Aquat. Living Resour., 1995, **8**, 303-307

Note

Milt-egg ratio in artificial fertilization of the Asian freshwater catfish, *Clarias macrocephalus*, injected salmon gonadotropin-releasing hormone analogue and domperidone

Ma. Victoria P. Tambasen-Cheong [1], Josefa D. Tan-Fermin [1], Luis Maria B. Garcia [1] and Rodolfo B. Baldevarona [2]

[1] *Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC/AQD), 5021 Tigbauan, Iloilo, Philippines.*

[2] *Institute of Aquaculture, College of Fisheries, University of the Philippines in the Visayas, 5023 Miag-ao, Iloilo, Philippines.*

Accepted March 10, 1995.

Tambasen-Cheong M. V. P., J. D. Tan-Fermin, L. M. B. Garcia, R. B. Baldevarona. *Aquat. Living Resour.*, 1995, **8**, 303-307.

Fécondation artificielle du silure asiatique d'eau douce Clarias macrocephalus *suite à l'injection d'une hormone gonadotrope et de dompéridone.*

INTRODUCTION

Catfish *Clarias macrocephalus* are artificially fertilized by inducing females to spawn using various hormones (Mollah and Tan, 1983; Ngamvongchon *et al.*, 1988; Thalathiah *et al.*, 1988; Tan-Fermin, 1992; Tan-Fermin and Emata, 1993). However, the necessity of administering hormones to males and the quantity of eggs that can be effectively fertilized per unit volume of milt have not been investigated in these studies. In catfish, males need to be sacrificed because milt cannot be stripped by pressing the abdomen of the fish. Sacrificing male catfish to obtain the milt can cause further depletion of the wild population of *C. macrocephalus*. A standard procedure maximizing the use of available gametes to ensure the maximum effectiveness of artificial fertilization may contribute to the development of a breeding programme for re-stocking catfish in the natural habitat.

Dopamine antagonists like pimozide (PIM) and domperidone (DOM) block the action of an endogenous gonadotropin-release inhibitory factor (GRIF) identified as dopamine in the goldfish (Chang and Peter, 1983). DOM, in combination with salmon gonadotropin-releasing hormone (sGnRHa), is now packaged in one preparation (Tradename: Ovaprim, Syndel Laboratories, Canada), and is popularly used for inducing ovulation and spawning of freshwater fishes (Peter *et al.*, 1988; Nandeesha *et al.*, 1990). An increase in milt volume after hormone treatment was reported in carp (Billard *et al.*, 1983; Takashima *et al.*, 1984; Courtois *et al.*, 1986; Ngamvongchon *et al.*, 1987; Billard *et al.*, 1987; Saad and Billard, 1987) and in the rabbitfish (Garcia, 1991). This paper investigates the effect of Ovaprim on milt production and fertilizing ability of *C. macrocephalus*, and determines the optimal milt-egg ratio required for artificial fertilization.

MATERIALS AND METHODS

Fish collection and handling

Sexually mature hatchery-bred catfish, with mean body weight (BW) of 148 g, were fed trash fish daily

at 5% BW. Fish were kept in 9.5 × 1.5 × 1 m concrete tanks with mud substrate under natural photoperiod (Iloilo, Philippines). A day before the experiment, male and female catfish were anaesthetized in 100 ppm 2-phenoxyethanol (Merck), weighed, and transferred individually to 60 l fibreglass tanks containing 20 l freshwater and mud substrate. Water temperature in the fibreglass tanks ranged from 28° to 29°C.

Hormone administration

Male catfish were given a single intramuscular injection of graded doses of Ovaprim in experiment 1 and the optimal dose in experiment 2. Ovaprim contains 0.02 mg sGnRHa and 10 mg DOM/ml. Eggs from mature females were biopsied using a silastic medical grade tubing (OD = 1.96 mm, ID = 1.47 mm, Dow Corning Corporation, Michigan, USA), and then injected 0.5 ml Ovaprim/kg BW or 0.05 mg LHRHa + 1 mg PIM/kg BW. PIM was first dissolved in dimethylsulfoxide, to which propylene glycol was added at 1:9, v/v (Omeljaniuk *et al.*, 1987). PIM and LHRHa were injected simultaneously at 1 ml/kg BW on either side of the dorsal musculature (Tan-Fermin and Emata, 1993). Injections were administered to males at 07.00 h with a disposable plastic tuberculin syringe. Three groups of females were injected at 1700 h either on the same day, a day before, or 2 days before the males to synchronize ovulation with milt extraction at 12, 24, and 48 h post-injection.

Preparation of testicular homogenate

Testes from freshly-sacrificed fish were dissected and cleansed of blood and other adhering tissues with 0.9% NaCl (saline). Excess saline was blotted on absorbent paper before testes were weighed. Each pair of testes was macerated on a Petri dish and mixed with 1 ml saline. Since catfish milt is limited to a few drops, adding saline to the tissue homogenate facilitated collection and quantification of the milt. Excess tissue was separated from the milt homogenate (dilution: 1:1.9-6.0), which was then collected and measured (to the nearest 0.01 ml) with a 1 ml tuberculin syringe. The volume of neat semen was the difference between the total amount of milt homogenate collected and saline added. The milt homogenate was stored in a refrigerator (4-6°C) and was used within 8 h after collection.

Measurement of sperm density

Milt was aspirated into a blood diluting pipette and diluted 1:260 with saline. Sperm density (per ml milt), was counted under 400x magnification following a standard hemacytometer method (American Optical Scientific Division, 1975). Four replicates per milt sample were counted.

Dry fertilization

The dry fertilization protocol and incubation procedure of Tan-Fermin and Emata (1993) was followed with some modifications. Tap water (0.5 ml/g egg) was added to the egg-milt mixture and then gently stirred for another 1-2 s. Determination of fertilization, hatching and survival rates up to yolk resorption were described by Tan-Fermin and Emata (1993).

In experiment 1, 36 mature male catfish equally divided into 4 groups were injected 0.5, 1, and 2 ml Ovaprim/kg BW. Control fish were given 1 ml/kg BW of the injection vehicle consisting of dimethyl sulfoxide (DMSO) and propylene glycol (PG) (1:9, v/v). Three fish from each treatment group were sacrificed at 12, 24, and 48 h post-injection and milt extracted. Milt volume and sperm density were determined for each fish.

Fertilizing capacity was determined by mixing 0.15 ml of the diluted milt homogenate (mean dilution of 1:3.5 in saline) with a 0.001 kg aliquot of ovulated eggs pooled from 3.5 females. Mean egg diameter was determined from the short and long axes of 60 phosphate-buffered formalin-fixed eggs, which were measured (to the nearest 0.01 mm) with a micrometer eyepiece within 120 h post-fixation (Tan-Fermin, 1991). Fertilization runs were conducted in 3 replicates per male.

In experiment 2, male fish were injected 2 ml Ovaprim/kg BW and sacrificed at 24 h post-injection to obtain milt. Three fertilization runs were conducted by mixing diluted milt pooled from 3 males and ovulated eggs combined from 5-10 females. Sperm density of the milt homogenate was measured as described previously. In addition, 1 g egg samples ($n = 65$) were weighed, fixed, and counted to determine the number of eggs/g ovary. In all fertilization runs, 2, 5, 10, 25, 50, 100, and 200 ($\times 10^{-3}$) ml milt homogenate were each tested on 2.5, 5, and 10 ($\times 10^{-3}$) kg egg samples. Fertilization, hatching, and survival rates were recorded for each run.

Statistical analysis

Differences among group means were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at 0.05 level of significance (Sokal and Rohlf, 1981). Fertilization, hatching, and survival rates were arcsine-transformed before statistical analyses.

RESULTS

Mean milt production (5.84 ml/kg BW) peaked, while mean sperm density significantly decreased (2.55×10^3 spermatozoa/ml) 24 h after injection of 2 ml Ovaprim/kg BW in experiment 1 (*fig.* 1). Fertilization, hatching, and survival rates at 12, 24, and 48 h after injection were similar among Ovaprim- and

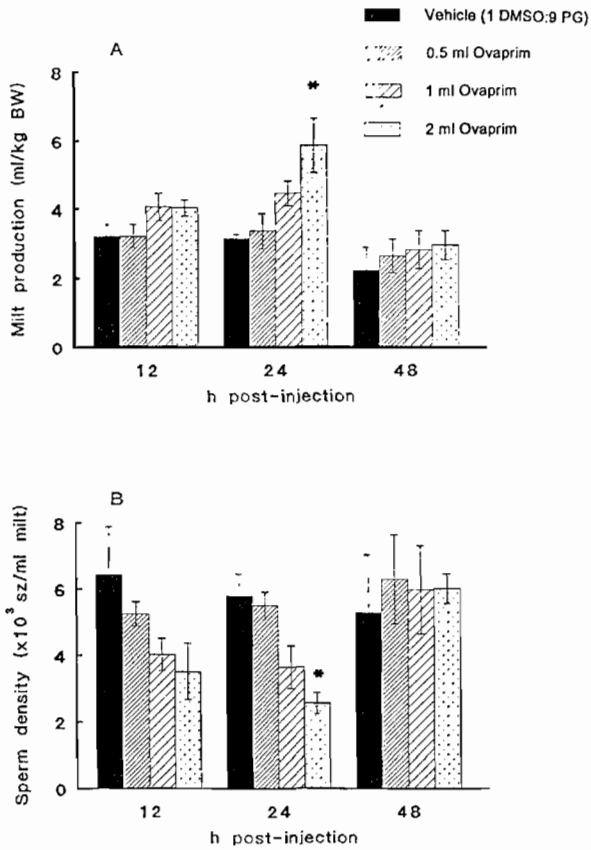

Figure 1. – Milt production (A) and sperm density (B) of mature male catfish *Clarias macrocephalus* (Gunther) in response to graded doses of Ovaprim (active components: 0.02 mg sGnRHa + 10 mg DOM/ml). Asterisk indicates significant difference in the mean values ± SEM.

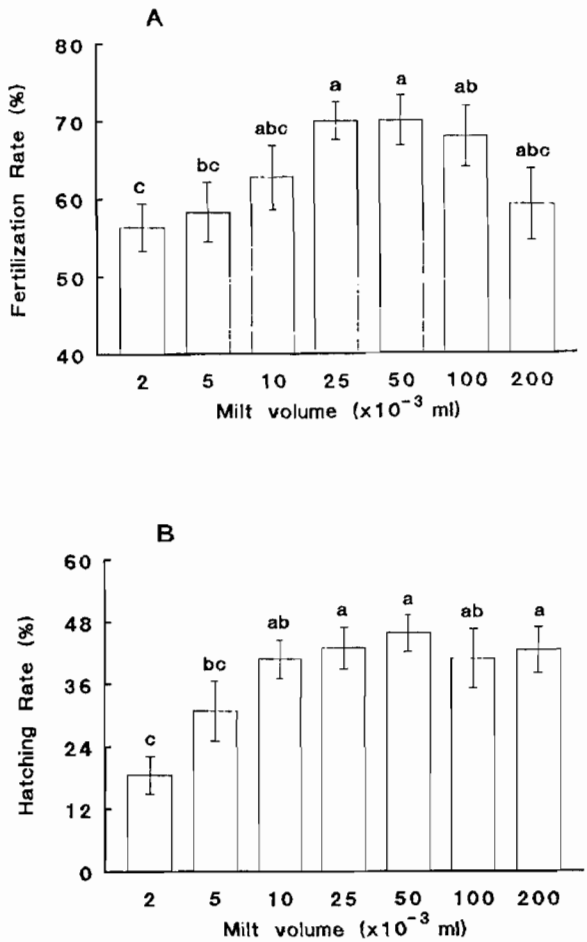

Figure 2. – Fertilization (A) and hatching (B) rates of catfish *C. macrocephalus* (Gunther) eggs inseminated by different volumes of milt from Ovaprim-treated fish. Each bar represents pooled data from 2.5, 5, and 10 ($\times 10^{-3}$) kg egg samples. Values are mean of three trials ± SEM. Means with the same letter are not significantly different ($p > 0.05$).

vehicle-injected fish, with means ranging from 67.3 to 89.5%, 36.7 to 85%, and 62.0 to 98.3%, respectively.

Milt was obtained from male catfish 24 h after injection of 2 ml Ovaprim/kg BW in experiment 2. Since egg weight did not have a significant effect on fertilization and hatching, the data from each milt volume tested were pooled. Fertilization (70%) and hatching (41-46%) rates using data pooled from 2.5, 5, and 10 ($\times 10^{-3}$) kg egg samples were highest when inseminated with 25 and 50 ($\times 10^{-3}$) ml of diluted milt (*fig.* 2).

Sperm density of diluted milt was 0.77×10^3 spermatozoa/ml. A 1-g sample contained approximately 470 eggs, with diameter ranging from 1.3 to 1.8 mm.

DISCUSSION

The present study is the first report that determined the effects of sGnRHa and DOM on milt production, and quantified the amount of eggs that can be effectively fertilized per unit volume of milt in this species. In previous studies, an assessment of the viability of catfish eggs from hormone-treated females (Mollah and Tan, 1983; Ngamvongchon *et al.*, 1988; Tan-Fermin and Emata, 1993) was done by inseminating ovulated eggs with an arbitrarily set volume and dilution of milt. Using the optimal milt-egg insemination ratio established in the present study, 25-50 ($\times 10^{-3}$) ml diluted milt: 0.01 kg eggs, 0.2 ml milt can therefore fertilize 0.04-0.08 kg eggs instead of only 0.01 kg, as practised by Tan- Fermin (unpublished). Dilution of milt also resulted in increased fertilization and hatching rates in brook trout (Plosila and Keller, 1974), salmonids (Billard, 1977), tilapia (Rana and McAndrew, 1989), and Atlantic croaker (Gwo *et al.*, 1991).

The optimum milt-egg ratio can be used to determine the optimum sex ratio required for artificial fertilization. Since average milt production in Ovaprim-treated fish is 5.84 ml/kg BW, a 0.1-kg male can produce 0.584 ml of milt. Diluting the milt 1:3.5 in saline and using 0.025-0.05 ml

of it to inseminate 10 g eggs, a 0.1-kg male catfish can theoretically fertilize 286-572 g eggs from 13-26 females (mean egg output = 220 g/fish, Tan-Fermin and Emata, 1993). Moreover, since sperm density of diluted milt is 0.77×10^3/ml, 0.025-0.05 ml contains approximately $19.2\text{-}38.5 \times 10^3$ spermatozoa. With an average of 470 eggs in 1 g sample, the effective gamete insemination ratio is about $4\text{-}8 \times 10^3$ spermatozoa per egg.

Highest milt production and a corresponding decrease in sperm density 24 h post-injection of 2 ml Ovaprim/kg BW (0.04 mg sGnRHa + 20 mg DOM/kg BW) indicate that this treatment is effective in stimulating milt production in *C. macrocephalus*. These observations may reflect the testicular hydration response of catfish to Ovaprim, resulting in the dilution of the semen (Donaldson and Hunter, 1983; Nagahama, 1987). Domperidone probably potentiated the gonadotropin-releasing activity of sGnRHa. PIM, another dopamine antagonist, was also reported to potentiate the effects of low doses of LHRHa on milt production in male carp 24 h after injection (Billiard *et al.*, 1987).

Fertilization and hatching rates in this study were lower than those reported by Mollah and Tan (1983) and Tan-Fermin and Emata (1993). Nonetheless, the range is still acceptable for artificial fertilization. The relatively low fertilization rate in the present investigation may be attributed to parthenogenesis, that results from a delay in inseminating the eggs caused by pooling eggs from 5 to 10 females. Spontaneous development may have occurred immediately after stripping even before insemination, thereby preventing the fertilization of a considerable number of eggs. Parthenogenesis in tropical carp and catfish may proceed to the morula or gastrula stage, followed by decomposition (Withler, 1980). Spontaneous development of eggs prior to insemination may also have caused low hatching rates in *C. gariepinus* (Steyn and Van Vuren, 1987).

Acknowledgments

We thank R. Pagador, M. de los Santos, E. Leaño, E. Chavoso, R. M. T. Cheong, R. D. P. Tambasen, M. Fujas, A. B. Maravilla, F. Solano, F. Alba, R. Liberan, and the SEAFDEC/AQD Fish hatchery staff for technical assistance, and L. A. Espada for the data analysis.

REFERENCES

American Optical Scientific Instrument Division 1975. AO Bright-line Hemacytometer Counting Chamber. Manufacturer's Manual. Buffalo, New York, NY, 19 p.

Billard R. 1977. A new technique of artificial insemination for salmonids using a sperm diluent. *Fisheries* **2**, 24-25.

Billard R., J. P. Choisis, P. Reinaud 1983. Stimulation of spermiation in carp in response to LH-RH and D-Ala^6-LH-RH ethylamide. *Aquaculture* **35**, 173-176.

Billard R., K. Bieniarz, W. Popek, P. Epler, B. Breton, K. Alagarswami 1987. Stimulation of gonadotropin secretion and spermiation in carp by pimozide-LHRHa treatment : effects of dose and time of day. *Aquaculture* **62**, 162-270.

Chang J. P., R. E. Peter 1983. Effects of dopamine on gonadotropin release in female goldfish, *Carassius auratus*. *Neuroendocrinology* **36**, 351-357.

Courtois F., F. Takashima, R. Billard 1986. Stimulation of spermiation following injection of carp pituitary homogenates in carps. *Bull. Jpn. Soc. Sci. Fish.* **582**, 995-997.

Donaldson E. M., G. A. Hunter 1983. Induced final maturation ovulation and spermiation in cultured fish. In: Fish Physiology, Vol. IXB. Hoar W. S., D. J. Randall, E. M. Donaldson eds. Academic Press, New York, NY, 351-403.

Garcia L. Ma.B. 1991. Spermiation response of mature rabbitfish, *Siganus guttatus* Bloch, to luteinizing hormone-releasing hormone analogue (LHRHa) injection. *Aquaculture* **97**, 291-299.

Gwo J. C., K. Strawn, M. T. Longnecker, C. R. Arnold 1991. Cryopreservation of Atlantic croaker spermatozoa. *Aquaculture* **94**, 355-375.

Mollah M. F. A., Tan E. S. P. 1983. HCG-induced spawning of the catfish, *Clarias macrocephalus* (Gunther). *Aquaculture* **35**, 239-247.

Nagahama Y. 1987. Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. *Zool. Sci.* **4**, 209-222.

Nandeesha M. C., K. G. Rao, R. N. Jayanna, N. C. Parker, T. J. Varghese, P. Keshavanath, H. P. C. Shetty 1990. Induced spawning of Indian major carps through single application of Ovaprim-C. The Second Asian Fisheries Forum, Tokyo, Japan, 581-586.

Ngamvongchon S., L. Y. Kok, F. Takashima 1987. Changes in endocrine profile and spermiation response in carp after LHRH analogue injection. *Nippon Suisan Gakkaishi* **53**, 229-234.

Ngamvongchon S., O. Pawaputanon, W. Leelapatra, W. E. Johnson 1988. Effectiveness of an LHRH analogue for the induced spawning of carp and catfish in Northeast Thailand. *Aquaculture* **74**, 35-40.

Omeljaniuk R. J., S. H. Shih, R. E. Peter 1987. In *vivo* evaluation of dopamine receptor-mediated inhibition of gonadotropin secretion from the pituitary of the goldfish, *Carassius auratus*. *J. Endocrinol.* **114**, 449-458.

Plosila D. S., W. T. Keller 1974. Effects of quantity of stored sperm and water on fertilization of brook trout eggs. *Progress. Fish-Cult.* **34**, 179-181.

Rana K. J., B. J. McAndrew 1989. The viability of cryopreserved tilapia spermatozoa. *Aquaculture* **75**, 335-345.

Saad A., R. Billard 1987. Spermatozoa production and volume of semen collected after hormonal stimulation in the carp, *Cyprinus carpio*. *Aquaculture* **63**, 67-77.

Sokal R. R., F. J. Rohlf 1981. Biometry, 2nd edn. W.H. Freeman, San Francisco, CA, 859 p.

Steyn G. J., J. H. J. van Vuren 1987. The fertilizing capacity of sharptooth catfish (*Clarias gariepinus*) sperm. *Aquaculture* **63**, 187-193.

Takashima F., C. Weil, R. Billard, L. W. Crim, A. Fostier 1984. Stimulation of spermiation by LHRH analogue in carp. *Bull. Jpn. Soc. Sci. Fish.* **50**, 1323-1329.

Tan-Fermin J. D. 1991. Suitability of different formalin-containing fixatives for the eggs of *Clarias macrocephalus* (Gunther). *Isr. J. Aquac. Bamidgeh* **43**, 57-61.

Tan-Fermin J. D. 1992. Induction of oocyte maturation and ovulation in the freshwater Asian catfish *Clarias macrocephalus* (Gunther) by LHRHa and pimozide. *J. Appl. Ichthyol.* **8**, 90-98.

Tan-Fermin J. D., Emata A.C. 1993. Induced spawning by LHRHa and pimozide in the freshwater Asian catfish *Clarias macrocephalus* (Gunther). *J. Appl. Ichthyol.* **9**, 89-96.

Thalathiah S., A. O. Ahmad, M.S. Zaini 1988. Induced spawning techniques practiced at Batu Berendam, melaka, Malaysia. *Aquaculture* **74**, 23-33.

Withler F. C. 1980. Chilled and cryogenic storage of gametes of Thai carps and catfishes. *Can. Tech. Rep. Fish. Aquat. Sci.* **943**, 15 p.