"The Effect of Staleness of the Sexual Cells on the Development of Echinoids." By H. M. VERNON, M.A., M.D., Fellow of Magdalen College, Oxford. Communicated by W. F. R. WELDON, F.R.S. Received June 27, 1899.

The effect of varying degrees of staleness of the ova and sperm of an organism upon subsequent development appears to have been very little studied, though such a condition must obviously be a factor of frequent occurrence under natural conditions. Thus in most of the Cœlentera, Echinoderms, and in some of the worms, it would seem to be a matter of chance whether the ova and spermatozoa come into contact when freshly shed, or only many hours after extrusion. In some mammals also, especially in man, the relative degree of freshness of ovum and spermatozoon at the time of fertilisation is entirely a matter of chance.

The chief connection in which the question of staleness has been hitherto studied is that of polyspermy. Thus O. and R. Hertwig found* that on crossing certain species of Echinoderms, as the ova of *Sphærechinus granularis* with the sperm of *Strongylocentrotus lividus*, more and more of the ova were fertilised up to a certain point if they were kept for an increasing number of hours in sea water, but that after this point they began to undergo polyspermy in an increasing degree, and to develop abnormally. To what precise extent is this tendency present, however, and how is it affected by the staleness of the ova on the one hand, and of the sperm on the other? Also, do the normally developing ova of stale sexual products continue to develop equally well with those from fresh products, or not? Such are the questions it is attempted to answer in this paper.

The method of experiment was very simple. The ovaries and testes of ripe specimens of the Echinoid *Strongylocentrotus lividus* were shaken in jars of water, and portions of the contents of these were mixed, either immediately, or after a given number of hours. The mixed solutions were allowed to stand for an hour, and were then poured into beakers and diluted with about ten times their volume of water. Twenty-four hours later, some of the stirred up contents were introduced into a small glass cell, and a drop of corrosive sublimate solution added to kill the blastulæ and make them sink to the bottom. The numbers of normally developing blastulæ, and of abnormally developing and unsegmented ova were then counted, 300 to 500 being usually enumerated, in order to get an accurate estimate. In all cases the mixed ova from two or more ripe specimens were used, and were

* 'Experimentelle Untersuchungen über die Bedingungen der Bastardbefruchtung.' Jena, 1885.

fertilised by the mixed sperm of two or more specimens, in order to get as average results as possible. In the experiments to be subsequently described, however, in which the stale ova and spermatozoa were mixed several different times at a few hours' interval with fresh sperm and ova, as often as not only one fresh specimen was used in each case.

In the subjoined table are given the results obtained in one of the most complete experiments. In this case parallel series of determinations were made, in which the ova and sperm were kept in, and after fertilisation diluted with, respectively tank water from the Aquarium, and pure water collected several kilometres from the shore of the Bay of Naples.

| Time of fertilisation. | Tank water. | | Pure sea water. | |
|---|---|---|---|---|
| | Per cent. blastulæ. | Per cent. diminution per hour. | Per cent. blastulæ. | Per cent. diminution per hour. |
| Directly .... .... | 98·5 | .. | 96·9 | |
| After 6 hours .... | 95·3 | 0·5 | 95·6 | 0·2 |
| ,, 21 ,, .... | 83·2 | 0·8 | 97·2 | nil |
| ,, 24 ,, .... | 77·9 | 1·8 | 92·7 | 1·5 |
| ,, 27 ,, .... | 73·2 | 1·6 | 66·5 | 8·7 |
| ,, 30½ ,, .... | 55·7 | 5·0 | 0·25 | 18·9 |
| ,, 33 ,, .... | 36·0 | 7·9 | 0·0 | 0·1 |
| ,, 35½ ,, .... | 2·2 | 13·5 | | |
| ,, 46 ,, .... | 0·0 | 0·2 | | |

It will be seen that the ova survived better in the tank water than in the pure sea water, though in two other similar series of experiments the reverse relationship, which one would naturally expect, showed itself. Of the ova fertilised immediately after shedding, one may see that respectively 98·5 and 96·9 per cent. developed to normal blastulæ. On keeping, the ova in the tank water began at first to deteriorate more rapidly than those in the pure sea water, but between the 24th and 27th hours, those in pure sea water suddenly began to fall away, and after 30½ hours, only 0·25 per cent of the ova remained to undergo normal development. The ova kept in tank water, on the other hand, postponed their rapid degeneration till the 27th to the 35½th hours, or more especially till the 33rd to the 35½th hours. In order to show more strikingly the suddenness of the onset of this abnormal development on keeping the ova, another column has been added to each half of the table, giving the percentage diminution of normally developing ova per hour. For instance, after six hours development in tank water, 3·2 per cent. less of the ova developed normally, or, on an

average, 0·5 per cent. per hour for each of the first six hours. This value is put, for convenience, against the "after six hours" line in the table, though it should rightly be placed between the "directly" and "after six hours" lines. The other values are arranged in the same way. We see then, that of the ova developed in tank water, from 0·5 to 1·8 per cent. per hour underwent abnormal development up to the 27th hour, but that then the percentage rapidly increased, till from the 33rd to the 35½th hours, it reached to 13·5 per cent. In the case of the ova kept in pure sea water, the result was still more striking. Thus till the 24th hour only 1·5 per cent. or less per hour developed abnormally, but from the 27th to 30½th hour, no less than 18·9 per cent.

| Time of fertilisation. | Per cent. diminution per hour. | | |
|---|---|---|---|
| | Tank water (1,000,000 per litre). | Tank water (49,000 per litre). | Pure water (680,000 per litre). |
| Directly ..... | (71·6) | (83·9) | (86·0) |
| After 9 hours | 0·7 | 0·7 | 0·2 |
| „ 20 „ | 3·3 | 6·6 | 0·3 |
| „ 24 „ | 5·9 | 1·1 | 13·8 |
| „ 29 „ | 0·5 | 0·05 | 3·5 |
| „ 32½ „ | 0·2 | 0·0 | 1·5 |
| „ 46 „ | 0·06 | .. | 0·2 |

In this next table a similar series of observations is recorded, but in addition a third series of determinations was made, in which ova and sperm were kept in about twenty times as great a volume of water as was used in the other experiments. Thus it was thought that perhaps the keeping together of very large numbers of ova and of spermatozoa in small volumes of water might tend to increase the rapidity of their deterioration. As far as this single result can show, however, just the reverse is the case. Thus when only 49,000 ova per litre were kept together, the maximum rate of deterioration was reached between the 9th and 20th hours, whilst when 1,000,000 per litre (about the usual state of dilution) were kept, the maximum rate was not reached till the 20th to 24th hours.

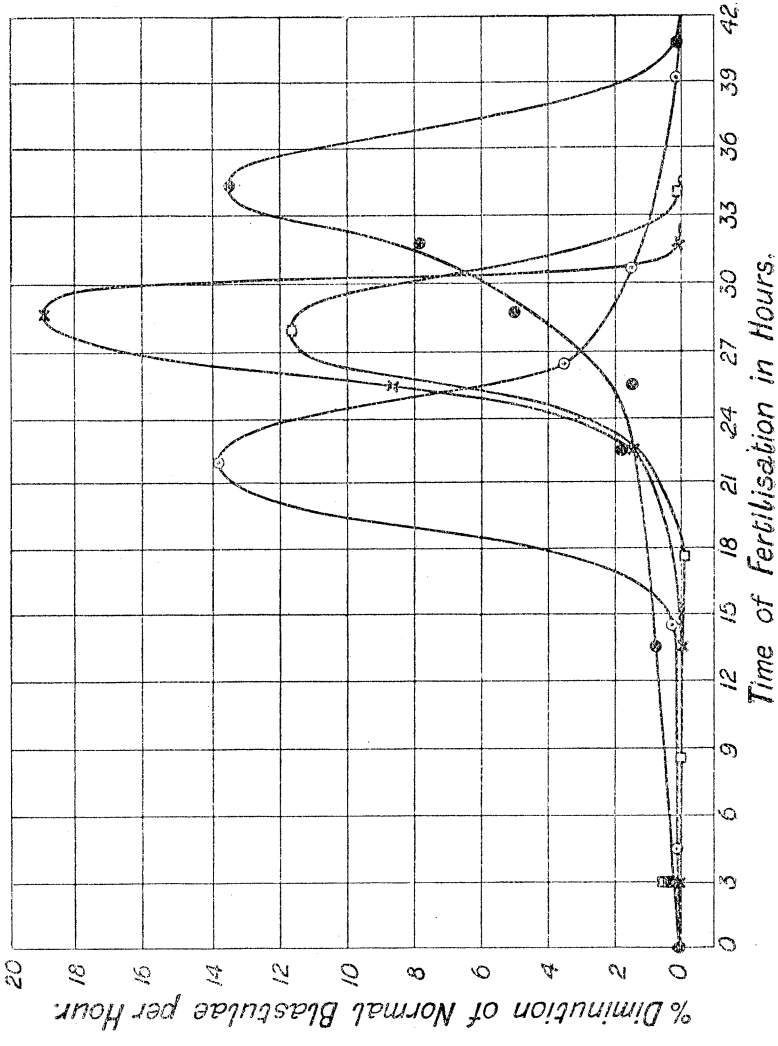
In pure sea water, with a dilution of 680,000 ova per litre, the maximum rate was also between the 20th and 24th hours, but a fair number also degenerated between the 24th and 29th hours. In this table it will be noticed that the actual percentages of blastulæ have been omitted, and only the percentage numbers of ova per hour undergoing abnormal development given. The numbers given in brackets against the "fertilised directly" line indicate the percentages of normal blastulæ produced on immediate fertilisation.

| Time of fertilisation. | Per cent. diminution per hour. | | Time of fertilisation. | Per cent. diminution per hour. | Time of fertilisation. | Per cent. diminution per hour. |
|---|---|---|---|---|---|---|
| | Tank water. | Pure water. | | Tank water. | | Tank water. |
| Directly.... | (99·7) | (98·8) | Directly.... | (100·0) | Directly.... | (98·2) |
| After 6 hrs. | 0·7 | 0·4 | After 9 hrs. | 1·6 | After 6 hrs. | 0·7 |
| „ 10 „ | 0·6 | 0·4 | „ 24 „ | 1·0 | „ 11 „ | nil |
| „ 22 „ | 5·8 | 1·3 | „ 33 „ | 6·7 | „ 24 „ | nil |
| „ 31 „ | 2·2 | 8·6 | „ 45 „ | 0·8 | „ 32 „ | 11·7 |
| „ 35 „ | 0·8 | 0·2 | .. | .. | „ 36 „ | 0·1 |

In this next table the results of four series of observations are included. In the first two the relative effects of tank and of pure sea water, were again compared. In this case the pure water had a much better preservative effect, the ova undergoing their maximum deterioration some ten hours later than those kept in tank water. In the remaining two series of observations, the sexual products were kept in tank water, the maximum rate of deterioration being between the 24th and 33rd hours, and the 24th and 32nd hours respectively. This last experiment is in some ways the most striking one made, as 94·8 per cent. of the ova developed to blastulæ until the 24th hour, whilst by the 32nd hour only 0·8 per cent. so developed.

As a whole, therefore, these observations show a fair amount of constancy. The mean times of the period of maximum deterioration in the various series are respectively $34\frac{1}{4}$, $28\frac{3}{4}$, 22, $14\frac{1}{2}$, 22, 18, $26\frac{1}{2}$, $28\frac{1}{2}$, and 28 hours, or, on an average, $24\frac{2}{3}$rd hours after the shedding of the ova and sperm. The reason of this constancy may have been the similarity of the conditions of experiment. Thus all the observations were made in the latter half of March and the first half of April, and throughout the temperature of the water only varied between 13·5° and 15·3°.

The chief conclusion to be gathered from these experiments seems to me to lie in the comparative suddenness of the onset of the increased rate of deterioration of the sexual cells. Thus in all but two out of the nine experiments, the rate of increase of abnormal development remained at about 1 per cent. per hour till the 20th to the 27th hour, and then became so rapid, that within about nine hours the capacity for normal development had almost entirely disappeared. Thus rapid increase is well shown by the graphic method in the accompanying figure. Here the four most striking results obtained are reproduced, the values obtained in each of the different experiments being distinguished by different signs.




The probable reason of this increase readily suggests itself. Thus supposing that animals developing from ova which are very nearly, but not quite stale enough to avoid normal fertilisation and development, are for that reason less strong and vigorous than those arising from fresh sex cells, it follows that the period during which the sex cells remain normal ought to be as long as possible, but that, once these have begun to deteriorate, they ought to absolutely lose their functional capacity as rapidly as possible, in order that the number of enfeebled organisms which we have supposed to arise may be as small as can be.

In all the experiments thus far described, both ova and sperm were kept for similar periods before fertilisation. In order to determine whether the onset of abnormal development depended more especially on the staleness of the one element or of the other, experiments were also made in which either stale ova were fertilised with fresh sperm, or fresh ova with stale sperm. At the same time some of the stale ova were fertilised with the stale sperm, so that properly comparative results were obtained. These are collected in the subjoined table.

| Time of fertilisation. | Percentage of normal blastulæ. | | |
|---|---|---|---|
| | ♀ stale. ♂ stale. | ♀ stale. ♂ fresh. | ♀ fresh. ♂ stale. |
| After 9 hours ..... | 88·0 | 81·0 | 97·0 |
| „ 9 „ ..... | 85·6 | 70·0 | 65·2 |
| „ 20 „ ..... | 29·0 | 4·7 | 95·5 |
| „ 22 „ ..... | 91·0 | 95·0 | 93·0 |
| „ 24 „ ..... | 45·3 | 87·3 | 96·2 |
| „ 24 „ ..... | 70·1 | 87·6 | 19·7 |
| „ 24 „ ..... | 94·4 | 95·0 | 81·1 |
| „ 27 „ ..... | 73·3 | 67·3 | 68·6 |
| „ 29 „ ..... | 2·7 | 94·3 | 5·9 |
| „ 33 „ ..... | 10·6 | 48·1 | 73·6 |
| „ 34 „ ..... | 0·0 | 23·8 | 36·0 |
| Mean ..... | 53·6 | 68·6 | 66·5 |

On comparing the three columns, one can see that there is no regularity in the figures. In two cases the maximum number of normal blastulæ was obtained from stale ova and stale sperm, in four cases from stale ova and fresh sperm, and in five cases from fresh ova and stale sperm. In the last line of the table are given the mean percentages of all the observations made. From these one may perhaps conclude that whilst on an average just as many blastulæ are obtained with stale ova as with stale spermatozoa, yet that when both the sex cells are stale, the proportion is slightly less. Probably, however, one is more justified in concluding that within certain limits it is a matter of indifference whether one or other or both of the sex cells is stale. Thus if the last three observations in the table, made between the 29th and 34th hours, be omitted, the average percentages of blastulæ obtained in the remaining eight experiments are respectively 72·1, 73·5, and 77·0, *i.e.*, nearly as great with both sexual cells stale as with only one. These last three experiments would, however, seem distinctly to indicate that when the sexual cells have reached their period of rapid deterioration, it is a distinctly more favourable condition if only one and not both of the cells be stale.

The conclusion we have arrived at is not altogether an expected one, as far as one could form any expectation from indirect evidence. Thus, as before mentioned, O. and R. Hertwig found that the ova of certain Echinoids, if kept ten to twenty hours, underwent fertilisation in very much larger numbers than freshly shed ova. The fresher and better the condition of the sperm, however, the better the chance of cross fertilisation. Again, in a former paper,* I showed that hybrid larvæ from the ova of *Strongylocentrotus lividus* and the sperm of *Sphærechinus granularis* could only be obtained in any number during the months of July and August, when the sexual products of *Strongylocentrotus* were found to reach their minimum maturity. Those of *Sphærechinus* were, on the other hand, in a mature condition. One would therefore be inclined to conclude that in the present experiments, stale ova fertilised by fresh sperm would yield a larger proportion of blastulæ than fresh ova fertilised by stale sperm. It must be remembered, however, that the conditions are essentially different. Thus in direct fertilisation, it is the natural property of every ovum, whether fresh or stale, to undergo fertilisation by any spermatozoon which still preserves its vitality; whilst in cross fertilisation it is, as a rule, the natural property of every ovum to resist such impregnation, and this resistance is only overcome when the vitality is diminished by keeping the ovum in water, or by other means.

We have thus far examined only how far the staleness of the sexual cells affects the number of normally developing blastulæ. Does it have any more permanent effect, and do the larvæ developed from stale products differ either in form or size from those developed from fresh products? In the paper just mentioned, a few experiments upon this subject were recorded, and these showed a very distinct effect to be produced: but as they were not very numerous, no great stress was laid upon them. They have since been repeated, and sufficient confirmation of them obtained. The method of experiment was, as usual, to mix portions of the liquids containing the stale or fresh ova and sperm, and then, after an hour, pour them into jars containing about $2\frac{1}{2}$ litres of sea water. These jars were kept in a tank of running sea water at a practically constant temperature for eight days, and the larvæ were then killed by the addition of corrosive sublimate, and preserved in 80 per cent. alcohol. They were then mounted in glycerin, and measured under the microscope in groups of fifty, by means of a micrometer eye-piece.† In a complete experiment, five series of measurements had to be made, viz.: (1) of the normal larvæ obtained from the fresh ova fertilised with fresh sperm, (2) those from stale ova and stale sperm, (3) from stale ova and fresh sperm obtained from another freshly opened Echinoid, (4) from fresh ova and stale sperm,

* 'Phil. Trans.,' B, 1898, p. 465.

† For fuller details of the method, *vide* 'Phil. Trans.,' B, 1895, p. 577.

(5) and, lastly, those from the ova and sperm of the freshly opened Echinoids. It is of course impossible to get an exact basis of comparison for the larvæ obtained from one stale and one fresh sexual product. The best possible is to take a mean between the size of the original normal larvæ and that of the larvæ obtained from the fresh sexual products used for fertilising the stale products. The larvæ obtained with both sexual products stale are of course accurately comparable with the original normal larvæ.

In the accompanying table are given the mean percentage differences in the size of the larvæ obtained with fresh and stale products, from the original normal larvæ in the one case, and from the mean between the original and the fresh normal larvæ in the other two cases. The actual body length measurements of the original normal and fresh normal larvæ are also given, these being in micrometer eye-piece scale units.

| Condition of sexual cells. | Fertilisation made after | | | | | |
|---|---|---|---|---|---|---|
| | 9 hrs. | 24 hrs. | 33 hrs. | 45 hrs. | 24 hrs. | 34 hrs. |
| Stale ♀, stale ♂ ..... | −0·2 | +1·9 | + 1·1 | − 1·9 | −3·6 | nil |
| Fresh ♀, stale ♂ ..... | +7·1 | +3·7 | +10·9 | + 1·5 | +3·9 | − 2·7 |
| Stale ♀, fresh ♂..... | −2·8 | −3 0 | + 2·0 | −15·9 | −5·2 | −13·0 |
| Body length of normal larvæ ..... | 29·94 | .. | .. | .. | 30·72 | |
| Body length of fresh normal larvæ ..... | 26·12 | 30·59 | 25·85 | 30·34 | 30·99 | 31·01 |

In the first group of observations given in the table these series of measurements were repeated after keeping the sexual products respectively 9, 24, 33, and 45 hours. When both the sexual cells were stale, it may be seen that the size of the larvæ was only slightly, if at all, affected, the average variation from the original normal larvæ being only 0·2 per cent. With fresh ova and stale sperm, on the other hand, the larvæ were considerably increased in size, even those obtained with sperm forty-five hours stale being slightly larger than the mean normal. With stale ova and fresh sperm, there was in three out of the four experiments a distinct diminution in the size of the larvæ, this amounting to no less than 15·9 per cent. in the case of the ova kept forty-five hours. This experiment thus affords a most satisfactory confirmation of the conclusion tentatively put forward in the above-mentioned paper, *i.e.*, it shows that *whilst larvæ obtained from stale ova and stale sperm are of the same size as those obtained from fresh sexual products, those from fresh ova and stale sperm are distinctly larger than the normal, and those from stale ova and fresh sperm distinctly smaller.*

The next experiment in the table is not so satisfactory, as in the one instance in which stale ♀ stale ♂ larvæ were obtained, there was a distinct diminution in size; whilst in one of the two sets of fresh ♀ stale ♂ larvæ, there was also a diminution, instead of the expected increase. In the two experiments with stale ova and fresh sperm, the diminution was in each case very considerable, so that the somewhat abnormal results obtained in this series of experiments may perhaps be put down to the fact that all the larvæ, whatever the conditions under which they were obtained, showed a tendency to undergo diminution in size.

In all the experiments thus far described, the fresh normal larvæ were measured as well as the original normal larvæ. In another series of observations, however, and in all the observations described in the former paper, only the original normal larvæ were measured; hence, though the values obtained for stale ♀ stale ♂ larvæ are just as accurate as before, those for stale ♀ fresh ♂ and fresh ♀ stale ♂ larvæ are presumably less accurate, as they are also compared against the original normal larvæ, and not the mean of the original and fresh larvæ. All these observations are collected in the subjoined table. In

| Condition of sexual cells. | Fertilisation made after | | | | | Means of all observations. |
|---|---|---|---|---|---|---|
| | 24 hrs. | 18 hrs. | 9 hrs. | 22 hrs. | 9 hrs. | |
| Stale ♀, stale ♂ ..... | −1·8 | .. | .. | + 2·0 | −3·4 | −0·74 |
| Fresh ♀, stale ♂ .... | +5·5 | +11·0 | +9·6 | + 3·5 | −9·5 | +4·05 |
| Stale ♀, fresh ♂ ..... | −0·4 | −17·6 | .. | −11·3 | −1·8 | −6·90 |
| Body length of normal larvæ ..... | 30·22 | 30·72 | 29·41 | .. | 30·95 | |

the first series, the one not recorded in the former paper, there is a slight diminution in the size of the stale ♀ stale ♂ larvæ, but a considerable increase in that of the fresh ♀ stale ♂ larvæ. In the next experiment no stale ♀ stale ♂ larvæ were obtained, but the fresh ♀ stale ♂ larvæ showed a very marked increase in size, and the stale ♀ fresh ♂ larvæ showed the maximum decrease of 17·6 per cent. Most of the rest of the observations conform more or less to the rule above laid down, but there is one very marked exception, the fresh ♀ stale ♂ larvæ being in one case 9·5 per cent. smaller than the normal, instead of larger, as one would expect.

Taking means of all the observations in both series, we find that as an average of eight observations, the stale ♀ stale ♂ larvæ were diminished 0·7 per cent. in size; as an average of eleven observations, the fresh ♀ stale ♂ larvæ were increased 4 per cent., and as an average

of ten observations, the stale ♀ fresh ♂ larvæ were diminished 6·9 per cent. These values, being means of a fairly large number of observations, may be regarded as trustworthy within certain limits, and will, I think, be held sufficient to justify the rule above laid down.

Confirmation of the conclusion that larvæ derived from stale ova and fresh sperm are smaller than the normal was obtained from quite another source. Thus in one case, it was found that the hybrid larvæ obtained on crossing the twenty-four hours stale ova of *Sphærechinus granularis* with fresh *Strongylocentrotus* sperm were 5·4 per cent. smaller than those from the cross of the fresh ova and sperm, whilst those from some of the same stock of ova after keeping an additional nine hours, and then crossing with fresh sperm, were 9·3 per cent. smaller. Again, in another experiment, larvæ from twenty-four hours stale *Sphærechinus* ova crossed with fresh *Strongylocentrotus* sperm, were 3·8 per cent. smaller than those from the fresh ova and sperm. None of the repeated attempts at obtaining crosses with stale ova and stale sperm succeeded, and in the only case in which fresh ova were crossed by stale sperm, the cross of fresh ova and fresh sperm failed, and so prevented any comparison being made.

In a previous part of the paper it was shown that the proportion of blastulæ obtained with both sexual products stale was nearly as great as that with only one of them stale, and a similar relationship was found to extend to the proportion of larvæ. Thus, excluding the series of observations made after forty-five hours, in which only 0·4 per cent. or less of the ova arrived at the larval stage, and including only those series in which all three of the fertilisations were attempted, it was found that on an average stale ova with stale sperm yielded 43·5 per cent. of larvæ, stale ova with fresh sperm 55·5 per cent., and fresh ova with stale sperm 49·0 per cent. In this case, therefore, as in that of the blastulæ, the stale ova with stale sperm yielded the least proportion, and the stale ova with fresh sperm the greatest, but the extreme limits of variation are only comparatively slight.

It may perhaps be asked whether this somewhat curious result as to the effect of staleness of one or other of the sexual products on the size of the larvæ is at all likely to be a factor of any importance under natural conditions. To me it seems that in at least one respect its value may be considerable, viz., it may be a very potent cause of variation. As has been already stated, it seems probable that the condition of relative staleness of the sexual products at the time of fertilisation may very frequently occur in several phyla of the animal kingdom, and hence it is by no means improbable that the average variability of each generation may be increased considerably by this means. As has been shown elsewhere,* the mean probable error of variation in the body length of these *Strongylocentrotus* larvæ is 6·1 per

* 'Phil. Trans.,' B, 1895, p. 615.

cent. If, then, with a particular sea-urchin a third of the ova did not undergo fertilisation till some twenty hours stale, whilst another third underwent fertilisation by twenty hours stale spermatozoa, and only the remaining third underwent fertilisation at once by fresh spermatozoa, then the probable error of variation of all of the larvæ so arising would roughly speaking be doubled. Of course this is an extreme instance, which could never occur in the case of Echinoids, but might easily occur in the case of, for instance, man. Whether the variations so produced would be in any degree transmissible by inheritance, is quite another point, but supposing it to be only the size of the offspring which is thus influenced, it may be merely a question of varying degrees of nutrition, and so be directly transmissible. In any case, whether inheritable or not, variation of itself may be in many cases of value, as it may give a better chance to natural selection and other agencies of picking out those individuals more adapted to their environment, and rejecting those less adapted. Thus, if all the individuals are nearly alike the evolutionary process must needs be exceedingly slow.

Finally, these results are of interest in that they prove the inequality of the sex cells. The diminution in the size of larvæ obtained from stale ova and fresh sperm may perhaps be looked upon as one of diminished nutrition, the result of the staleness of the yolk, but it is difficult to imagine why the staleness of the spermatozoon used to fertilise an ovum should produce a larva larger than the normal, unless one holds that the part played by the sex cells differs in some essential particular.

*Summary.*

The following are the chief conclusions arrived at in this paper:—

(1) If the ova and sperm of the Echinoid *Strongylocentrotus lividus* be kept for various times in sea water before fertilisation, then for about the first twenty to twenty-seven hours the number of normal blastulæ formed diminishes only about 1 per cent. per hour. After this abnormal development sets in rapidly, so that generally after a further nine hours or so, no blastulæ at all are obtained. The rate of falling off in the number of normal blastulæ may increase to as much as 18·9 per cent. per hour.

(2) If ova not more than twenty-seven hours stale be fertilised with equally stale sperm, practically as many blastulæ are obtained as when stale ova are fertilised by fresh sperm, or fresh ova by stale sperm. After twenty-seven hours, however, the number of blastulæ obtained with both products stale falls off more rapidly.

(3) Larvæ obtained from stale ova and stale sperm are of practically the same size as those from fresh sexual products, but those from fresh ova and stale sperm are distinctly larger than the normal, whilst those from stale ova and fresh sperm are distinctly smaller.