

Localized Production of the Hatching Substance in *Rana pipiens*.¹

GENE A. KALLAND, Indiana University²

Introduction

The hatching of an amphibian embryo from its jelly capsule is aided by a hatching substance that is secreted by the embryo. The nature of this substance, sometimes called a hatching enzyme, has not been completely characterized although it has been shown to be heat labile (1) and to have a general proteolytic activity (4). This substance has been demonstrated in both urodele and anuran embryos (1, 3, 4, 5, 6, 7, 10) and acts by digesting a hole in the jelly capsule surrounding the embryo and thereby allows the wiggling embryo to escape.

It has been shown in an urodele, *Ambystoma opacum*, that the production of the hatching substance is restricted to the head region (6, 7). In this region there are unicellular epidermal glands that are large and intact prior to hatching but collapsed and empty after hatching. This phenomenon is true whether the hatching is natural or induced by treatment with pilocarpine. For this reason these cells have been given the name hatching gland cells.

Similar unicellular glands have been described in anurans (2, 11) but there has been no demonstration of a localized production of the hatching substance. The following experiments were performed to determine whether the hatching substance in *Rana pipiens* embryos is produced in a specific area or over the entire body surface.

Methods and Results

The basic approach taken in this work was to isolate portions of pre-hatch embryos, culture alike pieces together, and then determine if the media in which they were cultured contained any hatching substance. Hatching substance was detected by the ability of the culture media either to raise the vitelline membranes of gastrulae or to dissolve pieces of jelly capsule.

In the first experiment 70 pre-hatch embryos, Shumway stage 16 (8), were demembranated by removing all jelly capsules and membranes with watchmakers forceps. The embryos were then placed in full strength Steinberg's solution (9) containing antibiotics at the following concentrations: Elkosin 0.1%, streptomycin 0.04%, and penicillin 0.04%. (This solution from here on will be referred to as the standard solution.) They were then put through three washes of fresh standard solution by transferring them with a pipette from one watch glass of fresh media to another. The embryos were cut into quarters with iridectomy scissors as shown in Fig. 1. Alike quarters were put together in standard solution in petri dishes and allowed to sit for two hours in order to heal.

1. Contribution #780 from the Zoology Department, Indiana University.

2. The author wishes to express his appreciation to Dr. R. Briggs for his helpful suggestions during the investigation.

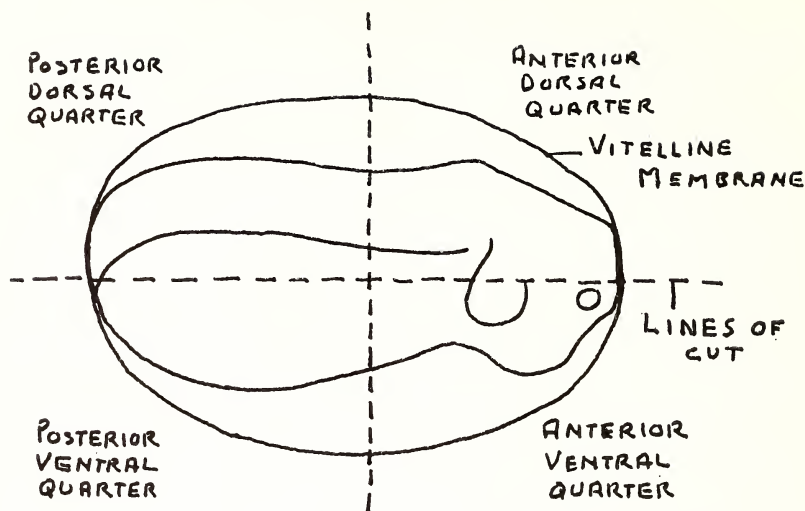


Figure 1. Stage 16 *Rana pipiens* embryo showing lines of cut and quarter designation in the first experiment.

They were then washed the same way as the whole embryos and finally placed in small plastic petri dishes containing standard solution. The volume in the dishes was adjusted to 3.2 ml. Seventy embryos from the same clutch of eggs were demembrated, washed, and placed in standard solution and the volume was adjusted to 4.0 ml. Twenty-one control embryos with membranes were put in standard solution. After 21 hours at room temperature, 22° C., the controls were at late stage 19 and all 21 had hatched. The producers, that is the demembrated whole embryos and the quarter embryos, were removed from their culture media 10 hours later. The petri dishes of culture media were stored at room temperature until 12 hours later when two stage 10 embryos (early gastrulae) were put in each of the five culture media and in some stand-

TABLE I

	3 hrs.	6 hrs.	9 hrs.	23 hrs.
Standard Solution	—	—	—	—
Whole Embryos	+	+	+	+
Posterior Dorsal	—	—	—	—
Posterior Ventral	—	—	—	—
Anterior Dorsal	—	±	+	+
Anterior Ventral	—	—	—	—

Results of observations in the first experiment on the ability of these solutions to raise the vitelline membranes of gastrulae. Time is from the time the gastrulae were placed in the solution. + indicates raising of membrane.

ard solution. The test embryos were checked periodically for raising of the vitelline membranes. The results are shown in Table I. From this it is apparent that the anterior dorsal quarter of the embryo is the only quarter producing any significant amount of hatching substance.

The second experiment was performed to localize, if possible, the area producing the hatching substance. In stages 16 and 17, just prior to hatching, the most anterior blunt end of the embryo is in direct contact with the vitelline membrane. An anterior view of the embryo (Fig. 2), shows a triangular shaped dark area which generally coincides with the area of contact. This area was considered, therefore, as a possible production site of the hatching substance.

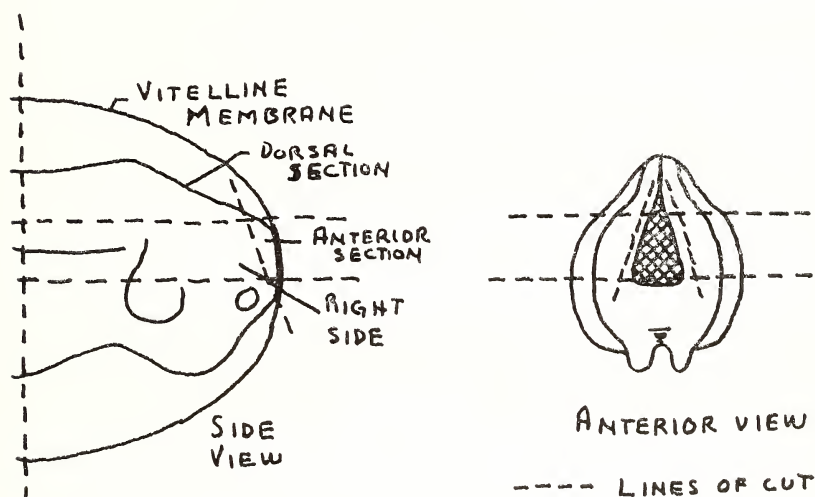


Figure 2. Stage 16 embryo showing dark area on anterior end and lines of cut in second experiment.

Ten stage 17 embryos were demembranated, quartered, and washed, as in the previous experiment, but only the anterior dorsal quarters were retained. These were then cut with glass dissecting needles, as shown in Fig. 2, and the following pieces were retained: the section containing the dark area, the right side, and the dorsal section. These were washed and alike pieces were put together in standard solution in a small depression in wax contained in a watchglass. It was felt that the manipulation of these small pieces of tissue would decrease their ability to function so the volume of media was kept as small as feasible. Each culture contained, therefore, between 0.02 and 0.03 ml. of standard solution. These small volumes precluded the use of gastrulae as test subjects. Instead, small pieces of jelly capsule, taken from the experimental embryos during demembranation, were placed in the cultures along with the pieces of tissue. A fourth culture of jelly capsule alone in standard solution was set up in the watchglass. The softening or solution of the pieces of jelly capsule would indicate the presence of the hatching substance. The watchglass was then put in a moist-chamber to prevent evaporation.

Twenty-one control embryos with membranes were put in standard solution and these and the cultures were left at room temperature, 25° C. The pieces of jelly capsule were checked periodically for softening. The results are shown in Table II. At 13 hours all of the control embryos were at early stage 19 and had hatched. From these results it can be concluded that the hatching substance is produced in the most anterior region containing the dark area and probably to a lesser extent along the dorsal part of the embryo.

TABLE II

	13 hrs.	19 hrs.
Standard Solution	—	—
Right Side	—	—
Anterior	±	+
Dorsal	—	+

Results of observations in the second experiment on the ability of these solutions with their producers to dissolve pieces of jelly coat. Time is from time of placement of embryo tissue and jelly coat in the solution. + indicates softening or solution of the piece of jelly capsule.

Discussion

The results of these experiments agree with the work previously done with *Ambystoma opacum* (6, 7) and go further in showing a relatively restricted region of production which coincides with a visible landmark on the embryo. The descriptions of the distribution of hatching gland cells in certain anurans indicate that most of the cells are located in this frontal area and to a lesser extent along the dorsal midline (2, 11). If it is assumed that the distribution of the hatching gland cells in *R. pipiens* is similar to that described for other anurans then there appears to be a correlation between the demonstrated production site and the distribution of the hatching gland cells. Obviously histological work should be done to determine the location of these cells in *R. pipiens*.

Summary

Pre-hatch embryos were stripped of their jelly capsules and membranes and cut into quarters, or smaller sections, and alike pieces were cultured together. The culture media were then tested for hatching activity by determining their ability either to raise the vitelline membranes of gastrulae or to dissolve pieces of embryo jelly capsule. From the results of these experiments it was concluded that the hatching substance was produced mainly in the most anterior portion of the embryo and to a lesser extent along the dorsal midline.

Literature Cited

1. COOPER, K. W. 1936. Demonstration of a hatching secretion in *Rana pipiens*. Proc. Nat. Acad. Sci. **22**:433-434.
2. KOBAYASHI, H. 1954. Hatching mechanism in the toad, *Bufo vulgaris formosus*; 1. Observation and some experiments on perforation of gelatinous envelope and hatching. Jour. Fac. Sci. Univ. of Tokyo **7**:79-87.
3. MINGANTI, A., and G. AZZOLINA. 1955. Proteolytic activity of the hatching enzyme of *Bufo* and *Discogloss*. Ricera Sci. **25**:2103-2108.
4. NOBLE, G. K. 1926. The hatching process in *Alytes*, *Eleutherodactylus* and other amphibians. Amer. Mus. Novit. **229**:1-7.
5. ————. 1931. The Biology of the Amphibia. McGraw-Hill Book Co., New York, p. 131.
6. NOBLE, G. K., and M. K. BRADY. 1930. The mechanism of hatching in the marbled salamander. Anat. Rec. **45**:274.
7. ————. 1933. Observations on the life history of the marbled salamander, *Ambystoma opacum* Gravenhorst. Zoologica **11**:89-132.
8. SHUMWAY, W. 1940. Stages in the development of *Rana pipiens* I. The external form. Anat. Rec. **78**:139-147.
9. STEINBERG, M. 1957. In: Carnegie Institution of Washington Year Book, 56, p. 347.
10. WINTREBRET, P. 1928. L'eclosion par digestion de la coque chez les poissons, les amphibiens et les cephalopodes dibranchiaux decapodes. Compt. rend. Assoc. Anat. **23**:496-503.
11. YANAI, T. 1958. Notes on the hatching glands of the frog, *Polypedatus buergeri*. Annot. Zool. Japon. **31**:222-224.