THE INSEMINATION OF BODY CAVITY AND OVIDUCAL EGGS OF AMPHIBIA

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After exposure to sperm suspension, body cavity eggs of *Hyla crucifer*, *Bufo fowleri*, and *Rana pipiens* frequently exhibit various cortical changes such as crinkling, indentation, pigment migration, or flattening; but in all cases the eggs subsequently deteriorate. Oviducal eggs, however, which have passed through the expanded infundibulum (essentially a part of the body cavity) and the ostium (the abdominal orifice) may develop when inseminated (Born, 1892; Rugh, 1935, 1948; Good and Daniel, 1943; Aplington, 1949).

Inability to obtain normal development in amphibian eggs removed from the body cavity and exposed to sperm suspension has puzzled investigators since the late 1800's. Although a reservoir of unpublished information exists, published literature on the subject is meager. Barthélémy (1922) reported the initiation of development in appropriately treated[1] body cavity eggs of *Rana fusca* by pricking them with a needle dipped in blood or sperm although immersion in sperm suspension was unsuccessful. Born (1892) stated that an inseminated body cavity egg of *Triton taeniatus* attained the morula stage of development. Good and Daniel (1943), working with *Triturus torosus*, obtained irregular cleavage in a small percentage of fertilized coelomic eggs, one of which reached the neurula stage. Bearing on the problem is the work of Bataillon and others concerned physiologically with the activation of oviducal eggs (Bataillon and Tchou Su, 1930). Rugh (1948) comments that whether the answer to this dilemma rests within the jelly, or with changes in the egg, or with both, has not yet been determined. Indications are that both are concerned, as Good and Daniel (1943) found that the artificial addition of oviducal jelly to coelomic eggs markedly increased the number of eggs showing segmentation. They also found that between ovary and cloaca there is a rearrangement of yolk platelets in the egg, and that in fertilized coelomic as contrasted with fertilized oviducal eggs cleavage is much delayed and slower.

If amphibian eggs are regarded as mature when they have been surrounded with their full complement, *i.e.*, several layers of oviducal jelly, eggs taken from the body cavity or from the upper half of the oviduct are immature. Nevertheless, the transition is so abrupt between jellyless, non-fertilizable body cavity eggs and oviducal eggs fertilizable when only one layer of jelly is present that certain questions are warranted, particularly, are amphibian body cavity eggs immature either chromosomally or cytoplasmically as compared with oviducal eggs, and what is the role of the oviduct? The present paper is an attempt to supply answers to these questions on a morphological basis.

It is hoped that this material may be of use to other investigators who cooperatively may find the complete solution to this problem.

CHROMOSOMAL MATURITY

For the identification of chromosomes during maturation, five to twenty eggs from each of several representative regions of the reproductive tract of normally ovulating *Hyla crucifer*, *Bufo fowleri*, and *Rana pipiens* were fixed in P.A.F.,

[1]Immersion for 20–30 hours at laboratory temperature either in aerated grenouille serum or in a solution of NaCl, 7 parts in 1000 parts of dist. water, or the latter alkalinized with NaOH to n/200,000.

sectioned at 8 microns, and stained in Delafield's hematoxylin. Slides were then washed, decolorized in 1.0 percent HCl, washed, stained with eosin, washed, immersed in 1.0 percent NH_4OH, washed, and dehydrated. This procedure colors the yolk platelets pink in contrast to the chromosomes, which retain the hematoxylin.

Stages of maturation attained by *H. crucifer*, *B. fowleri*, and *R. pipiens* eggs here studied, during their passage from ovary to lower oviduct (includes uterus) are reported in table 1.

TABLE 1

Maturation of Bufo fowleri, Hyla crucifer *and* Rana pipiens *eggs in different regions of the reproductive tract*

Amphibian	Resolution of germinal vesicle	First polar spindle	First polar body formed	Second polar spindle
Bufo fowleri	ovary			
	ovulation	body cavity	body cavity	body cavity
		upper oviduct	upper oviduct	upper oviduct
Hyla crucifer	ovary	ovary	ovary	ovary
		body cavity	body cavity	body cavity
Rana pipiens	ovary			
	ovulation	body cavity	body cavity	
		upper oviduct	upper oviduct	upper oviduct
			middle oviduct	middle oviduct

Prophase changes, including resolution of the germinal vesicle, occur when the egg is still in or just leaving the ovary. In the several ovulating *Hyla* utilized, all eggs formed the second polar spindle either in the ovary or in the body cavity (fig. 1 and 2); eggs of *Bufo* attained this condition either in the body cavity or in the oviduct. In *Rana pipiens*, about one egg in ten formed a first polar body in the body cavity, usually near the infundibulum. The great majority formed the first polar body and second polar spindle in the upper or middle oviduct.

Once initiated, nuclear changes from germinal vesicle to second polar spindle appear to proceed quite independently of the location of the egg in its passage to the lower oviduct. There is clearly no inhibition of maturation in the body cavity of *Hyla*. Conversely, there is no apparent oviducal impetus to maturation in *Rana* because when the oviducts of *Rana* are ligated, eggs forced to remain in the body cavity go on to form a second polar spindle.

The heading "second polar spindle" is included in table 1 chiefly because it is employed in other studies on maturation reported in the literature. While there is an appreciable interval between resolution of the germinal vesicle and the expulsion of the first polar body (total time 2 to 4 hours, Le Brun, 1902) an interphase stage between the formation of the first polar body and the second

EXPLANATION OF FIGURES IN PLATE I

All figures X1550. Longitudinal axes of spindles 0.02 to 0.03 mm.

1. Ovarian egg of ovulating *Hyla crucifer*, showing first polar spindle and follicle cells. (The follicular sheath was torn at this point. These cells were superimposed from another section.)
2. Body cavity egg of *Hyla crucifer*, showing first polar body and second polar spindle.
3. Uterine egg of *Rana pipiens* (induced ovulation), abnormally in first polar spindle. This egg was fixed in P.A.F. 6 minutes after insemination.
4. Normal uterine egg of *Rana pipiens*, showing first polar body and second polar spindle.

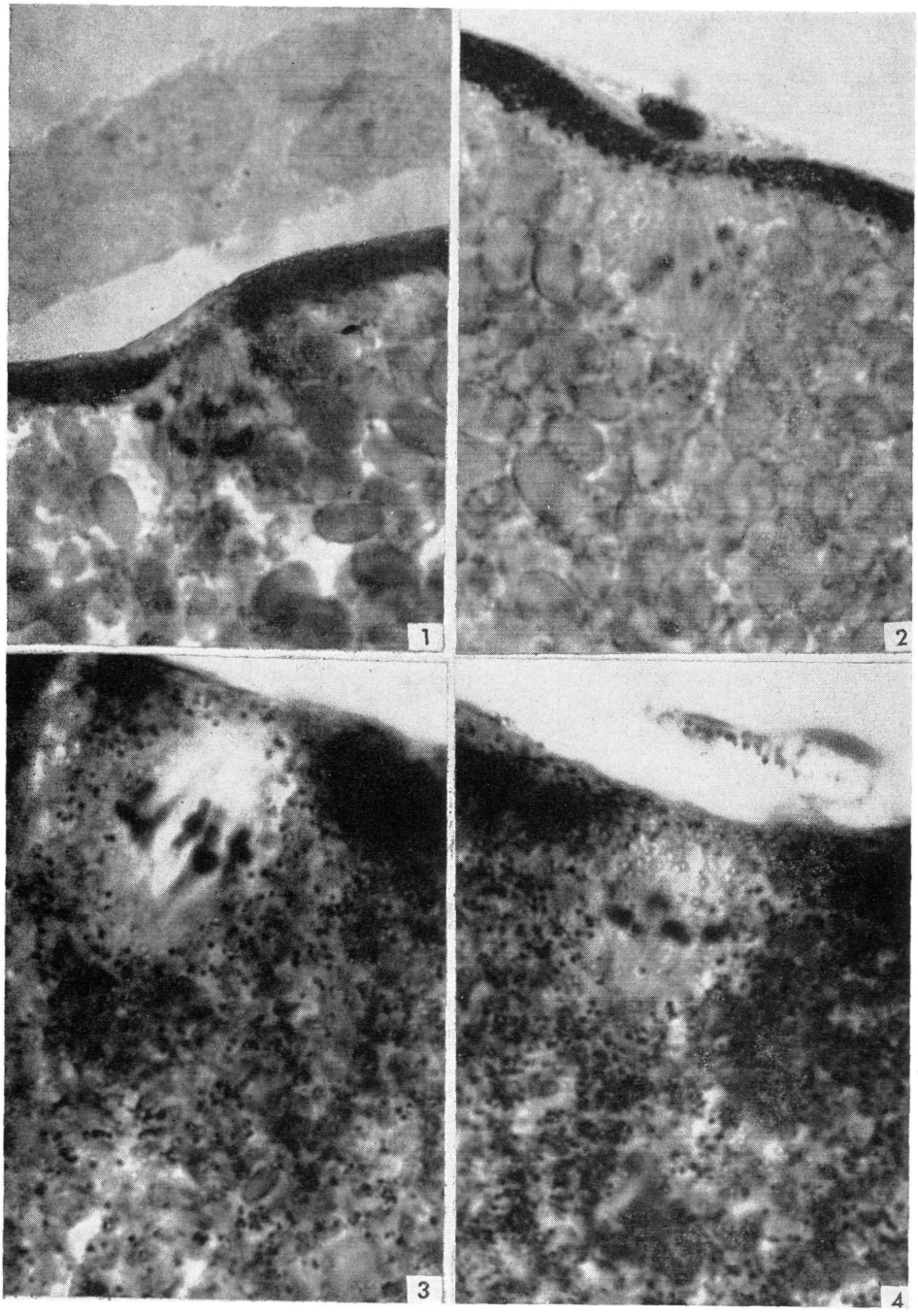
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TABLE 2

Maturation of amphibian eggs in different regions of the reproductive tract

Amphibian	Resolution of germinal vesicle	First polar spindle	First polar body	Second polar spindle	Investigator
Axolotl (*Ambystoma tigrinum*)		body cavity? upper oviduct	upper oviduct middle oviduct	lower oviduct	Jenkinson, 1905
Bombinator igneus	ovulation	body cavity	upper oviduct		Le Brun, 1901, 1902
Bufo americana	ovary	body cavity	upper oviduct middle oviduct		King, 1901
Bufo vulgaris	ovary	ovary	ovary	ovary	Le Brun, 1901, 1902
Cryptobranchus allegheniensis	ovulation	ovary body cavity oviduct	ovary body cavity oviduct	lower oviduct	Smith, 1912
Hynobius retardus	ovulation	body cavity? upper oviduct	upper oviduct	lower oviduct	Makino, 1934
Rana fusca (*temporaria*)	ovary	ovary body cavity	body cavity	oviduct	Wagner, 1923
Rana fusca (*temporaria*)	ovulation	body cavity upper oviduct		lower oviduct	Le Brun, 1901, 1902
Rana pipiens	ovulation	body cavity	oviduct	uterus	Rugh, 1948
Triton alpestris	ovary	body cavity upper oviduct	upper oviduct middle oviduct	lower oviduct	Carnoy and Le Brun, 1899
Triton alpestris	ovary	upper oviduct	upper oviduct		Le Brun, 1901, 1902
Triton cristatus	ovary	upper oviduct	upper oviduct		Le Brun, 1901, 1902
Triton taeniatus		body cavity	body cavity upper oviduct		Born, 1892
Triton taeniatus	ovary	body cavity upper oviduct	middle oviduct	lower oviduct	Le Brun, 1901
Triton torosus	ovulation	body cavity upper oviduct	body cavity upper oviduct	body cavity upper oviduct lower oviduct	Le Brun, 1902
Triton torosus	ovary	body cavity	body cavity upper oviduct	body cavity upper oviduct	Good and Daniels, 1943
Triturus viridescens	ovulation	body cavity	upper oviduct		Jordan, 1893

polar spindle is apparently of short duration. Such a stage is seldom figured in the literature, and when it is (Carnoy and Le Brun, 1899, fig. 14 and 15; Le Brun, 1902, fig. 38) there is merely a transient massing of the chromosomes without the formation of a nuclear membrane. Moreover, in the hundred or more body cavity and upper oviduct eggs here studied, no interphase condition following polar body 1 was found. For experimental purposes it seems reasonable to regard an egg from which polar body 1 has been expelled as being already in the second polar spindle, and these headings in table 1 are considered as essentially identical.

A summary of published accounts of egg maturation in amphibia with reference to the location of the egg during its reproductive passage is presented in table 2.

As in table 1, prophase changes are seen to occur uniformly when the egg is still in or just leaving the ovary. Beyond this point the data are best interpreted in the light of other facts concerning ovulatory phenomena. First, although the time of onset of ovulation is sudden in a given individual, the liberation of eggs from the ovary occurs gradually. Eggs ovulated later in the reproductive period are found to be in later stages of maturation than eggs ovulated early (Smith, 1912, *Cryptobranchus*).[2] Second, chromosomal maturation is proceeding in body cavity eggs which travel different distances to reach the ostia and which often must wait their turn at the crowded infundibulum before entering the oviduct.

The *Hyla crucifer* (table 1) and *Bufo vulgaris* (table 2), in which all maturation stages occurred in the ovary, were probably individuals examined late in their ovulatory cycle. In cases where the same species has been studied by two investigators and observations differ (*R. fusca*, *R. pipiens*, *T. alpestris*, *T. taeniatus*, *T. torosus*), the reported differences are probably a function of the variables outlined; and where no variability is reported (*B. igneus*, *B. vulgaris*, *R. pipiens* (Rugh), *T. alpestris* (Le Brun), *T. cristatus*, *Triturus viridescens*), it is likely that if larger numbers of eggs or individuals were examined differences in maturation time would be found also in these forms.

From an overall point of view it seems clear that in ovulating amphibia, some eggs mature to second polar spindle after entering the oviduct and are thus chromosomally immature in the body cavity while others mature to second polar spindle before entering the oviduct. It is the latter which are of particular interest because in this group are body cavity eggs which never develop following insemination but are chromosomally indistinguishable from oviducal eggs which usually fertilize and develop.

CYTOPLASMIC MATURITY

Although chromosomal maturation proceeds continuously from ovary to oviduct, by contrast, body cavity eggs exhibit cortical or cytoplasmic immaturities which are alleviated only after the egg enters the oviduct.

Unlike the eggs of *Triton*, *Triturus*, and other salamanders which are normally polyspermic (Frankhauser, 1941), oviducal eggs of Anura are monospermic. In *Rana* and *Hyla*, however, body cavity eggs in sperm suspension may be penetrated by many spermatozoa (up to 22 detected), visible with the Feulgen technique against the background of pigment granules and colorless yolk platelets. As observed under the dissecting microscope, puncture points can sometimes be identified in the eggs of *Rana pipiens* by minute streams of yolk platelets which flow outward into the water. It might be expected, a priori, that fertilization of coelomic eggs in the normally polyspermic salamanders would have a greater chance of success but such is not the case (Good and Daniel, 1943). Evidently

[2]There is some indication, unsubstantiated by definite proof, that in induced as contrasted with normal ovulation, the overall ovulation period is shorter and the stage of chromosomal maturation more uniform.

there is a change in the cortical properties of both Urodele and Anuran eggs after they enter the oviduct.

Following insemination a few eggs in any batch (*Rana, Hyla*) may undergo a brief period of abnormal and superficial "cleavage" confined to the upper hemisphere of the egg. Such eggs when sectioned show irregular concentrations of pigment granules reminiscent of sperm penetration paths, and vacuolization (apparently a degenerative phenomenon) of the cytoplasm. Several of these "cleavage eggs" (*Rana*) contained nuclear centers with atypical masses of chromosomes, while in others the egg chromosomes remained in second polar spindle metaphase, becoming atypical as the egg deteriorated. Chromosomes, if any, evolved from penetrating spermatozoa could not be identified. Other visible changes in these eggs (*Rana, Hyla*) are the downward migration of pigment until the egg (uncleaved) approximates the appearance of an early yolk plug stage, and flattening or mushrooming of the whole egg. The latter condition is somewhat less pronounced when the eggs are supported on jelly. Generally speaking, the smaller, lighter eggs of *Hyla* gave fewer abnormalities than eggs of *Rana*. Eggs of *Bufo fowleri* are less satisfactory to use because their heavy pigmentation cuts down visibility.

One *Hyla* egg attained what can possibly be regarded as an advanced stage of cleavage (fig. 5 and 6) because a number of the cells contained nuclear centers. But even in this case, the cleavage was abnormal in its largely peripheral distribution and irregularity in cell size.

Thus, macroscopic and microscopic evidence would seem to indicate that the failure of body cavity eggs to fertilize should be attributed to cortical or cytoplasmic rather than to chromosomal immaturity.[3]

THE ROLE OF THE OVIDUCT

In contrast to body cavity eggs, eggs taken from any region of the oviduct and fertilized in pond water or .1 Ringer's solution may develop (Rugh, 1935, 1948). It is my observation that in the critical region of the oviduct, *i.e.*, in the upper several centimeters where conditions are transitional, the number of eggs which fertilize diminishes as the ostium is approached. In this region the eggs are much crowded, the first layer of jelly is scarcely complete, and any manipulation is damaging. When immersed in sperm suspension, the surface of these eggs crinkles as does that of body cavity eggs, and in non-fertilizable eggs punctures are occasionally produced. When the first layer of jelly is completed and the second added as the egg proceeds down the oviduct, the cortex (? egg membrane) becomes firmer, crinkling disappears, puncture points never occur, and the pigment appears more sharply delineated. While some eggs abruptly acquire the capacity to be fertilized, others are somewhat slower to do so.

It may be argued that in *R. pipiens* and *B. fowleri* the lowered percentage of

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EXPLANATION OF FIGURES IN PLATE II

Figures X130

5 and 6. Two sections through an inseminated body cavity egg of *Hyla crucifer*. Nuclear centers (not visible at this magnification) were present in a number of the cells. Note irregular and peripheral cleavage and pseudo-cleavage.

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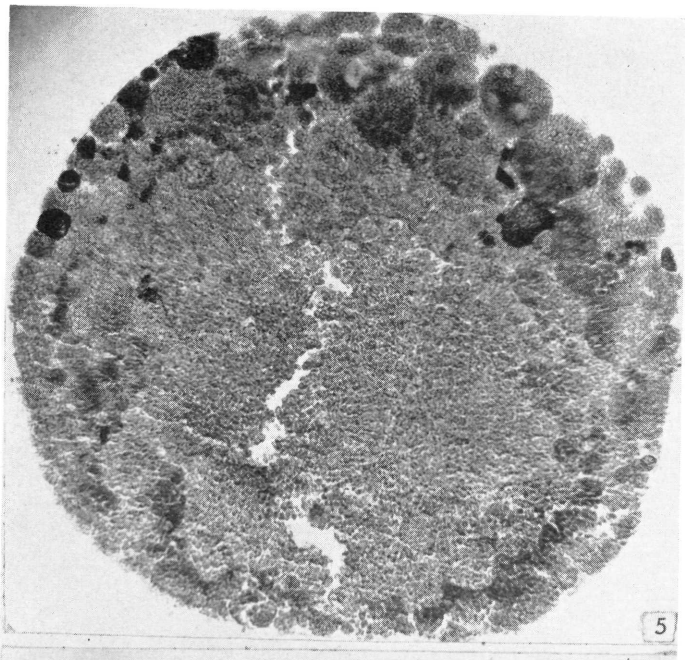
[3]It is known that histochemical changes, *e.g.*, the redistribution of sulphydryl compounds and RNA occur in the egg following the breakdown of the germinal vesicle, but at the present time these changes are not well understood. Whether, as seems likely, they contribute to the cytoplasmic maturity of the egg including the establishment of bilateral symmetry, whether their distribution is aided by egg rotation in the oviduct, etc., remains hypothetical. See Fankhauser (1948).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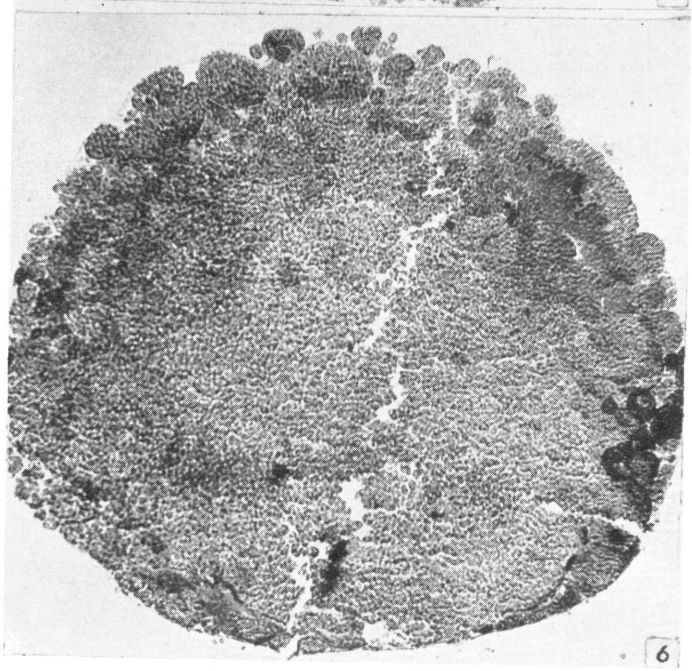
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fertilization in the upper few centimeters of the oviduct is traceable to eggs which are chromosomally immature, but this is certainly not the case in *H. crucifer* where body cavity and oviducal eggs were chromosomally alike. Furthermore, in *Rana pipiens* uterine eggs aberrantly in an earlier stage of maturation than many body cavity eggs will fertilize and develop normally. This was accidentally discovered in one batch of eggs (induced ovulation) of which half were stripped into fixative, the rest inseminated and allowed to develop. On examination the former exhibited not the expected second polar spindle (fig. 4) but a first polar spindle (fig. 3). The chromosomes were clearly tetrads; no polar body was present.

Whatever the conditioning effect of the oviduct on the egg may be, it is not species specific, at least between *B. fowleri* and *R. pipiens*. When ovaries are removed from a non-ovulating *R. pipiens* and body cavity eggs from a normally ovulating *B. fowleri* are transferred by pipette to the *pipiens* body cavity, these eggs on passing through the oviduct fertilize and develop after insemination with *fowleri* sperm suspension. They will also develop when taken from different regions of the oviduct. Coelomic eggs of *R. pipiens*, placed in the *B. fowleri* body cavity, recovered from the oviduct and inseminated with *R. pipiens* sperm suspension, will also develop normally.

SUMMARY

1. After exposure to sperm suspension, body cavity eggs of *Hyla crucifer*, *Rana pipiens*, and *Bufo fowleri* subsequently deteriorate. Oviducal eggs, however, may develop following insemination. The transition between jellyless, non-fertilizable body cavity eggs and upper oviduct eggs fertilizable when only one layer of jelly is present is rapid.

2. On a morphological basis, it is suggested that the failure of body cavity eggs to fertilize is attributable to cytoplasmic rather than to chromosomal immaturity.

3. Cytoplasmically: inseminated body cavity eggs may exhibit polyspermy instead of normal monospermy (anura), yolk platelet extrusion, abnormal and superficial cleavage, unusual pigment migration, crinkling, and flattening. These conditions are remedied as the egg receives its first coat of jelly in the upper oviduct.

4. Chromosomally: in *Hyla crucifer*, body cavity eggs may complete their maturation before entering the oviduct; in at least 6 other species of amphibia some body cavity eggs are in the same stage of maturation as some oviducal eggs, and in *Rana pipiens*, although normally fertilized when in second polar spindle, eggs in the first polar spindle will fertilize only after they have entered the oviduct.

5. The role of the oviduct is shown to be non-specific. Between *B. fowleri* and *R. pipiens* reciprocal transfer of body cavity eggs followed by their recovery from the oviduct and fertilization in parental sperm suspension results in normal development.

6. Published accounts of maturation in amphibia are summarized and discussed.

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