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Evidence of sexual transition in Leopard Grouper (*Mycteroperca rosacea*) individuals held in captivity

Evidencia de transición sexual en individuos de cabrilla sardinera (*Mycteroperca rosacea*) mantenidos en cautiverio

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ABSTRACT

This study describes histological observations of the gonads of 12 captive leopard grouper, *M. rosacea* maintained in captivity. Monthly gonad samples during February to April 2003, were obtained by catheterization and analyzed to determine sex and degree of ovarian development. Oocytes were classified into 5 stages of development and the frequencies were obtained to describe the oocyte distribution in the ovary. Two fish that were females in February were in a bisexual stage in March and functional males in April. The transitional stage was observed during the reproductive season and included degeneration of primary oocytes and proliferation of spermatogonia.

Key words: Gonad development, leopard grouper, captivity.

RESUMEN

Este estudio describe las observaciones histológicas del desarrollo gonadal de 12 individuos de la cabrilla sardinera, en condiciones de cautiverio. De febrero a abril del 2003 se obtuvieron muestras mensuales de la gónada mediante un catéter flexible, las cuales fueron analizadas para determinar el sexo del organismo y el estadio de desarrollo gonadal. Los ovocitos fueron clasificados en 5 etapas de desarrollo y se calcularon las frecuencias para determinar su distribución dentro del ovario. Dos individuos que fueron identificados como hembras en febrero, se encontraron en estadio bisexual en marzo y fueron machos funcionales en abril. Este estadio de transición se observó durante el periodo reproductor y se caracterizó por la degeneración de ovocitos primarios y la proliferación de espermatogonias.

Palabras clave: Cabrilla sardinera, cautiverio, desarrollo gonadal.

INTRODUCTION

The leopard grouper *Mycteroperca rosacea* (Streets 1877) is one of five species of the genus *Mycteroperca* in the eastern Pacific (Rosenblatt & Zahuranec, 1967; Heemstra & Randall, 1993). *M. rosacea* is distributed throughout the Gulf of California as far south as the state of Jalisco, Mexico (Allen & Robertson, 1998). It inhabits rocky areas and sargasso beds near the shore and islands in depths < 50 m (Gracia-López *et al*., 2004a).

Over-exploitation has led to listing as a vulnerable species in the "IUCN Red List of Threatened Species" (VU A1d+2d), high risk

of extinction in the wild in medium-term future (IUCN, 2006). In the last few years, some research about the biology, natural feeding habits and ecology has been carried out (Peláez-Mendoza, 1997; Díaz-Uribe *et al.*, 2001; Mendoza-Bustamante, 2002) being the first support for studies on reproduction technology and larval rearing (Gracia-López *et al.*, 2004a; Gracia-López *et al.*, 2004b; Gracia-López *et al.*, 2005; Kiewek-Martínez, 2004) with a possibility of re-population of natural stocks.

Groupers belong to Serranidae family (Subfamily Epinephelinae). They mainly inhabit coastal waters in tropical, subtropical and temperate seas (Abu-Hakima, 1987; Brusle & Brusle, 1975; Lee *et al.*, 2002). They primarily display protogynous hermaphroditism (Devlin & Nagahama, 2002; Lee *et al.*, 2002; Sadovy *et al.*, 1994), but some have been described as gonochoric where juveniles pass through a bisexual stage of gonad development like *Epinephelus striatus* (Bloch, 1972) (Sadovy & Colin, 1995) and most recently *M. rosacea* (Erisman *et al.*, 2006). Sex change is common among marine fish. The best-known cases have been described for labrids, serranids and sparids (Robertson & Justines, 1982; Warner, 1982), among others. It is part of some species life history and it seems to occur in one direction and in some serranid species it is socially controlled (Robertson, 1972; Ross *et al.*, 1983).

Most studies of gonad development and sex change using histological evidence were achieved with wild sacrificed fish (Abu-Hakima, 1987; Bhandari *et al.*, 2003; Erisman *et al.*, 2008; McGovern *et al.*, 1998; Smith, 1965). Therefore, the sequence of gonad changes taking place during the sexual transition is described based on different individuals. In fish aquaculture, sex ratio in maturation tanks is essential to obtain fertilized eggs. Therefore, sex change of individuals has notable repercussions on stock configuration and reproduction of broodstock maintained in captivity (Hong *et al.*, 2006). Previous studies with tagged *M. rosacea* individuals that were maintained in captivity changed sex from female to male between breeding seasons (Gracia-López personal communication), but there were no observations or any histological evidence of the time required to achieve complete gonad transformation or if it occurred during the breeding season or out of it. Therefore, this study was carried on to describe gonad development of leopard grouper held in captivity and to identify individuals that could change sex under these conditions.

MATERIALS AND METHODS

During June 2002, twelve leopard groupers (0.32 to 0.84 Kg) were captured by hook and line with live bait and different types of lures at San Evaristo, B.C.S. (25.9°N, 110.7°W) and transferred to research facilities (CIBNOR) located in La Paz, Mexico. Fish were anesthetized with 100 mg/L tricaine (MS222, Western Chemical), tagged in the dorsal muscle with Spaghetti Floy Tags, and weighed. Fish were maintained during 13 months in a cylindrical 1-m depth and 16-m^3 capacity tank with filtered (< 1 µm) flow-through seawater, supplemental aeration and natural photoperiod and temperature. Fish were fed frozen sardine, squid, or mackerel *ad libitum* on alternate days.

Temperature (Submersible Temperature Logger, HOBO TidbiT, WI), salinity (refractometer S/M1II-E, ATAGO CO. LTD.) and dissolved oxygen (YSI 85, OH) were daily measured. Photoperiod was calculated by the method described by Rodriguez *et al.*, (2001). Water temperature was between 25 and 29 °C (Fig. 1), salinity between 40 and 41 ppt. and dissolved oxygen 7.2-7.6 mg/L.

From February to July 2003 gonad samples were obtained monthly, by gonad catheterization with a polyethylene catheter inserted into the gonoduct of anaesthetized individuals (Gracia-López *et al.*, 2004a; 2004b). Samples were fixed for 48 h in Karnovsky fixative (20% glutaraldehyde, 40% sacarose and 40% sterilized seawater) (Karnovsky, 1965), dehydrated, embedded in paraffin, sectioned (< 5 µm) and stained with Harris hematoxylin-eosin. Each sample was examined under an optical microscope (Olympus Bx-41). Images were captured using a digital camera (Coolsnap-Pro color MediaCybernetics, San Diego, CA, USA) and processed with Image-Pro Plus software (version 5.0, MediaCybernetics), which is designed for high-resolution image analysis.

Gonads of each female were classified into 5 development stages based on the description made by Kuo *et al.* (1988) and Carrillo *et al.* (1989) for teleost fish which is based on easily recognizable morphologic characters like primary growth (stage I), early vitellogenesis (stage II), late vitellogenesis (stage III), ovulation (stage IV) and atretic follicles (stage V). Oocyte frequency in each development category was obtained from the number of oocytes in each stage in relation to the total number of analyzed oocytes (Fig. 2A-H; Table 1). Fish in sexual transition or bisexual (stage VI) were identified according to the description made for *Epinephelus fario* and males by the observation of testis in the histological slides or the presence of sperm when gentle abdominal pressure was applied (Kuo *et al.*, 1998).

Total length of individuals was calculated by the function $y = a\ x^b$, (y = weight; a = 1.43×10^{-5}; x = total length; b = 2.970) based on the study for wild leopard grouper, *M. rosacea* (Díaz-Uribe *et al.*, 2001).

RESULTS

Weight of broodstock during the study ranged from 0.34 to 1.00 Kg (Fig. 3).

Description of different ovarian stages, diameter of oocyte and germinal vesicle in each stage of development is shown in Table 1. Oocytes in primary growth were the most abundant throughout the study (66-90%). In February, an increase in the percentage of oocytes at early vitellogenic stage occurred (7%) and in April, near the spawning season oocytes in primary growth decreased and an increase of the percentage of the oocytes in

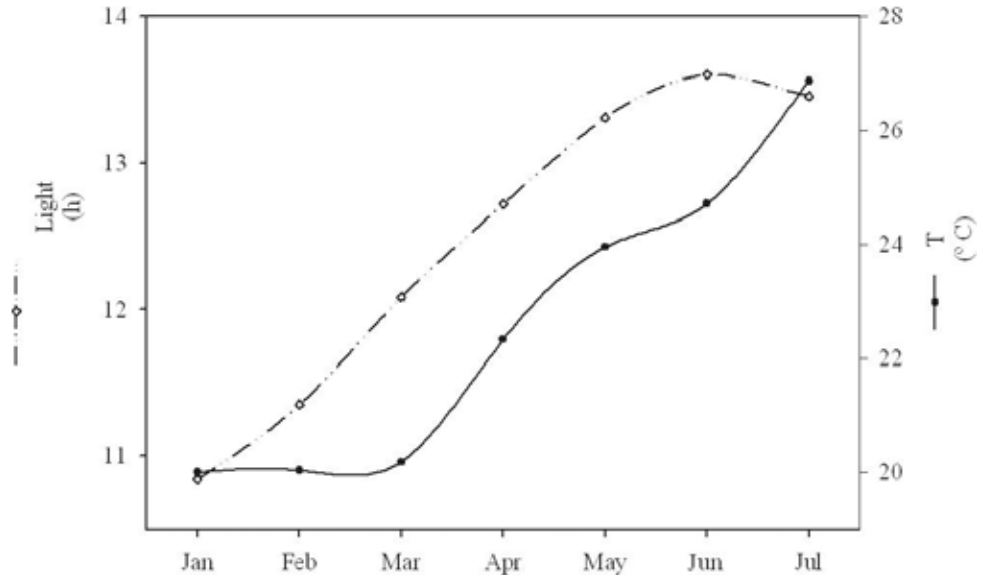

Figure 1. Photoperid and water temperature during experimental period.

Table I. Female gonadal development for *M. rosacea* in captivity. OD = oocyte diameter (mean ± standardeviation)

Category	Development stage	Description	OD (µm)*
I. Primary growth	Chromatin nucleus	Central nucleus and large nucleolus surrounded by a thin and basophilic ooplasm layer (Fig. 4A).	27 ± 6
	Perinucleolar	Multiple nucleoli in the interior nuclear periphery. Ooplasm less basophils without cytoplasmic inclusions. The follicular cells form a thin layer. The follicular membrane as a simple layer of 3-4 flat cells surrounding each one of the oocyte (Fig. 4B).	65 ± 3
II. Early vitellogenesis	Cortical alveolus	The cortical alveoli begin to appear into the oocyte. Small lipid globules dispersed in the ooplasm are observed. It presents a thin zona radiata (Fig 4C).	97 ± 14
	Early vitellogenic class oocytes	Small yolk and lipid globules fill the ooplasm. Follicle consists of single layer of flattened follicle cells (granulose cells) (Fig. 4D)	127 ± 28
III. Late vitellogenesis	Late vitellogenic class oocytes	The layers of the granulose and the thecal cells are observed clearly. The zona radiata is evident and thicker. Eosinophilic yolk globules increase in size. Start the coalescence of the oil globules (Fig. 4E).	307 ± 58 79 ± 12
IV. Maturation and Ovulation	Germinal vesicle migration (GVM)	Event associated with the final oocyte maturation (FMO). Nucleus begins to migrate from the centre towards the animal pole (GV migration). After the migration, nucleus breakdown (GVBD) and spills its content towards the cytoplasm of the animal pole. The coalescence of lipid globules leads to the formation of oil droplets (Fig. 4F).	369 ± 48 89 ± 12
	Oocyte hydration	Hydration begins at the end of the maturation and right before the ovulation. Oil droplets fusion forming an only oil drop. The yolk protein is fused completely forming a continuous mass of fluid. This process grants transparency and buoyancy to the egg increasing its size due to the great amount of water incorporation. The hydrous egg is completely homogeneous and finely granulate (Fig. 4G).	
V. Atresia	Atresia	The phagocytosis of the granulosa cells off the follicular epithelium begins after the breaking of the zona radiata. Disorganization of the layer of follicular cells and is replaced by conjunctive tissue. The nuclei and the yolk globules disintegrate. This stage is evident during the period of post-egg-laying although it can be observed throughout the development (Fig. 4H).	176 ± 59

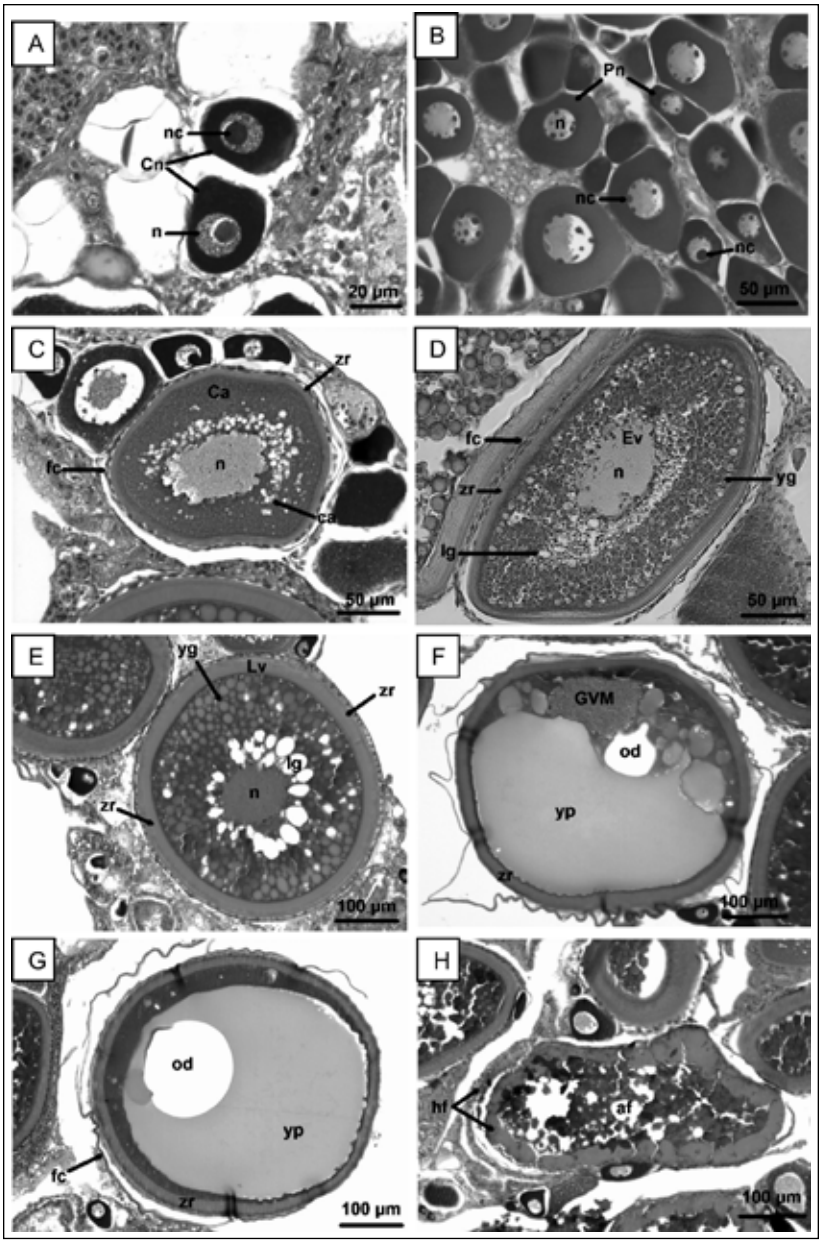

Figure 2 A-H. Leopard grouper (*Mycteroperca rosacea*) oocyte development stages in captivity. I. Primary growth (previtellogenesis); A) Cn, chromatin nucleolar oocyte (100x); B) Pn, perinucleolar oocyte (40x). II. Ev, early vitellogenic class oocytes (20x); C) Ca, cortical alveoli oocyte (20x); D) Small yolk and lipid granules (10x). III. Lv, late vitellogenic class oocytes; E) Yolk and lipid granules (10x). IV. Ovulation (maturation); F) GVM, germinal vesicle migration (10x); G) Hydrated oocyte (10x). V. Atresia; H) af, atretic follicle (10x); n, nucleus; nc, nucleolus; ca cortical alveolus; fc, follicle cell;lg, lipid globules; zr, zona radiata; yg, yolk globules; yp, yolk protein; od, oil droplet; hf, hypertrophic follicular layers. Hematoxylin-eosin stained section (4 µm).

other stages of ovarian development was observed; the percentages of early vitellogenic, late vitellogenic, and ovulated oocytes increased to 12, 7 and 6%, respectively (Fig. 4, Table 1).

In February, ten females had oocytes in primary growth and two females had oocytes in every stage of development, including atretic oocytes. In March, the two females that had the most advanced stages of gonad development were found in sexual transition. The rest of the fish remained females. In April, there were no more transitionals individuals and this sex ratio was maintained throughout the experiment. (Table 2). In March when the bisexual fish were observed, broodstock weight was from 0.32 to 1.02 Kg. The two transitional fish weighed 0.52 and 0.86 Kg (Fig. 2).

Individuals in sexual transition had ovaries with advanced stages of development in February (Fig. 5A) characterized mainly

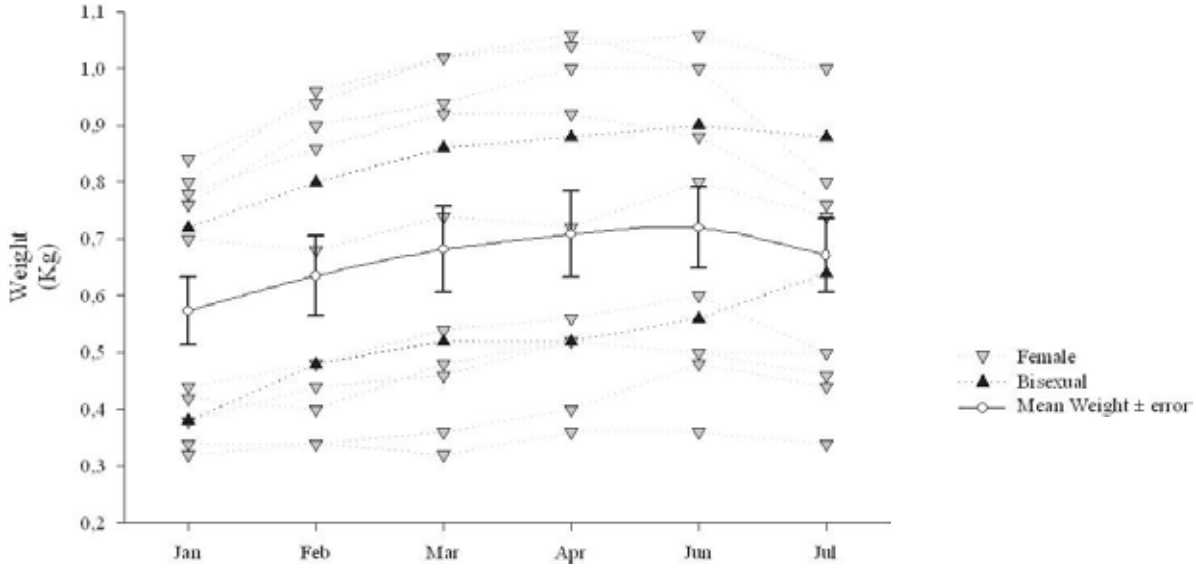

Figure 3. Weight of leopard grouper, *Mycteroperca rosacea* broodstock maintained in captivity during January to July 2003.

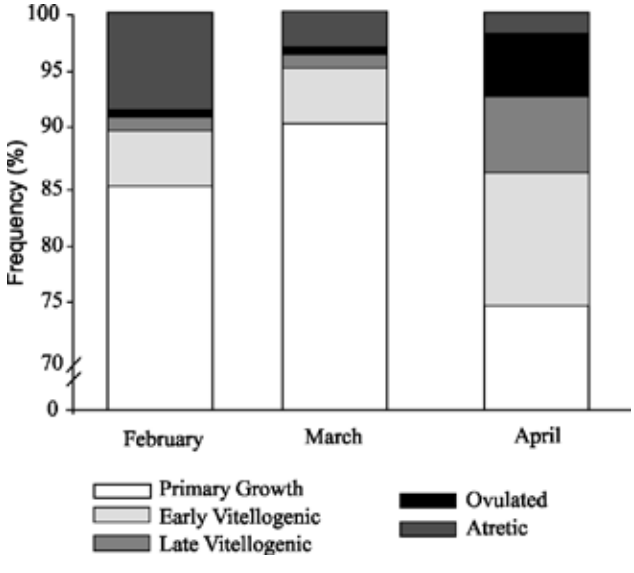

Figure 4. Oocyte development stages distribution of *Mycteroperca rosacea* held in captivity.

by primary oocytes in the perinucleolar stage 74%, early vitellogenesis 9%, late vitellogenesis 2%, ovulated 1% and atretic follicles 14% (Table 3). In March, the analysis of the histological slides revealed the presence of ovotestis, gonad with both testicular and ovarian aspects (Fig. 5B). The transitional stage was characterized by the presence of oocytes in primary growth in the perinuleolar stage, followed by degenerated primary oocytes and the proliferation of spermatogonia, spermatocytes and spermatozoa with flagellum (Fig. 5 B-C). At this stage, the differentiated male cysts proliferated to the interior of the lamellae as the primary oocytes degenerated and were reabsorbed.

Males were characterized by the presence of spermatogenesis. A large number of spermatogonia, spermatocytes, spermatids and spermatozoa were observed in functional testes in April (Fig. 5C).

DISCUSSION

In this study, the gonad development of the leopard grouper *M. rosacea* was described with the use of histological techniques. As observed in nature, broodstock had the most advanced gonad development in April (Erisman *et al.*, 2008). Late vitellogenesis was observed in February indicating the beginning of gonad development and in April, the highest amount of oocytes in secondary growth was observed. The smallest mature individual was 3+ years (360 mm), coinciding with maturity of *E. tauvina* (Abu-Hakima, 1987).

It was recently demonstrated that wild leopard grouper, *M. rosacea* is a gonochoric specie that goes through a juvenile immature bisexual stage in sizes smaller than 220 mm (2 years old) (Erisman *et al.,* 2008). In this study, the individuals that changed sex were older than 3 and 4 years, 351 and 416 mm respectively (the age was obtained based on a backward estimate (Diaz-Uribe, 2001), and had mature ovaries with all the development stages one

Table 2. Total number of brookstock of *M. rosacea* and sexual proportion for each weight category of the fish.

Weight category	Total	Female	Male	Individual/ category
(Kg)	(n)	(%)	(%)	(%)
0.00-0.50	54	56	44	19
0.51-1.00	136	70	30	47
1.10-1.50	38	71	29	13
1.51-2.00	23	65	35	8
2.10-2.50	14	57	43	5
2.51-3.00	5	60	40	2
3.10-3.50	3	67	33	1
3.51-4.00	9	44	56	3
4.10-4.50	7	43	57	2

month prior to the sexual transition. There are some grouper species were the largest individual in a group is the one that changes sex. In other species, sex change could be observed in small size individuals like *Epinephelus tauvina* (Forsskal 1772) (Abu-Hakima, 1987), *Epinephelus aeneus* (Geoffroy Saint-Hilaire 1817) (Hassin *et al.*, 1997) and *Epinephelus merra* (Bloch 1793) (Bhandari *et al.*, 2003). However, it is usual that average female size in a population is smaller than mean size of males. This differs from *M. rosacea* were the size of females and males overlapped.

Table 3. Sexual distribution of *M. rosacea* in captivity during 2003.

Month	Female	Bisexual	Male
February	12	0	0
March	10	2	2
April	10	0	2
May	10	0	2
June	10	0	2

Individuals of *M. rosacea* changed sex to male eight months after their capture and the transition was complete in less than two months. These individuals had mature ovaries with every stage of development in February, later the presence of ovo-testis was observed with ovarian tissue reminiscences one month previous the presence of sperm and the complete transformation to functional male in March. Captivity could influence the time for sex change, because in wild populations it is often observed out

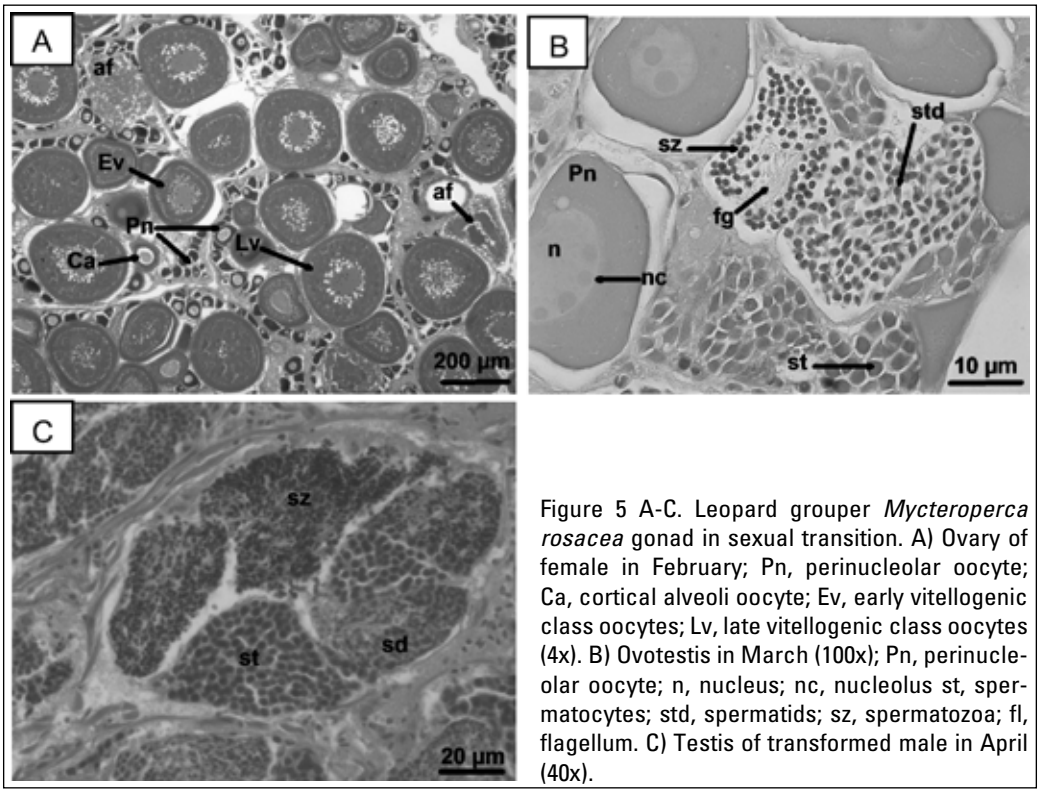

Figure 5 A-C. Leopard grouper *Mycteroperca rosacea* gonad in sexual transition. A) Ovary of female in February; Pn, perinucleolar oocyte; Ca, cortical alveoli oocyte; Ev, early vitellogenic class oocytes; Lv, late vitellogenic class oocytes (4x). B) Ovotestis in March (100x); Pn, perinucleolar oocyte; n, nucleus; nc, nucleolus st, spermatocytes; std, spermatids; sz, spermatozoa; fl, flagellum. C) Testis of transformed male in April (40x).

of the breeding season (Brusle & Brusle, 1975; Bhandari *et al*., 2003; Bhandari *et al*., 2004a; McGovern *et al*., 1998; Smith, 1965). The time required for a captive individual to change sex varies according to the specie like *E. coloides* that changed sex after 7 years of captivity (Yeh *et al*., 2003). In studies of captive *E. merra*, sexual transition was completed in a shorter period during the reproductive season which related with the high levels of sex steroids produced during this period (Alam *et al*., 2005; Alam *et al*., 2006).

In some grouper species the social unit consist of several small individuals and a large dominant male, when the male disappears or no longer can maintain control of subordinates one of the largest females could change sex assuming the male roll (Yaron & Sivan, 2005). In this study, the two sex transition fish were not the largest suggesting that captive *M. rosacea* has the potential to change sex in a wide size range. This has been reported in other captive grouper species like *E. akaara* where size of females that change sex is different to size found in wild populations (Tanaka *et al*., 1990) and *E. striatus*, which is a species described as gonochorist with the potential of sex change under natural or controlled conditions (Sadovy & Colin, 1995). There are also other species like *Bostrichthys sinensis* (Lacepède, 1801) that have been characterized as gonochoric in wild populations, but in culture conditions 10 to 15 % of individuals are hermaphrodites suggesting that captivity conditions (high water temperature or chemicals introduced to rearing tanks) have an effect in sexual behavior (Hong *et al*., 2006).

Some grouper species like *Epinephelus marginatus, E. coioides* and *E. merra* have hormonally induced to change sex with methiltestosterone (MT), a mixture of hormones or aromatase inhibitors (Alam *et al*., 2006; Bhandari *et al.,* 2004a; Bhandari *et al.,* 2004b; Bhandari *et al*., 2005; Glamuzina *et al*., 1998; Yeh *et al*., 2003) to obtain a higher sperm production. Induction of adult sex reversal of *E. merra* with aromatase inhibitor produced males in a period of two and a half months with larger testes than in wild males, a great sperm production and high egg fertilization rate (Bhandari *et al*., 2004b; 2005) but the induction to sex reversal to juveniles of the same specie, produced males with small volume of sperm suggesting that sex reversal success could depend on the size and age of individuals (Candi *et al*., 2004; Glamuzina *et al*., 1998; Robertson & Justines, 1982).

In this study, the same individuals identified by their tags, were repeatedly sampled and maintained in captivity as suggested by Bhandari *et al*. (2003), therefore we can assure that results presented here belong to the same individual. These findings must be considered in stock configuration of broodstock for aquaculture production. Further studies on reproductive behavior of females and males in captivity along with sex reversal induction trials are needed to corroborate leopard groupers reproductive behavior in captivity.

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