The Fertilization of Onoclea¹.

BY

WALTER R. SHAW.

With Plate XIX.

THE details of the process by which the sexual cells and their nuclei unite have not been exhaustively studied in very many plants. Most of the contributions to the subject are the results of investigations into the development during extended periods of the plants under observation, so that necessarily the particular subject of cell-conjugation and nuclear fusion has received proportionally less time and attention. This is more especially true with regard to the Characeae, the Bryophytes, and the Pteridophytes.

LITERATURE.

The accounts of fertilization in plants all indicate that the process consists in the fusion of two nuclei in the resting condition. This has been described by Wager² for *Cystopus*, by Harper³ for *Sphaerotheca* and *Erysiphe*, by Oltmanns⁴ for *Vaucheria*, by Klebahn⁵ for *Oedogonium*, and by Farmer and

¹ Prepared under the direction of Professor Douglas H. Campbell in the Botanical Laboratory of Leland Stanford Junior University.

² Wager '96, p. 331.

³ Harper '96, pp. 656 and 659.

⁴ Oltmanns '95, p. 401.

⁵ Klebahn '92, p. 252.

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Williams¹ and by Strasburger² for *Fucus*. The approaching sexual nuclei and the resulting spore-nucleus have been described by Klebahn³ for *Closterium* and *Cosmarium* and for *Rhopalodia*⁴, and by Fairchild⁵ for *Basidiobolus*. In *Vaucheria* the sperm-nucleus becomes nearly like the egg-nucleus before the two come in contact. In *Oedogonium* the chromatin granules of the sperm-nucleus are larger than those of the egg-nucleus at the time when fusion begins. In *Fucus* the sperm-nucleus becomes closely appressed to the egg-nucleus before the granular structure of the sperm-chromatin appears. From Strasburger's⁶ work on the Gymnosperms it is evident that the sexual nuclei in these plants are alike and nearly equal at the time of fusion, and in this respect the sexual nuclei of *Lilium Martagon* are similar, according to the account of Guignard⁷. Mottier⁸ has recently found that, contrary to the description by Guignard of *L. Martagon*, the constituents of the male and female nuclei of *Lilium candidum* cannot be distinguished in the nucleus formed by the fusion.

The general character of the sexual organs and the sexual cells of the Ferns is too well known to need reviewing here. The way in which the sexual organs open and the multiciliate spermatozoids make their way from the antheridium to the archegonium is described in many text-books⁹. It is with the development and structure of the spermatozoid with reference to its constituents and their distribution and activities that investigation has lately been mainly concerned. Guignard¹⁰ had in 1889 concluded that the body of the spermatozoid is formed from cytoplasmic as well as nuclear substance: and, according to Strasburger¹¹, Belajeff had in the same year advanced so far as to describe the spermatozoid

¹ Farmer and Williams '96, p. 482.

² Strasburger '97, p. 351.

³ Klebahn '91, p. 440.

⁴ Klebahn '96, p. 639.

⁵ Fairchild '97, p. 292.

⁶ Strasburger '78, pp. 50-51; see also Vines '95, p. 472.

⁷ Guignard '91, p. 198; see also Wilson '96, p. 161.

⁸ Mottier '97, p. 149.

⁹ See Strasburger and Hillhouse '89, pp. 292-295; also Atkinson '94, p. 20.

¹⁰ Guignard '89, p. 379.

¹¹ Strasburger '92, p. 105.

as consisting of a chromatic body formed from the nucleus of the mother-cell, and an achromatic band formed from the cytoplasm of the mother-cell. Strasburger himself described in detail the development of the spermatozoid of *Osmunda*¹, and the mature spermatozoid of *Phegopteris Giesbrechtii*² as it appeared when stained with fuchsin-iodine green. He found the two anterior coils to stain red, and these he considered to be of cytoplasmic origin; while the posterior coil took the blue colour characteristic of the nucleus. The cilia he found distributed along the forward coil some distance from the end, and the middle coil, he suggested, might have contained the centrosome, if there had been one. At present³ he is inclined to think that there is no individualized centrosome in the spermatozoid of Pteridophytes. He found by treating the spermatozoid of *Marsilia*⁴ with the same stain and allowing it to fade, that only the hinder and larger of the ten or twelve coils contain the nucleus, which extends forward to the region where the cilia are attached. In following the development of the spermatozoid of *Onoclea*, Campbell⁵ was unable to find that so large a part of the forward end is of cytoplasmic origin as Strasburger⁶ described for *Osmunda* and *Phegopteris*. 'The body of the free spermatozoid,' the former writes, 'has the form of a flattened band with thickened edges, which tapers to a fine point at the anterior end, but is broader and blunter behind⁷.' This description fits the living spermatozoid as it is to be seen when caught in the slime about the open archegonia.

The principal accounts of the spermatozoid after its entrance into the egg are those of Campbell. In studying the development of *Pilularia*⁸ he found the spermatozoid within the egg close to the egg-nucleus. It had become a spherical granular nucleus darker than the egg-nucleus and with little more than

¹ Strasburger '92, p. 114.

² l. c., p. 116.

³ Id. '97, p. 420.

⁴ Id. '92, p. 122.

⁵ Campbell '95, p. 312.

⁶ Strasburger '92, pp. 115-116.

⁷ Campbell '95, p. 313.

⁸ Id. '88, p. 249; '95, p. 405; for fig. cf. also Wilson '96, p. 106.

half the diameter of the latter. In following the development of *Osmunda*¹ he found that the spermatozoid loses its pyramidally coiled form after it comes in contact with the egg-nucleus and before the two fuse. In his work with *Marattia*² he found the spirally coiled spermatozoid in contact with the egg-nucleus. In a later stage he found two nuclei present which seemed to be the male and female nuclei. They were not very different in size or appearance. He concluded that the female nucleus had become smaller, and that the spermatozoid had changed into a similar nucleus. 'The two nuclei,' he writes, 'then gradually fuse, but all the different stages could not be traced. Before the first division takes place, however, but one nucleus can be seen, and this nucleus resembles the nucleus of the unfertilized egg.' From these accounts it has been concluded that the spermatozoid of the Ferns enters the egg in the condition in which it swims free, and then, on coming near to or in contact with the egg-nucleus, it undergoes a change in structure which makes it more like the latter and also like the resting nucleus in the sperm-cell of the antheridium. In this respect the details of fertilization are much alike in many plants, and the behaviour of the animal spermatozoön is strikingly similar. This is shown by Van Beneden's account of fertilization of *Ascaris*³, in which the spermatozoön, after entering the egg, rapidly changes and forms a typical nucleus exactly similar to the egg-nucleus.

PRELIMINARY INVESTIGATION.

The differences in size, structure, and habits between the male and the female gametes are greatest in those plants in which the condition of the gametes during fertilization has been least studied: viz. the Archegoniatae and the Characeae. In the fall of 1895 the writer made some microtome-sections

¹ Campbell '92, p. 70; '95, p. 348.

² Id. '94, p. 9; '95, p. 261.

³ Wilson '96, p. 133 (refers to Van Beneden 1883-87-88).

of the oogonium of *Chara* with a view to finding the spermatozoid on its way to the egg-nucleus. In the species which were studied, no sign could be found to indicate at what stage the spermatozoid entered the oogonium, and no spermatozoids were found in the sections. At that time Dr. D. H. Campbell suggested that some of the Ferns would be better subjects for following the stages which were sought, and he kindly supplied spores of *Marsilia vestita* and also another species of *Marsilia* (*M. Drummondii*?) from Australia and *Onoclea sensibilis*.

When the spores of *Marsilia* are sown in water, the prothallia become fully developed in about fifteen hours¹ at ordinary temperatures; at the end of that time the spermatozoids are set free, and make their way to the female prothallium and through the large mucilaginous funnel to the archegonium, where they sometimes arrive before that organ is open. The first division of the egg takes place within about an hour² after the entrance of the spermatozoid into the archegonium. It was found difficult to mark the exact time at which the spermatozoid enters. While endeavouring to become sufficiently familiar with the plant to overcome this difficulty the writer found that in some cases the oospores of the Australian *Marsilia* developed into embryos without any evidence of the presence of spermatozoids about the archegonia. The spores had been sown about ten o'clock at night, and were first examined about half-past eight the next morning. No spermatozoids were seen during that morning, and there were no remains of spermatozoids about the mucilaginous funnel in front of the archegonium. The prothallia were kept, however, and after a few days they were found to bear embryos of considerable size. This suggested the idea that the embryos might have been developed without fertilization, and a short series of experiments³ was made with isolated macrospores for the purpose of testing the matter, and indicated that such was

¹ Campbell '95, p. 405.

² l. c., p. 407.

³ Shaw '97, p. 114.

the case. In the light of this result the writer's experience with *Chara* affords ground for questioning whether parthenogenesis in that genus is confined to the dioecious species, *C. crinita*.

The spores of *Onoclea sensibilis* had been brought by Dr. Campbell from Massachusetts. They were sown in the laboratory, some in September, 1895, and others in January, 1896. When the earlier culture was examined in January, some of the female prothallia were found to have formed mature archegonia, but when they were placed in water most of the archegonia were slow to open. Spermatozoids were set free in a few minutes after male prothallia were placed in water. On February 26 spermatozoids crowded into and about the open archegonia within eight minutes after the prothallia were placed in distilled water. In *Marsilia* the first division of the embryo takes place within an hour after fertilization, and in *Pilularia* after about three hours¹. Dr. Campbell, by his extensive studies of Fern embryology, was led to believe that the first division of the egg of *Onoclea* occurred about twenty-four hours after fertilization. It was desired to obtain a series of prothallia which would represent as completely as possible the period intervening between the entrance of the spermatozoid into the archegonium and the first division of the egg, for it was the intention at the outset to describe the nuclear and cytoplasmic structures and changes within the egg during that period. On February 26 and the two following days, prothallia were left on the surface of distilled water for a variety of periods of from about ten minutes to fourteen hours and then fixed. On March 4 a similar series was made, which extended the period to twenty-four hours. From the cultures sown in the middle of January, prothallia were used on March 23 to extend the period to thirty hours, and on April 23 to seventy-two hours. Prothallia from each series were sectioned, but in none of them were any embryos or signs of

¹ Campbell '95, p. 407.

division of the egg found. Finally, a double series was begun on May 11 by flooding some of the prothallia in the saucers in which they had grown, and by transferring others from the soil to the surface of distilled water, as had been done with the specimens of all the previous series. Those left on the soil were flooded for a time sufficient for the impregnation of all the mature eggs and then drained. Specimens from the soil were fixed at the end of every twenty-four hours for fifteen days, and from the water at the same times for the first seven days. It was thought, naturally enough, that seven days would be more than enough for the development of embryos, and it was intended that this double series should show whether the growth of the embryos was prevented or retarded by removing the prothallia from the surface of the soil and leaving them on the surface of distilled water. The material was preserved in alcohol, and when sections were made, in the following September, no embryos were found in the prothallia fixed at the end of seven days, and the writer made the mistake of not sectioning a large number of those from the fifteen-day period.

Dr. Campbell then obtained from Michigan spores of *O. Struthiopteris*, and several cultures were started, some in the saucers used in the preceding season, some in a box on soil already in the laboratory, and some in a similar box on a fresh supply of black soil, a mixture of leaf mould and adobe. The plants in the saucers were not well lighted, and for this and other reasons they did not grow so rapidly or uniformly as those in the boxes. Each box was covered with a pane of glass. The spores on the old soil were sown on November 3, and the box was inclined at an angle of 30° before a deep south window about 60 cm. distant. Those on the fresh soil were sown on November 17, and placed in the same way before an east window. The tilted position of the boxes secured a better illumination, and kept the surface of the soil well drained. It also prevented the water which condensed on the inside of the glass cover at night from dripping on the mature prothallia and thus fertilizing

the ripe archegonia. Many of the plants of the culture of November 3 were ready for fertilization in less than two months. The soil of this culture was poorly mixed, so that the plants on different parts of its surface did not develop uniformly, but they were quite capable of producing embryos.

The first series (No. I) of *O. Struthiopteris* was begun December 29, 1896. Three lots were fertilized in as many ways: lot 1 included prothallia which were removed from the soil, fertilized under the microscope, and replaced on moist soil in watch-glasses; in lot 2 were plants which were removed from the box with soil attached to their root-hairs, and fertilized by flooding them in watch-glasses, where they were drained and left; lot 3 was fertilized by flooding the prothallia on the soil. Others were treated like the first lot, except that they were not passed under the microscope. Ten specimens from lot 2 were placed in water under the microscope eight days afterwards, and archegonia opened on only two of them. This indicated that the formation of mature archegonia had ceased on most of the prothallia of this lot, probably because the fertilization was effective. Plants from different lots were fixed on each successive day for ten days. Specimens representing the whole series were sectioned, but no embryos were found.

On February 15, 1897, the culture of November 17, 1896, was well advanced. The prothallia were the greenest and most regularly formed of all that had been raised. The box was placed in a horizontal position, and filled for about fifteen minutes with water enough to flood half of the culture. The box was then drained and returned to its original position so as to minimize the geotropic disturbance. A series (No. III) was then taken from the box after varying intervals up to nine days and twenty-three and a half hours. When the oldest of these were sectioned, one egg with the nucleus in the anaphase of the first division, and another with the first division complete, were found. In the next younger lot of this series, which was six days and nineteen hours old, no dividing egg or embryo was found. With this clue to

the fact that the first division of the egg takes place nine or ten days after fertilization, more of the older stages from Series I were sectioned and the following young embryos were found:—

Embryos of *Onoclea Struthiopteris* from Series I.

Fertilized 9 days, 3 embryos of 2 cells.

		{	2	„	2	„
„	IO	„	2	„	4	„
			I	„	8	„

On March 18 both box cultures were flooded (one had been half-flooded before), and from each specimens were fixed daily to form a series, one covering fifteen and the other twenty days. With thermometers, which hung near the cultures, the temperature of the room was noted each day when plants were fixed, and recorded with the maximum and minimum temperatures for the preceding twenty-four hours. It ranged from 10° C. to 25° C. The material yielded the following:—

Embryos of *Onoclea Struthiopteris* from Series IV and V.

					2 embryos of 4 cells.
		{	1	”	6 ”
Fertilized 10 days			{	1	” 15 ”
				2	” 16 ”
				1	” 17 ”
” 12 ”				1	” 16 ”

The results were enough to satisfy the writer that, under favourable conditions in the laboratory, the egg of *Onoclea Struthiopteris* is not likely to divide sooner than a week after fertilization; and they indicated that uprooting, handling, and replanting the prothallia did not retard the formation of the embryo.

After the embryos of *O. Struthiopteris* had been found, attention was again turned to the longest series of prothallia which had been killed in 1896, and by sectioning a large number of prothallia the following were obtained:—

Embryos of *Onoclea sensibilis* from Series G.

Fertilized 8 days, 1 embryo of 2 cells.

„	10	„	2	„	2	„
			{ 2	„	4	„
„	12	„	{ 3	„	8	„
			{ 3	„	16	„
„	15	„	{ 1	„	32	„
			{ 1	„	130 μ long.	

This showed clearly that we should not expect to find the first division of the egg in these prothallia less than a week after fertilization, and, conversely, that the absence of embryos in the shorter series which had been prepared does not by itself indicate that they were abnormal. It was unfortunate that the specimens kept on the surface of the water did not cover a period longer than seven days. This failure was repaired as far as possible by fixing such a series from the cultures of the second species studied, but these plants were killed too late to be available for present purposes. It is also regretted that the long series of *O. sensibilis* was obtained after the prothallia had grown to a large size, and bore such large numbers of archegonia as to make the study of fertilization in these plants a more complex problem; for at some certain time after reaching maturity the egg, if unfertilized, becomes atrophied to such an extent as to render normal fertilization impossible. Yet spermatozoids enter such eggs, and it has been suggested that their presence may then have some retarding influence on the development of the normally fertilized eggs on the same prothallium. On account of the long period occupied by the stages which it was proposed to follow, these Ferns are less suitable for the research than was expected.

METHODS.

The cultures of *Onoclea sensibilis* were obtained by sowing spores on sterilized soil in earthenware saucers. Cultures

started in September were ready for fertilization-experiments in the following February, and those started in January were sufficiently mature for the same purpose two months after the spores were sown. A series of trials showed that when female prothallia bearing mature archegonia were placed in water, the archegonia opened within five minutes, and when male prothallia were present the open archegonia were all entered by spermatozoids within three minutes. It was found convenient to carefully transfer large numbers of prothallia from the soil to the surface of water in watch-glasses. A few minutes or an hour later, the prothallia were examined, and those with no open archegonia were rejected. Then, after the lapse of the desired periods, specimens were taken from the watch-glasses and placed in the fixing agent. All of the series used, except Series G, were fertilized in the manner described. The plants of the latter series, as already explained (p. 267), were fertilized on the soil and not removed until the time for fixing. Unless otherwise specified the observations refer to *Onoclea sensibilis*. In the following table of the series of prothallia killed, the italics show the material used :—

Sowing of culture.	Series.	Beginning of Series.	Periods between fertilization and fixation.	Chronic acid fixing solution.	Hours in fixing agent.
Sept. '95	B	Feb. 26, '96	10, 20, 30 minutes, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14 hours.	1%	18-24
Sept. '95	C	March 4, '96	6, 8, 10, 12, 14, 24 hours.	1%	24
Sept. '95	D	March 9, '96	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 hours.	1%	6-8
Jan. 10-17, '96	E	March 23, '96	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 hours.	$\frac{1}{2}\%$	8-24
Jan. 10-17, '96	F	April 23, '96	1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 60, 72 hours.	1%	24
Jan. 10-17, '96	G	May 11, '96	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 days.	1%	24

For fixing¹ the prothallia they were placed whole in $\frac{1}{2}\%$ or 1% chromic acid. The best results were obtained by leaving the specimens in $\frac{1}{2}\%$ chromic acid for eight to twenty-four hours. In most cases dilute sulphurous acid was used for removing the last traces of chromic acid.

Most of the material was stained with Czokor's alum-cochineal, in which the prothallia were allowed to remain for eighteen to twenty-four hours. Some of the plants were stained in alum-carmine, and others in diluted Delafield's haematoxylin. Some of the sections of plants which were stained *in toto* in alum-cochineal were counter-stained on the slide with Bismark brown. A few sections were restained on the slide with Flemming's triple stain², safranin-gentian violet-orange G, and a few others with Heidenhain's iron-haematoxylin.

The prothallia were sectioned in paraffin. At first turpentine was used as a medium between alcohol and paraffin, but it was soon discarded, and xylene used according to the method recommended by Zimmermann³ in his handbook. The wings were cut off with a razor before the prothallia were imbedded. The sections were cut 10μ thick on a Minot microtome of the older style. They were cleared with clove oil and mounted in Canada balsam.

INVESTIGATION.

The spermatozoids, which were usually held in large numbers in the slime before the mouth of the archegonium, remained unchanged for a long time, and were favourable objects for study. They were in a position to be acted upon very quickly by the fixing agent, and also by the stain. Those fixed after fourteen, eighteen, and twenty-four hours had the same appearance as those fixed after a few minutes. They had all lost the nutritive vesicle, and become a little

¹ For fixing the series of *O. Struthiopteris* most of the agents given in Zimmermann's Botanical Microtechnique were used.

² Zimmermann '93, p. 186.

³ l. c., pp. 32-33.

drawn out on entering the slime. With all the stains used the darkest part of the body is a corkscrew-shaped homogeneous rod, oval in cross-section, which tapers gradually forward and abruptly backward. It does not terminate in a sharp point at either end. In all the specimens which were noted closely the spiral turns of the body are in the direction of a left-hand screw. Webber¹ finds that the helicoid ciliated band in the spermatozoid of *Zamia* is coiled in the same direction. The number of turns varies with the length to which the corkscrew is extended: when shortened, like a watch-spring, it makes two turns (Fig. 10 *b*); just outside the archegonium it usually makes about three and a half turns (Fig. 5); in the canal, when it becomes most extended, it may make five turns (Fig. 1). In all the forms an almost stainless band can be seen attached to the forward edge of the corkscrew, and extending beyond its forward point. This band is broadest toward the forward end, and it tapers backward to the thickest part of the corkscrew rod. The outer edge of the band appears to be thicker than the rest, and the thickening is greatest near the forward end. The dark rod is assumed to be the nucleus, and the wing-like band the cytoplasmic portion of the spermatozoid. The cilia did not usually stain enough to be visible; but when Heidenhain's iron-haematoxylin was left rather dark they could be seen distinctly enough to show that some extended forward and others backward. They could not be traced to their respective points of attachment, and so they were omitted from the drawing (Fig. 8) which was made from such a specimen. No centrosome was seen.

The nucleus of the spermatozoid has the same form as that figured by Campbell² for the developing spermatozoid of *O. Struthiopteris*. It extends through the greater part of the length of the body. In this respect it differs from the nucleus which Strasburger³ found in the spermatozoid of *Phegopteris*. In the latter it occupied only the larger pos-

¹ Webber '97, p. 17.

² Campbell '95, p. 131.

³ Strasburger '92, p. 116.

terior turn of the corkscrew. The spermatozoid of *O. sensibilis* is like that of *Chara*, as described by Belajeff¹, in having the cytoplasm extending along the nucleus. The thickened outer edge of the cytoplasmic band may correspond to the 'Rückfäden' which Belajeff was able to distinguish in the forward cytoplasm of the spermatozoid of *Chara* until a late stage in its development.

Long before the archegonium opens the egg-nucleus comes to the resting condition, and contains one or more nucleoli. The ventral canal-cell is the smallest cell in the archegonium. After it is formed, the pressure of the egg makes it still smaller and concave, and its nucleus becomes flattened. In later stages the walls of the ventral canal-cell swell up, and by pressure cause the egg to become concave on the outer side, which later forms the receptive spot. In these stages the egg-nucleus also is flattened and concave. The egg is in this condition when the archegonium opens. In the living sections, under the microscope, the writer observed the egg to swell as soon as the canal was cleared of its dissolving contents, and fill up the venter. If many spermatozooids were near, they swarmed into the canal, and a large number made their way into the venter, where they swarmed about freely, quite differently from those in the close quarters of the neck, which were motionless or moved slowly. Often spermatozooids which had entered the venter found the canal again, and made their way out, slowly through the narrower portion of the canal, but rapidly in the wider part. On first entering the slime discharged by the archegonium, the spermatozooids left their trophoplasmic vesicles behind, and their motion was retarded. It was the resistance of the slime which pulled off the vesicles. In the canal the slimy mucilage seemed to be denser, and when a spermatozoid entered it, the corkscrew spiral became drawn out, and the number of turns increased, and the forward motion of the spermatozoid was accompanied by a rotation which corresponded to the pitch of the screw.

¹ Belajeff '94, p. 43.

All the sections of prothallia that were killed within an hour after the entrance of the spermatozoids into the archegonia, show the eggs in a collapsed condition (Figs. 1 and 2), concave on the outer side, and the nucleus in each conforms to the shape of the cytoplasm. In this state the shape of the egg is about the same as it is in the unopened archegonium after the canal-cells have swelled. The return to this form may have been due to the action of the fixing or imbedding agents, the egg in this stage being more susceptible to their shrinking influence, either because it is not at this time in the state of tension which it acquires later, or because the open canal permits the rapid access of the plasmolizing agents. There are reasons to believe, however, that the collapse is not an artificial plasmolysis, but that it takes place as soon as the spermatozoid enters the egg. The mature egg has been described (for the other species) as having a large hyaline receptive spot¹. The concavity of the collapsed egg occupies the position of that spot. That it was formed before the plants were killed seems evident from the movement of a number of spermatozoids in the venter. This can be seen in the living plants. That the number of these spermatozoids is large is shown by the specimens stained and sectioned. They can hardly have been carried into the venter by the fixing agent, for those in the canal were fixed first, in the extended condition, and those in the venter afterward, in the contracted form. From the evidence at hand it appears that as soon as the egg is entered by a spermatozoid it loses its turgidity, and the spermatozoids which come into the venter afterward meet with little or no resistance from the egg. It may be that the turgid condition of the egg, in the first place, offers mechanical facility for the screw-like spermatozoid coming through the narrow base of the neck to force itself into the cytoplasm of the receptive spot; and that the plasmolytic condition of the egg afterward deprives the following spermatozoids of this advantage, and protects

¹ Campbell '95, Fig. 159.

the egg from injury or from multiple fertilization by them. A few careful experiments in fixing archegonia before and after the first spermatozoid enters the egg ought to reveal the truth of the matter.

Within half an hour after the entrance of the spermatozooids into the archegonium the canal is practically closed by the expansion of the four proximal neck-cells and the four just beyond them. The egg gradually recovers its turgidity and forces the free spermatozooids against the outer wall of the venter (Fig. 3). The membrane which, from analogy with the development of the eggs in other plants, we would expect to form around the egg immediately after the entrance of the spermatozooids, was not seen in any of the earlier stages. If the membrane is of the nature of cellulose it ought to be brought out distinctly by the Bismark-brown with which the subject of Fig. 1 was stained on the slide. This stain colours even the cytoplasm in this case. In all the preparations which were examined there was no evidence that a membrane of appreciable thickness is formed immediately after the entrance of the spermatozoid or for some time afterward. A conclusion so contrary to analogy must remain in doubt. It may be that the chromic acid used for too long a time destroyed the membrane.

The early history of the spermatozoid inside the egg was not satisfactorily followed. After the ten- and twenty-minute periods the collapsed state of the egg interfered with the study of the enclosed spermatozoid, and the stains used for these stages were not the best. The difficulty was increased by the free spermatozooids crowding into the concavity of the egg. So the mode of entrance of the spermatozoid into the egg cannot now be described. In one case the spermatozoid appeared to be still outside the egg-nucleus, against which it lay in an open coil after an hour, but in other cases it was found inside the egg-nucleus within thirty minutes. The nucleus of the spermatozoid undergoes no visible change in structure while in the egg-cytoplasm. Whether the cytoplasmic wing and the cilia of the spermatozoid are taken

into the egg-nucleus could not be shown with the faint cytoplasmic stains used at these stages. We may reasonably expect to find that they are left outside in the egg-cytoplasm, but it is a question which will require to be settled by actual observation. In Figs. 3 and 11, which were drawn before the structure of the free spermatozoid was understood, there is something in the egg-cytoplasm on the outer side of the egg-nucleus which strongly suggests by its general appearance that it is the remains of the sperm-cytoplasm, accompanied in the case of Fig. 3 by the cilia. The writer has lately observed, in a specimen stained with Heidenhain's haematoxylin, something that looks like a loose bunch of cilia in the same position; and in some horizontal sections the outer part of the egg-cytoplasm, when seen from the outer side, shows radiations which have a spiral twist, such as the cilia of the spermatozoid sometimes show when that body is viewed from in front¹.

The egg-nucleus at the beginning of fertilization, and all through the process, is in the typical resting condition. The nucleoli, for there are generally several, are the most conspicuous structures in the stained sections. They vary in size and present a peculiar porous structure. The linin appears as a delicate network which bears the very small chromatin bodies. This was not demonstrated for all stages, but the general appearance of the female nucleus is the same throughout the process, and in later stages some well-stained examples made it possible to observe the linin network and chromatin bodies minutely (Fig. 12). Nothing was seen to indicate that the whole nuclear 'membrane,' if it may be called such, was dissolved. There were indications that it dissolves or is ruptured at the place where the sperm-nucleus enters. The most remarkable fact observed, and one about which there is no doubt, is that the sperm-nucleus enters the egg-nucleus before it changes in form or visible structure. This is clearly shown in the section represented

¹ Webber has shown ('97, 2, p. 227) that in the fertilization of *Zamia* the cilia are left behind soon after the entrance of the spermatozoid into the egg-cell.

in Fig. 1 and sections of the other eggs on the same slide. In Fig. 1 the small end of the spermatozoid is directed toward the base of the archegonium. The lower portions of two coils were cut off by the razor, and are to be seen in the next section on the slide. The sperm-nucleus within the egg-nucleus becomes granular and the granules slowly separate. Thirty minutes after the entrance of the first spermatozoid into the archegonium the sperm-nucleus may show traces of the granular structure, as in Fig. 2, but usually it is not evident until two hours have elapsed. It shows plainly in Fig. 3, from a specimen fixed after three hours. Fig. 4, after twenty-four hours, shows little, if any, advance in this respect. Fig. 6, after twenty-four hours, shows the sperm-chromatin distributed in one quarter of the egg-nucleus very much as it was found in Fig. 9 after sixty hours. In most of the preparations the chromatin of the two nuclei can be seen distinctly, but in only a few cases was the linin also clearly distinguished. Among the best of these is one represented in Fig. 12, which was fixed in $\frac{1}{2}\%$ chromic acid for twelve hours, washed two days in water, one and a half days in dilute sulphurous acid, and three hours in water, and then stained with alum-carmin. The linin threads connecting and supporting the chromatin granules of the female nucleus are especially distinct, and had one the time one might almost construct a complete map of the network system. In the sperm-nucleus the granules and the threads are so closely packed that the courses of the threads cannot be followed in detail. The sperm-nucleus often retains the spiral arrangement of its substance for a long time, as in Fig. 13, after twelve hours. It may lose this arrangement early, as in Fig. 7, after fourteen hours. Here the larger and smaller end can still be identified. In the latest stages in which the sperm-substance could be recognized it had become distributed through a larger part of the female nucleus (Fig. 9).

The different eggs fertilized on one prothallium at the same time do not have the sperm-nucleus in the same

condition. This makes it impossible to say without further study what is the rate of normal nuclear fusion. Among the few early stages of *O. Struthiopteris* that were sectioned and successfully stained, one after two days showed the male nucleus but little further advanced than that of Fig. 14, which was killed after thirty-six hours. Another egg of that species contained, after three days, a nucleus which had slightly enlarged but contained nothing that could be identified as male chromatin, although there were outside of this egg the crowded remains of free spermatozooids which must have entered the venter when the canal was open and the egg in a receptive condition. So it appears that after three days the nuclear fusion may be complete.

The cytoplasm of the egg becomes vacuolated as the cell becomes turgid, and may after a time be pretty evenly distributed around the lumen (Fig. 11); or it may be denser on two sides of the nucleus (Fig. 4). In the material stained with alum-cochineal and Delafield's haematoxylin, the cytoplasm appeared to be composed mainly of spherical bodies. But in the beautiful alum-carmin preparations the cytoplasm, although very slightly stained, showed, with the most favourable illumination, a reticular or alveolar structure resembling that of the nuclear network, but with larger meshes. This was not exhibited distinctly enough to be represented in the drawing. No centrosome or radiations, such as are formed about one, were seen in the egg-cytoplasm.

In most of the eggs that are fertilized the protoplasm decreases in quantity after two or three days, and retreats to the inner side of the venter with the nucleus, which becomes smaller. Whether any such as these afterward divide is not known. A large proportion of the fertilized eggs never divide. It might be supposed that the rather peculiar treatment of the prothallia in these experiments was responsible not only for an increase in the proportion of sterile eggs, but also for a wider variation in the rate of nuclear fusion. Opposed to such a supposition is the fact that many of the prothallia left on water showed more

nuclei in various stages of mitosis than those which were not disturbed until they were fixed.

In an early stage of the segmentation of the egg, an exception was found to the order of the divisions usually described¹. This was in an embryo in which the epibasal octants had already formed, but the hypobasal quadrants were separated by a median instead of a transverse wall. On one side of this two octants had formed, and on the other the nucleus was in the metaphase of division. In other cases the quadrant walls were more or less oblique.

The absence of radiations in the cytoplasm of the egg during fertilization is a character in which the Fern resembles the lower plants in which the process has been described. Strasburger's suggestion² that their absence in *Fucus*, where they occur during mitosis, may be due to the fact that the cell division does not immediately follow fertilization, will be equally applicable to this Fern if the radiations can be found during mitosis. We were led to expect from the accounts of fertilization in other plants that the sperm-nucleus would become more or less like the egg-nucleus before the two united. The entrance of the unchanged sperm-nucleus into the egg-nucleus in *Onoclea* is so notably different from what has been said to occur in the eggs of other Ferns, that a further study of these is very desirable. *Marsilia* and *Pilularia*, while presenting some difficulty with regard to regulating and marking the time at which the spermatozoids enter the archegonia, have the great advantage that the nutrition of the eggs is well provided for, and there are not several eggs to contest for the food from one prothallium. It was thought that the disadvantages of working with the *Onoclea* prothallia could be avoided by selecting young ones on which only one archegonium was ripe, but this was not found practicable when a large number were required. The unsuspected fact that the egg did not divide for more than a week after fertilization was the greatest hindrance to the

¹ Campbell '95, p. 316.

² Strasburger '97, p. 419.

completion of this account of fertilization. The quiescent period may be partly or entirely due to imperfect nutrition, and if so it is likely to vary in different closely related species, and in the same species under different conditions. We have found the period to be long in three cultures and two species.

Many of the prothallia used for the present study probably bore, in addition to the eggs fertilized at the recorded times, others which matured later and were then fertilized. This might have been prevented by draining the prothallia, as was done with those which were fixed during the next season. This may be accomplished when the prothallia are removed from the soil to be fertilized, as must be done if they are to be carefully examined, by placing them on moist filter paper in a moist chamber. It will be desirable not only to prevent the fertilization of archegonia which mature later, but also any which have matured earlier than the beginning of the experiment. The most trustworthy account of fertilization will be based on eggs on prothallia on which only one egg is fertilized and that at maturity. Such specimens can be obtained by fertilizing large numbers of prothallia and selecting after ten minutes those on which only one archegonium opens and attracts spermatozoids.

SUMMARY OF RESULTS.

1. The body of the free spermatozoid consists of a long corkscrew-shaped nucleus which stains homogeneously, and a lateral band of cytoplasm which extends a short distance in front of the nucleus.
2. The sperm-nucleus enters the egg-nucleus before it changes in form or visible structure.
3. Within the egg-nucleus the chromatin-granules of the sperm-nucleus slowly separate as the meshes of the linin-network slowly enlarge.
4. Throughout the process of fertilization the female nucleus is in the resting condition.

5. The first division of the egg was in no case found until more than a week after fertilization.

6. It is suggested that the egg becomes plasmolysed as soon as the first spermatozoid enters it, and that this serves as a provision against injury by following spermatozooids.

The work which forms the basis of this paper was done in the Botanical Laboratory of Leland Stanford Junior University, with the kind advice and encouragement of Professor Douglas H. Campbell, for which the writer takes pleasure in here expressing his thanks. The paper was written and most of the figures were drawn during the summer of 1897, in the Hopkins Seaside Laboratory, a branch of the University located at Pacific Grove, California.

A detailed account of the development and structure of the spermatozoid of the Ferns was given by Belajeff¹ before the above paper was written, but it had not then been seen by the writer. In that account Belajeff brought out the fact that there is a specially differentiated body in the cytoplasmic band of the spermatozoid which gives rise to the cilia. He described the development of this body (the 'Nebenkern'), from a small body near the nucleus of the 'spermatozoid mother-cell,' into a thread-shaped body in the mature spermatozoid. The writer² of the present paper subsequently found these 'Nebenkerne' in the antheridia of *Onoclea* before and during the last cell-division by which the so-called 'spermatozoid mother-cells' are formed. But the cilia-bearing portion of the spermatozoid of the Fern, like that of the Cycad as described by Webber³, takes no active part in what may be regarded as the essential process of fertilization, and therefore no extended reference to these works need be appended to the foregoing account of fertilization.

¹ Wl. Belajeff, Three preliminary papers in the *Berichte d. deut. Bot. Gesell.*, 1897, p. 337 ff.

² W. R. Shaw, *Über die Blepharoplasten bei Onoclea und Marsilia*, 1898. Soon to appear.

³ Webber, '97, 2, 227.

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EXPLANATION OF FIGURES IN PLATE XIX.

Illustrating Mr. Shaw's paper on *Onoclea*.

Fig. 1 was drawn with a Leitz objective No. 7, and a Leitz drawing-ocular; all the others were drawn with a Zeiss apochromatic 2.0 mm objective, apert. 1.40; Figs. 5 and 12 with a Zeiss compensation ocular No. 12; the others with a Zeiss compensation ocular No. 8. For sketching the figures (except Fig. 1) the prism section of a Leitz 45° drawing-ocular was screwed to the Zeiss compensation-ocular in place of the upper diaphragm holder.

Magnification determined with a stage micrometer:—Fig. 1 $\times 500$; Fig. 5 $\times 2,000$; all others $\times 1,200$.

All figures are from sections 10 μ thick.

The *arrow* indicates the direction of the growing point of the prothallium.

The *time* given is that which elapsed between the entrance of the first spermatozoids and the fixing of the prothallium.

Onoclea sensibilis.

Fig. 1. One per cent. chromic acid: alum-cochineal and Bismark brown. Vertical section through an open archegonium probably within ten minutes after the entrance of the first spermatozoid. One unchanged spermatozoid is inside the egg-nucleus. Parts of two coils of this spermatozoid were cut off, and appear in the next section of the series.

Fig. 2. One per cent. chromic acid: alum-cochineal. Thirty minutes. Vertical section of the venter of an archegonium containing spermatozoids, and the collapsed egg with a spermatozoid within the nucleus. The canal of this archegonium is almost closed. Two spermatozoids in the section were not drawn.

Fig. 3. One per cent. chromic acid: alum-cochineal. Three hours. Nearly vertical section of the egg. The outside spermatozoids are forced against the venter wall by the expanding egg. Portions of the egg-nucleus, one containing a nucleolus, are in each of the adjoining sections of the series.

Fig. 4. One per cent. chromic acid: safranin and orange G. Twenty-four hours. Vertical section of an egg in which fertilization is not much in advance of the preceding case.

Fig. 5. Half per cent. chromic acid: Delafield's haematoxylin. Eighteen hours. Spermatozoid caught in the slime outside an archegonium showing the kinoplasmic band extending as a wing along the forward side of the two anterior coils of the corkscrew-like nucleus.

Fig. 6. One per cent. chromic acid: safranin, gentian-violet, and orange G. Twenty-four hours. Two vertical sections of an egg. The male chromatin partly distributed in the egg-nucleus. The lightly shaded spermatozoid is in a lower focus.

Fig. 7. Half per cent. chromic acid : Delafield's haematoxylin. Fourteen hours. Horizontal sections of an egg. The large nucleolus is shaded lightly because it occurs in a lower focus than the adjacent male chromatin.

Fig. 8. One per cent. chromic acid : Heidenhain's haematoxylin. Fourteen hours. Two spermatozooids which were held in the slime outside an open archegonium. The cilia could be seen, but were not drawn because they could not be traced to their points of attachment with certainty : some were directed backward, but most of them forward. One nucleus was broken into two and the other into three pieces by the razor.

Fig. 9. One per cent. chromic acid : Heidenhain's haematoxylin. Sixty hours. Egg-nucleus from an oblique horizontal section. The large nucleolus is shaded lightly because it is behind the male nucleus.

Fig. 10. Half per cent. chromic acid : alum-carmin. Twelve hours. Spermatozooids held in the slime outside an archegonium, showing the nucleus and kinoplasm.

Fig. 11. Half per cent. chromic acid : Delafield's haematoxylin. Twelve hours. Vertical section of an egg with the enclosed spermatozoid still showing the coiled spiral form.

Fig. 12. Half per cent. chromic acid : alum-carmin. Twelve hours. Horizontal section of an egg. Both the male and the female nucleus have a linin-network bearing chromatin bodies. The cytoplasm under most favourable illumination shows an alveolar or reticulate structure.

Fig. 13. Half per cent. chromic acid : alum-carmin. Twelve hours. Vertical section through an egg-nucleus. $\times$ marks the direction of the archegonium canal.

Fig. 14. Half per cent. chromic acid : Heidenhain's haematoxylin. Thirty-six hours. Nucleus in a horizontal section. The distribution of the male nucleus is not much further advanced than in Fig. 12 after twelve hours. The lighter large nucleolus is not in the same plane as the end of the spermatozoid.

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