Research Article

Reproductive cycle of leopard grouper *Mycteroperca rosacea* (Streets, 1877) held in captivity: relationship between gonad development and sex steroid concentration

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ABSTRACT. In this study, sex steroids (E2, T, and 11-KT) concentrations in blood of captive leopard grouper (*Mycteroperca rosacea*) and their relation to gonad development were determined during a reproductive cycle. Each month, gonad samples from 24 fish were obtained to analyze gonadal maturation and blood was extracted to determine steroid levels. Throughout the year, ovaries showed a higher percentage of primary oocytes. In January, oocytes at early and late vitellogenesis stages increased progressively; from March to June, ovulation rates were high. In females, estradiol levels increased from March and decreased after the spawning season. Lower levels of testosterone were found during the spawning season and 11-KT remained without significant changes throughout the year. Males exhibit a significant increase in E2 from March until July; testosterone remained without significant variation throughout the year, and 11-KT plasma levels increased from March until July and decreased after the spawning season. In this study, we verified the sex change of an organism and the concentrations of sexual steroids were determined and discussed.

Keywords: *Mycteroperca rosacea*, sex steroids, leopard grouper, sex change, hermaphroditism.

INTRODUCTION

Leopard grouper, *Mycteroperca rosacea* (Streets, 1877) is distributed near the coasts of the eastern central Pacific (southwest coast of Baja California Peninsula and throughout the Gulf of California to Jalisco, Mexico) (Heemstra & Randall, 1993; Allen & Robertson, 1998). This species inhabits areas where the water temperature is between 20 and 30°C and salinity are between 34 and 35, in depths less than 50 m (Heemstra & Randall, 1993; Gracia-López *et al.*, 2004a). It can be easily adapted to captivity and are resistant to diseases and handling. Leopard grouper has a high market value and high demand for local consumption (Gracia-López *et al.*, 2004b). It has recently been described as gonochoristic in the wild and that some juveniles pass through a juvenile bisexual immature phase of gonad development (Erisman *et al.*, 2008) but sexual transition was found in the wild on 2011 (Estrada-Godínez *et al.*, 2011) as well as in capti-

vity (Kiewek *et al.*, 2010). Over-exploitation has led to be listed as a vulnerable species in the "IUCN Red List of Threatened Species" (VU A1d+2d), with a high risk of extinction in the wild, in the near future (IUCN, 2006). A considerable effort has been made in research on reproduction as well as on larvae and juveniles. All of this has generated a lot of basic knowledge of the species as well as allowing for culture improvements that have led to increased fecundity, fertilization rates, larval and juvenile survival, among others. Wild mature individuals were induced to spawn with HCG producing >40,000 eggs/female (Gracia-López *et al.*, 2004a) and hormonal induction on captive fish with HCG and LHRHa was successful producing over 2×10^6 eggs and fertilization rate of 62% (Kiewek-Martínez, 2004; Kiewek *et al.*, 2010). Embryonic development and egg and larval morphology and larval enzyme activity until the first feeding were described (Gracia-López *et al.*, 2004a; Martínez-Lagos & Gracia-López, 2009; Martínez-Lagos *et al.*, 2014) and the effects of

temperature and salinity on eggs and larvae also were studied (Gracia-López *et al*., 2004b). More than 1.7×10^6 eggs and 1.2×10^6 newly hatched larvae were achieved with a survival rate of 2.66% (Gracia-López *et al*., 2005).

Steroid hormones play a key role in gametogenesis, regulation of reproductive behavior and development of secondary sexual characteristics (Aida, 1988; Nagahama, 1994; Schulz & Miura, 2002; Yaron *et al*., 2003). Estradiol (E2) is responsible for female sex differentiation (Strüssmann & Nakamura, 2002; Bhandari *et al*., 2004a). It is produced by the ovary and transported to the liver where it stimulates hepatic secretion of vitellogenin (Nagahama, 1994). It is related to reproductive behavior (Kroon & Liley, 2000) and the decrease of blood levels stops vitellogenesis and produces oocyte degeneration (Bhandari *et al*., 2004a; Sarter *et al*., 2006). Androgens (T and 11-KT) also play a key role in fish sex differentiation (Baroiller *et al*., 1999; Rohr *et al*., 2001; Devlin & Nagahama, 2002). Testosterone is produced by the theca cells in the gonad (Kagawa *et al*., 1982) and is the precursor of 11-KT and E2 (Kroon & Liley, 2000; Devlin & Nagahama, 2002; Barannikova *et al*., 2004). Another important hormone, 11-Ketotestosterone (11 KT) is usually found in higher concentrations in males, although it has been quantified in females (Lone *et al*., 2001; Alam *et al*., 2005). It is synthesized in the testis of all teleost fish and plasma levels correlate with the gonadosomatic index (IG) and spermiation (Cuisset *et al*., 1994; Nagahama 1994; Lone *et al*., 2001; Rodríguez *et al*., 2001; Rohr *et al*., 2001; Alam *et al*., 2005). In females, this androgen is believed to be involved in the morphologic changes during sexual maturation (Rohr *et al*., 2001; Barannikova *et al*., 2004). There is great interest in the aquaculture industry to produce grouper species (Marino *et al*., 2003; Gracia-López *et al*., 2004b; Sarter *et al*., 2006). However, there are still some major constraints for raising grouper, including egg production, egg fertilization, and larval survival rates, as in the Hong Kong grouper, *Epinephelus akaara* (Okumura *et al*., 2002). The purpose of this study is to perform an accurate description of captive leopard grouper reproduction including the analysis of the key sexual steroids (E2, T, and 11-KT) and gonad development.

MATERIALS AND METHODS

Leopard grouper individuals (n = 24), were captured by hook and line in May 2004 (Gracia-López *et al*., 2004a). After capture, fish were transported to the laboratory, weighed and tagged (Floy Tag, Seattle, WA, USA) for further identification. Initial weights were between 0.4 to 1 kg for females and between 0.6 to 3.4 kg for males. Final weights were between 0.8 to 2.2 kg for females and between 0.82 to 4.4 kg for males. Fish age was determined by back calculation (Diaz-Uribe *et al*., 2001) being the age between 1 and 3 years. Twelve fishes were placed in each of the two circular 16 m^3 tanks, provided with a recirculation system (Kiewek-Martínez, 2004). They were maintained under natural photoperiod (max = 10.8 L: 13.2 D and min = 13.6 L: 10.4 D), and water temperature from 18.4 to 26.1°C, with the exception of summer 2005 when the water was cooled whenever it exceeded 26°C to avoid thermal stress (Fig. 1d). Fish were fed frozen sardines and squid *ad libitum* on alternate days.

The study started seven months after capture and lasts for a year. Fish samples were taken monthly, where fish were anaesthetized (50 mg L^{-1}, MS222 Tricaine-S, Western Chemical, WA) to obtain gonad samples by catheterization (inner diameter: 0.8 mm) of the oviduct (Gracia-López *et al*., 2004b) and fixed in Davidson's solution (glycerin, formaldehyde 37-40%, ethyl alcohol 96%, filtered seawater, and acetic acid). Blood (3 mL) was taken from the caudal vein and plasma separated by centrifugation at 2,500 rpm for 20 min at 4°C. Samples were stored at -80°C and analyzed to obtain sex steroid concentrations.

Males were identified either by examination of the samples from gonad catheterization when possible or by observation of fluid sperm obtained by gentle abdominal stripping. Weight (kg) and length (cm) were recorded. Gonad samples were embedded in paraffin, sliced (<5 µm) and stained with Harris Hematoxylin-Eosin. Each slide (n = 232) was analyzed under a compound microscope (Caliolimpus Bx-41) at 10x magnification and photographed (Image Pro-Plus). The relative abundance of each cell type was determined by counting the total number of cell types included in 140-280 oocytes per female.

Ovarian development was classified into five categories described for teleost fish (Carrillo *et al*., 1989). These were: I Primary oocytes; II Early vitellogenic; III Late vitellogenic; IV Ovulated and V Atretic. All oocytes in the photographs were counted and assigned a development category. The percentage of oocytes in each category of development was estimated for each female to obtain the frequency histogram of gonad development. Fish in sexual transition or bisexual (stage VI) were identified according to the description made for *Epinephelus fario* and males (Kuo *et al*., 1998). Estradiol and T plasma concentrations were determined by specific immu-noassay (EIA) (Rodríguez *et al*., 2001) and 11-KT plasma levels were analyzed using EIA developed for the Siberian sturgeon (Cuisset *et al*., 1994), except that

primary antibodies were used at a final dilution of 1:320,000 and the tracer (Cayman Chemicals, MI, USA) was diluted at 1:10 Ellman Units (EU mL^{-1}).

Specifications for sensibility and specificity of the assay were taken from Rodríguez *et al*. (2001). Sex steroid levels (E2, T, and 11-KT) were quantified for every fish. Sex steroid concentrations are presented as mean ± SE. To determine statistical differences between steroid concentrations, one-way ANOVA was performed, followed by Duncan's multiple-range test and critical ranges ($P < 0.05$). Development categories in the ovary were expressed as percentages. Results were analyzed using Statistica software v. 5.5 (StatSoft, Tulsa, OK, USA) and illustrated with a graphics program (Sigma Plot v. 8.0, SPSS, Chicago, IL, USA).

RESULTS

Females

Sixteen females were studied. Oocytes in primary growth stage were the most abundant throughout the study (Fig. 1a). In January, a small proportion of vitellogenic oocytes (>1.4%) were observed and in the following months, an increase of the percentages of oocytes in advanced stages of maturation was observed. In March, ovulated eggs were observed coinciding with the increase of water temperature and daylight (Figs. 1a, 1d). In the following months, the proportion of ovulated eggs increased attaining the most abundant in June (6%). From March to June the spawning season occurred; in July, the post-spawning season started, which was characterized by the increase of the percentage of oocytes in the primary stage (>95%). Throughout the year atretic oocytes were observed (<1%) (Fig. 1a).

Lowest concentrations of E2 were observed from November to March (0.32-0.47 ng mL^{-1}). A significant increase was observed in April, the highest concentration of the reproductive cycle (1.08 ± 0.19 ng mL^{-1}) coinciding with gonad recrudescence. Estradiol concentrations remained high in May and June, and also during the post-spawning season. Testosterone levels increased through the year from January and significant differences are shown in Figure 2c. Levels of 11-KT were low throughout the study (<1.1 ng mL^{-1}).

Males

Results were based on the study of seven males. Estradiol concentrations were achieved and significant differences between months were found. Estradiol increased from the start of the spawning season and the highest concentration (<0.9 ng mL^{-1}) was observed in June related to the highest number of spermiating males

(Fig. 2a). After June, estradiol concentration decreased and significant differences were found between June and August and the following months. Results of testosterone did not show significant differences throughout the year (<2 ng mL^{-1}) (Fig. 2b). A steady increase of plasma 11-KT was observed from January to July, the highest concentration observed. This progressive increase of 11-KT coincided with the highest rates of spermiating males (Fig. 2c).

One individual (0.66 kg) was found in the bisexual stage of development. Results showed us that primary oocytes were the most abundant among the different categories of oocyte development throughout the year. In April, the frequencies of oocyte stages were 90.5% primary oocytes; 2.6% early vitellogenic, 4.2% late vitellogenic and 2.6% atretic oocytes. In the following reproductive period (April 2006), fluid sperm was found. Estradiol was from 0.48 to 1.47 ng mL^{-1} until July and low values (<0.06 ng mL^{-1}) were detected from August to November. Testosterone was from 1.21 to 1.74 ng mL^{-1} from January through June. A decrease in concentrations occurred until August (0.60 ng mL^{-1}) and increased from 1.32 to 2.01 ng mL^{-1} from September through November. Plasma levels of 11-KT remained low until May (0.12-0.37 ng mL^{-1}). In June, the highest concentration was observed (4.12 ng mL^{-1}) and the second increase in concentration was found in November (3.76 ng mL^{-1}).

DISCUSSION

Results of this study showed the relationship between sex steroids and gonad development of the leopard grouper *M. rosacea* held in captivity during a reproductive cycle. In this study, broodstock was not sacrificed and individuals were sequentially monitored. This work differs from other sex steroids studies in which fish were sacrificed to obtain average values of a group. As done for red grouper, *Epinephelus morio* (Valenciennes, 1828) (Johnson *et al*., 1998); honey-comb grouper *Epinephelus merra* (Lee *et al*., 2002; Bhandari *et al*., 2004a, 2004b, 2005; Alam *et al*., 2005); orange-spotted grouper *Epinephelus coioides* (Yeh *et al*., 2003); and blackeye goby *Rhinogobiops nicholsii* (Kroon & Liley, 2000), among others.

Additionally, this is the first information regarding sex steroid levels and gonad development in a bisexual immature captive fish. The histological analysis provided an accurate assessment of the sex of each fish and also confirmed that leopard grouper ovary is group-synchronous that contains batches of oocytes in different stages of development. This type of ovary is common in serranid fish, such as the red grouper *E. morio* (Johnson *et al*., 1998) and present in fish belongs
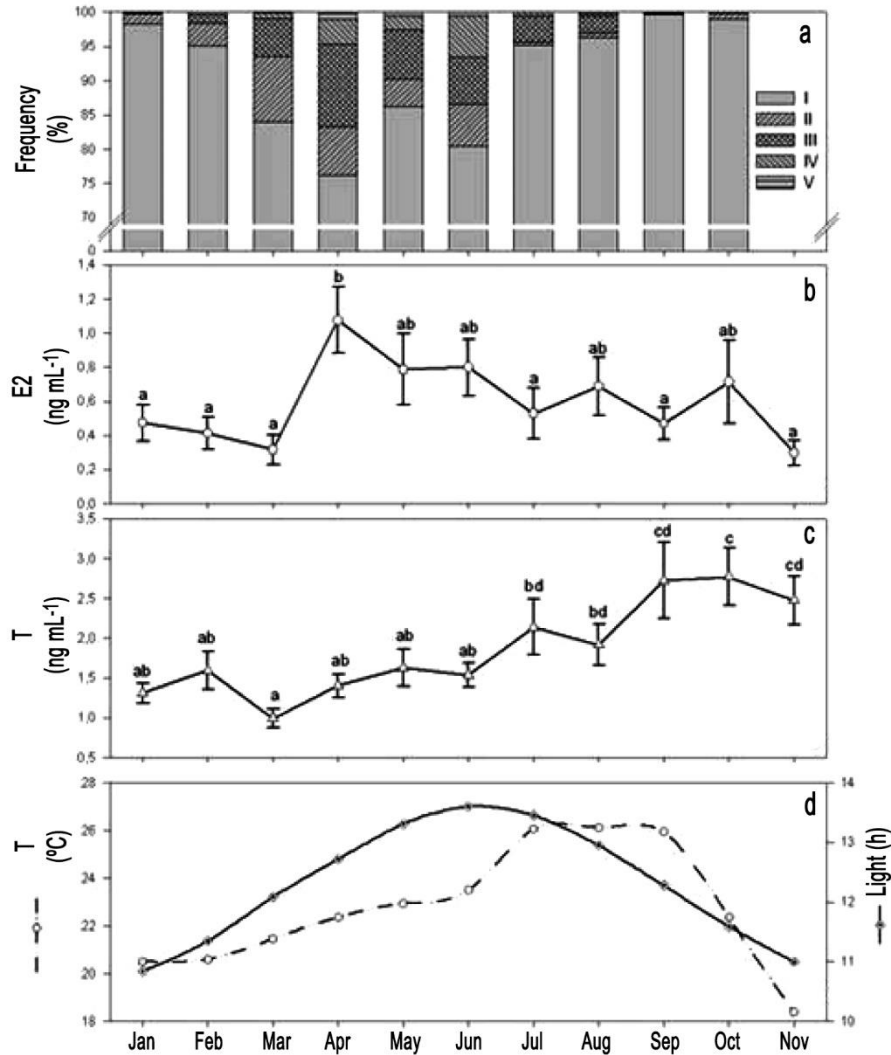
Figure 1. a) Percentages of the different maturity stages of oocytes. b) Monthly concentrations of Estradiol (E2). c) Concentration of plasma levels of testosterone (T). d) Temperature and photoperiod during the experimental period. Monthly values of sex steroids are depicted as mean ± SE (n = 16). Different letters represent significant differences ($P < 0.05$) between months.

to other families as the European sea bass *Dicentrarchus labrax* (Asturiano *et al*., 2002). In species with group-synchronous ovaries, there are differences in the sex steroid dynamics; decrease of T in the pre-ovulatory period has been reported; in other species, and there was a decrease in plasma E2 in pre-spawning period; and in others, E2 and T concentration remained high for most of the pre-ovulatory period (Peter & Yu, 1997).

In this study, levels of E2 in females increased as the spawning season approached and the highest concentration related with the high proportion of oocytes in late vitellogenic stage in April suggesting the role of this estrogen in the release of hepatic vitellogenin (Patiño & Sullivan, 2002) and the later

incorporation into the oocytes (Jalabert, 2005). This has been previously described for *E. merra,* where E2 is responsible for the maintenance of the female stage and the ovarian development (Bhandari *et al*., 2005). External factors, particularly daylight and temperature, are activators of the brain-pituitary-gonad axis involved in the regulation of reproduction in fish (Rodriguez *et al*., 2001). E2 changes were related to increasing daylight and temperature. The relationship between the increase in E2 levels, when daylight and temperature increase, and the advanced stages of gonad development have been shown in studies on other grouper species, such as *E. morio* (Johnson *et al*., 1998), *E. merra* (Lee *et al*., 2002; Bhandari *et al*., 2004b, 2005, 2006), dusky grouper, *Epinephelus*
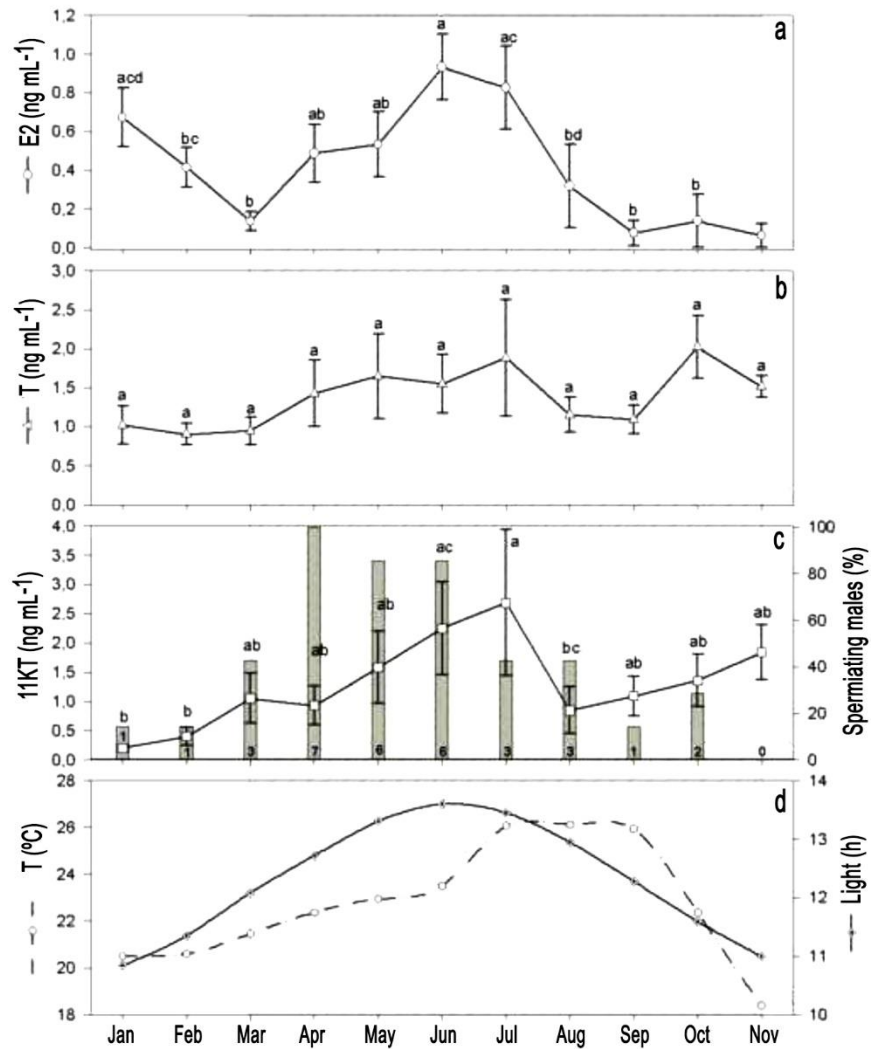
Figure 2. a) Results of monthly plasma levels of estradiol in males. In Figures, 2b and 2c the results of testosterone and 11-ketotestosterone (11-KT) concentrations are shown along with the percentage of spermiating males. Temperature and photoperiod are shown in Figure 2d. Monthly values of sex steroids are depicted as mean ± SE (n = 7). Different letters represent significant differences ($P < 0.05$) between months.

marginatus (Marino *et al.*, 2003), and other fish, like *Eleotris acanthopoma* (Wang *et al.*, 2001). In the present study, females had higher T concentrations than males. This steroid increased constantly during the reproductive period indicating the role of T as a precursor of E2, which presents variations throughout the vitellogenic period in *M. rosacea*.

During post-spawning period, gonads are in regression and T is not converted to E2 or 11-KT leading to T elevation in the blood (Schulz & Muira, 2002; Devlin & Nagahama, 2002; Yaron & Sivan, 2005). Similar results have been obtained for other fish, such as sea bass (Prat *et al.*, 1990) and sleeper *E. acanthopoma* (Wang *et al.*, 2001); on which high correlation between T plasma levels with advanced

gonad development has been described with further increase of T during the post-spawning period. Sex steroid 11-KT is usually higher in males than females (Devlin & Nagahama, 2002) because androgens and FSH are involved in regulating spermatogenesis (Schulz & Muira, 2002). Females studied had low levels of 11-KT, as reported for other fish species, such as *E. morio* (Johnson *et al.*, 1998), *R. nicholsii* (Kroon & Liley, 2000) and sobaty, *Sparidentex hasta* (Lone *et al.*, 2001), among others. Androgen production in males is maintained high during maturation (Devlin & Nagahama, 2002; Barannikova *et al.*, 2004). There were two E2 elevations, the first operated early at pre-gametogenesis and could be attributed to the cellular proliferation of germinal cells preparing the onset of

meiotic divisions which was likely E2 dependent as has been described for another teleost (Miura & Miura, 2003). The second surge of E2 occurs in parallel with 11-KT, the major steroid for gametogenesis in male fish (Borg, 1994). Changes of 11-KT during the active gametogenesis period (March-July) fit well with the claimed role of this hormone in males because there is a clear correlation between levels of 11-KT in plasma and the rates of spermiating males. In other grouper species like red grouper, *E. merra,* E2 is usually found at low concentrations (<1 ng mL^{-1}) during spermato-genesis (Johnson *et al*., 1998).

The analysis of the steroid profiles of the bisexual immature fish seems to be similar to the steroid (E2, T, and 11-KT) patterns of males. A bimodal pattern of E2 with no changes of T and a significant elevation of 11-KT during reproductive season seem to be a common pattern for leopard grouper sex change in captivity. In studies with *E. morio*, a drop in E2 is also observed and is attributed to degeneration of ovarian tissue (Johnson *et al*., 1998). Results for *E. merra* (Bhandari *et al*., 2004a, 2004b, 2005), *R. nicholsii* (Kroon & Liley, 2000) and the threespot wrasse, *Halichoeres trimaculatus* (Higa *et al*., 2003) indicate that the sex differentiation towards male phase is achieved when there is a decrease in estrogens (E2) and an increase in androgens.

Observations of several fishes showed a dramatic increase in the plasma levels of 11-KT (reviewed by Devlin & Nagahama, 2002), as in the wild grouper *E. merra* which suggests that this steroid plays an important role in sex differentiation (Bhandari *et al*., 2004a, 2004b). But in other species, such as tilapia, sex differentiation towards the male is triggered by the absence of E2 rather than the presence of androgens (Yaron & Sivian, 2005).

In the present study, an individual in sexual transition, specifically from female to male: *i.e.*, a protogynous hermaphrodite organism was observed and in the next reproduction season, was a functional male. Kiewek-Martínez *et al*. (2010) also reported the sexual transition for the same species kept also in captivity.

Erisman *et al.* (2008) did not obtain evidence of sex change in any individual collected throughout their study and, consequently, the leopard grouper was classified as a gonochorist species, but Estrada *et al.* (2011) found one individual at sex change. In these two last studies, 680 wild individuals were analyzed and only one protogynous hermaphrodite fish was observed. The results of the last studies on this species revealed that leopard grouper could exhibit sex change, with a low percentage of incidences.

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