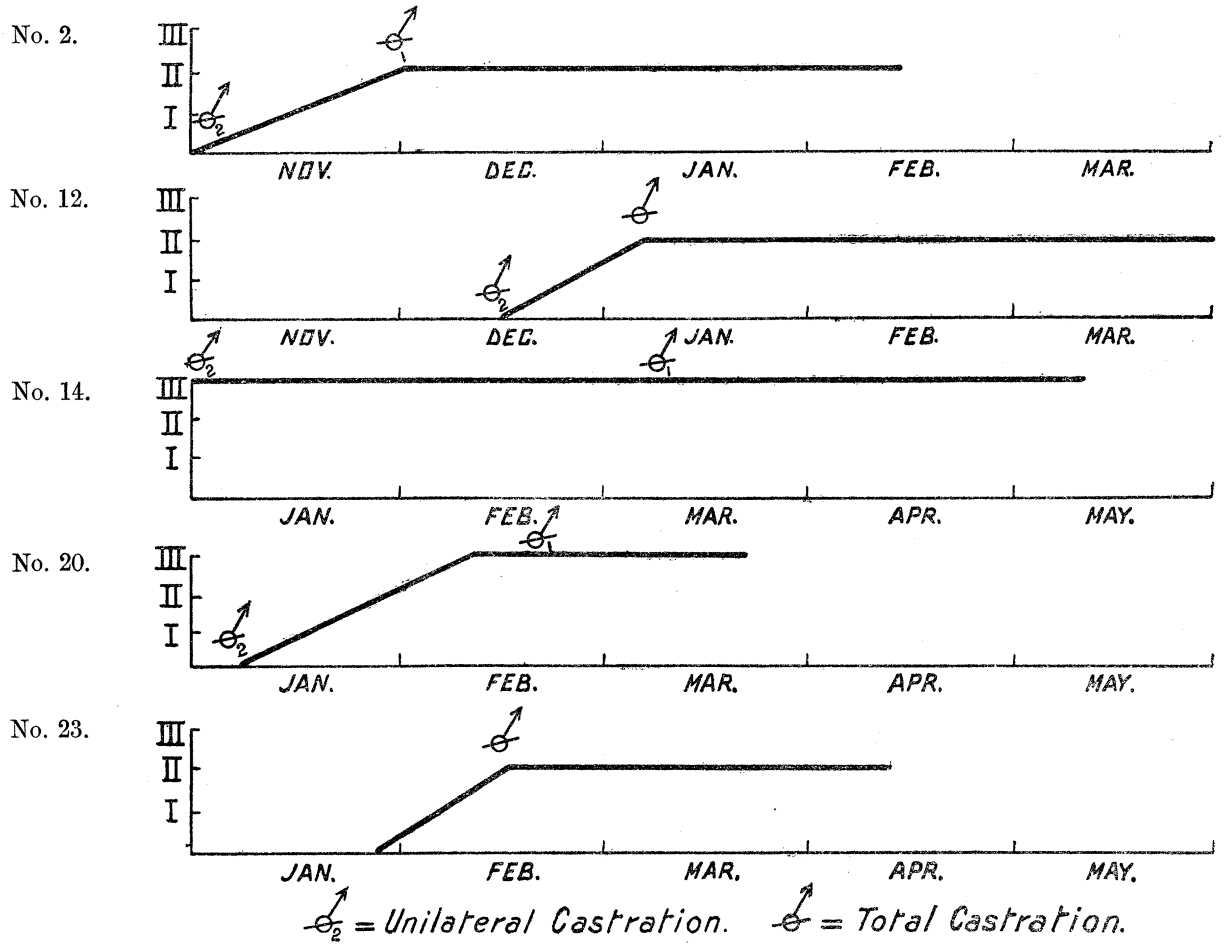
On the other hand, if the testis at the time of total castration has the interstitial tissue in the condition medium wide or wide (p. 231), then the fish will develop the secondary sexual characters subsequent to the operation if it is subjected to a rise of temperature (Operation Nos. 32, 33, Graph *b*I). The control fish subjected to a rise of temperature develop the secondary sexual characters at precisely the same rate, while the fish remaining in their natural habitat do not develop these characters since they are not subject to a rise of temperature during that time.

Such fish developing the secondary sexual characters after total castration are exactly similar to those recorded by KOPEČ (1927).

The results of total castration when the secondary sexual characters are developed show that there is no rapid loss of the secondary sexual characters, although in a few cases they showed signs of reduction (Operation Experiments No. 14, 20, etc., Graph *b*II).

GRAPH *b*II.—Effects of Total Castration on the Secondary Sexual Characters.

Secondary Sexual Characters partly or wholly developed at time of operation.



A criticism of these operations is that in no case did the fish survive the full length of the breeding season corresponding to the temperature of the experiment.

If the secondary sexual characters have been partly developed (Stage II, p. 217) by a rise of temperature, and the fish is then totally castrated, this stage persists. An examination of the testis removed shows that only a part of the interstitial tissue is secretory. Under these conditions therefore the interstitial tissue bears a quantitative relation to the secondary sexual characters.

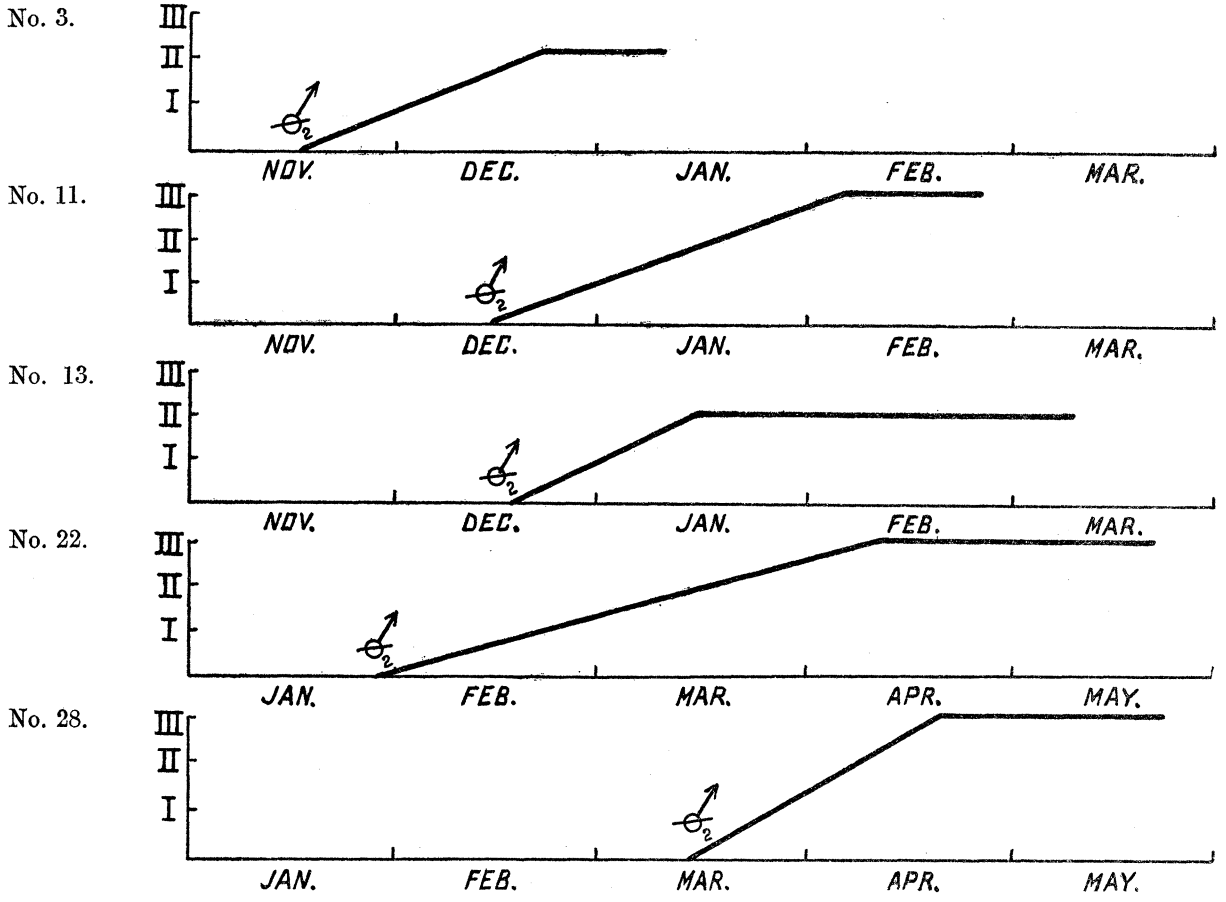
The comparatively short survival of fish totally castrated in Stage III suggests a dependence on the testis. The explanation is more probably that the fish are in a less suitable condition to withstand the shock of the operation when the secondary sexual characters are developed than when they are absent.

Results of Unilateral Castration.

In contrast to total castration, unilateral castration makes little difference to the development of the secondary sexual characters (Graph *b*III).

GRAPH *b*III.—Effects of Unilateral Castration on the Secondary Sexual Characters. Series I.

Unilateral Castration prior to the Development of the Secondary Sexual Characters.



The rate of development of the secondary sexual characters after the fish has been unilaterally castrated and subjected to a rise of temperature, is frequently slower than that of the normal fish. The fact that sometimes the rate of development in unilateral castrates is equal to that of normal fish, and at times considerably slower, points to the shock of the operation as being the probable cause of this slowing down of the rate of development.

If the fish is unilaterally castrated with the secondary sexual characters fully developed, these may be reduced in certain cases. Operation No. 5 was a case in which the secondary sexual characters were lost after unilateral castration. This fish was one

of Experiment III, in which the breeding season, experimentally produced, only lasted one month. The unilateral castrated fish behaved exactly like the normal fish.

The changes undergone by the remaining testis in unilaterally castrated fish are interesting. First it must be stated that there is no marked " compensatory hypertrophy " of the interstitial tissue in the remaining testis. A small amount of hypertrophy may occur. The nature of the interstitial tissue of *Gasterosteus*, the fluctuations in volume which normally occur, the individual variations due to very slight differences in technique, result in it being impossible to make accurate comparisons of the total amount of interstitial tissue in one testis with that of another, unless the differences are considerable.

The interstitial tissue of the remaining testis develops at a rate corresponding to the development of the secondary sexual characters, if the fish has been subjected to a rise of temperature. In most cases the development of the interstitial tissue is the only difference between the testis removed and the testis remaining after the operation. This forms very conclusive evidence that the secondary sexual characters are a function of the interstitial tissue.

In those cases in which the fish was unilaterally castrated with the sexual characters developed, the testis removed shows a well-developed interstitium. An examination of the testis remaining, after the secondary sexual characters have been lost (Operation No. 5), shows that the only change which has occurred in the gonad is that the interstitial tissue is degenerating. This is of interest in showing (i) that the reduction of the interstitial tissue accompanies the reduction of the secondary sexual characters, under experimental conditions as in nature, and (ii) that the reduction of the interstitial tissue may occur without any change in the germ cells.

In experiments Operations Nos. 22, 28, 29 and 30, the fish were subjected to a rising temperature after the operation, rising at a rate of 5° C. per week to 27° C. After two months, the testis remaining in the fish showed marked differences from that removed by the operation. In addition to a well-developed interstitial tissue, which was accompanied by the appearance of the secondary sexual characters, the tubules showed a fresh spermatogenesis, which had resulted in the distention of the anterior tubules particularly. So great was this distention that in some cases the normal limits of the testis had been burst through and small outpushings occurred. The fresh spermatogenesis was interesting in that the tubules had, as a result, the appearance of a permanent germinal epithelium; in fact, that had the appearance which is found in animals with a continuous spermatogenesis, such as mammals (Plate 22, fig. 17).

By comparison with Experiment VIII (p. 245), there can be little doubt that this fresh spermatogenesis, which occurred while the tubules were already distended by pre-existing spermatozoa, resulted from the exposure to high temperatures.

The occurrence of spermatogenesis in these fish was accompanied by the well-developed interstitial tissue and the secondary sexual modifications. It is clear therefore that the growth phase of the spermatocyte may be independent of the reduction of the

interstitial tissue. Taking this in conjunction with the case described above, of the loss of the interstitial tissue unaccompanied by any change in the germ cells (Operation No. 5), it will be seen that the natural relations between the development and loss of the interstitial tissue and the commencement of spermatogenesis, is completely reversible experimentally. It is impossible to consider therefore that in *Gasterosteus*, the interstitial tissue bears a trophic relation to the germ cells (see p. 232 above).

Series C.—X-ray Experiments.

The recent work of BOUIN and ANCEL (1929) shows conclusively that by suitable dosage of X-rays all the germinal cells of the gonad may be destroyed by X-rays, leaving the interstitial cells unaltered, and that very intense radiations destroy both the germ cells and the interstitial cells.

Previous investigators on the effect of X-radiation on the testis of fish (CHAMPY, 1923) have been unable to bring about definite sterilisation.

There is a strong absorption of X-rays by the water containing the fish. I am indebted to Dr. A. E. BARCLAY, Department of Radiology, Cambridge, for having measured the X-ray absorption of water and of the fish, and so making it possible for me to give the dosage at the level of the testis in unit skin doses (or pastille doses). In the X-radiation experiments performed by Dr. BARCLAY, the fish were irradiated by a tube vertically over them at a distance of 22·5 cms., the current 95 k.v. at 2 m.a. An exposure of 9 minutes gave a dose of $1\frac{1}{4}$ unit skin dose.

The earliest X-ray experiments (Nos. 1, 2 and 3) were made at the Department of Anatomy, University College, London. The conditions of exposure rendered it impossible to determine the actual dose received by the fish. From the results produced in comparison with experiments where the dosage was determined accurately, it is possible to estimate the dose received by the fish approximately.

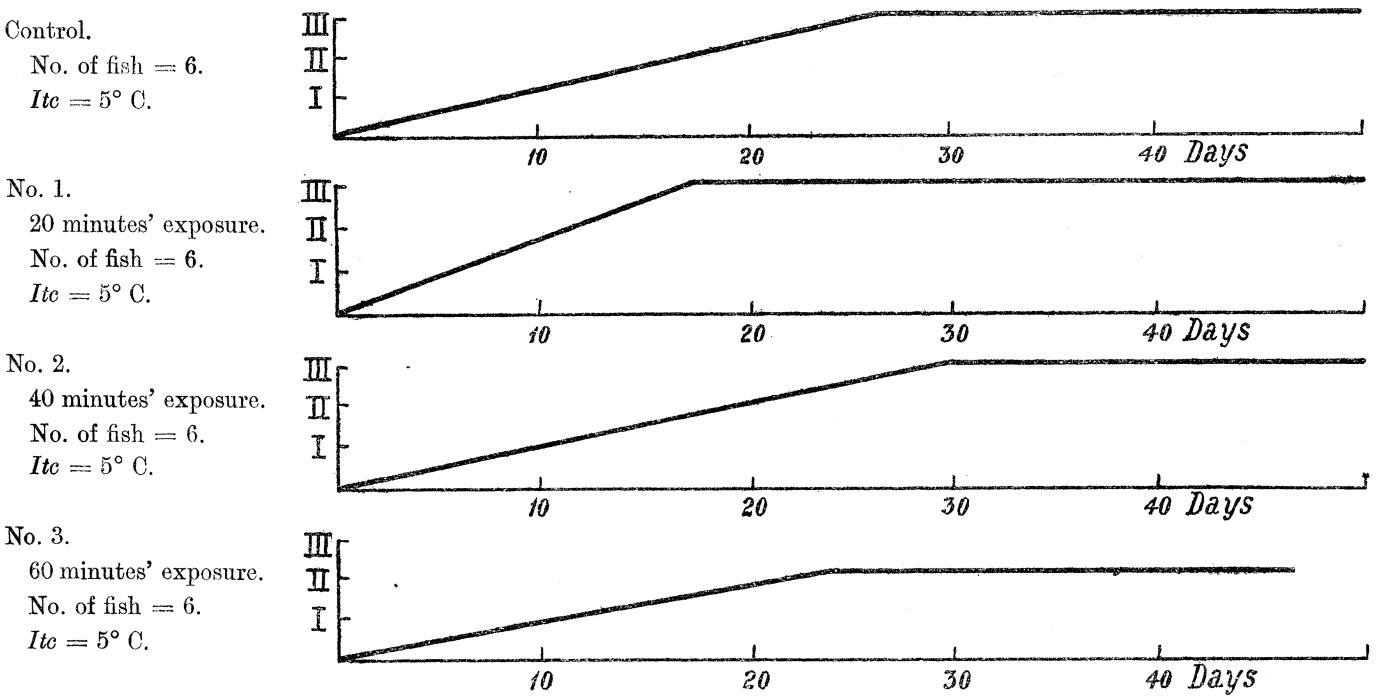
In the course of catching and keeping in the aquaria the fish were in all cases exposed to an initial rise of temperature of 5°–6° C.

The X-ray experiments are divisible into three types :—

1. Light sub-sterility doses	1–$1\frac{1}{2}$ unit skin dose.
2. Medium doses causing partial destruction of spermatozoa..	2–5 unit skin dose.
3. Heavy doses causing sterility	7–8 unit skin dose.

X-ray Experiments, Type 1.—Experiments 1, 5, 7 and 8 are of this type. When the dose is 1 u.s.d. there is a rapid development of the secondary sexual characters, and a hypertrophy of the interstitial tissue (Plate 22, fig. 21). The secondary sexual characters developed at nearly twice the rate of the fish in the control. This dose caused no observable change in the condition of the germ cells (Graph *c*I and *c*II).

GRAPH cI.—X-ray Experiments Nos. 1, 2, and 3.
Rate of Development of the Secondary Sexual Characters after Exposure to X-rays.

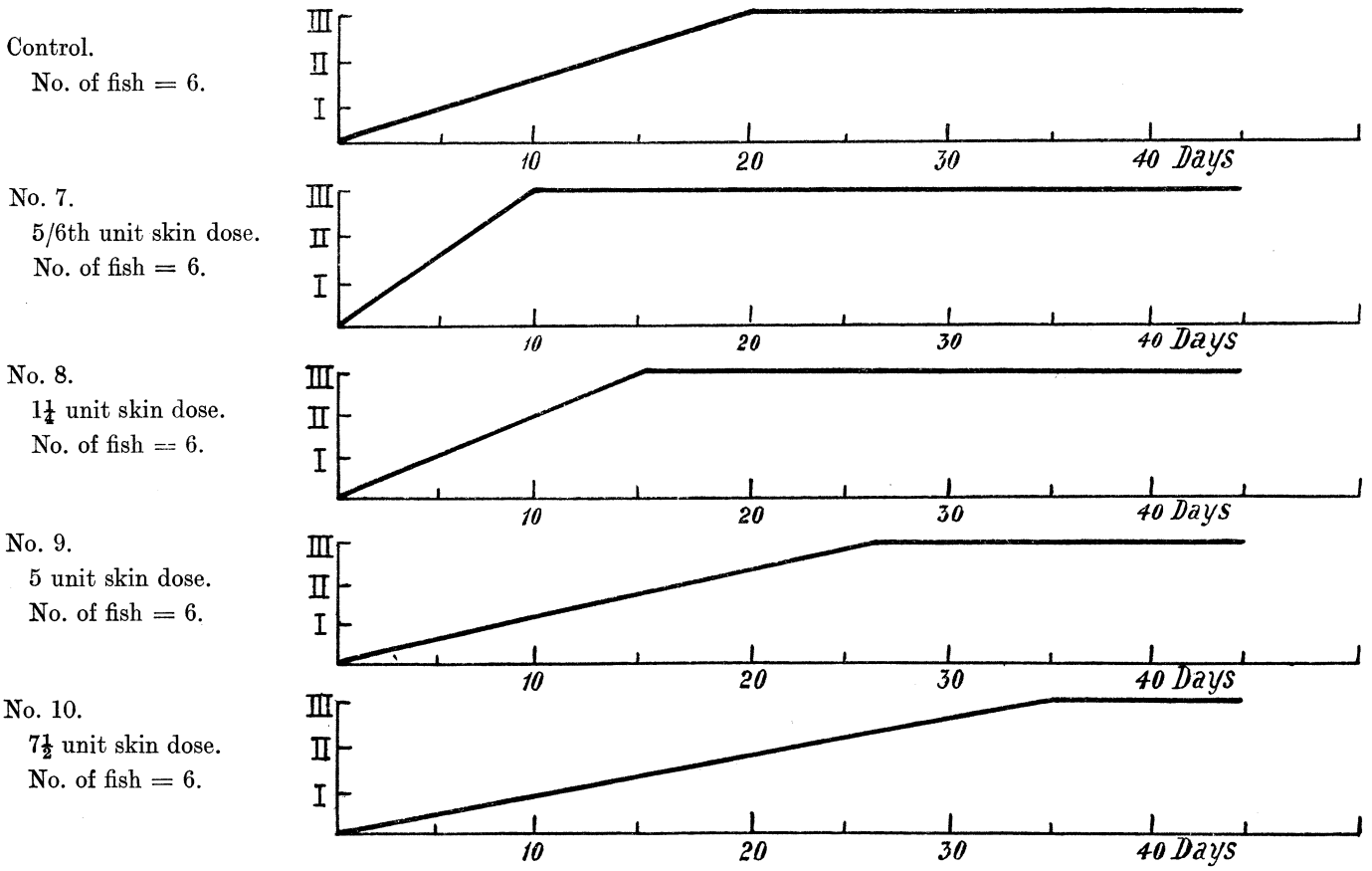


X-ray Experiments, Type 2.—In fish subjected to this dosage (Experiments 2, 4, 6), the secondary sexual characters developed more slowly than in the controls, the rate of development decreasing as the dosage increased. Doses exceeding 2 u.s.d. were given in repeated exposures. In this case the fish developed the early stages of the secondary sexual characters rapidly, followed by a period in which they showed no change. There was no marked hypertrophy of the interstitial tissue of these fish. After one month the larger tubules contained at their centre masses of dead spermatozoa. These dead spermatozoa stain very much lighter with iron hæmatoxylin than the normal sperm. They also aggregate in an even mass with large spaces between them, not in small groups. When the secondary sexual characters are developed the interstitial tissue is very similar to that of the control.

X-ray Experiments, Type 3 (*Graph cII*).—These fish were given repeated doses, at intervals of three days (Experiments 9 and 10). The secondary sexual characters developed rapidly up to Stage II. There was then a considerable period (20 days) during which the secondary sexual characters underwent no change. The sexual characters finally developed normally.

No change in the germ cells was observable until after the first fortnight. Thereafter a complete destruction of the spermatozoa set in, commencing with the occurrence of masses of dead sperm in the larger tubules, and spreading to every part of the testis. The cells embedded in the cells of Sertoli remained normal longest, but after one month

GRAPH cII.—X-ray Experiments Nos. 7, 8, 9, and 10.
Rate of Development of Secondary Sexual Characters after Exposure to X-rays.



no motile sperm could be observed in teased preparations of the testis.* (Plate 22, fig. 22).

The interstitial tissue of these fish contained patches of normal interstitial tissue. There were also groups of interstitial cells which either had not developed or had developed into a fibrous condition.

Effects of Extracts of Anterior Lobe of Pituitary.

By the courtesy of Dr. A. S. PARKES, I was able to investigate the effect of injection of extract of the anterior lobe of the pituitary which he used in his experiments (PARKES, 1925, 1929).

As a control a similar extract was made of the testis of a fish in which the secondary sexual characters had just developed.

* GATENBY (1929) has described a similar protective influence of the cells of Sertoli to the destruction of spermatozoa by X-rays in the testis of the Cavy.

The fish taken for the experiments were some which had lost the secondary sexual characters about two weeks before the experiments were made. The extracts were injected into the epiaxial musculature of the fish.

The experiments did not show a high order of uniformity. The testicular extracts gave in one or two cases out of ten fish a transient redevelopment of the secondary sexual characters. The injection of the extract of the anterior lobe of the pituitary gave a redevelopment of the secondary sexual characters lasting for about three weeks in five cases out of eight. A second injection prolonged the fully developed state for ten days.

The most significant result of these experiments is the fact that the injection of the extract of the anterior lobe of the pituitary caused a redevelopment of the interstitial tissue of the testis.

Artificial Fertilisation of Gasterosteus.

Methods.—The method used in the artificial fertilisation experiments was essentially the dry-sperm method used at most fish hatcheries. The eggs of a "ripe" female were stripped into a clean watch-glass, and the testis, removed from the male, by dissection, was broken over and the contents mixed with the eggs. After removal of the remains of the testis 1 to 2 c.c. of water were added to the dish. The watch glass was placed in a bowl containing clean tap-water at the required temperature, 10 to 15 minutes after the experiment was begun. The water in this bowl was aerated with a very fine stream of filtered air. When the incubation took longer than a week, the water in the bowl was changed.

The incubation time varied with the temperature (*cp.* GRAY, 1928). After hatching, the fish were fed with the water from aquaria containing *Daphnia* and other small invertebrates, the larger animals being removed by filtering the water. The technique described is essentially the same as that used by ANTHONY (1918). ANTHONY gives a table of the time of incubation at different temperatures, and his results and mine are combined in the accompanying table.

TABLE VII.—Time of Incubation at Different Temperatures, *Gasterosteus*.

Temperature. °C.	Number of Days of Incubation.	
	BENNETT.	ANTHONY.
25·9°	—	5·3
25°	5	—
18·8°	—	6·6
15°	9	—
10°	13	—
8°	—	15

These artificial fertilisation experiments are of importance in that they show that the sperm from a male without the secondary sexual characters will fertilize the eggs of a mature female (see p. 261). It was found that the male in Stage II does not show any signs of the breeding instincts when placed with a mature female, but the testis is capable of fertilising the eggs of the female. In this connection the remarks of ANTHONY are interesting. He states his criteria of maturity in the sexes (p. 261) and concludes : " Notons par parenthèses qu'il résulte de mes observations que l'un des meilleurs de ces caractères sexuels secondaires serait l'aspect de l'œil qui est vert émeraude chez le mâle seulement."

The Regulatory Mechanisms of the Reproductive Cycle.

From the observations on the natural reproductive cycle and the above experimental results, a tentative analysis can be made of the various internal and external factors influencing the reproductive cycle and conditioning its various phases.

The critical periods of the reproductive cycle have been defined above (p. 201, *et seq.*) and these critical phases will be examined in sequence.

Sexual Maturity.

The beginnings of the transformation of the gonia into the auxocytes, the spermatocytes and the oocytes, are difficult to recognise, but judging from the time of appearance of the definitive auxocytes this transformation always occurs during the summer months.

In any one source there are during the winter a varying number of fish whose length exceeds that of the smallest adult, but which are sexually immature or *young fish* as defined on p. 202. The proportion of these fish was small in the habitats Gc, but large in habitats Ga, Gb and Gd (p. 205).

If the fish are reared in the laboratory at a temperature about that of the natural habitat during the summer, they reach sexual maturity at the end of four months. The above-mentioned large *young fish*, which are found in December and January, are clearly at least five or six months old.

Sexual maturity therefore does not depend on the age of the fish exclusively. Nor is it dependent on a developmental stage, since of the above-mentioned groups of fish, some over 4·5 cms. (in the case of the source Gc) and not sexually mature, and others 4·5 cms. sexually mature, must both be of approximately the same age, and since they are of the same species from the same habitat, a habitat where the race of fish is approximately pure (p. 206), both groups of fish must be in the same developmental state.

That, however, the age or developmental state does in part condition sexual maturity is obvious, and an idea of the exact developmental state in the case of the race of fish from the source Gc, is given by the fact that none of these fish reach sexual maturity below the length of 4·5 cms.

In the case of the adult animal, the evidence points conclusively to the fact that the annual onset of gametogenesis is conditioned by the temperature of the habitat approaching its maximum, and it seems very probable from the above evidence that this is also true of the assumption of sexual maturity.

In a habitat in which the high summer temperature is of relatively short duration, and is followed by a rapid fall of temperature, only a small proportion of the fish would be expected to reach sexual maturity by their first winter. These physical conditions are fulfilled by the habitat Ga (see p. 223), and in this habitat the proportion of large *young fish* was unusually high.

In the reproductive cycle of the adult there are four *critical points* (p. 201).

Critical Point 1. *The commencement of gametogenesis.*

(*a*) *The Male.*—In the male this critical point occurs during a restricted time during the year, in nature, from the end of July to the end of August.

In the experiments the transformation of the spermatogonia into primary spermatocytes was brought about in two sets of experiments, the conditions of the two sets differing slightly.

Condition (*i*).—In the experiments in which the fish were rapidly acclimatised to a high temperature (25° C.) there was no change in the condition of the interstitium at the end of two months, but within the tubules there were spermatocytes undergoing maturation. It is not known how soon after the beginning of the experiment the spermatocytes were developed (Experiment VIII, p. 245).

Condition (*ii*).—In this case the fish had been unilaterally castrated at the commencement of the experiment (Operation Experiments 22, 28 and 29). The evidence of unilateral castration experiments in which the temperature was maintained at or below 15° C. shows that this operation by itself causes no change in the germ cells of the testis remaining in the fish (p. 253). In the experiments in question, however, the fishes were slowly raised to a temperature of 27° C. at a rate of 5° C. per week. In contrast to the fish subjected to Condition (i) above, in these fish there was a marked change in the interstitium, the interstitial and connective tissues becoming well developed, and the fish developing the secondary sexual characters. In these fish the new spermatogenesis occurred in outgrowths of the anterior tubules. A careful examination of the testis with these anterior outgrowths has convinced me that this apparent special hypertrophy of the anterior tubules is due, not to any specific effect of the experiment, but to the fact that there is in the testis with the interstitium developed, no space for the increased volume consequent on fresh spermatogenesis, and the tubules burst out of the usual limits of the testis at its weakest point. This weak point is situated anteriorly where the connective tissue sheath of the testis is thinnest. In the case of the fish subjected to Condition (i), the lymph spaces of the interstitium provided the necessary space for the expansion of the tubules.

The fresh spermatogenesis which is induced by either of the conditions is apparently incomplete in that the new spermatozoa resulting would not fill the tubules of the testis, in the absence of the pre-existing sperm. It will be recalled, however, that when maturation commences in the testis of the adult fish in its natural habitat, the volume of the testis is at its minimum, and the small amount of new spermatogenesis induced by the experimental conditions is only relatively small.

This effect of a high temperature, causing the commencement of maturation in the adult male, is perhaps a relationship acquired by *Gasterosteus* and other fish living under conditions which subject them to a cycle of temperature changes, and not a characteristic relation in the maturation of the germ cells of vertebrates in general. I am unaware of any observations on the physiology of the germ cells at the commencement of maturation which would permit a closer analysis of this effect. These experimental observations are supported by an examination of a considerable number of adult male fish from different sources (over 300), and also by the records of VAN OORDT (1924), in that the only cases in which maturation was observed in commencement were those which were taken when the temperature of the habitat of the fish was at its maximum, or very soon after this time.

The critical temperature below which this effect does not occur is 15° C. for the fish from the source Gc (Baitsbite, River Cam, Cambridge), from the observations of experiments and of the fish from their natural habitat. There can be little doubt that this temperature varies with the habitat of the fish, and probably annually, with variations of the annual temperature cycle of the environment.

(*b*) *The Female.*—The commencement of maturation of the adult female was only observed in fish from their natural habitat, female fish not being included in the experiments on the effect of high temperatures. In their natural habitat the commencement of maturation of the egg occurs at the same time as the commencement of maturation of the male. If the commencement of the primary growth phase of the oogonia is comparable with the formation of the spermatocyte (see p. 202), then the commencement of maturation in the female bears the same relation to the temperature cycle of the environment as in the male.

Critical Point 2. i. *The Completion of Maturation in the Adult Male.*

In the adult male there is a considerable variation in the time of completion of spermatogenesis, and this variation is further increased if fish from different habitats are compared (see p. 223).

The probable cause of the varying time of completion of maturation has been considered above (p. 223).

In view of these observations of the variation of the time of completion of spermatogenesis in different habitats, it is impossible to consider that the completion of spermatogenesis bears any direct relation to the breeding season, nor can it be considered even as an important critical point of the reproductive cycle.

That the spermatozoa of the fish from the habitat Gc, Cambridge, were functionally mature at the time of completion of spermatogenesis was shown by the fact that they fertilised the eggs of a female which had been artificially brought to maturity (see p. 257).

2. ii. *The Completion of Maturation of Female.*

When the fish egg is nearing maturity there is, as far as I am aware, no change in the microscopic structure, and it follows that the only criterion of maturity in the case of the female is the fertilisability of the eggs. As far as could be judged from negative artificial fertilisation experiments, the eggs of the female *Gasterosteus* do not mature until very shortly before the natural breeding season. These experiments may, however, not afford a trustworthy guide to maturity, since it is well known in experiments on the artificial propagation of fish that the eggs of females in apparently the same state of maturity differ greatly in their fertilisability.

About two weeks before the natural breeding season commenced, however, some 15 per cent. of the females were with ovaries sufficiently developed to allow the eggs to be artificially fertilised. Further, when placed with a male which had been kept under experimental conditions and which had the secondary sexual characters developed precociously, these females behaved as if they were in the natural spawning season. It may be assumed, therefore, that some part of the total adult female population develop full maturity before the breeding season.

The actual breeding season of the fish is marked therefore by the passing of the potentially mature male, with the spermatozoa completely developed but without the breeding instincts, to the functionally mature state. The time during which the male is potentially mature varies from source to source, with the varying time of completion of spermatogenesis, although the entire completion of spermatogenesis is not essential to potential maturity (p. 224).

Critical Point 3. *The Beginning of the Spawning Season.*

There are three criteria of the spawning season in fish :—

(i) The direct observation of the fish spawning and the observation of the young.
(ii) The condition of the gonads.
(iii) The state of the seasonally developed secondary sexual characters.

The first of these criteria, which is clearly the most accurate, is somewhat difficult to make use of in the natural habitat of the animal. On the other hand, it may readily be observed in aquaria. It was decided therefore to use either the second or third criteria and to check its accuracy by observations on the fish in aquaria.

The gonads of mature fish during the spawning season are frequently in a condition termed "running," that is, the milt or roe is readily discharged when the fish is handled. In *Gasterosteus* the male never reaches this condition, and there is little

difference between the potentially mature and the functionally mature testes in this respect.

The running condition of the ovary of *Gasterosteus* is readily observed, and the death of a mature female which has not yet spawned is accompanied by the extrusion of a large number of eggs.

The occurrence of fish which have spawned is also a criterion of the breeding season. The spent female is easily recognised, but the changes in the volume of the testis which accompany spawning are so small that they cannot be taken as an accurate guide to the condition of the male, the full reduction of the testis occurring nearly a month after the actual spawning.

The seasonal secondary sexual characters of the male are readily observable and have been taken as an index of the breeding season in the male. The justification of this assumption is that throughout a large number of fish examined both in nature and under experimental conditions, no fish has been observed by me to exhibit the nesting or other breeding instincts when the secondary sexual characters are absent; on the other hand, when these characters are developed experimentally at times other than the normal breeding season, the fish always shows these breeding instincts.

In the female the only secondary sexual modification I have observed is a copper-green sheen present on the lateral parts of the body of the fish. This character is, however, not constant, and would seem to be due directly to the expanded state of the abdominal walls by the distended ovaries, and therefore is an accidental sexual modification.

Reviewing these three criteria of the breeding season when applied to *Gasterosteus*, it is seen that the breeding season may be recognised in the female by the state of the gonads and in the male by the presence of the secondary sexual characters.

As shown above, the annual breeding of the species is dependent initially on the change in the male from the potentially mature condition to functional maturity. This change is coincident with the development of the secondary sexual characters of the male.

The Mechanism of the Development of the Secondary Sexual Characters of the Male.

It may be considered *a priori* and from the experimental results that there are at least three factors affecting the development of the secondary sexual characters :—

(1) The germ cells.
(2) The interstitial tissue.
(3) The change of temperature of the habitat.

1. *Rôle of the Germ Cells.*—In the adult fish the development and loss of the secondary sexual characters is independent of the state of the germ cells (pp. 225, 253). The first appearance of the secondary sexual characters in the life of the fish is also independent of the initial spermatogenesis. Some immature fish will show spermatogenesis practically

completed and no secondary sexual characters, while others will develop these while the spermatogenesis is in full progress (p. 258).

Lastly, the X-ray sterilization experiments show that the secondary sexual characters may be developed after the spermatozoa in the testis have been destroyed.

2. *Rôle of the Interstitial Tissue.*—In every male *Gasterosteus* I have examined which developed the secondary sexual characters, the interstitial tissue was abundant in the testis immediately before this development. The number of such fish examined (over 100), which include fish whose secondary sexual characters were developed at times outside the normal breeding season, is sufficient to justify the generalisation that this is a definite relationship.

In its fully developed condition the interstitial tissue has the cytological structure of a gland of internal secretion (p. 226).

When the fish is living in its natural habitat the cycle of development of regression of the interstitial tissue does not, however, agree exactly with the cyclical changes of the secondary sexual characters, in that the development of the interstitial tissue may precede the development of the secondary sexual characters by one or two months (p. 228). The changes in the interstitial tissue, however, under natural conditions do agree very closely with the changes of the germ cells, and this agreement falls into line with the hypothesis that the interstitial tissue bears a trophic relation to the germ cells (p. 231).

Experimental observations, however, show that this relationship between the germ cells and the interstitial tissue is accidental. In Operation Nos. 28, 29 and 30, the interstitial tissue remained developed, although maturation commenced in the testis. In Experiment III, Operation No. 5, the reduction of the interstitial tissue occurred without any change in the condition of the germ cells, but coincided with the loss of the secondary sexual characters.

Further, provided the fish is subjected to the necessary change of temperature, the secondary sexual characters will always develop rapidly if the interstitial tissue is developed (p. 242, *et seq.*). Therefore, provided the necessary temperature changes are present, the interstitial tissue bears an exact relation to the secondary sexual characters. When the interstitial tissue is developing and the secondary sexual characters are also developing, and the fish has been subjected to appropriate temperature changes, there is a strong suggestion that there is a quantitative relationship between the state of development of the interstitial tissue and the secondary sexual characters. This cannot be determined accurately owing to the difficulties of measuring quantitatively the interstitial tissue (*cf.* p. 253).

Yet another point of evidence of the relationship between the interstitial tissue and the secondary sexual characters is given by the unilateral castration experiments (p. 253). These operations show that the only change which may occur in the testis when the fish develops the secondary sexual characters is the development of the interstitial tissue.

Almost every experiment which in the writer's opinion might afford evidence as to the rôle of the interstitial tissue in *Gasterosteus* has been performed. With one doubtful exception, the effects of testicular extracts, these experiments have uniformly yielded positive results showing that the interstitial tissue is the source of a hormone leading to the development of the secondary sexual characters.

It will be seen that the evidence of VAN OORDT against this hypothesis depends upon an accidental relationship which occurs in nature, conditioned by the temperature cycle of the natural habitat and that if this temperature cycle is destroyed experimentally, then the relationship disappears.

It has already been demonstrated that VAN OORDT'S conclusion of the relationship between the completion of spermatogenesis and the development of the secondary sexual characters, arises from an examination of a small number of fish and from a habitat in which spermatogenesis is completed late (p. 225). The experimental observations show further, that any stage of spermatogenesis may be present in a fish which has the secondary sexual characters developed (p. 253).

3. *The Rôle of Changes of Temperature.*—Experiments XIX and XX, and Operation Experiments 32 and 33 provide the key to the understanding of the action of a rise of temperature on the development of the secondary sexual characters. These fish were potentially mature (p. 259) and had the interstitial tissue at its maximum state of development. On being subjected to a rise of temperature of 5° C. these fish developed the secondary sexual characters rapidly. The fish in their natural habitat did not develop these characters until a month later (Experiment XIX). It may be recalled that these experiments subjecting the fish to a rise of temperature in the laboratory were paralleled by transferring the fish from the river to a shallow stream at a temperature 4°–5° C. higher than the river, and here the fish also developed the secondary sexual characters rapidly.

The Operation Experiments 32, 33, etc., show that the removal of the testis, prior to the temperature change, does not affect the development of the secondary sexual characters. This is clearly due to the fact that the testes of these fish contained well-developed interstitial tissue when they were totally castrated. Other experiments show that if the fish is totally castrated with the interstitial tissue undeveloped, there is no appearance of the secondary sexual characters if the fish is subjected to a suitable temperature rise. The secretion of the interstitial tissue has therefore been produced and is available after the removal of the testes of the fish.

The secretion of the interstitial tissue alone is not sufficient to cause the development of the secondary sexual characters, nor is its activity conditioned by a threshold temperature (p. 245). The fact that it is not necessary to maintain the temperature above a critical value is significant, since it will be recalled that in many of the experiments the fish was subjected to a rise of temperature prior to the full development of the interstitial tissue.

It is evident therefore that the rise of temperature affects directly neither the inter-

stitial tissue nor the interstitial hormone, but it must act on some organ or organs to cause them to react with the interstitial hormone.

Such organs may be either those which are subjected to the secondary sexual modifications, or they may be glands of internal secretion.

A priori, the diversity of the organs which undergo the secondary sexual changes is so great that it seems improbable that they are all affected in the same manner by this critical rise of temperature.

A comparison of Amphibian metamorphosis and these secondary sexual changes, which include the hypertrophy of muscles and the kidney tubules, suggests that it is not unlikely that the pituitary and thyroid bodies are concerned. There is at present no experimental basis for this speculation. (The experiment of injection of extracts of the anterior lobe of the pituitary (p. 256) indicate that this hormone acts by way of the interstitial tissue.)

Summary of the Mechanism of the Development of the Secondary Sexual Characters of the Adult Male.

The order of events leading to the development of the secondary sexual characters is as follows. During or after the completion of spermatogenesis, the interstitial tissue of the testis begins to develop. The development of the interstitial tissue is completed in a fish in its natural habitat from two weeks to two months before the natural breeding season of the fish. The secondary sexual characters do not appear until the rate of rise of temperature of the habitat reaches the order of about 1° C. per week (p. 245). When this condition is fulfilled, there is a rapid development of the secondary sexual characters and a coincident acquisition of the breeding instincts.

By appropriate experimental conditions this order of events may be disturbed. Thus it has been shown that completion of spermatogenesis is not essential to the development of the interstitial tissue. Again, the fish may be subjected to the temperature rise prior to the development of the interstitial tissue, in which case the development of the secondary sexual characters develop at the same rate as the interstitial tissue.

The temperature cycle of the environment conditions the time of appearance of the secondary sexual modifications in the adult fish in its natural habitat, but under experimental conditions the rhythm of the interstitial tissue may become the limiting factor.

The Cyclical Changes in the Interstitial Tissue.

The Natural Cycle begins with the development of the interstitial tissue during the winter and early spring. This development of the interstitial tissue may coincide with the later stages of spermatogenesis or it may occur after this is completed (p. 228).

The interstitial tissue reaches its full development prior to the natural breeding

season, but internal secretion produced does not act until the fish has been subjected to a temperature rise (see pp. 263, etc.).

The reduction of the interstitial tissue coincides with the loss of the secondary sexual characters, the loss of these sometimes occurring shortly after the reduction of the interstitial tissue.

By appropriate variations of the temperature of the fish this cycle may be varied experimentally.

Thus the fully developed state of the interstitial tissue may be prolonged by keeping the fish at a constant medium temperature, in place of the natural rising temperature which accompanies its fully developed condition (Experiment I).

The fully developed condition may be re-established when the degeneration has commenced by reducing the temperature of the fish (Experiment II).

Shortly after the reduction has occurred the interstitial tissue is induced to redevelop by subjecting the fish to a rise of temperature (Experiment III).

The natural development of the interstitial tissue is accelerated by raising the temperature of the fish. If, however, the rise of temperature is rapid there is no development of the interstitial tissue (Experiment VIII). An examination of the rate of development of the secondary sexual characters on subjecting the fish to the optimal initial rise of temperature, after the end of October, shows that this change of temperature has no specific action on the interstitial tissue. The increased rate of development of the interstitial tissue in comparison with that of the fish in its natural habitat is due solely to the fish being at a higher average temperature in the experiment than in the natural habitat.

It will be seen that by means of the experimental variations of temperature made, it is possible to cause a *redevelopment* of the secondary sexual characters, but that changes of temperature do not stimulate the normal development of the tissue.

The three significant phases of the interstitial tissue are :—

i. The development.
ii. The duration of the fully developed state.
iii. The degeneration of the tissue.

i. There are no positive experimental results which indicate the action of any external stimulus to cause the development of the interstitial tissue when it is at its minimum development. This development is independent of any phase of the germ cells (pp. 253, 262).

ii. The duration of the fully developed interstitial tissue varies directly with the presence of the secondarv sexual characters (p. 253). On the other hand, there is no immediate loss of the secondary sexual characters after total castration (p. 250, also below).

iii. The loss of the fully developed interstitial tissue may occur unaccompanied either by any change in the light, food and temperature of the fish, or by any change in the state of the germ cells.

The commencement of the cycle of the interstitial tissue in *Gasterosteus* therefore is not conditioned directly by either the cycle of the germ cells, or by the environment, in as far as environmental changes have been investigated in this work (*i.e.*, changes of food, light and temperature).

It has already been shown that the development of the secondary sexual characters is dependent on the secretion of the interstitial tissue. It remains to examine whether the continuance of these characters in their fully developed state is dependent on the interstitial tissue, and whether the ultimate degeneration of the secondary sexual modifications is to be considered as a specific inhibition or, on the other hand, the degeneration is by an endogenetic process arising from the loss of the secretion which conditions the development of these characters (and thus comparable, for example, with the involution of the mammalian uterus).

Total castration when the secondary sexual characters are fully developed does not bring about any rapid loss of these modifications (p. 250), but the animals survived the operation for only one to one and a half months. It might be concluded from this that once the secondary sexual characters have been developed they are independent of the testis.

In homiothermic mammalia the regression of the secondary sexual characters occurs soon after postpuberal castration (TANDLER und GROSZ, 1913). On the other hand, in poikilothermic mammalia, COURRIER (1927) has found that the rate of regression of the secondary sexual modifications after similar castration varies directly with the temperature of the animal. It is not improbable therefore that the slow rate of regression of the secondary sexual characters of *Gasterosteus* after castration is due to the fact that the animals were kept at a uniform moderately low temperature.

In this connection it is suggestive to notice that the state of regression of the interstitital tissue at which the animal passes into Stage V, varies with the temperature. In the fish in their natural habitat the state of regression of the interstitial tissue at Stage V varies, and in some cases the interstitial tissue is but a little fibrous. On the other hand, in Experiment III, the interstitial tissue was markedly reduced when the fish were in Stage V.

Taking these considerations with the facts known in mammalia (LIPSCHUTZ, 1924), it will be seen that the duration of the secondary sexual characters of *Gasterosteus* is dependent on the continuance of the interstitial hormone, and that the rate of regression of these characters after the supply of interstitial hormone has ceased is directly variable with the temperature of the fish.

General Review of the Reproductive Cycle.

The attainment of sexual maturity by the young fish depends on both its size and the temperature of the habitat (p. 258). It seems probable that a moderately high temperature is necessary for the initial maturation of the young fish (about 14°C).

In the adult male the commencement of maturation is conditioned by the temperature of the habit being over 15° C. (p. 259). A male fish may become mature in respect to the germ cells ("*potentially mature*," p. 261) without breeding. The transformation of the *potentially mature* male to the *functionally mature* condition is dependent on the development of the secondary sexual characters (p. 259).

The development of the secondary sexual characters arises from (i) the development of the interstitial tissue, and (ii) the fish being subjected to a rise of temperature, the rate of which has definite limits (p. 259).

In the natural habitat the development of the interstitial tissue of the adult male precedes the rise of temperature (p. 228).

The external factors, if any, conditioning the development of the interstitial tissue, have not been identified in the course of the experiments recorded in this paper. There is both cytological (p. 228) and experimental evidence (p. 262 *et seq.*) that the internal secretion of the tissue is developed before the cells assume the state typical of the fully developed tissue.

The presence and the amount of light and ultra-violet light, and variations of these do not have any specific action in causing the development of the interstitial tissue. The commencement of the development is independent of any change temperature. The rate of development of the interstitial tissue varies directly with the temperature of the fish. High temperatures do not inhibit the development of the interstitial tissue unless the rate of change of temperature is fast (p. 245).

As to the internal factors conditioning the development of the interstitial tissue, the experiments make it possible to conclude that this is completely independent of the state of the germ cells. The reversal of degeneration of the interstitial tissue and presumably its development may be brought about by extracts of the anterior lobe of the pituitary.

By subjecting the fish to small doses of X-rays a marked stimulus to the development of the interstitial tissue is given. The nature of this reaction is unknown. Strangeways and Hopwood (1926) have found no trace of stimulating action on exposure of tissues *in vitro*. It may be pointed out that in the X-ray experiments the whole fish was subjected to exposure, and it is conceivable therefore that the stimulus on the interstitial tissue was indirect.

The effect of the specific rise of temperature is not to "activate" the interstitial hormone, nor does it act directly on the testis itself (p. 264).

The progress of maturation in the adult female is accelerated by a rise of temperature. There is no period of potential maturity in the female (p. 261).

The spawning migration of both sexes only occurs when the fish is mature (p. 210). In the male therefore the spawning migration is directly connected with the secondary sexual characters, and is conditioned by the factors which determine the development of the secondary sexual characters.

The discharge of the genital products occurs only under conditions which normally

lead to fertilisation. The male builds its nest either when placed directly with a mature female or under the stimulus of the sight of a mature female.

The duration of the spawning season varies inversely with the temperature at which the fish is maintained.

The loss of the secondary sexual characters of the male is brought about by the cessation of production of the interstitial hormone (p. 267). The factors conditioning the reduction of the interstitial tissue are unknown. This reduction may be reversed by either injection of extract of the anterior lobe of the pituitary or by the reduction of the temperature of the fish. The redevelopment of the interstitial tissue and the secondary sexual characters brought about in this manner is, however, of limited duration only (p. 257, Experiment III).

A review of the above shows that of the physical factors influencing the reproductive cycle, temperature is the most important. It is significant, however, that the critical phases of the temperature cycle which affect the reproductive cycle are not those which occur during the breeding season ; the action of the temperature changes on the breeding season is indirect. The commencement of maturation, conditioned by the fish being at its maximum temperature, occurs after the completion of the breeding season. The rate of spermatogenesis which determines the commencement of the state of potential maturity in the male is governed by the rate of change of temperature in autumn and winter. The development of the secondary sexual characters which initiates the actual breeding season is conditioned by the fish being subjected to a rise of temperature.

Except that in the female there is no specific commencement of the breeding season such as is marked in the male by the development of the secondary sexual characters, the female reproductive cycle is conditioned by the temperature cycle of the environment in precisely the same manner as that of the male.

Discussion.

It is hoped to bring together elsewhere some of the numerous but scattered observations on the different phases of the reproductive cycle of fish, and reference to such records will here be restricted to those which indicate on the one hand that certain of the features of the reproductive cycle of *Gasterosteus*, as elucidated above, are typical for Teleost fish living under temperate climatic conditions, or, on the other hand, that some of these features are characteristic only of the case in point.

The relationship between the maximum temperature of the environment and the commencement of spermatogenesis which was shown to exist in the adult fish is conditioned in the young animal by the necessity for the fish having reached a certain developmental state.

This rôle of a developmental state as a conditioning factor in the initial maturation of the young fish appears to hold good for a number of fishes living in Temperate and

Boreal waters. The male *Cottus* does not commence maturation until its second year (HANN, 1927). The Haddock (*Gadus æglefinus*) in Icelandic waters spawn at an age at least one year older than those in the North Sea (THOMPSON, 1929). This is also true for the Plaice (*Pleuronectes platessa*) of the Barents Sea in comparison with the North Sea (ATKINSON, 1908). My own observations show that the Grey Mullet (*Mugil cephalus*) of the Ægean Sea reach maturity at the end of their first year, while those of the English Channel do so at an age of three years.

The time of commencement of maturation of the adult fish has been determined by a histological examination of the gonad in comparatively few cases. In both *Cottus* and *Perca* this maturation of the adult commences at the time of the maximum temperature of the habitat, agreeing therefore with the observations made on *Gasterosteus* (HANN, 1927; TURNER, 1919). The commencement of maturation may be approximately estimated in the case of fish living under temperate climatic conditions from the observation of the beginning of the increase in volume of the gonads, this occurring two or three months after the commencement of maturation. My own observations confirm this in respect to *Carassius carassius* and *Salmo fario*. It is probable that maturation commences at the beginning of autumn in a large number of species of fresh-water fish in temperate climates.

The data for sea fish is difficult to analyse, but it seems probable that there is a considerable variation in different species in respect to the commencement of time of maturation. In the Herring (*Clupea herangus*) in the Norwegian Sea, however, the commencement of maturation and the time of spawning coincide respectively with the maximum temperature and the vernal rise of temperature of the habitat (HJORT, 1910, and LEA, 1913).

There is a general coincidence between the vernal rise of temperature of the habitat and the commencement of spawning of a number of fishes and Invertebrates (ORTON, 1920), and in *Gasterosteus* this was found to be an obligatory relationship. It is possible that there may be a threshold temperature below which a given species of fish will not spawn, and it is reasonably certain that this is the case in a number of species of Invertebrates (ORTON, 1920). The many species of fish which spawn in autumn form an exception to the generalisation that a rising temperature conditions the spawning of fish living under temperate climatic conditions. The mode of action of this temperature rise in *Gasterosteus* is to set in action a chain of events which leads to the spawning season. If this chain develops slowly there will be a considerable lag between the breeding season and the temperature rise which has conditioned it. This, it is suggested, may possibly be the explanation of the fact that many species which spawn in spring or summer in temperate climates, spawn in late summer in higher latitudes. (For example, *Coregonus*, which spawns in spring in Central Europe and in autumn in North Canada.)

Spermatogenesis may be in progress or it may be completed at the beginning of the spawning season. In *Gasterosteus* it is usually completed prior to the spawning season, but there are numerous exceptions.

The state of spermatogenesis at time of spawning varies with the length of the spawning season in a number of cases I have examined. In *Salmo fario*, which has a short spawning season, spermatogenesis is completed prior to this. In species such as *Calliomomus lyra* and *Carassius carassius*, in which the spawning season lasts three to four months, spermatogenesis is in progress at its commencement.

There is a wide variability in the duration of the spawning season of fish living under temperate climatic conditions. The investigations on *Gasterosteus* indicate the manner of regulation of this in this species, but throw no light on the origin of this adaptation.

The duration of the spawning season in *Gasterosteus* varies with the temperature of the habitat. The general effect of a low latitude is to advance the time of the spawning season, but since in any one species this will commence at about the same temperature in different localities, the duration of the spawning season will not vary greatly with the latitude unless the duration is normally long, or if the temperature rises very rapidly.

Tropical conditions vary the reproductive cycle considerably. Maturation frequently commences while the fish is very small (HJORT, 1912). This suggests that there may be a difference in the temperature coefficient of the growth of the fish and that of the development of the germ cells; indications of this were found in *Gasterosteus* (p. 262). Spermatogenesis and oogenesis are frequently continuous, and there is a continuous breeding season (SEMPER, 1883; WORTHINGTON, 1929).

The coincidence of early and continuous maturation under tropical conditions and the commencement of maturation at the maximum temperature of the habitat under temperate conditions, taken with the experimental observations of *Gasterosteus*, suggest that there is frequently a relationship between high temperatures and maturation in fish.

On the other hand, there are some fish such as the Greenland Halibut (*Platysomatichthys* (*Reinhaddtius*) *hippoglossoides*) live during their adult life in water at a practically constant low temperature, in the case of this species about 1° C. (JENSEN, 1925), and the beginning of maturation in such species cannot be associated with any very considerable temperature change.

The contrast between the temperate and the tropical reproductive cycles may be demonstrated by a consideration of the reproductive cycle of such a species as *Gasterosteus* under uniform temperature conditions. The experiments (such as No. V, p. 242) suggest that under these conditions, if the temperature is uniformly low, there will be neither a beginning of maturation nor of a breeding season. If, however, we suppose that these could be initiated, there would be a prolonged period of spermatogenesis extending into the breeding season. On the other hand, if the temperature was uniformly high, then there would be a commencement of spermatogenesis, and this spermatogenesis might perhaps be continuous (p. 94). There would, however, be either a restricted breeding season or none (Experiments VIII, XVIII, XXII, etc., pp. 242, 245–254). If the spermatogenesis and breeding season be supposed to begin at the normal times during the reproductive cycle, then at a high temperature there would be a rapid

spermatogenesis, completed long before the beginning of the breeding season. The spermatogenesis of a fish living under temperate climatic conditions is thus a discontinuous process. Its commencement is initiated, not as might be supposed by the discharge of the mature germ cells, but by a specific external influence.

Of the two specific effects of changes in temperature of the habitat on the reproductive cycle of such a fish as *Gasterosteus*, that conditioning the beginning of maturation may thus be considered as being common to a number of fish, whereas the conditioning of the beginning of the spawning season by a temperature rise is an adaptation restricted to a smaller number of cases.

The conditioning of a process by a temperature rise is to some extent an unusual hypothesis. In Invertebrates spawning is frequently limited by a threshold temperature below which this does not occur. It may be noticed that in many cases where observations of the natural habitat have lead to the hypothesis of a threshold temperature, these observations are frequently insufficient to distinguish the effect of a temperature rise from that of an absolute temperature. A process which is dependent on an absolute temperature may become by adaptation, dependent on a temperature rise.

Many fish are extremely sensitive to changes of temperature (HJORT, 1910, POWERS, 1921). In the course of its spawning migration the Atlantic Tunney, *Thynnus thynnus*, becomes increasingly sensitive to the temperature of its habitat, until the species congregate within the limits of one particular isotherm (DE BUEN, 1925). Two very striking examples are recorded by CAHN (1927). The spawning of *Labidesthes sicculus* coincides very accurately with a high temperature (20° C.); on the other hand, *Leucichthys artedi*, which is found in the same region, spawns when the temperature is exactly 3·5° C.

The euryhalinity and distribution of the genus *Gasterosteus* indicate that this fish is a recent immigrant from the sea and it seems very probable that the temperature-rise relationship may have arisen from a pre-existing threshold temperature relation.

The mechanism by which the different phases of the reproductive cycle are controlled by temperature appears to be by way of differences of temperature coefficients of varying processes. GRAY (1928) has found a difference of temperature coefficient between the development of *Salmo fario* and the hatching enzyme. A difference of temperature coefficient between the interstitial tissue and the secondary sexual modifications in degeneration has been found by COURRIER (1927) in *Vesperugo serotinus* and a similar difference exists in *Gasterosteus* (p. 262).

Further, in comparison with the specific effect of a high temperature conditioning the commencement of maturation in *Gasterosteus*, there are numerous cases of a direct effect of high temperature on the germ cells of Amphibia (*e.g.*, WITSCHI, 1929).

Although further observations are required on the reproductive cycle of tropical fish, it appears from the experiments recorded in this paper that the natural rhythm of a fish living in nature under temperate climatic conditions cannot be so altered by experimental conditions as to become like that of a tropical fish.

Summary.

1. The problem considered in this paper is the relationship of the phases of the reproductive cycle of a fish living under temperate climatic conditions to one another, and to the external environment of the animal.

2. The Three-spined Stickleback, *Gasterosteus aculeatus* L. was used in the investigation because it has a well-marked breeding season, and it is convenient for laboratory experiments. The observations were supplemented by the examination of *Pygosteus pungitius*, *Carassius carassius* and *Salmo fario.*

3. No significant differences were observed between the major aspects of the reproductive cycle of *Gasterosteus aculeatus* and *Pygosteus pungitius*; in the male *Carassius carassius*, spermatogenesis is in progress during the long spawning season, but in *Salmo fario* it is always completed prior to the short spawning period.

4. In its natural habitat near Cambridge, *Gasterosteus aculeatus* has a well-marked spawning migration from the river to the shallow streams. This spawning migration is confined to fish in advanced stages of maturity and is coincident with the development of the secondary sexual characters.

5. Stages in the development and loss of the secondary sexual characters of the male can be recognised.

6. The germ cells, the interstitial and connective tissues of the testis undergo an annual cycle of development and regression. In samples from different habitats, a river, a pond and a canal in the same latitude, the commencement of maturation and of the breeding season occurred at about the same dates, maturation in August and September and breeding in April or May. In the male the maximum development of the interstitial tissue in the testis occurred in all cases either prior to or coincident with the beginning of the breeding season. There was, however, a considerable variation in the times of completion of spermatogenesis.

7. The variation in the rate of completion of spermatogenesis is due to the variation in rates of fall of temperature of the habitat in autumn. When the rate of fall is rapid, spermatogenesis is continued into the breeding season; where the fall is slow, spermatogenesis is completed two to three months prior to the breeding season.

8. In those male fish in which spermatogenesis is completed prior to the beginning of the breeding season, there is a period of "*potential maturity*," during which the spermatozoa are able to fertilise mature eggs. The development of the secondary sexual characters is coincident with the acquisition of the breeding instincts of the fish, and therefore coincident with the assumption of the state of "*functional maturity.*" There does not appear to be a corresponding stage of potential maturity in the female.

9. The fat-bodies of *Gasterosteus* undergo a cyclical development and regression, being absent during the breeding season. This relationship to the breeding season is, however, accidental and not obligatory.

10. The reproductive cycle of such a fish as *Gasterosteus* may be divided into phases marked by the following critical points :—

i. The first maturation of the young fish.
ii. The annual commencement of maturation.
iii. The completion of maturation.
iv. The beginning of the breeding season.
v. The close of the breeding season.

11. These stages are more readily recognised in the male than in the female. The following criteria have been used to distinguish the critical points in the reproductive cycle of the male.

For Points i and ii above, the presence of the growth phase of the spermatogonia in the testis.

The completion of maturation is marked by the absence of spermatogenesis in the testis.

The commencement of the breeding season by the development of the secondary sexual characters, and its close by the reduction of these modifications.

The justification of this criteria is considered.

12. These critical points may be considered to be brought about by either (*a*) a specific change in the external environment of the animal, or (*b*) by an internal mechanism. Such an internal mechanism may be a specific initiation or inhibition of the phase, or the process may be self-regulating.

13. *Phase i.*—The initial maturation of the young fish is due directly to the environment of the fish being at a high temperature, conditioned by the fish having reached a certain developmental state (or in the case of fish from any one habitat, it is conditioned by the fish having reached a certain size).

14. *Phase ii.*—The commencement of maturation of the adult fish is brought about when the temperature of the environment of the fish is at its maximum.

15. *Phase iii.*—The completion of maturation is not specifically brought about by an external or internal process. The rate of spermatogenesis is controlled by the temperature of the habitat of the fish. If the temperature is high, the completion of spermatogenesis follows soon after maturation.

16. *Phase iv.*—The development of the secondary sexual characters is conditioned by an external factor and a specific internal factor. Under appropriate conditions either of these factors may become limiting.

The external factor is a rise of temperature. To produce the maximum rate of development of the secondary sexual characters when the external factor is the limiting factor, the rate of change of temperature is of the order of 1° C. per week. It is not necessary that either the change of temperature be continued or that the maximum temperature reached is maintained.

The internal factor is the secretion of the interstitial tissue of the testis. When the interstitial tissue has been developed the hormone is liberated, since its action occurs subsequent to the removal of the testes at this stage.

17. *Phase v.*—The loss of the secondary sexual characters is not due to any direct influence of the external environment or to any specific internal inhibition. The duration of the secondary sexual characters varies according to the temperature of the habitat of the fish, and appears to be dependent on the rate of utilisation of some substance or substances, the amount of which available at the commencement of the breeding season is fixed.

18. Those phases which are conditioned by the external environment directly, viz., Phases i, ii and iv may be brought about at any time in the year by appropriate experimental conditions. Different phases may also be superimposed on one another. Thus it is possible to bring about the commencement of spermatogenesis during the breeding season.

19. Variations of food and light have no direct influence on the reproductive cycle.

20. By suitable experimental conditions it has been shown that there is no relation between the cycle of the germ cells in the male and the interstitial tissue.

21. The rôle of the internal factors in the reproductive cycle have been investigated by experimental castration, both total and unilateral, by X-ray sterilisation, and by injection of extract of the anterior lobe of the pituitary.

22. The factors conditioning the cycle of the interstitial tissue have not been fully established, but it has been found that the redevelopment of the interstitial tissue can be brought about by means of injection of extract of anterior lobe of the pituitary. The development of the interstitial tissue may also be markedly stimulated by sub-sterility doses of X-rays. The fertility of the fish did not appear to be impaired by means of such sub-sterility doses of X-rays.

23. The state of the interstitial tissue was judged by its cytological condition, not by its quantity. There is some variation in the amount of interstitial tissue in the testis, but it is in general at its maximum development at the time of the commencement of the breeding season.

24. A comparison with other fish suggests that there is a relationship between the commencement of spermatogenesis and a high temperature in many fresh-water Teleost fish. On the other hand, the temperature-rise relationship of the beginning of the breeding season is perhaps a singular feature peculiar to the case in point.

25. With the exception that the secondary sexual characters are absent in the female, the reproductive cycle of the female is conditioned by the same factors as that of the male.

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EXPLANATION OF PLATES.

Except where otherwise stated, all microphotographs are of material fixed in BOUIN'S Alcoholic Picro-Formol-Acetic mixture and stained with Iron Hæmatoxylin.

Key to Lettering on Plates.—B.C., blood corpuscle; *c.t.*, connective tissue; *d. sp.*, dead spermatozoa; *gra.*, granules in interstitial cells; *Int.*, interstitial tissue; *It.*, interstitium testis; *it. c.*, interstitial cell; *it. nu.*, nucleus of interstitial cell; *lu.*, lumen of tubule; *lym.*, lymph space; *P. Me*, peripheral melanophore; *sp.*, spermatozoa; *sp. cy.*, spermatocyte (2ry, secondary do.); *spg.*, spermatogonium; *v.d., vas deferens*.

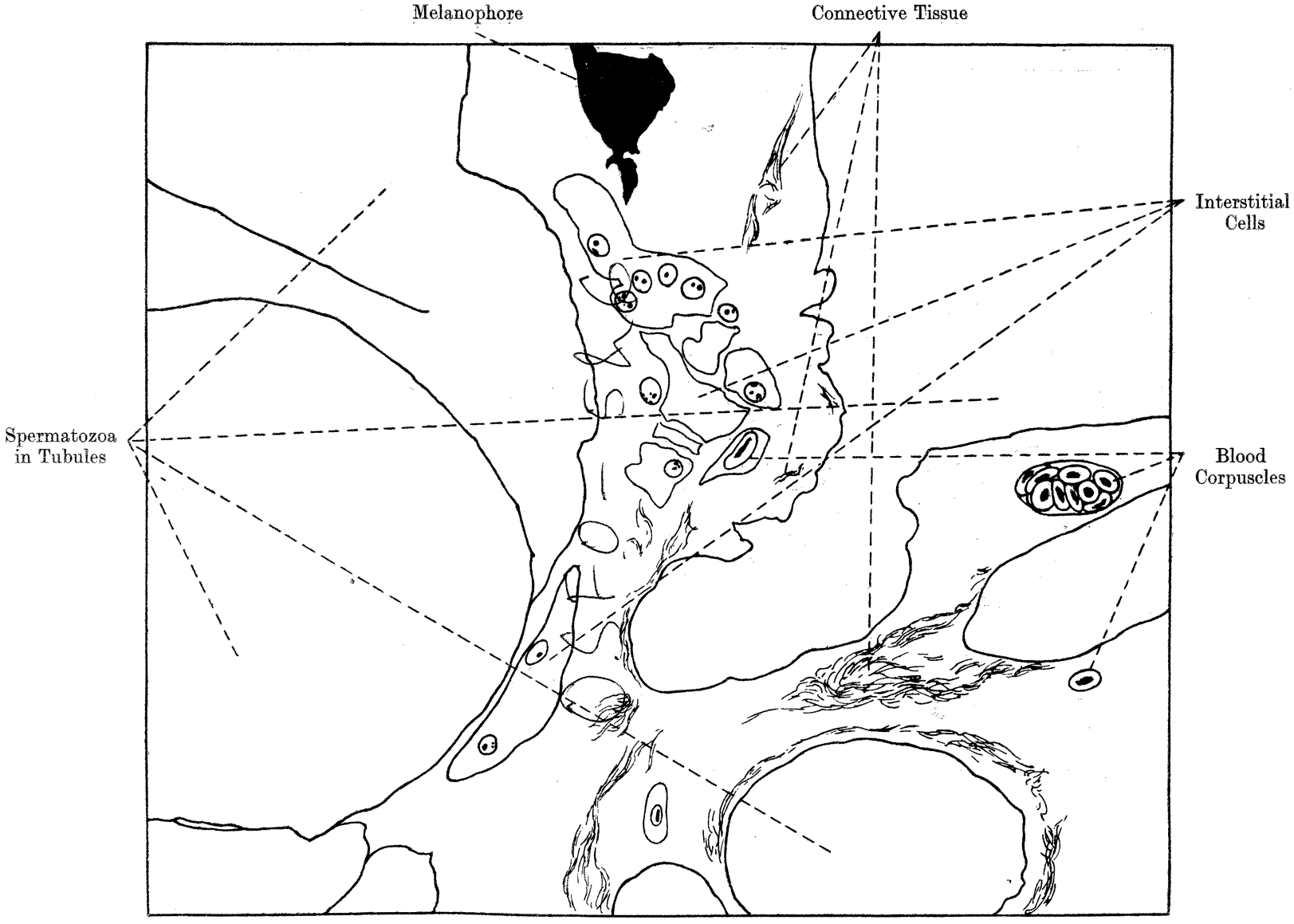
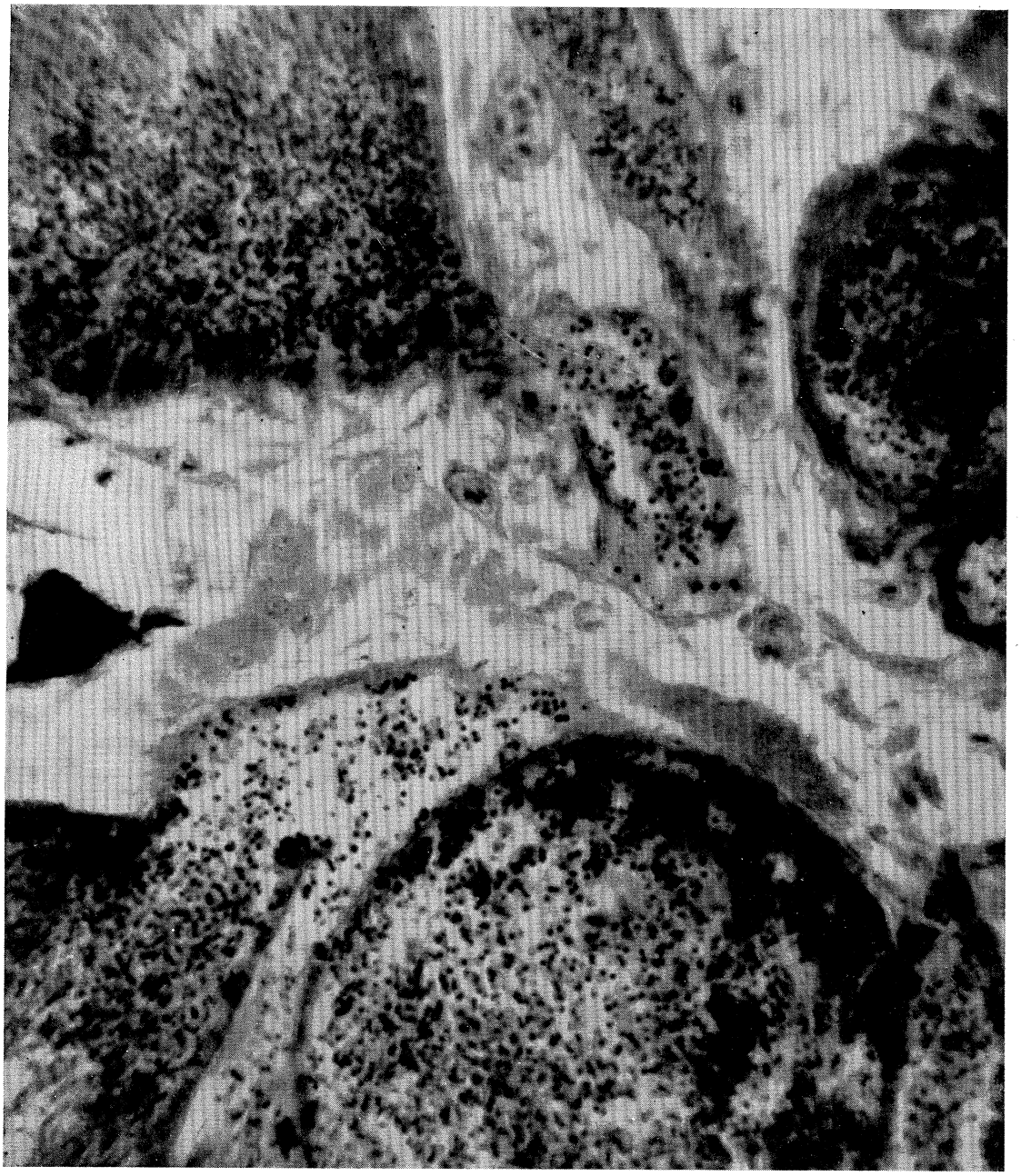
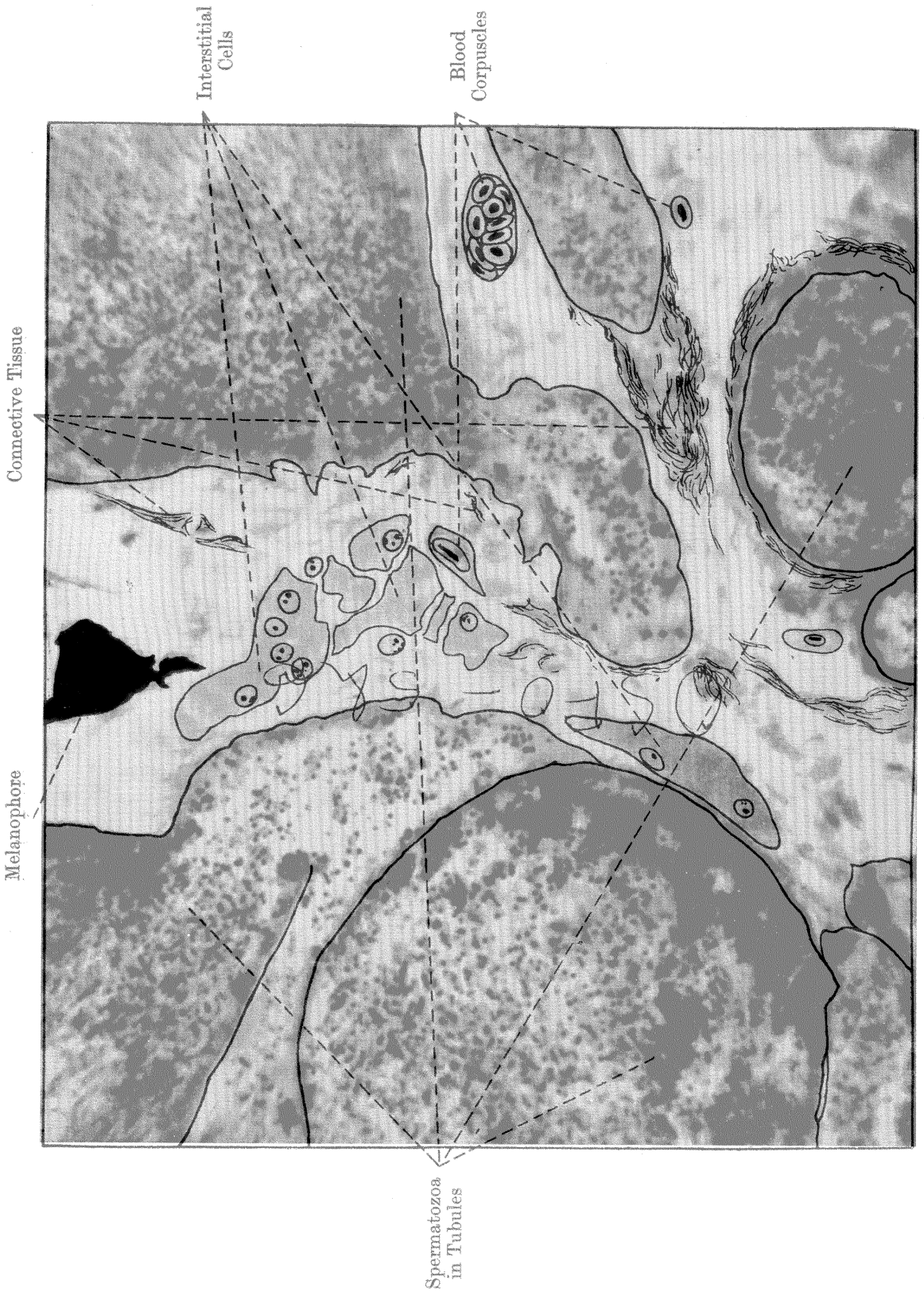


PLATE 19, FIG. 4.—Portion of the interstitium of a testis with interstitial elements fully developed.
Fixation: Alcoholic Bouin Mixture. Stain: Masson's Trichromic.





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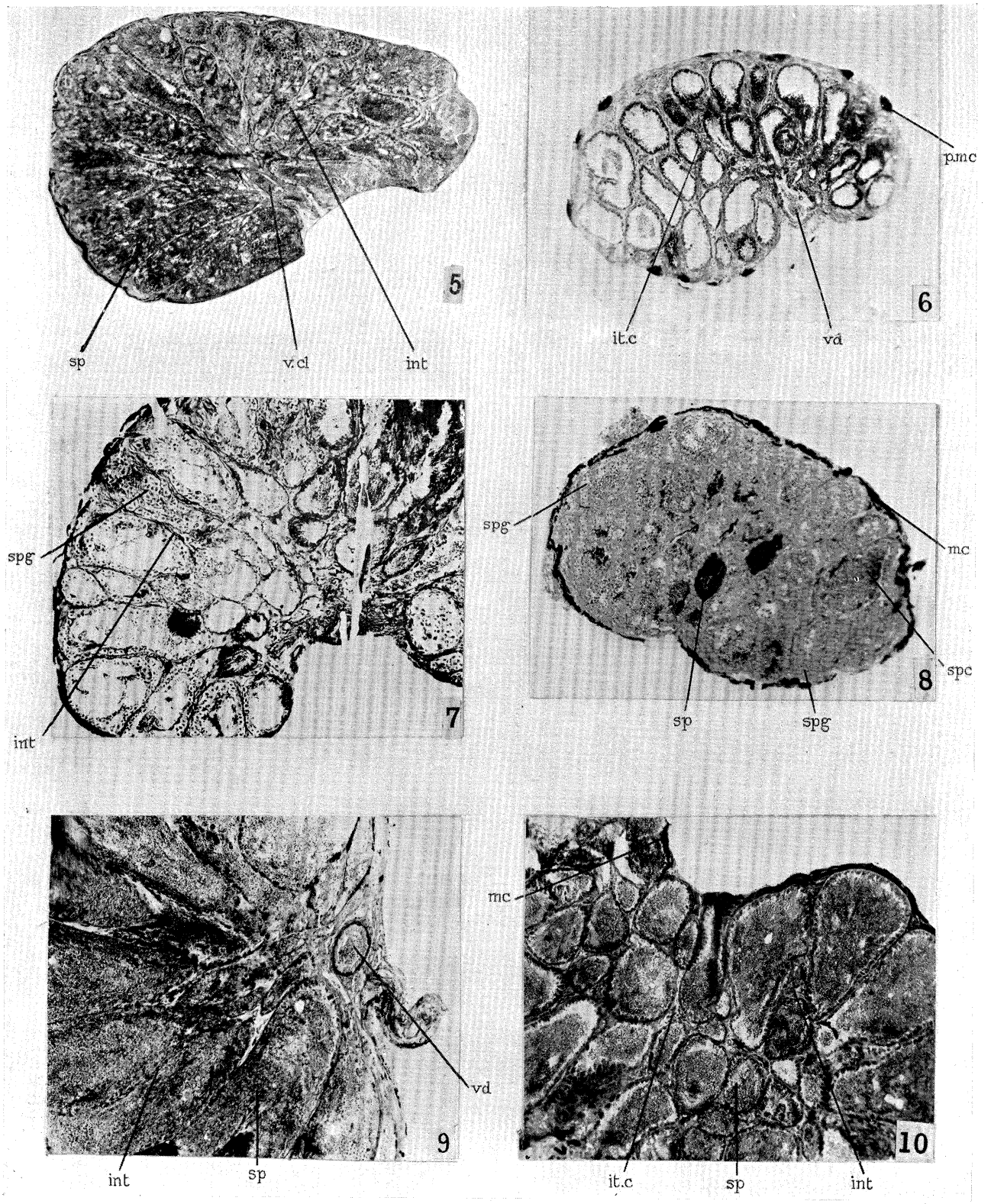
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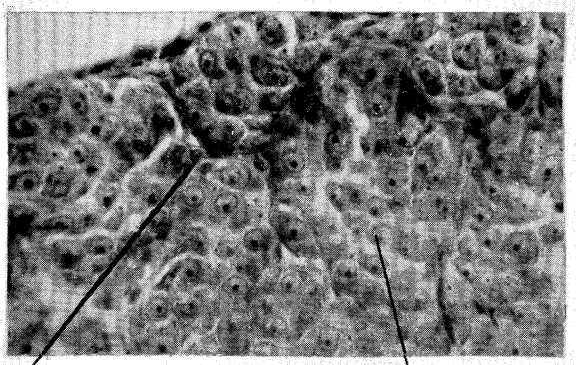

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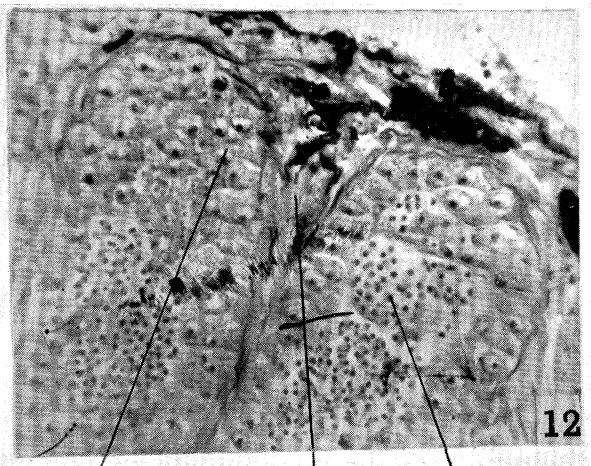

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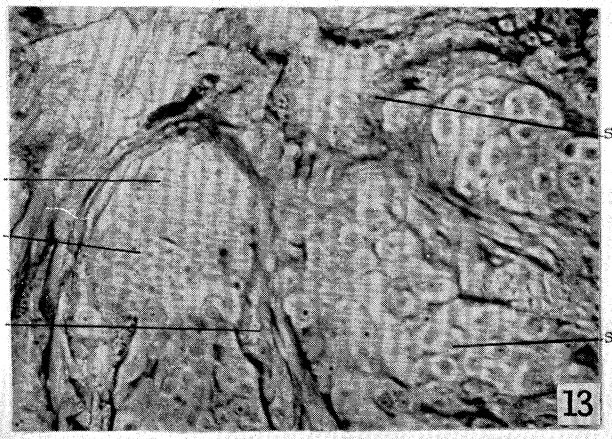

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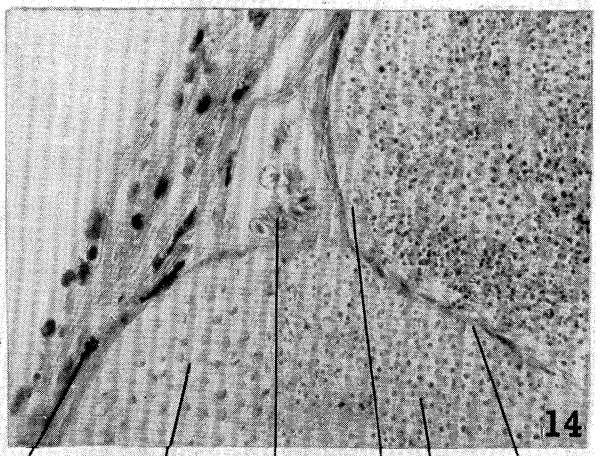

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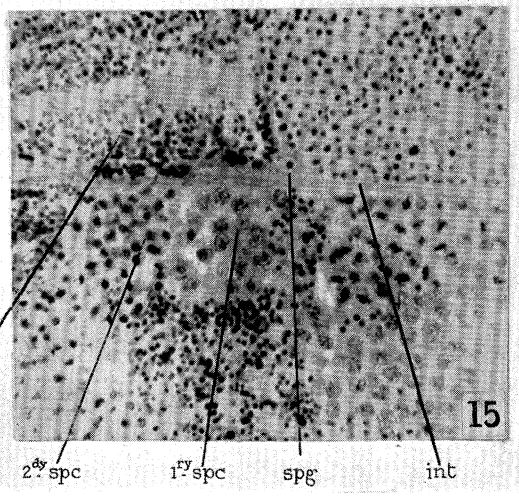

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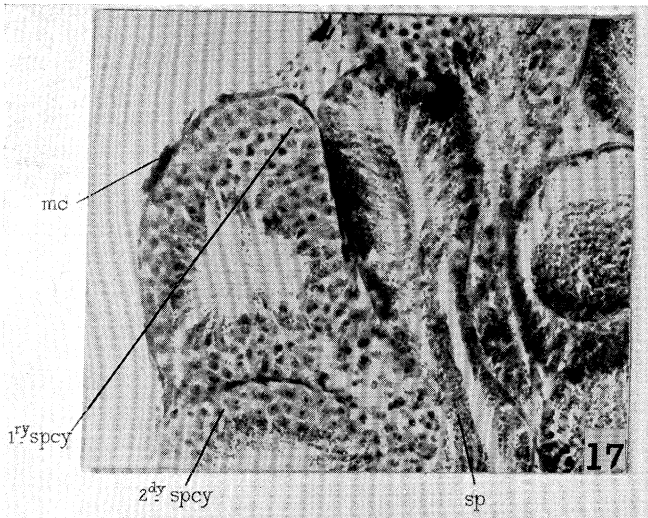
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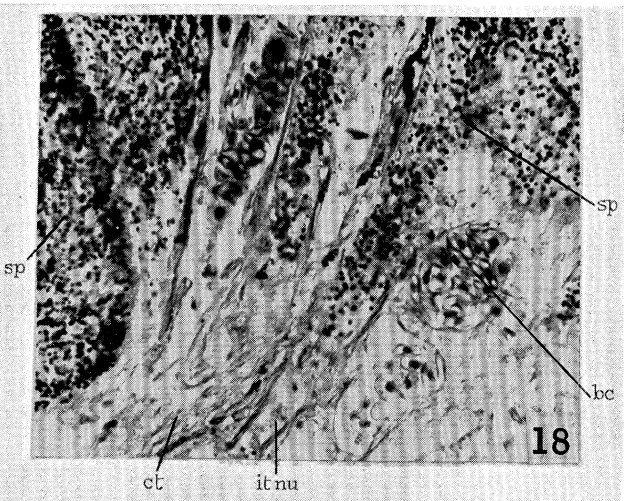
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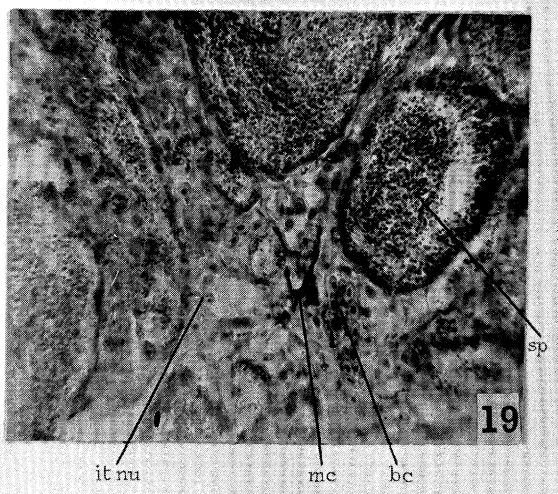
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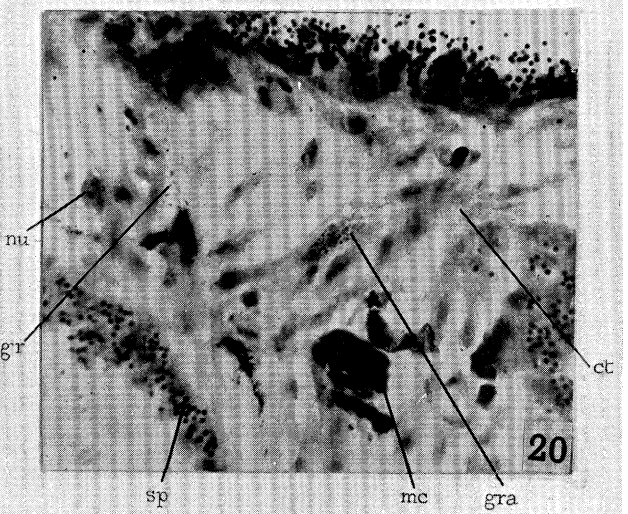
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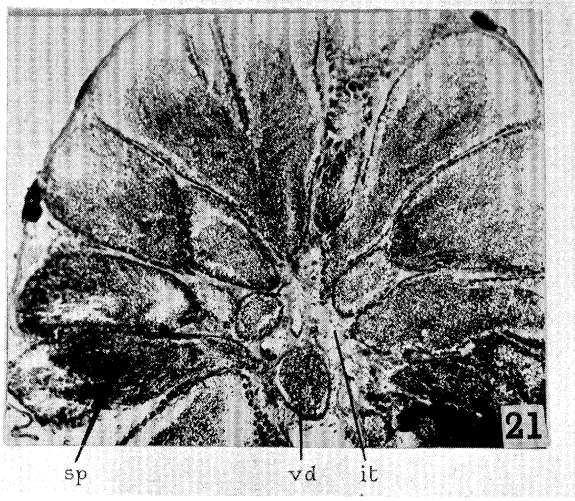
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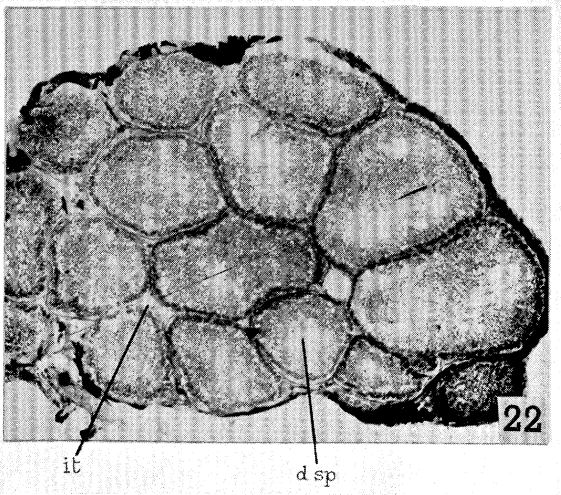
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ILLUSTRATIONS IN TEXT.

FIG. 1.—Portion of testis near hilus showing the migration of spermatogonia and their transformation into spermatocytes (p. 221).

FIG. 2.—Portion of testis at commencement of spermatogenesis showing interstitial tissue at its minimum development (p. 227).

FIG. 3.—Small portion of testis in late stages of spermatogenesis, showing the developing interstitial tissue (p. 230).

PLATE 19.

FIG. 4.—Portion of the interstitium testis of a male in which spermatogenesis is completed but prior to the discharge of the spermatozoa. Stain: Masson's Trichromic (Acid Fuchin and Aniline Blue). × 1,000 (p. 225).

PLATE 20.

FIG. 5.—Testis just prior to spawning, interstitial tissue well developed ("*Wide*"). × 100 (p. 231).

FIG. 6.—After discharge of spermatozoa. Interstitium now showing very clearly ("*Very Wide*"). × 150.

FIG. 7.—Commencement of spermatogenesis, spermatogonia lining the tubules. Interstitium greatly reduced. ("*Thin*"). × 200.

FIG. 8.—Early phases of spermatogenesis, interstitium completely reduced. × 150.

FIG. 9.—Portion of testis in which spermatogenesis is very nearly completed. Interstitium thin. Note germ cells lining *vas deferens*. × 250.

FIG. 10.—Portion of testis with spermatogenesis completed, interstitial tissue well developed. × 250.

PLATE 21.

FIG. 11.—Portion of testis of an immature young fish showing spermatogonia. Note complete absence of interstitial tissue. Compare fig. 12. × 550.

FIG. 12.—Portion of testis of an adult fish showing spermatogonia. Note the characteristic condition of the interstitium of the adult. × 550.

FIG. 13.—Portion of middle of testis of an adult in early phases of spermatogenesis. Note the complete reduction of the interstitial cells. × 550.

FIG. 14.—Portion of testis of an adult in later phases of spermatogenesis. Note the reduced interstitium. × 550.

FIG. 15.—Portion of testis to show spermatogenesis. × 550.

FIG. 16.—Part of testis with spermatogenesis in progress. Note the abundant lymph spaces. × 200.

PLATE 22.

FIG. 17.—Hypertrophy accompanied by spermatogenesis of the anterior tubules resulting from exposure to high temperatures. Note completion of spermatogenesis in neighbouring tubules (p. 253). × 550.

FIG. 18.—Interstitial tissue in development ("*Medium Wide*") (p. 231). × 600.

FIG. 19.—Interstitial tissue fully developed. × 500.

FIG. 20.—Interstitial cells showing osmophil granules after fixation with FLEMMING'S Strong Mixture without Acetic acid (p 228). × 1,000.

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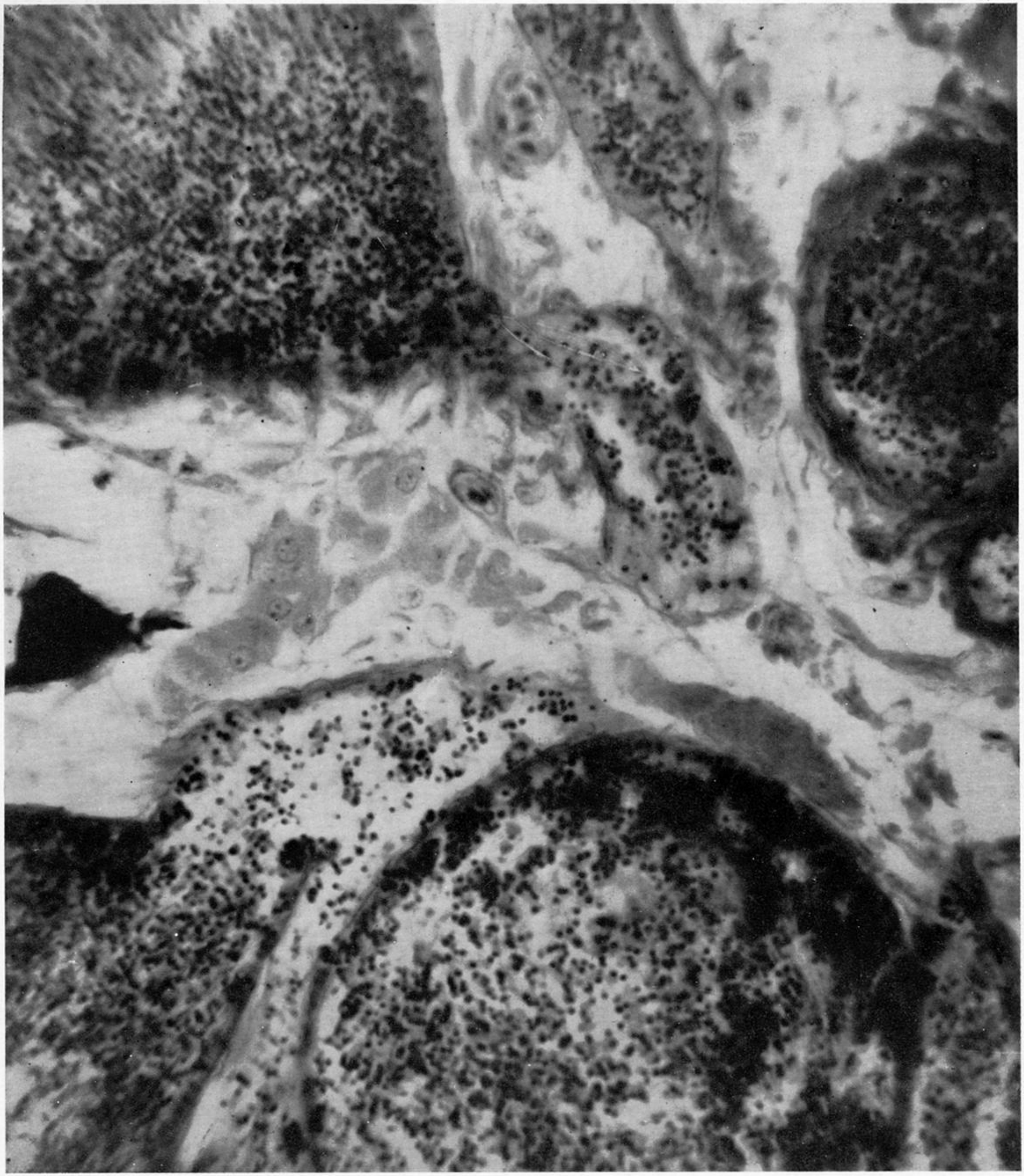
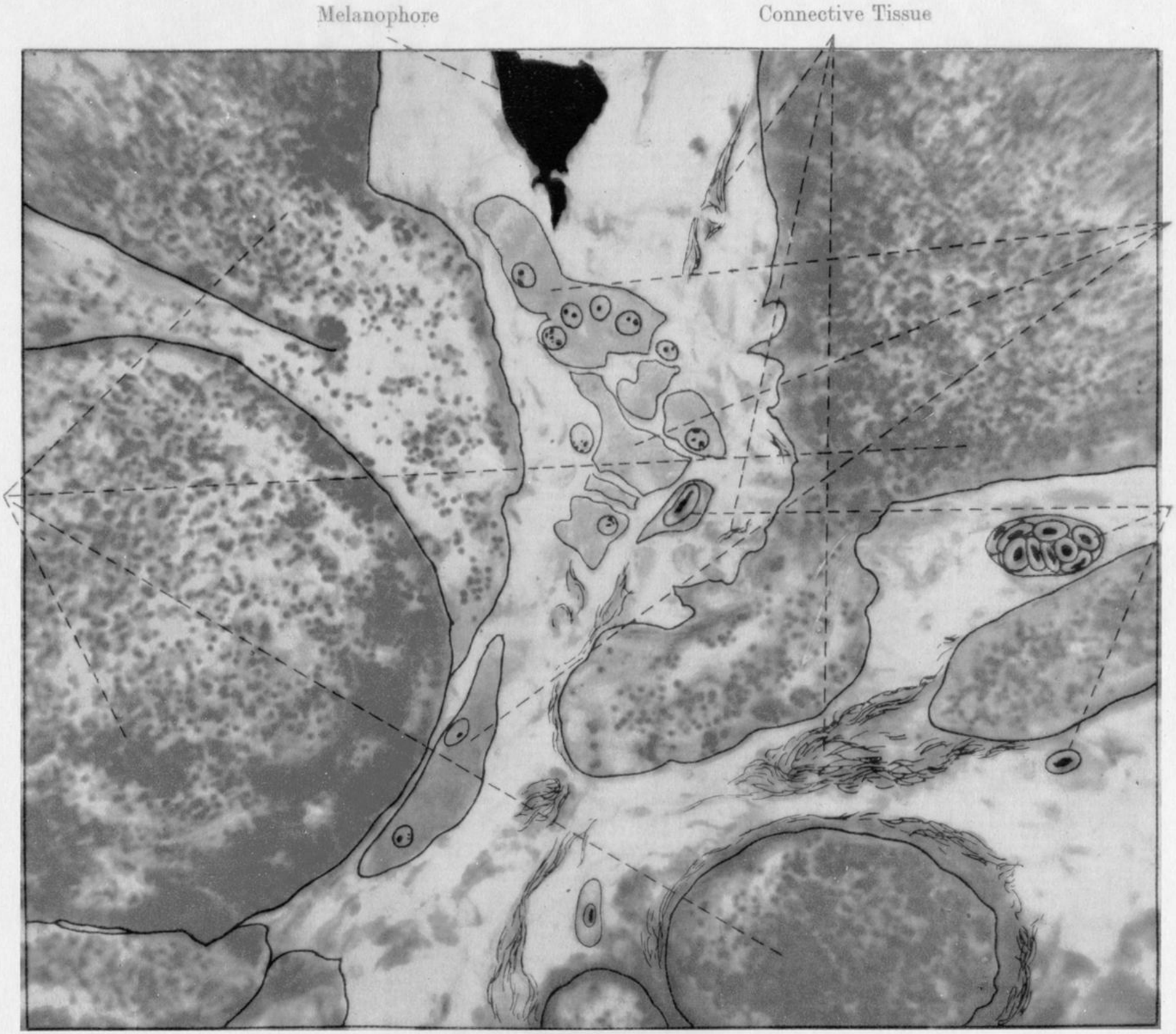
FIG. 22.—Testis after very nearly complete sterilisation by repeated doses of X-rays. Note the agglutination of the dead spermatozoa leaving spaces in the tubules. The sperm attached to the cells of Sertoli lining the tubules have not been affected (p. 256). × 150.





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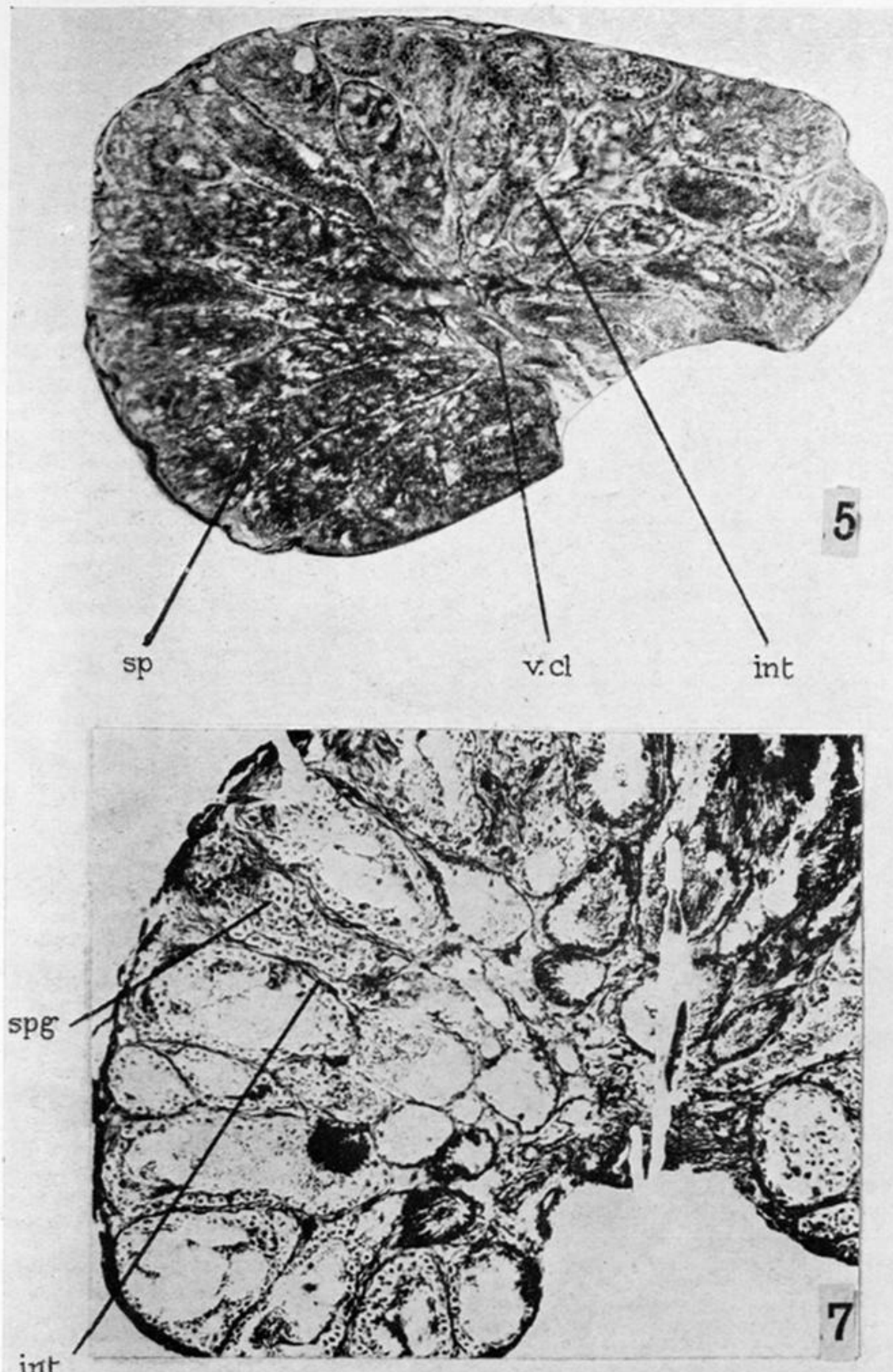

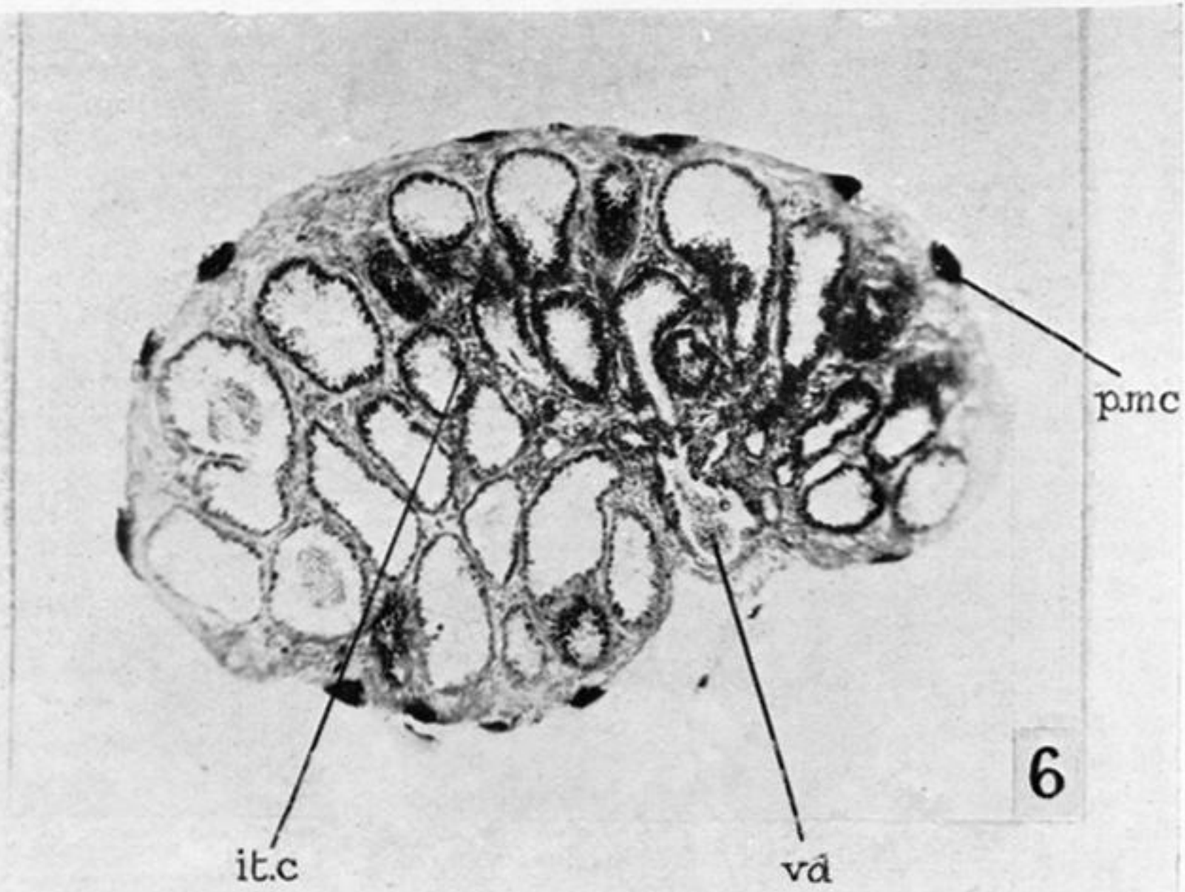

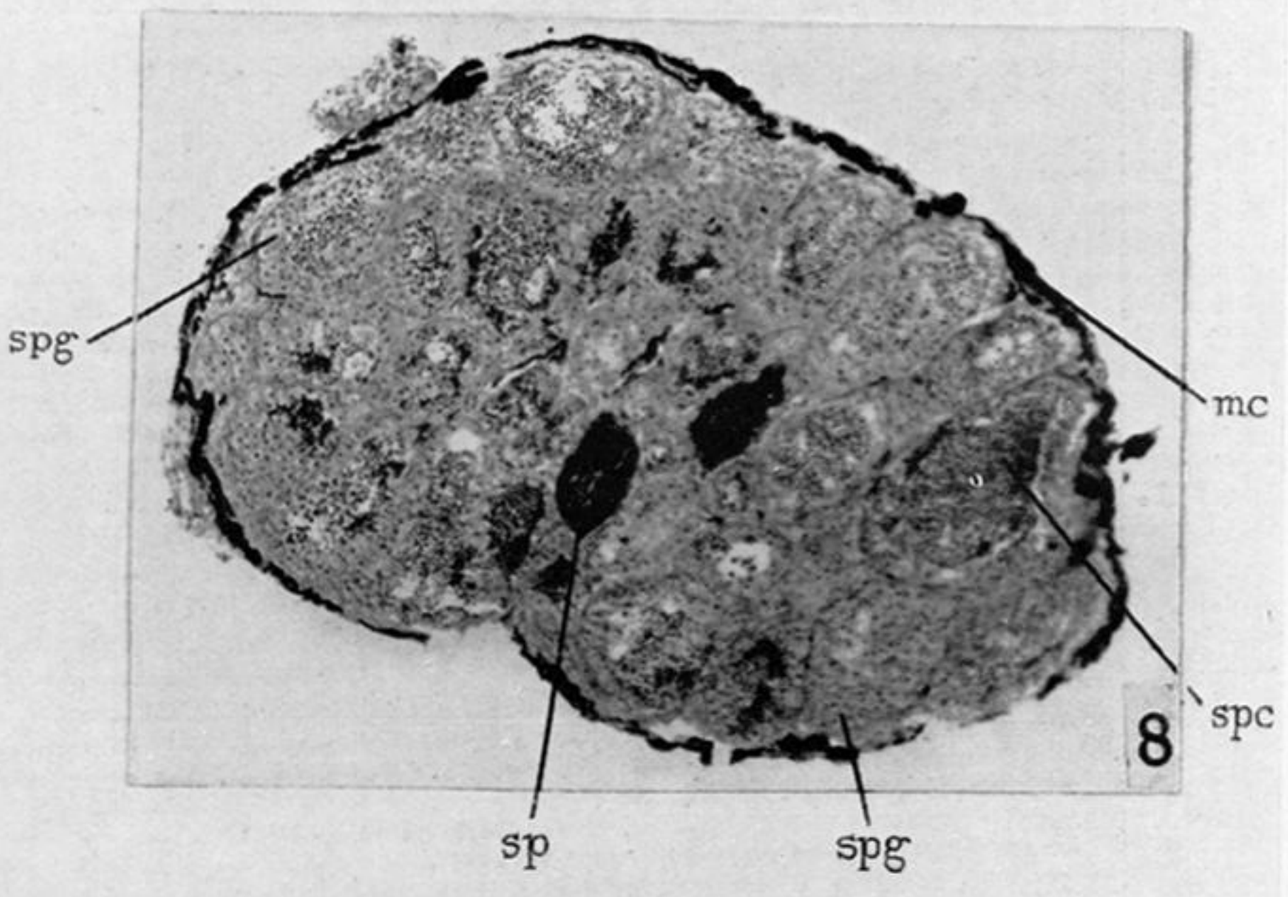

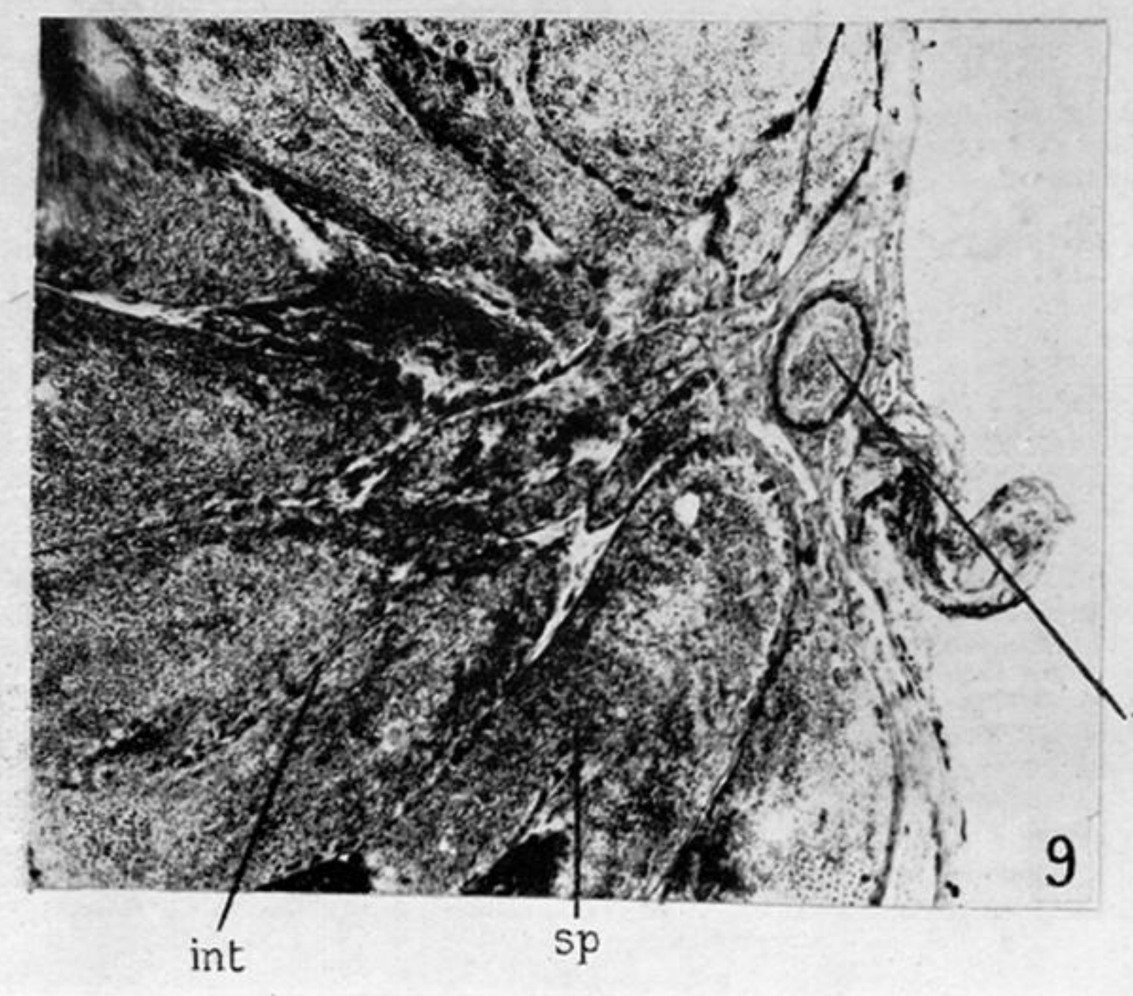

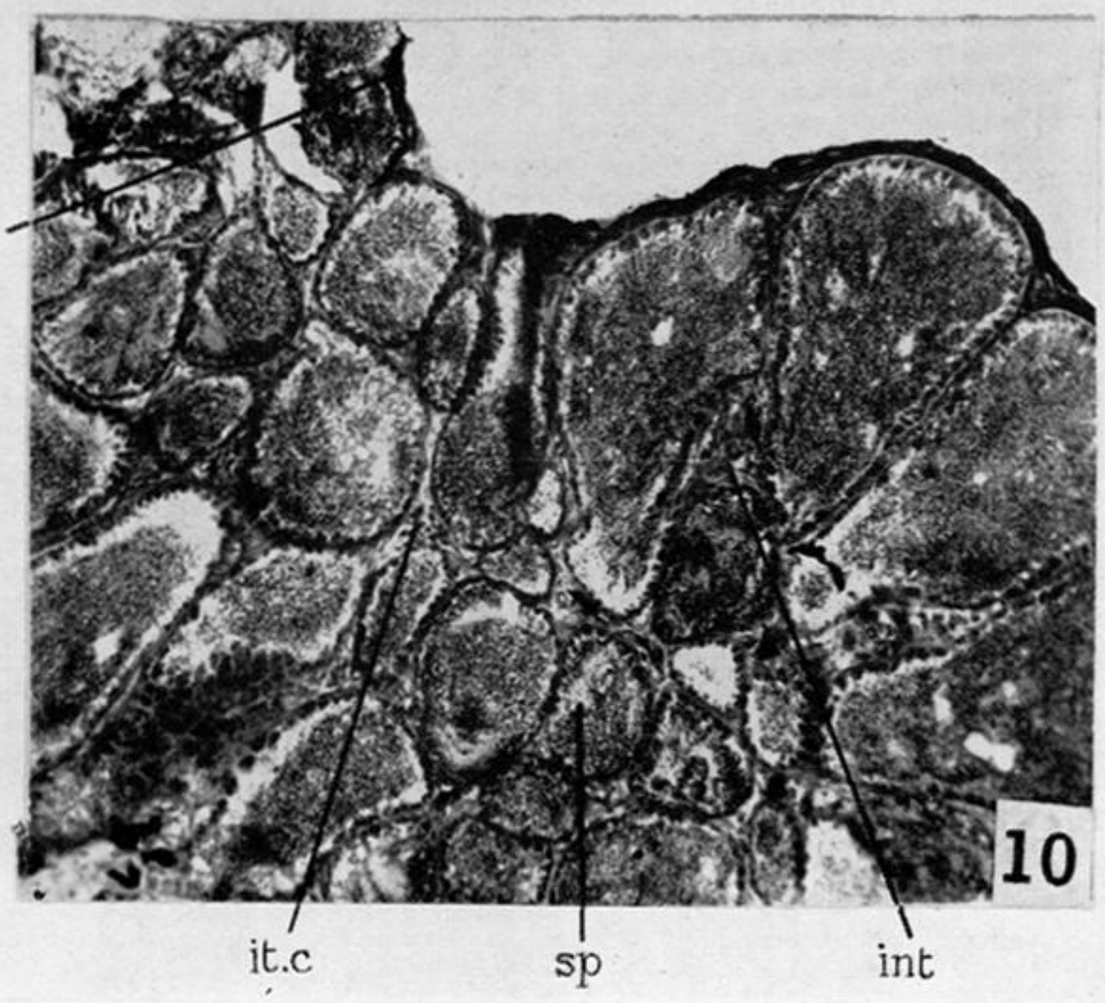

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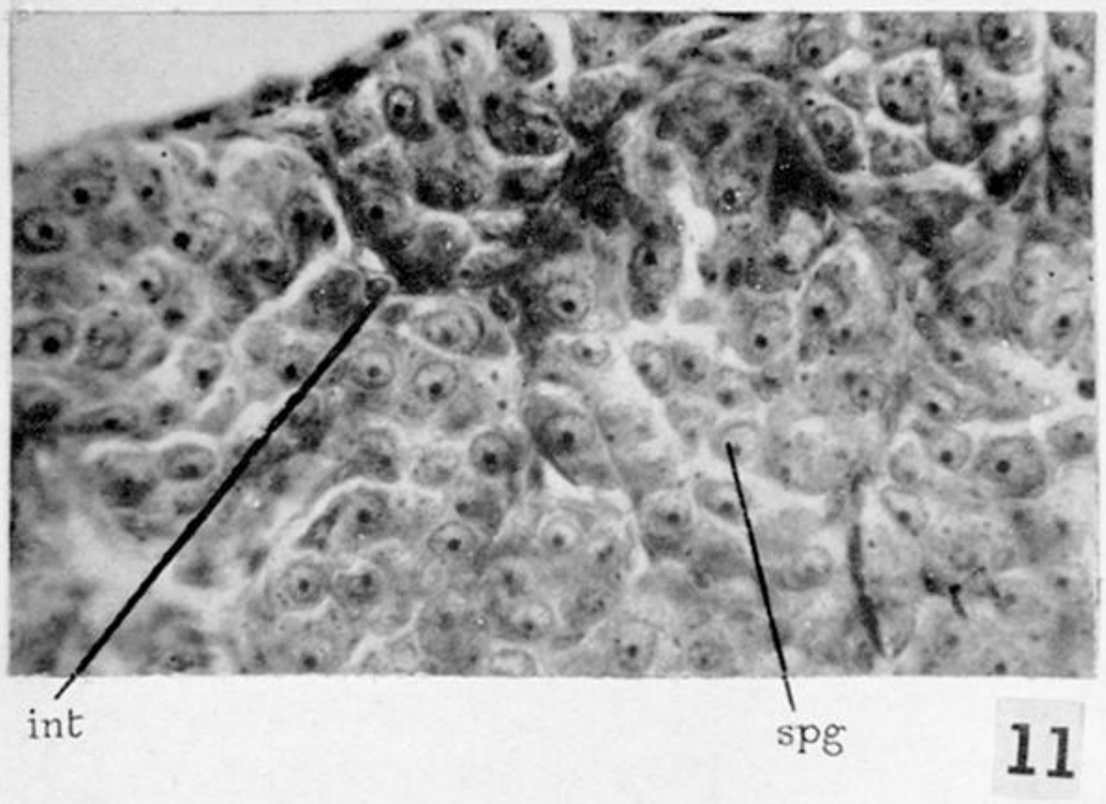

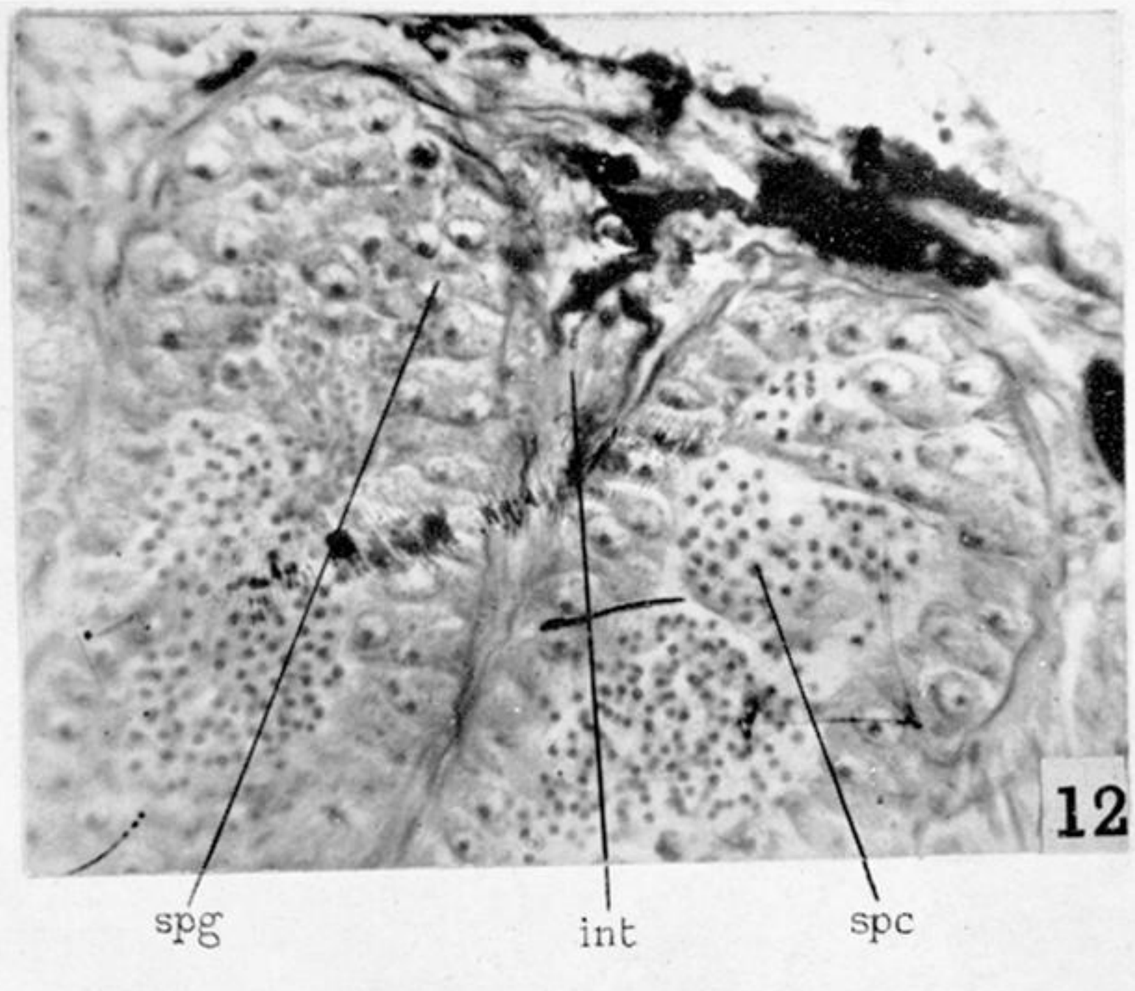

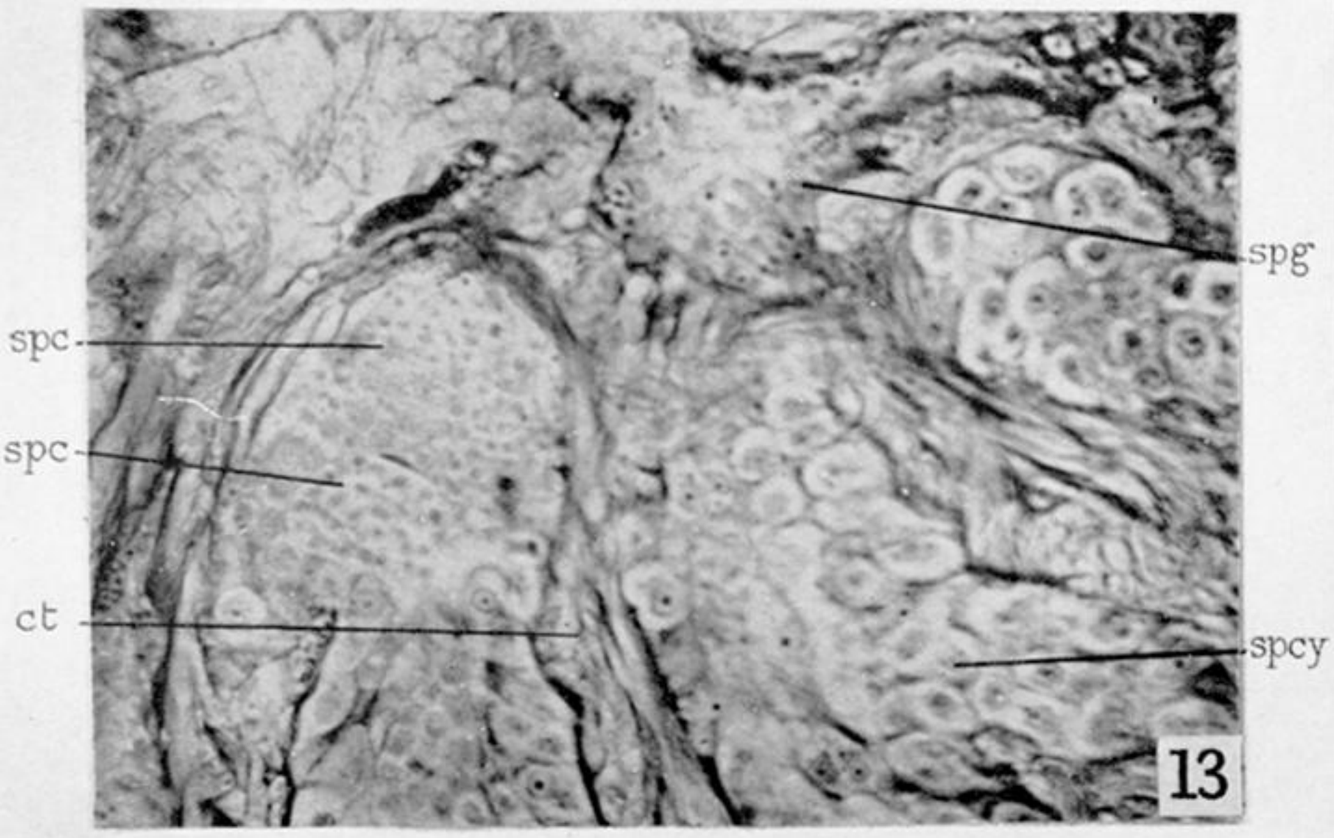

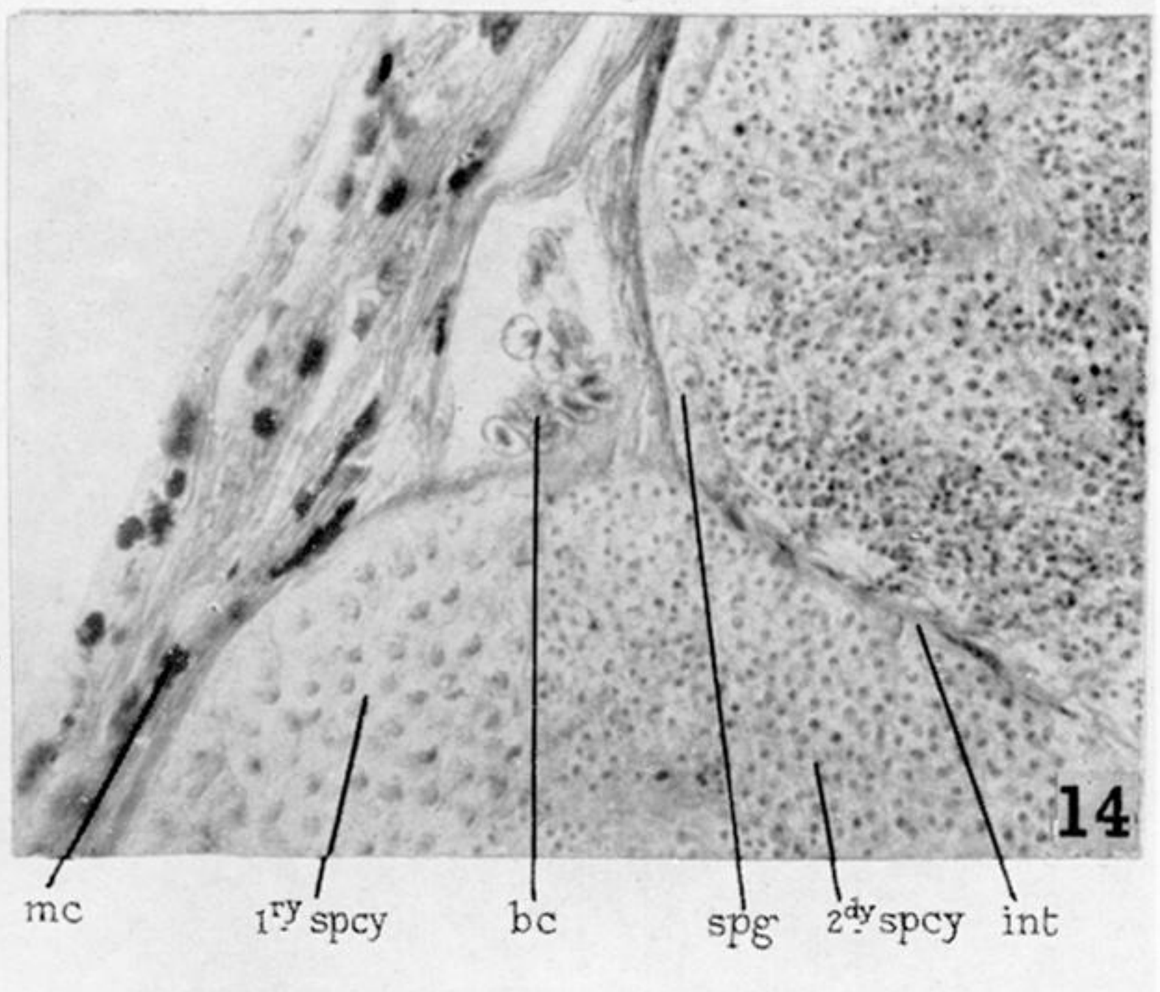

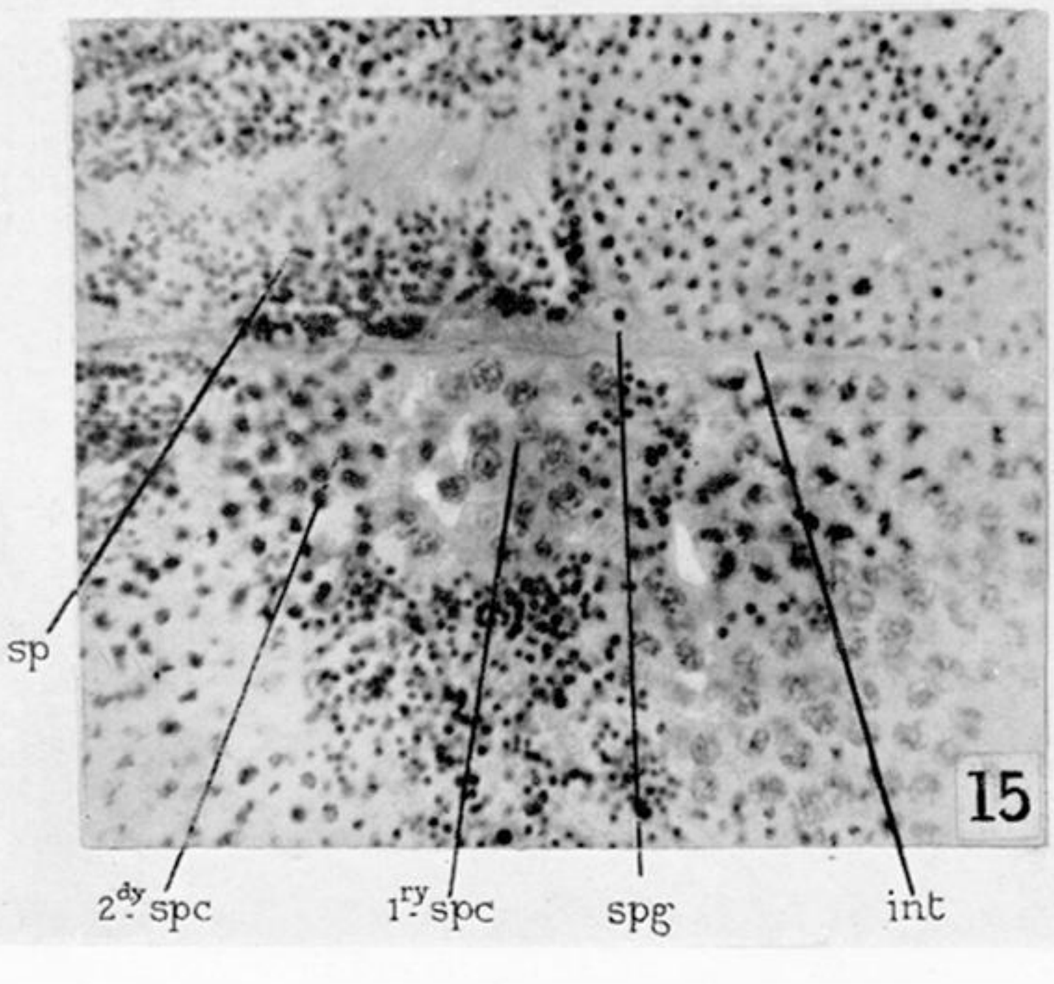

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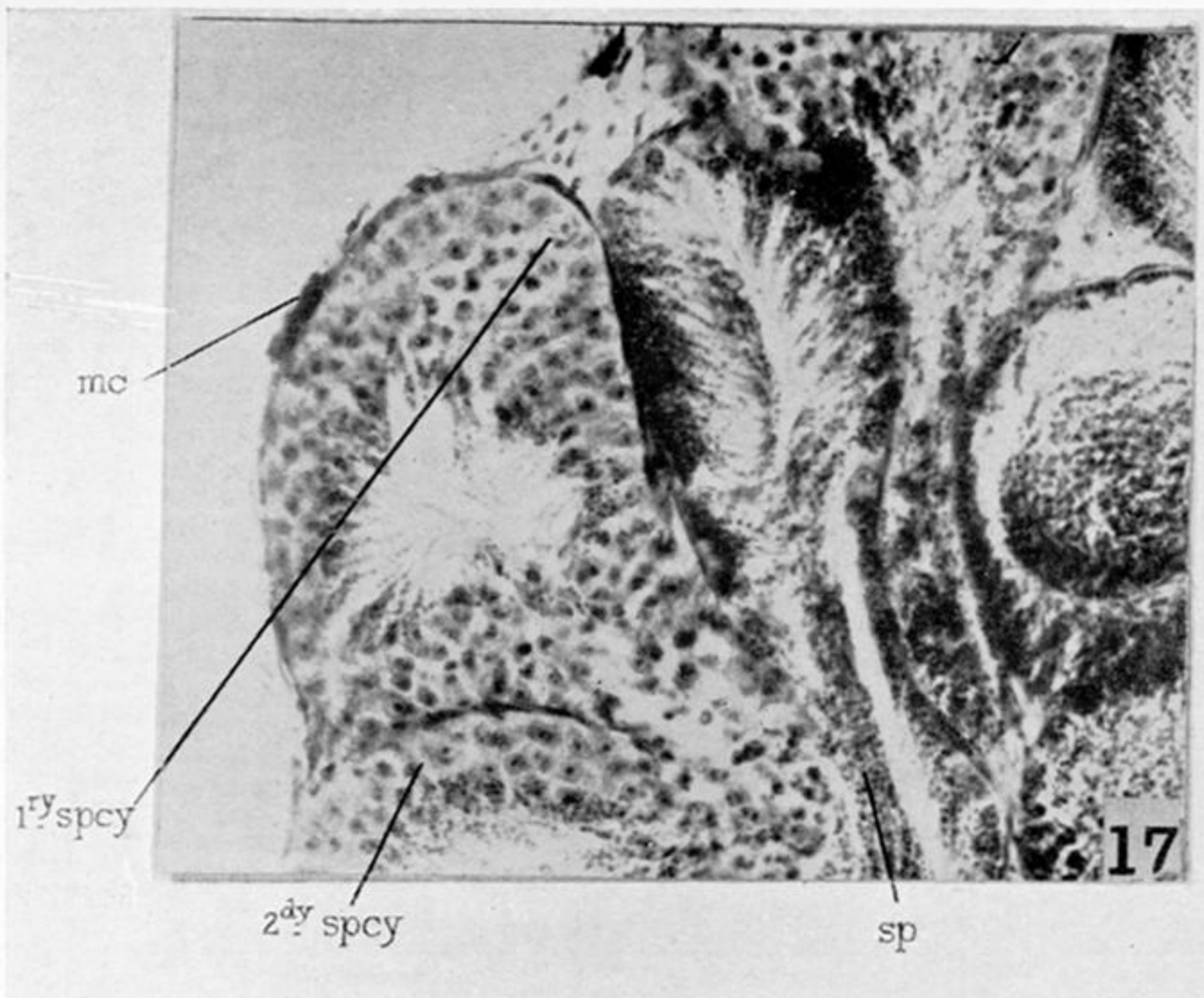

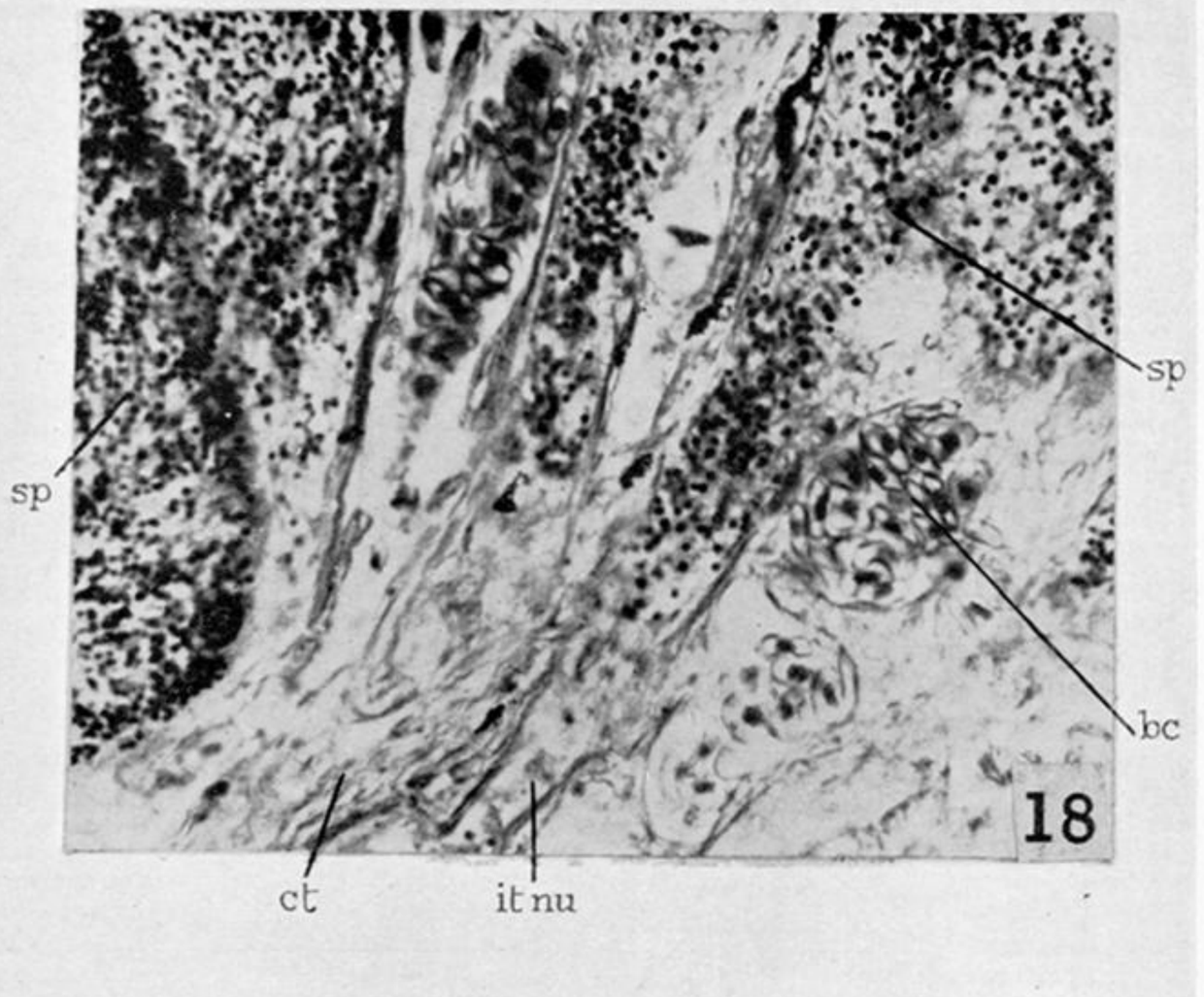

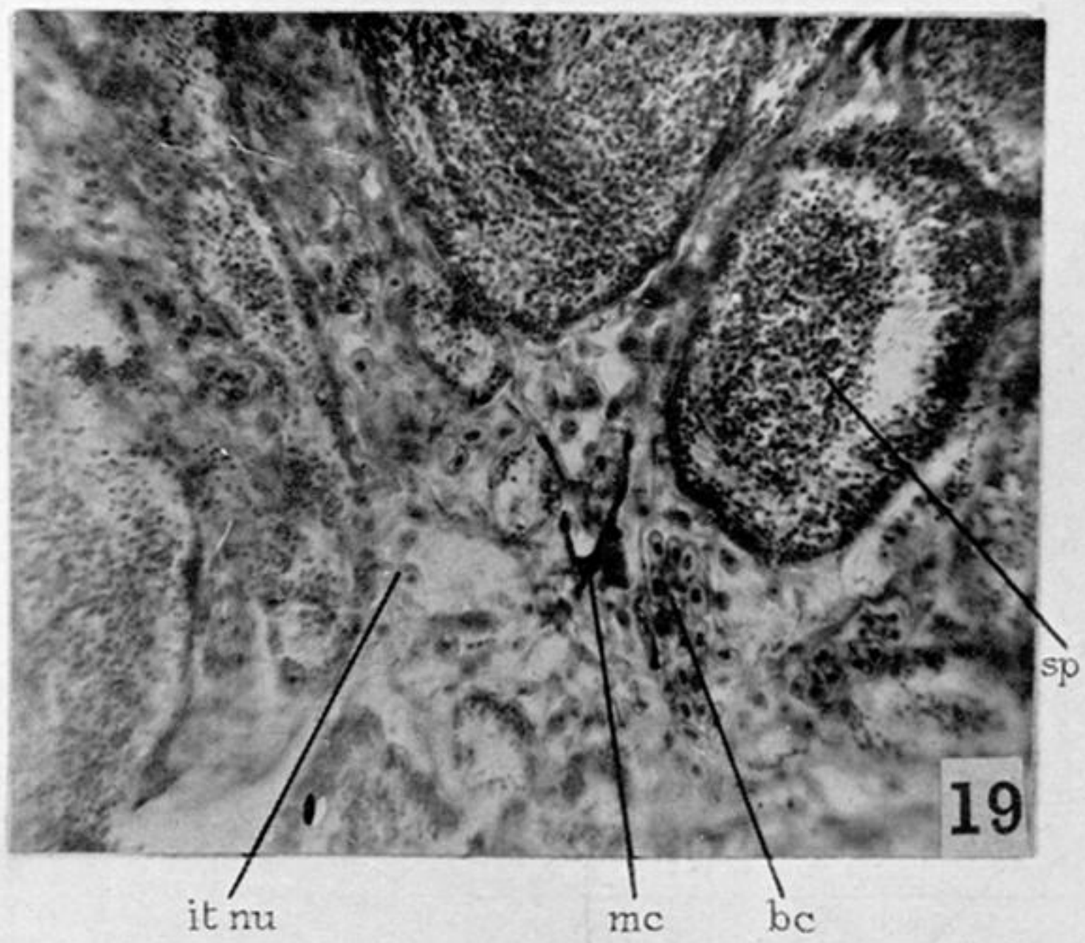

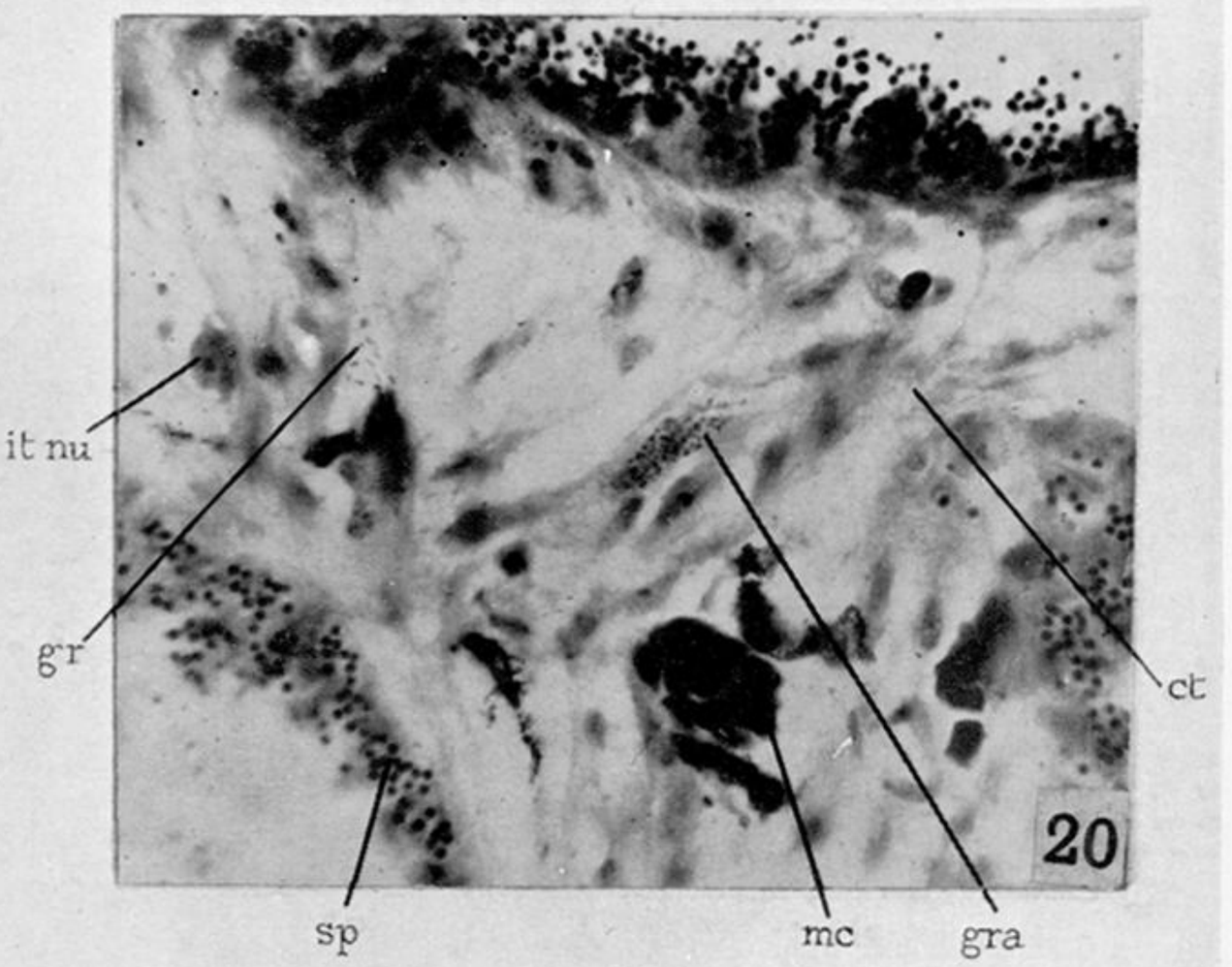

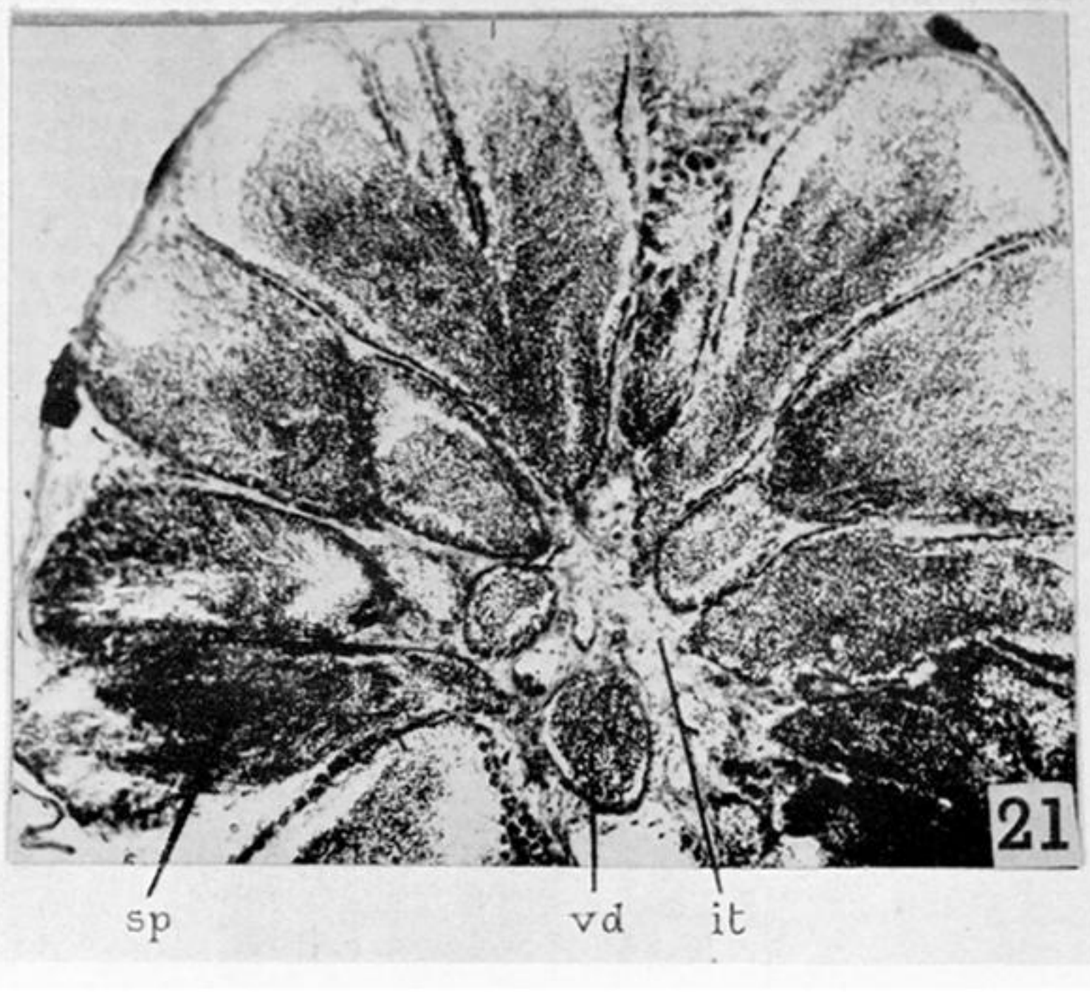

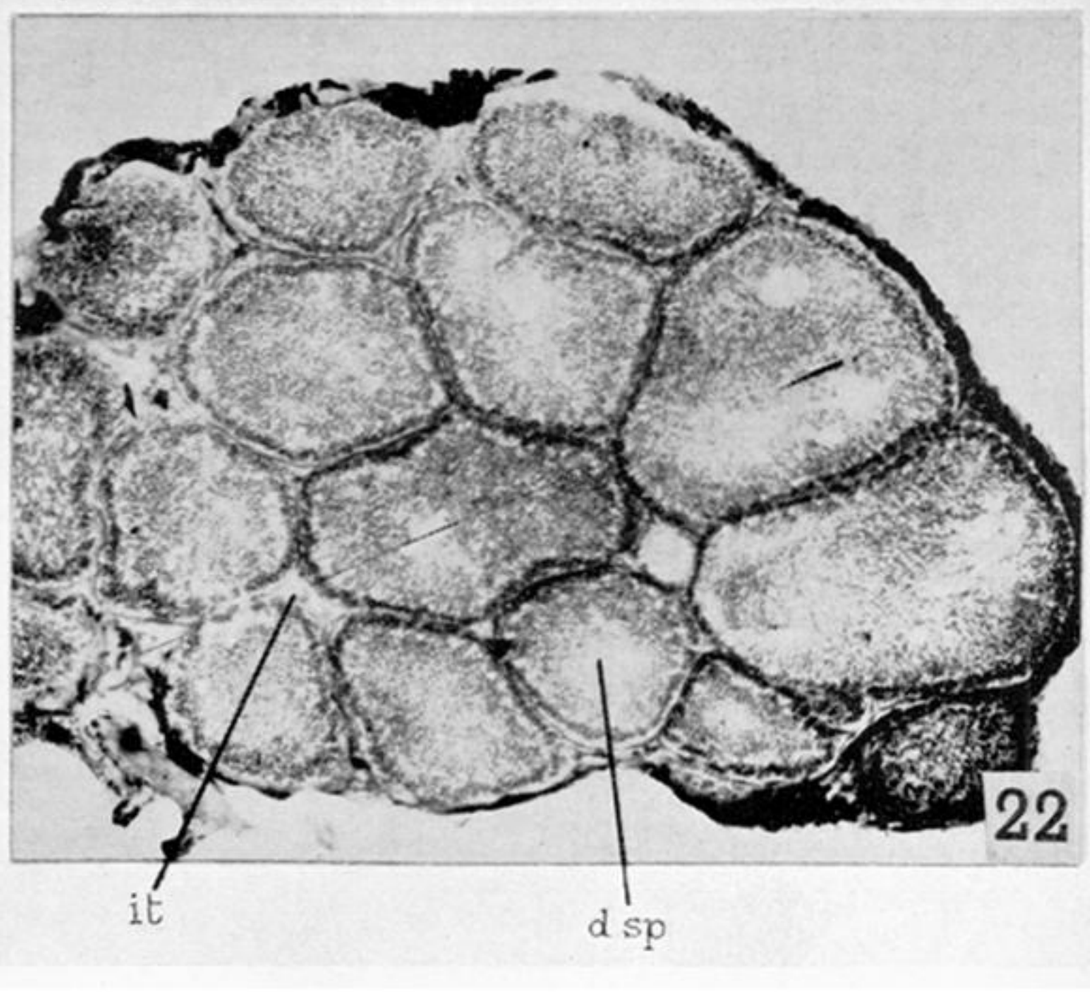

PLATE 22.

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