Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 3: 385–398 (1978)

# Production of Gynogenetic Haploids by Treatment of Spermatozoa with Toluidine Blue in *Rana japonica*

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(With 5 Text-figures)

## INTRODUCTION

The establishment of methods for producing gynogenetic haploids most easily and most reliably in an amphibian species is important to investigators working in the border area between embryology and genetics by making use of this species. While haploid amphibian embryos usually fail to eat and die before becoming tadpoles, a small number of haploids are able to survive beyond the metamorphosis stage. MIYADA (1960, 1977) produced metamorphosed frogs by inseminating eggs with toluidine blue-treated sperm. He was the first to describe the life histories as well as the external and internal characters of haploid frogs. The method for incapacitating the sperm nucleus with toluidine blue was devised by BRIGGS, GREEN and KING (1951). BRIGGS (1952) measured the amount of this dye bound per sperm nucleus and presented the quantity required for complete inactivation of the nucleus without injury to extranuclear structures.

On the other hand, the establishment of methods for incapacitating the frog sperm nucleus alone was necessary for producing gynogenetic diploids in which almost all the genes contained in each female pronucleus became homozygous and revealed their functions. Although various kinds of radiation have the same action upon spermatozoa as toluidine blue does, the chemical dye is often more useful than radiation, as equipment is hardly necessary.

The present authors carried out an experiment to establish the best utilization of toluidine blue in producing gynogenetic haploids in the most common Japanese brown frog species, *Rana japonica*.

## MATERIALS AND METHODS

Many male and female brown frogs, *Rana japonica* GUENTHER, were collected from the suburbs of Hiroshima in the fall of 1966 and kept in a terrarium for hibernation. Early in 1967, as the females began spontaneously to ovulate, ten of them were selected as materials for producing gynogenetic haploids. These females (Nos. 1 ~ 10) were excellent in fertilizing capacity when a test was per-

formed by making use of a small number of eggs obtained from each female; more than 95% of the eggs cleaved normally. The remaining eggs of each female were utilized for experiments within 24 hours after the test. Ten males (Nos. 1～10) were used in obtaining sperm suspensions. The testes of each male were crushed in 3 ml of frog RINGER's solution (phosphate buffer, pH 7.8). A stock solution of toluidine blue was made by dissolving 0.5 gr of toluidine blue O (Merck) in 100 ml of RINGER's solution. At the beginning of experiments, one part of the stock solution of the dye was mixed with 100 parts of sperm suspension. Thus, spermatozoa were immersed in the toluidine blue solution at the concentration of 0.005%, and were then left there for eight different periods of time, that is, 5, 10, 15, 20, 25, 30, 40 and 60 minutes. After each period the mixture of spermatozoa and the dye was diluted 1 to 5 by adding distilled water and used at once for artificial insemination. Control spermatozoa were prepared by diluting the sperm suspension 1 to 5 by adding distilled water after the toluidine blue treatment of spermatozoa in the experimental series was over, and used at once for insemination in the control series.

The eggs of each of the ten females were divided into nine parts. Eight of the latter were inseminated with spermatozoa treated with toluidine blue for eight different periods of time, respectively, while the remaining part of the eggs was fertilized with the control spermatozoa. Each part of the eggs was reared in a small glass dish at $17\pm0.5$°C until the hatching stage and then placed at room temperature until completion of metamorphosis.

The identification of haploidy was done on embryos at the hatching stage by

TABLE 1

Development of eggs inseminated with sperm exposed

| Exposure of sperm to dye (min.) | No. of eggs | No. of normally cleaved eggs | No. of gastrulae | | No. of neurulae | |
|---|---|---|---|---|---|---|
| | | | Normal | Abnormal | Normal | Abnormal |
| 0 (Control) | 604 | 540 | 539 (99.8%) | 1 ( 0.2%) | 535 (99.1%) | 4 ( 0.7%) |
| 5 | 737 | 686 | 648 (94.5%) | 38 ( 5.5%) | 600 (87.5%) | 48 ( 7.0%) |
| 10 | 747 | 679 | 607 (89.4%) | 72 (10.6%) | 468 (68.9%) | 139 (20.5%) |
| 15 | 734 | 651 | 516 (79.3%) | 135 (20.7%) | 369 (56.7%) | 147 (22.6%) |
| 20 | 637 | 573 | 484 (84.5%) | 89 (15.5%) | 329 (57.4%) | 155 (27.1%) |
| 25 | 702 | 580 | 461 (79.5%) | 119 (20.5%) | 302 (52.1%) | 159 (27.4%) |
| 30 | 685 | 610 | 566 (92.8%) | 44 ( 7.2%) | 517 (84.8%) | 49 ( 8.0%) |
| 40 | 680 | 597 | 578 (96.8%) | 19 ( 3.2%) | 557 (93.3%) | 21 ( 3.5%) |
| 60 | 724 | 626 | 614 (98.1%) | 12 ( 1.9%) | 597 (95.4%) | 17 ( 2.7%) |

observing the shape and external structure as well as by counting the chromosome number of mitotic figures and measuring the size of resting nuclei in the preparations which had been made from their tail tips by the squash method.

## OBSERVATION

### *I. Control series*

In the breeding season of 1967, ten matings (Nos. 1～10) were performed between 10 females (Nos. 1～10) and 10 males (Nos. 1～10). Of 604 eggs in total, 540 (89.4%) cleaved normally. The rate of normally cleaved eggs in each of the ten matings was 81～96%. During the embryonic stage, 506 (93.7%) of the normally cleaved eggs developed normally and hatched, while the others died of various abnormalities. The rate of normally hatched tadpoles to normally cleaved eggs in each of the ten matings was 87～100%. The total number of normal and abnormal embryos which attained the hatching stage was 533 (98.7%) of the normally cleaved eggs. At the tadpole stage, 37 individuals died of ill-development or some other abnormalities, and 469 (86.9%) metamorphosed normally (Table 1, Fig. 1).

As 175 normally shaped tadpoles in total were obtained from three (Nos. 1～3) matings, their ploidy was examined by using their tail-tips. As a result, it was found that all of them were diploids (Table 2). On the basis of this finding, 331 normally shaped tadpoles produced from the remaining 7 matings were

to toluidine blue for different lengths of time

| No. of tail-bud embryos | | No. of hatching embryos | | No. of 40-day-old tadpoles | No. of metamorphosed frogs |
|---|---|---|---|---|---|
| Normal | Abnormal | Normal | Abnormal | | |
| 533 (98.7%) | 2 ( 0.4%) | 506 (93.7%) | 27 ( 5.0%) | 490 (90.7%) | 469 (86.9%) |
| 508 (74.1%) | 92 (13.4%) | 377 (55.0%) | 131 (19.1%) | 343 (50.0%) | 314 (45.8%) |
| 284 (41.8%) | 184 (27.1%) | 163 (24.0%) | 121 (17.8%) | 146 (21.5%) | 138 (20.3%) |
| 171 (26.3%) | 198 (30.4%) | 74 (11.4%) | 97 (14.9%) | 57 ( 8.8%) | 48 ( 7.4%) |
| 96 (16.8%) | 233 (40.7%) | 15 ( 2.6%) | 81 (14.1%) | 15 ( 2.6%) | 11 ( 1.9%) |
| 133 (22.9%) | 169 (29.1%) | 8 ( 1.4%) | 125 (21.6%) | 5 ( 0.9%) | 5 ( 0.9%) |
| 385 (63.1%) | 132 (21.6%) | 0 | 385 (63.1%) | 0 | 0 |
| 530 (88.8%) | 27 ( 4.5%) | 0 | 530 (88.8%) | 0 | 0 |
| 586 (93.6%) | 11 ( 1.8%) | 0 | 586 (93.6%) | 0 | 0 |

TABLE 2

Normally and abnormally shaped embryos at the hatching stage

| Exposure of sperm to dye (min.) | No. of eggs | No. of normally cleaved eggs | Embryos at the | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total no | Percentage to normally cleaved eggs | Normally shaped | | |
| | | | | | No. | Percentage to normally cleaved eggs | Percentage to hatching embryos |
| 0 (Control) | 604 | 540 (89.4%) | 533 | 98.7 | 506 | 93.7 | 94.9 |
| 5 | 737 | 686 (93.1%) | 508 | 74.1 | 377 | 55.0 | 74.2 |
| 10 | 747 | 679 (90.9%) | 284 | 41.8 | 163 | 24.0 | 57.4 |
| 15 | 734 | 651 (88.7%) | 171 | 26.3 | 74 | 11.4 | 43.3 |
| 20 | 637 | 573 (90.0%) | 96 | 16.8 | 15 | 2.6 | 15.6 |
| 25 | 702 | 580 (82.6%) | 133 | 22.9 | 8 | 1.4 | 6.0 |
| 30 | 685 | 610 (89.1%) | 385 | 63.1 | 0 | 0 | 0 |
| 40 | 680 | 597 (87.8%) | 530 | 88.8 | 0 | 0 | 0 |
| 60 | 724 | 626 (86.5%) | 586 | 93.6 | 0 | 0 | 0 |

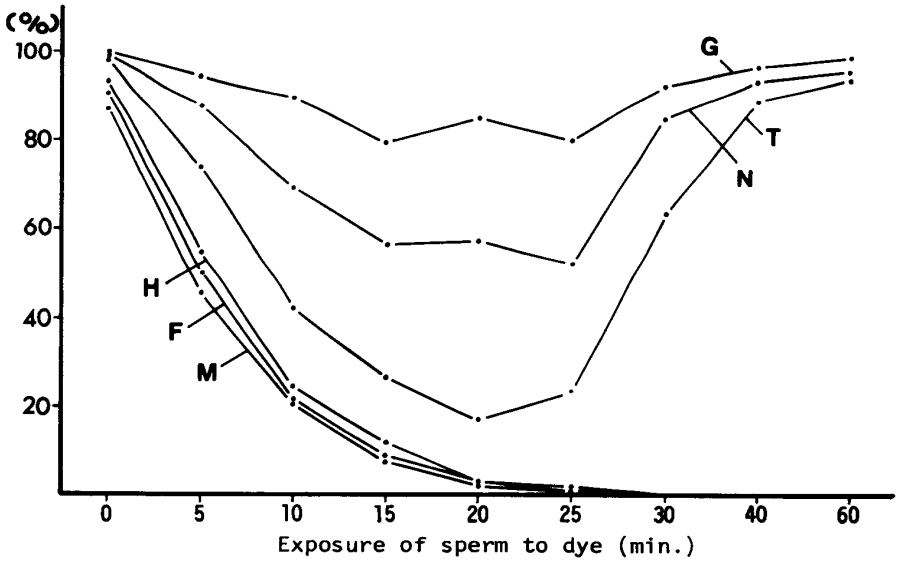


Fig. 1. Proportions of individuals at different developmental stages to normally cleaved eggs.
G: Gastrulae N: Neurulae T: Tail-bud embryos
H: Hatching embryos F: Feeding tadpoles M: Metamorphosed frogs

assumed to be diploids without observing their mitotic figures and resting nuclei. Besides these normally shaped tadpoles, 27 abnormally shaped embryos at the hatching stage were produced from the ten matings. Examination of their ploidy revealed that each of them was nearly a diploid, and neither haploids nor mosaics with haploid cells were found among them.

## *II. Experimental series*

### 1. Sperm treated for 5 minutes

Of 737 eggs in total obtained from females Nos. 1~10, 686 (93.1%) cleaved

raised from eggs inseminated with toluidine blue-treated sperm

| hatching stage | | | | | | |
|---|---|---|---|---|---|---|
| | Abnormally shaped | | | | | |
| Total no. | Haploid type | | | Non-haploid type | | |
| | No. | Percentage to normally cleaved eggs | Percentage to hatching embryos | No. | Percentage to normally cleaved eggs | Percentage to hatching embryos |
| 27 | 0 | 0 | 0 | 27 | 5.0 | 5.1 |
| 131 | 12 | 1.7 | 2.4 | 119 | 17.3 | 23.4 |
| 121 | 16 | 2.4 | 5.6 | 105 | 15.5 | 37.0 |
| 97 | 23 | 3.5 | 13.5 | 74 | 11.4 | 43.3 |
| 81 | 44 | 7.7 | 45.8 | 37 | 6.5 | 38.5 |
| 125 | 110 | 19.0 | 82.7 | 15 | 2.6 | 11.3 |
| 385 | 371 | 60.8 | 96.4 | 14 | 2.3 | 3.6 |
| 530 | 518 | 86.8 | 97.7 | 12 | 2.0 | 2.3 |
| 586 | 583 | 93.1 | 99.5 | 3 | 0.5 | 0.5 |

normally after artificial insemination by toluidine blue-treated spermatozoa of males Nos. 1～10 (Table 1). In each of 10 matings (Nos. 1～10), 84～100% of the respective number of eggs cleaved normally. At various embryonic stages, many individuals died of various abnormalities, differing from the control series; 38, 48, 92 and 131 died at the gastrula, the neurula, the tail-bud and the hatching stage, respectively. A total of 377 embryos (55.0%) of the normally cleaved eggs hatched normally. The percentage of normally hatched tadpoles in each of the 10 matings was 39～71%.

Normal and abnormal hatching embryos totalled 508, that is, 74% of the normally cleaved eggs. At the tadpole stage, 63 individuals died of ill-development, edema or some other abnormalities, and eventually 314 (45.8%) of the normally cleaved eggs metamorphosed normally. The rate of normally metamorphosed frogs in each of the 10 matings was 28～64%.

Twelve (9.2%) of the 131 abnormal embryos at the hatching stage had a typical haploid syndrome, that is, they were microcephalic and edematous and had a short tail with rather high tail fins (Table 2). After examining the mitotic figures and resting nuclei in their tail tips, all the 12 abnormal embryos were found to be haploids. This number of haploids corresponded to 2% of the total number of normal and abnormal embryos at the hatching stage. The remaining 119 abnormal embryos at the hatching stage had no haploid syndrome, although they were edematous. They had curved body or blisters on the skin. After examining their mitotic figures and resting nuclei in the tail-tip preparations, there were no haploids among them.

Mitotic figures and resting nuclei were observed in 111 normally shaped tadpoles produced from 3 matings Nos. 1～3. As all of them were nearly diploids, all the 266 normally shaped tadpoles produced from the other 7 matings were also presumed to be nearly diploids.

## 2. Sperm treated for 10 minutes

Artificial insemination was made between a total of 747 eggs from females Nos. 1～10 and toluidine blue-treated spermatozoa from males Nos. 1～10. In each of 10 matings (Nos. 1～10), 83～100% of the respective number of eggs cleaved normally; a total of 679 (90.9%) eggs did so in the 10 matings (Table 1). Although this rate of cleaved eggs was not lower than that of the controls, most of them became abnormal and died during the embryonic stage; 72, 139 and 184 died of various abnormalities at the gastrula, the neurula and the tail-bud stage, respectively. At the hatching stage, 121 other embryos became abnormal and died, while 163 or 24% of the normally cleaved eggs were normal in appearance and became normally shaped tadpoles. The rate of normally hatched tadpoles in each of the ten matings was 9～47%; the normal and abnormal hatching embryos totalled 284 (41.8%).

After the hatching stage, 25 tadpoles died of ill-development or edema, and eventually 138 (20.3%) of the normally cleaved eggs attained completion of metamorphosis. The rate of normally metamorphosed frogs in each of the 10 matings was 6～40%.

Sixteen of the 121 abnormal embryos at the hatching stage had a typical haploid syndrome (Table 2, Fig. 2b). They were 6% of the total number of normal and abnormal embryos at the hatching stage. After examining the mitotic figures and resting nuclei in their tail-tips, all of them were confirmed to be actually

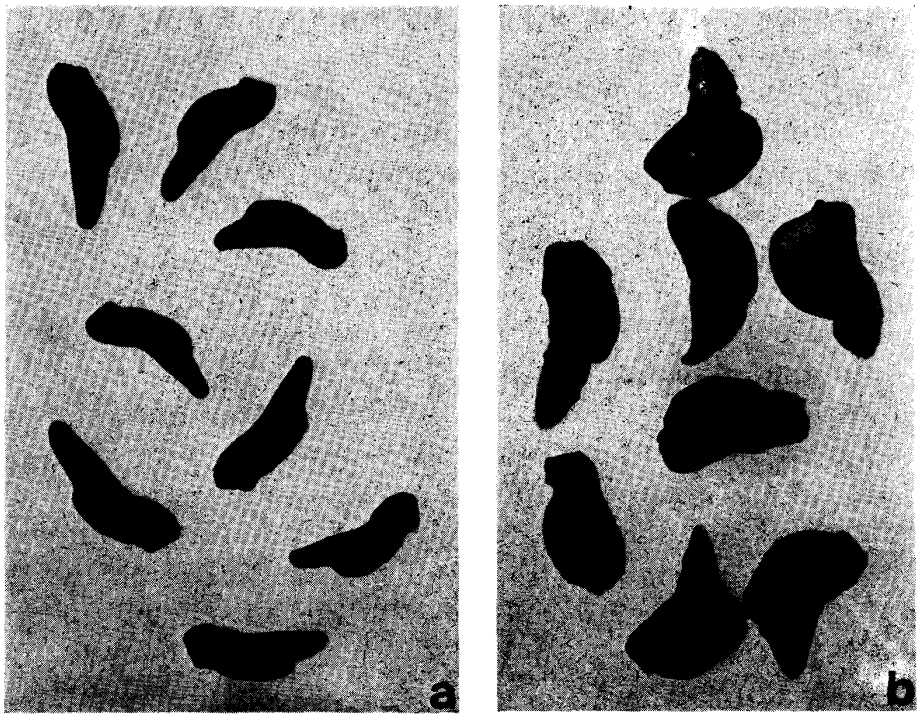


Fig. 2. Haploid embryos raised from spermatozoa treated with toluidine blue for 10 minutes. ×3.5

a, Control diploids. b, Haploid embryos at the hatching stage.

haploids (Fig. 3b). Contrarily, there were no haploids among the remaining 105 embryos, except three which were mosaics containing haploid cells. There were also no haploids among 35 normally hatched tadpoles produced from three matings Nos. 1～3. All of them were nearly diploids except one tadpole which was a mosaic of diploid and triploid cells.

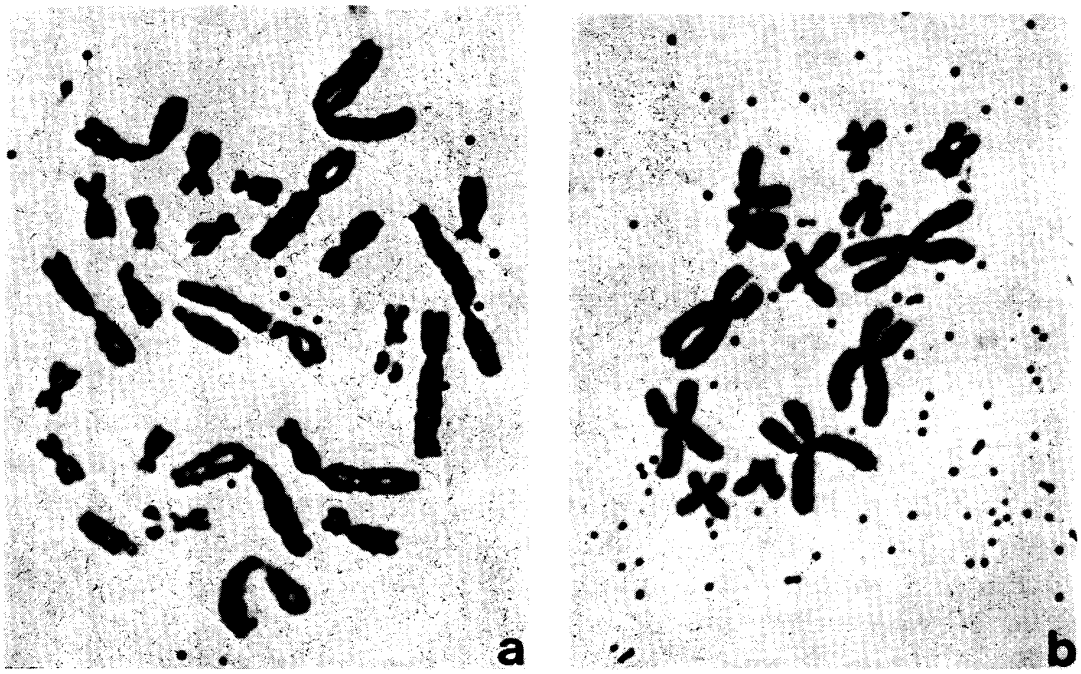


Fig. 3. Metaphase plate of mitotic chromosomes in the tail-fin of a haploid embryo raised from a toluidine blue-treated spermatozoon. ×1600

a, Control diploid. b, Haploid embryo at the hatching stage.

### 3. Sperm treated for 15 minutes

A total of 734 eggs of 10 females (Nos. 1～10) were used for insemination with toluidine blue-treated spermatozoa of 10 males (Nos. 1～10). It was found that 73～100% of the respective total number of eggs in each of 10 matings cleaved normally; 651 (88.7%) of the total eggs did so (Table 1). The development of the normally cleaved eggs was much worse than that in the experimental series from sperm treated with toluidine blue for 10 minutes. During the embryonic stage, 135, 147 and 198 of the normally cleaved eggs died of various abnormalities at the gastrula, the neurula and the tail-bud stage, respectively. At the hatching stage, 97 became abnormal and were dying, while 74 (11.4%) were normal in appearance and hatched normally. The normal and abnormal embryos at the hatching stage totalled 171 (26.3%). In each of 10 matings, normally hatched tadpoles corresponded to 1～20% of the respective number of normally cleaved eggs. By completion of metamorphosis, 26 tadpoles died of ill-development or edema, and eventually 48 (7.4%) metamorphosed normally; in each of the 10 matings, 0～17% of normally cleaved eggs did so.

Of the 97 abnormal embryos at the hatching stage, 23 had a typical haploid syndrome (Table 2). They were 14% of the total number of normal and abnormal embryos at the hatching stage. Their haploidy was later ascertained by observing mitotic figures and resting nuclei in the tail-tip preparations. Among the remaining 74 embryos, there were no haploids except six which were mosaics containing haploid cells. Sixteen normally hatched tadpoles produced from matings Nos. 1~3 were all confirmed to be nearly diploids by observing mitotic figures and resting nuclei.

### 4. Sperm treated for 20 minutes

Of 637 eggs in total obtained from females Nos. 1~10, 573 (90.0%) cleaved normally after artificial insemination by toluidine blue-treated spermatozoa of males Nos. 1~10 (Table 1); in each of 10 matings, 78~96% of the respective number of eggs did so. In spite of the high percentages in cleavage, the normally cleaved eggs were very low in developmental capacity. Only 15 (2.6%) of them hatched normally and became normally shaped tadpoles, while 89, 155, 233 and 81 died or were dying of various abnormalities at the gastrula, the neurula, the tail-bud and the hatching stage, respectively. The normal and abnormal embryos at the hatching stage totalled 96 (16.8%). The 15 normally hatched tadpoles were produced from 3 of the 10 matings, while no normal tadpoles were obtained from the other 7 matings. During the tadpole stage, 4 died of edema or ill-development, and 11 metamorphosed normally.

Forty-four of the 81 abnormal embryos at the hatching stage had a typical haploid syndrome (Table 2). They were 46% of the total number of normal and abnormal embryos at the hatching stage. Their haploidy was later confirmed by observing mitotic figures and resting nuclei in the tail-tip preparations. Although two others were mosaics containing haploid cells, there were no haploids among the remaining 35 abnormal embryos. All the normally hatched tadpoles were nearly diploids.

### 5. Sperm treated for 25 minutes

Spermatozoa of males Nos. 1~10 were used to inseminate 702 eggs in total obtained from females Nos. 1~10. In each of 10 matings (Nos. 1~10), 73~93% of the respective number of eggs cleaved normally; in total 580 (82.6%) eggs did so (Table 1). While eight (1.4%) of the normally cleaved eggs hatched normally, all the others became abnormal at various embryonic stages; 119, 159, 169 and 125 died or were dying at the gastrula, the neurula, the tail-bud and the hatching stage, respectively. The total number of normal and abnormal embryos at the hatching stage was 133 (22.9%). Five of the 8 normally hatched tadpoles completed metamorphosis.

Of the 125 abnormal embryos at the hatching stage, 110 had a typical haploid syndrome (Table 2). They were 82.7% of the total number of normal and abnormal embryos at the hatching stage. The haploidy was confirmed in 18 of them produced from three of the 10 matings by observing mitotic figures

and resting nuclei in the tail-tip preparations, while the haploidy of the other haploid-type embryos was not confirmed. Among the remaining 15 abnormal embryos there were no haploids, although three of them were mosaics containing haploid cells. The 8 normally shaped tadpoles were all nearly diploids.

### 6. Sperm treated for 30 minutes

As a result of artificial insemination of 685 eggs obtained from females Nos. 1～10 by toluidine blue-treated spermatozoa, 610 (89.1%) of them cleaved normally (Table 1). In each of 10 matings, 74～95% of the respective number of eggs cleaved normally. Of the normally cleaved eggs, 44, 49 and 132 died at the gastrula, the neurula and the tail-bud stage, respectively. The remaining 385 (63.1%) embryos became abnormal and were dying at the hatching stage.

Of these abnormal embryos at the hatching stage, 371 (96.4%) had a typical haploid syndrome (Table 2). Their haploidy was confirmed in 91 embryos produced from 3 matings Nos. 1～3 by observing mitotic figures and resting nuclei in the tail-tip preparations. While five of the remaining 14 abnormal embryos were mosaics with haploid cells, there were no haploids among the other 9 embryos.

### 7. Sperm treated for 40 minutes

Of 680 eggs in total obtained from females Nos. 1～10, 597 (87.8%) cleaved normally by artificial insemination with toluidine blue-treated spermatozoa of males Nos. 1～10 (Table 1). In each of 10 matings, 71～97% of the respective number of eggs cleaved normally. While 19, 21 and 27 of the normally cleaved eggs died at the gastrula, the neurula and the tail-bud stage, respectively, the other 530 (88.8%) became abnormal and were dying at the hatching stage. Of the latter embryos, 518 (97.7%) had a typical haploid syndrome (Table 2). Their haploidy was confirmed in 175 haploid-type embryos produced from 3 matings Nos. 1～3 by observing mitotic figures and resting nuclei in the tail-tip preparations. While a few of the other 12 embryos were mosaics with haploid cells, no haploids were found among these embryos.

### 8. Sperm treated for 60 minutes

Artificial insemination was performed between 724 eggs in total obtained from females Nos. 1～10 and toluidine blue-treated spermatozoa of males Nos. 1～10. As a result, 626 (86.5%) of the eggs cleaved normally (Table 1); in each of 10 matings, 68～95% of the respective number of eggs did so. After 12, 17 and 11 of the normally cleaved eggs died of various abnormalites at the gasturla, the neurula and the tail-bud stage, respectively, all the remaining 586 (93.6%) became abnormal and were dying at the hatching stage.

Of the abnormal embryos at the hatching stage, 583 (99.5%) had a typical haploid syndrome (Table 2). Their haploidy was confirmed in 178 embryos produced from 3 matings Nos. 1～3 by observing mitotic figures and resting nuclei in the tail-tip preparations. The remaining 3 abnormal embryos which did not reveal a haploid syndrome were all mosaics containing haploid cells.

### 9. Function of sperm treated with toluidine blue

When eggs were inseminated by sperm treated for 5~25 minutes with toluidine blue, the yield of normally hatched tadpoles decreased with the increase of the length of treatment. While normally hatched tadpoles were always produced by sperm treated for 5~15 minutes, such tadpoles were never produced by sperm treated for 30 minutes or more. However, there were slight differences among different matings whose sperm had been treated for an equal length of time. When sperm treated for 20 minutes were used, no normally hatched tadpoles were produced from 7 matings, while some were produced from the other 3 matings. When sperm treated for 25 minutes were used, no such tadpoles were produced from 7 matings, too.

The rate of both normal and abnormal hatching embryos to normally cleaved eggs was the lowest on the whole when eggs were inseminated by sperm treated with toluidine blue for 20 minutes. However, there were slight differences in this respect among different matings. In matings Nos. 3, 6 and 7, this rate was the lowest when eggs were inseminated by sperm treated for 15 minutes, while in matings Nos. 1, 5, 8, 9 and 10 it was the lowest when eggs were done by sperm treated for 20 minutes. In the other two matings, Nos. 2 and 4, the rate was the lowest when eggs were inseminated by sperm treated for 25 minutes.

When eggs were inseminated by sperm treated for 20 minutes with toluidine blue, 44 (45.8%) of 96 hatching embryos were haploids. The rate of haploids became sharply higher with the increase of the length of treatment; 83%, 96%, 98% and 99% of the respective number of hatching embryos became haploids, when eggs were inseminated by sperm treated for 25, 30, 40 and 60 minutes, respectively. These percentages of haploids corresponded to 19%, 61%, 87% and 93% of respective number of normally cleaved eggs. Thus, it was found that sperm treated for 40 or 60 minutes with toluidine blue were most useful for producing haploids abundantly, when the dye was used in the concentration of 0.005% at pH 7.8.

## DISCUSSION

Since more than 60 years ago, haploid embryos have abundantly been produced from amphibian eggs by artificial parthenogenesis, gynogenesis, androgenesis or merogony. Of these artificial methods, the gynogenesis seems to be the most efficient in obtaining numerous haploids easily and reliably in anurans at least. HERWIG, O. (1911) and HERTWIG, G. (1913) produced haploids by inseminating eggs with sperm exposed to radium bromide. RUGH (1939), BRIGGS, GREEN and KING (1951) and LEHMAN (1955) utilized X-rays, while SELMAN (1958), and POGANY (1971, 1973, 1976) used UV-rays in order to incapacitate the sperm nucleus. These kinds of irradiation were also used to incapacitate the egg nucleus and to produce androgenetic haploids by HERTWIG, G. (1911), RUGH and EXNER (1940), BRIGGS, GREEN and KING (1951), GURDON (1960) and HAMILTON (1963).

On the other hand, some chemicals have been found to give the same effect on eggs as irradiation does in incapacitating the sperm nucleus. HERTWIG, G. (1924) and DALCQ (1931) observed gynogenetic development of frog eggs by sperm treated with trypaflavine. BRIGGS, GREEN and KING (1951) and BRIGGS (1952) have confirmed that toluidine blue is more effective than trypaflavine as a dye for incapacitating the sperm nucleus in *Rana pipiens*. By making use of toluidine blue MIYADA (1960) produced numerous gynogenetic haploid *Rana nigromaculata* which were viable beyond the hatching stage. Eighteen of these haploids became completely metamorphosed or metamorphosing frogs. A haploid frog produced by him later was 3 years old (MIYADA, 1977). Recently, JONES, JACKSON and WHITING (1975) produced gynogenetic haploid embryos from *Xenopus laevis* eggs by inseminating with sperm treated with ethyleneurea. Although the so-called HERTWIG effect has so far been recognized by all the investigators who participated in producing gynogenetic or androgenetic haploids, the most recent studies on gynogenesis by JONES, JACKSON and WHITING (1975) and POGANY (1971, 1973, 1976) seemed to be focussed on the elucidation of the mechanisms resulting in the HERTWIG effect.

The results obtained by the present authors showed the HERTWIG effect very clearly (Fig. 4). The rate of embryos at the hatching stage was the lowest, that is, about 17% of normally cleaved eggs when eggs were inseminated by sperm treated with 0.005% toluidine blue solution for 20 minutes, while it was about 94 percent by sperm treated with the same solution for 60 minutes. The proportions of eggs developing abnormally and of eggs developing as haploids were similar on the whole to those found by BRIGGS (1952), JONES, JACKSON and WHITING (1975) and POGANY (1976). However, the results of the present reseach

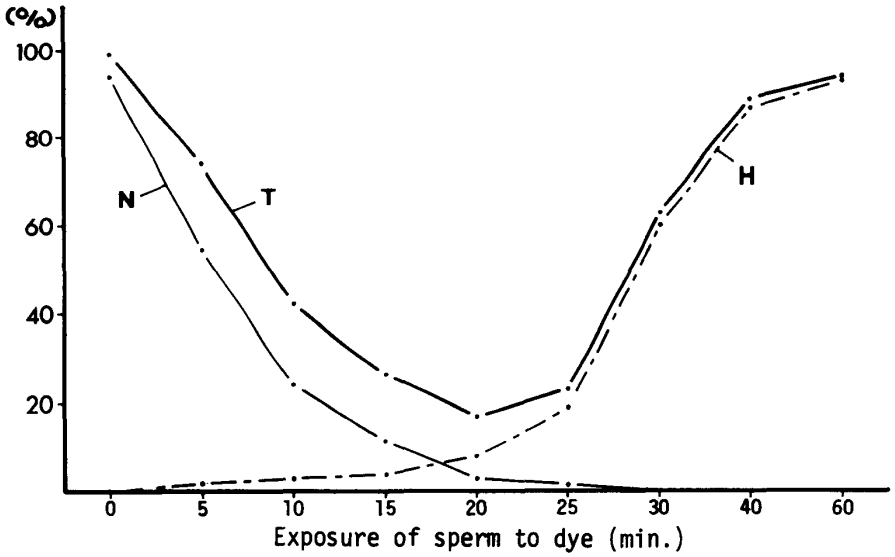


Fig. 4. Proportions of total, normally shaped and haploid type embryos at the hatching stage to normally cleaved eggs.

H: Haploid type embryos N: Normally shaped embryos T: Total embryos

somewhat differed from BRIGGS and POGANY's results in that the rise and drop in the number of eggs developing abnormally were not so sharp as found in the experiments of these authors (Table 1, Fig. 5).

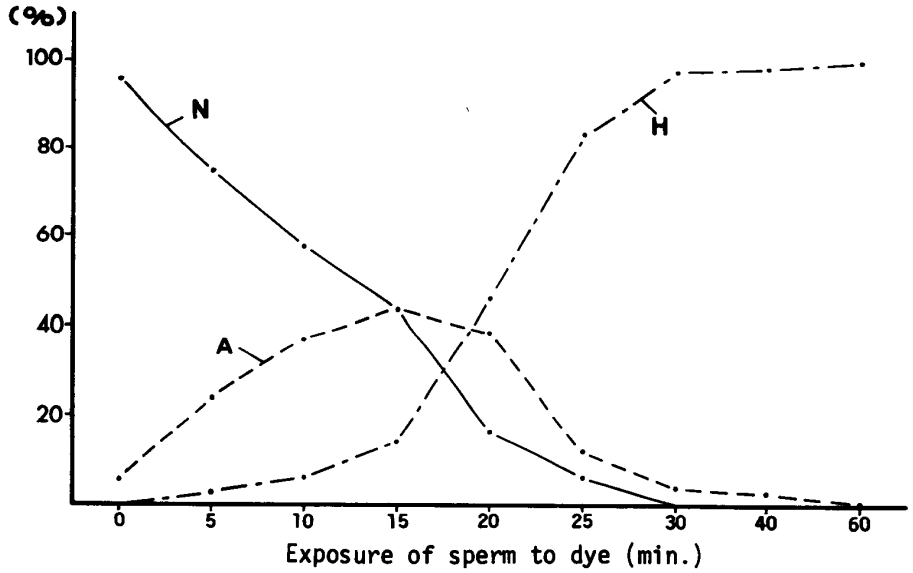


Fig. 5. Proportions of normally shaped, haploid type and abnormal non-haploid type embryos to total ones at the hatching stage.

A: Abnormal, non-haploid type embryos H: Haploid type embryos N: Normally shaped embryos

POGANY suggested two consecutive types of reactions to UV-irradiation. The first type is manifested by highly abnormal growth in spite of the retention of the diploid condition, while the second one is manifested by rapid loss of affected chromosomes. In the present study, the rates of abnormal, non-haploid embryos revealed a comparatively low normal curve between zero and 60 minute exposure to toluidine blue (Fig. 5). Thus, the first type of radiation suggested by POGANY seemed to be lacking. However, it was not determined whether the abnormal embryos were diploids or aneuploids, although they were confirmed to be non-haploid embryos. The proportion of normally shaped embryos which were almost diploids decreased linearly and became nearly zero at the exposure of 30 minutes. In contrast with this, the rates of haploids revealed a gentle sigmoidal curve between zero and 60 minute exposure.

As clearly shown in Fig. 5, haploid embryos can be produced most reliably and efficiently from *Rana japonica* eggs by inseminating with sperm treated with 0.005% toluidine blue solution for 40 or 60 minutes. From the three curves shown in Fig. 5, it seems probable that the abnormal, non-haploid embryos are individuals transitional from diploids to haploids, that is, hypodiploids which were produced by damage or loss of some of the sperm chromosomes exposed to toluidine blue. The karyotypes of such abnormal embryos are now being examined at the authors' laboratory.

## SUMMARY

Eggs of the Japanese brown frog, *Rana japonica,* were inseminated with toluidine blue-treated spermatozoa of the same species in order to establish the best method for producing gynogenetic haploids by making use of this dye. The results of experiments showed very clearly the HERTWIG effect. The rate of embryos at the hatching stage to cleaved eggs was the lowest when eggs were inseminated by spermatozoa treated with 0.005% toluidine blue solution for 20 minutes. When eggs were inseminated by spermatozoa treated with the dye for 40 or 60 minutes, they cleaved normally and nearly all the normally cleaved eggs became typical gynogenetic haploids.

## ACKNOWLEDGMENTS

The authors express their gratitude to Emeritus Professor Toshijiro KAWAMURA for his helpful advice given during the course of this study and for his critical review of the original manuscript.

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