Long-term Maintenance of Egg-fertilizability in Water in the Salamander *Hynobius nigrescens*

MASATO HASUMI,
YOSHINORI HASEGAWA,
AND HISAAKI IWASAWA

Abstract: Eggs in an egg sac of *Hynobius nigrescens* have nearly 100% fertilizability even after three hour-long immersion in water. Promptly inseminating in water seems to be extrinsic embryologically in this species.

Key words: Salamander; *Hynobius nigrescens*; Egg-fertilizability

Usually, the fertilizability of anuran eggs is lost within a few minutes in water (Ishihara et al., 1984; Katagiri, 1987). Also, in the loach *Misgurnus fossilis*, the fertilizability of eggs is maintained for only five min in water at 19–23°C (Gamo et al., 1960). Artificial fertilization in fresh-water fishes and amphibians is conducted mostly by the dry method without immersion of eggs in water (Kume, 1929; Brunst, 1955; Gamo et al., 1960; Gurdon, 1967; Moriya, 1976; Dabagyan and Sleptsova, 1991). We report here in *Hynobius nigrescens* a new finding contradicting the traditional view that the fertilizability of eggs is lost shortly after spawning in water.

MATERIALS AND METHODS

Aquatic phase males and gravid females of *H. nigrescens* were captured on 7 June 1993 from a breeding pond in Mt. Ninoji-dake, Niigata Prefecture, Japan (37°54'N, 139°29'E; 1100 m elevation). We performed artificial fertilization using these animals on the night of the same day, because the females were ready to oviposit. An egg sac in the right, distal part of the ovisac (=dilatable ovisac; for terminology, see Hasumi, 1993) of a female was taken out through her cloacal opening, and immersed in 400 ml of aged tap water at 8°C, the mean water temperature of the breeding pond. Each egg sac from a total of 14 females was immersed in the water for a different length of time, from 1 to 180 min. After having been immersed in the water, eggs in the egg sac were inseminated for five min with 0.4 ml of seminal fluid collected from the vasa deferentia of one male. That is, a

total of 28 males were used for the seminal fluid collection, including the control groups mentioned below. The egg sac was thereafter maintained in aged tap water at 8°C. As a control for the experiment, eggs in an egg sac from the female's left, dilatable ovisac were inseminated without prior immersion in the water.

Fertilization rates, i.e., percentages of cleaved eggs (Moriya, 1976; Ishihara et al., 1984), could not be determined. Instead, we examined percentages of developing embryos at the stages of pre-tail bud I–III (stage numbers 21–23; Iwasawa and Yamashita, 1991) 12 days after insemination. The percentage of developing embryos for each experimental group was adjusted, by dividing by that for its control. The rates of developing embryos between experimental and control groups were compared with the chi-square test ($\alpha=0.05$).

RESULTS

The mean number of eggs in the egg sacs examined was 27.3 (SD=4.2, N=28), ranging from 14 to 34 (Table 1). Eggs in an egg sac had nearly 100% fertilizability even after three hours of immersion in water. In fact, the fertilizability of eggs did not seem to decrease with prolonged immersion in water before insemination. However, the percentages of developing embryos in experimental groups of 12, 30, 45, and 60 min were significantly lower than in their control groups, but not in others.

DISCUSSION

The male behavior called "midwifing" (Nussbaum, 1985) is designed to assist a female in the delivery of egg sacs (Sasaki, 1924) and results in a prompt release of the sacs from the female's cloaca (Hasumi and Iwasawa, 1994). This behavior certainly makes an important contribution to prompt fertilization in water. However, the surprising result of this study clearly shows that prompt insemination in water is extrinsic embryologically in *H. nigrescens*. In the field, a number of males crowd around the spawning female and the eggs are inseminated as the egg sacs emerge from the female's cloaca. Competition between males for a genetic contribution to the offspring seems to be a primary factor facilitating prompt insemination.

Further analysis is needed to determine when the fertilizability of eggs is lost in water. At any rate, such a long persistence of egg-fertilizability in water is not known in fresh-water fishes or anurans. It might be interesting to examine differences in the role of oviducal secretions and

TABLE 1. Time of immersion of egg sacs in water, number of developing embryos in the egg sacs, total number of embryos in each, percentage of developing embryos, and adjusted percentages for each experimental group in *Hynobius nigrescens*. Abbreviations C and E represent control and experimental groups, respectively.

Time in water (min)	N of embryos		Percentage of developing embryos	Adjusted percentage
	Developing	Total		
1 C	25	29	86.2	
E	23	30	76.7	89.0
2 C	19	23	82.6	
E	18	25	72.0	87.2
5 C	25	30	83.3	
E	21	28	75.0	90.0
6 C	28	28	100.0	
E	33	33	100.0	100.0
8 C	23	27	85.2	
E	22	30	73.3	86.0
9 C	22	24	91.7	
E	23	27	85.2	92.9
12 C	20	21	95.2	
E	19	27	70.4	73.9
15 C	27	27	100.0	
E	21	22	95.4	95.4
30 C	28	31	90.3	
E	19	28	67.8	75.1
45 C	26	28	92.8	
E	19	33	57.6	62.1
60 C	34	34	100.0	
E	20	28	71.4	71.4
90 C	12	14	85.7	
E	29	29	100.0	100.0
150 C	21	22	95.4	
E	29	30	96.7	100.0
180 C	25	25	100.0	
E	29	31	93.5	93.5

the mechanism of sperm penetration between anurans (Ishihara et al., 1984; Katagiri, 1987) and hynobiid salamanders.

The small clutch size is a weakness in the present analysis, because the occurrence of only a few unfertilized eggs or dead embryos considerably decreased the percentage of developing embryos. However, this does not diminish the reliability of our results, because the rates of developing embryos in most of the experimental groups did not differ significantly from those of their control groups.

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- Biological Institute, Faculty of Science,
Niigata University, Niigata, 950-21 JAPAN*

要旨 クロサンショウウオ卵の水中における長時間の受精能力保持

羽角 正人・長谷川嘉則・岩澤 久彰

クロサンショウウオの卵嚢中の卵は卵嚢を水に3時間浸した後でも100%に近い受精能力を持つ。従って本種では水中で一刻を争って卵嚢に注精する雄の行動に発生学的必然性は認めら

れない。

(950-21 新潟市五十嵐 新潟大学理学部生物学教室)