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A Preliminary Study on the Maturation and Reproduction of *Spinibarbus denticulatus* (Oshima, 1926), an Indigenous Species of Northern Vietnam

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Abstract

These preliminary studies were conducted to understand some of the basic reproduction parameters of the indigenous carp, *Spinibarbus denticulatus* (Oshima 1926*)* as a prelude to more specific research studies, and the subsequent development of hatchery technology. Gonad and oocyte development was assessed over a 12 month period. Observation of the annuli rings of the fish scale was found to be a reliable means of measuring fish age. Mature males were smaller and matured earlier (4 years) than females (5 years). The gonadosomic index revealed two peaks (April and October). Oocytes, developing at various stages were examined from January to March. In January the oocytes sizes were uniformly small. Two distinct oocyte-size groups were observed in the February sampling and three size groups were observed in March. The proportion of large-size oocytes (55%) was higher compared to mid-size (26%) and small-size (19%) oocytes during the near peak spawning months. The average number of oocytes in the ovaries in a female was 31,041. The mean sperm concentration was 8.42±0.36 million cells per ml with only a small amount (3.3±0.2 ml) of total expressible milt per male. However, when induced with LHRHa the milt production increased to 6.2±0.5 ml without an increase in the total number of sperm cells. The species shows potential for mass production; however, low fecundity and late puberty could present obstacles to artificial seed production.

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Introduction

Chinese carps, Indian major carps and tilapias make up over 90% of freshwater species cultured. Over 95% of these come from Asia (FAO 2003_a), and most of these are exotic species in Southeast Asia (Liste and Chevey 1932). Although the culture of introduced species is profitable, they have also been implicated in either displacement of indigenous species or introgression with local species (Ogutu-Ohwayo and Hecky 1991; De Long and Van Zon 1993; Na Nakorn et al. 1999). Moreover, exotic species are more susceptible to local diseases (e.g., grass carp is prone to red spot disease; Kim et al. 1999). Grass carp, a primary cultured species for the rural poor, and a major source of animal protein in their diet, has been severely affected by this pathogen that many poor farmers of North Vietnam have abandoned its culture (Bart 2000). There is a need to identify an alternative species to grass carp which can be fed on readily available and low-cost grass, preferably an indigenous species.

Southeast Asia has one of the largest diversities of freshwater fish species in the world (FAO 2003b). Unfortunately, comparatively few species from this region are widely cultured, partly due to the lack of sufficient knowledge of their reproduction. The carp, *Spinibarbus denticulatus* (Oshima 1926) has significant potential to become a more widely farmed species, particularly for the low-input system relevant for developing countries, if hatchery produced seed is available. With the local name 'ca bong', *S. denticulatus* is indigenous to North Vietnam with a distribution from upstream to the middle reaches of the Red, Lo and Gam river systems. This species contributed nearly 25-30% of the total wild fish capture in the Lo and Gam river systems in the past, and 20-30% of that in the Ba Be reservoir (Hao 1993). There is distinct cold and warm seasonal variation with water temperature ranging from 9-16 °C during winter and 25-30 °C during the summer months (Dan and Little 2000). This wide range of temperature tolerance indicates potential for this species to be cultured in regions with cooler temperatures. *S. denticulatus* belongs to the sub-family, Barbinae of the Cyprinidae family. The largest *S. denticulatus* recorded was 30.0 kg (Dau and Le 1971). It is a macrophagous herbivore with diets very similar to that of the grass carp (Bau 1998). Although this species has the potential to be cultured in some temperate environments, studies on the life history of *S. denticulatus* and its habitat requirements have not been published to date.

An attractive feature of this species is its resistance to some local pathogens that cause red spot disease, even when raised in the same cage with infected grass carp (personal communication, Mr. Pham Bau, 2002, Research Institute for Aquaculture No. I, Vietnam). Spawning is thought to take place during the spring and autumn months. Natural stocks are declining because of increasing fishing pressure, habitat destruction through construction of hydropower dams and improper capture practices e.g., use of dynamite and poison. A thorough study is needed to better understand the reproductive biology in order to produce juveniles for stocking. The preliminary observations of basic reproductive biology reported here may lead to the development of hatchery production techniques for this species.

Materials and Methods

Study animals

Male and female *S. denticulatus* (n=270) ranging from 3 to 7 years of age were acquired from fish cages in Ha Giang and Tuyen Quang provinces, transported to Me Linh Research Station, Vinh Phuc province and held in nine earthen ponds (300m^2) for the study. Fish were fed a combination of grass and rice sprouts *ad libitum*. Water quality (pH, dissolved oxygen, total ammonia and nitrite) was monitored weekly throughout the study period.

To assess gonadal development and maturation, 10 fish (5 year or older) were sacrificed monthly (December 2001 to November 2002) and fish weight and gonad weight were measured to the nearest 0.01g. To determine age at sexual maturation 3 (n=8), 4 (n=10), and 5-year old females (n=19) were harvested in April. All fish in the three age groups were dissected to assess the condition of the gonads. Gonadosomic Index (GSI) was calculated using the following equation: GSI=Wt. of gonads/Wt. of fish x 100.

Age was determined by counting the scale annuli rings. Scales were removed from above and below the lateral line, washed and visual observation was made under the microscope to assess annual rings (Fig. 1). Verification of the age through scale reading was cross-checked with farmer information on the size and date of stocking. Monthly observation of scales from a male and a female fish over the 12-month period were

also made to determine timing of annuli deposition. Visual observations of the experimental animals were made during the peak and off spawning seasons for color changes and sexual dimorphic characteristics.

Figure 1. Scales of *Spinibarbus denticulatus* removed from below the lateral line of three females of *S. denticulatus* of age 4, 5 and 6 (left to right, respectively). Dark rings represent the annual growth

Oocytes and fecundity

Nine females were dissected each month and their ovaries were removed to assess fecundity. The number of oocytes in the ovary was estimated by first removing all oocytes from the connective tissue of the ovary, and by sampling approximately 15 g of mixed oocytes from each female. From the 15 g of mixed oocytes, 50 oocytes were randomly removed, and each oocyte was measured once to the nearest 0.01 mm using the ocular/eye piece micrometer of a compound microscope. The diameter of various size oocytes was enumerated starting from January (prior to the spawning period), and ovarian oocyte size-frequency distribution was determined over a 3-month period (January to March). Oocytes belonging to the same size group were separated and classified into three categories, and the monthly change in size composition was determined.

Spermatological assessment

Male fish (4 to 7 years of age) were removed from the water, dried and gentle abdominal pressure was applied using a thumb and index finger to remove milt. Pressure was applied starting below the pectoral fins and moving down towards the genital pore. This process was repeated until sperm stopped flowing. Total expressible milt was pooled from an individual male and drawn into a 5.0 ml syringe to assess total volume. Sperm motility was assessed by adding a droplet of distilled water on the freshly stripped sperm and observing under the microscope. Two stages of motility, progressive (vigorous movement) and vibration (movement *in loco*) were observed. Sperm concentration (spermatozoa mL^{-1} of milt) was estimated using Neubaur's counting chamber following Vutiphandchai and Zohar (1999).

To increase the amount of expressible sperm, mature males (n=13) of similar size (2.6±0.3kg) were harvested and six were induced with Luteinizing hormone-releasing hormone analogue (LHRHa) at 10µg kg^{-1}, while the other seven were injected with 0.9% saline solution as blank controls. Males were stripped after 6h of injection and volume was measured using a 10.0ml standard syringe. The number of sperm cells per ml of milt was estimated using a Neubaur's counting chamber.

Statistical analysis

Gonadosomatic index for females (3, 4 and 5-year) were analyzed by one-way ANOVA. Mean values for different age groups were compared with LSD and differences were considered significant at $P<0.05$. Data in percentage were arcsine transformed prior to analysis.

Results

Ovary and testis

During the peak spawning season, mature bi-lobular ovaries appeared turgid and brownish in color. Prior to spawning, ovaries made up over 60% of the abdominal cavity. Similarly, the mature bi-lobular testis appeared pink in color and comprised a smaller portion (<10%) of the abdominal cavity.

A bi-modal gonadosomatic index (GSI) was apparent in females observed over a 12-month period, with a major spike occurring in April (3.6±0.7%) and a minor spike (2.6±0.2%) in October. The largest ovary sampled in April was 130 g at 5.0% GSI. The lowest GSI were found in December (0.5%) and in June (0.8%). Visual inspections (January, February and March) revealed an increasing GSI paralleled by an increased proportion of the maturing large oocytes, although no statistical analysis was carried out.

A similar bi-modal GSI was observed in males (Fig. 2), but of a lower magnitude compared to the females. A major peak occurred in April (1.4±0.2%) and a minor peak (1.4±0.1%) in October. The largest testis sampled in April was 40.0 g at 1.4%. The lowest GSI values were found in December (0.8 GSI) and in June (0.6 GSI).

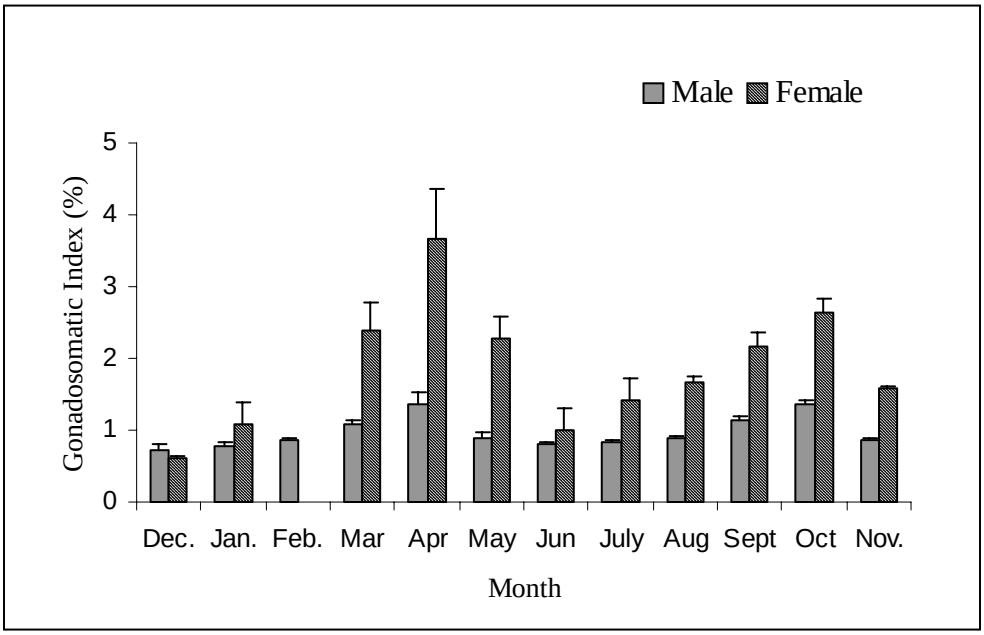

Figure 2. Monthly changes in gonadosomatic index - GSI (gonad weight/body weight x 100) of male and female *S. denticulatus* (>5-year old) over a 12-month period. Error bars indicate S.D.

Oocyte development and fecundity

Oocyte size distribution in the ovary revealed distinct size classes of developing oocytes rather than a continuous size distribution (Fig. 3).

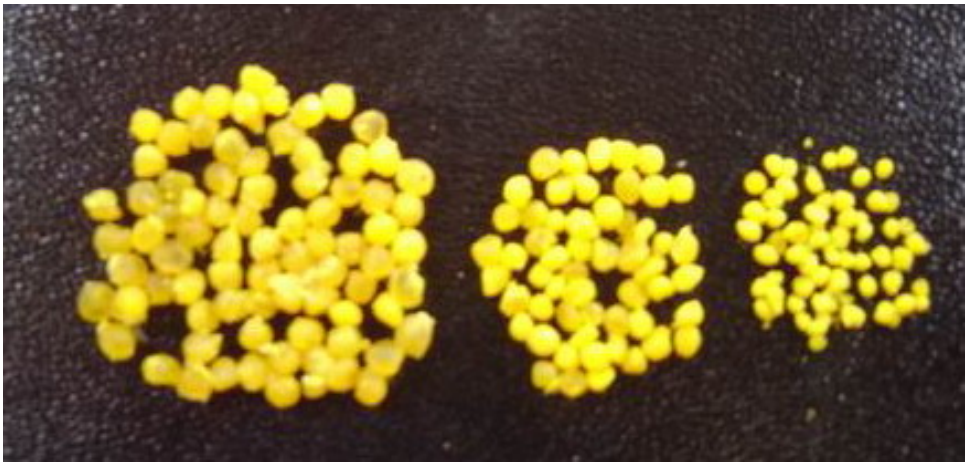

Figure 3. Three class size eggs: 1.1(0.8-1.4 mm), 1.6(1.4-1.8 mm), and 2.1(1.9-2.4 mm) in the ovary of a 5-year old female examined in March-April

The mean diameter of oocytes during the early spawning season (January) was found in a single class, 0.7 mm (0.6-0.8 mm). These oocyte size variations added a second class size in February, 1.0 mm (0.9-1.2 mm). Approximately 36% of the eggs were small while the remainder (54%) was a larger size during this sampling. In March, three class sizes were observed, 1.1 mm (0.8-1.4 mm), 1.6 mm (1.4-1.8 mm), and 2.1 mm (1.9-2.4 mm), with relative proportions of 18.7, 26.4 and 54.9%, respectively. A progressive color change from opaque white to translucent yellow was observed during early February to March sampling. The smallest size class was pale yellow while progressively larger eggs were dark-yellow to light brown in color.

Fecundity ranges widely (13,000-45,000 oocytes $fish^{-1}$) among the females sampled. Oocyte density (number of oocytes per kg of fish) ranged from 6,000 to 14,000, with a mean of 9,873±1,185 kg^{-1}. However, there was no correlation (r= 0.129) between weight and fecundity among the eight females (mean weight 3.1± 0.4 kg) examined.

Sperm characteristics

Microscopic observation of sperm cells (100x–oil emersion) indicated that they were similar to those of other teleosts with a head, mid-piece and a single tail. While progressive or vigorous motility duration was only 1.99±0.03 min, total motility (progressive motility and vibration *in*

loco) was longer, 2.70±0.03 min. Fish injected with hormones expressed a considerably higher volume of milt (6.2±0.5 ml) compared with those injected with saline only (3.3±0.2 ml). The density of sperm in fish without hormonal induction ($1.6x10^7$ ml^{-1}) was double compared to that of hormone injected males ($0.8x10^7$ ml^{-1}) indicating that the total sperm number was unchanged by hormone treatment. While sperm remained motile over a 5-hour period (sampled every 30 min from the time of stripping), there was a progressive decline in the duration of motility after 2 hours.

Male and female characteristics

Although males and females, 3 to 7 years were held in nine ponds for a 12-month period, neither mating behavior nor recruitment was observed. A clear and distinctive annual ring was observed on the scales of mature fish (Fig. 1). The annular rings were irregular in shape with varying distances between the rings and between stocks. While most rings were complete circles, there were a few that were partly complete. The monthly observation of rings between males and females did not differ. Deposition of annuli was first observed in January.

Male broods tended to be smaller (2.3±0.2 kg) than the females (3.3±0.4 kg) in the 5, 6, and 7-year age class (Fig 4). It was difficult to ascertain sexual dimorphism before maturation and out of the spawning season, sampled over a 12-month period. However, during the spawning season males tended to be more colorful with an iridescent green appearance. Males possessed a rough texture (pearl organs) along the exterior of the operculum running below the eyes towards the mouth during the peak spawning period. One of the most obvious signs of a mature male during the spawning season was the release of milt. Milt flowed readily out of the genital pore with slight digital pressure to the abdomen.

During the spawning period, mature females had an enlarged abdomen. Both spawning females and males became more colorful with the females slightly lighter in color than males. Unlike the genital papilla which is often swollen and pink during the spawning season in most fish, the female genital opening was covered with a white fleshy protrusion. Males appeared to have only a simple opening without a protrusion as in females.

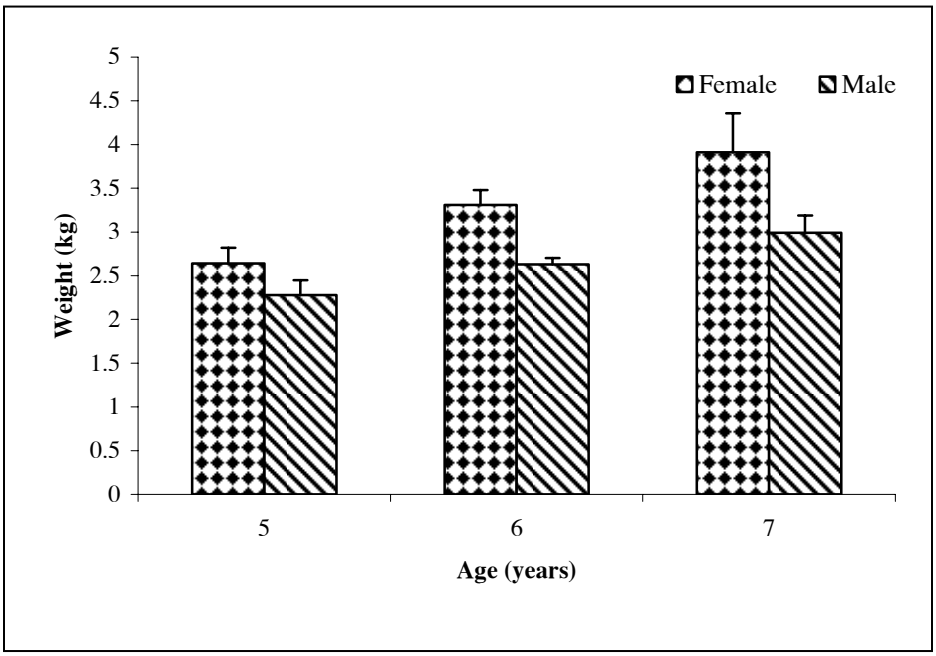

Figure 4. Mean weight of females (n=24) and males (n=19) at age of 5; 6; and 7 years fish over 12 month-culture. Error bars represent S.D.

Age at maturation

The mean weight of a 5-year old fish ovary was significantly higher (81.4±8.2 g) than in 3 (15.9±0.7 g) or 4-year old (25.1±0.9 g) females (Table 1). The existence of a large variation in gonad size was observed between different age groups with approximately a two fold increase in 4 than in 3-year old, but five times higher in the 5-year old group. The GSI of a 5-year old fish was also higher (2.8±0.3) than of 3 (1.1±0.0) and 4-year old (1.2±0.0) females. No observable difference was found between the GSI of 3 and 4-year old fish. The mean weight of females varied with age, where 3, 4 and 5-year old females were 1.4±0.1, 2.1±0.0 and 2.9±0.1 kg, respectively.

Table 1. Gonad weight, body weight and gondosomatic index of 3, 4 and 5-year old females during April (the peak spawning period)

Descriptions	Age of female		
	3-year[1]	4-year[1]	5-year[1]
Gonad weight (g)	15.9±0.7[b]	25.1±0.9[b]	81.4± 8.2[a]
Weight of female (kg)	1.4±0.1[c]	2.1±0.0[b]	2.9± 0.1[a]
Gonadosomatic index (%)	1.1±0.0[b]	1.2±0.0[b]	2.8 ±0.3[a]
Number of individuals	8	10	19

[1]Values are mean ± SE; mean values with different superscripts letters in the same row were significantly different ($P<0.05$).

Discussion

Monitoring oocyte size in dissected ovaries over a period of three months (January, February and March) clearly showed that oocytes matured in stages. Three distinct size classes of oocytes observed out of the spawning season suggests possible multiple spawning within a season. The gonadosomatic index monitored over a 12-month period showed that the ovary and testis increase in size twice a year with two major peaks occurring in April and October. Furthermore, the relatively high GSI observed from March to May and July to October indicated continuous maturation of oocytes in the ovary throughout the warmer months in ponds. Overall, low maximum GSI (3.65%) also indicated that they may spawn over a several month period. Oocytes developing in the ovary at various stages during and in the off spawning season suggest that this species, although typically thought to spawn twice a year, could be induced to spawn more frequently during the warmer months of the year.

Despite relatively low fecundity (9,873±1,185 kg^{-1}) compared with grass carp, the yolk-filled oocyte size was larger (2.01±0.03 mm diameter) than that typically found in other cyprinids indicating possibly more robust larvae with a potentially high survival rate; these are both important traits in an aquaculture species. Lower fecundity rate per spawn could be compensated for by the potential for multiple spawns per year and high fry survival rates.

Sperm characteristics of *S. denticulatus* were similar to other cyprinids with a short duration (1.99±0.3 min) of progressive motility followed by vibration *in loco* (2.7±0.3 min) before complete cessation of motility. The volume of expressible milt however, was low (3.3±0.3 ml)

from 2.6±0.5 kg^{-1} males. An attempt to increase total sperm by administration of LHRHa resulted in doubling the expressible milt to 6.2±0.5 ml, but the total number of spermatozoa remained the same. Previous studies on induction of males have shown similar results (Kwantong and Bart 2003). While the increased volume of milt would make artificial fertilization more convenient, whether there is any change in the quality of sperm from induced males needs to be determined.

The lack of recruitment in ponds where mature broodfish were held for 2 years suggested that perhaps pond-based husbandry lacks the triggers for natural spawning. Further studies on the natural spawning, egg incubation and larval rearing conditions are required as well as induced spawning trials by manipulating the environment and/or endocrine hormones.

The annual rings of fins, otolith or scales are commonly used to assess the age of finfish (Geffen 1992; Ikejima et al. 1998). Otolith reading requires sacrificing the animal, and pectoral fin assessment requires cross sectioning and the use of a stereo microscope. However, the examination of scales in this study required only simple observation of the rings against a light source for them to be clearly visible to the naked eye. It is therefore the least invasive, and appears to be an excellent means to quickly assess age under field conditions.

Since the natural habitat of *S. denticulatus* has distinct cold and warm seasons and the rate of feeding and growth slowed in the ponds during the coldest periods, it was assumed that the dark rings indicated this seasonal growth variation. This was then verified by observing a scale taken from the same male and female over a 12 month period. This preliminary study attempted to estimate the age of fish by collecting the broods from a known source and matching the age with presumed annuli rings. Moreover, observation of scales from the same male and female over a 12 month period suggested that annuli are in fact annual rings. Dark rings could also be caused by a number of other events including environmental stress or poor feeding during harvest and transport. Since study animals were collected from cages, further validation of age should be experimentally determined.

These preliminary observations indicated that males were smaller than females as commonly observed in many other teleosts. This assessment was based on both sexes having the same number of annual rings and farmers' accounting of the date and size at stocking. However, this needs to be verified using experimental data. If such dimorphic characteristics do

in fact persist, it would be important to understand when they become apparent in the life of this species.

Typically, the age of sexual maturation in tropical and subtropical species does not exceed two to three years. Observations of 37 females (2.9±0.5 kg) sampled during the peak spawning period indicated that only females of age 5 had sufficiently mature ovaries. The implication of this is that a long term investment would be required to develop and maintain broodstock which could present an obstacle for low-input aquaculture. The determinants of precocious sexual maturation are thought to be the environment, endocrine hormones and domestication (Le Bail 1988; Holland et al. 1996). Studies have also shown that long term hormone therapy may also reduce the time to puberty in some species (Gur et al. 1995). This provides an opportunity for exploring means to reduce the time to maturation by manipulating hormones, feed and nutrition, and husbandry practices.

Conclusion

To culture a new species requires years of coordinated and sustained research. An early decision on the selection of an appropriate species for further targeted study minimizes failure and waste of resources. These preliminary observations on some of the important maturation and reproductive parameters of this promising species for more widespread culture provide essential information on the age of sexual maturation (>4 years), gonadal development and reproductive cycle (April and October peak periods), male and female characteristics as well as fecundity (13,000-45,000 oocytes $fish^{-1}$) and gamete characteristics. Clearly, more specific studies on hormonal regulation of gamete production and maturation, effects of domestication and husbandry practices on maturation, environmental and/or hormonal manipulation and subsequent mass production of seed are needed before a more widespread culture of *S. denticulatus*.

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