Determination of age in honey-bees.

Determination of Age in Honey-Bees.

By

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With Plate 11.

It is of the utmost importance in the study of certain bee diseases to be able to separate definitely bees dying of a specific disease from those which are merely dying of old age. In the height of the honey-flow worker bees are said literally to work themselves to death in about six weeks after hatching. Consequently during the summer the normal daily mortality is very high—from a hive of fifty thousand workers being at least several hundreds. The last bees hatched out in autumn hibernate with the queen and thus attain an age of several months. It is popularly supposed and stated in some books on apiculture that an old bee may be recognised by her worn appearance, her body tending to be hairless and her wings frayed. The inaccuracy of this general statement and the uselessness of such a diagnosis for scientific purposes was soon apparent. I have carefully examined bees of many months in age (e. g. autumn bees flying in May) and compared them with those of a few weeks without finding any constant external differences. Conversely, relatively young bees may become hairless and their wings frayed under exceptional circumstances.

As a rule old bees die away from home, possibly during a foraging expedition, but they sometimes appear to return,

especially to a weak colony, and have ultimately to be turned out of the hive by its younger and more vigorous members. Bees having so few ways of showing their symptoms, it will be readily understood that there is much difficulty in differentiating disease from senescence at sight. If a bee is ill or very old it will first be unable to fly and later hardly able to crawl. Many "bee-experts" diagnose a crawling bee to be suffering from "Isle of Wight"[1] disease if its rectum is distended with an accumulation of undigested pollen, wax, etc., which can be readily squeezed out by slight pressure. It must be remembered, however, that, since bees normally defæcate on the wing, there will tend to be a collection of such undigested matter in any bees unable to fly.

Before proceeding further it will be well to explain briefly how far the investigation of the common diseases of adult bees has progressed in this country. Among diseases which can be diagnosed microscopically or bacteriologically are:

(1) Microsporidiosis or Nosema disease due to the protozoan parasite Nosema apis, Zander, which has been described by Fantham and Porter (**7**). Much has been written on this disease (**7a, 20**), which is not at present at all common in England. No doubt the activities of the Board of Agriculture since 1906 have helped to reduce the frequency of its occurrence.

(2) A form of dysentery or inability to retain fæces appears to be due to a small oval yeast—possibly of more than one species. The contents of the colon in affected bees are often watery, and always show enormous numbers of actively budding yeasts which are also present in the ventriculus. These organisms, which stain readily by the Gram or Claudius method, have never been found in the walls of the alimentary canal nor in other tissues. They are readily stained and therefore presumably killed by methylene blue, so that no doubt this and other mild antiseptics help to reduce their numbers. In these disorders, therefore, spraying and medicated feeding are likely to have some beneficial effect.

[1] See footnote to p. 193.

It must be remembered that yeasts are very widely distributed organisms, so that hardly a bee examined will be free from small numbers taken in with pollen grains, etc. They are usually, of course, quite harmless, and it is only when conditions are suitable for them to pullulate that they appear to have any pathogenic effect.

(3) After a bee has been free in a room for even a short time I have found that it almost invariably develops one or more colonies of a mould. Branches of the mycelia of these often penetrate the walls of the alimentary canal, causing adhesions between its various parts and rapidly proving fatal. The spores of these fungi are no doubt ingested by the bee with dust from the room.

(4) The black shiny condition occasionally met with in some bees of a stock owing to loss of the normal covering of hairs has been associated by Cheshire (**2**, ii, p. 570) with the presence of an organism—Bacillus gaytoni—in the intestine. This finding has not so far been confirmed. Cheshire states that in such cases the queen is generally badly affected and that re-queening is the best way of checking the disease.

In addition to these there would seem to be at least one other disease for which a specific organism has not yet been separated. This I shall refer to provisionally as malignant disease.[1] Rennie (**17**) has recently published some interesting

[1] It is better to avoid the use of the term "Isle of Wight" disease owing to the confusion associated with it. This mysterious unknown disease (malignant disease) has been much confused with microsporidiosis. Further, one or two instances in which yeasts have been mistaken for Nosema by incompetent observers posing as experts have come under my notice. Thus at times at least three of the above disorders have been called "Isle of Wight" disease, and it is now, I fear, impossible to determine with certainty to which the term was originally applied.

Much has been written also about bee paralysis, but I can see no reason for considering this to be a distinct disease. Supposed isolated instances are doubtless often due to accident—the result of fighting amongst themselves or with wasps, etc.—age and cold, or, as Malden (**7a**, p. 135) points out, many so-called cases are in reality cases of the above (malignant disease).

experiments on the infectiousness of this disease. On no occasion have I been able to obtain any bacteria from the blood, muscles or fat body of bees examined bacteriologically. From the alimentary canal so vast is the number of bacterial colonies usually obtained with ordinary nutrient media that the difficulty is to separate them for identification. No organisms have so far been demonstrated actually inside the cells of the alimentary canal although I have stained numerous serial sections with almost every conceivable stain, including Gram, Van Gieson, Claudius, etc. As might be expected, the intestines of bees from almost every different locality contain different bacteria which will grow on various media, some aërobically, others anaërobically. So long as I was uncertain whether a crawling bee was merely senescent or really diseased, the problem of finding whether any special bacterium was associated with the disease was practically impossible to solve.

The possibility of determining the age of bees is likely to be of further importance in estimating the chances of survival of a stock during the winter. There is no doubt that age and suitable general conditions of life have much to do with successful wintering, and that loss of stock owing to lack of these conditions is often quite wrongly attributed to disease.

Being then convinced of the necessity of being able to determine the age of a bee, I carefully examined, in the first place, such exterior parts of the body as the hairs, glossa, gonapophyses, etc. On concluding that nothing definite was to be determined from these, the study of the brain and head glands was begun.

The work of Minot (**15**) and others, together with the fact that a bee appears to work incessantly during its short life, suggested that the nerve-cells would give a good indication of age. The results so far obtained show that a great deal may be learnt from a study of the brain and glands of the head.

Normal Life-history of a Worker Bee in Summer.

After laying of fertilised egg	1st–3rd day	Incubation of egg.
	3rd–8th day	Larva is hatched and fed by workers (nurse bees).
	9th day	Cell containing larva is sealed with a capping of wax and pollen.
	9th–22nd day	Larva spins a cocoon and becomes a pupa or chrysalis, then a perfect insect (imago).
	22nd day	Bites her way through capping.
After emergence from cell	For 1st two weeks	Young bees remain in hive acting as nurses, etc.
	From 2nd to ? 6th week	Fly backwards and forwards to hive on foraging expeditions.

For the experimental work a revolving observatory hive was fixed on the laboratory bench with its passage for exit communicating with the outside air by a small hole in the mullion of a window. The hive itself held only two combs taken with brood and a queen from an ordinary hive. The observatory hive was connected above with a glass-fronted "super" containing four sections and room for more combs. The bees were fed, when necessary, through holes in the top of this "super." One of the ordinary glass sides of the hive was replaced by a specially designed one, consisting of four glass doors in narrow frames, each opening independently and commanding one-half of a comb. By means of these doors bees at required stages could be removed without disturbing the whole hive. A record kept of the date of sealing of some of the larvæ enabled me to remove imagines on the eve of hatching. Other bees were taken out with padded forceps as they bit their way through the cappings of their cells, and either used for experiment or marked and put back.

Marking proved to be a decided difficulty. A dab of white or light-coloured oil-paint on the thorax would sometimes remain visible for two or three weeks if it dried quickly enough and was of the correct consistency. Pigment mixed with gum-arabic was no good at all, for on drying it came

off in a lump with any hairs of the thorax that it touched.[1] Attempts were made to tinge the wings by applying a dye dissolved in water, alcohol, chloroform or ether, but no penetration could be effected.

The least unsatisfactory method of marking in some ways is to clip the tip of one or more wings. This does not interfere with flight so long as only the very tip is removed. By clipping different combinations of the four wings any number at a time (2^4–1), i. e. fifteen different dates of hatching could be indicated. The drawback to this method is, of course, the difficulty in recognising a clipped bee except at very close quarters.

For keeping bees under special observation I use large glass bell-jars in which they can take short flights, and will sometimes live for three or four weeks. Each jar is provided with a large piece of crumpled paper in which the bees can hide from the light, and honey, syrup or candy and pollen are supplied at intervals. By adding various drugs to the food it is easy to test their action, but on the whole wasps or bumble-bees are better for feeding experiments since they live longer in captivity.

Technique.

After some preliminary experiments the brains were found to be best fixed for the routine study of nerve-cells in the following way: The whole head is cut from a living bee, after subduing with chloroform, if necessary. The upper part of the chitin (just above the three simple eyes) is quickly

[1] Since writing this I have heard from Mr. Bullamore that at the Cambridge Institute of Bee-keeping they have added to the pigment used some of the preparation known as "new skin," and in this way managed to distinguish bees for three or four weeks. Certainly the addition of the celloidin solution is an improvement, appearing to give elasticity and so prevent the pigment from coming off so quickly. However, even this has a tendency to peel off and bring the thoracic hairs with it, so that I am afraid it may still be difficult to recognise bees after four or more weeks, and these are the ages at which specimens are now required for study.

sliced off and the head placed in a small tube of Petrunkewitsch fixing-fluid for about fourteen hours. After thoroughly washing in 70 per cent. alcohol containing iodine, the head is transferred to 90 per cent. alcohol for some hours. It is then easy to remove the remainder of the chitin from the posterior part of the head, and to lift out the entire brain (Pl. 11, figs. 1 and 2) with the subœsophageal ganglion and some of the head-glands attached. After further washing the brain is prepared for embedding and cut into serial sections as required.

Sagittal sections were cut of some brains, but generally transverse sections are more useful. These are cut from above downwards, starting through the three simple eyes. By leaving the optical pigment attached to the brain it is easy to orientate it when embedded.

For routine examination of individual bees transverse sections 7 μ thick are preferred, and they are stained with Ehrlich's hæmatoxylin and orange G. Many other stains were tried. Among them carbolthionin was good for brain-cells, and alcoholic carmine with picronigrosin as a counter-stain was useful for picking out membranes and connective tissue. Perfect fixation of the material immediately on death is, of course, most important in order to exclude error arising from post-mortem changes.

Ohlmacher's fixative penetrated very well, but owing to the large percentage of absolute alcohol (80) and chloroform (15) present, all the lipoids of the cells were dissolved, producing intense vacuolation throughout. Fleming's solutions, on the other hand, were not sufficiently penetrating for the whole brain. No doubt by employing some of the osmic acid mixtures without acetic acid on small portions of the brain or other ganglia and staining by the more elaborate methods, much more interesting detail might be made out in the cytoplasm. I have not, however, myself felt justified in spending time on these methods, since all that is required for the pathological work undertaken for the Board of Agriculture is to discover whether a crawling bee sent for examination is

senescent or not. If the nerve-cells are found to be in sufficiently good condition to rule out the possibility of senescence, it is then worth while to spend some weeks on the complete investigation of the bacteria of the bee, on the chance of finding organisms which are possibly pathogenic.

Attempts were made to do away with the necessity of fixation at all and to examine the nerve-cells in a fresh state. For this purpose the abdominal as well as the cerebral ganglia were investigated. Injection with methylene-blue has not proved successful with bees (I cannot find any instances in which it has succeeded with adult insects). It must be remembered that the ganglia are surrounded with a considerable thickness of connective-tissue, and in addition a close net-work of tracheæ makes their opacity very great.

The abdominal ganglia were sometimes rapidly removed from a bee opened under Ringer's solution (Locke's modification) as usual and then flooded with a dilute solution of methylene-blue in the same solution (·001 per cent.), but penetration was always difficult, and the cells could only be made clear after a considerable amount of teasing of the ganglionic tissue. Consequently these processes had to be abandoned in favour of a constant method of fixation as described above.

Experimental Results.

In the serial transverse sections cells of several different sizes are to be found (**11, 18**). The cells that I have studied are the large ones with a considerable amount of cytoplasm when young, and a nucleus measuring 8 by 10 microns or more. No definite Nissl bodies are to be seen after the fixation used, but some of the older cells are hyperchromatic, having a varying number of irregular masses of deeply-staining substance in the cytoplasm (Pl. 11, fig. 8). These large cells are found chiefly in the following four main regions:

(1) Almost at the centre of the brain is a wedge-shaped mass of cells pointing towards the anterior surface, and

having as a base a group of giant fibres. In each of the dozen or so sections, 7 μ thick, passing through this region there are generally four or five large cells—rather more in very young brains.

(2) Cells of the antennal lobes. Each of these lobes consists of a convoluted spherical mass of fibres giving rise to the antennal nerve and surrounded by nerve-cells. These are the cells studied by Hodge (**9, 10**) and Smallwood and Phillips (**18**).

(3) Just anterior to the inner fibrillar body of the optic lobe is a mass of cells, chiefly small ones, but with a few giant ones among them.

(4) Cells of the subœsophageal ganglion. The mass of fibres forming the centre of the ganglion and giving rise to the ventral nerve-cord is surrounded by a layer of cells which is especially thick on its lower surface. It has been usual to consider the subœsophageal ganglion as part of an insect's brain (**16, 8**), since Faivre's physiological experiments (**6**) appeared to show that the power of co-ordinating the movements of the body is there situated. This ganglion therefore appears to correspond to the cerebellum of vertebrates.

The large cells, with nuclei 8–12 μ in diameter, from these four regions in one and the same brain show great constancy in appearance. They are also very similar to those from other bees of the same age, but exhibit constant differences from those of bees at other ages.

On the whole it is most convenient to study the cells from the fourth region, i. e. the subœsophageal ganglion. The large cells here are very numerous, forming several layers below the mass of fibres going to the ventral nerve-cord, and bounded on each side by small groups of fibres, from which the mandibular, maxillary and labial nerves arise (Pl. 11, figs. 3 and 4). These layers of cells appear in about ten serial sections, and are therefore about 70 microns thick. In young bees they consist chiefly of large plump cells, only separated from each other by narrow strands of connective tissue with occasional connective-tissue cells (Pl. 11, fig. 3). As the

bee increases in age, vacuoles appear at the periphery of the cytoplasm of the nerve-cells, so that even at the end of the first week of toil most of the cells have somewhat the appearance shown in Pl. 11, fig. 6. During the exertions of foraging for nectar, pollen, etc., the brain-cells age still further; so that a bee returning to the hive at mid-day laden with pollen showed the condition represented in Pl. 11, fig. 7.

The cell shown in Pl. 11, fig. 8, came from the sub-œsophageal ganglion of a bee which was unable to fly, and had lost nearly all the hairs on its thorax. There is thus good reason to think that it was senescent. The final stage of disintegration of the cytoplasm is shown in Pl. 11, fig. 9, one of the cells from the ganglion drawn in Pl. 11, fig. 4. This belonged to a bee which came from a hive on a fine day in March, but was too weak to effect a cleansing flight and soon became moribund. It will be seen that the nerve-cells are quite worn out, there being in this part of the ganglion only a framework of connective tissues, with here and there the nucleus of a nerve-cell in a more or less necrotic condition, with only a trace of cytoplasm round it.

It will be noticed that the nuclei of bee nerve-cells contain several nucleoli as described by Binet (**1**) for most insects. Dolley (**3**), however, figures for the crayfish a single large nucleolus such as is found in typical vertebrates. I cannot agree with Mann (**13**), Hodge (**9**), and some others that there is a marked shrinkage of the nucleus due to fatigue. In well-fixed brains the nuclei appear to retain their normal shape and also their size, as shown by Smallwood and Phillips (**18**). In very old bees, however, the chromatin seems generally to leave the nuclear membrane and tends to form a single clump just inside.

A reduction of cytoplasm due to fatigue has been previously described by Dolley (**3**) in the nerve-cells of the crayfish after artificial stimulation. This he refers to as an œdematous condition, since the so-called vacuoles left at the periphery of the cell as the protoplasm disintegrates become, of course,

filled with fluid. In crayfish and other animals which live for some years with intervening periods of rest one would expect to find alternating signs of fatigue and recuperation in the cells such as he describes. In bees, as far as can be ascertained, there is no nocturnal rest. The specimens for experiment were, however, taken from the hive about noon. In this way it was hoped to eliminate any differences which might possibly arise from daily fatigue. It seems very doubtful, however, in spite of Hodge's results (**9**), whether there is any recuperation during the night. Except that bees do not fly after dusk, the various activities appear to go on as usual. Certainly wax secretion and building of comb, transference of honey or sugar from one part of the hive to another, ventilating, guarding, etc., are carried on throughout the night. It would be most surprising to obtain constant differences in the nerve-cells of bees caught on the wing in the morning and evening without taking their age into account as first attempted by Hodge.

Dolley (**4**) has shown that incessant exercise has a very decided effect on the nerve-cells of artificially stimulated dogs as well as of the crayfish as described above. One is therefore not surprised to find that the incessant work done by a bee produces a pronounced cumulative effect, and further, since the bees normally work themselves to death in so few weeks, that the condition of the nerve-cell gives a good indication of age in bees.

During successful hibernation no doubt a certain amount of repair takes place in the brain and other tissues of the bee. I hope to study these recuperation stages in more detail during the winter.

Some bees which appear to be dying of disease have been found to have nerve-cells rather less aged than those of the active forager shown in Pl. 11, fig. 7. It seems likely that loss of activity consequent on attack by disease would prevent, if anything, the usual disintegration of the nerve-cells, so that a diseased bee would appear, if studied from this point of view, to be younger than it really is.

If these determinations of age are to be made of general use, it is important to eliminate as far as possible the personal equation. This can be done to some extent by comparing the number of giant nerve-cells in some constant region of the brain. There appears to be a small decrease in the number of these cells with age, as stated by Hodge (**10**), and as has been shown also for the Purkinjë cells of the human cerebellum by Ellis (**5**). However, the counting of them entails much labour, and consequently the tables with these numbers will be held over until next year, when I hope to have a perfect series of brains at all ages.

Further, the condition of the glands gives some clue to age, for in normal healthy bees their physiological condition corresponds to actual age.

The glands of the head that are being studied are the pharyngeal, salivary, mandibular and maxillary as described by Snodgrass (**19**) and Cheshire (**2**). In addition, a small pair of glands has been discovered of which I can find no previous mention (Pl. 11, fig. 2). These occur one on each side of the œsophagus as it emerges from between the supra- and subœsophageal ganglia—that is, they are just in front of the commissures. These œsophageal glands are almost spherical, about 120 microns in diameter. From each proceeds what appears to be the remains of a convoluted duct to meet its fellow of the opposite side at an angle of 60° above the œosphagus and in close contact with the central portion of the supra-œsophageal ganglion.

For the full investigation of these various head glands much study is still necessary, and experiments with intra-vitam stains are being carried on. Some of the glands vary enormously with the immediate function of the bee. The pharyngeal glands, for example, become distended with secretion soon after hatching and remain so during the two weeks that the bee functions as a nurse. By the time that pollen gathering is undertaken, these glands appear to be exhausted and in an advanced hyperchromatic state which gives the collapsed glands a necrotic appearance. The different histological

appearances at the various ages promise to give most interesting results as to the cause and effects of disease, as well as the elucidation of many points in the life-history of bees, such as the process of elaboration of the varying bee foods—royal jelly, etc.,—and the ages at which these activities are carried on.

It is a pleasure here to express thanks to Prof. G. C. Bourne, in whose department this work is being carried on, and to my husband, Mr. E. S. Goodrich, for their interest and valuable advice during the progress of the work. Mr. J. B. Gatenby has also made many helpful suggestions in connection with the treatment of insects for cytological examination. I also wish to record that this work on bees is being carried on by means of a grant from the Development Commissioners of the Board of Agriculture. Much assistance in obtaining bees suspected of disease has been given by Mr. A. G. L. Rogers, and more recently by Dr. Keeble and his assistants in the Horticultural Section of the Food Production Department.

Summary.

For the study of those bee diseases with which no specific organisms have so far been identified, it is important to be able to eliminate bees dying of old age, and this cannot be done with certainty by observing outward symptoms. However, the age of bees, which normally work almost incessantly for about six weeks and then die, may be determined with some accuracy from a study of the brain-cells. With advancing age the cytoplasm of these cells undergoes gradual reduction peripherally, until in senescence only a vestige is left surrounding the nucleus.

The condition of the head-glands, including a pair of œsophageal glands which do not appear to have been previously recorded, gives some indication of age in normal healthy bees.

University Museum,
Oxford;
September, 1919.

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EXPLANATION OF PLATE 11,

Illustrating Mrs. Helen L. M. Pixell-Goodrich's paper on "The Determination of Age in Honey-bees.

[The figures were drawn with the aid of a camera lucida at an approximate magnification of 2000 unless otherwise stated.]

Ant. Antennal nerve proceeding from antennal lobe of brain. *c.* Commissures. *E.* Compound eye. *e.* Simple eyes. *lab.* Labial nerve. *max.* Maxillary nerve. *md.* Mandibular nerve. *n.c.* Ventral nerve-cord. *œs.* Œosphagus. *œs. gl.* Œsophageal glands. *phar.* Pharynx. *s.g.* Sub-œsophageal ganglion.

Fig. 1.—Front view of the whole brain after removal of the pharyngeal glands. × 20.

Fig. 2.—Right postero-lateral view of brain showing alimentary canal with œsophageal glands. × 23.

Fig. 3.—The right half of a section through the lower part of sub-œsophageal ganglion of a young bee immediately after hatching, showing the central mass of ganglion-cells between the fibres going to the mandibular, maxillary and labial nerves. Orth's alcoholic carmine and picro-nigrosin. × 400.

Fig. 4.—A corresponding half section through the same ganglion of a bee five or six months old after hibernation, showing the necrotic condition of the cells. Iron-hæmatoxylin and orange G. × 400.

Fig. 5.—A large cell from the subœsophageal ganglion of an imago immediately before hatching, showing its general plump appearance

and freedom from highly chromatic substance in the cytoplasm. Iron-hæmatoxylin and picro-nigrosin.

Fig. 6.—A similar cell from the subœsophageal ganglion of a bee seven days after hatching showing the periphery of the cytoplasm already becoming slightly vacuolated. Ehrlich's hæmatoxylin and orange G.

Fig. 7.—A corresponding cell from a foraging bee on returning to the hive laden with pollen. The cytoplasm is decidedly vacuolated at the periphery. Ehrlich's hæmatoxylin and eosin.

Fig. 8.—A corresponding cell from a bee unable to fly, apparently senescent. Ehrlich's hæmatoxylin and orange G.

Fig 9.—A corresponding cell from a bee dying in March after hibernation. Iron-hæmatoxylin and orange G.

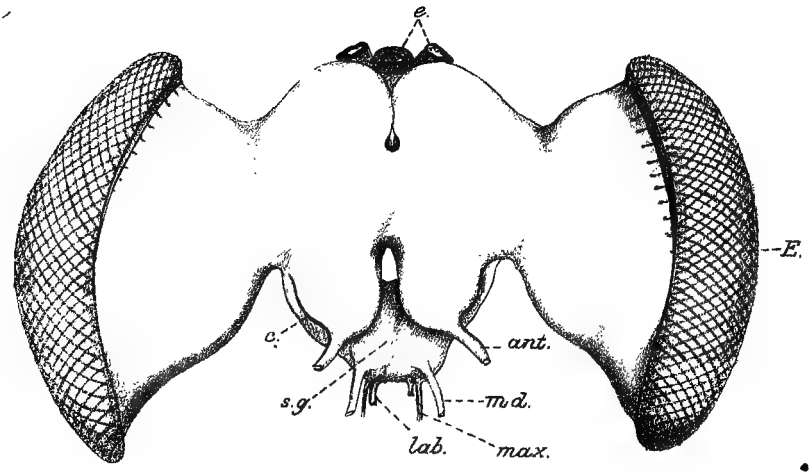

Fig. 1.

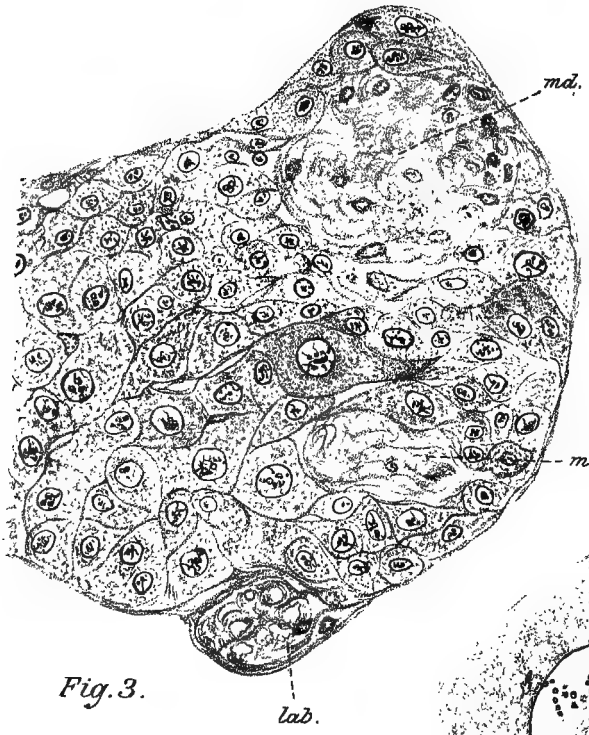

Fig. 3.

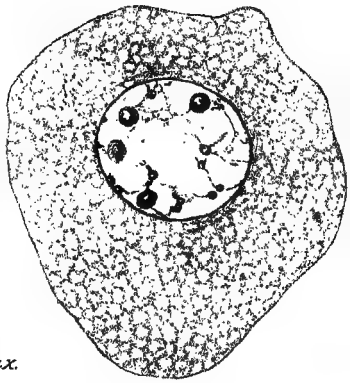

Fig. 5.

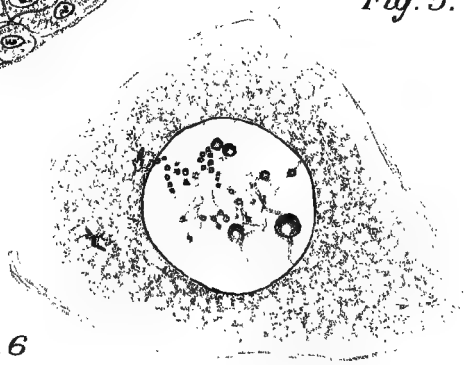

Fig. 6

H.L.M.P.G. del.

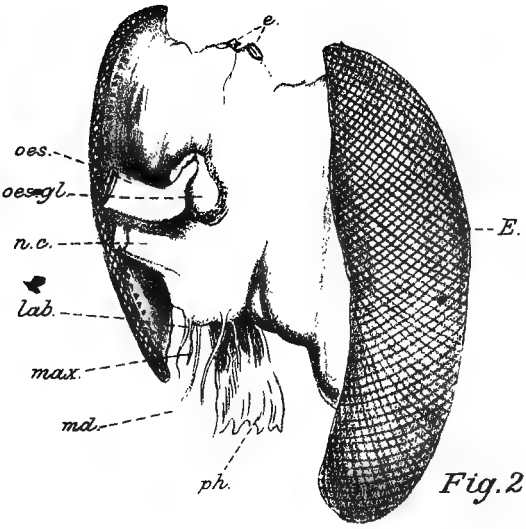

Fig. 2.

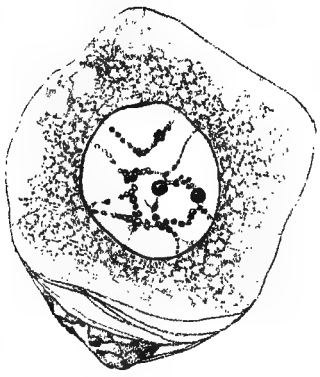

Fig. 7.

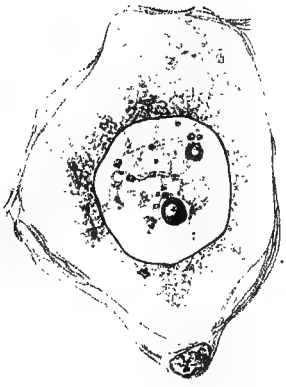

Fig. 8.

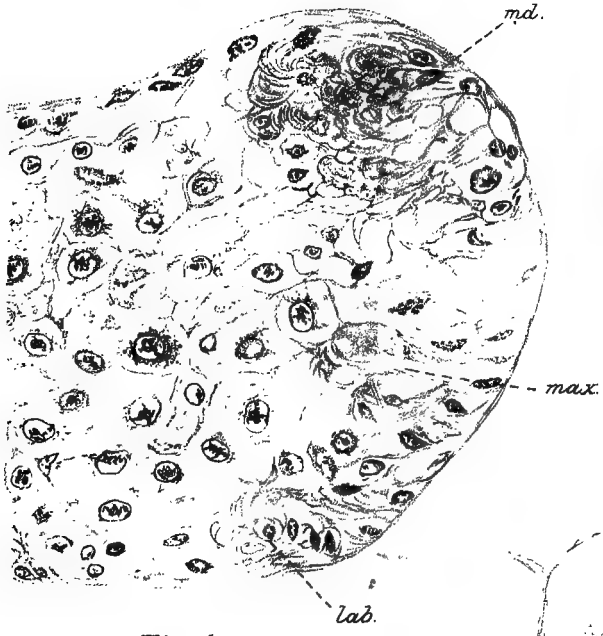

Fig. 4.

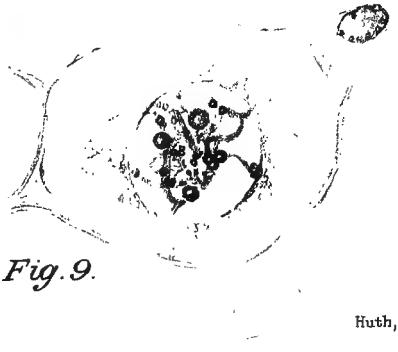

Fig. 9.

Huth, London.