Some Results of Investigations into the European Bitterling, *Rhodeus amarus* Bloch

Request for Assistance from Asiatic Zoologists for the Further Development of our Bitterling Research Scheme

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1. Introduction

The purpose of this publication is to draw attention to investigations carried out on the European bitterling, *Rhodeus amarus* Bl.

The results thus far obtained make it advisable that Asiatic species should also be included in a much broader research scheme in which special attention will be paid to comparative embryology, endocrinology, ethology and also taxonomic problems.

This article will close with a request to all interested Asiatic zoologists, and especially ichthyologists, for cooperation in realising this scheme.

2. Historical Data

The symbiosis between freshwater mussels and bitterlings was discovered in W. Europe in the following way.

In 1857 the German investigator Kraus, without knowing its function, first noticed the long ovipositor of the female bitterling during the spawning time. Shortly after this Leydig described this organ as a greatly elongated urogenital-papilla. Cavolini, however, as early as 1787, noticed the presence of fish ova in bivalves. In 1818 Döllinger found fish eggs at various stages of development in the gills of a freshwater mussel in the river Main in Germany.

It was many years, however, before it was found that these eggs were in reality those of bitterlings. Noll was the first to discover the relation between the freshwater mussels and the bitterings. In 1870 Schott, at the direction of Noll, did the following experiment. He deposited 20 freshwater mussels which presumably contained eggs, in a pond containing no other fish. From them a school of about 100 bitterlings hatched. Noll, therefore, concluded that the ovipositor was used for depositing the eggs in bivalves and these then serve as incubator for the fertilized eggs.

We would be most interested to learn the history of the discovery of this symbiosis in Asiatic countries.

3. The Female Bitterling as Test-Animal for the Diagnosis of Pregnancy

In 1935 American and German publications proclaimed the bitterling as a suitable test-animal for the diagnosis of pregnancy.

A few cc urine of a supposedly pregnant woman may be injected into the fishes or simply added to the aquarium water. Ovipositor growth would then result if it should be urine from a pregnant woman. In a non-pregnant case there would be no ovipositor growth, or only a slight one.

We decided to test this assumption as follows:

Gynecologists supplied us with the urine of 113 women and it was later possible to compare the clinical reports as to whether the individuals were pregnant with the results obtained with the ovipositor test. It then appeared that the ovipositor test was of no value for the diagnosis of pregnancy (Duyvené de Wit, 1939).

The advantage of these negative results was that it forced us to develop a standardized test method for the qualitative and quantitative analysis of the hormones causing ovipositor growth (Duyvené de Wit, 1939, 1940, 1941).

4. Ovipositor Growth and Ovary

It was found that the humans urine contains a substance causing ovipositor growth. Chemically pure steroid hormones were then tested in an attempt to establish which hormone was responsible for the marked activity of the urine of woman (especially during the corpus luteum-phase). This also led to negative results and the hormone has not yet been identified. The substance appears to be of a luteoid character, but the interference of the pregnane-diole-complex has thus far prevented its chemical analysis, (BRETSCHNEIDER & DUYVENÉ DE WIT, 1947).

During these investigations the following peculiarity was noticed :- When urine is added to the aquarium water, ovipositor growth results, but 6 hours after the addition of urine (Fig. 1). The follicular fluid obtained from pig ovaries causes ovipositor growth after 1 hour. This discrepancy in *latent period* was then believed to be due to the follicular fluid acting directly on the ovipositor and the urine acting via another organ. This possibility was investigated by comparing the ovaries of female bitterlings treated with hormones with those which had not had any hormone treatment. It was then found that very remarkable changes occurred in the ovaries of the treated fish; viz. a number of medium sized ova had transformed into *corpora lutea*. This disproved the statement of SEITZ (1939) that corpora lutea are "the privilege of mammals and man."

These corpora lutea are *pre-ovulation* corpora lutea (Fig. 2) as they develop from follicles before ovulation. As its histological structure resembles that of mammalian corpora lutea, it was assumed that the corpora lutea of *Rhodeus* also exert a hormonal function.

Castration was unsuccessful as the animals succumbed to Saprolegnia infections. Castration was then carried out by means of radiation with X-rays. When these animals are then treated with urine, testosterone, oestrogens, corticoids and even progesterone, there was *no* ovipositor growth. It was therefore apparent that these hormones act via the ovary. However, when these same animals were injected with an extract of ovaries from fishes containing corpora lutea, ovipositor growth did occur. This proved that the former substances do not act directly on the ovipositor. Only the corpus luteum hormone of *Rhopeus*, and probably of all fishes, has a direct action.

The mammalian corpus luteum hormone (progesterone) is therefore not identical with that of fishes.

As the corpus luteum hormone of fishes acts on the oviduct, it was called *oviductin*. It is hoped that the chemical structure of this substance will be determined in the near future.

5. The Relationship between Hypophysis, Ovary and Ovipositor

The ovary under normal conditions causes ovipositor growth via the pre-ovulation corpus luteum hormone. This growth effect is due to increased mitosis and vascularisation which is accompanied by a swelling of the collagenous fibres.

As the ovary of mammals is under direct control of the hypophysis, it was thought that the hypophysis might possibly be concerned in the reaction to steroid sex hormones. The hypophysis of *Rhodeus* is so small that it cannot be removed operatively and it was therefore decided to bore it out via the palate with a dentist drill.

Most of these attempts, however, failed as was shown by the positive reactions to hormones in the treated fishes and subsequent histological examination. One fish, however, gave no positive reactions and it was histologically found that the hypophysis had been *completely* destroyed in this animal. It therefore seemed probable that this gland has an influence on the ovipositor growth, e. g. the development of corpora lutea in the ovary. To determine this, hypophyseal extracts of carp were injected into intact bitterling females. This caused marked corpus luteum development and ovipositor growth.

The question then arose as to which part of the hypophysis is responsible for the production of the gonadotrophic hormone. A number of fishes were fixed at regular intervals after the administration of urine or pure hormones. It was then found that an island of cells in the anterior pituitary differed considerably in staining capacity. This could be correlated with the time that had elapsed between hormone treatment and fixation. It was, therefore, concluded that these cells secreted their hormone very shortly after the beginning of the treatment and after this the hormone as again formed in the same cells and again secreted periodically (Fig. 3).

It was apparent that these cellular changes correspond with the corpus luteum formation in the ovary. There is, therefore, a *chain-reaction:*– The hormone added to the water enters the bloodstream via the mucus membrane of the gills, stimulates a hypothetical sexual centre in the hypothalamus which again stimulates the hypophysis via the hypophyseal stalk; the hypophysis then secretes a gonadotrophic hormone which causes corpus luteum formation and this again causes ovipositor growth (see diagram. Fig. 4).

The hypothesis of a sexual centre in the hypothalamus is based on the following :– It was found that the addition of an anæsthetic such as chloroform, ether, urethane etc., to the aquarium water also caused ovipositor growth. The growth curves obtained in this way were unusual. There was linear growth for a short period and then the curves became horizontal for a time and this again was followed by linear growth. This phenomenon may be explained by assuming that the narcotic first has an influence in the brain including the hypothetical sexual centre and that the hypophysis comes in autonomical action, i. e. it is no longer "blocked." (The central nervous system not only has an activating action on organs under its control, but also exerts a retarding or "blocking" effect.)

In the next phase the hypophysis itself seems to be affected: secretion of gonadotrophic hormone and oviductin production stop. When the fishes are returned to fresh water they recover and the hypophysis becomes active once more and ovipositor growth again sets in (Fig. 5).

6. Testis and Ovipositor Growth

It is probable that the endocrine chain reaction under normal conditions in the female is as follows: External stimuli such as light, temperature etc. activate the hypophysis via the central nervous system, thereby causing secretion of gonadotrophic hormones; this in turn activates the testes causing spermatogenesis and hormone secretion which is accompanied by spawning behavior.

The male bitterling also secretes a hormone into the water as is proved by the fact that water which contained sexually activated males only, is also capable of producing ovipositor growth when females are placed in it afterwards. According to JASKI, *Lebistes* males produce a similar substance called copulin. It is remarkable that copulin, unlike testosterone and other androgenic mammalian hormones, does not act via the hypophysis, but acts directly on the ovipositor. It, therefore, resembles oviductin and has not yet been chemically identified.

7. Reversion of Season and Spawning Activity

It is generally assumed that a rise in temperature and increasing daylight length in the temperate zones cause animals to mate once a year, generally in spring. This is undoubtedly of importance in birds, but we doubt whether it is of primary importance in *Rhodeus* and other fishes.

In November 1951 we obtained a number of bitterlings from the Nederlandse Heidemaatschappij (Arnhem, Netherlands), which arrived in Bloemfontein (S. Africa) within 24 hours by plane. These fish were, therefore, moved from the northern autumn to the southern spring within a very short time. Within a week the fish started spawning and by January 1952 produced a school of young bitterlings.

It is remarkable that these same fish spawned again in the next S. African autumn, in spite of the decreasing daylight length. There were, therefore, two spawning seasons in one year.

The same phenomenon occurred with the second consignment of fish which reached us in the S. African autumn (April, 1952). They continued their interrupted spawning immediately and again spawned six months later in the S. African spring.

In both cases spawning activity, therefore, occurred at increasing as well as at decreasing daylight lengths. The temperature in these spawning seasons varied between 60–70° F.

We hope to investigate in which season the young fish which were hatched in the spring and autumn will spawn and whether they too will have two spawning seasons instead of one. It is important to know whether the time of spawning depends primarily on inherited or external factors.

8. Influence of Mussel on Oviposition

A mussel with closed valves has no attraction for bitterlings, but when the valves open and a current of water comes from the exhalent sipho, bitterlings of both sexes immediately become interested in it and inspect the sipho until chased away by a territory holding male.

Like Tinbergen and his coworkers (1938) we found that it is the water current which attracts the fish. When a current of water was sent through a glass tube between the 2 valves the fish immediately became interested in it, but this interest disappeared as soon as the current stopped. Ovipositions occurred in these dummies, and a number of large, yellow, oval eggs were removed from them.

A water current issuing from between the valves is, therefore, of great importance to *Rhodeus* with regard to oviposition. It is not known to what extent a current without valves will attract the fishes as well.

9. Influence of Male and Mussel on Ovipositor Growth

The presence of a male and a mussel has another influence on the ovipositor growth.

Meltzer (1947) showed that the ovipositor of a female does not retain the same length throughout all the days of the spawning season, but that it undergoes periodical fluctuations. On some days the ovipositor reaches beyond the tip of the tail and on these days oviposition occurs. This is then followed by a quick reduction of the ovipositor length and during 6 or 7 days no eggs are deposited. After this full ovipositor length is again attained. There is, therefore, a cyclic periodicity in the development of the ovipositor and this is graphically demonstrated in Fig. 6.

The problem now arises as to what extent the cyclic ovipositor growth is influenced by the presence of mussels and/or males.

There are authors which claim that no ovipositor growth occurs if the male or mussel should be absent. To determine this, four combinations of males, females and mussels were examined.

I.	Aquarium	with	2	females,	1 male and 1 mussel.
II.	〃	〃	2	〃	, no 〃 〃 1 〃 .
III.	〃	〃	2	〃	, 2 males 〃 no 〃 .
IV.	〃	〃	2	〃	, without males or mussels.

Ovipositor growth occurred in all four combinations. The cyclic ovipositor growth during the spawning season is therefore independent of external factors and depends on an intrinsic factor. There was, however, a marked difference in the length of time between two successive oviposition days.

In group I, the two females had a very regular cycle of 19 oviposition days with an average interval of 6 days. Their spawning season therefore continued for about 4 months which is at least a month longer than in Holland.

In group II, fish A started with one oviposition day, but after this the ovipositor became invisible and the female sexual behaviour disappeared completely. Her partner B produced an oviposition-cycle with an interval of 7 days between peaks. From this it would appear that the male has no influence on ovipositor growth, but this proved not to be the case, as will be seen from the results in group III and IV. In group II the following occurred. After the one and only oviposition day of fish A she adopted the male pattern of behaviour. She established a territory around the mussel and on the oviposition days of B she led her to the mussel in the same way as is usually done by the male. A ruttish female, therefore, apparently does not distinguish between a genetic male and a female behaving like a male.

In group III, an oviposition peak occurred on approximately every 13th day. The absence of a mussel apparently doubles the intervals in the cycle.

In group IV, in absence of males and mussels, the intervals are increased fourfold in comparison with those in group I. In this group the absence of males and mussels also affects the behaviour and constitution of the fishes. Unlike the fishes of the other groups, these fishes are hypersensitive to vibrations. They were most of the time hiding in a corner of the aquarium and very pale.

10. Oviposition Mechanism

The slack ovipositor is introduced into the small exhalent sipho of the mussel as follows. Both the oviduct and ureter open in a common cavity which is surrounded by a muscular wall. This structure has a conical appearance from the outside and is visible during the spawning season. The anus is situated at the tip of this genital papilla while the ovipositor is attached to the ventrocaudal side of it. This conus may be moved vertically downwards. When a female has taken in position above the exhalent sipho of the mussel, usually with the tail directed towards the ligament, she suddenly swoops downwards and inserts the conus vertically into the sipho and the ovipositor still temporarily remains outside the sipho. By this time, or already before, the cavity in the muscular conus has been filled with urine and one or more eggs. Contraction of the muscular wall then forces the urine to the outside with the ova in front of it, thereby forming a "water axis" which then leads to a stiffening of the ovipositor over an increasing length and this forces it further into the mussel.

When the ova have been ejected the urine flows out and the tube again becomes slack and is pulled out of the mussel.

The developing eggs are attached to the gills of the mussel by lateral processes on the head of the embryo's and remain there for a further 3-4 weeks when they leave and lead an independent life. Cochlidiae of the mussels may attach themselves to the young bitterlings and thus be dispersed. The symbiotic relation between bitterling and mussel is therefore that of mutual commensalism (Fig. 7).

11. Requests for Assistance from Asiatic Zoologists for the Development of our Bitterling Research Scheme

From the above summary of some results obtained with the European bitterling it is apparent that comparative embryological, endocrinological, ethological and taxonomical investigations of the available Asiatic species have many promising aspects, especially with regard to the phylogenetic origin of the very specialized symbiosis between bitterlings and mussels.

We therefore request the assistance of zoological colleagues in the Far East with regard to the following:–

1. If they would make available any published or unpublished data regarding these fishes.
2. If they would be prepared to collect the representatives of the 30–40 Asiatic species occurring in their countries for transport to S. Africa.
3. If they could inform us of Rhodeinae which reproduce without mussels or where the symbiosis is not yet obligatory.

It is due to the kind assistance of the Head of the S. E. Asia Scientific Cooperation Office of Unesco, Djakarta, Indonesia, that we are in contact with a number of Asiatic zoologists in Japan, China and Korea, who have already offered and partly given their cooperation. Due to the kind assistance of Prof. David BARKER, University of Hongkong, and of Prof. Yaichiro OKADA, Dr. Katsuzo KURONUMA and Mr. N. ASANO in Japan we already have a number of *Pseudoperilampus ocellatus*. These fishes were transported gratis by the Royal Interocean Lines Ltd. We also wish to thank the Hon. Consul of the Netherlands in Japan for the arrangement of the fish already sent to us.

Pseudoperilampus ocellatus reproduces extremely well in S. African mussels although the latter have no "phylogenetic experience" regarding the incubation of bitterling eggs. It was found that spawning between a European bitterling female and an Asiatic male in the presence of a S. African bivalve is possible.

12. Summary

1. A short summary of recent investigations on the European bitterling, *Rhodeus amarus* BL., is given.
2. It is thought desirable to continue this work with Asiatic species, and an appeal to Asiatic zoologists for assistance is made regarding the collection of these species.

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References

DUYVENÉ DE WIT J. J. 1939: Onderzoekingen over de sexueel-endocrine organisatie van *Rhodeus amarus* BLOCH en de betekenis van de legbuistest voor de endocrinologie in het algemeen. Thesis, Utrecht.
——1940: A quantitative and qualitative test for steroid hormones based on the ovipositor reaction of the female bitterling. Journ. of Endocr., ii, 141.
——1941: Eine neue Testmethode für endokrinologische Zwecke. Pflüg. Arch. f. d. ges. Physiol. ccxlv, 2.
BRETSCHNEIDER L. H. & J. J. DUYVENÉ DE WIT 1947 : Sexual Endocrinology of Non-Mammalian Vertebrates. Monogr. on the Progress of Research in Holland during the War, Elsev. Publ. Comp. Inc., Amsterdam-New York.
SEITZ L. 1939: Wachsum, Geschlecht und Fortpflantzung, Springer. Berlin.
JASKI C. J. 1939: De elevatie van *Lebistes reticulatus.* Thesis. Utrecht.
TINBERGEN. N. c. s. 1938: De bittervoorns en hun mossels, De Levende Natuur, v.
MELTZER J. 1947: The oestrus cycle of *Rhodeus amarus* BLOCH. Proc. Kon. Ned. Akademie v. Wetensch., l, vi.
NOLL F. C. 1870 : Bitterling und Malermuschel, I und II. Der Zool. Garten x, 257, 1869, and xi, 131.
—— 1877: Gewohnheiten und Eierlegen des Bitterlings. Der Zool. Garten, xviii.

Photograph A: *Rhodeus amarus* pair near a mussel.

〃 B: Female bitterling in oviposition.

〃 C: Different stages of the oviposition act.

Fig. 1: Ovipositor growth curve after addition of urine to the aquarium water. After 14 hours fresh water has been given.

〃 2: Transverse section through the ovary of *Rhodeus amarus*. Apart from small, medium and large eggs, all stages in the formation of corpus luteum are seen.

〃 3: Medial section through hypophysis of *Rhodeus amarus*. The dark parts are composed of basophilic cells.

〃 4: Diagram showing the concatenation: stimulating agent→hypophysis→ovary→ovipositor.

〃 5: Action of narcotics on the central nervous system of *Rhodeus amarus* according to the disinhibition hypothesis.

Above: the sexual centre is blocked; hypophysis in the secretion phase, resulting in ovipositor growth (I).

Centre: hypophysis also blocked; no secretion of gonadotrophic hormone and no ovipositor growth (II).

Below: sexual centre alone is blocked; hypophysis is uninhibited again; secretion phase; ovipositor growth (III).

〃 6: Periodical fluctuations in ovipositor growth during spawning time. Oviposition takes place at each of the peaks.

〃 7: Showing the embryos of *Rhodeus amarus* in the gill-cavities of *Unio*. (From Olt).

Photograh A

Photograh B

Photograh C

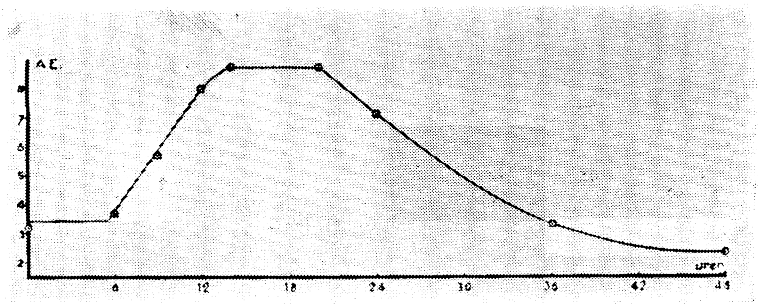

Fig. 1

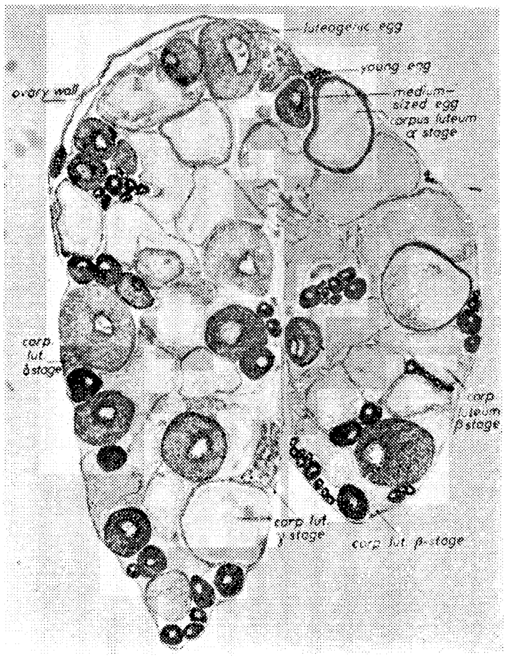

Fig. 2

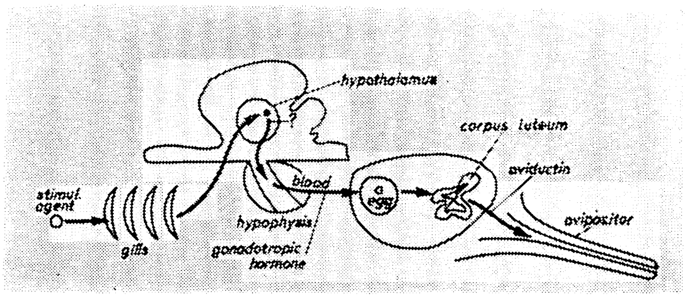

Fig. 4

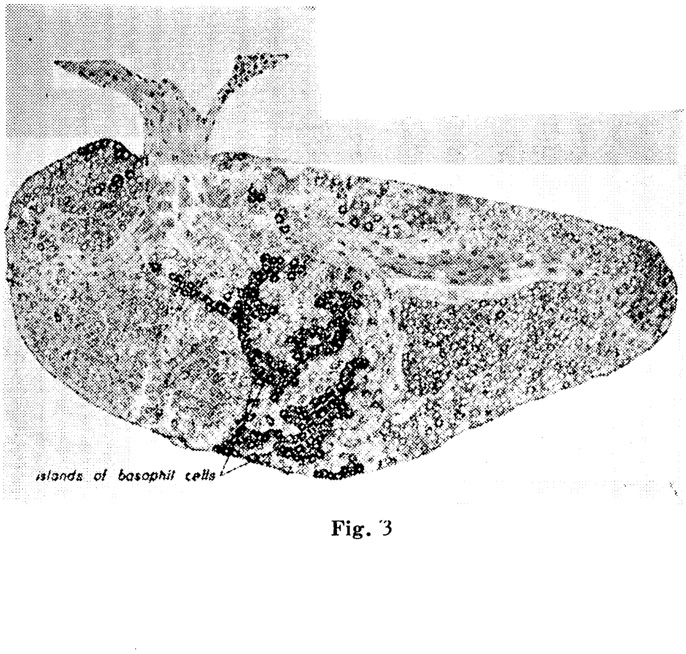

Fig. 3

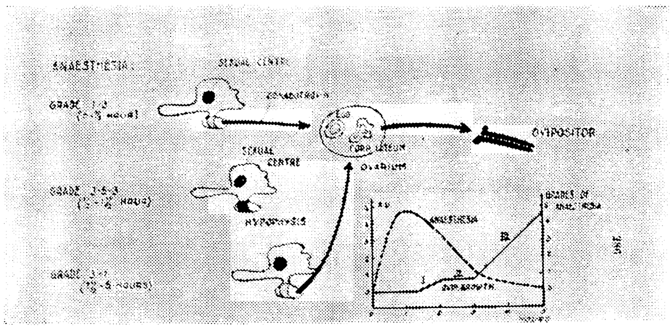

Fig. 5

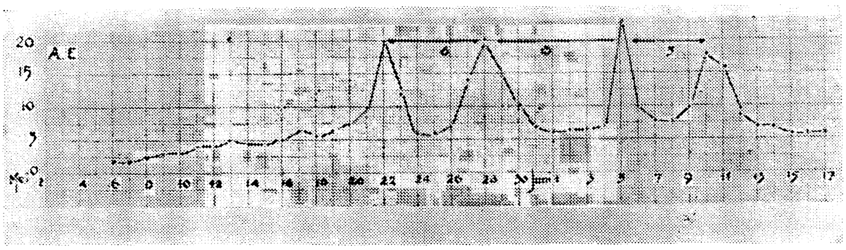

Fig. 6

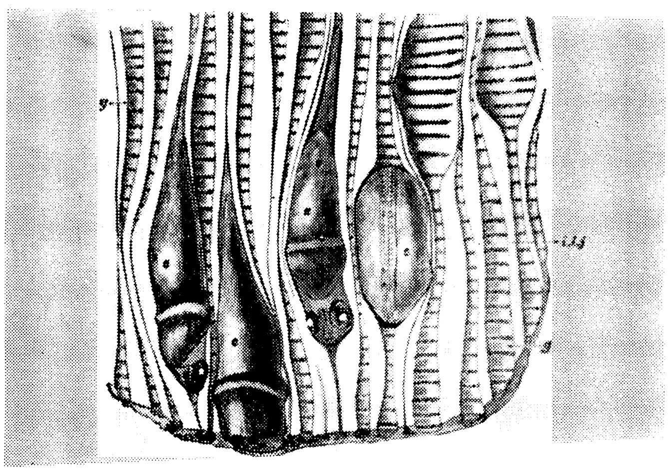

Fig. 7