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Induction of Female-to–male Sex Change in the Honeycomb Grouper (*Epinephelus merra*) by 11-ketotestosterone Treatments

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The honeycomb grouper, *Epinephelus merra*, is a protogynous hermaphrodite fish. Sex steroid hormones play key roles in sex change of this species. A significant drop in endogenous estradiol-17β (E2) levels alone triggers female-to–male sex change, and the subsequent elevation of 11-ketotestosterone (11KT) levels correlates with the progression of spermatogenesis. To elucidate the role of an androgen in sex change, we attempted to induce female-to–male sex change by exogenous 11KT treatments. The 75-day 11KT treatment caused 100% masculinization of pre-spawning females. Ovaries of the control (vehicle-treated) fish had oocytes at various stages of oogenesis, while the gonads of the 11KT-treated fish had transformed into testes; these contained spermatogenic germ cells at various stages, including an accumulation of spermatozoa in the sperm duct. In the sex-changed fish, plasma levels of E2 were significantly low, while both testosterone (T) and 11KT were significantly increased. Our results suggest that 11KT plays an important role in sex change in the honeycomb grouper. Whether the mechanism of 11KT-induced female-to–male sex change acts through direct stimulation of spermatogenesis in the ovary or via the inhibition of estrogen synthesis remains to be clarified.

Key words: honeycomb grouper, *Epinephelus merra*, protogynous hermaphrodite fish, sex change, 11-ketotestostrone

INTRODUCTION

Sex change is widespread in nature, occurring in plants, invertebrates, and teleost fishes (Policansky, 1982; Warner, 1984; Grafe and Linsenmair, 1989). There is, however, no evidence that any amniote vertebrate (mammals, birds, and reptiles) exhibits this reproductive pattern. Sex change is a purely ontogenetic event in some species, but in others, it can be triggered by environmental stimuli such as interactions with conspecifics. The size-advantage model for sequential hermaphroditism remains the most widely accepted evolutionary explanation of the adaptive significance of sex change (Ghiselin, 1969; Warner, 1988; Allsop and West, 2003). This model posits that if an individual can reproduce more effectively as one sex when small or young and as the other sex when larger or older, it should change sex at some point in its life history.

Groupers of the genus *Epinephelus*, one of the valuable food fish in the Southeast Asian region, are protogynous hermaphrodites (Smith, 1965; Tan and Tan, 1974; Brusle-Sicard *et al.,* 1992). Their sex change strategy follows the size-advantage model; they can change sex from female-to-male after exceeding a certain age and body size. Most groupers of commercial importance are relatively large and difficult to handle in the laboratory. The honeycomb grouper (*Epinephelus merra*), which inhabits coral reefs in the tropical regions of Japan and the Pacific, is a good model for grouper research because of its relatively small body size, availability, and similarity of reproductive characteristics with other groupers, including the sex-change strategy (Kobayashi *et al*., 2000; Bhandari *et al*., 2003). Moreover, the events that occur during natural as well as artificial sex change in this species have been intensively studied.

Female-to-male sex change in the honeycomb grouper is associated with a drop in estradiol-17β (E2) levels followed by an increase in androgen levels (Bhandari *et al.,* 2003). Aromatase inhibitor (AI) treatments block E2 production and induce sex change in both sexually immature and mature individuals, whereas E2 supplementation inhibits AI-induced sex change (Bhandari *et al.,* 2003, 2004a, b, 2005), indicating that estrogen is critical for maintaining the female

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sex. We have recently found that androgen-producing sites exist in the ovary of honeycomb grouper (Alam *et al.,* 2005). In addition, naturally occurring sex change is accompanied by significant increases in the size of androgen-producing cells and production of androgen by the gonads (Alam *et al.,* 2006). This suggests that androgens play an important role in sex change. However, whether or not exogenous androgen treatments mimic natural sex change in this species has not been examined.

11-ketotestosterone (11KT) is considered a principal androgen in teleost fishes (Borg, 1994), with females containing detectable levels of 11KT in their blood plasma (Bhandari *et al*., 2003). In many sex-changing fish, the levels of circulating 11KT have been reported to increase with the progression of sex change (Nakamura *et al*., 1989; Johnson *et al*., 1998; Kroon and Liley, 2000; Bhandari *et al*., 2003; Perry and Grober, 2003), and exogenous 11KT treatments have been shown to induce female-to-male sex change (Kroon and Liley, 2000; Yeh *et al*., 2003). Our previous studies suggest that a drop in E2 levels alone is the key to the onset of sex change in honeycomb grouper, and that increasing testosterone (T) and 11KT levels following gonadal transformation could be stimulatory to male germ-cell proliferation and differentiation in the ovary. In the present study, we examined whether or not exogenous 11KT treatment mimics the natural sex change in the honeycomb grouper.

MATERIALS AND METHODS

Animals

Our previous study showed that honeycomb groupers with a total length less than 20 cm are females (Bhandari *et al*., 2003). For the present study, wild honeycomb groupers with total lengths less than 20 cm were purchased from fishermen at Nakijin village, Northern Okinawa, Japan. All of the fish (n=40) were acclimatized in 1000-liter polyethylene tanks for 3 days with flow-through sea water, natural photoperiod, and aeration. Chopped squid meat was used for feeding the fish twice daily throughout the experiment. The experiment was conducted during the pre-spawning season of the honeycomb grouper, from 25 March to 8 May, 2003.

Hormone preparation and treatments

11-ketotestosterone (Wako, Japan) was mixed with melted cocoa butter (40°C), dissolved by vortexing, and kept at the same temperature until implantation. Initial blood samples were taken from 10 untreated fish with a 1-ml heparinized syringe (Terumo, Japan) and then kept on ice. Blood samples were centrifuged at 6000 rpm for 8 minutes, and plasma was collected from each sample separately and stored at –30°C until further analysis. The gonads were then carefully removed and fixed in Bouin's solution and 18 hours later preserved in 70% ethanol until further analysis.

The remaining 30 fish were divided into two groups: control and 11KT-treated. Following anaesthetization with 0.5% phenoxyethanol, 15 fish were implanted with 11KT (10 mg/1-kg body weight of fish) into the body cavity, while the remaining 15 were implanted with cocoa butter alone. Each group was reared in floating cages (1m×1m×1m) placed in 10-ton indoor cement tanks with flow-through seawater and aeration. Out of 30 fish, five died from the control and three from the 11KT-treated groups.

Sample collection

On the 75th day of implantation, 10 fish from the 11KT-treated group were sacrificed, and blood and gonad samples were collected using the same procedures as described above. Fixed gonads were dehydrated by graded ethanol series and embedded in paraffin. Seven-micrometer sections were prepared and standard histological techniques were used to stain gonadal sections with hematoxylin and eosin. Sex hormone levels in the plasma were determined by ELISA by the method of Rahman *et al.* (2000), with inter- and intra-assay variation below 12%.

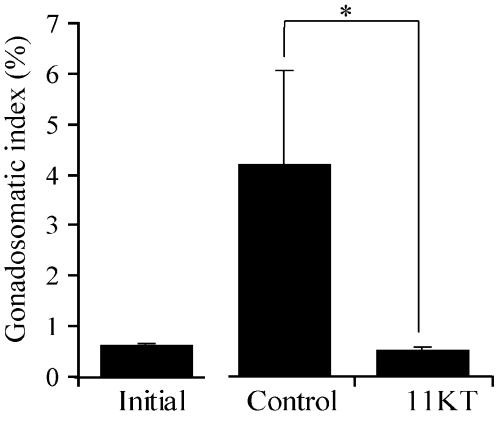

Fig. 1. Gonadosomatic indices (GSI) of fish before and after 11KT treatment. 11KT-treated fish had significantly low GSI (*, P<0.05). Data given as mean±SEM.

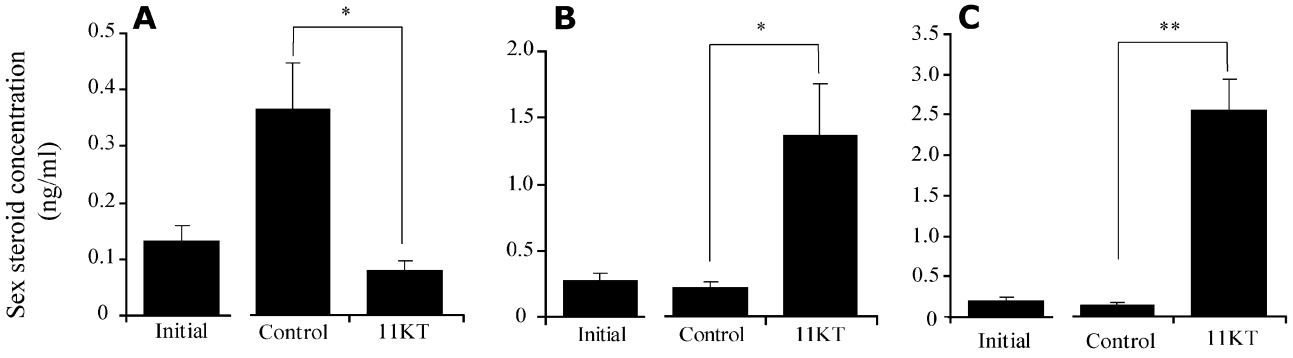

Fig. 2. Plasma levels of sex steroid hormones in the honeycomb grouper following 11-ketotestosterone (11KT) treatment. A, estradiol-17β (E2); B, testosterone (T); and C, 11KT. 11KT treatment resulted in significant a decrease in plasma E2, and increases in T and 11KT levels. Data given as mean±SEM. (*, P<0.05; **, P<0.001)

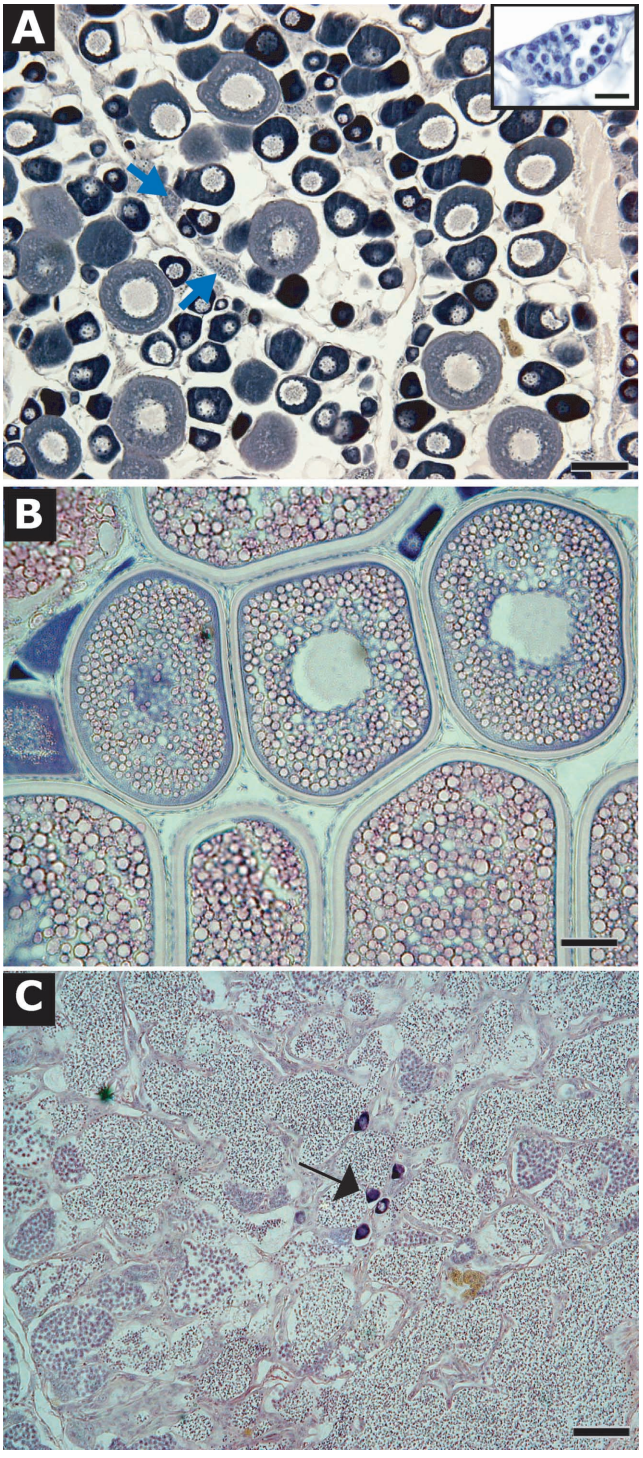

Fig. 3. Photographs of gonadal sections prior to or following 11-ketotestosterone (11KT) treatment of female honeycomb groupers. (A) Before treatment. Ovaries of initial control fish contained pre-vitellogenic oocytes and some spermatogenic crypts in the ovigerous lamellae (arrow) (also see inset picture; scale bar=25 μm). (B) Vehicle-treated gonad. (C) 11KT-treated gonad. 11KT treatment caused sex reversal of sexually mature ovaries. Approximately 80% of the treated fish had gonads completely transferred into testes, and 20% had a low number of residual oocytes undergoing atresia (arrow). Sperm was exuded from all testes during sampling. Scale bar=100 μm.

Fertility Check

Since honeycomb groupers are lunar spawners (Lee *et al*., 2002; Soyano *et al*., unpublished data) and prefer a very calm and quiet environment for mating, the sex-changed individuals were allowed to mate at midnight around the time of a full moon. Two 11KT-treated fish were kept with two untreated females in a separate 500-liter polyethylene tank to ensure functional sex change. Fertilized eggs were collected by placing a fine net at the end of the outlet pipe, and the total numbers of eyed eggs were counted under a light microscope.

Statistical analysis

The effects of 11KT treatment were analyzed by subjecting 11KT-treatment data to independent-sample t-test against control data. Data are given as mean±SEM.

RESULTS

Morphological changes, survival and gonadosomatic indices (GSI)

Control fish exhibited bulged bellies due to the accumulation of eggs in the ovaries. 11KT-treated fish had reduced bellies that released sperm upon gentle pressure on the abdomen. The GSI (100 x gonad weight/body weight) of 11KT-treated fish was significantly lower ($P<0.05$) than that of the control fish (Fig. 1).

Plasma sex hormones

Plasma levels of sex hormones in all the samples were determined by ELISA. Plasma E2 levels were significantly lower in 11KT-treated fish (Fig. 2A), whereas both T and 11KT levels were significantly higher (Fig. 2B & 2C).

Changes in the gonadal structures

Before treatment, ovaries contained oocytes at the primary, perinucleolus, and pre-vitellogenic stages. Small spermatogenic crypts were seen in the peripheral part of the ovigerous lamella of all ovaries (Fig. 3A, arrow and inset at higher magnification). During routine check-up or terminal sampling, handling caused control females to ovulate. On day 75, five of the 10 control fish had already ovulated. Histologically, these ovaries contained oocytes with post-ovulation follicles. The remaining five females contained oocytes in the vitellogenic stage (Fig. 3B). Conversely, the gonads of 11KT-treated fish had completely transformed into testes. Spermatozoa were present in addition to germ cells in advanced stages of spermatogenesis (Fig. 3C). Of the 12 surviving fish, three contained testes with traces of atretic oocytes.

Fertility of sex-changed males

We were able to collect eggs only on the fourth day of mating. Light microscopy of the eggs collected revealed 55% fertility (total number of fertilized eggs x 100/total number of eggs examined), and the eggs contained embryos at various embryonic stages.

DISCUSSION

Our previous studies focused on estrogen regulation of female-to-male sex change in the honeycomb grouper. In all cases, the E2 concentration first decreased at the onset of sex change. Subsequently, androgen (T and 11KT) levels

increased and accompanied the gonadal transformation into testes. Following mass immuno-histochemical studies of the sex-changing gonads, a new site of androgen production in the ovary was also discovered, which could contribute to the natural sex change in this species (Alam *et al*., 2005). In the present study, we attempted to induce sex change in sexually mature females with exogenous 11KT treatments to clarify whether or not androgens play any role in sex change in this species. The 75-day 11KT treatments caused complete transformation of ovaries into functional testes. The sex-changed fish were able to mate as males and to fertilize the eggs of wild-type females, indicating a functional sex change. This 11KT-induced sex change appears to mimic both natural and AI-induced female-to-male sex change in the honeycomb grouper, *Epinephelus merra*.

During natural sex change, circulating E2 levels significantly decline, resulting in rapid degeneration of primary/perinucleolar-stage oocytes (Bhandari *et al*., 2003). A simple suppression of endogenous E2 synthesis via AI treatments causes dramatic degeneration of oocytes, with spermatogonial proliferation starting from the periphery of the ovigerous lamellae. In the saddleback wrasse, *Thalassoma duperrey*, a significant drop in E2 levels was found to be an initial trigger for sex-change initiation (Nakamura *et al.,* 1989). In the three-spot wrasse, *Halichoeres trimaculatus*, a simple *in vitro* incubation of ovarian fragments in L-15 medium resulted in oocyte degeneration, followed by proliferation of presumed spermatogonia on the periphery of the lamella (Higa *et al*., unpublished data). In both the grouper and the wrasse, this rapid transformation started and proceeded without involvement of any exogenous androgens, indicating that suppression of the female pathway may allow the male pathway to activate in the same gonad.

A treatment with 11KT suppressed endogenous E2 levels and simultaneously stimulated T and 11KT levels. It is unknown whether 11KT-induced sex reversal is due to the direct stimulatory effects of 11KT on male germ cell proliferation, or whether the effects are propagated via blockage of E2 synthesis and oocyte degeneration. For many fish, an endogenous estrogen-androgen balance is required for maintaining sexual phenotypes. During the female phase, endogenous E2 levels are high and decline precipitously with the onset of sex change. Endogenous androgens start to increase only after spermatogenesis begins, and they remain high throughout the male phase. Based upon these findings, we hypothesize that when endogenous E2 levels fall below a physiological threshold, oocytes no longer survive, and the spontaneous androgen levels, which normally do not show significant effects on the female phase, now can initiate spermatogonial proliferation in the ovary. The dramatic increase in T and 11KT levels could then maintain spermatogenesis in the gonad.

Most studies focusing on androgen-induced sex change in other protogynous hermaprodites suggest an important role of 11KT on both behavioral (Perry and Grober, 2003) and gonadal sex change (Kroon and Liley, 2000; Yeh *et al.,* 2003). In all cases, E2 levels were shown to decline during the onset of 11KT-induced sex change; however, the mechanism by which 11KT treatments suppress E2 production remains to be discovered. Molecular characterization of genes encoding steroidogenic enzymes, steroid receptors, and transcription factors that regulate steroidogenesis during sex change would help elucidate this mechanism.

Gonochoristic fish, which do not naturally undergo sex change, have been shown to exhibit a stable sex determination system (Yamamoto, 1969). Androgen treatments at the embryonic stage or prior to gonadal sex differentiation cause female-to-male sex change (Devlin and Nagahama, 2002). The mechanisms by which androgens masculinize ovaries are not clearly understood. However, all the studies on androgen-induced masculinization suggest that it acts via androgen-suppression of P450-aromatase gene/protein expression (Govoroun *et al*., 2001; Bhandari *et al*., 2006; Nakamura *et al*., 1998; Devlin and Nagahama, 2002). In the Nile tilapia, it was shown that transcripts of androgen receptor β are present in XX gonads 15 days after hatching. The 5'-flanking region of the ovarian form of P450-aromatase contains one copy of androgen responsive element (ARE). 11KT treatment reduces P450-aromatase promoter activity in cells co-transfected with androgen receptors α and β. *In vivo*, exogenous 11KT treatment suppresses the expression of P450-aromatase gene in steroid-producing cells of XX gonads (Ikeuchi *et al*., unpublished result; see review by Nagahama *et al*., 2004). Taken together, it is likely that androgen-induced masculinization involves suppression of steroidogenic enzyme expression, including P450-aromatase, and the that action of the exogenous androgen that masculinizes germ and somatic cells is probably mediated by androgen receptors. Moreover, doublesex- and mab-3-related transcription factor 1 (DMRT1) that shows distinct expression during testicular differentiation in the rainbow trout (Merchand *et al*., 2000), tilapia (Guan *et al*., 2000; Nagahama *et al*., 2004), medaka (Kobayashi *et al*., 2004), and black porgy (Wu *et al*., 2005) may play an important role in androgen-induced sex change in both gonochoristic and hermaphrodite fish.

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REFERENCES

Alam MA, Komuro H, Bhandari RK, Nakamura S, Soyano K, Nakamura M (2005) Immunohistochemical evidence identifying the site of androgen production in the ovary of the protogynous grouper *Epinephelus merra*. Cell Tissue Res 320: 323–329

Alam MA, Bhandari RK, Kobayashi Y, Nakamura S, Soyano K, Nakamura M (2006) Changes in androgen-producing cell size and circulating 11-ketotestosterone level during female-male sex change of honeycomb grouper *Epinephelus merra*. Mol Reprod Dev 73: 206–214

Allsop DJ, West SA (2003) Changing sex at the same relative body size. Nature 425: 783–784

Bhandari RK Komuro H, Nakamura S, Higa M, Nakamura M (2003) Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper,

Epinephelus merra. Zool Sci 20: 1399–1404
Bhandari RK, Higa M, Nakamura S, Nakamura M (2004a) Aromatase inhibitor induces complete sex change in a protogynous honeycomb grouper, *Epinephelus merra*. Mol Reprod Dev 67: 303–307
Bhandari RK, Komuro H, Higa M, Nakamura M (2004b) Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool Sci 21: 305–310
Bhandari RK, Alam MA, Higa M Soyano K, Nakamura M (2005) Evidence that estrogen regulates the sex change of honeycomb grouper (*Epinephelus merra*), a protogynous hermaphrodite fish. J Exp Zool 330A: 497–503
Bhandari RK, Nakamura M, Kobayashi T, Nagahama Y (2006) Suppression of steroidogenic enzyme expression during androgen induced sex reversal in Nile tilapia, *Oreochromis niloticus*. Gen Comp Endocrinol 145: 20–24
Borg B (1994) Androgens in teleost fishes. Comp Biochem Physiol C109: 219–245
Brusle-Sicard S, Debas L, Fourcault B, Fuchs J (1992) Ultrastructural study of sex inversion in a protogynous hermaphrodite, *Epinephelus microdon* (Teleostei, Serranidae). Reprod Nutr Dev 32: 393–406
Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364
Ghiselin MT (1969) The evolution of hermaphroditism among animals. Q Rev Biol 44: 189–208
Govoroun M, McMeel OM, D'Cotta H, Ricordel MJ, Smith TJ, Fostier A, Guiguen Y (2001) Steroid enzyme gene expressions during natural and androgen-induced gonadal differentiation in the rainbow trout *Oncorhynchus mykiss*. J Exp Zool 290: 558–566
Grafe TU, Linsenmair KE (1989) Protogynous sex change in the reed frog *Hyperolius viridiflavidus*. Copeia 1989: 1027–1029
Guan G, Kobayashi T, Nagahama Y (2000) Sexually dimorphic expression of DM (Doublesex/Mab-3)-domain genes during gonadal differentiation in tilapia. Biochem Biophys Res Comm 272: 662–666
Johnson AK, Thomas P, Wilson RR (1998) Seasonal cycles of gonadal development and plasma sex steroid levels in *Epinephelus morio*, a protogynous grouper in the eastern Gulf of Mexico. J Fish Biol 52: 502–518
Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Suzuki A, Saito N, Nakamoto M, Shibata N, Nagahama Y (2005) Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. Dev Dyn 231: 518–526
Kobayashi Y, Lee YD, Takemura A, Takano K, Soyano K (2000) Histological observation of sex change in honeycomb grouper, *Epinephelus merra*. In "Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish" Ed by B Noberg, OS Kjesbu, GL Taranger, E Andersson, SO Stefansson, Bergen, p 269
Kroon FJ, Liley NR (2000) The role of steroid hormones in protogynous sex change in the Blackeye goby, *Coryphopterus nicholsii* (Teleostei: Gobiidae). Gen Comp Endocrinol 118: 273–283
Lee YD, Park SH, Takemura A, Takano K (2002) Histological observations of seasonal reproductive and lunar-related spawning cycles in the female honeycomb grouper *Epinephelus merra* in Okinawa waters. Fish Sci 68: 872–877
Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre J, Bernot A, Laudet V, Guiguen Y (2000) DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biochim Biophys Acta 1493: 180–187
Nagahama Y, Nakamura M, Kitano T, Tokumoto T (2004) Sexual plasticity in fish: a possible target of endocrine disrupter action. Environ Sci 11: 73–82
Nakamura M, Hourigan TF, Yamauchi K, Nagahama Y, Grau EG (1989) Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. Env Biol Fish 24: 117–136
Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost fish. J Exp Zool 281: 362–372
Perry AN, Grober MS (2003) A model for social control of sex change: interactions of behavior, neuropeptides, glucocorticoids, and sex steroids. Horm Behav 43: 31–38
Policansky D (1982) Sex change in plants and animals. Annu Rev Ecol Syst 13: 471–495
Rahman MS, Takemura A, Takano K (2000) Correlation between plasma steroid hormones and vitellogenin profiles and lunar periodicity in the female golden rabbitfish, *Siganus guttatus* (Bloch). Comp Biochem Physiol 127B: 113–122
Smith CL (1965) The pattern of sexuality and the classification of serranid fishes. Am Mus Novit 2207: 1–20
Tan SM, Tan KS (1974) Biology of the tropical grouper. *Epinephelus tauvina* (Forskal) I. A preliminary study on hermaphroditism in *E. tauvina*. Sing J Pri Ind 2: 123–133
Warner RR (1984) Mating behavior and hermaphroditism in coral-reef fishes. Am Sci 72: 128–136
Warner RR (1988) Sex change and the size advantage model. Trends Ecol Evol 3: 133–136
Wu GC, DU JL, Lee YH, Lee MF, Chang CF (2005) Current Status of Genetic and Endocrine Factors in the Sex Change of Protandrous Black Porgy, *Acanthopagrus schlegeli* (Teleostean). Ann N Y Acad Sci 1040: 206–214
Yamamoto T (1969) Sex differentiation. In "Physiology of Fishes Vol 3" Ed by WS Hoar, DJ Randall, Academic Press, New York, pp 117–175
Yeh SL, Kuo CM, Ting YY, Chang, CF (2003) Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. Comp Biochem Physiol 218: 375–382

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