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Source: Zoological Science, 14(4) : 623-628

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.623

ZOOLOGICAL SCIENCE **14**: 623–628 (1997)

Effect of Micropylar Morphology and Size on Rapid Sperm Entry into the Eggs of the Medaka

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ABSTRACT—An experimental study on sperm entry into the micropyle of the medaka egg was carried out in order to analyze the effect of micropylar size on fertilization. The eggs and micropyles of *Oryzias latipes* were larger than those of *Oryzias melastigma*. When eggs of *O. latipes* and *O. melastigma* were simultaneously inseminated by spermatozoa of *O. latipes*, *O. melastigma* or *Aplocheilus panchax*, *O. melastigma* spermatozoa rapidly entered the conspecies eggs as well as the large micropyles of *O. latipes* eggs, while most of the *O. latipes* and *A. panchax* spermatozoa failed to rapidly enter the small *O. melastigma* micropyles. Moreover, when *O. latipes* eggs with a small or a large vestibule were simultaneously inseminated by *O. melastigma* spermatozoa, the size of the micropylar vestibules (range of 17-23 μm in diameter) also affected rapid sperm entry into the egg. However, this effect was not recognized when the eggs were inseminated by conspecies spermatozoa. On the other hand, when *O. latipes* eggs were inseminated by a mixture of *O. latipes* and *O. melastigma* spermtozoa or *O. latipes* and *A. panchax* spermatozoa, the faster swimming spermatozoa fertilized significantly more frequently than the slower *O. latipes* spermatozoa. These results suggest that rapid sperm entry into the micropyle is not only affected by differences in the morphology and size of the micropyles, but also might be accelerated by the linear swimming velocity of the spermatozoa.

INTRODUCTION

In teleostean eggs, there is a single narrow pore, the micropyle, at the animal pole side of a thick egg envelope (chorion). The micropyle functions to permit the entry of only one spermatozoon into the egg and to prevent superabundant or foreign spermatozoa and bacteria from entering the egg during fertilization. The spermatozoon which will penetrate into the ooplasm uses the micropyle to reach the surface of the egg plasma membrane. The egg and the spermatozoon lose their ability to fertilize a short time after they are released from the reproductive ducts into external water (Yamamoto, 1961; Ginsburg, 1972). Therefore, spermatozoa must rapidly find and efficiently enter the narrow micropylar canal before their fertilizability is lost. Substances secreted from the egg activate and prolong the movement of spermatozoa, which generally increases the probabilty of contact between gametes. So far, the presence of the following substances has been reported: a sperm attractant in the bitterling egg (Suzuki, 1958), a sperm activating substance in the herring egg (Morisawa *et al.*, 1992; Yanagimachi *et al.*, 1992; Pillai *et al.*,1993), and a sperm guiding substance in salmonid and herring eggs (Yanagimachi *et al.*, 1992) and the medaka egg (Takano and Onitake, 1989; Iwamatsu *et al.*, 1993, 1997). Various species-specific structures of the chorion surface and the micropyle have also been distinguished in teleostean fishes (Ginsburg, 1972; Riehl and Kock, 1989). According to Amanze and Lyengar (1990), Riehl and Patzner (1991) and Riehl and Kokoscha (1993), the chorions of *Barbus conchonius*, *Sturisoma aureum* and *Luciocephalus* sp. eggs, possess radially arranged grooves around the micropyle. The structures of the chorion and micropyle seem to play a role in assisting the substances that trap spermatozoa in the micropyle. This view point has not been experimentally verified in eggs of fishes (Hart, 1990), although fertilization between different genera or species of teleosts has been investigated (see Suzuki and Fukuda, 1971). To investigate the relationship between the micropylar structure and rapid sperm entry, we experimentally examined the effect of different micropylar sizes on rapid sperm entry. The results suggest that rapid sperm entry may be affected not only by the differences in morphology and size of micropyles, but also by the swimming velocity of the spermatozoa or other factors such as sperm guiding substances.

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MATERIALS AND METHODS

Preparation of gametes

Mature Japanese medakas *Oryzias latipes* (orange-red type), Indian medakas *O. melastigma* and the killifish *Aplocheilus panchax* were bred in our laboratories. Unfertilized eggs of the *Oryzias* species were isolated in saline (6.5 g NaCl, 0.4 g KCl, 0.113 g $CaCl_2$ and 0.15 g $MgSO_4 \cdot 7H_2O$ in one liter of distilled water, adjusted to pH 7.3 with $NaHCO_3$) within 2 hr after ovulation. Females of these species spawned around the onset of light every day under light (14 hr)- and temperature (26–28°C)-controlled conditions. Sperm suspensions were prepared from isolated testes of mature males of all these species according to a routine procedure, then used within a few minutes.

Scanning electron microscopic observations

A fresh sperm suspension was fixed with Karnovsky's fixative (4°C) for at least 12 hr. The samples were washed once with 0.1 M phosphate buffer (pH 7.3) and dehydrated in a graded series of ethanol solutions. After immersion in isoamyl acetate, they were dried in a critical point apparatus (Hitachi, HCP-2). These samples were then mounted on brass and coated with gold vapor using a fine coating apparatus (JOEL, JEC-1100 Fine Coat). Observations were performed using a scanning electron microscope (JOEL, JEM T-20)

Observation of sperm movement and entry into the micropyle

All experimental observations were performed at 23–26°C. Spermatozoa swimming in saline were observed on a television monitor. The swimming velocity of spermatozoa was measured from the image in the monitor.

Examination of fertilization rates

Insemination of unfertilized *O. latipes* or *O. melastigma* eggs was performed by adding a fresh suspension (final sperm concentration, 0.4–7.7 $\times 10^7$ spermatozoa/ml) of *O. latipes*, *O. melastigma* or *A. panchax* spermatozoa, and was stopped after 1-300 sec by rinsing the eggs in 0.005% SDS-saline for 5-10 sec. The eggs were then transferred into fresh saline for development. Sixty minutes after insemination, the fertilization rate was calculated as the percentage of fertilized eggs among the total eggs inseminated. Fertilization of *O. latipes* eggs by *O. melastigma* or *A. panchax* spermatozoa was ascertained by the appearance of melanophores on the embryo.

In one experiment, *O. latipes* eggs with small or large micropylar vestibules were sorted by measuring the vestibule diameters under an ordinary light micro-scope (× 200). Only the eggs with a small micropylar vestibule were marked by cutting off the attaching filaments. Eggs of both types were mixed and inseminated together with a diluted sperm suspension (5.9–7.7 $\times 10^6$ *O. latipes* spermatozoa/ml or 1.1–2.9 $\times 10^6$ *O. melastigma* spermatozoa/ml). Each experiment was repeated 5 times.

The data were statistically analysed by the Student's t-test.

RESULTS

Morphology of gametes of *O. latipes* and *O. melastigma*

The eggs and micropyles of *O. latipes* were larger than those of *O. melastigma* (Table 1). The micropyle of *O. melastigma* eggs consisted of a small vestibule with a marginal ridge and a short canal (Fig. 1). In unfertilized eggs of this species, the average diameter of the vestibule was about 10 μm, and the overall length of the micropyle measured about 40 μm. The micropylar canal widened at the outer end where it joined the basal region of the vestibule.

The overall length (ca. 22 μm) of *O. latipes* spermatozoa

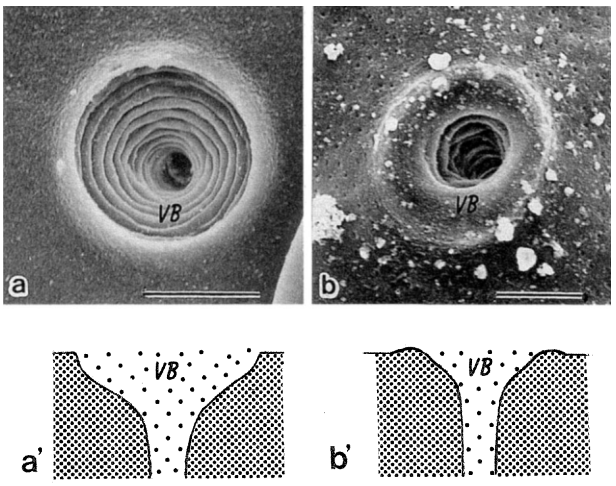

Fig. 1. Micropyles of *Oryzias latipes* and *Oryzias melastigma* eggs. **a**: *O. latipes*, **b**: *O. melastigma*. VB, vestibule. Bar, 5 μm. **a'** and **b'**: Diagrams of longitudinal sections through the micropyles (**a**, **b**).

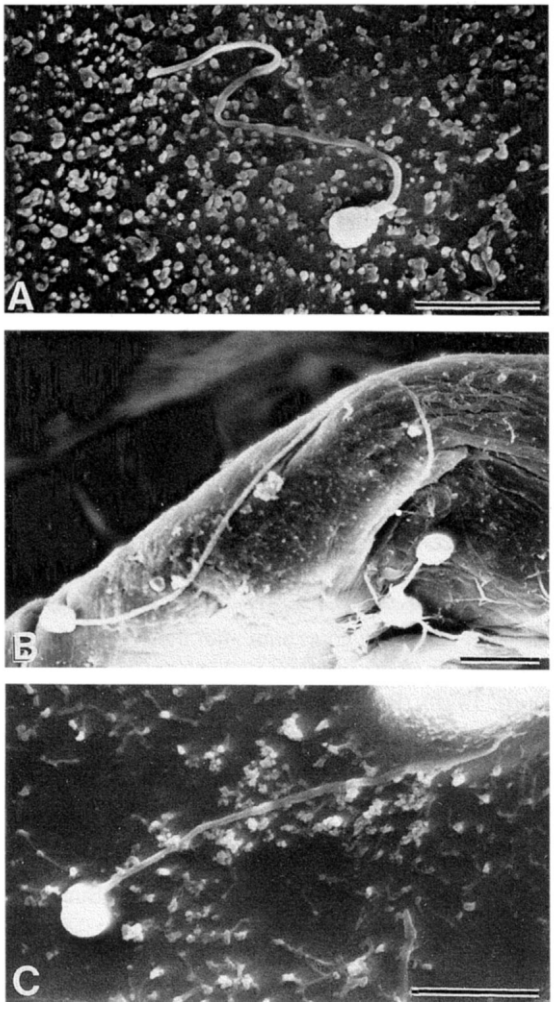

Fig. 2. Micrographs of spermatozoa of *Oryzias latipes*, *Oryzias melastigma* and *Aplocheilus panchax*. **A**: *O. latipes*, **B**: *O. melastigma*, **C**: *A. panchax*. Bar, 5 μm.

Table 1. Morphology of gametes of *Oryzias latipes*, *O. melastigma* and *A. panchax*

species	*O. latipes*		*O. melastigma*		*A. panchax*
	BF(μm)	AF(μm)	BF(μm)	AF(μm)	(μm)
eggs					
Overall diameter	1223.4 ± 7.8	1295.3 ± 8.7	1020.1 ± 9.6	1067.2 ± 5.6	–
of the chorion	(26)	(25)	(19)	(18)	–
Overall diameter of the vitellus	1161.0 ± 9.1	1116.0 ± 8.8	967.2 ± 7.4	918.5 ± 8.3	–
Thickness of the chorion	22.6 ± 1.1	19.2 ± 0.3	15.5 ± 0.5	13.3 ± 0.3	–
Diameter of the vestibule	20.0 ± 0.6	26.6 ± 0.8	9.8 ± 0.4	11.9 ± 0.6	–
Diamter of inner opening of micropylar canal	3.5 ± 0.1	0	3.2 ± 0.1	0	–
Overall length of micropyle	50.2 ± 1.8	34.7 ± 1.5	39.7 ± 1.5	–	–
Spermatozoon*					
Overall length	21.6 ± 0.4		26.3 ± 1.0		16.7 ± 1.4
Width of head	1.7 ± 0		1.4 ± 0		1.5 ± 0
Length of head	2.4 ± 0.1		1.9 ± 0		1.8 ± 0
Swimming velocity**	100.2 ± 0.1 (21)		175.2 ± 7.5 (21)		42.5 ± 2.1 (12)

AF, Sixty minutes after fertilization; BF, Before fertilization. Numbers in parentheses indicate the numbers of eggs examined. *Fixed. **μm/sec (No. of spermatozoa).

was less than that (ca. 26 μm) of *O. melastigma* spermatozoa. The width and length of the heads of *O. latipes* spermatozoa were significantly ($p<0.005$) greater than those of *O. melastigma* spermatozoa (Fig. 2, Table 1). These sperm measurements corresponded to the inner diameters of the micropylar canals in eggs of the two species.

Swimming velocity of *O. latipes*, *O. melastigma* and *A. panchax* spermatozoa

When spermatozoa of *O. latipes*, *O. melastima* and *A. panchax* were released from testes into saline, they began to actively swim straight forward. The maximum swimming velocity (26°C) of fresh *O. melastigma* spermatozoa was significantly greater than that of *O. latipes* or *A. panchax* spermatozoa (Table 1).

Fertilization rate in *O. latipes* eggs with different sizes of micropylar vestibules

O. latipes eggs with a large or a small micropylar vestibule (Fig. 3) were simultaneously inseminated for 10 sec by *O. latipes* or *O. melastigma* spermatozoa. The rate of fertilized eggs with a large micropylar vestibule was significantly ($P<0.01$) greater than that with a small micropylar vestibule upon insemination by *O. melastigma* spermatozoa (Fig. 4). However, when these eggs were inseminated by the conspecies spermatozoa, no significant difference in fertilization rates was recognized between the eggs with different sizes of micropylar vestibules.

Interspecific and intraspecific fertilization between *O. latipes*, *O. melastigma* and *A. panchax*

Unfertilized eggs of *O. latipes* and *O. melastigma* were mixed and inseminated for 5–300 sec (23–25°C) using a sperm

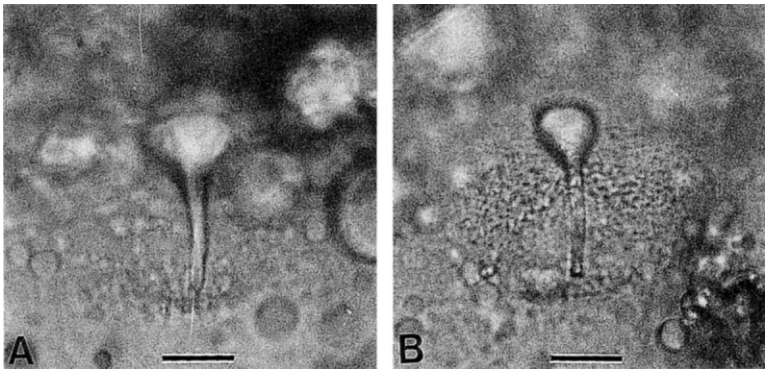

Fig. 3. Micrograghs of micropyles of *Oryzias latipes*. **A**: Micropyle with a large vestibule. **B**: Micropyle with a small vestibule. Bar, 20 μm.

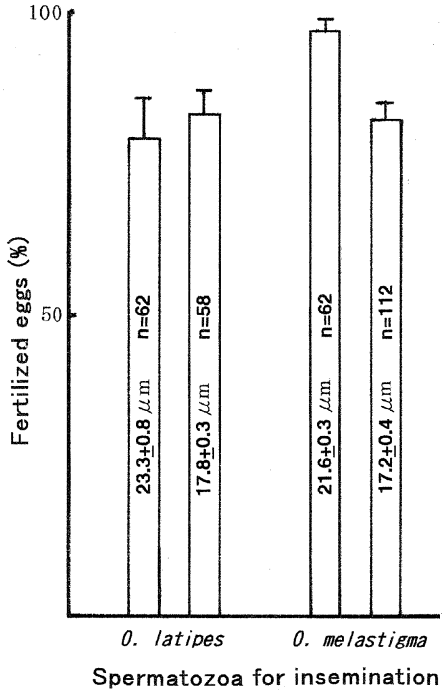

Fig. 4. Fertilization rates of *Oryzias latipes* eggs with different-sized micropylar vestibules. *O. latipes* eggs with a large or small micropylar vestibule were simultaneously inseminated by a diluted suspension of *O. latipes* or *O. melastigma* spermatozoa. The sizes of micropylar vestibules (mean ± SE) and the numbers of eggs used are given in each column.

suspension from *O. latipes* ($0.5–1.4 \times 10^7$ spermatozoa/ml), *O. melastigma* ($0.4–1.0 \times 10^7$ spermatozoa/ml), or *A. panchax* ($2.3–3.6 \times 10^7$ spermatozoa/ml). The eggs were quickly rinsed in saline containing 0.005% SDS, which has spermicidal action, and then transferred into fresh saline. The rates of cleaved eggs among the total eggs inseminated were calculated about 60 min after insemination. As presented in Figs. 5, 6 and 7, the results revealed that the thinner *O. melastigma* spermatozoa induced activation of both *O. melastigma* and *O. latipes* eggs within 10 sec after insemination, whereas the thicker spermatozoa of both *O. latipes* and *A. panchax* failed to induce activation of *O. melastigma* eggs. The vigorous motility of these spermatozoa gradually declined within 30 sec after insemination.

Embryos of *O. latipes* lack melanophores, while those of *O. melastigma* and *A. panchax* possess melanophores. When eggs of *O. latipes* were fertilized by spermatozoa of *O. melastigma* or *A. panchax*, they developed to form interspecific hybrid embryos identified by the presence of melanophores. In order to examine the difference in rates of rapid sperm entry, *O. latipes* eggs were inseminated by a mixture of sperm suspensions from *O. latipes* (3.6×10^7 spermatozoa/ml) and *O. melastigma* (7.7×10^6 spermatozoa/ml) for 5 or 10 sec. The number of the hybrids with melanophores was significantly greater than the number developing into embryos without melanophores (Fig. 8). When the insemination mixture contained *O. latipes* ($2.3–3.4 \times 10^7$ spermatozoa/ml) and *A. panchax* ($3.1–6.1 \times 10^7$ spermatozoa/ml) spermatozoa, 74% of fertilized eggs developed to normal embryos without melanophores.

DISCUSSION

The present experiments show that *O. latipes* spermatozoa enter quickly into large micropyles of conspecific eggs, but not into the smaller and morphologically different micropyles of *O. melastigma* eggs. The same result was also obtained on the entry of spermatozoa from a different genus of fish, *A. panchax*, into *O. latipes* and *O. melastigma* eggs. Also,

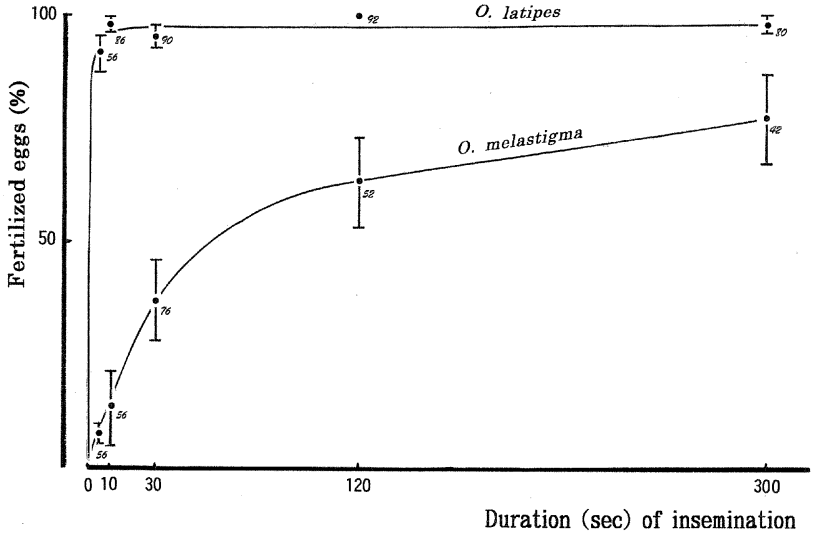

Fig. 5. Fertilization rates of *Oryzias latipes* and *Oryzias melastigma* eggs inseminated by *Oryzias latipes* spermatozoa. The number of eggs used is given at each data point.

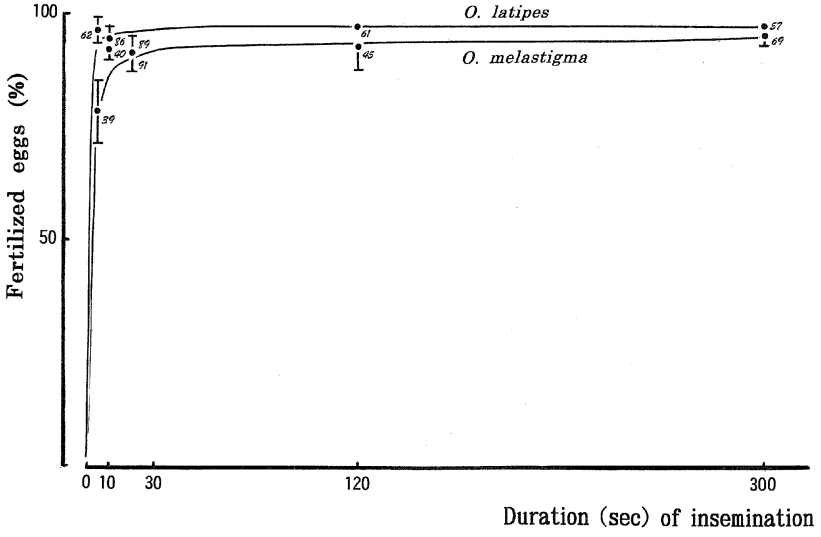

Fig. 6. Fertilization rates of *Oryzias latipes* and *Oryzias melastigma* eggs by *Oryzias melastigma* spermatozoa. The number of eggs used is given at each data point.

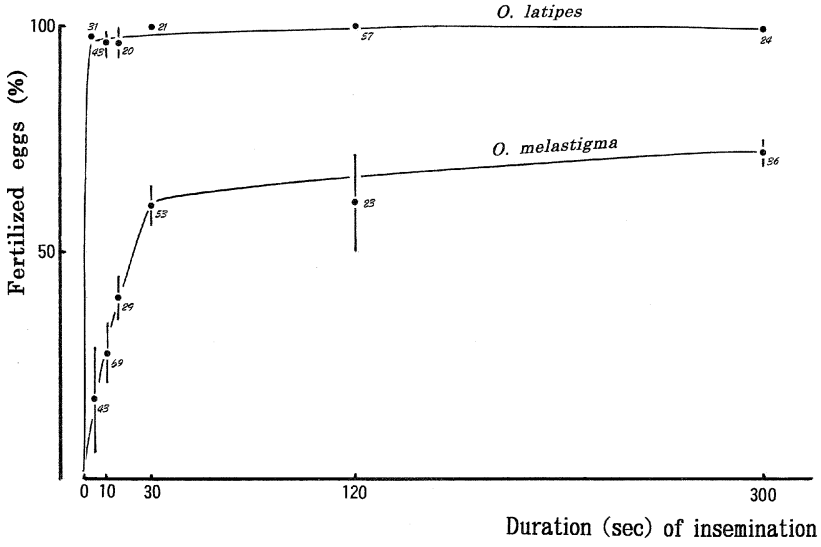

Fig. 7. Fertilization rates of *Oryzias latipes* and *Oryzias melastigma* eggs by *Aplocheilus panchax* spermatozoa. The number of eggs used is given at each data point.

O. melastigma spermatozoa could enter more rapidly into *O. latipes* eggs with a large micropylar vestibule than into those with a small micropylar vestibule. These data on interspecific and intergeneric fertilizations seem to indicate that rapid sperm entry into the micropyle may be affected by the size and morphology of the vestibules. On the contrary, another experiment on intraspecific fertilization in *O. latipes* failed to reveal that rapid sperm entry was affected by different micropylar sizes. This contradictory finding suggests that in *O. latipes*, factors other than the diameter of the micropylar vestibule within the range of 17–23 μm may facilitate the rapid sperm entry into the micropyle. Such factors may include sperm guiding substances. Recently, we found sperm guiding substances (chorionic glycoproteins) that were distributed as a diluted mucous area (DMA) on the chorion surface and within the micropyle of *O. latipes* eggs (Iwamatsu *et al.*, 1997). The *O. melastigma* eggs may also have species-specific substances to guide spermatozoa since *O. melastigma* spermatozoa can rapidly enter the small micropyles of conspecific eggs. *O. melastigma* spermatozoa, which have a small head and a long tail, may be capable of effecting rapid entry into the micropyle due to their high swimming speed.

Spermatozoa of the different fishes used in the present experiment swim in saline with the velocity of *O. melastigma* > *O. latipes* > *A. panchax*. The swimming velocity of *O. latipes* spermatozoa in the present data agrees with the velocities reported previously (Ishijima *et al.*, 1993; Iwamatsu *et al.*, 1993). When *O. latipes* eggs were inseminated by a mixture

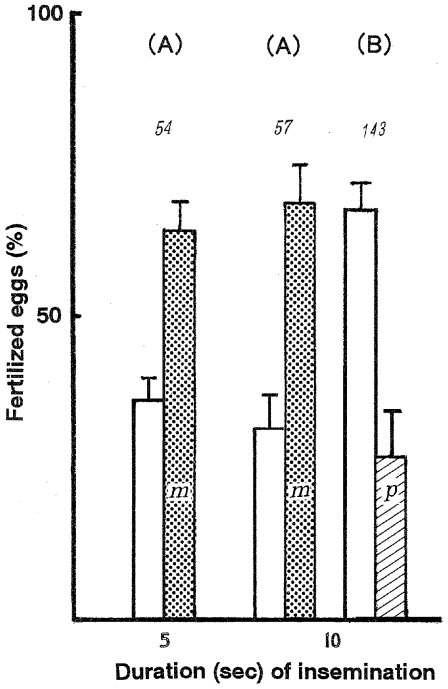

Fig. 8. Interspecific fertilization of *Oryzias latipes* eggs by mixtures of (A) *Oryzias latipes* and *Oryzias melastigma* spermatozoa or (B) *O. latipes* and *A. panchax* spermatozoa. *O. latipes* eggs that were fertilized by *O. melastigma* or *A. panchax* spermatozoa all developed to embryos with melanophores, so that hybrids were easily identified by the appearance of melanophores. Open columns: Eggs fertilized by *O. latipes* spermatozoa. m: Eggs fertilized by *O. melastigma* spermatozoa. p: Eggs fertilized by *A. panchax* spermatozoa. The number of eggs used is given above each column.

of *O. latipes* and *O. melastigma* spermatozoa, *O. melastigma* spermatozoa entered the micropyle faster than *O. latipes* spermatozoa. On the other hand, when *O. latipes* eggs were inseminated by a mixture of spermatozoa from *O. latipes* and *A. panchax*, the former spermatozoa entered the micropyles faster than the latter spermatozoa. These results suggest that high swimming velocity may be important for rapid entry of spermatozoa into the micropyle if the diameter of the vestibule is more than 10 μm. In addition to activators (substances and temperature) of sperm movement, sperm guiding substances, and morphological characteristics of the egg surface during fertilization, the concentration of spermatozoa also plays a role. If a less concentrated suspension of spermatozoa is applied, the time required for fertilization of all the eggs is greatly prolonged (Iwamatsu *et al.*, 1991). The time required for all unfertilized eggs to make contact with spermatozoa is 3-6 sec in the trout and 10–15 sec in the sturgeon when the sperm concentration is more than 10^7 spermatozoa/ml (cf. Ginsburg, 1972). In order to eliminate sperm concentration as a factor in the present experiments, high concentrations of spermatozoa (10^7/ml) were used with the result that most eggs were fertilized within 12 sec (Iwamatsu *et al.*, 1991). Therefore, this effect seems to be unimportant in the present data.

Thus, the present data indicate that in *Oryzias latipes* eggs, rapid sperm entry into the micropyle seems to be accelerated by a larger micropylar vestibule and by a high velocity of straight forward swimming by the spermatozoa, in addition to such chemical factors as sperm guiding substances (Iwamatsu *et al.*, 1997).

REFERENCES

Amanze D, Lyengar A (1990) The micropyle: a sperm guidance system in teleost fertilization. Development 109: 495–500

Ginsburg AS (1972) Fertilization in Fishes and the Problem of Polyspermy. Akad Nauk, SSSR, pp 366

Hart NH (1990) Fertilization in teleost fishes: Mechanisms of sperm-egg interactions. Intrn Rev Cytol 121: 1–66

Ishijima S, Hamaguchi Y, Iwamatsu T (1993) Sperm behavior in the micropyle of the medaka egg. Zool Sci 10: 179–182

Iwamatsu T, Onitake K, Yoshimoto Y, Hiramoto Y (1991) Time sequence of early events in fertilization in the medaka egg. Develop Growth Differ 33: 479–490

Iwamatsu T, Ishijima S, Nakashima S (1993) Movement of spermatozoa and changes in micropyles during fertilization in medaka eggs. J Exp Zool 266: 57–64

Iwamatsu T, Yoshizaki N, Shibata Y (1997) Changes in the chorion and sperm entry into the micropyle during fertilization in the teleostean fish *Oryzias latipes*. Develop Growth Differ 39: 33–41

Morisawa M, Tanimoto S, Ohtake H (1992) Characterization and partial purification of sperm activating substance from eggs of the herring, *Clupea palasii*. J Exp Zool 264: 225–230

Pillai MC, Shields TS, Yanagimachi Y, Cherr GN (1993) Isolation and partial characterization of the sperm motility initiation factor from eggs of the pacific herring, *Clupea pallasi*. J Exp Zool 265: 336–342

Riehl R, Kock K-H (1989) The surface structure of antarctic eggs and its use in identifying fish eggs from the southern ocean. Polar Biol 9: 197–203

Riehl R, Kokoscha M (1993) A unique surface pattern and micropylar apparatus in the eggs of *Luciocephalus* sp. (Perciformes, Luciocephalidae). J Fish Biol 43: 617–620

Riehl R, Patzner RA (1991) Breeding, egg structure and larval morphology of the catfish *Sturisoma aureum* (Steindachner) (Teleostei, Loricariidae). J Aquaricult Aqua Sci 6: 1–6

Suzuki R (1958) Sperm activation and aggregation during fertilization in some fish. I. Behavior of sperm around the micropyle. Embryologia 4: 93–102

Suzuki R, Fukuda Y (1971) Growth and survival of F_1 hybrids among salmonid fishes. Bull Freshwater Fish Res Lab 21: 117–138

Takano M, Onitake K (1989) On the mode of sperm entry into the micropylar canal in the medaka, *Oryzias latipes*. Zool Sci 6: 1169 (Abstract)

Yamamoto T (1961) Physiology of fertilization in fish eggs. Intrn Rev Cytol 12: 361–405

Yanagimachi R, Cherr GN, Pillai MC, Baldwin JD (1992) Factors controlling sperm entry into the micropyle of salmoid and herring eggs. Develop Growth Differ 34: 447–461

(Received January 6, 1997 / Accepted April 14, 1997)