

The Effect of Exogenous Hormone Treatment on Spermiation in *Rhynchocypris oxycephalus* (Sauvage and Dabry)

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Abstract.—For the evaluation of hormonal control of spermiation in fish, a method to quantify the spermiation response of mature *Rhynchocypris oxycephalus* (Sauvage and Dabry) to hormonal therapy is described. Spermatoцит was determined after 7 min centrifugation at $18,000 \times g$ and sperm density was estimated by a standard hemocytometer method. Sperm density can be predicted from spermatoцит since their relationship is linear as described by the regression equation, $Y = 3.68X - 27.18$ ($R^2 = 0.82$, $N = 50$), where Y is spermatoцит and X is sperm density. Milt production by mature *R. oxycephalus* was highest at 24 h after injection of 1,000 IU human chorionic gonadotropin (HCG) and 50 μg luteinizing hormone-releasing hormone analogue (LHRHa) per kg body weight. Increased milt production coincided with low spermatoцит and sperm density levels. These results demonstrate that spermiation in mature *R. oxycephalus* can be reliably evaluated by a spermatoцит method and that HCG and LHRHa are effective in stimulating of spermiation in this species.

The ability to control the reproductive activities of fish is advantageous in aquaculture and aquariology. Control of timing of reproduction within fish stocks allows for more efficient seed production by either synchronizing populations to spawn simultaneously or enabling multiple spawnings of stocks throughout the year. It also makes possible the development of interspecific hybrid species whose spawning seasons do not normally overlap (Lam 1982; Park et al. 1994). One method for controlling reproduction in fish is by the use of exogenous hormone treatments (Patino 1997).

Such treatments have been shown to be effective in inducing spermatogenesis and spermiation in immature males, and inducing final maturation and ovulation in immature females.

The use of luteinizing hormone-releasing hormone analogue (LHRHa) has been shown to be effective in inducing spermiation in fish. Weil and Crim (1982) successfully induced spermiation in immature landlocked Atlantic salmon *Salmo salar* by interperitoneal injection of 750 μg LHRHa/kg body weight (BW), implantation of 1,500 μg LHRHa/kg BW, and oral treatment with 25 μg LHRHa/kg BW. Injection of common carp *Cyprinus carpio* with 10 μg LHRHa/kg BW has been demonstrated to increase sperm production (Takashima et al. 1984). Of the various mammalian gonadotropins, the human chorionic gonadotropin (HCG) is the most effective at inducing spermiation in fish (Billard 1976; Stacey and Peter 1979; Donaldson and Hunter 1983). Human chorionic gonadotrophin has been used successfully in goldfish *Carassius auratus*, rainbow trout *Oncorhynchus mykiss*, seabream *Sparus aurata*, miltfish *Chanos chanos*, European eel *Anguilla anguilla*, and Japanese eel *Anguilla japonica* (Donaldson and Hunter 1983). Two methods have been used to evaluate the effect of hormone treatment on spermiation in fish. Early studies quali-

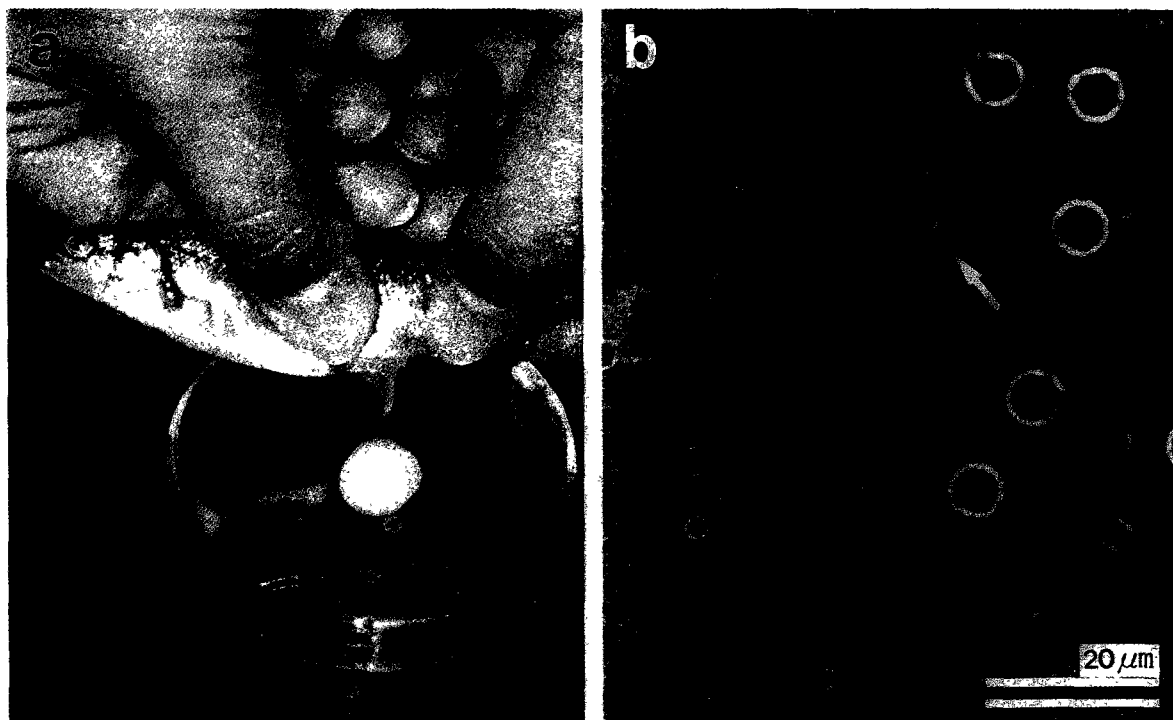


FIGURE 1. a. Milt collection by hand-stripping. Fish were anesthetized with 300 ppm lidocaine-HCl/NaHCO₃ (Park et al. 1998c) and then each fish was weighed and laid on its dorsal side. The urogenital area was wiped clean and dried with absorbent paper. Urine and some feces were initially forced out by gentle massage of the abdomen. b. External morphology of *Rhynchocypris oxycephalus* (Sauvage and Dabry) spermatozoa (arrow).

tatively measured success of treatment by the quantity and density of stripped sperm (Yamazaki and Donaldson 1968; Shehadeh et al. 1973; Juario et al. 1980). More recent studies have quantified the effect of treatment on sperm production by determining the spermatocrit (Baynes and Scott 1985; Munkittrick and Moccia 1987; Garcia 1991; Park et al. 1992).

Rhynchocypris oxycephalus (Sauvage and Dabry) (Leucisinae, Cyprinidae) is a widely distributed freshwater fish in Korea (Kim 1997; Park et al. 1998a, 2001). This species is particularly suitable for use in laboratory studies, as it is hardy and can withstand cold temperatures (Park et al. 1998b, 1999). Furthermore, there is good background information on husbandry techniques and aspects of its biology (Park et al. 1998a, 1998b, 1998c, 1999). In this study we report the effects of intraperitoneal injection with HCG and LHRHa on

spermiation in *R. oxycephalus*. Conditions for determining spermatocrit and the relationship between spermatocrit values and sperm densities are also reported.

Materials and Methods

Rhynchocypris oxycephalus (Sauvage and Dabry) were obtained from the Genetics and Breeding Laboratory of the Division of Ocean Science, Korea Maritime University, Busan, Korea. The average size of the fish used was 10.1 ± 1.2 cm total length and 18.4 ± 0.6 g body weight (BW). Water temperatures were maintained at 18.0 ± 0.5 C during the study. Prior to handling, fish were anesthetized by bathing them in a solution of 300 ppm lidocaine-HCl/NaHCO₃ at 20 C (Park et al. 1998c). To obtain milt, the urogenital area was wiped clean and dry, and urine and some feces initially forced out by gentle massaging of the abdomen. As shown in Fig. 1a, milt was then

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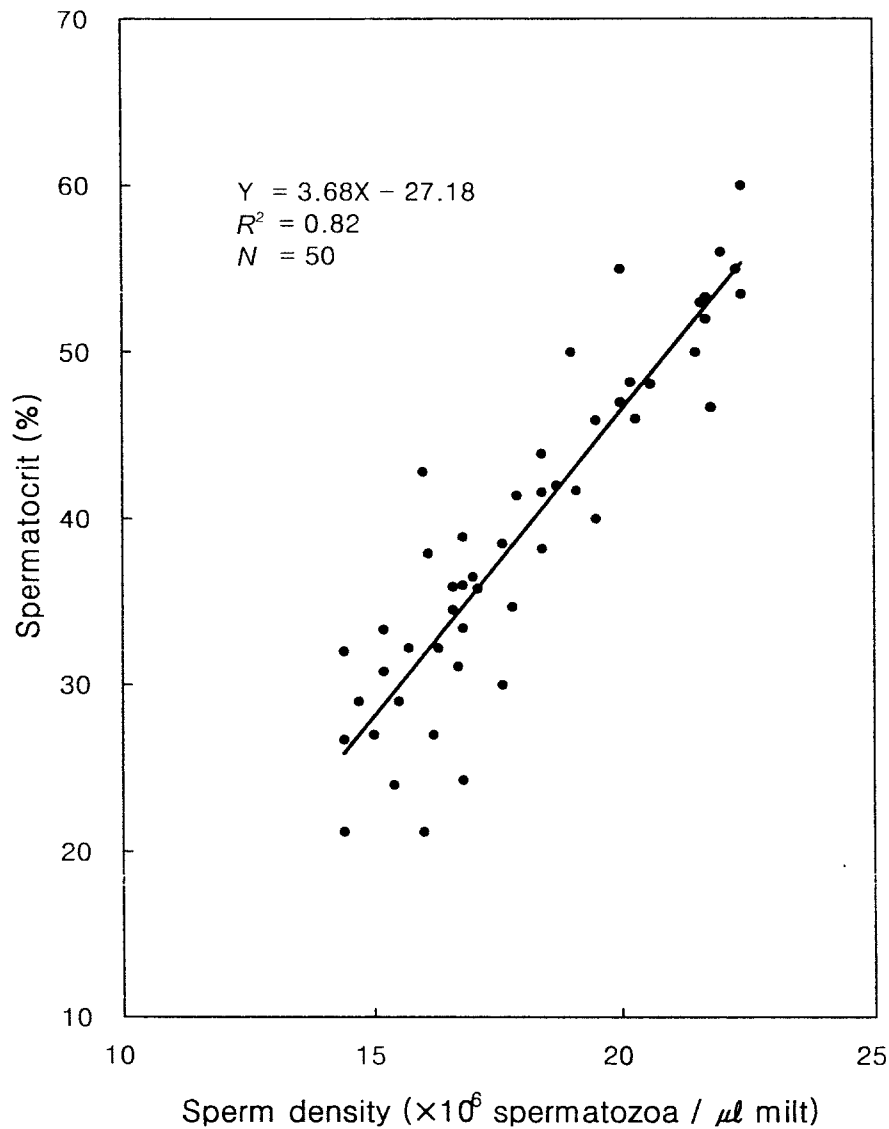


FIGURE 2. The relationship between sperm density (X) and spermatocrit (Y) of *Rhynchocypris oxycephalus* (Sauvage and Dabry).

TABLE 1. Mean change in spermatocrit, sperm density, and milt production levels of mature *Rhynchocypris oxycephalus* (Sauvage and Dabry) after an injection of 1,000 IU human chorionic gonadotropin (HCG)/kg BW and 0.5 mg analogue of mammalian luteinizing hormone releasing hormone (LHRHa)/kg BW. N = 30. Means \pm SEM. Means with different superscripts in the same column are significantly different at P < 0.05.

Hours after injection	Milt production (μ L per g BW)			Spermatocrit (%)		
	Control	HCG	LHRHa	Control	HCG	LHRHa
0	26.2 \pm 2.8	24.2 \pm 0.9a	23.0 \pm 1.5a	30.8 \pm 1.8a	29.2 \pm 2.1a	31.4 \pm 3.2a
24	33.5 \pm 2.9	71.8 \pm 7.9b	88.9 \pm 6.8b	28.7 \pm 4.9b	19.9 \pm 2.0b	24.5 \pm 3.7b
48	24.3 \pm 3.0	59.1 \pm 4.7c	53.3 \pm 5.2b	24.9 \pm 3.7c	21.4 \pm 1.7b	21.0 \pm 1.8c
72	29.7 \pm 2.7	27.6 \pm 1.5a	32.3 \pm 3.0a	22.8 \pm 2.0d	24.1 \pm 2.5c	23.4 \pm 1.7b

hand stripped, collected using an automatic pipette or a 1-mL plastic syringe, and stored on chipped ice until analysis.

Because of the potential import of determining the optimal centrifugation condition for spermatocrit determination, we have undertaken a study of determining the optimal centrifugation time for constant sedimentation. Triplicate milt samples from 10 individuals were loaded into individual nonheparinized microhematocrit capillary tubes (i.d. int. 1.2×75 mm, Fisher Scientific) and sealed with tube sealing compound. Capillary tubes were centrifuged at $18,000 \times g$ for 1-min intervals for a total of 12 min. To determine the optimal centrifugation time for constant sedimentation, the spermatocrit of each tube was measured using a hematocrit reader.

The spermatocrit of *Rhynchocypris oxycephalus* (Sauvage and Dabry) milt samples loaded in nonheparinized capillary tubes decreased with increasing centrifugation time at $18,000 \times g$ until it remained constant after 7 to 12 min. Based upon these results, we suggest that 10 min of centrifugation at $18,000 \times g$ is an appropriate condition for the determination of the spermatocrit. After determination of the optimal conditions for determining spermatocrit, sperm samples were collected from 50 individuals and their spermatocrits determined by centrifugation at $18,000 \times g$ for 10 min. Sperm density for each of these samples was determined by diluting the sperm with 0.9% NaCl using a blood dilut-

ing pipette and counting the sperm cells in a hemocytometer at $400\times$ magnification. The relationship of sperm density ($\times 10^6$ spermatozoa) to spermatocrit (% of milt volume) was determined by regression analysis.

Three groups of 30 male *R. oxycephalus* were used to determine the effects of administration of human chorionic gonadotropin (HCG, Sigma, USA) and an analogue of mammalian luteinizing hormone releasing hormone (LHRHa, des-Gly¹⁰[D-Ala⁶]LHRH-Ethylamide, Sigma, USA) on spermiation. One group received intraperitoneal injections of 1,000 IU/kg BW HCG, the second group 50 μ g/kg BW LHRHa, and the third control group 0.1 mL of sterile physiological saline per kg BW. After injection, the groups were placed in individual tanks and maintained at 18.0 ± 0.5 C. Prior to injection, and at 24, 48 and 72 h post-injection, milt samples were hand stripped from all individuals.

Spermatocrits, sperm densities, and total milt volumes were determined for these samples. Percentage data were arcsine transformed. Data were analyzed by ANOVA test followed by Duncan's multiple range test to determine the differences in spermatocrits, sperm densities, and total milt volumes between groups. Data were considered significant at the 0.05 probability level. The relationship between spermatocrit and sperm density was examined by simple linear regression.

Results

Fig. 1b shows an external morphology of *R. oxycephalus* spermatozoa. Sperm density of hormone induced spawners ranged from 14.4 to 22.4×10^6 sperm/ μ L milt which corresponds to spermatocrit values ranging from 21 to 60%. The relationship between sperm density and spermatocrit was linear and is described by the linear regression equation, $Y = 3.68X - 27.18$ ($R^2 = 0.82$, $N = 50$), where Y is spermatocrit and X is sperm density (Fig. 2).

Administration of HCG and LHRHa to

TABLE 1. *Extended.*

Sperm density ($\times 10^6$ spermatozoa per μ L milt)		
Control	HCG	LHRHa
15.2 ± 1.0	$15.7 \pm 1.1a$	$16.2 \pm 1.3a$
14.3 ± 2.7	$9.5 \pm 2.4b$	$12.9 \pm 3.2b$
13.9 ± 1.7	$12.4 \pm 1.5c$	$11.5 \pm 2.0b$
14.1 ± 1.5	$13.9 \pm 1.0c$	$13.2 \pm 0.8b$

R. oxycephalus resulted in a significant increase in milt production at 24 and 48 h postinjection (Table 1). Milt production in these groups returned to control levels at 72 h. The effects of hormone administration on sperm density and spermatocrits were not as apparent. In the control and both hormone-treated groups, there were significant decreases in the spermatocrit levels at 24 h postinjection. Spermatocrit levels remained low and were variable within each group at 24, 48 and 72 h postinjection. There was no significant difference in sperm density in the control group over time (Table 1). In the hormone-treated groups there were significant reductions in the sperm density at 24 h postinjection ($P < 0.05$). Sperm density in the group which received the HCG injection increased significantly at 48 and 72 h postinjection ($P < 0.05$), however the levels attained were significantly less than the preinjection levels. In the group that received the LHRHa injection there was no significant difference in sperm densities between 24, 48, and 72 h postinjection.

Discussion

Based upon our results, we suggest that 10 min of centrifugation at $18,000 \times g$ is an appropriate condition for the determination of the spermatocrit in *R. oxycephalus*. The duration of centrifugation is less than previously reported for other species using comparable procedures. Spermatocrits of rabbitfish *Siganus guttatus* were obtained after 45 to 75 min of centrifugation at $15,000 \times g$ (Garcia 1991). Spermatocrits of olive flounder *Paralichthys olivaceus* were obtained after 30 to 60 min of centrifugation at $12,000 \times g$ (Park et al. 1992).

The relationship between sperm density and spermatocrit in *Rhynchocypris oxycephalus* (Sauvage and Dabry) can be described using linear regression described by the equation, $Y = 3.68X - 27.18$. The relationship between sperm density and spermatocrit has been described for both the rabbitfish and the olive flounder to also be linear; however, the slopes of the regression

lines are considerably lower in these species (Garcia 1991; Park et al. 1992). This result could be due to a variety of factors, such as differences in sperm size between these different species. Smaller sperm size would account for the longer centrifugation times necessary to determine spermatocrits in these species. Since the relationship between sperm density and spermatocrit is highly correlated, spermatocrit readings are a fast and reliable method for the assessment of spermiation in *R. oxycephalus*. The applicability of this method for determining spermatocrit should be assessed for a wider variety of fish species.

Administration of HCG and LHRHa resulted in marked increases in milt production and decreased sperm density and spermatocrits compared to those of control at 24 and 48 h postinjection. Milt production levels returned to postinjection levels at 72 h postinjection. Such responses are not unexpected since both HCG and LHRHa have a long history of use to stimulate reproduction in fish (Lam 1982; Kim et al. 1992; Kelly and Kohler 1994; Park et al. 1997). Increased milt production and decreased spermatocrit and sperm density have been previously reported in LHRHa-injected rabbitfish and HCG-injected olive flounder (Garcia 1991; Park et al. 1992).

Increased production of diluted milt is the normal response to maturation hormones of fish having lobular-type testicular structure such as *R. oxycephalus* due to hydration of the testis (Billard et al. 1982; Garcia 1991). Thinning of the milt has been noted in milkfish *Chanos chanos* administered HCG and grey mullet *Mugil cephalus* administered 17α -methyltestosterone (Shehadeh et al. 1973; Juario et al. 1980). Milt with thick consistency often fails to disperse well in seawater and can result in low fertilization success. Thinning of *R. oxycephalus* milt by administration of HCG and LHRHa may result in improved fertilization success as was the case for milkfish (Juario et al. 1980). Further studies are required to ensure that hormone administration does

not have a negative effect on sperm survival, fertilization rate, and survival at hatch in *R. oxycephalus*.

In summary, the present results demonstrate that milt collection in *R. oxycephalus* during spawning season was easily possible after administration of HCG or LHRHa. Improvement of this kind of technique should be expected to contribute to reducing the cost for seed production and to develop the hybridization with *R. steindachneri* (Park et al. 1998c).

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