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EFFECTS OF AGING UPON GERM CELLS AND UPON EARLY DEVELOPMENT.

PART III. CHANGES IN VERY AGED EGGS

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In the previous two studies it was shown that, beginning with maturation, definite progressive morphologic and physiologic changes took place in the egg, both within and without the body, such as (1) an increase in volume of the egg, (2) a loss of surrounding jelly layer, (3) a retardation in the rate and change in the manner of forming the fertilization membrane, (4) a retardation and inhibition in cleavage. These changes served as measurable and corroborative evidences of the physiologic condition or vitality of the eggs of a female at any time, and served to measure the deterioration or loss of vitality with age.

There were other symptoms or evidences of physiologic deterioration, especially in late stages of ageing, or overripening, which will be discussed briefly in this paper. These changes include: (1) Agglutination of eggs, (2) fusion of eggs, (3) abnormal cleavage, (4) separation of blastomeres and (5) cytolysis of the eggs.

The same technique, the same nomenclature, the same precautions and the same three species of sea urchin were used as in Parts I. and II. For details of these I must refer to these studies.

AGGLUTINATION OF EGGS.

In Part II. it was shown that freshly removed eggs in good physiologic condition formed fertilization membranes within two minutes, at a rate accelerated with age, and with a wide peri-

vitelline space. With increasing age, or in freshly liberated eggs in poor physiologic condition, the rate of membrane formation was correspondingly retarded, and the perivitelline space was correspondingly diminished. In late stages of physiologic deterioration, the membrane-forming substance was entirely gone, and no membranes were formed. Synchronous with these changes in the fertilization membrane, other changes took place, of which the most important for my present purpose were, first a progressive loss of surrounding jelly layer of the eggs, and secondly a change in permeability of the cortical layer of the eggs which permitted an increasing volume of sea water to penetrate into the egg, increasing its volume and rendering the cytoplasm increasingly viscous.

When the eggs reached this stage of aging or physiologic deterioration, agglutination took place. Such aged or stale eggs had actually reached the condition that experimenters (Loeb, Driesch, Goldfarb and de Haan) have sought to produce experimentally.

Loeb used hypotonic sea water, which swelled the eggs and burst their membranes. Later he used hyperalkaline dilute sea water, which in addition made the cortical layer viscous. Driesch, Goldfarb, and de Haan removed the surrounding jelly and the fertilization membranes by mechanical treatment and then centrifuged the eggs in sea water or in hyperalkaline sea water. Goldfarb later used only such eggs as had been kept in the laboratory for about five hours, and without other treatment obtained much larger numbers of agglutinations. These investigators as well as Morgan, Nussbaum, Wilson, Zur Strassen and others had observed from time to time the spontaneous agglutination (and fusion) of eggs in different species of urchins, as well as in other animals. It is very probable indeed that in these instances spontaneous agglutinations occurred in the eggs that had sufficiently aged, and hence were in the condition most favorable for their ready agglutination. Certain it is that in all of my cultures of aging eggs, and in all three species, agglutination took place in every experiment in which the eggs were allowed to age over a sufficiently long period. The facts concerning the agglutination in aging eggs may be summarized as follows:

Agglutination sometimes occurred in freshly liberated eggs. But in every such instance when suitably tested, as described in Study I. and II., they were found to be in poor physiologic condition. Thus in experiment 1, agglutination was observed in one female, 9 minutes after the liberation of her eggs, in another, 29 minutes after liberation. But these eggs, in spite of their freshness, were in very poor physiologic condition.

Agglutination of the eggs of different females of an experiment often began at very different ages, depending upon the physiologic condition of the eggs when liberated. The more deteriorated the eggs, the earlier the agglutination. For example, the eggs of female 1, in experiment 1, first agglutinated in 9 minutes, female 3 in 29 minutes, female 2 in 225 minutes. These eggs were all in varying degrees of poor physiologic condition, those of female 4 which were in good physiologic condition did not agglutinate in the 309 minutes that the eggs were under observation. In experiment 3, the eggs of female 3 did not agglutinate until 8½ hours old, and the eggs of females 1 and 2 first agglutinated when six hours old. In experiment 4, the eggs of female 1 and 2 first agglutinated when 2½ hours old, female 5 and 6, when 8½ hours old; female 3 did not agglutinate in the 11 hours under observation, etc.

Once begun, agglutinations were observed throughout the remainder of the experiment. I am not certain to what extent there was, with increasing age, an increase in the number of agglutinated clusters, and to what extent there was an increase in the number of eggs to each cluster. Both kinds of increases took place in experiment 1, 2, 3, 4, etc. (*Toxopnuestes*), experiment 14 (*Hipponoë*) and experiment 17 (*Arbacia*), etc.

Agglutination occurred in fertilized as well as in unfertilized eggs, and in both groups each of the eggs in the cluster developed in a normal manner. The later regulations and the different types of larvæ resulting from such clusters I have elsewhere described in detail (Goldfarb, '13, '15). For the present I merely wish to emphasize the fact that agglutination took place long before the death and before extreme deterioration of the eggs.

Agglutination was independent of the age or physiologic condition of the sperm. Eggs in suitable condition agglutinated

whether fertilized or unfertilized. And when fertilized they agglutinated equally well whether old or fresh sperm were used, subject only to the following qualification, viz., when fresh sperm in high concentration were used, they caused the eggs to revolve rapidly in various directions, thereby preventing adjoining eggs from agglutinating, and separating many of the eggs already agglutinated. This was observed in *Toxopneustes*, experiment 1, 2, 3, 4; in *Arbacia*, experiment 16, 17; in *Hipponoë*, experiment 14, etc.

Clusters of 2 to 40 and more eggs were agglutinated to one another. Large clusters died in early cleavage. Clusters of 3 and 4 eggs often reached the blastula stage, and sometimes the gastrula stage, and then died. Clusters of 2 frequently reached the larva stage. The various types of gastrula and larvæ resulting from such agglutinated pairs of eggs and the processes involved, I have described in a previous publication (Goldfarb, '13, '15).

Agglutination occurred in every experiment in which the eggs had aged sufficiently, *i. e.*, in which physiologic deterioration had proceeded to a sufficient degree, as determined by the various tests enumerated in Study I. and II. Such deterioration gave rise without any other treatment, to exactly the conditions that were found to be most successful in the experimental agglutination of eggs, namely, loss of jelly layer, loss of membrane and a viscous condition of the naked protoplasm of the egg.

The rate of physiologic deterioration and hence the rate of agglutination depended upon the condition of the eggs at liberation, the species of egg used, the temperature of the sea water, and age.

Fusion of Eggs.

Some of the agglutinated eggs fused more or less completely together in all cultures of aging eggs. Only a small per cent. however fused, partly because clusters of more than 4 eggs died in early cleavage, while clusters of 3 or 4 eggs rarely survived beyond the blastula stage; and partly because clusters of 2 or 3 eggs often separated as a result of the uncoördinated ciliary activity of the different component blastula, gastrula or larvæ. Therefore only some of the closely agglutinated clusters of 2 or 3

eggs, fused together permanently, either in the egg stage, or in the course of subsequent early development. The processes involved in the fusion of eggs or embryos, and the various types of larvæ resulting from such fusions I have descibed elsewhere (Goldfarb, '13, '15).

For the present my interest lies not in the fusion process, per se, nor in the various correlated phenomena, but rather in fusion as another evidence or symptom of aging or deterioration of the eggs.

The observations may be summarized as follows:

"Spontaneous" fusion of eggs occurred in all three species of eggs, and in all cultures in which the eggs had aged sufficiently.

"Spontaneous" fusion occurred in unfertilized as well as in fertilized eggs.

In unfertilized eggs fusion was usually complete, giving rise to "giant" eggs which measured 8, 10 and 13 units diameter, whereas the control eggs measured but 6 units. These volumetric differences indicate, as Driesch pointed out, the number of eggs which were united into the single giant egg.

A number of observers have recorded the "spontaneous" occurrence of giant eggs or fused larvæ (Herbst, Morgan and Wilson, Nussbaum and Oxner). These spontaneously formed giant eggs or fused larvæ, it is very probable, arose as in my cultures by the natural deterioration of aging eggs, affecting the different structures of the egg in a manner so favorable to the fusion process.

Fusion occurred also among fertilized eggs. The resulting degree of completeness of fusion was conditioned largely, if not exclusively, by the stage in development when fusion first began. The earlier the fusion the more complete, and vice versa, the later, the less complete. Early fusion gave rise to "single" larvæ, either giant or normal. Late fusion gave rise to double larvæ of many types (Goldfarb, '15).

Fusion began at widely different ages, *i. e.*, at widely different periods after the liberation of the eggs. In *Toxopneustes*, experiment 6, the eggs of one female fused when 2 hours old, another when 5 hours old; in experiment 8, when 5 hours old; experiment 2, when 6 hours old; experiment 11, when 23 hours

old; experiment 9, when 25 hours old; experiment 12, when 50 hours old. In *Hipponoë*, the eggs fused when 23 hours old, and 42 hours in experiment 14. In *Arbacia* when 48 hours, experiment 17; 63 hours, experiment 20; 65 hours, experiment 19, etc. The explanation for this wide range in the onset of fusion within the same species, lies in the correspondingly wide diversity in the physiologic condition of the eggs of different females at the time of liberation, and hence correspondingly wide difference in the rate of deterioration.

Abnormal Cleavage.

Besides agglutination and fusion, there were other evidences of late aging, which might conveniently be grouped together under the term abnormal cleavage. Such abnormality included an increasing irregularity of shape and size of the blastomeres, an increasing lack of cohesion of the blastomeres, increasing retardation of mitosis, increasing numbers of atypic blastulæ, gastrulæ and larvæ, and an increasing inhibition of development.

These symptoms of extreme physiologic deterioration occurred essentially alike in all three species.

The onset of abnormality in cleavage occurred at widely varying ages, due to correspondingly wide variation in the physiologic condition of the eggs at liberation. For example, in experiment 4, the eggs of one female first showed marked irregular cleavage when 2⅔ hours old, another female when 5 hours old, others when 8½ hours old, etc.

After a long initial period, during which cleavage was regular, there was with further and extreme deterioration a progressive increase in the degree of irregularity and in the numbers of irregularly cleaving eggs. For example, in experiment 2, some of the eggs of females 2 and 3 became decidedly irregular, when 99 minutes old. From this time on, the number increased continuously until the eggs were 214 minutes old. Beyond this age, it was increasingly difficult to ascertain the ratio of irregular to the whole number of eggs because of increasing cytolysis and fragmentation of both unfertilized and fertilized eggs. In experiment 16, when the eggs were 1½ hours old, the average number of irregularly cleaving eggs of the 3 females was 7 per

cent. of the total. When 5 hours old, the number had increased to 46 per cent. of the total. In *Arbacia*, experiment 16, the average per cent. of irregularity of the 7 females when their eggs were 1½ hours old was 12 per cent. of the total. When 18½ hours old, 12 per cent. of the non-cytolized and non-fragmented eggs cleaved irregularly. When 24 hours old, the number of irregular eggs had increased to 40 per cent. of the non-cytolized eggs.

These deteriorated and irregularly cleaving eggs were short lived. With increasing age they died in increasing numbers, and at earlier and earlier stages in their development. *The more deteriorated the eggs, the less completely did they develop.* This differential mortality served as another index of the vitality or physiologic age of the eggs of any female.

These results give substantial support to Pearl's hypothesis of a varying degree of virility of the different eggs of an individual.

Irregular cleavage, with the consequences above enumerated, was due to several causes. In the first place the loss of jelly and the absence of the membrane made it increasingly possible for polyspermy to occur, particularly when fresh sperm in high concentration was used. Polyspermy in old eggs had been observed before by the Hertwigs, '86. These polyspermic eggs gave rise to irregular mitosis and irregular cleavage. In the second place the changed permeability, with its consequent excessive inflow of sea water, gave rise to mechanical or physical interference, dissolved certain of the protoplasmic granules, caused an excessive metabolism and an accumulation of metabolic products, gave rise to a very viscous condition of the protoplasm, etc., all of which were contributory agents in producing the various and increasing irregularities in cleavage.

Separation of Blastomeres.

Another quite characteristic manifestation of extreme age or physiologic deterioration was the more and more complete separation of the blastomeres. As the eggs increased in age, there was, as I have pointed out, an increasing viscosity of the cytoplasm and membrane, ultimately a complete loss of the fertilization membrane, or membrane-forming substance, with a

consequent enlargement and partial separation of the first two blastomeres, into an elongated double ball; and finally a more and more complete separation. The blastomeres fell apart completely.

There can be no doubt that F. R. Lillie ('14) and Loeb's observations that blastomeres do fall apart in old eggs is correct.

Separation of blastomeres occurred in every experiment in which the observations were made over a sufficiently long period. And once begun the blastomeres continued to separate with successive cleavages until there was in place of the egg, a flattened mound of minute blastomeres, stuck to the bottom of the dish or to one another. And with increasing age, more and more eggs passed through this history.

As might have been anticipated, the onset of separation varied with different females according to the physiologic condition of the eggs at liberation. Separation was first observed in experiment 3, when the eggs were 6 hours old; in experiment 4, when 5 hours old (1 female) and 8 hours old (in the other 2 females); in experiment 5, 8 hours old in one female, later, in the other 5 females; in *Arbacia*, the eggs of one female, experiment 16, showed the separation phenomenon when 18 hours old, 2 other females when 24 hours old, and 2 others when 42 hours old. In experiment 15, separation was observed in 3 females when eggs were 24 hours old, in experiment 17 when 23 and 28 hours old, etc. In *Toxopneustes*, and *Hipponoë*," separation usually occurred in 8 to 10 hours after liberation, in *Arbacia* usually not before 24 hours.

With the continued and complete separation of the blastomeres in successive cleavages, an increasing number of the fertilized eggs died and with their disintegration many of the non-separating eggs also died.

There can no longer be any doubt that there were no fertilization membranes about these aging eggs. Glazer ('14) urged that separation was due to the absence of the fertilization membrane. It is far more probable that the absence of the membrane together with the physiologic changes in the protoplasm (the result of aging) are both responsible for the separation of the blastomeres.

Cytolysis of Ageing Eggs.

The consummation of the various deteriorating changes in aging eggs is cytolysis and death.

Cytolysis of sea-urchin eggs under the influence of various experimental conditions, such as saponin, salycyl aldehydes. propyl alcohol, distilled water, etc., have been carefully described by Loeb. Loeb speaks of two methods of cytolysis, which he calls "white" and "black."

Aging eggs cytolize in the same two ways. In the "white" or cytolysis by liquefaction, a changed permeability of the cortical layer permits an increasing volume of sea water to enter the egg with a corresponding enlargement of the egg, a more viscous condition of the cytoplasm, a diminution in size of the protoplasmic granules, and an increasingly hyaline and translucent appearance of the egg. In the second type of cytolysis, there is either far less increase in size of the egg or no increase at all; the central mass remains opaque and becomes increasingly opaque; the cytoplasm is far less viscous; the outer surface which is hyaline is fragmented, and sometimes the inner mass as well, is fragmented, and the outer fragments fall off, with a consequent diminution in size of the eggs, even far below the norm.

I was unable satisfactorily to establish whether these two are independent methods of cytolysis, possibly associated with different degrees of virility of the eggs or whether they are sequential phenomena. In most cultures, both types of cytolysis are seen at the same time.

The onset differs in the eggs of different females, and, as in other evidences of aging, this variation is due to differences in the physiologic condition of the eggs at liberation. Those eggs which were in good physiologic condition at liberation, cytolized late; those in poor condition, early. Eggs in relatively similar physiologic condition cytolized at a similar rate at the same temperature. The greater the temperature, the greater the rate.

In *Toxopneustes*, cytolysis in any considerable numbers, was first observed when $\frac{1}{6}$ hour old in experiment 1, $\frac{1}{6}$ hour old in experiment 3, 6 hours old in experiment 2 and 5, 11 hours old in experiment 4, 20 hours old in experiment 9. In *Hipponoë*, the rate of cytolysis is essentially the same as in *Toxopneustes*.

In *Arbacia* it was much slower. Beginning, in the given experimental conditions, in about 28 hours as in experiment 17, and extending to 42 hours as in series 19, 46 hours in experiment 18, and later in other experiments. This difference in rate of cytolysis is in part due to differences in temperature of the sea water in the two localities, but it is also due, and is another evidence of, a protoplasmic difference in the two species of eggs.

Correlation of the Changes in Ageing Eggs.

I have for the sake of clearer exposition described each type of change in aging eggs, such as agglutination, irregular cleavage, etc., as though each type of change was independent and unrelated to the other changes. *I wish now to emphasize the entirely dependent, related and correlated character of the changes I have heretofore treated separately.*

Seven types of changes and tests have been enumerated, whereby the physiologic condition of the eggs may be accurately determined. These tests are (1) size of the egg, (2) per cent. possessing jelly layer, (3) rate and width of membrane, (4) rate, total and character of cleavage, (5) rate and degree of agglutination, (6) rate and degree of fusion of eggs, (7) and per cent. of cytolysis. *Each test serves as a verifiable measure of qualitative and quantitative changes in the vitality or physiologic condition of the eggs under the given conditions.*

But more significant for my present purpose is the fact that the variation in any one of these seven tests is associated with corresponding variation in the others. *So highly correlated are they that a knowledge of one enables one to predict with a remarkable degree of exactness, the degree of variation in the others.* If the cleavage rate for example, is known, one could predict the size of the eggs, the per cent. with jelly layers, the rate of fertilization membrane, the per cent. of agglutination, etc. This somewhat sweeping statement is made with the understanding that it is subject to the qualifications referred to in the text.

It is sometimes inconvenient or difficult to take certain precautions with respect to one or another of the seven groups of tests, and without such precautions any one test may give a large error. But the ensemble of the tests or any considerable

group of them rectifies any such error, and gives an accurate measure of the physiologic condition of the eggs. The *determination by several or all of the tests serves as the most exact method so far recorded, whereby the vitality of any batch of eggs may be accurately ascertained and whereby the rate and nature of the manifold changes, with age or under any set of experimental conditions, may be determined.*

The correlated changes are given below in tabular form.

	Physiologically Good Eggs. "Fresh" Eggs.	Physiologically Poor Eggs. "Aged" Eggs.	Physiologically Bad Eggs. "Very Aged" Eggs.
Size:	Small deviation from norm.	Increasing deviation. Increasing size.	Further deviation. Further increase above or decrease below norm.
Jelly:	All or nearly all eggs with jelly	Increasing per cent. without layer.	Further per cent. without layer.
Membrane formation:	(*a*) Formed within 2 minutes.	Increasingly retarded.	None.
	(*b*) Wide space between eggs and membrane.	Increasingly narrow.	None.
Cleavage:	(*a*) Maximum per cent. of eggs cleave.	Increasingly lower per cent.	Further reduction.
	(*b*) Maximum rate of cleavage.	Increasingly retarded.	Further retardation.
	(*c*) Minimum irregular cleavage.	Increasing per cent. irregular.	Further irregular.
	(*d*) No separation of blastomeres.	Increasing per cent. separate.	Further separation.
Agglutination:	None of the eggs agglutinate.	Increasing number agglutinated.	Further agglutination.
Fusion:	None of the eggs fuse.	Increasing number fuse.	Further fusion.
Cytolysis:	None of the eggs cytolized.	Increasing number cytolyze.	Further cytolysis.

This remarkably close correlation of the various changes associated with aging may be expressed graphically as in Fig. 1. The ordinates represent the degree of change or physiologic deterioration, the abscissas represent age of eggs. There are a number of independent tests which demonstrate unequivocally that freshly liberated eggs (in good physiologic condition) improve with age, and after a definite period begin to deteriorate. Such eggs upon liberation produce their membranes increasingly fast, and cleave more rapidly, and with increasingly large per cents, as the eggs become older. This gradual improvement or

super-ripening is represented in the heavy lined curve (Fig. 1).[1] The process continues until an optimum is finally reached many hours after liberation at a stage marked *B*. Beyond this age there occurred a definite progressive deterioration, indicated by

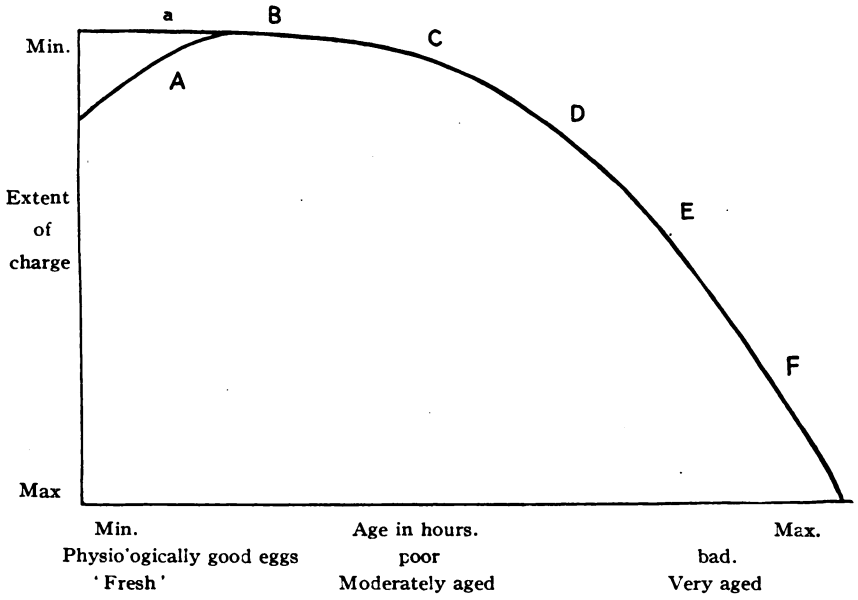

FIG. 1. Showing extent of morphologic and physiologic changes during ageing or deterioration of eggs. *AB* represents the period of superripening; *BCD*, the period of overripening; *DEF*, the period of extreme deterioration and death. *ABCDE* represents change in volume, rate of membrane formation, rate of cleavage, total cleavage (*Hipponoë*). *aBCDE* represents loss of jelly, total cleavage (*Arbacia*), width of membrane formation. *CDEF* represents increase in agglutination, fusion, abnormal cleavage, separation of blastomeres, cytolysis, and increase in oxidation.

the progressive retardation in the rate of membrane formation, in the rate of cleavage, in the decrease in total cleavage, etc. With further aging, there appeared other symptoms that marked further deterioration, such as agglutination, fusion, irregular cleavage, separation of blastomeres, and finally cytolysis and death. Some of these symptoms first appeared at *C* of the curve, such as agglutination, others at *D* such as irregular cleavage, etc.

[1] *I. e.*, part A of the curve.

Discussion.

A number of investigators have been struck by the unexpectedly large variability in the eggs of different females of sea urchins, even when the eggs were liberated at the same time, and kept under apparently identical conditions. A few examples and the explanations offered to account for this diversity may be cited.

Stockard observed "that a number of eggs when subjected to the same solutions do not all respond in a like manner," and held that such variability was due to "differences in individual resistance and vigor."

F. R. Lillie also noted "the failure to obtain exactly the same curve . . . ," and believed the result "was due in part to the natural variability of different lots of eggs and sperm."

Loeb, J., and Wasteneys, H., also believed that this variability was due in large part to differences in the eggs.

Wasteneys later came to the conclusion that this variability was due to differences in sensitiveness of eggs of different females, and perhaps to temperature.

R. S. Lillie also held that variability was due to the condition of the eggs.

F. R. Lillie came closer to an understanding of the matter when he pointed out that "the condition of the eggs whether ripe or immature, fresh or stale, with or without jelly, is more important than concentration of the eggs. The condition of the gonad" he adds, "is the most variable thing in summer sea urchins."

Loeb ('02) came still closer to the truth, when he stated that "this (variability in maturation of asterias eggs) probably depends on the fact that the eggs of different females are not all in the same condition of ripeness."

Many other observations might be mentioned in which the authors have either ignored this large variability or, having noted it, ascribed the result to individual resistance, sensitiveness, natural variability and other equally indefinite causes.

In this connection one must mention Loeb and Chamberlain's work. These authors distinguished between the primary variation in the eggs of a given female, from the secondary variation

in the eggs of different females. They ascribed the primary variation to a difference in the amount of enzyme present in the different eggs of the same parentage.

In earlier studies, I showed that the ripe eggs from different freshly collected females varied far beyond Loeb's individual or primary variation. I determined *the exact range of variability of the freshly removed eggs, not merely in one but in several morphologic and physiologic entities, and for three species of sea urchin. I showed moreover that these variations were measurable and corroborative indices of differences in the vitality or physiologic condition of the different lots of eggs. And hence it was possible not only to ascertain with exactitude the condition of the eggs but to sort them out according to their physiologic condition.*

This unusual diversity in freshly liberated eggs of different females is due, firstly to the original or primary variability of Loeb and Chamberlain already referred to; secondly, to injurious changes that take place within the body of the female before liberation, and even as early as the maturation of the egg. For as soon as maturation is completed the eggs are susceptible to injurious agencies in the body fluid of the mother, particularly changes in ionic concentration and oxygen content. Whether these be the sole injurious agents or whether accumulated toxic (metabolic) substances also contribute, I cannot say. In any event, the equilibrium is upset and the *cycle of physiologic deteriorative changes which begins with maturation, proceeds at varying rates depending upon the length of time the eggs are held within the body, and the nature of the body fluids.*

Since the eggs of different females may mature at different times, and the eggs of a given female mature at different intervals, and since the body fluids either cause or permit deleterious changes to take place, and finally since the time between maturation and liberation may vary very widely in different females, it is not surprising then that such marked differences obtained in the different lots of eggs. These marked variations occurred in the two southern species probably throughout the breeding season; in the northern species the variation was less marked during the height of the breeding season.

Hence to assume that ripe eggs of different females, or that

all the eggs of a given female are in the same or nearly the same physiologic condition, when freshly liberated, is misleading and frequently very inaccurate.

It has been held by Minot that senescence of the individual began, not after sexual maturity, but far back in the history of the individual, even before birth. My studies on aging eggs lead me to the conclusion that senescence of the individual may be traced as far back in the history of the individual as the maturation of the egg. The aging eggs show numerous evidences of senescence, not only during the egg stage but throughout their development including their larval stage. And it is very probable indeed that the effects are carried into the adult stage of the individual.

The value of the results depends upon the acceptance of the technique used. The freshly liberated eggs were placed under conditions that preliminary tests had shown were optimum for that particular species of sea-urchin egg, namely: (1) the sea water used had been collected at the height of the incoming tide, and so obtained a more constant grade of sea water, and more free of detritus, etc., (2) the sea water was filtered (with certain precautions) and stored in glass; (3) standard flat bottom bowls were used, in which the concentration of eggs was far below the injurious limit, and nearly constant for the different series; (4) the volume of sea water was large enough to prevent interference by evaporation, etc. (250 c.c.); (5) the supernatant sea water as well as the bowls were changed at least once a day; (6) the temperature was kept fairly constant and close to that of sea water in the open, etc.

Under these conditions the freshly liberated eggs (whatever their physiologic condition may have been at the time of liberation) underwent a graded series of morphologic and physiologic changes, which were not new processes or changes, but the continuation of the same changes which took place within the body before liberation, namely, loss of jelly, retardation of membrane formation, increase in size of the egg, decreased rate of division and decreased ability to cleave, etc.[1]

The rate of change was however accelerated. Such accelera-

[1] And it is very probable indeed, that these changes occur in a state of nature.

tion depended upon three factors, namely, the condition of the eggs when liberated, the time after liberation (age), the temperature. The more deteriorated the eggs at liberation, the greater the rate of change at any given interval thereafter. Likewise the longer the period (age) after liberation, the greater the rate of change or senescence. And finally the higher the temperature of the sea water, the faster the deterioration, closely approximating the expectation from Van't Hoff's law. The sea water at the Tortugas was approximately 10° C. higher than at Woods Hole, and the rate of deterioration was more than twice as great in the former as in the latter.

With further aging and increasing rate of change, other symptoms of deterioration became manifest and were increasingly intensified, such as agglutination, fusion, abnormal cleavage and cytolysis. These have been described and measured for the three species.

If the germ cells of other tropical organisms behave as those of the two tropical species of sea urchins, *i. e.*, die or become infertile, or are unable to develop beyond an early stage, even a few hours after liberation, one may account, at least in part, for the relatively few individuals that reach maturity in so many tropical species.

The eggs that do survive show profound changes in nearly all and possibly all the parts of the egg. A full appreciation of this fact enables one to correlate and to find a common explanation in apparently unrelated experiments.

A number of investigators have noted a decreased productivity with increasing age of the mother. Minot ('91) came to this conclusion from his experiments with guinea pigs. Marshall's observations upon women, Pearl's upon fowls and lambs, Hammond's upon rabbits and pigs, and recently King's upon rats, all led to the same conclusion. Two explanations appear to me to account for the results, both of them involving the deterioration of the egg. Either the gonads of old mothers produce eggs in lower physiologic condition than younger mothers, or the eggs are of the same or similar physiologic condition at any age, but the interval between maturation and fertilization is greater in older mothers and hence the eggs in poorer physiologic condition

when fertilized. In either event the end result seems to be essentially the same as in the sea-urchin eggs, namely fewer develop, more become irregular or abnormal, with the age of the mother.[1] The early history of the changes in the sea-urchin egg and sperm as they deteriorate, we now know, and it is very probable that the eggs of aging mothers undergo fundamentally similar changes.

The experiments with aging eggs throw light upon, and indicate the probable cause of seemingly conflicting results in hybridization experiments.

The Hertwigs ('86) affirmed in contradistinction to the conclusions of Pfluger ('82) and Born ('83) that hybridization took place the more readily when the eggs were of lower vitality. Vernon ('98) also noted that the chances of hybridization were increased when the eggs had previously been kept for about nine hours in sea water, which, I have shown, lowers the vitality of the eggs. Hagedorn ('09) discovered another method for increasing the chances of hybridization, namely by increasing the HO concentration of the sea water. Herbst, Tennent, Godlewsky-Loeb and Kupelweiser used various modifications of Hagedoorn's method and obtained similar results. But as will be later shown, the increased alkalinity of the sea water only served to increase the rate of deterioration of the eggs, and hence prematurely aged them; these eggs showed the same changes which I have described in aging eggs, changes which permit the ready entrance of multiple autogenous sperm, or heterogenous sperm, and hence give rise to abnormal development or to hybridization.

Similarly, Doncaster, Vernon, and Herbst observed that hybridization could be increased by raising the temperature of the sea water. This also I have shown serves merely to precociously age the eggs and hence makes greater hybridization possible.

Less effective was the use of dilute sea water used by Tennent and Herbst. Here again, dilute sea water increased ionization, and hence increased the metabolism of the egg, which in turn increased the rate of aging.

[1] The age of the sperm or the father is far less important than the age of the egg or of the mother.

All these apparently diverse methods have this in common, viz., that they all induce the same changes in the eggs, i. e. accelerate the aging of eggs, and thus permit the ready entrance of autogenous or heterogenous sperm. Instead of emphasizing the one or the other external agent or change, as Herbst has done, I prefer to emphasize that which is common to all, namely, the very definite morphologic and physiologic changes within the egg, which I have termed physiologic deterioration or aging.

There are on record other phenomena which may now be interpreted in terms of the known changes in aging eggs, and which may be briefly summarized as follows:

Vernon ('95, '98) observed a seasonal change in the size of the larvæ, in the per cent. of hybridization, in the per cent. reaching the larval stage, in the maternal or paternal type of hybridized larvæ. These seasonal changes are in all probability due, as he himself suggested, to the varying physiologic conditions of the eggs, at different periods of the breeding season.

Fuchs ('14) observed that the per cent. of self-fertilizations in *Cione* increased with the age of the eggs. This is exactly what one should expect from the known changes in aging eggs and of the ready entrance of sperm in old eggs.

Matthews ('01) could induce parthenogenesis by shaking (in *Asterias forbesii*), most readily, when the eggs were at least three hours old.

De Vries, according to Vernon, obtained a larger per cent. (40) of mutants in old seeds, whereas fresh seeds gave only 1 to 5 per cent. This suggested that not only may the protoplasmic parts of the egg be radically modified by aging, but that the nuclear parts may be correspondingly affected.

Other experiments serve to strengthen this conclusion, particularly those upon sex determination.

R. Hertwig ('06 and '07) and Kuschekewitch were first to note that sex could be altered by aging the eggs of the frog. More recently Riddle ('12–'14), experimenting with pigeons, concluded that the sex of the offspring was conditioned by the physiologic activity of the reproductive organs or by the age of the mother. Both hyperactivity and aging of the mother causes an excess of male-producing eggs.

Stout has reported similar results in *Cichorium*.

King experimented with toad eggs and succeeded by drying the eggs in materially changing the sex ratio.

If it should be established that in these instances a change in sex was really induced by the experimental condition, it could be readily shown that in all these instances a physiologic deterioration of the eggs was the primary cause and affected profoundly not merely the cytoplasm but the nuclear substance of the eggs as well. It may be possible that aging modifies or destroys the chromosomes in the same way that particles of the cytoplasm are modified or destroyed.

For, recent studies of Marine and Manley, of Patten, of MacNider and others point unmistakably to the conclusion that in aging adult individuals there occur definite changes in different tissues of the body, as well as in the blood stream, changes which are profound in extent and correspond very closely to those I have described in aging eggs. In view of these observations I am led to believe that the reproductive tissues and the eggs of older mothers would be correspondingly modified. And it is also probable that the cyclical change in the aging eggs correspond with and are caused by corresponding cyclical changes in the parental tissues. For it will be recalled that the eggs showed an initial period of increasing ripening, increasing fertilizability, increasing rate of development, etc., until an optimum condition was attained, and then there followed a long period of progressive deterioration, modification and ultimate disintegration.

Returning now to the consideration of the changes in aging eggs, the question at once suggested itself. What then is the fundamental nature of the physiologic deterioration, which began with maturation and ended in the complete cytolysis of the egg. The changes are twofold. There is, on the one hand, a dissolving of the surrounding jelly layer, and, on the other hand, a gelatinization and dissolution of the membrane-forming substances in the cortical layer of the egg. These twofold changes are parallel. I find myself at variance with Harvey who held that the loss of jelly under "normal" conditions was independent of aging of the egg. My observations clearly show that if proper precautions against mechanical shaking and changed HO

ion concentration be made, the loss of jelly is concomitant with physiologic deterioration or aging of the eggs.

The second and more important change is concerned with the cortical layer of the egg. With age the dissolution of membrane-forming substances progresses and the permeability of the cortical layer increases, as a consequence of which a number of other changes occur, namely: (1) An inflow of sea water and an increase in size of the egg; (2) increasing mechanical interference due to the excess sea water; (3) thinning and final disappearance of fertilization membrane; (4) gradual solution of protoplasmic granules; (5) increasing viscosity of the cytoplasm; (6) increasing metabolism and metabolic products.

By aging is meant the ensemble of these and other changes in the egg.

It is not to be wondered then that with protecting jelly, cortical layer, cytoplasm and probably nucleus as well, chemically and physically altered, profoundly altered in some instances and entirely gone in others, that the subsequent history of the egg should show evidences of these changes, and be correspondingly altered in development. These changes in development may be categorically summarized as follows: (1) Increasing retardation and final loss in membrane formation; (2) increasing modification in character of membrane; (3) increasing retardation and final cessation of cleavage; (4) increasing irregularity in cleavage; (5) increasing viscosity of cytoplasm and cortical layer; (6) increasing agglutination and fusion of eggs; (7) increasing separation of the blastomeres; (8) increasing modified larvæ, etc.

The problem of physiologic deterioration or "aging" is reducable then to two main factors (1) a change in permeability of the cortical layer, (2) a change in the metabolism of the egg. I have already outlined the nature and conseqences of the first of these two factors. A few words may be added concerning the second.

Loeb's researches on oxidation of eggs had led him to the conclusion that with "aging," the eggs had increasing difficulty in disposing of the accumulated store of toxic metabolic products. Wasteneys subsequently by direct experimentation showed that there was a definite increase in metabolism with aging of eggs.

My own observations, still unpublished, extend and corroborate Wasteneys's results.

But to what extent was the change in permeability of the cortical layer, with its train of events, responsible for the deterioration of the eggs. If it was an important factor, it should be possible experimentally to retard these cortical changes, and hence correspondingly to increase the longevity and to retard the deterioration of the eggs. Such experiments were made repeatedly and successfully, an account of which will appear later. I mention these experiments merely to emphasize the fact that there appears at present two methods by which physiologic deterioration and "aging" may be controlled, namely, by retarding or preventing either the metabolism, or the change in permeability of the eggs.

By reducing the metabolism of the eggs, Lyon, Loeb and Wasteneys retarded the deterioration of the eggs. By retarding the cortical changes I have retarded the deterioration of eggs as well. By a summation of both methods far greater control of aging or deterioration was obtained.

Both methods are closely related. A change in the cortical layer induced a corresponding change in the metabolism of the egg, and it is probable that the reverse obtains, namely a change in metabolism is associated with a change in the cortical layer.

This leads directly to the possibility urged by Loeb, Child and, particularly in view of the unpublished work of E. J. Cohen,[1] that ageing and hence longevity of the germ cells is a function of the rate of metabolic activity of the protoplasm of the egg or sperm, the greater the rate of metabolic activity, the shorter the life of the germ cell, and vice versa, the lower the rate of metabolism the longer lived the germ cell.

Hence eggs in sea water are longer lived than dilute concentrations of sperm, both of the same chronologic and physiologic age. For the same reason eggs in sea water are shorter lived than concentrated or dry sperm. For in this condition the sperm are inactive, and hence lower metabolic rate. How far the result is due to the greater metabolism on the one hand as

[1] The first study has since appeared in the BIOLOGICAL BULLETIN, Vol. 34, 3, 1918.

urged by Child, or to the greater accumulation of metabolic toxic products as urged by Loeb, or to both of these factors, we cannot in our present state of knowledge determine.

Summary.

Aging eggs show progressive measurable morphologic and physiologic changes. Besides those described in Study II., there are a number particularly evident in later stages of aging eggs, such as agglutination and fusion of eggs, irregular cleavage, separation of the blastomeres and cytolysis.

A. 1. The agglutination phenomenon occurred only in physiologically very deteriorated or "aged" eggs, as evidenced in part by the low per cent. of the jelly layer, inability to develop a fertilization membrane, a more viscous condition of the cytoplasm and cortical layer, and by numerous other tests. These are exactly the conditions that have been sought in the experimental agglutination of eggs.

2. The onset of agglutination occurred at different ages for the eggs of different females, such variations being due to differences in physiologic condition of the eggs at the time of liberation from the parent. The more deteriorated the eggs at liberation the earlier the agglutination, and vice versa. Agglutination occurred in every experiment in which the eggs had aged sufficiently.

3. Some eggs remained agglutinated throughout their subsequent developmental history, others were secondarily separated at varying swimming stages in development, others died precociously, due to asphyxiation.

4. Fertilized as well as unfertilized eggs were agglutinated.

5. Clusters of 2 to 40 or more eggs were thus agglutinated.

6. Agglutination is not determined by the condition of the sperm except in so far as concentrated fresh sperm may, by revolving the eggs, separate agglutinated eggs or prevent their so doing.

B. 1. When the eggs were in sufficiently poor physiological condition, as determined by suitable tests, they tended not merely to agglutinate but many subsequently fused more or less completely.

2. Such fusion may occur in the egg stage or during subsequent development, with corresponding complete or incomplete fusion. The degree of fusion determined the various types of fused embryos and larvæ described in previous publications.

3. Complete spontaneous fusion of not more than three eggs occurred not infrequently and gave rise to giant eggs.

4. Fusion occurred in all experiments in which the eggs were allowed to age sufficiently.

5. The variation in the time of fusion was determined by the physiologic condition of the eggs at liberation. The more deteriorated the sooner the fusion.

6. Fused eggs and embryos, and giant eggs occurred in all three species of sea-urchin eggs, namely, *Arbacia*, *Hipponoë* and *Toxopneustes*.

7. It is probable that the "spontaneous" fusions of larvæ and embryos, described by many workers, find their explanation in similar aging or deterioration of the eggs.

C. 1. With increasing age and physiologic deterioration of the eggs, cleavage was increasingly irregular. This irregularity was manifested in a change in size and shape of the blastomeres, in retardation in the rate of cleavage, in increasing inhibition at progressively earlier stages of development, increasing numbers and types of atypic embryos and larvæ, and in extreme stages, in total lack of cleavage.

2. In every batch of eggs irregular cleavage occurred as soon as the eggs had aged sufficiently. The onset and the degree of irregularity varied with the physiologic condition of the eggs when liberated. Once begun there was a progressive increase in the numbers and in the degree of abnormality.

3. Irregular cleavage which is a consequence of aging is due in part to excessive intake of sea water, and in part to polyspermy. The quantitative relations of these two factors was not determined. Both are due to a change in the cortical layer of the egg.

4. Deteriorated or irregularly cleaving eggs, were shorter lived than physiologically fresh eggs. The greater the irregularity (or physiologic age) the short lived the eggs, and vice versa, the more virile the egg the greater the longevity and the less irregular development.

D. 1. With increasing physiologic deterioration, there occurred another type of irregularity, namely, more or less complete separation of the blastomeres.

2. Such separation occurred in every experiment that was carried over a sufficiently long period, and with further aging separation of blastomeres was increasingly complete.

3. The separation of the blastomeres occurred with successive cleavages.

4. In *Toxopneustes* and *Hipponoë*, separation usually occurred eight to ten hours after physiologically good eggs were removed from the female. In *Arbacia* about 24 hours were required. The variation in onset in different females was due to variation in the physiologic condition of the eggs at liberation.

E. 1. With still further physiologic deterioration (or "ageing") cytolysis set in. There were two types of cytolysis, namely cytolysis by liquefication or enlargement, and by fragmentation or reduction.

2. The onset of cytolysis differs in the eggs of different females depending upon the physiologic condition of the eggs at the time of liberation.

3. Cytolysis occurred three or more times as rapidly in *Toxopneustes* and *Hipponoë* than in *Arbacia*. This difference is in part due to accelerated metabolic rate at the higher temperature of the southern species, partly due to increased HO ion concentration of sea water at Tortugas and partly to protoplasmic differences of the different species of eggs.

F. Agglutination, fusion, abnormal cleavage, separation of blastomeres and cytolysis of the eggs are phenomena correlated with intense physiologic deterioration of the eggs, and were observed in all cultures in which the eggs were sufficiently deteriorated.

Seven independent groups of tests were used to determine the degree of deterioration. These tests corroborate one another. Any one test, with suitable precautions, measures the vitality of the eggs. From any one, the other manifestations of aging may be predicted. A group of tests offers the most convincing means of measuring exactly the degree of senescence of any sample of of eggs.

These seven groups of tests or symptoms of senescence also indicate the fundamental nature of the chemical and physical changes involved in the aging process, namely a change in the cortical layer of the egg, and a change in oxidation. The change in the cortical layer, *i. e.*, the change in permeability, affects the membrane, the cleavage, and all the other consequences of aging above enumerated.

It is very probable that the changed cortical layer with its train of consequences, as well as the change in respiration, are fundamentally reducible to the one phenomenon, namely, changed metabolism.

These results afford a common explanation of apparently diverse phenomena, such as change of sex with age (Riddle, Hertwig, etc.), senescence (Minot), reduced productivity (Pearl, King, etc.), physiologic differences in cross fertilizations (Tennant, etc.), etc. (For details see discussion.)

TABLE I.

SHOWS VARIATION IN ONSET AND INCREASE IN AGGLUTINATION, FUSION, IRREGULAR CLEAVAGE, AND CYTOLYSIS WITH DETERIORATION AND AGE. A STANDS FOR AGGLUTINATION; F, FOR FUSION; S, FOR SEPARATION OF BLASTOMERES; I, FOR IRREGULAR OR ABNORMAL CLEAVAGE; C, FOR CYTOLYSIS; N, FOR NORMAL.

Exp. No.	Date.	Age of Germ Cells in Minutes.		Female Number.						Remarks.
		♀.	♂.	1.	2.	3.	4.	5.	6.	
1	7/2	9	9	* A	N	f * C I	N			*Toxopneustes*
		29	29	* * A I	N	* f * A C I	* I			
		64	64	* A I	N	* * A C I	* * C I			
		209	19	A I	C I	* * A C I	* * C I			
		225	35	v A I	A C I	* A I	* I			
		250	60	v A I	A C I	* A I	* I			
		309	120	v A I	A C I	* A I	* I			
2	7/12	17	17	N	N	N				
		42	42	N	N	N				
		99	99	N	* I	* I				
		161	161	N	* I	* I				
		214	214	N	* I	* I				
		294	294	N	* I	* A I				
		360	360	A C	* A I	* A F I				
		420	420	A C	* A I	* A F I				
		436	60	I	* I	* I				
3	7/14	20	20	N	N	N				
		80	80	N	C	N				
		140	140	N	C	N				
		210	210	N	C	N				
		280	280	N	C	N				
		370	370	A S	A S	S I				
		440	440	* A S	* A S	S I				
		500	500	* A S	* A S	* A S I				
		600	2	S I	S I	S I				
4	7/16	160	160	* * A I	* * A I	N	N	N	N	
		300	300	* * A I	* * A I	S I	N	N	* I	
		500	500	* * A S I	* * A I	S I	I	A I	A I	
		670	19	* S I	* C I	* S I	* I	* I	* I	

* means many; *v* means very many; *f* means few individual specimens were agglutinated or cytolized, etc.

TABLE I.—*Continued.*

Exp. No.	Date.	Age of Germ Cells in Minutes.		Female Number.						Remarks.
		♀.	♂.	1.	2.	3.	4.	5.	6.	
5	7/19	130	130	N	N	N	N	N	N	
		240	240	N	N	N	N	N	N	
		350	350	N	N	N	C I	C	I	
		470	470	S	C I	C I	C I	C	C I	
		580	15	S	C I	C I	C I	I		
6	7/21	120	120	I	A F					
		330	330	F I	A					
7	7/7	75	75	N	N	N	N			
		110	110	N	N	N	N			
		470	10	S	N	N	N			
8	7/5	300	120	A S F I						
9	7/4	1200	20	A C I	A C I					Cooled 10° F.
		1200	20	A C I	A C I					75° F.
		1380	103	C	C I					
		1400	230	C	C I					
		2750	52	C	C					
		1500	20				F			
10	7/7	2790	20	S C	S C	S C	S C			
11	7/8	1390	21	S F I	S C I	S I	S I			
12	7/9	3040	21	F	F	F	F			
		hrs.	hrs.							*Hippo-*
13	6/19	23	23	S F I						*noë.*
		18	24	S						
14	6/24	24	12	F I						
		42	29	A S I						
		42	0	A S I						
		29	29	A S						
		29	0	A S						
15	8/14	1½	1½	I	I	I	I	I	N	*Arbacia.*
		5	5	I	I	I	I	I	N	
		24	1	S I	S I	I	I	S I	A I	
		49	1¼	A S I	A S I	A S I	A S I	A S I	A S I	

TABLE I.—*Continued.*

Exp. No.	Date.	Age of Germ Cells in Hours.		Female Number.						Remarks.
		♀.	♂.	1.	2.	3.	4.	5.	6.	
16	8/16	½	1½	I	I	I		I	I	
		18	18	I	I	I		I	S I	
		23	23	A I	A I	I		A F I	A S	
		24	4	I	S I	I		S I	S I	
		42	1	S I	S I	S I		A F C S I	A F C S I	
		42	⅙	S I	S C	S C I		A F C S	C	
		48	⅑	S I	S I	S I		A F C S		
		65	¾	F S I	S I	S		A F		
		70	1/11	S I	S I	F				
17	8/13	23	23	N	S	A	A			
		28	4	A S I	A S I	A S I	A	S I		
		28	28	A C	A C	A C	A	C		
		48	½	A F	A F C	A	A	A		
		49	½	A I	A	A	A	A		
	8/13	4	4½	I	I	I	I	I		
18	8/12	48	48	I						
		48	48	I						
		18	48	I						
		18	18	I						
		1/10	48	*f* I						
		1/10	18	*f* I						
19	8/18	1	23	N	N					
		1	45	N	N					
		1	68	N	N					
		5	73	N	N					
	8/18	42	⅛	S I	C	C I	A F C I	A F C I	C I	
		48	⅕	S I	C S I	S C I	S C I	C I	C I	
		65	¾	F S I	C S I	S C I	S C I	C I	C I	
		70	1/11	F S I	C S I	F S C I				
	8/18	7	25	I S	S I	S I				

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