

Half The Frog I Should Have Been: Making Haploid Frogs for Teaching High School Biology

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ABSTRACT

I am interested in creating unusual labs to stimulate scientific inquiry in high school biology students. The phenotypic consequences of haploidy (only one set of chromosomes instead of two) were studied in frogs (*Rana pipiens*) by irradiating sperm with ultraviolet irradiation. Ploidy was initially examined by comparing nuclear sizes in diploid and haploid tadpoles. Chromosome squashing technique using silver-stained Nucleolar Organizer Regions (NOR) was used to confirm ploidy in these animals. I found that UV light has extreme effects on the viability and mobility of sperm cells. Nuclear size of the haploids was significantly smaller than that of the diploids, reflecting the differences in chromosome number and DNA amount. This difference could be used in the classroom as a convenient way to distinguish haploids from diploids for studies of things like behavior, development rate, viability, and the effects of ploidy on phenotype. These results indicate the potential for induced haploidy in frogs as a useful hands-on lab project for K-12 biology classes.

INTRODUCTION

In teaching High School Biology it is important to foster scientific inquiry in students. Activities that occur on the genetic level are difficult for students to understand simply because they can't see them. A hands-on experiment that would show them that what occurs on the cellular level affects the whole organism would be extremely helpful in fostering their comprehension of molecular biology. The differences between diploids (2n) and haploids (1n) on the genetic level could be used to distinguish variance in behavior, viability, metamorphosis, and development; all of which students are able to observe and see with their own eyes. To achieve an understanding of biology students must be able to truly comprehend how the micro level of an organism affects the macro level. In turn, it is useful for students to realize haploidy can have real consequences on biological systems. For instance, if the females of a species were able to reproduce viable organisms without any contribution of DNA from the male, the importance of sexual reproduction would drastically change. Evolutionary drifts would vary greatly within one species, because individual organisms would not be able to mask lethal genes with only one copy of DNA. This could lead to an increase in the number of mutations of a species that would normally occur at a slower rate. Thus, evolutionary concepts could be learned through the study of haploid frogs.

The phenotypic consequences of haploidy were studied in frogs (*Rana pipiens*) by exposing sperm to UV light. UV irradiation destroys DNA, yet leaves the sperm mobile so that fertilization is carried out. The resulting developing embryo is haploid. The focus of the experiment became creating haploid frogs. There were three questions asked to accomplish this goal. Can haploid *Rana pipiens* (n=13) be created by UV irradiation sperm? How are haploid frogs detected? Finally, is there an optimal level of UV irradiation to produce haploids?

It was hypothesized that UV irradiating sperm could produce haploids but it would have an effect on the viability of the sperm, and thus effect fertilization of the eggs. There would be an optimal UV irradiation where haploids would be produced. Lastly, haploidy would have an effect on the nuclei of amphibians. The average nuclear size of the haploid would be significantly smaller, reflecting half the amount of DNA and differences in chromosome number.

It was found that haploids could be created by UV irradiating sperm. Haploidy can be determined by examining chromosome squashes. In addition since the average surface area of haploid nuclei is considerably smaller than that of the diploids, nuclear size may be used to confirm haploidy. UV irradiation does have effects on the viability and haploidy of amphibians. The amount of tadpoles that developed from irradiated sperm was significantly lower than that of the non-irradiated sperm. There was an optimum level of UV exposure to produce haploid *Rana pipiens*, in which below no haploids were produced and above fertility rates declined. The optimum dosage of UV irradiation was a 15-watt germicidal lamp, 38 cm away from the sperm, for a period of 8 minutes.

MATERIALS AND METHODS

Three separate trials were performed in which the eggs of *Rana pipiens* were in vitro fertilized with irradiated and non-irradiated sperm. Two male *Rana pipiens* were euthanized in a solution of MS222 (0.8g of MS222: 400ml of tap water). The males were dissected and the testes (yellowish ovoid bodies) were removed. The testes were rolled on a paper towel to remove adhering blood and then placed in three small petri dishes with 10ml of aged tap water. The testes were minced in the tap water and allowed to stand for 15 minutes (Johnson 1973). The sperm were then examined under a compound microscope to confirm mobility. Two of the petri dishes with sperm suspension were placed under a 30-watt germicidal UV light. The third petri dish was left un-irradiated for a control. One of the petri dishes was placed 20 cm away from the light source and irradiated for 4.5 minutes, while the other dish was placed 38 cm away and irradiated for 7.5 minutes. The sperm suspensions were occasionally swirled during irradiation to ensure equal exposure. After exposure to the UV light the sperm were covered and placed aside while the eggs were removed from the female. In trial two and three the above procedure was repeated except the UV irradiation exposure was different. In trial two both sperm suspensions were irradiated with a 30-watt lamp, at 38 cm away from the petri dish. One of the dishes was exposed to the UV light for 8 minutes, while the other was irradiated for 15 minutes. In trial three both of the sperm suspensions were irradiated with a 15-watt lamp, at 38 cm away from the petri dish. However, one of the dishes was exposed to the UV rays for 8 minutes, while the other for 15 minutes. The differences in wattage, distance, and time of UV irradiation were used to determine the optimum level of UV irradiation to produce haploid frogs. A total UV exposure was calculated using the Inverse Square Law ($UV \text{ exposure} = \text{Watts (Joules/sec)} / 4\pi (\text{distance [cm]})^2$).

Thirty petri dishes were labeled as follows; 10 with 2N, 10 with 1N*, and 10 with 1N[^]. The female frog was held against the palm of one hand, while the other hand held the limbs. The female's abdomen was massaged continually until all the eggs were stripped into the 30 petri dishes. The controlled (un-irradiated) and experimental (irradiated) sperm was pipetted over the eggs in the 30 dishes. The eggs were allowed to sit for 15 minutes to ensure fertilization, and then the petri dishes were flooded with aged tap water. An hour later, when the jelly membranes had enlarged, the eggs were separated from each other using a plastic ruler and spoon (Johnson 1973). The embryos remained in the petri dishes and their embryonic development was observed until 240 hours after fertilization using a dissecting microscope. (The normal stages of *Rana pipien* development may be found in the appendix- Table A). When the embryos reached the tail bud stage (66 hours after fertilization) ploidy was confirmed by squash preparations.

Embryos from the three respective groups were placed in a dilute 0.1% colchicine solution for 24 hours. The embryos were then anesthetized in MS222 for 5 minutes. They were soaked in glass distilled water for 20 minutes and then fixed in 3:1 fixadent (3 parts 100% ethanol and 1 part glacial acetic acid). To make the chromosome preparations, the embryos were finally soaked in 45% acetic acid for 1 to 2 minutes. The whole embryo was then transferred to a slide and was minced with a razor blade. A cover slip was placed on top of the minced tissue and then squashed between bibulous paper (Sessions 2000). The squash preparations were examined under a compound microscope and pictures were taken with the oil immersion lens (100X magnification). The slides were frozen in the freezer until the next experiment was performed.

While examining the chromosomes the nuclei length and width of 100 diploids and 100 haploids were measured using the ocular meter in the microscope lens. These measurements were later used to determine surface area. Using the data collected from the 200 nuclei the measurements were converted from ocular meters to micrometers with a calibrator. The data were utilized to calculate surface area [\square (a)(b)] of each of the 200 nuclei. Variable (a) represents half the length and (b) represents half the width of the nuclei. The average surface area of the haploids and diploids and the standard deviation were determined as well. These calculations were all done on Microsoft Excel spreadsheets. Student's t test [$\square = x \pm (ts/ n)$] helped achieve the confidence interval for the mean average surface area of the 2N and 1N tadpoles (Harris 1995). In addition, graphs of the data were created using Microsoft Excel.

The slides were taken out of the freezer and the coverslips were immediately removed. The slides were then soaked in 95% ethanol for 2 minutes and air-dried (Sessions pers. comm.). The dried slides were stained with AgNO₃ (see appendix for procedure) and the Ribosomal Gene Loci (NORs) were examined (Sessions 1990).

RESULTS

The normal development rate for haploid and diploid *Rana pipiens* is shown in Fig1a. Embryonic development of diploid tadpoles is completed 240 hours after fertilization. Growth occurs exponentially in the beginning and then gradually levels off as stage 25 (Operculum completed) is approached. Haploid and

diploid tadpoles develop at the same rate up to 66 hours after fertilization. (Note: haploid tadpoles were destroyed at 66 hours to determine ploidy, therefore, the development observations ended). It was found that haploids cannot be distinguished from diploids simply by their appearance. Illustrations of various important stages in the embryonic development of diploid tadpoles are depicted in Fig. 1b.

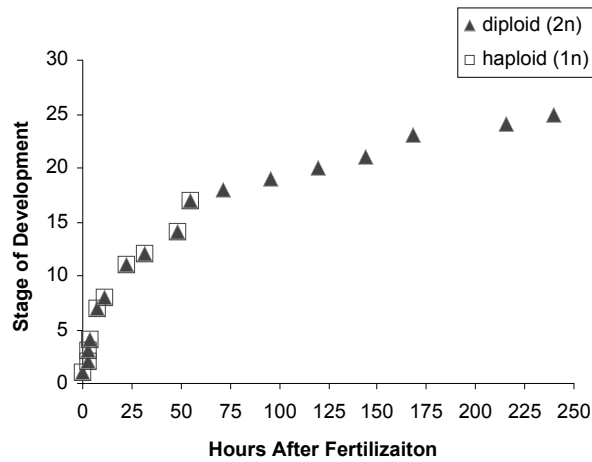


Figure 1a. Normal Development Curve for 2n and 1n *Rana pipiens*

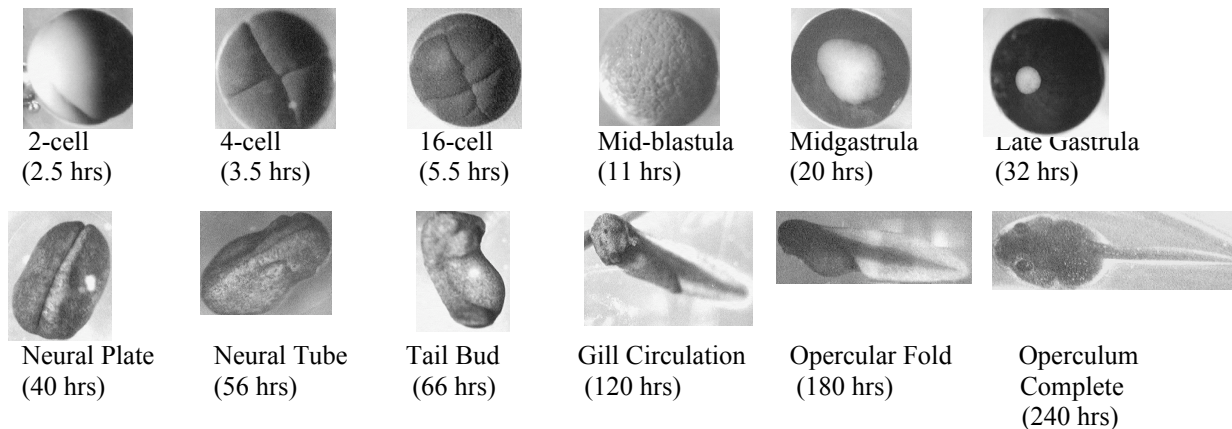


Figure 1b. Illustrations of representative stages in the normal embryonic development of *Rana pipiens*.

It was found that haploids could be distinguished from diploids by cytogenetics. Chromosome spreads for haploid (1n) and diploid (2n) tadpoles are shown in Fig. 2. The cells are arrested in the metaphase stage of mitotic cell division and can clearly be seen. It was apparent that the diploids have 26 chromosomes while the haploids only have 13.

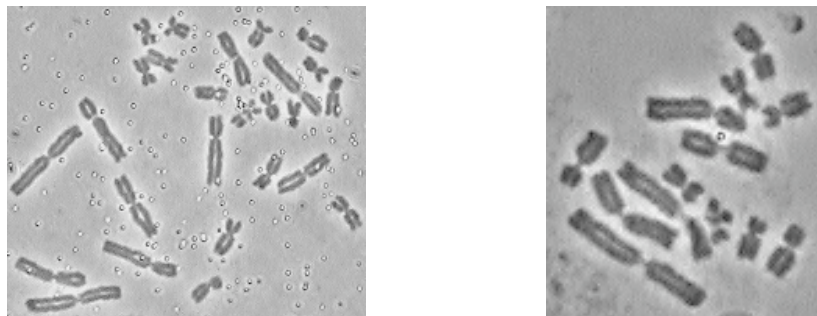


Figure 2. Squash preparations of cells from (from left to right) a diploid ($2n = 26$) and haploid ($n = 13$) *Rana pipiens* embryo; 100X magnification.

Below (Fig. 3) are the Ribosomal Gene Loci (NORs) for the haploid and diploid frogs. The NORs are the circles stained black on chromosome number ten. It was found that the diploids (left) had two ribosomal gene loci, while the haploids only had one locus.

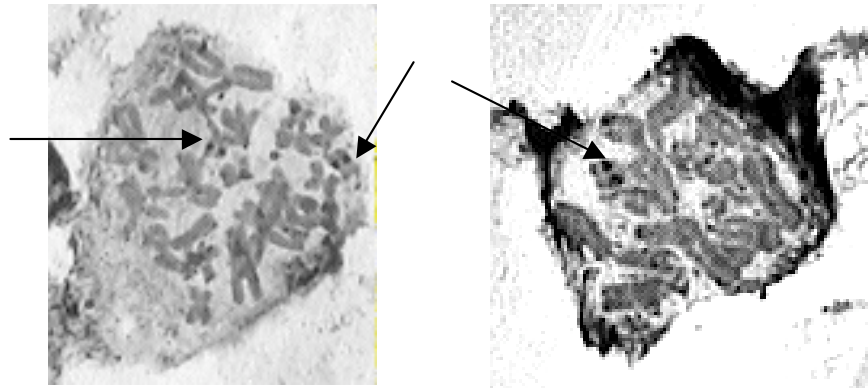


Figure 3. Nuclear Organizer Regions (NORs) [from left to right] a diploid ($2n=26$) and haploid ($n=13$) *Rana pipiens* embryo; 100X magnification

The data presented in Table 1 was collected from the 200 nuclei similar to the ones shown in Fig. 4. It was used to construct the graph shown in Fig 5. The data indicates that the average surface area of nuclei in diploids is double that of the haploids. Diploid nuclei have an average surface area of 365.5 um^2 while the haploid average surface area was 162.8 um^2 . The percent error for the $2n$ measurements was 19.2% and the error in the $1n$ measurements was 20%. The student's t-test indicates that the 95% confidence interval for the $2n$ average surface area of nuclei is between 351.6 and 379.4 um^2 . The student t-test for the $1n$ average surface area of nuclei demonstrates a range of 156.3 to 169.27 um^2 .

Table 1. Average surface area of nuclei in diploid and haploid *Rana pipiens*

	MEAN	STANDARD DEVIATION RANGE	STUDENT'S T-TEST
2n	365.5 um^2	295.3-435.7	351.6-379.4
1n	162.8 um^2	130.1-195.5	156.33-169.27

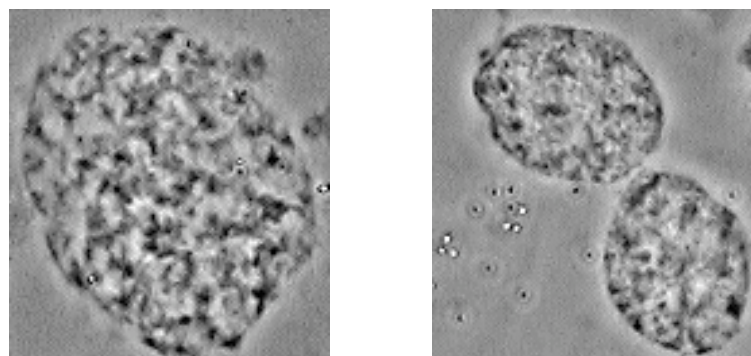


Figure 4. Nuclei of cells from (left to right) a diploid ($2n=26$) and haploid ($1n=13$) *Rana pipiens* embryo; 100x magnification

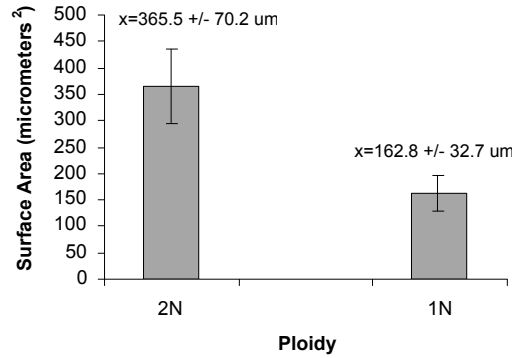


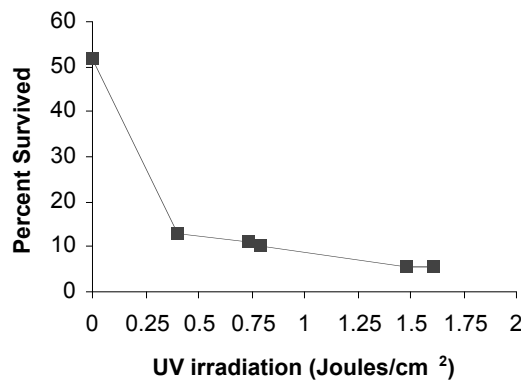
Figure 5. Average Nuclear Surface Area for diploid and haploid *Rana pipiens*

The data in Table 2 was used to construct the graph shown in Fig.6. The different amounts of UV exposure that the sperm were or were not subjected to are shown in Table 2. Total UV exposure (left column) was calculated using the inverse square law. It was found that the viability of the tadpoles decreases as UV exposure increases (Fig. 6).

Table 2. UV exposure and its effects on viability of tadpoles (*Rana pipiens*)

UV Exposure (Joules/cm)	UV Watt	Distance from Sperm (cm)	UV exposure (minutes)	Viability (Percent)
1.61	30	20	4 1/2	5.5
1.48	30	38	15	5.75
0.79	30	38	8	10
0.74	15	38	15	11
0.40	15	38	8	13
0.00	0	0	0	51.67

Figure 6. Percent viability of tadpoles exposed and not



exposed to UV irradiation

The graph shown in Fig. 7 displays the effect of UV light exposure on the production of haploid *Rana pipiens*. The optimum UV exposure to produce haploid amphibians is a 15-watt lamp, 38cm away from the sperm, for an exposure of 8 minutes (Fig. 7). An exposure of UV light for 0 or 15 minutes does not produce haploids. The percent haploids produced was 36.4% and their viability at 66 hours after fertilization was 13%.

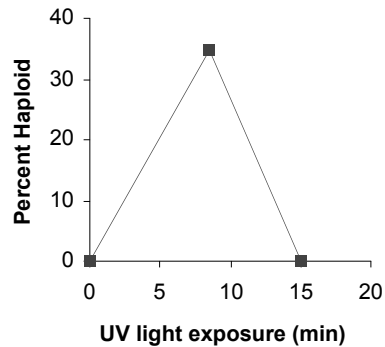


Figure 7. Effect of UV light on the production of haploid *Rana pipiens* (UV light = 15watts at 38 cm away)

DISCUSSION

Ultraviolet radiation, a genotoxic agent, has effects on the phenotypic and genotypic responses of an organism. UV light is the most harmful and mutagenic component of the solar radiation spectrum (Ravant et. al. 2001). It is divided into three forms according to wavelength; UVC, UVB, and UVA. UVC is extremely hazardous to biological systems, but it does not reach the earth's surface; because it is absorbed by atmospheric gases. On the other hand, UVB radiation reaches the earth's surface and is responsible for causing sunburns as well as mutagenic and carcinogenic effects. UVA radiation is able to pass unimpeded through the ozone layer, but it typically does not cause great injury to biological systems. The slightest change in the ozone layer can have great effects on organisms and the environment. For instance if the ozone layer is depleted by only 5%, then there is a 10% increase in the amount of UVB radiation reaching the earth's surface (Licht and Grant 1997). One of the consequences of UVB radiation is mutations in the DNA of organisms. DNA bases absorb UVB photons causing photooxidation reactions to occur. These reactions damage to DNA by forming thymine dimers. Thymine dimers prevent DNA from carrying on its normal functions and ultimately destroys it. Gametes exposed to UV light may become sterile or immobile (Ravant et. al. 2001).

It was demonstrated that there was not a noticeable difference in the development rate of haploids and diploids up to 66 hours after fertilization (Fig 1a). Both the experimental and controlled groups followed the normal stages of embryonic development (Johnson 1973). It has been shown in a similar study that gametes of sea bass irradiated with UV light produced haploids that develop at the same rate as diploids, even with the absence of half the genome (Felip et al. 1999). Therefore, it may be suggested that haploids and diploids, during embryonic development, can only distinguished from each other at the genetic level.

Determining haploidy can be done by examining chromosome squashings, ribosomal gene loci (NORs), and nuclear size. It is easy to detect haploidy by examining NORs, because these regions clearly stain black with silver nitrate. NORs are found on chromosome number ten in *Rana pipiens*. Diploid frogs have two homologous number ten chromosomes (one from the mother and one from the father) so that two ribosomal gene loci are seen. On the other hand, haploid tadpoles only have chromosome number ten from the mother. Consequently only one ribosomal gene locus is seen. The silver stained chromosomes from the diploid and haploid tadpoles in fact confirmed this.

The significant size difference in the nuclei of diploid and haploid *Rana pipiens* was also helpful in confirming ploidy. The results did support the hypothesis that haploids would have a smaller nucleus, reflecting half the amount of DNA and chromosomes. This suggests that the size of the nuclei is dependent upon the amount of genetic material located within it. The error in my measurements may be attributed to human error in reading the ocular micrometer as well as the analytical error in the ocular micrometer itself.

It was shown that as sperm were exposed to higher amounts of UV exposure the viability rates of the tadpoles declined. This suggests that the UV light is completely destroying or immobilizing the sperm. Therefore, sperm never fertilize the eggs, or create embryos that do not have adequate systems for survival. It was confirmed that there is an optimal level of UV exposure from which haploids can be created. Beyond this optimal level fertility rates decline. The viability of the haploids at the optimum level or beyond the optimum level is significantly low. This suggests that their low viability may be attributed to their homogeneity, which leads to the presence of deleterious recessive lethal genes that cannot be masked.

These findings correspond to previous studies. Prior research done on the liver enzymes of haploid and diploids shows differences in enzymatic activity (Kashawagi and Kashawagi 1996). It was concluded that the hydrogen peroxide system in the liver of the haploid frogs is abnormal. The differences found were attributed to the low viability rates of the haploids. Scientists stated that this abnormality would lead to cell death, which gave another possible explanation for low viability of haploids (Kashawagi and Kashawagi 1996). Other experiments were performed that further confirmed that UV irradiation significantly reduces the hatching success and thus effects the viability of the embryos (Blaustein et. al 1998).

The previous research does indicate there is a biochemical and physiological differences between haploids and diploids. Results of one study showed that the total body lengths of haploid tadpoles were significantly less than the diploids (Kashawagi and Kashiwagi 1996). The diploid tadpoles required about four days for completion of tail atrophy at metamorphosis. While, the haploids needed eight to nine days for tail atrophy (Kashiwagi and Kashiwagi 1996). Furthermore, it has been shown that haploid tadpoles do not have the capability of swimming like normal fish. "Tadpoles are veritable blobs, with a tail in two" (Wasserburg 1997). They tend to wobble when they swim, due to their larger frontal area (Wasserburg 1997). These studies confirm that there are observable differences between haploids and diploids that can be studied by High School Biology students in a lab. This hands-on lab activity will contribute to the students understanding of how the genetic level affects the whole organism.

In conclusion, haploids can be created by UV irradiating sperm and are easily detectable by chromosome squashing, ribosomal gene loci, and nuclear size. There is an optimal level of UV exposure to produce haploid frogs. This research contributes to the understanding of how you create and determine haploidy in amphibians. These results indicate the potential for induced haploidy in frogs as a useful hands-on lab project for K-12 biology classes. Once haploids have been created this experiment may be taken step further by observing biochemical and physiological differences in ploidy. The difference in ploidy will be especially useful in studying variance in viability, development rate, metamorphosis, tail atrophy, and body size of diploid and haploid *Rana pipiens*.

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APPENDIX

Protocol for AgNO₃ (Banding for NORs)

- 1) Mix 2 parts of 50% silver nitrate solution and 1 part developer in a glass vial and mix thoroughly
Part Developer: 2% gelatin and 1% formic acid. Mix gelatin powder in a 50ml of distilled water and heat to dissolve. Cool and add formic acid.
- 2) Add 3 drops to each preparation and quickly add a coverslip
- 3) Incubate at 90°C for 30-60 seconds (until staining solution has turned a muddy yellowish brown)
- 4) Rinse off coverslip with distilled water
- 5) Air dry slides and mount in oil or permanent mounting medium

Table A. Description of embryonic development stages of the frog, *Rana pipien*

STAGE	DESCRIPTION	AGE (HOURS)
1	Unfertilized	0
2	Gray Crescent	.5
3	2-cell	2.5
4	4-cell	3.5
5	8-cell	4.5
6	16-cell	5.5
7	32-cell	6.5
8	Mid-blastula	11
9	Late blastula	14
10	Dorsal lip	17
11	Midgastrula	20
12	Late gastrula	32
13	Neural plate	40
14	Neural folds	58
15	Late neural folds	52
16	Neural tube	56
17	Tail bud	66
18	Muscular response	76
19	Heart beat	96
20	Gill circulation	120
21	Mouth open	138
22	Tail fin circulation	156
23	Opercular fold	180
24	Operculum closed on right	210
25	Operculum complete	240