The Fertilizability of Coelomic and Oviducal Eggs of the Toad, *Bufo bufo formosus*[1),2)]

By

Chiaki Katagiri

Zoological Institute, Hokkaido University

(With 6 Text-figures and 5 Tables)

It has been shown by previous workers (Bataillon, '19; Good & Daniel, '43; for recent reviews see Shaver & Barch, '60) that coelomic amphibian eggs are not fertilized, and the eggs become fertilizable during their passage down the oviduct into the uterus. In the oviduct several layers of jelly envelopes, known to be essential for fertilization, are secreted progressively around the eggs (Rugh, '35; Barch & Shaver, '63). Further, it is in the coelom and the female genital tract that the eggs undergo maturation division from the germinal vesicle stage to the second meiotic metaphase (Aplington, '57). Presumably because of this complexity, data obtained for the fertilizability of oviducal eggs have varied considerably according to the technique and the species used (Kambara, '53; Tchou & Wang, '56; Nadamitsu, '57; Glick & Shaver, '63). In view of recent knowledge concerning the role of jelly envelopes in fertilization (Shaver & Barch, '60; Shivers & Metz, '62; Katagiri, '66), it seems important to determine at what level of the oviduct the eggs attain fertilizability, and which jelly layers are indispensable for fertilization. Research into the source of the unfertilizability of coelomic eggs may also provide information concerning the role of jelly envelopes and of the oviduct in fertilization.

The present paper deals with the fertilizability of toad eggs taken from different levels of the oviduct. Results of an attempt to fertilize coelomic eggs will also be presented.

Material and methods

The material used was the toad, *Bufo bufo formosus*, obtained from a dealer in Tokyo. Ovulation was induced by subcutaneous injection of toad pituitaries suspended in 1/2 strength De Boer's solution (1/2 DB). The time required for the occurrence of ovulation

1) This paper is dedicated to Professor Sajiro Makino, Zoological Institute, Hokkaido University, Sapporo, in honor of his sixtieth birthday, June 21, 1966.

2) Contribution No. 174 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. 15, 1965.

after injection depended, of course, upon the season of year, the amount of pituitaries injected, and the females used. From November to January two successive injections, each of 2 pituitaries, were needed at an interval of 24 hr. In February and March a single injection of 1 or 2 pituitaries was sufficient to induce ovulation. In the cases where a large number of coelomic eggs was needed, the uppermost region of the oviduct was ligated before pituitary injection, without anesthetization of the female. On keeping the injected females at 20°C for 17–24 hr., eggs were found in the coelom, oviduct and uterus of an individual. Coelomic eggs were washed several times either with 1/2 DB or De Boer's solution (DB) to remove excess debris, and stored in DB at 5°C. Oviducal eggs were obtained in the following manner. An oviduct filled with eggs in line was cut into several segments and each segment was placed separately in DB. The eggs were removed by breaking the oviducal wall with watchmaker's forceps. In most experiments fully jellied uterine eggs served as a control for the viability of eggs. Artificial insemination was performed by the ordinary method, with a sperm suspension prepared by macerating a testis in 10 ml of 1/20 strength De Boer's solution (1/20 DB). The results were expressed as the percentage of cleavage by counting fertilized eggs at blastula or gastrula stage. The method of insemination in the presence of dialyzed jelly or polyvinylpyrrolidone (PVP) solution has been given elsewhere (Katagiri, '66).

Results

Fertilizability of eggs taken from different levels of oviduct. The jelly envelopes, when fully deposited, are composed of 4 layers. The inner two layers constitute concentric layers, and the outer two form a continuous tube which encases the former in a row[1]. In the present paper the jelly layers will be designated J_1, J_2, J_3 and J_4, from the inner to the outer ones.

In the first experiment, the fertilizability of eggs taken from different parts of the genital tract was studied. Out of 18 batches, the results obtained in 2 females are shown in Table 1. It is clear from the table that none of the coelomic eggs or the eggs taken from the uppermost oviducal region, invested with J_1 only, are fertilizable. Fertilization invariably occurs in the eggs taken from the lower oviducal region and the uterus. In the bacthes A and B of Table 1, however, there is a considerable difference in the fertilizability of eggs taken from the upper and middle oviducal regions. In batch A, the fertilizability is very much low, even if J_3 is added around the eggs. In batch B, on the other hand, the eggs taken from the upper oviducal regions are highly capable of fertilization when the layer J_2 is added. The eggs, taken from the upper and middle oviducal regions and not fertilized upon insemination, often showed remarkable wrinkling of the egg surface and pigmentation changes. The difference observed between batches A and B was that in the former most eggs remained in the coelom, whereas in the latter most eggs had passed through the oviduct into the uterus, with few eggs remaining in the coelom. This may indicate the difference of time after the onset of ovulation in these batches. Repeated experiments employing several batches often resulted in similar variances in fertilizability of the eggs taken from the upper and middle

1) For detailed description of the structure of jelly envelopes, see Kobayashi ('54).

oviducal regions. In some cases the fertilizability tended to increase as the amount of the layer J_2 increased, as reported by Kambara ('53).

Table 1. Fertilizability of eggs taken from different levels of oviduct.

Batch No.	Location of eggs	Distance from ostium (cm)	Jelly layer*				No. of eggs used	Percentage cleaved
			J_1	J_2	J_3	J_4		
A	Oviduct (I)	0 - 5	(+)	−	−	−	29	0
	Oviduct (II)	5 - 8	+	(+)	−	−	4	0
	Oviduct (III)	8 - 11	+	+	−	−	10	0
	Oviduct (IV)	11 - 14	+	+	−	−	11	0
	Oviduct (V)	14 - 20	+	+	+	−	24	0
	Oviduct (VI)	20 - 25	+	+	+	−	18	5.6
	Oviduct (VII)	25 - 31	+	+	+	+	27	48.1
	Oviduct (VIII)	31 - 39	+	+	+	+	30	86.7
	Oviduct (IX)	39 - 47	+	+	+	+	26	100.0
	Oviduct (X)	47 - 52	+	+	+	+	28	100.0
	Coelom	——	−	−	−	−	37	0
	Uterus	66 -	+	+	+	+	21	100.0
B	Oviduct (I)	0 - 5	+	(+)	−	−	27	29.6**
	Oviduct (II)	5 - 9	+	+	−	−	17	100.0
	Oviduct (III)	9 - 15	+	+	(+)	−	33	100.0
	Oviduct (IV)	15 - 23	+	+	+	−	45	100.0
	Oviduct (V)	23 - 32	+	+	+	+	35	100.0
	Oviduct (VI)	32 - 40	+	+	+	+	28	100.0
	Coelom	——	−	−	−	−	32	0
	Uterus	76 -	+	+	+	+	35	100.0

* +, present; −, absent; (+) present in some eggs.

** 19 eggs with J_1 uncleaved, 8 eggs with J_1 & J_2 cleaved.

The next experiment was designed to determine whether the presence of the layer J_2 around eggs is primarily indispensable for the occurrence of fertilization. The uppermost region of an oviduct was ligated before pituitary injection. When the arrival of the eggs at the uterus through the unligated oviduct was confirmed, the animal was pithed to collect coelomic eggs. At the lower level, about 10 cm from the uterus, an incision was made in the wall of the ligated oviduct, into which coelomic eggs were introduced by pipette. After keeping the animal at 10°C for 17 hr., the eggs, invested only with the outermost layer J_4, were collected from the uterus. The result of inseminating these eggs is summarized in Table 2. It proves that the presence of J_2 is not indispensable for fertilization. The layer J_4 behaves similarly to the layer J_2 with respect to the occurrence of fertilization.

Analysis of variances in the fertilizability of oviducal eggs. The source of the variances obtained in the fertilizability of oviducal eggs was then analyzed from several points of view. Observations of inseminated oviducal eggs proved that no spermatozoa are found around eggs which are invested with the innermost jelly

layer, J_1 (Fig. 1). With eggs invested with the layer J_2, however, there are a number of spermatozoa which have penetrated into the jelly and make perpendicular contact with the vitelline membrane (Figs. 2 & 3). Sometimes eggs were collected which had just entered into the transitional part of the oviduct between J_1- and J_2 secreting regions. In these eggs, a part was invested with J_1 only, with the remaining area invested with a small amount of J_2 in addition to J_1 (Fig. 2, arrow). A remarkable features observed in such eggs was that a great number of spermatozoa penetrated through J_2 and J_1 into the vitelline membrane. In the area where only J_1 was present, no spermatozoa were observed. Penetrability of spermatozoa into J_1 through J_2 and not J_1 only was found in all the eggs irrespective of the occurrence of cleavage of the egg proper. These observations suggest that the fertilizability of oviducal eggs cannot be interpreted simply as a function of the amount of jelly.

Table 2. Result of an experiment to decide the role of jelly envelopes in fertilization

Eggs derived from	Jelly layers*				No. of eggs used	No. of eggs cleaved	Percentage cleaved
	J_1	J_2	J_3	J_4			
Coelom	−	−	−	−	56	0	0
Coelom**	−	−	−	+	61	52	85.2
Upper oviducal region	+	+	−	−	27	25	92.6
Uterus	+	+	+	+	44	44	100.0

* +, present; −, absent.

** Introduced into lower oviducal region to allow passage through oviduct for 10 cm.

This is confirmed by the next experiment. In this experiment eggs taken from the lower oviducal region and uterus were placed in 1/20 DB for 3 hr. at 5°C to allow hydration of the jelly. Then the outer jelly layers J_4, J_3 and a part of J_2, were mechanically removed to the extent that the depth of the jelly was approximately the same as in eggs located in the upper oviducal region. Along with this series, eggs taken from the upper oviducal region were placed in 1/20 DB for 3 hr. at 5°C. The results of inseminating these experimental lots are shown in Table 3. Since the amount of jelly around the eggs is approximately the same, it is reasonable to conclude that the difference in fertilizability presented in Table 3 is based on the difference in the maturity of the egg proper.

The state of the egg nucleus was then studied in the sectioned materials. All the eggs taken from the lower oviducal region and uterus contain the metaphase spindle of the second maturation division. In the eggs taken from the upper oviducal region and coelom, the situation is somewhat variable among the batches observed, *viz.*, some eggs form the first polar spindle and others form the second one. This variance is attributable to the difference in the time of observation after the

onset of ovulation, since an examination of oviducal eggs forced to remain in the coelom by ligation of oviduct revealed that most eggs contain the second polar spindle. Although the microscopical observations here described were not made for all the experimental lots presented above, it is highly probable that most oviducal eggs presented in batch B of Table 1 formed the second polar spindle and some of those presented in batch A of Table 1 did not attain that stage of maturation division.

Table 3. Result of an experiment to decide the role of jelly envelopes in fertilization

Eggs derived from	Jelly layer*				No. of eggs used	No. of eggs cleaved	Percentage cleaved
	J_1	J_2	J_3	J_4			
Upper oviducal region	+	+	−	−	32	2	6.3
Lower oviducal region**	+	+	−	−	38	38	100.0
Uterus**	+	+	−	−	52	51	98.1
Uterus	+	+	+	+	35	35	100.0

* +, present; −, absent.

** Outer jelly layers were mechanically removed.

From an overall point of view it is reasonable to conclude that the fertilizability of oviducal eggs must be considered in the context of two variables; the maturity of the egg proper and the presence of jelly. In the case where the egg attains the maturity, fertilization may take place if only a small amount of J_2 is present around the egg. This is the case found in batch B of Table 1. However, the addition of a sufficient amount of J_2 and even J_3 does not ensure the occurrence of fertilization unless the egg attains maturity, as in the case presented in batch A of Table 1.

Fertilizability of coelomic eggs. It has been shown elsewhere that dialyzed jelly and PVP act as a substitute for the jelly envelopes in fertilization of dejellied uterine eggs (Katagiri, '66). The possibility thus arises that fertilization could be induced if coelomic eggs are inseminated in these substances. To test this, coelomic eggs were collected 20 hr. after pituitary injection and, starting from this time, were kept in DB for 0, 24, 48 hr. at 5°C. The eggs were then inseminated either in 1/20 DB, dialyzed jelly, or in 5.0% PVP dissolved in 1/20 DB. The results, summarized in Table 4, reveal that a percentage of coelomic eggs becomes fertilizable after prolonged immersion in DB. No significant difference is found between the dialyzed jelly and PVP in increasing fertilizability. Experiments of similar design employing several batches proved that 30–50% of fertilization is always obtained if coelomic eggs are kept in DB for 1–3 days at 5°C. In the most successful case, 38 out of 74 eggs (51.4%) kept *in vitro* for 26 hr. were fertilized upon insemination in PVP.

All the coelomic eggs fertilized in PVP are capable of normal cleavage and

blastula formation. During gastrulation the majority of these eggs exhibited abnormality, with more or less delayed yolk plug closure, and became arrested as exogastrulae (Fig. 4). A few embryos developed through the gastrular stage into tailbuds, with persistent yolk plug (Fig. 5). These abnormalities were, however, ameliorated to some extent by transferring the fertilized eggs into 1/20 DB, and to a greater degree by treating the embryos in the following way. The outer jelly layers were mechanically removed from uterine eggs after a prolonged hydration of jelly envelopes in 1/20 DB. In this hydrated jelly were buried the fertilized coelomic eggs at morula or blastula stage. In this environment, gastrulation proceeds normally in most eggs, and normal tailbud embryos are formed (Fig. 6). Thus, as pointed out by Subtelny & Bradt ('61), the abnormalities in gastrulation observed here are not due to intrinsic deficiencies in the coelomic eggs, but rather to external factors.

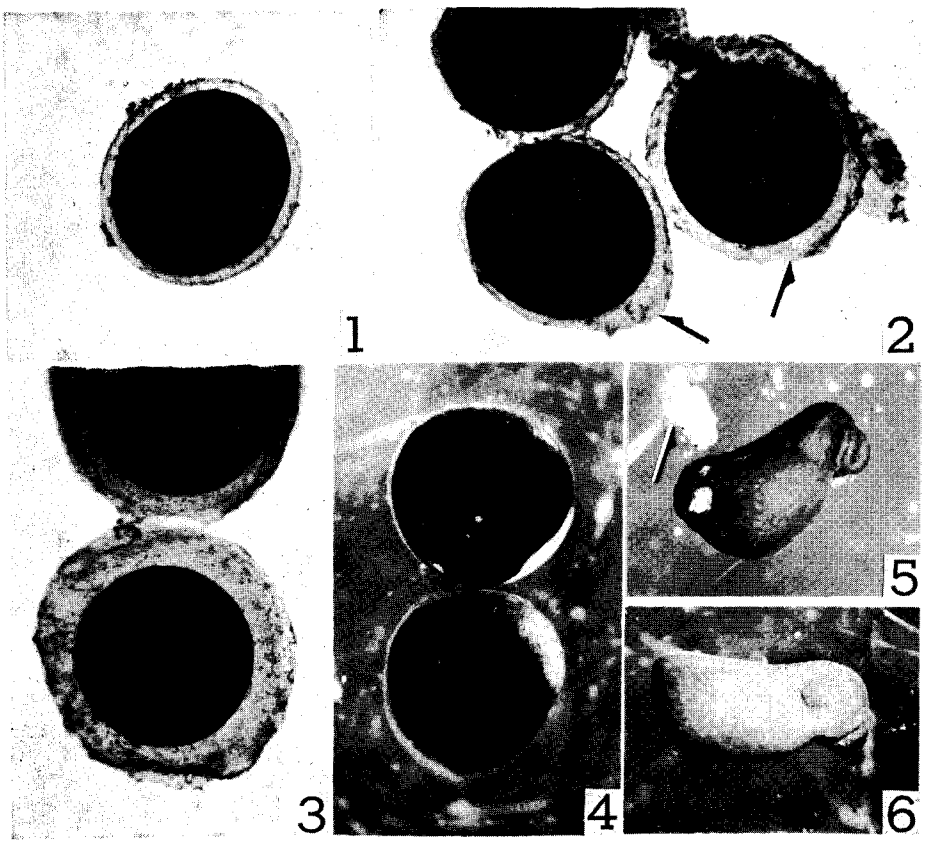
Table 4. Fertilizability of coelomic eggs after exposure to DB at 5°C

Time treated with DB (hr)	Inseminated in	No. of eggs used	Percentage cleaved
0	1/20 DB	65	0
0	5.0% PVP	69	2.9
24	1/20 DB	74	0
24	5.0% PVP	77	3.9
48	1/20 DB	58	0
48	5.0% PVP	82	37.8
48	Dialyzed jelly	75	32.0

What is the source of the lower percentage of fertilization obtained in the coelomic eggs? Microscopical examinations were made of the eggs derived from the bath given in Table 4. In eggs kept in DB for 24 hr., 11 out of 15 eggs observed formed the second polar spindle. Pricking of the same experimental lot further proved that activation, as defined by egg rotation and the second polar body emission, was clearly induced in 27 out of 30 eggs. This suggests that the lower fertilizability of coelomic eggs is attributable to factors which cannot be specified, presumably of cytoplasmic nature.

The next experiment was designed to confirm the role of the oviduct in establishing the cytoplasmic maturity of eggs for the occurrence of fertilization. Coelomic eggs were kept in DB for 45 hr. at 5°C until all the eggs were capable of activation upon pricking. Another ovulating female was pithed, the eggs located in the coelom removed, and the oviduct was ligated at the uppermost region near the ostium. Then two incisions were made in the oviducal wall at different levels; the one near the ostium and the other at a level about 10 cm lower than the ostium. The coelomic eggs kept *in vitro* for 45 hr. were introduced through the upper incision. From the lower incision the oviducal eggs thus rolled out, invested with the jelly layers J_1 and J_2 which were secreted by the upper oviducal

region. The results of inseminating these eggs are summarized in Table 5. The table shows that fertilization occurred in 36% of the eggs kept *in vitro*. It is further clear that the eggs become highly fertilizable during their passage through the oviduct of 10cm in length. Since PVP behaves as a perfect substitute for the jelly envelopes, as shown elsewhere, the eggs seem to have acquired some sort of cytoplasmic maturity other than the addition of jelly envelopes.



Figs. 1–3. Photographs of eggs taken from upper oviducal region. ca. ×9, respectively. Fig. 1, An egg from uppermost oviducal region, invested with J_1 only; Fig. 2, Eggs invested with either J_1 or J_1 & J_2. Arrows indicate J_2; Fig. 3, Eggs invested with J_1 & J_2.

Figs. 4–6. Photographs of coelomic eggs inseminated in PVP. Fig. 4, Gastrulae showing delayed yolk plug closure and exogastrulation. ca. × 9; Fig. 5, Tailbud embryo with persistent yolk plug. Arrow indicates a part of yolk plug broken during manipulation; Fig. 6, Normal tailbud embryo, transferred from PVP to hydrated jelly at blastula stage. ca. ×8.

Discussion

Variances of fertilizability of oviducal eggs presented above are similar to the results described in the recent report of Glick & Shaver ('63), who conducted their experiments with *Rana pipiens* eggs taken from various levels of the oviduct and the ovisac. Sensitivity to pituitary stimulation for ovulation evidently differs according to the batches used, the season of year, or other physiological conditions of unspecified nature in a female. These factors may cause variations of time before the onset of ovulation, and thus of the maturity of eggs derived from different females. The complicated events involved in the ovulatory phenomena as pointed out by Aplington ('57) may also cause variances of egg maturity in a given individual. For example, though the onset of ovulation is sudden, the liberation of eggs from the ovary occurs gradually. As a whole the present results indicate that the fertilizability of oviducal eggs must be considered as a function of at least two variables, *i.e.*, the maturity of the egg proper and the presence of jelly envelopes.

Table 5. Result of experiment to show the role of oviduct in increasing the fertilizability of eggs. Further explanation in text.

Treatment	Inseminated in	No. of eggs used	Percentage cleaved
DB 48 hr	1/20 DB	74	0
DB 48 hr	5.0% PVP	84	36.9
DB & oviduct 48 hr	1/20 DB	48	89.6

According to Aplington's ('57) review on egg maturation in amphibia with reference to the location of eggs during their reproductive passage, considerable variations in egg maturity are found by different workers even using the same species. Thus, some claim that eggs mature to the second polar spindle after entering the ovduct, while others say that the second polar spindle is formed before the eggs enter the ovdiuct. In an extreme case, uterine eggs are reported to be in various stages of maturation, from the germinal vesicle stage to the second polar spindle formation (Tchou & Chen, '42). That the second polar spindle is formed without passage through the oviduct has been confirmed in the eggs which were forced to remain in the coelom by ligation of the oviduct. Further support of this is found in eggs which are ovulated *in vitro* and kept in Ringer's solution for a relatively long period (Nadamitsu, '53). However, the maturity of egg implies the maturity of cytoplasm besides that of nucleus. Thus there is a possibility that the oviduct may prepare or offer conditions favorable for cytoplasmic maturity, as will be discussed later.

Since the penetrability of spermatozoa into jelly is quite separable from the maturation of the egg proper, the question as to which layer of jelly is necessary for fertilization must be discussed in cases where the oviducal eggs have attained uniform maturity. The present study shows that eggs invested with the innermost

layer J_1 only are not fertilizable and those with J_2 added become fertilizable. There is other evidence indicating that fertilization takes place when a small amount of the innermost jelly layer is present around the egg, as found in the case of *Hyla arborea* (unpublished observation). However, this is not necessarily contradictory to the present results. As shown in an extensive study by Salthe ('60), there are structural varieties in the jelly envelopes of several amphibian species. This may cause variable explanations for the problem as discussed here. That only a trace of J_2 is sufficient to induce sperm penetration may find support in the experiment where dejellied uterine eggs were successfully fertilized in the presence of a rather small concentration of dialyzed jelly (Katagiri, '66). The role played here by the layer J_2 is likely to be that of activating spermatozoa or facilitating them to adhere to the vitelline membrane.

In the present study, experiments to fertilize coelomic eggs in the presence of dialyzed jelly or PVP have not been fully successful. As has been pointed out repeatedly, the maturity of egg nucleus to be fertilized can be induced without a sojourn of the egg in the oviduct. Failure of fertilization observed in coelomic eggs is therefore attributable to the immaturity of the egg cytoplasm, of unspecified nature. With respect to egg maturity after ovulation, the experiment of Tchou & Wang ('64) on toad eggs indicated the progressive increase of fertilizability as well as of developmental ability during 10–30 hr. after pituitary injection. The experiments of these workers, however, involved the procedure of introducing eggs into the oviduct. On the other hand, Barthélémy ('22) obtained the cleavage of coelomic frog eggs by pricking in the presence of blood or sperm. Furthermore, the experiment of Subtelny & Bradt ('61) clearly demonstrated that coelomic *Rana pipiens* eggs activated and injected with blastula nuclei are highly capable of normal cleavage and yield young froglets. From these facts it is conceivable that the developmental ability of eggs, including the cytoplasmic maturity, can be induced successfully without passage of the eggs through the oviduct.

Evidently, however, the eggs become highly fertilizable during their short sojourn in the oviduct. Thus the cytoplasmic maturity as postulated above seems to be related to the penetrability of spermatozoa into the egg. In this respect a highly specific antigen, named as antigen A, demonstrated by Nace *et al.* ('60) in the oviducal epithelium of *Rana pipiens* is of particular interest. From the distribution of this antigen in the eggs and its behavior following fertilization, its possible roles as a sperm receptor or a meitoic inhibitor have been postulated. The lower concentration but not absence of this antigen in the coelomic eggs and its increase during passage down the oviduct, may correlate well with the fertilizability of eggs obtained in the present study. More recently, there have been demonstrations of other antigenic components in the oviduct which are related to the jelly substance and are immunologically distinguishable from antigen A (Shaver *et al.*, '62; Barch & Shaver, '63). Assuming that PVP acts as a perfect substitute for jelly material (*cf.* Katagiri, '66), then the lower fertilizability observed in coelomic eggs is due to

the limited concentrations of the oviducal antigen, antigen A. This assumption is however only hypothetical, and further studies are needed to clarify the role of the oviduct for the establishment of the cytoplasmic maturity of the egg.

Summary

Fertilizability was studied of the coelomic and oviducal eggs of the toad, *Bufo bufo formosus*.

1. Considerable variances from batch to batch was noted in the fertilizability of eggs taken from different levels of the oviduct. Analyses were then made on the maturity of the egg proper and the behavior of spermatozoa in relation to the fertilizability of coelomic and oviducal eggs.

2. Jelly-less coelomic eggs and the oviducal eggs invested only with the innermost jelly layer J_1 are not fertilizable.

3. If the egg is properly mature, fertilization, as defined by the occurrence of normal cleavage, takes place in eggs which have been taken from the upper region of the oviduct and are invested with the inner jelly layers J_1 and J_2.

4. Fertilization is possible if a small amount of J_2 is present around the egg. The eggs artificially invested only with the outermost jelly layer J_4 are also fertilizable. The layer J_4 behaves similarly to the layer J_2 with respect to the occurrence of fertilization.

5. When coelomic eggs are inseminated in the presence of dialyzed jelly or polyvinylpyrrolidone (PVP), 30–50% of the eggs are fertilized and develop into normal tailbuds. On the other hand, coelomic eggs of the same batch become highly fertilizable after a sojourn in the oviduct for a short period.

6. On the basis of these results, discussion has been offered with particular emphasis on the possible role of the oviduct for the cytoplasmic maturity of the egg.

The author wishes to express his most grateful thanks to Professor A. Ichikawa for his advice and careful revision of the manuscript. He is also indebted to Dr. T. S. Yamamoto for his valuable suggestions.

References

Aplington, H.W.Jr. 1957. The insemination of body cavity and oviducal eggs of amphibia. Ohio J. Sci. **57**: 91–99.

Barch, S.H., and J.R. Shaver 1963. Regional antigenic difference in frog oviduct in relation to fertilization. Amer. Zool. **3**: 157–165.

Barthélémy, M.H. 1922. Maturation *in vitro* et activation des oeufs de la cavité générale et des conduits chez *Rana fusca*. Compt. Rend. Acad. Sci. **175**: 1102–1105.

Bataillon, E. 1919. Analyse de l'activation par la technique des oeufs nus et la polyspermie expérimentale chez les batrachien. Ann. Sci. Nat. Zool. **3**: 1–38.

Glick, R.N., and J.R. Shaver 1963. The fertilizability of frog eggs from different levels of the female reproductive tract. Exp. Cell Res. **32**: 615–618.

Good, G.N., and J.F. Daniel 1943. Fertilization of coelomic eggs of *Triturus torosus*. Univ. Calif. Publ. Zoöl. **51**: 149–158.

Kambara, S. 1953. Role of jelly envelopes of toad eggs in fertilization. Annot. Zool. Japon. **36**: 78–84.

Katagiri, Ch. 1966. Fertilization of dejellied uterine toad eggs in various experimental conditions. Embryologia, **9**: (in press).

Kobayashi, H. 1954. Hatching mechanism in the toad, *Bufo vulgaris formosus* 1. Observations and some experiments on perforation of gelatinous envelopes and hatching. J. Fac. Sci. Univ. Tokyo (Zool.) **7**: 79–88.

Nadamitsu, S. 1953. Ovulation *in vitro* in several species of amphibians. J. Fac. Sci. Hiroshima Univ. (Ser. B) **14**: 1–7.

———— 1957. Fertilization of coelomic and oviducal eggs of *Triturus pyrrhogaster* (BOIE). *Ibid.* **17**: 51–53.

Nace, G.W., T. Suyama, and N. Smith 1960. Early development of special proteins. In Symposium on germ cells and development. Instit. Intern. Embryo. Fondazione A. Baselli. pp. 564–603.

Rugh, R. 1935. Ovulation in the frog. II. Follicular rupture to fertilization. J. Exp. Zool. **71**: 163–194.

Salthe, S.N. 1963. The egg capsules in the amphibia. J. Morph. **113**: 161–171.

Shaver, J.R., and S.H. Barch, 1960. Experimental studies on the role of jelly coat material in fertilization in the frog. Acta Embryol. Morphol. Exp. **3**: 180–189.

————, S.H. Barch, and C.A. Shivers 1962. Tissue specificity of frog egg-jelly antigens. J. Exp. Zool. **151**: 95–103.

Shivers, C.A., and C.B. Metz 1962. Inhibition of fertilization in frog eggs by univalent fragments of rabbit antibody. Proc. Soc. Exp. Biol. Med. **110**: 385–387.

Subtelny, S., and C. Bradt 1961. Transplantation of blastula nuclei into activated eggs from the body cavity and from the uterus of *Rana pipiens*. Develop. Biol. **3**: 96–114.

Tchou-Su, and Chen Chou-Hsi, 1942. Fertilization of artificially ovulated premature eggs of *Bufo*. Sci. Rec. China 1: 203–208.

————, and Y.L. Wang 1956. Etudes expérimentales sur le rôle du mucus des oviductes dans la fécondation chez le crapaud, et la considération générale sur le mécanisme de la pénétration spermatique. Acta Exp. Biol. Sinica **5**: 75–122.

———— 1964. The development of eggs of *Bufo bufo asiaticus* inseminated at different states of maturity. *Ibid.* **9**: 101–116.