5 **Permanent sex inversion in 1-year-old juveniles of the protogynous dusky grouper**
(*Epinephelus marginatus*) using controlled-release 17 α -methyltestosterone implants

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Abstract

Long-term treatments (April - June 2004) with 17 α -methyltestosterone (MT) and/or human chorionic gonadotropin (hCG) were used to induce precocious sex change in 1-year-old juveniles (mean $\pm$ SD body weight of 131 $\pm$ 25 g) of the protogynous dusky grouper (*Epinephelus marginatus*). The MT treatment was given once a month using controlled-release implants (1.5 mg per implant, $\sim$ 11 mg Kg⁻¹ body weight), whereas hCG was injected intramuscularly once a month (MT/hCG group) or every 2 weeks (hCG group). *In vitro* mean ($\pm$ SEM) daily and total MT release from the implants was 22.6 $\pm$ 5.4 μ g day⁻¹ implant⁻¹ and 686 $\pm$ 113 μ g implant⁻¹, respectively. *In vivo*, plasma MT levels of European sea bass (*Dicentrarchus labrax*) used as model fish (203 $\pm$ 41 g body weight) and given a single MT implant were 18.5 $\pm$ 0.5 ng ml⁻¹ one day after implantation, declined to 9.6 $\pm$ 2.7 ng ml⁻¹ by day 11 and remained stable between 7.9 and 5.5 ng ml⁻¹ until day 32. Plasma MT levels were significantly elevated (2-way ANOVA, $P < 0.01$) in dusky grouper juveniles in response to the MT treatment, both 6 and 12 weeks after beginning the treatment. On the contrary, no significant differences (2-way ANOVA, $P > 0.05$) between MT/hCG-treated and control fish were observed in plasma testosterone (T) or 11 ketoT (11-KT). Pituitary luteinizing hormone (LH) content was significantly lower in both the MT/hCG and hCG groups, compared to controls (2-way ANOVA, $P < 0.001$), while plasma LH was below detection limits in all treatments. At the onset of the experiment all but one fish were already differentiated into females, having ovaries with primary oocytes. No signs of vitellogenesis or sex inversion were observed in fish from the control and hCG groups during the study. On the contrary, the MT implants induced complete sex change after 12 weeks, with the gonads organized in lobules and cysts filled with germ cells at all stages of spermatogenesis, including spermatozoa. This sex inversion was permanent and fish underwent complete spermatogenesis again during the following year. The

50 results demonstrate that the produced MT-implants, but not the hCG injections, were highly efficient in inducing both sex inversion and complete spermatogenesis in pre-pubertal dusky grouper at the completion of their second year in life. Production of male fish at such an early age may dramatically reduce by many years the time required for natural puberty, sex inversion and the production of sperm by dusky grouper reared under culture conditions.

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Keywords: *Epinephelus*, dusky grouper, sex inversion, steroid implants

1. Introduction

The dusky grouper (*Epinephelus marginatus*) is a popular species both for recreational and commercial fisheries, and is heavily exploited in most Mediterranean areas (Zabala et al., 1997). It is the only member of the Serranidae family considered an endangered species by Annex 3 of the Bern (1995) and Barcelona Conventions (1995). Similar to all groupers (sub-family Epinephelinae), the dusky grouper is a protogynous hermaphrodite with a complex and socially structured reproductive behaviour (Zabala et al., 1997; Marino et al., 2001). First sexual maturity is reached when females are 5 years old and 40 - 50 cm in total length (2 - 2.5 Kg body weight) (Chauvet, 1988; Marino et al., 2001; Andrade et al., 2003), while sex inversion occurs when individuals are 9 to 16 years old and 70 - 90 cm in total length (6 - 10 Kg) (Chauvet, 1988).

With interest in commercial aquaculture or stock enhancement programs, several attempts have been made in the past decade to facilitate reproduction of the dusky grouper under controlled conditions (Glamuzina et al., 1998b; Glamuzina et al., 2000; Spedicato and Boglione, 2000; Marino et al., 2003). There are two potential approaches for establishing captive broodstocks from wild fish: (a) collecting mature individuals from the wild or (b) rearing wild-caught juveniles to sexual maturity. These methods are time-consuming, economically prohibitive or unfeasible for dusky grouper, due to the late puberty of females, the extremely long time it takes to sex inversion, and the rarity of dusky grouper males in the wild. Therefore, induction of precocious puberty and sex inversion is of great importance.

The ability of sex steroids and gonadotropins to regulate sex differentiation and gametogenesis has been successfully exploited in fish farming for the manipulation of the onset of puberty (Ohta et al., 1996; Cavaco et al., 1998; Henry et al., 1998) or the

induction of sex change in both gonochoristic and hermaphroditic fishes (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001). Estrogens, mainly 17 β -estradiol (E₂), are used for the feminization of fish prior to sex differentiation and the induction of male-to-female inversion in protandrous hermaphrodites (Chang et al., 1995a; Omoto et al., 2002; Gorshkov et al., 2004). Masculinization of fish prior to sex differentiation and the female-to-male sex inversion in protogynous hermaphrodites can be achieved by treatments with androgens, such as testosterone (T), 11-keto T (11 KT) and the synthetic 17 α -methyltestosterone (MT) (Kitano et al., 2000; Kroon and Liley, 2000; Yeh et al., 2003a; Yeh et al., 2003c; Yeh et al., 2003b). Gonadotropins, such as luteinizing hormone (LH) and human chorionic gonadotropin (hCG) have also been used with varying success in sex inversion and maturation of various fishes (Koulisch and Kramer, 1989; Yeung et al., 1993; Ohta et al., 1996; Reinboth and Brusle-Sicard, 1997; Baroiller et al., 1999; Devlin and Nagahama, 2002).

Steroid-induced feminization and masculinization in gonochoristic fishes has been successful mainly when the treatment takes place during the critical period of sex differentiation. At this time, the gonads start developing and the primordial germ cells are still bipotential and capable of developing to either oogonia or spermatogonia, depending on the surrounding hormonal milieu (Hunter and Donaldson, 1983; Kitano et al., 2000; Omoto et al., 2002; Hendry et al., 2003; Park et al., 2004). On the other hand, successful induction of sex inversion in hermaphroditic fishes has been reported mostly in reproductively mature individuals (Kuo et al., 1988; Yeung et al., 1993; Lee et al., 1995; Yeh et al., 2003a). For example, in the protandrous black porgy (*Acanthopagrus schlegelii*) sex inversion was induced after treatment of spermiating males with estrogens (Chang et al., 1995b; Lee et al., 2001), whereas in the protogynous potato grouper (*E. tukula*) sex inversion was effected after treatment of vitellogenic females with androgens (Yeh et al., 2003a). In the dusky grouper, on the other hand, sex

inversion has been reported in juvenile fish ranging in body weight from 150 g to 2.9 Kg (Glamuzina et al., 1998a; Marino et al., 2000). However, the methods used so far have important drawbacks, including (a) use of relatively large amounts of synthetic steroid hormones, (b) incomplete sex inversion (25 – 38 % of treated individuals), (c) excessive handling of the fish for the administration of the hormone or (d) transient sex inversion, as fish change back to females during the next reproductive period.

The objective of the present study was to develop a novel controlled-release MT implant and examine its potential in inducing sex inversion in 1-year-old juvenile dusky grouper. The steroid treatment was given in combination with hCG, which has been shown in some fishes to be successful in inducing sex inversion (Reinboth and Brusle-Sicard, 1997; Koulisch et al., 2002) or spermatogenesis and spermiation (Ohta et al., 1996; Ohta and Tanaka, 1997).

2. Materials and Methods

2.1 Preparation of MT-loaded controlled-release implants

The MT implants were produced using Ethylene-Vinyl Acetate copolymer (EVAc) according to the method of Mylonas and Zohar (2001) with some modifications. Briefly, 18 ml of a 15% EVAc solution in MeCl_2 were mixed with 1.1 g inulin (Sigma, Germany) and 0.9 g of MT (Sigma, Germany) in powder form. The solution was ground with a tissue grinder (RZR 2020, Heidolph, Germany) until no large chunks of the steroid/inulin powder were visible, and then sonicated for 30 s at 30 W (UP200S, Dr. Hielscher GmbH, Germany). The MT/polymer/ MeCl_2 solution was poured on a levelled aluminium cast (50x50x4 mm) on dry ice. The solidified plate was moved to -20°C for 2 d in order to evaporate the MeCl_2 and then put in a vacuum dessicator at 25°C for 2 d to remove all moisture. Implants were punched from the dried EVAc/inulin/steroid plate with a 2 mm dermal punch (Keyes Punch, Miltex GmbH, Germany) and stored at -20°C until usage.

135 Each implant was designed to contain 1.5 mg of MT, in order to provide the fish with an effective dose of approximately 10 mg kg⁻¹ body weight (Lee et al., 1995; Yeh et al., 2003a; Yeh et al., 2003c; Yeh et al., 2003b) for a period of 30 d.

2.2 *In vitro* release from the MT-loaded implants

140 The *in vitro* release kinetics of the MT-loaded implants were examined using the procedure of Mylonas et al. (1995a) with some modifications. Briefly, the implants (n = 4) were embedded in 300 µl of a 2% solution of low melting agarose (Sigma, Germany) dissolved in *in vitro* buffer (1.68 g KH₂PO₄, 5.625 g NaHPO₄, 0.2 g sodium azide, 0.2 ml Tween 80 in 1 l of dd H₂O, adjusted to pH 7.0), which was placed at the
145 bottom of 7 ml flat bottom polyethylene vials kept on ice. Once the agarose layer solidified around the implants, another layer of 400 µl of melted agarose was placed on top, in order to cover the implants completely. After 15 min on ice, 5 ml of the *in vitro* buffer was added in the vials, which were then placed on a rocking shaker in a controlled-temperature incubator at 18°C. Beginning 1 day after the start of the assay
150 and at various times until day 32, a 500 µl sample of the *in vitro* buffer was taken from each replicate and stored at -80°C until analysis of the loaded steroid hormone. The *in vitro* buffer was replaced with fresh one after each sampling.

2.3 *In vivo* release from the MT-loaded implants

155 Due to the scarcity of dusky grouper juveniles, the *in vivo* release kinetics of the MT implants were examined using 1-year-old European sea bass (*Dicentrarchus labrax*) of mean ± SD body weight of 203 ± 41 g. Fish were maintained in a 2-m³ tank, supplied with water from a recirculation system, that kept water temperature at 18°C. Feeding, handling and sampling was as described above for dusky grouper. Each MT-
160 treated fish (n = 6) received one implant, which was inserted into the epaxial muscle

just beneath the dorsal fin, using a special syringe-type applicator, whereas the control fish ($n = 6$) were punched with the syringe applicator only. Beginning 1 day after MT implantation and at various times until day 32, blood from the caudal vasculature was collected in heparinized syringes and stored over crushed ice. Blood samples were
165 centrifuged for 10 min at 13000 g, the plasma was removed and stored at -80°C until analysis.

2.4 Induction of sex-reversal

The experiments were carried out between April and July 2004 in the facilities of
170 the Institute of Aquaculture, Hellenic Centre for Marine Research, Crete, Greece, using 1-year-old dusky grouper (mean $\pm$ SD body weight of 131 ± 25 g and total length of 18.6 ± 2.9 cm) produced in July 2002 from wild-caught broodstock after hormonal induction of spawning (Marino et al., 2003). Fish were maintained during the year in 2- m^3 round tanks supplied with surface sea water, exposed to ambient photo-thermal
175 conditions and fed *ad libitum* with commercial feed (TROUVIT, Hendrix, Italy) using a demand-feeder. To assure a sufficient nutrition, fish were additionally fed three times a week with a mixture of raw fish and larval feed (NRD 3/5, INVE Aquaculture, Belgium). Before any handling, fish were starved for 2 d to reduce handling stress and were anaesthetized in groups of 2-3 individuals in a bath with clove oil (Mylonas et al.,
180 2005) at 0.05 ml l^{-1} (dissolved first 1:10 v/v in 96% EtOH). All experimental animals were individually marked with a passive integrated transponder (PIT) tag (AVID, Uckfield, East Sussex, UK). The water temperature ranged from 16 to 23°C during the course of the experiment, water renewal was $100\% \text{ day}^{-1}$ and dissolved oxygen $>92\%$ saturation. During the experiment, fish allocated to different treatments were kept
185 separately in 0.2-m^3 floating net cages within two 2-m^3 tanks. Handling of fish was

carried out according to the European Union Directive 86/609EEC for the protection of animals used for experimental and other scientific purposes (EEC, 1986).

The experiment was conducted for a period of 12 weeks beginning in April 2004, just prior to the start of the annual reproductive season for wild dusky grouper (Marino et al., 2001), when the fish were 21 months old. Fish were allocated randomly ($n = 16$) into three treatment groups. The MT/hCG group received an MT-loaded implant ($1.5 \text{ mg MT implant}^{-1}$, about **11.5** mg MT kg^{-1} body weight) and an intramuscular injection of hCG (6000 IU kg^{-1}) once a month, the hCG group received an hCG injection (3000 IU kg^{-1}) every two weeks, and the control group was sham implanted (Table 1).

At the beginning of weeks 6 and 12 after the onset of the experiment, fish ($n = 5$) were sampled for blood, gonads and pituitaries. An initial sampling ($n = 12$) was also taken just prior to the start of the experiment (initial control). Due to the extremely small size of the gonads, which were impossible to identify macroscopically, for histological evaluation the whole ventral section of the swim bladder was dissected together with the attached “presumed” gonad, after clearing the abdominal cavity of the digestive track and associated organs. Blood was collected from the caudal vasculature into heparinized syringes and stored over crushed ice. After centrifugation for 10 min at $13,000 \text{ g}$ (Microfuge, Heraeus, Germany), the plasma was removed and stored at -80°C until analysis. Pituitaries were removed and stored at -80°C until analysis. Gonads were removed, weighed and fixed in a 4% formaldehyde: 1% gluteraldehyde buffered saline (4F: 1G solution) (McDowell and Trump, 1976) for at least 24 hours, dehydrated in a 70-96% ethanol series and embedded in glycol methacrylate resin (Technovit 7100; Heraeus, Kulzer, Germany). Serial sections were obtained at a thickness of $3 \mu\text{m}$ on a microtome (Biocut 2035, Reichert Jung, Germany) using disposable blades. After drying, slides were stained with methylene blue/ azure II/ basic fuchsin (Bennett et al., 1976) and examined under a light microscope (BH-2, Olympus, Japan).

At the end of the experiment (July 2004), the remaining fish from all three treatments were maintained together in a 500-L tank for one more year, until being sacrificed and sampled during the reproductive season (August 2005). The fish (n=5; mean $\pm$ SD body weight of 218 ± 17 g) were checked for spermiation by manual stripping and were then sacrificed and the gonads were removed and processed for histological assessment, as described above.

2.5 Hormone measurement

Quantification of MT in the *in vitro* buffer and fish plasma was done using a commercial enzyme-linked immunoassay (ELISA) kit (Bio-X diagnostics, Belgium). The ELISAs used for the quantification of T and 11-KT in the plasma of fish were performed according to Rodríguez et al. (2000) and Cuisset et al. (1994), respectively, with some modifications and using reagents from SpiBio (France). Briefly, 300 μ l of plasma were extracted twice with 2 ml of diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 minutes. After centrifugation for 3 min at 1800 g and freezing for 10 min at -80°C , the supernatant was collected and placed in new tubes by decanting. Drying of the supernatant was done under a stream of nitrogen (Reacti-vap III, Pierce, USA) and the samples were reconstituted in 350 μ l of assay buffer.

The T, 11 KT, and MT ELISAs were validated for use with dusky grouper plasma, by demonstrating parallel displacement to the standard curve, of serial dilutions of plasma spiked with a known amount of the appropriate steroid (2-way ANOVA of concentration vs matrix; T, $P = 0.12$; 11 KT, $P = 0.15$; MT, $P = 0.28$). The spike recovery (n = 4) was 126, 19 and 89% for the T, 11 KT, and MT ELISAs, respectively.

The sensitivity of the T, 11 KT and MT ELISAs were 10, 10 and 50 pg ml^{-1} , respectively.

Measurement of LH pituitary and plasma levels were done using a heterologous ELISA for striped bass (*Morone saxatilis*) LH according to Mañanos et al.(2002), after
 240 demonstrating parallel displacement of serially diluted pituitaries from dusky grouper.

2.6 Statistical analysis

For analyzing the *in vitro* MT release from the implants, data were subjected to 1-way Analysis of Variance (ANOVA), followed by Duncan's New Multiple Range
 245 (DNMR) test. To assess differences in (a) plasma MT in the *in vivo* release experiment and (b) plasma MT, T and 11-KT levels, and pituitary and plasma LH levels in the induction of sex inversion experiment, data were subjected to 2-way ANOVA (time x hormone treatment), followed by DNMR test where appropriate. Statistical analyses were done using a linear statistics software (SuperAnova, Abacus Concepts, Inc., USA)
 250 at a minimum significance level of $P < 0.05$. All results are presented as mean $\pm$ SEM, unless indicated otherwise.

3. Results

The mean daily *in vitro* MT release increased significantly from day 1 to 4
 255 (ANOVA, DNMR, $P < 0.05$) to a peak value of $32.2 \pm 2.7 \mu\text{g day}^{-1}$, then decreased by day 11 and remained stable until the end of the monitoring period (Fig. 1A). The mean total amount of MT released from the EVAc implants was $686 \pm 113 \mu\text{g}$, which amounted to 45.7% of the estimated total MT loaded. *In vivo*, treatment with MT-loaded implants caused a significant (ANOVA, $P < 0.01$) elevation in plasma levels for
 260 at least 32 d (Fig. 1B). The highest plasma MT levels were detected on days 1-4, declining at day 11 and remaining stable until the end of the monitoring period.

No significant differences between MT/hCG-treated and control fish were observed in plasma T and 11-KT, either 6 or 12 weeks after the onset of the study (Fig.

2A and B). On the other hand, plasma MT levels were significantly elevated in response to the MT treatment, both at 6 and 12 weeks after the onset of the study (Fig 2C). Pituitary LH content increased significantly during the study (ANOVA, $P < 0.01$), regardless of hormonal treatment (Fig. 3). Both MT/hCG- and hCG-treated fish had significantly lower pituitary LH content compared to control fish, both at 6 and 12 weeks after the onset of the study (ANOVA, $P < 0.001$). Plasma LH levels were below the detection limits of the ELISA (0.08 ng ml^{-1}).

The gonads of dusky grouper sampled prior to the hormonal treatment (initial controls) were thread-like and difficult to identify macroscopically. All had an ovarian structure with a lumen and numerous ovigerous lamellae protruding into the lumen (Fig 4A). Ovaries were immature, with lamellae containing only primary growth oocytes at the (a) chromatin-nucleolus stage (Selman et al., 1993), (b) early perinucleolus stage (*i.e.*, oocytes with one or few large nucleoli and no cytoplasmic inclusions) or (c) late perinucleolus stage (*i.e.*, more and smaller nucleoli, appearance of a cortical alveolus and/or a lipid droplet) (Merson et al., 2004). The diameter of the biggest oocytes was $60 \mu\text{m}$. Gonia were located at the periphery of the lamellae. The gonad of one fish sampled at this time appeared to be still in the process of differentiation, as it was made up mostly of connective tissue with few scattered primary growth oocytes, cysts with oocytes in the chromatin-nucleolus stage and many gonidia (Fig. 4B). Unidentified germ cell cysts (typically 4-16 cells per cyst) were occasionally observed along the germinal epithelium of the lamellae (Fig. 4C). Although these cells appeared similar to spermatocytes, they were most probably diplotene oocytes prior to initiation of primary growth and follicle cell formation.

The dominating stage of oocyte development in the gonads of control fish 6 weeks into the experiment was the late peri-nucleolar stage, with an increase in the diameter to $100 \mu\text{m}$ (data not shown). Gonads from control fish after 12 weeks showed

290 no further advancement in oocyte stage, but with a slight increase in diameter to 120 μm
(Fig. 4D). Fish treated with hCG alone had similar morphology as control fish (data not
shown).

The gonads from all MT/hCG-treated fish sampled 6 weeks after treatment showed clear signs of transition to testes, with testicular tissue making up between 20
295 (Fig. 5A) and 80% (Fig. 5B) of the gonads. The testicular and ovarian tissues were not separated by connective tissue. The gonads retained the ovarian structure with a lumen and ovigerous lamellae and the male tissue was proliferating mainly at the periphery of the ovarian lamellae and around the ovarian cavity. In some parts, the ovarian structure was widely converged into testicular structure, consisting of lobules that contained cysts
300 with germ cells at different stages of spermatogenesis, and in all gonads leftover primary oocytes showed various stages of apoptosis. Twelve weeks after the onset of treatment with MT/hCG, gonads still retained the “ovarian” cavity (Fig. 5C), but the lamellar structure converted completely into the lobular structure of a testis (Fig. 5C). Lobules were filled with cysts containing spermatocytes I+II and spermatids, as well as
305 some spermatozoa (Fig. 5C). At this time, sperm could not be obtained using abdominal pressure. Based on macroscopic examination, gonads from MT/hCG-treated fish appeared to be smaller than the gonads from hCG-treated and control fish. However, the gonadosomatic index (GSI) could not be assessed, since the gonads of most fish sampled were so small that they had to be excised together with the
310 surrounding tissue.

One year after the hormone treatments ended and towards the end of the next reproductive period (August 2005), all fish from the MT/hCG group were males (Fig. 5D), whereas all control fish were immature females. The testes maintained the “ovarian” cavity and were filled with mostly spermatogonia, as well as spermatozoa in
315 sperm sinuses along the gonad wall (Fig. 5D). No sperm could be obtained using

abdominal pressure. The histological appearance of the testes was indicative of the completion of the spermiation period, when spermatogonia proliferation occurs.

4. Discussion

320 Several methods of hormone administration for sex inversion in fish have been applied in the past, including oral administration, immersion, intramuscular injection and implantation (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Beardmore et al., 2001; Piferrer, 2001). Administering various hormones via silastic, cholesterol or EVAc implants is a reliable and well-tolerated method for treating fish with hormones, 325 and application is very simple and not damaging to the fish (Mylonas and Zohar, 2001). The EVAc implant was originally developed for the sustained release of agonists of the decapeptide gonadotropin-releasing hormone (GnRH_a), in order to induce ovulation and spermiation (Mylonas et al., 1995b). Due to the significant differences in chemical properties between GnRH_a and MT (*e.g.*, molecular size, hydrophobicity, etc.), it was 330 expected that the release kinetics of the MT implants would also be different from the ones published for GnRH_a. Similar to previous studies using EVAc-GnRH_a implants in fish (Mylonas et al., 1997; Mylonas et al., 1998), maximum daily MT release *in vitro* and plasma MT levels *in vivo* were observed during the first 4 d after application. However, contrary to the reported declining GnRH_a release, MT release from the EVAc 335 implants remained relatively constant for the next 28 d. This is due to the hydrophobic nature of MT and the modifications in the implant fabrication process, compared to the EVAc-GnRH_a implants. The hormone release from the EVAc implants is a diffusion-controlled process, facilitated by the production during the fabrication process of channels within the polymer matrix (Sefton et al., 1984; Brown et al., 1986). Release 340 during the first few days is maximal, as large amounts of hormone are located near the surface of the implant, but the release in subsequent days decreases, since the diffusion

distance increases with time. The rate of release can be manipulated by the use of a mixture of two compounds (called bulking agents), one soluble and one insoluble in physiological fluids, such as albumin (soluble) and inulin (insoluble). In the case of GnRHa implants, a 1:1 ratio of albumin:inulin is usually used, whereas in the MT implants only inulin was employed. This has resulted in a slower MT release from the implant, which means smaller differences between maximum (1-4 d post application) and subsequent release levels, as well as longer total release period. Another reason for the different release kinetics of the EVAc-MT implants is the hydrophobicity of MT, which results in slower release rate through the channels created by the dissolution of the bulking agent.

The developed EVAc-MT implants compare favorably to other steroid-delivering systems employed recently in fish, which commonly are prepared by loading crystalline or solvent-dissolved steroids in silastic tubing (Antonopoulou et al., 1999; Zanuy et al., 1999; Cavaco et al., 2001; Yamaguchi et al., 2004). The major disadvantage of the silastic tubing implants is the very inefficient release of the loaded steroids, which means that very high amounts of hormone must be loaded in the implants in order to achieve significant increases in plasma steroid levels. For example, physiological levels of T were achieved for 72 d in the European sea bass after administration of T-loaded silastic implants at a dose of 100 mg Kg⁻¹ (Zanuy et al., 1999), whereas in the present study a dose of only 10 mg Kg⁻¹ was used. A similarly high dose of T in silastic implants was used in the goldfish (*Carassius auratus*) (Trudeau et al., 1993), whereas in the African catfish (*Clarias gariepinus*) a dose of 30 mg Kg⁻¹ produced significant plasma T elevations 2 weeks after administration of the silastic pellets (Cavaco et al., 2001). An improvement on the release efficiency of silastic steroid implants has been made recently with the dissolution of the steroid in a mixture of dimethyl-sulfoxide (DMSO) and castor oil (Yamaguchi et al., 2004). Using this method, implants loaded

with only 0.4 mg of E₂ or 11-KT were able to increase plasma levels of immature red sea bream (*Pagrus auratus*) weighing 708 g, to levels observed in mature fish. However, these levels never exceeded 1 ng Kg⁻¹ and the total amount of steroid released from the implants during the 30 d of the study was only 10% of total loaded, at best. Therefore, the EVAc-MT implants produced in the present study appear to be a very effective and efficient method for experimental elevation of plasma MT levels for a period of at least 32 d. These implants have recently been used successfully to induce spermiation with enhanced milt production in males of grey mullet (*Mugil cephalus*) (Aizen et al., 2005).

In the present study, 12-week treatment with MT implants together with hCG induced complete and permanent sex change in pre-pubertal, 1-year-old dusky grouper. This sex change was associated with the complete reorganization of the ovary into a lobular-structured testes, with cysts filled with germ cells at all stages of spermatogenesis, including spermatozoa. The lack of releasable milt at this time could be explained by the very small size of the gonads and the fact that the final sampling was done at the beginning of the reproductive season. Based on the histological appearance of the gonads, it appears that more spermatozoa and perhaps releasable milt could have been produced later in the reproductive season. However, due to the very limited number of MT-treated fish remaining at this time (5 individuals), it was considered important not to risk mortalities by further handling, and allow these fish to grow until the next reproductive season, in order to document if sex inversion was permanent. In regards to treatment with hCG alone, this did not induce sex inversion, indicating that the effects of the MT/hCG treatment were probably due exclusively to MT. Follow-up experiments, where 2-year-old dusky grouper were sex-reversed by treatment with MT implants alone (data not shown), confirmed this conclusion. To date, MT has been used extensively to induce sex change in various teleosts, including

both gonochores and hermaphrodites (Kuo et al., 1988; Piferrer et al., 1993; Blázquez et al., 1995; Lee et al., 1995; Chang et al., 1999; Lee et al., 2002; Omoto et al., 2002; Yeh et al., 2003b). In protogynous hermaphrodites, mainly members of the genus *Epinephelus*, the ability of MT to accomplish female-to-male sex change **has been demonstrated well** (Kuo et al., 1988; Tan Fermin et al., 1994; Lee et al., 1995; Marino et al., 2000; Lee et al., 2002; Yeh et al., 2003b). Endogenous androgens have been implicated in the female-to-male sex change, based on findings that plasma levels of T and/or 11 KT are low during the female phase and increase during sex change (Nakamura et al., 1989; Cardwell and Liley, 1991; Bhandari et al., 2003). Such data, however, do not distinguish if the rise in androgen levels is causing sex inversion or is the result of sex inversion and the functioning of the newly developed testes (Chan and Yeung, 1983). Treatment of protogynous groupers with P450 aromatase (P450arom) inhibitors (AI) or MT also resulted in enhanced plasma levels of 11KT and/or T in sex reversing fish (Kuo et al., 1988; Yeh et al., 2003b; Bhandari et al., 2004), suggesting that androgens are, at least partly, responsible for the onset or progression of female-to-male change. In the present study, T and 11 KT plasma levels were low and did not differ between MT-treated and control fish, even though sex inversion was effected by the MT treatment. The low T and 11 KT plasma levels suggest that the sex inversion effect of MT in dusky grouper is direct and that the small amount of endogenous steroids produced was due to the very small size of the gonads (which were so small they could not even be dissected from the fish).

In teleosts, E₂ is involved in the maintenance of the female state, and a decrease in E₂ levels, through inhibition by MT or AI, may stop vitellogenesis, induce oocyte degeneration and permit male development (Fostier et al., 1983). Recent studies have indicated that MT can suppress P450arom expression (Kitano et al., 2000), and that shutdown of P450arom expression and the subsequent decrease of E₂ synthesis is

420 responsible for the female-to-male sex change (Kitano et al., 2000; Kroon and Liley, 2000; Bhandari et al., 2004). In the present study, E₂ plasma levels were not measured due to the limited volume of plasma available from such small fish. However, the oocyte degeneration observed in gonads of fish treated with MT/hCG at the beginning of sex change suggests that MT may have a similar function in inhibiting E₂ production
425 in the dusky grouper.

In addition to the shutdown of P450arom gene expression, MT was shown to act directly on the brain-pituitary axis by increasing pituitary LH content and the number of cells expressing GnRH mRNA in the preoptic area of the brain (Amano et al., 1994). Elevation of GnRH production with its subsequent stimulation of pituitary LH release,
430 could explain the significantly lower pituitary LH content of the MT-treated fish in the present study. However, since plasma LH levels remained undetectable in all fish and pituitary LH content was also reduced in fish given hCG alone, it appears more likely that the low content of LH in the pituitary could have resulted from an inhibitory effect of hCG on the synthesis of LH. No information is available on the effects of hCG in the
435 regulation of gonadotropin synthesis/release in fish, therefore the existence of any feedback effects are only a matter of speculation at this stage. Studies in rabbits suggest the existence of a short-loop negative feedback control of LH secretion and production by hCG (Hirono et al., 1972; Molitch et al., 1976), whereas hyper-stimulation with hCG resulted in a rapid synthesis and release of LH in rats (Thomadakis and Kramer, 1999).
440 Further studies in elucidating similar effects in fish would be necessary.

In the present study, hCG was given in combination with the steroid treatment, because hCG has been successful in inducing spermatogenesis and spermiation in the Japanese eel (*Anguilla japonica*) (Miura et al., 1991; Ohta et al., 1996; Ohta and Tanaka, 1997), and in stimulating sex inversion in the protogynous wrasse (*Coris julis*)
445 (Reinboth and Brusle-Sicard, 1997) and bluehead wrasse (*Thalassoma bifasciatum*)

(Koulish and Kramer, 1989). The expectation was that hCG could (a) induce female-to-male inversion, firstly, followed after further treatment by stimulation of spermatogenesis and spermiation, or (b) enhance spermatogenesis and spermiation in fish induced to undergo sex inversion by the MT treatment. Sex inversion was not induced by hCG alone, and it is questionable that the observed spermatogenesis in the fish inverted by the MT treatment was enhanced by the hCG treatment. Together with the finding that plasma LH was not elevated in the MT/hCG treated fish, the data obtained in the present study suggest that LH or LH-like gonadotropin alone can not induce sex inversion and spermatogenesis in dusky grouper. The present results are different from previous findings in other protogynous fishes, in which treatments with hCG, LH or GnRH were effective in inducing female-to-male sex change (Koulish and Kramer, 1989; Kramer et al., 1993; Yeung et al., 1993; Ravaglia et al., 1997; Reinboth and Brusle-Sicard, 1997). For example, in the bluehead wrasse even a single injection of 20,000 IU hCG Kg⁻¹ was able to induce sex inversion, beginning only a week after treatment (Koulish and Kramer, 1989). This treatment was also associated with elevation of endogenous androgen production, as documented by the hCG enhancement of androgen-controlled color change. However, direct androgen treatment was ineffective in inducing female-to-male inversion in this fish (Kramer et al., 1988), suggesting that gonadotropin may be necessary for triggering sex inversion. Finally, in the protogynous ricefield eel (*Monopterus albus*), LH or GnRH α stimulation was effective in inducing sex inversion, which was associated with elevation and reduction in plasma T and E₂, respectively (Tang et al., 1974b; Tao et al., 1993), whereas steroid treatment alone was ineffective (Tang et al., 1974a).

It is apparent from the available information, that although androgens may be the final inducers of sex inversion in protogynous fishes, in some species gonadotropin must also be present for the effect to proceed. It is justified to speculate that the

differences observed in various studies in the effectiveness of gonadotropin-only treatments in inducing sex inversion may be related to the type of gonadotropins used (*i.e.*, hCG, LH or follicle stimulating hormone, FSH) and their natural role in the process in different fishes, as well as the ability of the gonad to respond to the gonadotropin therapy, *i.e.*, the existence of the available receptors. This, in turn, may be a function of the time during the year when the experiments were undertaken, *e.g.*, before, during or after the reproductive season (Yeung et al., 1993; Condeca and Canario, 1999), since natural sex inversion in hermaphroditic fishes occurs during specific times of the year, either pre- or post-spawning (Lee et al., 2001; Lone et al., 2001; Yeh et al., 2003b), or during the spawning season (Marino et al., 2001). Therefore, induction of sex inversion may be possible only during the time of natural sex inversion, as it has been shown for the protandrous gilthead seabream (*Sparus aurata*) (Zohar et al., 1995; Condeca and Canario, 1999) and black porgy (Lee et al., 2001).

We believe that the MT-implants produced in the present study have important advantages over the sex inversion methods used so far in the dusky grouper. Firstly, in the study of Marino et al. (2000) sex inversion was effected in only 25% of MT-fed individuals. This is a common problem with orally administered steroids, due probably to lack of uniformity of the MT-content of the feed (which is prepared on-site), degradation of the hormone during passage through the digestive track, as well as variations in the amount of feeding by each individual fish due to established hierarchies among broodstock (Beardmore et al., 2001). In the same study of Marino et al. (2000), sex inversion efficiency was improved to 100% when the MT-loaded feed was given by hand and individually to each fish, but this approach is cumbersome, labour intensive and depends on the behaviour and appetite of the fish. A second advantage of the MT-implant method presented here is the lack of frequent handling.

For example, sex inversion using MT injections was effective in only 38 % of the fish (Marino et al., 2000), and this after a series of 14 injections given every 7-10 days. In addition to being inefficient, this approach is again very labour intensive and can be damaging to expensive and painstakingly acquired broodfish, as fish have to be crowded, anaesthetized and handled repeatedly. On the contrary, 100% sex inversion was achieved in the present study with only 3 monthly administrations of the MT implants.

Further advantages of the developed MT-implant method include the fact that the total amount of MT used was about 33 mg Kg⁻¹ of fish, whereas it ranged between 85 and 113 mg Kg⁻¹ when the treatment was given in the feed (Glamuzina et al., 1998a; Marino et al., 2000). The significantly smaller amount of hormone (61 – 71% less) translates not only to savings in the cost of the hormone, but also to a reduction in the potential environment impact of this synthetic steroid (Beardmore et al., 2001; Ying et al., 2002). Finally, sex inversion using the MT implant was permanent, and the inverted males again underwent spermatogenesis and production of spermatozoa one year after the hormone treatments ended. On the contrary, in the study of Marino et al. (2000) sex inversion after orally-administered or injected MT was not maintained and fish inverted back to females during the next reproductive period. In the study of Glamuzina et al. (1998a) the permanency of the sex inverting treatment was not verified.

In conclusion, the major contribution of the present study is the development of an efficient MT administration method using EVAc implants, and the demonstration that a 12-week treatment given during the natural sex-inversion period is able to induce complete and permanent female-to-male change, as well as complete spermatogenesis in 1-year-old juvenile fish of <200 g in body weight. For comparison, the first natural sexual maturation of female dusky grouper occurs at the age of 5 and a bodyweight of about 2-2.5 kg (Marino et al., 2000; Andrade et al., 2003), whereas sex inversion occurs

when individuals are 9 to 16 years old and weigh 6-10 Kg (Chauvet, 1988). Production
525 of male fish at such an early age may dramatically reduce by many years the time
required for natural puberty, sex inversion and the production of sperm by dusky
grouper reared under culture conditions.

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Table 1. Experimental design for the hormone treatments and samplings of 1-year-old dusky grouper. The 17 α -methyltestosterone (MT) treatment was given once a month using controlled-release implants (1.5 mg MT). The human chorionic gonadotropin (hCG) treatment was given as intramuscular injections at 6,000 IU Kg⁻¹ once a month (MT group) or 3,000 IU Kg⁻¹ once every 2 weeks (hCG group).

		Week						
		1	3	5	7	9	11	13
Treatment								
830	Control	sham	sham	sham	sham	sham	sham	
	MT	MT	hCG	MT	hCG	MT	hCG	
	hCG	hCG	hCG	hCG	hCG	hCG	hCG	
Sampling		1						2

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Figure legends

Figure 1. (A) Mean ($\pm$ SEM) daily and cumulative 17α -methyltestosterone (MT) release *in vitro* from EVAc implants ($n = 4$) at 18°C . Different letter superscripts indicate significant differences in daily MT release (one-way ANOVA, $P < 0.05$). The cumulative amount of MT released from the implants during the 32 days of the study was 45.7% of the theoretical total loaded. (B) Mean ($\pm$ SEM) plasma 17α -methyltestosterone (MT) levels in European sea bass ($n = 6$) implanted with MT-loaded implants and maintained at 18°C . Significant differences in plasma MT levels in MT-treated fish during the study are indicated by different letter superscripts (one-way ANOVA, $P < 0.05$). In control fish, MT cross-reactivity was detected, but did not exhibit any significant changes during the experiment, and was significantly lower than in MT-implanted fish (two-way ANOVA, $P < 0.01$).

Figure 2. Mean ($\pm$ SEM) plasma androgen levels at 6 and 12 weeks after the onset of treatment of 1-year-old dusky grouper ($n = 5$) with 17α -methyltestosterone (MT) implants and human chorionic gonadotropin (hCG) injections on a monthly basis (Table 1). Different letter superscripts indicate significant differences in plasma steroid levels between treatment means within sample time, (two-way ANOVA, means comparisons, $P < 0.01$), whereas an asterisk indicates significant changes over the course of the experiment (two-way ANOVA, DNMR, $P < 0.05$).

Figure 3. Mean ($\pm$ SEM) pituitary luteinizing hormone (LH) content at 6 and 12 weeks after the onset of treatment of 1-year-old dusky grouper ($n = 5$) with 17α -methyltestosterone (MT) implants and human chorionic gonadotropin (hCG) injections

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on a monthly basis (Table 1). Different letter superscripts indicate significant differences in LH content in response to the hormonal treatment, whereas there was a significant increase in all treatments over time (two-way ANOVA, DNMR, $P < 0.05$).

865 **Figure 4.** Photomicrographs of gonads of 1-year-old dusky grouper prior to the onset
of hormonal treatment (A – C) and of control fish after 12 weeks (D). The hCG-treated
fish had a similar appearance to controls (data not shown). A: section through the ovary
showing the ovarian cavity (OC) and ovarian lamellae with oocytes at different stages
of primary growth (PN I: early perinucleolar, PN II: late perinucleolar) and nests of
870 gonia (G) situated at the periphery. B: still differentiating gonad (ovary) filled with
gonia, cysts of chromatin-nucleolus stage oocytes (CN) and few primary growth stage
oocytes. C: cyst with CN stage primary oocytes and unidentified germ cell cyst (UC).
D: gonads of control fish after 12 weeks, also containing only PN II stage primary
oocytes. Bar indicates 100 μ m.

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Figure 5. Photomicrographs of gonads of 1-year-old dusky grouper after 6 weeks (A -
B) and 12 weeks (C) of treatment with 17α -methyltestosterone (MT) and human
chorionic gonadotropin (hCG); and of fish from the same treatment group after 1 year
(D). A: lamellar structure of transitional gonad with cysts of spermatocytes (SC) and
880 apoptotic oocytes (AO). B: transitional gonad with ovarian cavity (OC), cysts of
spermatocytes, gonia (G) and primary growth oocytes (PO). C: lobular structure of
testes with remaining ovarian cavity and spermatocysts at different stage of
spermatogenesis, including spermatozoa (SZ). D: Testis mainly filled with
spermatogonia (SG) and spermatozoa (SZ), typical of the end of the reproductive
885 season. Bar indicates 100 μ m.

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890

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Sarter et

Figure 3
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895

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