Environmental Influence on Ovulation and Embryonic Development in *Rana pipiens* [1,2,3]

GRACE C. LEHMAN
The Division of Biological Sciences and The Center for Human Growth and Development, The University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT Environmental effects on ovulation and embryogenesis in *Rana pipiens* were assessed using both freshly-captured fall animals and laboratory-conditioned females which had undergone vitellogenesis in the laboratory. Frogs in both categories were divided into two groups. Ovulation was hormonally induced in one group of females prior to cold exposure and in the second group of animals following an 8-week period at 4°C with an 8L 16D photoperiod. The incidence of both ovulation and normal embryonic development was increased following exposure of the animals to low temperatures and short daylength. Those animals which only partially ovulated prior to cold treatment did not respond to hormone injections following the period of cold exposure. Examination of the ovaries of these females revealed a much greater degree of oocyte resorption than was found in frogs whose initial ovulation was induced only after exposure to cold temperatures. The administration of ovulation-inducing hormones prior to artificial hibernation may thus have initiated a phase of oocyte resorption which progressed even at 4°C.

The incidence of ovulation was similar in wild-caught and laboratory-conditioned females, but eggs from the latter showed a much lower percentage of development to Shumway stage 20. This effect may have been related to differences in the environmental factors to which the two groups were exposed during oogenesis.

A period of hibernation has been suggested as a prerequisite for successful ovulation and egg maturation in the toad, *Bufo asiaticus* (Tchou and Wang, '63a,b). Marchlewski ('58) has also mentioned that *Rana temporaria* requires 20 days at − 5°C in order for "normal reproduction" to occur. Prolonged cold exposure does not appear to be an absolute requirement for ovulation and oocyte maturation in the northern leopard frog, *R. pipiens*, and fertilizable eggs have long been obtained from prehibernatory fall animals (Rugh, '35) if the appropriate hormonal stimuli are administered (Rugh, '62; Wright and Flathers, '61). However, external factors such as temperature and light have apparent regulatory roles in amphibian reproduction (Salthe and Mecham, '74), and the present paper describes environmental conditions which influenced both the incidence of ovulation and the subsequent development of fertilized eggs.

MATERIALS AND METHODS

Animals

Freshly-captured adult *R. pipiens* females, received from Vermont in mid-April, 1975, were induced to ovulate (3,000-6,000 eggs) two days after their arrival. During subsequent vitellogenesis they were maintained at room temperature under conditions previously described (Nace, '68; Nace et al., '74) and were fed crickets three times per week. In late September, five of these laboratory-conditioned frogs were injected for ovulation.

[1] Contribution no. c-73 from the Amphibian Facility.

[2] This investigation was partially supported by National Institutes of Health grants RR 00572 and HD 07189.

[3] The animals used in this study were maintained in facilities that are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Forty-eight hours later they were stripped of ovulated eggs and both the injected and uninjected animals were placed at 16°C to begin acclimation to cooler temperatures.

A second group of females from the same source was obtained on 24 September, 1975. After five days at room temperature and two meals of live crickets, eight of these animals were administered hormones to induce ovulation. After 48 hours, ovulated eggs were expressed and both the injected and uninjected animals were first placed at 16°C, and then at 10°C and 4°C as described below.

Following exposure to cold both previously injected and non-injected animals received exogenous hormones to stimulate ovulation. Experiments using the spring- and fall-captured groups took place within a week of each other, and body weight and snout-vent length data were collected on all animals just prior to ovulation.

During the experimental period, observations were made of the condition of the oocytes in the ovaries of animals which died and of other animals which were pithed.

Hormone preparation and administration

Pituitaries from *R. pipiens* females were homogenized in distilled water and frozen in aliquots at a concentration equivalent to two pituitaries/0.2 ml. To induce ovulation each frog received an intraperitoneal injection of 0.2 ml of pituitary homogenate and 5.0 mg of progesterone.

Environmental conditions

Prior to cold exposure the animals were housed at room temperature (22-25°C) on a natural photoperiod. Temperatures were decreased gradually and the animals spent two days at both 16°C and 10°C before being finally maintained at 4°C for eight weeks. Photoperiod was fixed at 8L 16D at all temperatures below room temperature. While in the cold, the females were kept in plastic pans containing 8 cm of water which was changed every two weeks. Following exposure to cold the animals were subjected to increasing temperatures by spending two days at both 10°C and 16°C. After two days at room temperature and a natural photoperiod the animals were injected for ovulation.

Fertilizations

When ovulated eggs were to be fertilized, a sperm suspension was prepared from the excised testes (Rugh, '62) of a mature Vermont *R. pipiens* male. In each instance the sperm showed a high degree of motility. All ovulated eggs were counted and 100-200 eggs from each frog were used in test fertilizations. If fewer than 100 eggs were ovulated, all eggs were inseminated.

The embryos were maintained in dechlorinated water and observations were made of their development. At the time of hatching (Shumway stage 20) the number of normal embryos was recorded.

RESULTS

Animals captured in the fall (vitellogenesis in the field)

The attempt to induce ovulation in late September in freshly-caught *R. pipiens* was only partially successful (table 1, I). Although five out of eight animals ovulated, they produced few eggs. When re-injected after cold treatment, neither the animals which had previously ovulated nor the one which had previously failed to ovulate showed significant responses to hormonal stimuli (table 1, IIA). Of the 47 eggs artificially inseminated only 26 appeared to rotate and no cleavage was observed.

Following cold treatment, ovulation occurred in seven of eight previously uninjected females (table 1, IIB). Both the number of eggs ovulated per female and the percentage of eggs which developed to stage 20 increased significantly. Group comparison t-tests were used in the statistical analyses.

Among the eggs ovulated prior to cold exposure, many showed abnormal cleavage and exogastrulation. Following the cold

TABLE 1

Ovulation and embryonic development before and after cold exposure of Rana pipiens *females which underwent vitellogenesis in the field*

Group	No. of frogs	Preovulatory body wt (g) [1]	Snout-vent length (mm) [1]	No. which ovulated	No. of eggs ovulated [1]	% development to stage 20 [1]
I. Before cold exposure	8	53.4 ± 1.6	81 ± 1	5	238 ± 89 (ΣX = 1189) (R = 72-558)	15.2 ± 7.3
II. After cold exposure						
A. Previously injected frogs [2] (from I above)	5	54.1 ± 1.8	81 ± 2	2	24 (ΣX = 48) (R = 1-47)	0
B. Frogs with no previous injection	8	49.3 ± 1.1	79 ± 1	7	1478 ± 456 [3] (ΣX = 10,349) (R = 79-3005)	32.9 ± 6.4 [3]

[1] Data are expressed as mean ± standard error of the mean.
[2] All but one of the females had partially ovulated prior to cold exposure.
[3] $P < 0.01$ compared to Group I.

treatment such developmental aberrations were negligible.

Mortality only occurred among the animals which received hormones prior to cold treatment. Among these, one frog was found moribund at 4°C and two others died less than 24 hours after being returned to room temperature. Examination of the ovaries of these animals immediately after death revealed the presence of large numbers of resorbing oocytes which showed no indications of polarity.

Following ovulation assessment, two females from both groups IIA and IIB (table 1) were killed. Three of the four frogs had not ovulated and one (from group IIB) had given only 79 eggs. The ovaries from those animals with no hormone injection prior to cold treatment (Group IIB) contained normally pigmented eggs ranging from 1.6 to 1.8 mm in diameter. Although there were signs of pigment migration, polarity was still evident and the eggs retained their discrete structure. Those frogs with hormone injections before cold treatment (group IIA) contained resorbing oocytes ranging from 1.2 to 1.8 mm in diameter. The eggs were extensively mottled and showed little evidence of their original polarity. Some areas of the ovaries appeared as solid gray masses. These were regions of most extensive resorption in which the oocytes were no longer discernable as discrete structural units.

Animals captured in the spring (vitellogenesis in the laboratory)

Of the animals which had undergone a cycle of vitellogenesis in the laboratory, four of five frogs injected prior to cold treatment responded to the hormonal stimulus (table 2, I). Although one of these frogs ovulated over 2,500 eggs, an average of 75 eggs per animal (R = 11-152) were expressed from the remaining three females. While these eggs rotated, development to the blastula and ultimately to the hatching stage was negligible. After cold exposure none of these animals responded to a second hormone treatment (table 2, IIA) and the ovaries of one frog which died at this time contained large numbers of eggs undergoing resorption.

Following cold treatment the mean number of eggs ovulated by previously uninjected animals (table 2, IIB) was twice the number obtained from females which had not been cold treated (group I). The percentage of eggs which developed to stage 20 was also higher. However, individual frogs varied greatly and the increase in the

TABLE 2

Ovulation and embryonic development before and after cold exposure of Rana pipiens *females which underwent vitellogenesis in the laboratory*

Group	No. of frogs	Preovulatory body wt (g) [1]	Snout-vent length (mm) [1]	No. which ovulated	No. of eggs ovulated [1]	% development to stage 20 [1]
I. Before cold exposure	5	58.3 ± 6.7	85 ± 3	4	704 ± 699 (ΣX = 2814) (R = 11-2589)	0.3 ± 0.3 [2]
II. After cold exposure						
A. Previously injected frogs [3] (from I above)	4	58.8 ± 6.7	84 ± 4	0	—	—
B. Frogs with no previous injection	5 [4]	61.2 ± 4.9	88 ± 2	4	1576 ± 446 (ΣX = 6305) (R = 495-2601)	8.0 ± 3.8 [2]

[1] Data are expressed as mean ± standard error of the mean.
[2] Eggs from 3 females were inseminated.
[3] All but one of the females had partially ovulated prior to cold exposure.
[4] Following hormone administration two frogs appeared insensible and exhibited extreme darkening of the skin. One frog did not ovulate and upon recovery the second animal ovulated 495 eggs which were not inseminated.

two parameters following cold exposure was not statistically significant. In each instance the development of eggs from animals which had undergone vitellogenesis in the laboratory was less than that of eggs from frogs which had been captured in the fall.

DISCUSSION

Exposure of *R. pipiens* to the specified changes in temperature and daylength increased both the number of eggs ovulated per frog and the percentage of normal embryos which developed from sample fertilizations. Several factors must influence the interpretation of these results: the specified photoperiod was strictly held for temperatures at or below 16°C, while equally strict control of the photoperiod was not available for the frogs maintained at 22-25°C. Thus, the animals exposed to cold treatment and subsequently returned to room temperature experienced changes in daylength which could have affected their reproductive physiology.

Some data pertain directly to this question. In a preliminary study the preceding year, a group of newly-captured fall animals (N = 6) was maintained for eight weeks at room temperature with a photoperiod of 8L 16D (controlled by manually covering the containers with opaque material at the appropriate times). A similar group (N = 7) was exposed to reduced temperature with an 8L 16D photoperiod as described above. Those animals subjected to cold temperatures were not fed, while those at room temperature received three meals of live crickets/week. The frogs maintained at room temperature but exposed to eight weeks of reduced daylength ovulated few eggs and the percentage of development to stage 20 was slightly less than 1% (results comparable to those obtained using control animals induced to ovulate immediately after their arrival in the laboratory). Cold-treated females exposed to the 8L 16D photoperiod ovulated significantly greater numbers of eggs and the percentage of development was 33% These data suggest that the 8L 16D photoperiod had little effect on ovulation and development. However, the difference in feeding regimes for the two groups precludes a definitive conclusion.

Conceivably changes in temperature or prolonged cold exposure alone may have significantly affected the reproductive parameters described. In vitro studies have

indicated that hormone-induced ovulation and oocyte maturation increase progressively throughout hibernation and are maximal during the spring breeding season (Wright, '45; Masui, '67; Smith et al., '68). The spontaneous spawning of *R. temporaria* during an extended period at 3-4°C (Allison, '56) indicates that at low temperatures changes in pituitary and/or ovarian function can occur.

Tchou and Wang ('63b) have suggested that prolonged cold exposure increases the sensitivity of the oocytes of *B. asiaticus* to pituitary gonadotropic hormones. Evidence now indicates that the latter stimulate the follicle cells of the amphibian ovary to secrete the steroid hormone(s) responsible for (1) the initiation of ovulation, (2) the breakdown of the germinal vesicle, and (3) the changes in the nucleus and cytoplasm which are essential for fertilization, cleavage and embryogenesis (Redshaw, '72; Schuetz, '74). Thus, in the present study changes in follicle cell-oocyte function may have ultimately affected oocyte maturation, ovulation and embryonic development.

Although the fertilizing capacities of the sperm preparations undoubtedly differed slightly throughout the investigation, the repeatability of the results indicates that this variability was not a primary factor responsible for developmental differences observed between eggs from artificially hibernated and non-hibernated females. For any given experimental group used during preliminary and confirming studies, embryonic development was consistently enhanced following cold exposure of the females.

The incidence of ovulation was similar in both wild-caught fall animals and those which had undergone vitellogenesis in the laboratory. Eggs from the latter, however, showed a much lower percentage of development to stage 20. Although this effect may have been related to the different dietary elements and environmental factors to which the two groups were exposed during oogenesis, it nevertheless suggests that those aspects of oocyte-follicle cell function on which ovulation depends need not be affected by the physiological conditions leading to the disruption or inhibition of embryogenesis. The functional dissociation of specific processes within the oocyte-follicle cell complex has been previously demonstrated, and, in *R. pipiens*, ovulation and oocyte maturation are not mutually interdependent events (Subtelny et al., '68).

In neither the wild-caught fall frogs nor those which had undergone vitellogenesis in the laboratory was it possible to obtain significant numbers of eggs from cold-treated animals which had been injected for ovulation prior to cold exposure. The severe degenerative changes observed in the ovaries of such frogs suggested the possibility that the administration of gonadotropic or steroid hormones at that time initiated a phase of oocyte resorption which progressed even at 4°C. One wonders whether this was a pharmacological effect or whether in nature the hormones which stimulate ovulation also promote the resorption of those fully grown oocytes not extruded from the ovary. Little or no oocyte resorption would be expected during hibernation under natural conditions. In the laboratory even slight disturbance of the animals might elicit metabolic responses not found in over-wintering wild frogs.

Limited resorptive changes in the oocytes might also have contributed to the relatively low percentage (32.9%) of normal embryonic development observed in eggs from cold-treated females captured in the fall. Other factors may also have been involved, however, because in recent years both Amphibian Facility personnel and other investigators have observed reduced percentages of embryonic development both in the field (McKinnell: communications in Nace, '76, and Hine et al., '75) and in the laboratory (Smith, McKinnell, Dugan, personal communications to the Amphibian Facility).

The percentage of development in the present study might also have been higher had the animals spent more time in the

wild under conditions where they would have continued to feed. However, since little is known about environmental effects on reproduction and the sensitivity of the physiological system to external changes, it was advisable to utilize for this study animals which had not been exposed to the gradually decreasing temperatures which characterize the period of fall migration (Rittschof, '75).

The data thus obtained indicate that changes in the external environment significantly affect both ovulation and embryogenesis. The precise mechanisms by which these processes are enhanced is unknown, and the relative roles of light and temperature on the reproductive cycle as a whole have not been elucidated. Most in vitro and in vivo studies to date have suggested that hibernation itself is not simply a means of preventing the deterioration of fully grown oocytes, but represents a time of ovarian activity which perhaps results from changes in hypothalamic and/or pituitary function.

ACKNOWLEDGMENTS

The author would like to thank Dr. B. E. Frye and Dr. G. W. Nace for their critical readings of the manuscript.

LITERATURE CITED

Allison, R. M. 1956 Failure of enforced hibernation to inhibit breeding in the frog *(Rana temporaria)*. Nature, *177:* 342.

Hine, R. L., B. L. Les, B. F. Hellmich and R. C. Vogt 1975 Preliminary report on leopard frog *(Rana pipiens)* populations in Wisconsin. Department of Natural Resources Research Report No., *81:* 1-29.

Marchlewski, T. 1958 Thermal stadia in amphibia and their significance for evolution. Proc. Int. Congr. Zool., *15:* 136.

Masui, Y. 1967 Relative roles of the pituitary, follicle cells, and progesterone in the induction of oocyte maturation in *Rana pipiens*. J. Exp. Zool., *166:* 365-376.

Nace, G. W. 1976 Standards for laboratory amphibians. In: The Amphibian Visual System: A Multidisciplinary Approach. K. V. Fite, ed. Academic Press, New York.

——— 1968 The Amphibian Facility of the University of Michigan. BioScience, *18:* 767-775.

Nace, G. W., D. D. Culley, M. B. Emmons, E. L. Gibbs, V. H. Hutchison and R. G. McKinnell 1974 Amphibians: Guidelines for the Breeding, Care, and Management of Laboratory Animals. Sub-Committee on Amphibian Standards. ILAR (NAS/NRC). Washington, D. C.

Redshaw, M. R. 1972 The hormonal control of the amphibian ovary. Am. Zool., *12:* 289-306.

Rittschof, D. 1975 Some Aspects of the Natural History and Ecology of the Leopard Frog, *Rana pipiens*. Ph.D. Thesis. The University of Michigan.

Rugh, R. 1962 Experimental Embryology: Techniques and Procedures. Burgess Publishing Co., Minneapolis.

——— 1935 Ovulation in the frog. I. Pituitary relations in induced ovulation. J. Exp. Zool., *71:* 149-162.

Salthe, S. N., and J. S. Mecham 1974 Reproductive and courtship patterns. In: Physiology of the Amphibia. Vol. 2. B. Lofts, ed. Academic Press, New York.

Schuetz, A. W. 1974 Role of hormones in oocyte maturation. Biol. Reprod., *10:* 150-178.

Smith, L. D., R. E. Ecker and S. Subtelny 1968 *In vitro* induction of physiological maturation in *Rana pipiens* oocytes removed from their ovarian follicles. Devel. Biol., *17:* 617-643.

Subtelny, S. L., L. D. Smith and R. E. Ecker 1968 Maturation of ovarian frog eggs without ovulation. J. Exp. Zool., *168:* 39-48.

Tchou, Su and Yu-lan Wang 1963a La succession d'ovogenèse et l'impossibilité de la maturation ovulaire chez le crapaud femelle élevée dans le milieu à haute température pendant toute une année. Scientia Sinica, *12:* 1165-1168.

——— 1963b L'hibernation, facteur déterminant de la maturation ovulaire chez le crapaud *(Bufo bufo asiaticus)*. Scientia Sinica, *12:* 1161-1164.

Wright, P. A. 1945 Factors affecting *in vitro* ovulation in the frog. J. Exp. Zool., *100:* 565-575.

Wright, P. A., and A. R. Flathers 1961 Facilitation of pituitary-induced frog ovulation by progesterone in early fall. Proc. Soc. Exptl. Biol. Med., *106:* 346-347.