FERTILIZABILITY OF EGGS OF RANA PIPIENS AT VARIOUS OVIDUCAL LEVELS

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ABSTRACT

FERTILIZABILITY OF EGGS OF RANA PIPIENS AT VARIOUS OVIDUCAL LEVELS

BY ROBERT N. GLICK

These experiments concern the fertilizability of eggs taken from the upper, middle, and lower levels of the oviducts and the uterus at different time periods.

Female frogs were stimulated to ovulate by injection of pituitary glands. The oviducts were removed at forty-eight, fifty-four, sixty, and seventy-two hour intervals after initial stimulation by pituitary injection. The oviducts were removed and cut into three equal sections. The eggs were then removed from the three oviducal levels and the uterus and inseminated. The eggs were observed approximately twelve hours after insemination for cleavage. The results were recorded as percentage of eggs which cleaved out of the total number of eggs from each level.

Analysis of the results indicate differences in fertilizability of eggs taken from the different oviducal levels. In general, the eggs were fertilizable in low numbers in the upper oviducal level, and there

was an increase in fertilizability in the middle and lower levels with another increase in fertilizability in the uterus. The time lapse after initial stimulation by pituitary glands was very important in the acquisition of fertilizability.

The results are discussed in terms of egg jelly and the state of maturation of the eggs at insemination.

FERTILIZABILITY OF EGGS OF *RANA PIPIENS* AT VARIOUS OVIDUCAL LEVELS

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INTRODUCTION

It has long been known that amphibian eggs without their jelly coat are non-fertilizable. They can, however, be activated parthenogenetcally (Bataillon, 1919). Bataillon (1919) also found that eggs of a European frog when dejellied with potassium cyanide could be artificially stimulated if pricked with a small glass needle in the presence of blood. However, dejellying in this manner rendered the eggs non-fertilizable.

The eggs of toads when extruded are covered with four layers of jelly. The outer two layers form a common tube with which all the eggs are covered. In addition, each egg is individually covered by two layers of jelly. Kambara (1953) using the toad, Bufo vulgaris formosus, found that the second layer of jelly around the egg (C layer) was indispensable for fertilization. He also found that by covering dejellied eggs with jelly from toad eggs, with agar or with gelation, they become fertilizable. He concluded that the second layer of jelly is important for stimulating the thigmotatic response of the spermatozoa.

Tchou and Wang Yu-lan (1956) studied the role of

the egg jelly coats in the Asiatic toad, Bufo bufo asiaticus. Using both coelomic and dejellied eggs at various stages of maturation they showed that eggs without their jelly were non-fertilizable. By introducing coelomic or dejellied eggs into the oviducts, which thereby coats them with jelly, they become fertilizable. In order to test the importance of the inner and outer layers of jelly, eggs were removed from the oviducts at various levels and were found to be fertilizable at either the upper, middle, or lower portions of the oviducts. Jellyless eggs of urodeles will also become fertilizable if coated with jelly secretions taken from the oviduct. This was shown by Good and Daniel (1943) in the eggs of the newt, Triturus torosus.

It is clear that amphibian eggs, thus far studied, taken before they have acquired a jelly coat or after the jelly has been removed, are non-fertilizable and that they become fertilizable by passage through the oviducts where they acquire their jelly coats. This has also been shown to be true in the frog, Rana pipiens, by Arnold and Shaver (1962). However, there appears to be some question as to the level of the

oviduct at which fertilizability is acquired. (cf. Rugh, 1951, page 54, who states, "As soon as the egg (of the frog) enters the oviduct and begins to acquire an albuminous (mucin-jelly) covering, it becomes fertilizable.") No reference to the original data supporting this statement is cited, however.

The jelly coats of the eggs of the frog, Rana pipiens, consist of three distinct layers of jelly (Rugh, 1935). Each egg receives all three layers. There is no common tube of jelly as in the toads. As stated before, the jelly is put on the eggs as they descend the oviducts. The innermost layer is put on in the upper portion with the outer two layers being applied in the middle and lower portions of the oviducts.

Barch and Shaver (1963) have been able to demonstrate regional antigenic differences in the oviducts of the Rana pipiens using the agar diffusion technique of Ouchterlony. They have found some components common to the upper, middle, and lower thirds of the oviducts. There are also specific components found only in the upper or lower thirds and also components shared by the lower middle levels

and the upper middle levels. Shaver, Barch and Shivers (1962) have previously shown that frog egg jelly antigens are restricted to the oviducts, with the exception of the ovary in which occasionally components were found that were identical with certain egg jelly antigens. This exception may be explained by the possibility of contamination of the jelly with eggs, or more probably it is due to the release of an egg substance into the jelly.

Shivers (1961) demonstrated that several components are shared among the egg jellies of four species of Rana but that each species has specific egg-jelly components. This same author (Shivers, 1962) has also shown that the specific components are found in the two outer layers of the jelly coat.

In view of the antigenic differences found in the jelly components secreted by the different levels of the oviducts, it would be of interest to determine if any differences in fertilizability exist in eggs taken from the different levels. The data resulting from the experiments to be described indicate that differences in fertilizability of eggs of Rana pipiens do exist at different oviducal segments and also that the period of time ensuing from the onset of ovulation is of importance in the acquistion of fertilizability.

MATERIAL AND METHODS

Rana pipiens were used throughout the experiments. They were obtained from dealers in Vermont and Wisconsin.

The experiments were of two types. One type was designed to measure the fertilizability of eggs as a function of the elapsed time from the initial stimulation of the female to ovulation by means of injection of pituitary glands (time of initial injection equals zero time.) The other kind of experiment measured the fertilizability of eggs taken from different levels of the oviducts.

Female frogs were injected on each of two successive days, at the time when it was decided to test fertilizability, the frogs, were pithed, and the oviducts removed. The oviducts were then rinsed in 0.1% of full strength Holtfreters solution and put on a smooth glass plate. The oviducts were uncoiled and cut into three equal parts which were designated upper, middle, and lower segments. The eggs were removed by cutting each segment into sections not exceeding two cm. in length and forcing the eggs out by gently pulling a section under a pithing needle. The eggs were then put on glass

slides and inseminated. At the same time eggs taken from the uterus were fertilized in the same manner to serve as an indicator of the general viability of the eggs. After exposure to a normal sperm suspension for ten minutes, the eggs were transferred to finger bowls containing large amounts of areated tap water and kept in a constant temperature room at seventeen degrees Centigrade. The eggs were observed for cleavage approximately twelve hours after insemination. The results of inseminating eggs from each level and from the uterus were recorded as percentages of eggs cleaving of the total number in each class.

The procedure described above was repeated at 48, 54, 60, and 72 hours after initial stimulation with pituitary.

A concentrated sperm suspension was made by crushing two testes in five milliliters of 0.1% Holtfreters solution. The normal sperm suspension usually employed in the experiments was prepared by macerating one testes in five milliliters of fluid.

RESULTS

The Lindquist (1953) Type One design was used for the analysis of variance. In this design the levels of the oviduct represent one factor, and the time after the first injection of pituitary glands is the second factor. Eggs from each level of the oviduct (first factor) were tested for fertilizability at each time period (second factor). The data were then analysed to show if there were significant variations from chance in fertilizability of eggs due to either the time after pituitary stimulation or according to the level of oviducts from which they were taken. The interaction between the two factors was also tested. The raw data in percentages are presented in Table 1 (in the appendix).

The results of the statistical treatments indicated that the variation due to the two factors and the interactions between them were significantly different from chance at the .005 level. (See Table 2 of F values. Appendix) The statistical design of these analyses renders the variation due to differences between the eggs of different females irrelevant.

The sequential Q method (Snedecor, 1956) was used to compare the order of variation of fertilizability

of eggs from different levels of the oviducts within a specified time period as well as comparing the variation of levels between different time periods. (Shown graphically in Figure one)

The results of the sequential Q analysis indicated that at the forty-eight hour time period there was no significant difference in fertilizability between the upper, middle and lower levels. Eggs taken from the uterus at this time period had significantly higher fertilizability than those taken from the other three levels. Comparison of eggs taken from the upper oviducal level at four time periods(48, 54, 60, 72 hrs.) demonstrated that they are of the same level of fertilizability and significantly lower than those at any other level. However, the eggs taken at forty-eight hours from the middle, lower and uterine levels were significantly lower in fertilizability than eggs from the corresponding levels at fifty-four, sixty, and seventy-two hours after initial stimulation. This would indicate that eggs at the forty-eight hour time period were not as capable of fertilization as the eggs at the other

three time periods from there oviducal levels.

When eggs from all oviducal levels at the fifty-four hour time period were compared to each other, it was found that the upper level had a significantly lower number of eggs cleaving than the remaining three levels. The uterine eggs showed a higher degree of fertilizability than the upper, middle or lower levels, while the middle and lower levels were not significantly different from each other. This shows that after the eggs entered the upper level of the oviduct, fifty-four hours after initial stimulation, a small number became fertilizable, and as they passed through the middle and lower portions of the oviduct, their fertilizability increased, finally reaching usual levels of fertilizability.

The eggs taken from the fifty-four, the sixty and the seventy-two hour time periods are equally capable of being fertilized but, as shown above, eggs from the upper oviducal level are never fertilizable to the degree that those from the more posterior levels are. This is demonstrated in Figure I. Comparing the fifty-four hour middle,

lower, and uterine level eggs with those taken from the same levels in the sixty and seventy-two hour time periods, the fertilizability of eggs from the middle level of the fifty-four hour time period is not significantly different from the fertilizability of eggs from the middle levels of the sixty and seventy-two hour time period. The same is true of the lower and uterine levels when compared in the same manner, however, uterine eggs from 54 to 72 hours always cleaved in higher numbers than those at the more anterior levels.

When the eggs were observed, it was noticed that the eggs from the middle and lower levels of the oviducts were always two to four cleavage periods ahead of the eggs taken from the uterus (Plate one) although a significantly greater number of uterine eggs cleaved. Differences in the rate of cleavage were always observed regardless of the concentration of the sperm suspension used to fertilize the eggs or the time period from which the eggs were taken. This difference in cleavage rate persisted at least up to gastrulation.

FIGURE I

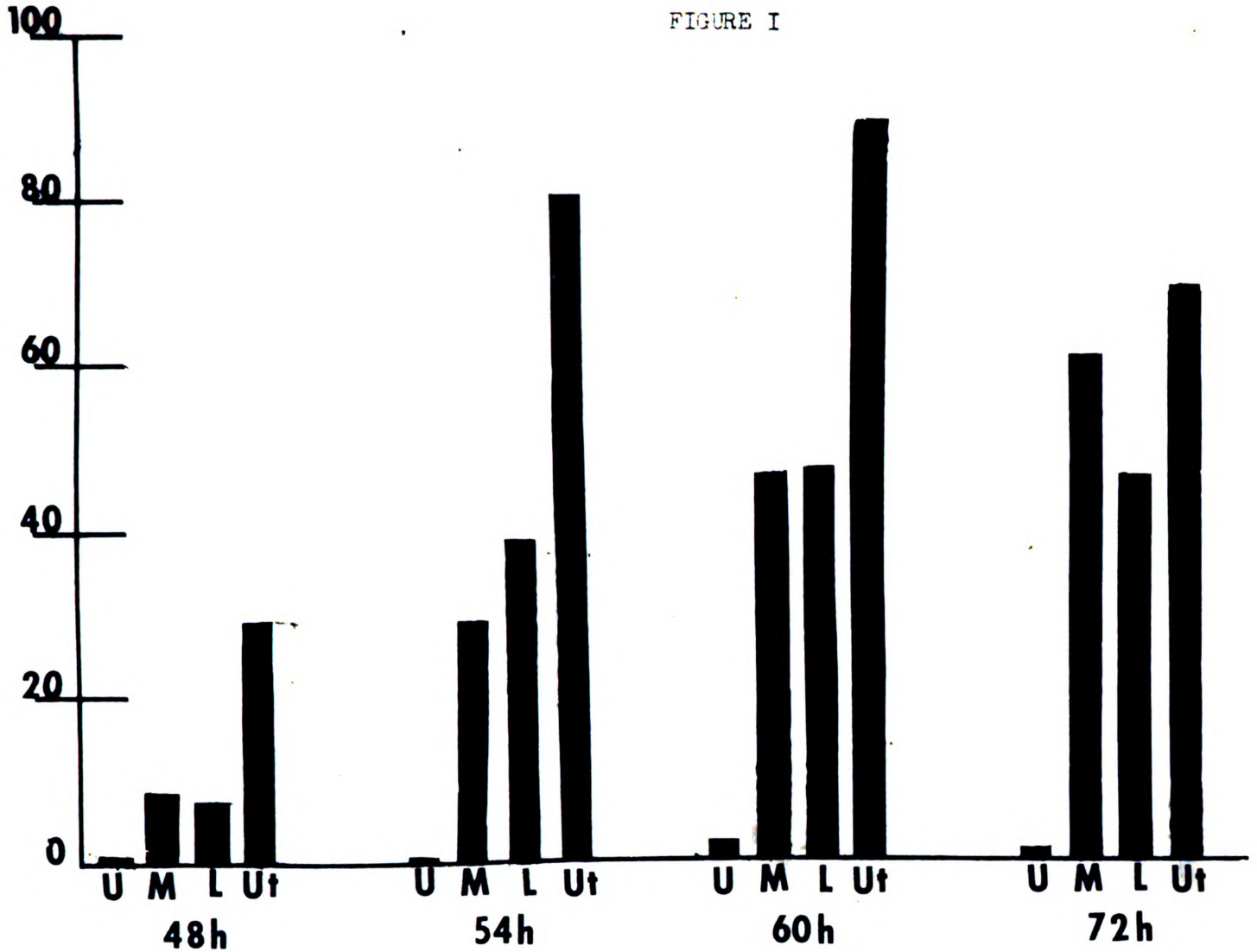


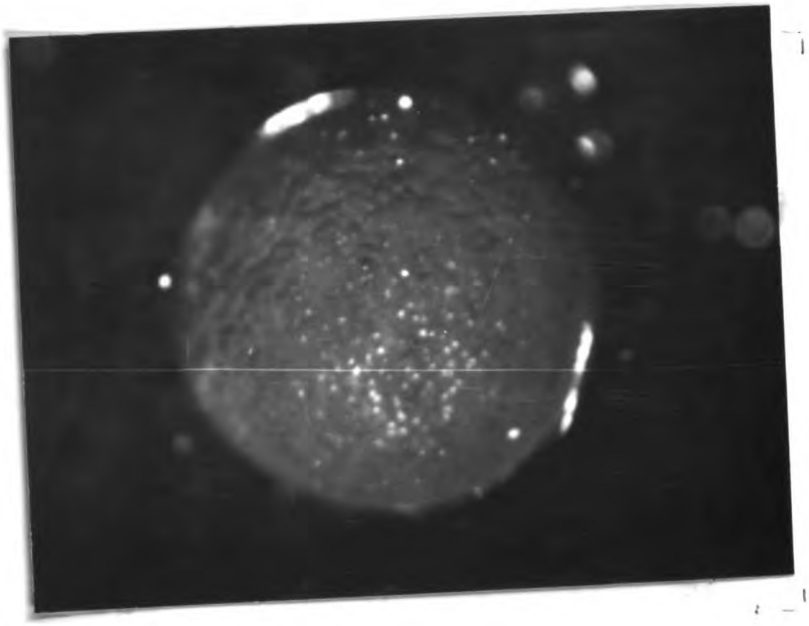
PLATE I

Pictures showing the difference in stage of cleavage of eggs from various levels of the reproductive tract.

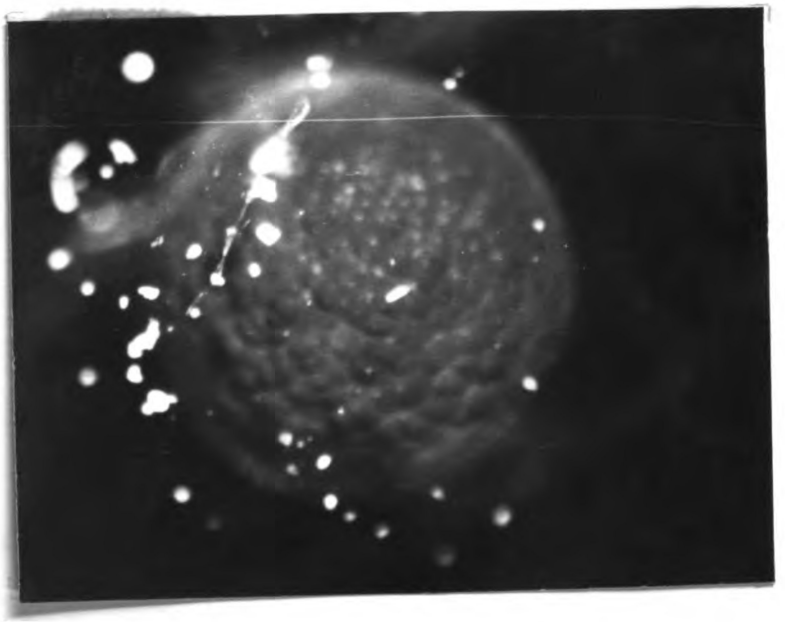
Picture A. Egg taken from the middle third of oviduct.

Picture B. Egg taken from the uterus.

PLATE 1



a.



b.

DISCUSSION

The importance of amphibian egg jelly in fertilization is well known, but its role in fertilization is not. It is apparent that in acquiring the jelly coat in the oviducts the eggs in some manner become capable of fertilization. However, this is not just a matter of maturation because body cavity eggs, which have never been exposed to jelly, are capable under certain conditions of being parthogenetically stimulated (Bataillon, 1919, Tchou-Su et Wang Yu-Lan, 1956). Subtelny and Bradt (1961) demonstrated the developmental capabilities of jelly-less body cavity eggs by transplanting nuclei of blastula cells into both uterine and body cavity eggs. They found that eggs from both sources developed equally well indicating again that body cavity eggs are capable of development.

Artificial stimulation by pricking with a needle, or by inoculation, however, imitates the penetration of the egg by a spermatazoön. Evidently, prior contact of the spermatazoa with jelly is necessary for normal fertilization. It is of interest that Shivers (personal communication) has found that body cavity eggs of Rana pipiens, when

inseminated with homologous sperm that had previously been exposed to egg water, (i.e. jelly components) were fertilizable.

The difference in fertilizability of eggs taken from the different levels of the oviduct may possibly be explained by considering the initial union of the gametes as an antigen-antibody mechanism with the jelly coat containing components capable of combining with the sperm. This possibility is suggested by data indicating that as the egg acquires its jelly coat, it becomes capable of being receptive to the spermatazoa. The increase in fertilizability of the eggs from the middle or lower portions of the oviduct as compared with the fertilizability of eggs taken from the upper level of the oviduct in a given time period may be due to specific sperm reception sites being put on in the outer two jelly layers. This interpretation would fit the work of Shivers, (1962) who demonstrated that the species specific components of egg jelly are found in the outer two layers of jelly. The antigenic differences in the jelly components secreted by the different levels of the oviduct as demonstrated by Barch and Shaver (1963)

may also be pertinent in this connection.

The idea of an antigen-antibody model of a mechanism for fertilization is not a new one. Frank Lillie (1912), working with sea urchins, used this concept to explain the agglutination of homologous sperm by sea water that had been exposed to eggs. Since then, considerable work has been done with sea urchins in connection with the role of fertilizin and antifertilizin in fertilization. (See Tyler, 1955, and Rothschild, 1956, for reviews)

Sperm agglutinins have been reported in Rana clamitans but not in Rana pipiens (Bernstein, 1952). He suggests that the absence of sperm agglutinins in R. pipiens is due to the relative insolubility of R. pipiens jelly or that the R. pipiens fertilizin (agglutinin) is univalent.

The uterus in the frog is a thin walled sac that serves as a storage place for the eggs and has no known secretory functions. Therefore, the large increase in fertilizability of eggs taken from the uterus as compared with eggs taken from the middle and lower thirds of the oviducts cannot be explained by the activity of specific jelly components, as they already have their complete complement of jelly when

deposited in the uterus. The data indicate that in some manner the fertilizability of the eggs is increased by storage in the uterus. Possibly, substances are secreted from the egg at this time or a period of storage in the uterus is necessary for molecular rearrangements at the egg surface or within the jelly. This is purely speculative at this time as there is no data to indicate these possibilities.

The fertilizability of eggs taken from the middle, lower and uterine levels forty-eight hours after initial stimulation is significantly lower than that of eggs taken from corresponding levels at the fifty-four, sixty, and seventy-two hour time period. Evidently, the factor of time is of importance since the eggs at forty-eight hours have not "matured" sufficiently. The data indicates that the eggs of the fifty-four through the seventy-two hour time periods are equally capable of fertilization. This indicates that fifty-four hours after the initial stimulation by pituitary injection the eggs are fully capable of fertilization, as far as the time factor is concerned.

The frog egg is usually considered "mature" when the egg spindle is in the metaphase of the second meiotic

division, and the egg is at this stage when it is fertilizable (Rothschild, 1956). The egg nucleus usually undergoes the first meiotic division in the upper segment of the oviduct or in the body cavity and continues to the metaphase of the second meiotic division at which it stays until fertilization. It might be argued that perhaps the eggs that did not fertilize at forty-eight hours had not reached the second meiotic metaphase. However, if this were the case, then there would be a progressive increase in fertilizability of eggs taken from the middle, lower and uterine levels at each succeeding time period as more eggs reached the second meiotic metaphase. This is not the case, for as stated before, eggs taken from the middle, lower and uterine levels of the fifty-four hour time period are not of significantly different fertilizability when compared to eggs from the corresponding levels of the sixty and seventy-two hour time periods. Thus, another "maturing" process must be involved, in view of the unfertilizability of body cavity eggs, or the low fertilizability of eggs from the upper oviducal level. The data from the present experiments suggest that this additional factor may

reside in the jelly layers deposited in more posterior levels. However, eggs removed from middle and lower oviducal levels, or from the uterus at forty-eight hours, are not as fertilizable as those taken at later periods, suggestion that still other factors may be involved in the final "maturing" process.

The data from the present experiments do not support the experiments of Bataillon and Tchou-Su (1930) in which non-fertilizability of eggs from the oviducts and eggs just arrived in the uterus was attributed to anesthetization by CO2. Assuming that their data were correct, in this case then, the eggs taken from the oviduct would become progressively non-fertilizable as they descended the oviducts into the uterus. This is not the case in these experiments as the data indicates the fertilizability of the eggs progressively increases as they descend the oviduct and reaches normal fertilizability in the uterus. Also, CO2 anesthetization would not explain the advanced cleavage stage of the oviducal eggs over the uterine eggs. There is no obvious explanation for the difference in cleavage rates observed between uterine and oviducal eggs. The problem of the advanced cleavage stage of the eggs taken from the oviduct has at least two aspects.

One is that the oviducal environment may enable the egg to cleave at a faster rate. The other aspect is that the environment of the uterus may cause an inhibition of cleavage rate.

SUMMARY

This project was undertaken to determine if any differences in fertilizability existed in eggs taken from the different levels of the oviduct, in view of the antigenic differences found in the jelly components secreted by the different levels of the oviducts.

The data indicates that there are differences in fertilizability between levels of the oviducts with the exception of the oviducal levels of the forty-eight hour time period. In this time period eggs of the upper, middle, and lower oviducal levels were not significantly different in fertilizability but were of significantly lower fertilizability compared to uterine eggs of the same time period. The eggs of the forty-eight hour time period were significantly lower in fertilizability than eggs taken from the oviducal levels of the fifty-four, sixty, and seventy-two hour time periods. But, the eggs from the upper level of all time periods were not significantly different. It was found that the eggs of the fifty-four through seventy-two hour time periods became fertilizable to a small degree on entrance into the upper oviducal level and became significantly more fertilizable in the middle and lower levels with another significant increase after entering the uterus. The

eggs taken from the middle and lower levels of the later three time periods were not significantly different from each other. There, the period of time ensuing from the initial stimulation by pituitary glands to between 48 and 54 hours was found to be of importance in the acquisition of fertilizability.

Eggs taken from the upper, middle, and lower levels, when fertilized, cleaved at a different rate that the eggs taken from the uterus. They were approximately two to four cleavage stages ahead of the uterine eggs.

The results of these experiments are discussed in terms of jelly layers applied to the eggs, and the state of maturation at the time the eggs were inseminated.

TABLE I*

No. of frogs	Time period	Level of oviduct	Percent cleavage
15	48hrs.	upper	0.6%
		middle	8.1%
		lower	7.8%
		uterus	28.8%
14	54hrs.	upper	1.14%
		middle	28.2%
		lower	38.8%
		uterus	80.1%
8	60hrs.	upper	2.75%
		middle	46.1%
		lower	47.8%
		uterus	88.5%
5	72hrs.	upper	1.8%
		middle	61.7%
		lower	46.4%
		uterus	69.6%

* Summary of data from six experiments

TABLE II

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-values	
Between subjects	62,694.76	41			
					d.f.-3/38
Hours	21,658.35	3	7,219.45	6.69	Significant at .005 level
Error (b)	41,036.41	38	1,079.91		
Total	158,354.62				
					d.f.-3/114
Levels A	56,233.67	3	18,744.56	89.54	Significant at .005 level
AB	90,453.50	15			
					d.f.-9/114
AB	15,561.48	9	1,729.05	8.26	Significant at .005 level
SwB	41,036.41				
ws	95,659.86	114			
Error (w)	23,864.71		209.34		

Analysis of variance Table

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