On the unsegmented ovum of Echidna (Tachyglossus).

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With Plate 3.

Introduction.

The account in the following pages deals with two eggs of Echidna (Tachyglossus) collected in Tasmania during the breeding season of 1929. They, with others, were acquired by the aid of a grant from the Grants Committee of the Royal Society, to which committee and to Professor J. P. Hill, who aided me in obtaining this grant, I express my appreciative thanks.

As is well known, material of the developmental stages of Echidna is not easy to get, and the matter is being made more complicated by the increasing scarcity of the animal. I have altogether some thirty intra-uterine stages, but even so the series can hardly be said to be complete. It is hoped to fill the existing gaps in the series, so far as possible, by further collecting during the season of 1930.

The known forms of Echidna[1] are regarded as belonging to one species, Echidna aculeata Shaw, of which there are three well-authenticated varieties, with a possible fourth. These are as follows: var. typica or aculeata, found on the mainland of Australia; var. lawesi, inhabiting Southern New Guinea; and var. setosa, confined to Tasmania. The fourth variety, multiaculeata, described by Rothschild in 1905, from the southern portion of South Australia, is of doubtful validity.

[1] I have used the widely known generic name Echidna in preference to the more correct term Tachyglossus.

Up to the present, as might be expected, practically all work dealing with the development and organogeny of Echidna, contained in the writings of Owen, Caldwell, Semon, Hill, Gatenby, and others, has been based on material of the variety typica.

My own material, being obtained wholly in Tasmania, is of the variety setosa, of which but one developmental stage has been recorded. This was a pouch egg, which, with the contained embryo, is now preserved in the collection of the Tasmanian Museum. It was briefly described by Morton in the 'Papers and Proceedings' of the Royal Society of Tasmania for 1887 (1888).

No detailed description of the unsegmented ovum of Echidna has yet appeared. Apparently Semon (1894) collected some material of this period of development, but it has not yet been investigated. Caldwell (1887, p. 472) states that an unsegmented ovum which he took from the lower end of the Fallopian tube measured 3·2 mm. in diameter, and of this egg he gives a description of the membranes. In his Pl. 31, fig. 1, he also gives a drawing of a vertical section through the germinal disc, but there is no description in the text, and it is not possible to make out from the figure any details of the structure of the disc or of the stage of development at which it has arrived.

As yet, the only full account published of the structure of the unsegmented monotreme ovum is that of Gatenby and Hill (1924), dealing with an intra-uterine egg of Ornithorhynchus. Previously to this, Gatenby had referred somewhat briefly to the same egg (1922). Gatenby and Hill, in addition to giving a full description of the arrangement of the white and yellow yolk and of the structure of the latebra, deal in detail with the germinal disc and with the distribution and significance of certain nuclear structures which they found in close association therewith. Since it will be necessary to refer to the work of these authors from time to time in the course of the present paper, it is advisable to do no more at this juncture than draw attention to the fact that they came to the important conclusion that the ovum of Ornithorhynchus, like that of Sauropsida, is polyspermatic, and further, they state

that the two polar bodies in Ornithorhynchus are of unequal size, the first being larger than the second, and undergoing division before the latter is completely separated off.

As will be seen from the results of the investigation of the two stages described in the present paper, Echidna agrees with Ornithorhynchus in that the first polar body is larger than the second, but it remains undivided. As for polyspermy, it is apparently not a normal occurrence in Echidna.

The egg of Ornithorhynchus examined by Gatenby and Hill was cut in sections in toto; but, with my own material, owing to the difficulties associated with the brittleness and general impermeability of the yolk, I felt that such a technique involved too much risk to the germinal disc. The two eggs here described were first fixed in Bouin's picro-formol-acetic mixture, after which, by the judicious use of needles and a small brush, all but the immediately underlying yolk was removed from the germinal disc. The latter, after being double-embedded in celloidin and wax, was cut into sections, each of 5 micra. The sections were stained in Heidenhain's iron-haematoxylin.

Ovulation in Echidna.

Owen (1881) describes and figures the left uterus of an Echidna containing three eggs, and Broom (1895), in his note on the period of gestation of this animal, records a total of two eggs as having been laid on successive days. Nevertheless, in the many specimens of the Tasmanian variety which I have had, there never has been more than one egg present at one time. Semon had a similar experience with the mainland variety, so that it would appear that any other condition is abnormal.

It has also been stated that the right uterus never becomes pregnant. Thus, in speaking of the group generally, Semon (1894, p. 62) says: 'Obwohl bei beiden Monotremengattungen sich sowohl im rechten als auch im linken Ovarium Eier entwickeln und ausbilden, werden doch nur die Eier des linken Ovariums vollkommen reif und gelangen nach Austritt aus dem Ovarium, wo die Befruchtung stattfindet, in den linken Ovi-

duct.' This statement, which has been adopted by Weber (1928, Bd. ii, p. 39), does not apply to the Tasmanian variety of Echidna. In this form, so far as my results go, about one-third of the developing eggs are to be found in the right uterus. It is possible that the case is different with the variety found on the mainland of Australia, but it must be remembered that, even here, Owen (1881, p. 1052) records an egg from the uterus of the right side, and Hill and Gatenby, only recently (1926, p. 746), have described a perfectly normal corpus luteum as being present in the right ovary of an Echidna which had just previously laid an egg.

Measurements of Eggs.

Gatenby and Hill (1924) have made some remarks on the variation in size of eggs of Ornithorhynchus and Echidna, particularly in the early intra-uterine stages, and have come to the conclusion that such variations are due partly to differences in the size of the ovum, partly to variations in the thickness of the albumen layer.

But it appears that few, if any, of these measurements have been made on living eggs. Such measurements are fairly constant, but they vary tremendously after fixation whatever be the fixative used. As will be seen hereafter, several types of fixing solution have been employed in this work, but the effect in all is practically the same. The shell becomes wrinkled at first, and later becomes separated from the yolk-mass by a space of variable extent, artificially caused by the absorption of fluid from the exterior. The albumen layer becomes greatly swollen and disorganized. That this is the case is shown by the following series of measurements taken before and after fixation. The eggs are all in early segmentation stages and the measurements of the living eggs are, I believe, the first to be placed on definite record.

I would look upon the egg of Ornithorhynchus of which a photograph is shown in Plate I, fig. 1, of Wilson and Hills's paper (1907), as a typical example of the kind. Here, the yolk-mass is seen to be separated from the outer shell membrane by a space which is more or less obliterated on one side, evi-

dently that on which the egg was resting during fixation and preservation.

Egg.	Total diameter.		Fixative.
	Fresh.	*After fixation.*	
	mm.	mm.	
i	4·5	6·0	Bles.
ii	4·0	5·5	Formol-bichromate-acetic.
iii	4·0	5·6	Formol-bichromate-acetic.
iv	4·0	5·6	Dubosc-Brasil.
v	4·4	5·3	Bles.

Under these circumstances, it is evident that measurements of the total diameters of eggs, taken after fixation, must be regarded with a large amount of caution.

Description of Material.

Egg No. 1.

The diameter of this egg, measured intact and in the living condition, was 4 mm. The shell, before removal, was seen to be extremely delicate and transparent, its thickness in sections being 0·0018 mm. The albumen resisted the swelling usually associated with fixation and varied somewhat in thickness, being as much as 0·03 mm. over the germinal disc, while it is as little as 0·017 mm. over parts of the remainder of the egg.

The removal of these two layers exposes the yolk-mass with the germinal disc on one side, enclosed in the very delicate zona. The diameter of the ovum deprived of its membranes is, therefore, as near as can be judged, 3·95 mm. This measurement is much in excess of that given by Caldwell (3 mm.) as the diameter of the oviducal ovum of Echidna (see also Hartman, 1929, Tables 6 and 8).

Below the zona, in the sections, is to be seen a somewhat vacuolated layer of varying consistency which appears to be a coagulum, possibly produced as a result of fixation.

It is usually stated that the yolk of the monotreme egg is yellowish in colour and that the blastodisc is therefore easily distinguishable from the remainder of the ovum. I have not

found this to be the case with the Tasmanian variety of Echidna. Here, when fresh, the yolk is perfectly white, and it is practically impossible to make out, through the shell, the position of the germinal disc. With fixing fluids containing picric acid, such as Bouin's solution, the yolk stains yellowish, and so the blastodisc stands out a little more prominently. But the use of most other types of fixatives makes it necessary to stain the egg in toto before the position of the disc can be determined.

The structure of the germinal disc in this egg and its relationship to the yolk, latebra, &c., are shown in fig. 1, Pl. 3. Externally is to be seen the shell (*sh.*), at this stage very thin and delicate, below which is the laminated layer of albumen (*alb.*).

Underlying this again is the zona pellucida (*z.p.*).

The arrangement of the yolk is similar to that found in the unsegmented egg of Ornithorhynchus (Hill and Gatenby, 1924) with some differences in detail. As in Ornithorhynchus, the ' yellow ' yolk is disposed in two zones, an outer and an inner, while there is present over the whole surface, except at the germinal disc, an extremely delicate layer of very fine yolk-spheres. The germinal disc (*g.d.*), more flattened and of greater extent than is the case in Ornithorhynchus, overlies a much vacuolated portion of the ovum, the nucleus of Pander (*nu.P.*), composed of finely granular material which does not stain with haematoxylin and which is very similar to the material of the disc itself. The nucleus of Pander is continuous below with the latebra (*lat.*), which is like that of Ornithorhynchus as described by Hill and Gatenby. Surrounding the nucleus of Pander is a ring-like mass of fine grained ' white ' yolk. Into the upper side of this mass projects a circular thickening of the lower side of the germinal disc to be referred to later.

The germinal disc differs greatly in appearance and shape from that described for Ornithorhynchus. Compared with that of the latter genus, the disc of Echidna is much more definite in outline, more flattened from above down, and more expanded laterally.

Viewed from the surface in the unsectioned ovum, the disc

is quite circular and presents a central transparent area surrounded by a darker ring. Its diameter, measured over all after being embedded and cut into sections, is 0·55 mm., more than ten times the surface diameter in the only comparable stage of Ornithorhynchus. Great as this discrepancy is, it is perhaps not surprising if we take into account the very remarkable changes which may take place in the disc of meroblastic eggs during maturation processes. These have been described in some detail by Harper for the pigeon (1904, see particularly figs. 4*a*, 5, and 8, Pl. 1).

In Echidna, the upper side of the disc is gently convex. On its lower side the disc, although unmistakably defined, is markedly irregular in its contour. It thins out at its outer edge and is of no great thickness (0·016 mm.) in the centre, but, about half-way between the two, there is an annular zone where the disc projects downwards into the yolk, its thickness here being as much as 0·043 mm.

These differences in the thickness of the disc account for the appearance of its division into a lighter area surrounding a darker one.

The disc is composed of a very fine, granular, homogeneous material from which yolk is practically entirely excluded, except along the lower edge and the margins, where the disc is to some extent invaded by yolk-spheres. The substance of the disc is entirely similar in structure and staining qualities to the material which constitutes the basis of the much vacuolated nucleus of Pander.

Nuclear Phenomena.—The ovum is in the last stage of maturation, the second polar body having just been given off. The two polar bodies are situated near the centre of the disc and are much smaller than in Ornithorhynchus. They are also very unequal, the second being quite minute. The first polar body measures 0·022 mm. × 0·012 mm. and is found to occupy three consecutive sections each of 5 micra; the second measures 0·0075 × 0·0055 mm. and is found in two sections only. Fig. 3, Pl. 3, is a drawing, much enlarged, of the two polar bodies and the female germ nucleus.

The female germ nucleus is a flattened mass of deeply

staining chromatic material, the chromosomes being clumped. It measures 0·005 × 0·0027 mm. and is almost in contact with the surface of the ovum which, at this point, has a slight outbulging. It would appear as if the separation of the second polar body had but just been completed. In the latter body, the chromatic material is arranged somewhat more openly, but it is impossible to make out details.

I am unable to find any trace of the sperm nucleus in the disc. The series of sections is quite complete, so that there is a possibility that the ovum has not been fertilized. This is very unlikely since numbers of spermatozoa are to be found in the albumen layer. It is rather more likely, as occasionally happens at this stage, that the male sperm nucleus is indistinguishable from the general mass of the disc.

Nor are there to be found in the disc any accessory sperm nuclei, marginal or otherwise. Examination of this and the next succeeding stage has convinced me that polyspermy does not normally occur in the fertilization processes of Echidna.

Egg No. 2.

This egg was obtained by one of my students (Mr. F. D. Cruickshank, B.Sc.), who was good enough to take charge of the removal of eggs from animals during my temporary absence from Hobart. The egg was not measured fresh, but in its swollen condition after fixation had a diameter of 4·6 mm. Its diameter after removal of the envelopes was 4 mm.

Sections of the disc show that development is at a stage not before described for any monotreme, that of conjugation of the germ nuclei.

Structure of the Egg.—The shell membrane (fig. 2, *sh.*, Pl. 3) is thin, being on the average about 0·003 mm. in thickness. Thin as it is, it has gained much in strength and in consistency since the last stage. It is much more definitely contoured and, being firmer, is more brittle, sometimes causing considerable trouble in the process of sectioning. Owing to alterations brought about during fixation, the thickness of the albumen layer (*alb.*) varies greatly, but in places is reduced to 0·0075 mm.

The germinal disc.—Viewed superficially the disc is no longer circular but is elliptical, with a long diameter of 0·49 mm. and a short one of 0·37 mm. It therefore occupies a surface area less than that of the disc of the stage previously described. This is to be attributed, no doubt, partly to individual variation, partly to alterations in the distribution of the substance of the disc itself. The loss of surface area is associated with an increase in the depth of the disc, which now measures in thickness in the centre 0·05 mm.

The disc (fig. 2, *g.d.*, Pl. 3), in section, is definitely biconvex in shape, the centre being its deepest portion. Its upper surface fits against the zona (*z.p.*) while the lower one is unevenly edged, passing over somewhat abruptly into the much vacuolated nucleus of Pander (*nu.P.*) below. The somewhat profound alterations which have taken place in the shape and size of the disc call to mind once again the changes which take place in the germinal disc of the pigeon's ovum as described by Harper (1904).

The substance of the disc is finely granular and is quite homogeneous except at the margins, where there is some slight penetration by yolk granules. At one end of the disc, however, that to which the pronuclei are nearer, the upper portion of the disc contains a well-marked and distinctive layer of yellow yolk-spheres, or, in other words, the end of the disc is here buried beneath a layer of such spheres. Such a phenomenon occurs in the pigeon, as can be seen by referring to Harper's paper (1904, figs. 12 and 13). As will be seen later, the difference in the composition of the two ends of the disc, together with the position of the germ nuclei and of the polar bodies, gives a definite and unmistakable bilateral symmetry to the undivided germinal disc.

In sectioning, an endeavour was made to keep parallel to the long axis, with the result that the conjugating pronuclei and the polar bodies are in the same sections. Altogether there are 75 sections in the series. The pronuclei are to be found mainly in sections 36 to 38, so that their position is quite median, but they are situated nearer one end of the disc than the other. They are placed one-third the distance from that end of

the disc which is superficially invaded by the yellow yolk-spheres.

There is, therefore, a definite bilateral symmetry and, in shape and size, the disc approximates closely to that of Ornithorhynchus at an early stage of segmentation. Such a stage has been described and drawn by Wilson and Hill (1907, Text-figs. 1 and 2). Their drawings show an elliptical disc divided into eight cells arranged symmetrically, four on each of a median axis which obviously corresponds to the long axis of the unsegmented disc of Echidna. The eight-celled stage of Ornithorhynchus would appear to measure approximately $0{\cdot}6 \times 0{\cdot}5$ mm. It is, therefore, comparable in size with the disc of Echidna.

From these considerations, supported by conclusions to be drawn from the situation of the pronuclei, it is reasonable to suppose that the first segmentation division in the ovum of Echidna takes place along the longitudinal axis, dividing the disc into two equal areas. Unless, however, there is a decided rearrangement of the nuclear material of the disc, it is obvious that the succeeding division must be unequal. This point will have to be settled on a subsequent occasion by reference to early segmentation stages.

The Pronuclei.—These are in the stage of conjugation and of partial fusion. Each is in the shape of a flattened bean-shaped body, the two being closely attached along the flattened sides where there is a tendency towards disorganization along the line of union. This line of attachment is tangential to the surface of the disc, so that one of the nuclei is nearer to the surface than the other. The more superficial is larger and paler. It measures $0{\cdot}01 \times 0{\cdot}021$ mm. The second pronucleus, situated more deeply, measures $0{\cdot}007 \times 0{\cdot}019$ mm.

The pronuclei are similar in appearance to those of the pigeon as depicted by Harper in his fig. 12, but the stage of Echidna here considered is somewhat more advanced. Of the two germ nuclei (fig. 5, Pl. 3), the larger is invested by a definite membrane which, however, shows signs of breaking down at each end. In the case of the smaller germ nucleus, the nuclear membrane has largely lost its contour and has

become wrinkled and shrunken. The region of the ooplasm immediately surrounding this nucleus is loosened, contains irregular spaces, stains rather lightly, and in general presents evidence of a distinct loss of nuclear fluid through the disorganized membrane.

There are appearances under the high power which suggest the emission, from both nuclei, of chromidia in the way described for the mouse and the rat (Kremer, 1924, figs. 12 and 13, 21 and 22), but of this one cannot be certain.

In the large pronucleus the chromatin takes the form of a loose network beset with rather coarse granules. The chromatin of the small pronucleus has the same arrangement but, owing to the loss of nuclear fluid which has taken place, the whole nucleus stains darkly.

Polar Bodies.—These are two in number, the first body remaining undivided. They are situated at the centre of one end of the disc, the end farthest from the pronuclei. As in the last stage they are of unequal sizes, the larger measuring $0{\cdot}017 \times 0{\cdot}0093$ mm. and occupying three consecutive sections of 5 micra each while the smaller measures $0{\cdot}0095 \times 0{\cdot}0075$ mm. and is found in two consecutive sections. Each is somewhat flattened and consists of material of similar composition to the plasm of the disc, with an ill-defined nucleus.

There is no trace, in any part of the disc, of accessory sperm nuclei or of the results of their division.

Summary of Conclusions.

1. In this communication is described for the first time the unsegmented germinal disc of the monotreme Echidna (Tachyglossus), and measurements are also given of living intra-uterine eggs at early stages.

2. Segmentation is not initiated in Echidna until the egg has arrived in the uterus.

3. The polar bodies are much smaller than those described for Ornithorhynchus. The first is much larger than the second but, contrary to what has been reported to occur in Ornithorhynchus, remains undivided.

4. The germ nuclei, immediately before the formation of the first cleavage spindle, undergo conjugation and fusion.

5. Contrary to what is described for Ornithorhynchus, polyspermy does not normally occur in the fertilization of the ovum of Echidna.

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Explanation of Plate 8.

Lettering.

alb., albumen; *g.d.*, germinal disc; *g.n.*♀, female germ nucleus; *lat.*, latebra; *nu.P.*, nucleus of Pander; *pb* 1, first polar body; *pb* 2, second polar body; *sh.*, shell; *w.y.*, white yolk; *y.y.*, yellow yolk; *z.p.*, zona pellucida.

Fig. 1.—A vertical median section through the germinal disc and adjoining parts in Egg No. 1. The second polar body is to be seen at the

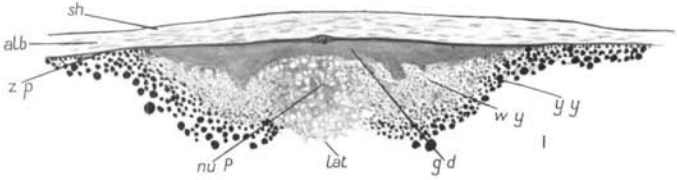
centre of the surface of the disc while the female germ-nucleus is included in the disc just below and to the right of the polar body. × 40 (approx.).

Fig. 2.—A similar section through Egg No. 2. At the extreme margin of the disc (at the right side in the figure) can be seen the minute polar bodies while, within the disc towards the other end are the conjugating germ nuclei. × 40 (approx.).

Fig. 3.—A drawing composed from five consecutive sections showing the female germ-nucleus and the polar bodies of Egg No. 1. In this and the next figure *coag.*, coagulated material between the zona pellucida and the surface of the ovum. × 400 (approx.).

Fig. 4.—The two polar bodies of Egg No. 2. × 400 (approx.).

Fig. 5.—The conjugating germ-nuclei of Egg No. 2. × 400 (approx.).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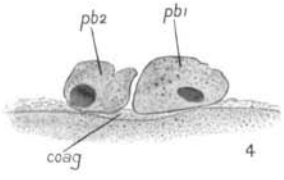
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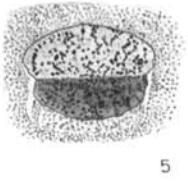
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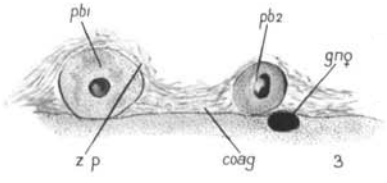
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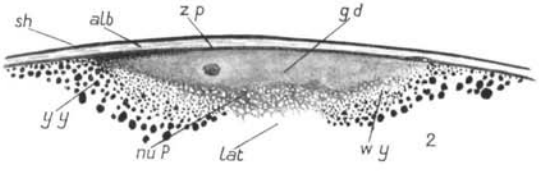
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T. T. Flynn, del.