Japan. J. Ichthyol.
40(4): 413–420, 1994

魚類学雑誌
40(4): 413–420, 1994

Induction of Sex Inversion in Juvenile Grouper, *Epinephelus suillus*, (Valenciennes) by Injections of 17α-Methyltestosterone

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(*Received May 12, 1993; in revised form July 19, 1993; accepted September 20, 1993*)

Abstract Four groups of two-year old juvenile groupers (*Epinephelus suillus*), each with 8–9 individuals of mean body weight (BW) 1.2 kg, were treated with 17α-methyltestosterone (MT). MT was injected intramuscularly within the range of 0.5–5.0 mg kg^{-1} BW every 15 days. Gonadal biopsy and stripping of the abdomen was done every 15 days, the fish being sacrificed after six or twelve injections. Initial controls had immature ovaries containing primary oocytes in lamellae that extended into the central lumen. After six injections, proliferation of stromal and gonial cells were observed in all fish sampled. Regardless of treatment, gonad sections of fish with a minimum BW of 1.2 kg showed degeneration of primary oocytes and the presence of spermatogenic cells. Milt was also present in larger-sized fish (BW: 1.5 kg) given 0, 0.5 and 1.0 mg MT kg^{-1} BW, after such fish had received an accumulated dose of 5 or 12 mg MT kg^{-1} BW. However, gonad sections of smaller-sized fish following these treatments contained only primary oocytes and gonial cells after six (BW: 0.7–1.0 kg) or twelve (BW: 0.6–1.3 kg) injections. In contrast, all fish treated with 5 mg MT kg^{-1} BW had testes in active spermatogenesis after six (BW: 1.2–1.6 kg) or twelve (BW: 0.8 kg) injections. Gonad weight and gonadosomatic index values decreased during consecutive sampling. Induction of female-to-male sex inversion in juvenile *E. suillus* by MT was probably synergistic with age and size.

Grouper (family Serranidae), locally known in the Philippines as "lapu-lapu" is one of the commercially important marine fish that abounds in coral reef areas. Highly esteemed for its taste, it is a good candidate for culture. Cage culture of grouper is practised in several Southeast Asian countries especially Hongkong, Singapore, Malaysia, Thailand and Indonesia. In the Philippines, grouper represents 2% of the total fish catch (Kohno et al., 1988). Traditional grouper culture is presently being done on a pilot scale by some private farms in three areas: Pangasinan, Manila and Panay Island. Fingerlings are collected for rearing by hook and line, bamboo trap and dipnet. However, the availability of fingerlings at specific places varies with the time of year. Therefore, to ensure an adequate and consistent supply of grouper fry, it is necessary to develop artificial propagation techniques. A major constraint in seed production work is the availability of mature males.

Groupers may be simultaneous or sequential (protogynous) hermaphrodites (Shapiro, 1987). Natural sex inversion in groupers occurs between 2 and 11 years of age depending on the species (Chen et al., 1980; Kuo et al., 1988). The scarcity of mature males occurring in the wild and the length of time necessary for female groupers to become males underline the need for studies on induced sex inversion.

Induction of sex inversion and control of sex differentiation using sex steroids, have been carried out by injection, implantation, incorporation in the diet or addition to the rearing medium (Yamamoto, 1969; Donaldson and Hunter, 1982; Chan and Yeung, 1983). In *Epinephelus tauvina* (Chen et al., 1977) and *E. fario* (Yeh et al., 1986; Kuo et al., 1988), sex inversion was successfully induced by oral administration of 17α-methyltestosterone (MT), a synthetic steroid. This study reports on the induction of sex inversion of juvenile grouper, *Epinephelus suillus*, the most popular cultured species in the Philippines (Kohno et al., 1988), by intramuscular injections of MT.

Materials and Methods

Fish

Two-year old juvenile groupers, *Epinephelus suillus*, with mean body weight of 1.2 kg, were used. These had been reared from wild-caught fry being grown in earthen ponds for three months until they were about 500 g each, and thereafter maintained in a 50-ton experimental concrete tank. Several cylindrical concrete blocks placed randomly in the tank had provided cover for the fish. They were fed on commercially-unimportant fishes to satiation on alternate days.

Seawater temperature and salinity ranged from 21–31°C and 28–31‰, respectively.

Hormone preparation and injection

17α-methyltestosterone (MT; 17α-methyl-4-androsten-17β-ol-3-one), was purchased from Sigma (St. Louis, Missouri, U.S.A.). Prior to each injection session (every 15 days), three sets of hormone solutions corresponding to the three doses tested were freshly prepared. For doses of 0.5 and 1.0 mg kg^{-1} body weight (BW), MT was initially dissolved in absolute ethanol (Merck) to obtain a solution of 100 mg MT ml^{-1}. Corn oil was then added to the dissolved hormone to obtain a solution containing 7% ethanol. For the 5 mg kg^{-1} BW dose, a solution of 140 mg MT ml^{-1} of ethanol was prepared. Corn oil was added until the hormone solution contained 37% ethanol, the level necessary for dissolving MT at 5 mg kg^{-1}. Corn oil solution containing only 7% ethanol served as control.

The hormones were injected into the dorsal musculature of the fish just above the lateral line using a plastic tuberculin syringe fitted with a 24 gauge × 25 mm needle. A series of injections was given on alternate sides of each anaesthetized fish using an injection volume of 0.1 ml kg^{-1} BW.

Experimental protocol

Juvenile groupers were individually weighed and marked using a numbered nylon anchor tag attached to the opercular bone (Garcia and Gapasin, 1988). Fish were then randomly separated into four groups of 8–9 individuals each and injected with MT every 15 days at dosages of 0.5, 1 or 5 mg kg^{-1} BW. Control fish received a corn oil-ethanol solution. The experiment began in late January and continued until mid-July.

To examine gonadal histology, two fish were sacrificed at the start of the experiment. Body weight (BW) and gonad weight (GW) were taken for the computation of gonadosomatic index (GSI = GW/BW × 100). Similarly, two and one fish from each treatment group were sacrificed after six and twelve injections, respectively. A 10-mm thick piece of tissue was sliced from the anterior, middle and posterior regions of the dissected gonads, fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm, stained with Mallory's trichrome (Humason, 1972), and examined using light microscopy.

The effectiveness of the hormone treatment was monitored every 15 days. Prior to injection, each fish was anaesthetized with 450 ppm of 2-phenoxyethanol (Merck). Gentle pressure was then exerted on the ventral abdominal region of the fish to determine the presence or absence of milt. In addition, the fish was cannulated by inserting a polyethylene tubing (PE 100, I.D. = 0.86 mm, O.D. = 1.52 mm, Clay Adams) into the gonoduct through the genital pore, followed by gentle aspiration of gonadal tissue. The nature of the biopsied tissue was further checked under a light microscope.

Relative counts of spermatogenic cells

Relative abundance of the stromal cells, primary oocytes and spermatogenic cells observed in all gonad sections was arbitrarily designated as none (−), few (+), intermediate (++) and abundant (+++).

Results

Initial sampling

The range of initial GW, BW and GSI of the two untreated fish were 1.10–4.71 g, 0.80–1.50 kg and 0.14–0.31%, respectively. Immature ovaries of these fish contained only primary oocytes, which were arranged along lamellae extending into the central lumen. Few gonial cells were present in the periphery of the ovarian lamellae (Fig. 1).

Second sampling

After the sixth injection, gonad sections of all sacrificed fish showed a proliferation of stromal and

gonial cells, consisting of oogonia and spermatogonia, or degeneration of primary oocytes, leading to a marked reduction in size of these cells (Fig. 2).

Male and female tissues were not clearly separated in the gonad. The presence of spermatogenic cells, in addition to primary oocytes, was observed only in fish with a minimum BW of 1.2 kg, regardless of treatment (1242, 2250, 3207; Table 1). However, spermatogenesis was most advanced in two fish sacrificed after six injections of 5 mg MT kg^{-1}, as shown by the type and abundance of gametic cells present and organization of the gonads. Although primary oocytes were still observed in gonad sections of all sacrificed fish, one fish injected with 5 mg MT kg^{-1} BW contained only spermatogenic cells (0152). The organization of the gonads was intermediate between a lamellar and lobular arrangement. Furthermore, fish treated with the highest dose of MT had a well-developed sperm duct system filled with spermatozoa.

In all gonad sections, the presence of a central cavity or lumen, lamellar organization of primary

Fig. 1. Section of gonad showing immature ovaries, from juvenile grouper sampled as initial control. *PO*—primary oocyte, *GC*—gonial cells. Scale bar = 50 μm.

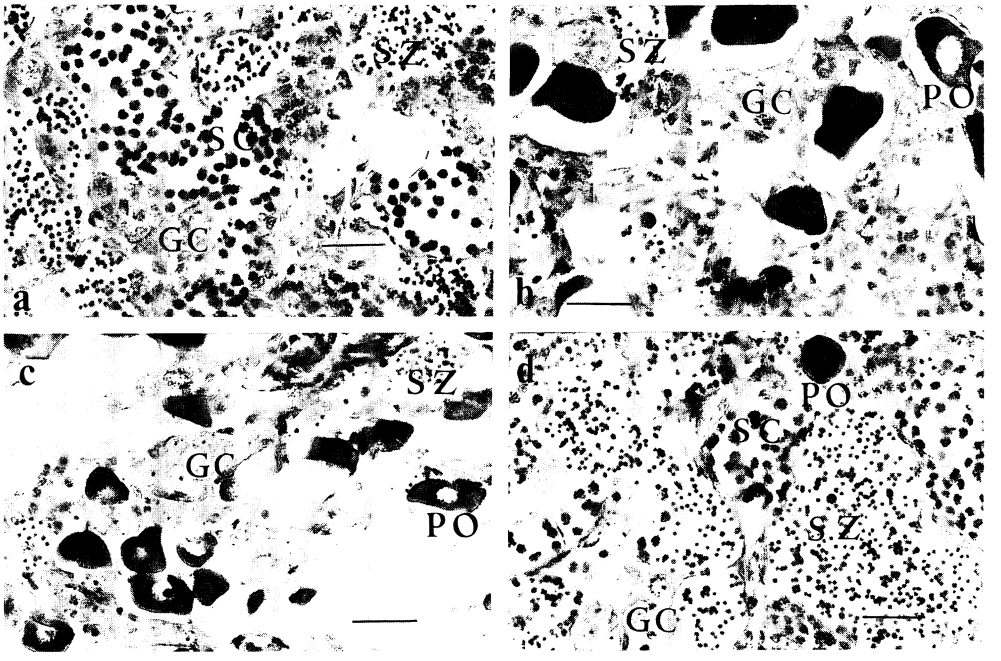

Fig. 2. Gonad histology of grouper after six injections of various doses of 17α-methyltestosterone (MT), showing different stages of spermatogenesis. a) 0 mg MT kg^{-1} BW (control); b) 0.5 mg MT kg^{-1} BW; c) 1.0 mg MT kg^{-1} BW; d) 5.0 mg MT kg^{-1} BW. *GC*—gonial cells, *PO*—primary oocyte, *SC*—spermatocytes, *SZ*—spermatozoa. Scale bar = 50 μm.

oocytes and secondarily formed sperm ducts were noted.

Terminal sampling

Five months after the start of the experiment, spermiation was observed in one control fish (1250) and one fish (2313) treated with 0.5 mg MT kg^{-1} BW (accumulated dose of 5 mg MT kg^{-1} BW; Table 2). Spermiation continued for one month thereafter, until the termination of the experiment. Milt was aspirated from a fish (3220) treated with 1 mg MT kg^{-1} BW, (accumulated dose of 12 mg MT kg^{-1} BW) after six months of treatment. Milt was noted in all fish weighing 1.5 kg.

Although milt was present in larger-sized fish given 0, 0.5 and 1.0 mg MT kg^{-1} BW, gonad sections of smaller-sized fish (BW range 0.6–1.3 kg) sampled from each of the three treatments contained only primary oocytes and gonial cells (1251, 2307, 3201). In contrast, a fish weighing 0.8 kg, which had been injected with the highest MT dose, had testis in active spermatogenesis (0.155; Table 2).

Gonad sections of fish treated with 0.5 mg and 1.0 mg MT lacked germ cells in the anterior regions (Table 2). Gonial cells and primary oocytes in these fish were present only in the middle and posterior regions.

Table 1. Relative abundance of stromal and gametic cells in juvenile *Epinephelus suillus* after six injections of various doses of 17α-methyltestosterone (MT) (second sampling; see text)

Dose (mg MT/kg)	Fish code	BW (kg)	Stromal cells	Gametic cells					Remarks
				GC	PO	SC	SD	SZ	
0	1253	0.7	+++	+	+++	−	−	−	
	1242	1.4	−	+++	++	++	++	++	spermatogenesis occurring
0.5	2305	0.8	+	+++	+++	−	−	−	
	2250	1.5	++	++	+++	+	−	+	spermatogenesis occurring
1.0	3203	1.0	+	+++	+++	−	−	−	
	3207	1.2	+	+	+++	+	−	+	spermatogenesis occurring
5.0	0152	1.2	−	+++	−	++	+	+++	few PO and spermatogenic
	0156	1.6	−	+++	+	++	−	+++	cells in anterior region; predominance of SZ in the middle and posterior regions

Abbreviations used: BW, body weight; GC, gonial cells; PO, primary oocytes; SC, spermatocytes; SD, spermatids; SZ, spermatozoa; −, none; +, few; ++, intermediate; +++, abundant.

Table 2. Relative abundance of stromal and gametic cells in juvenile *Epinephelus suillus* after twelve injections of various doses of 17α-methyltestosterone (MT) (terminal sampling; see text)

Dose (mg MT/kg)	Fish code	BW (kg)	Stromal cells	Gametic cells					Remarks
				GC	PO	SC	SD	SZ	
0	1250	1.5							spermiating for 1 month
	1251	1.3	−	+++	+++	−	−	−	
0.5	2313	1.5							spermiating for 1 month
	2307	0.8	−	+++	−	−	−	−	no germ cells in anterior region; PO and SG in the middle and posterior regions
1.0	3220	1.5							milt cannulated
	3201	0.6	−	+++	+++	−	−	−	no germ cells in anterior region; PO and SG in the middle and posterior regions
5.0	0155	0.8	−	+++	+	++	−	+++	spermatogenesis occurring

Abbreviations used: BW, body weight; GC, gonial cells; PO, primary oocytes; SC, spermatocytes; SD, spermatids; SZ, spermatozoa; −, none; +, few; ++, intermediate; +++, abundant.

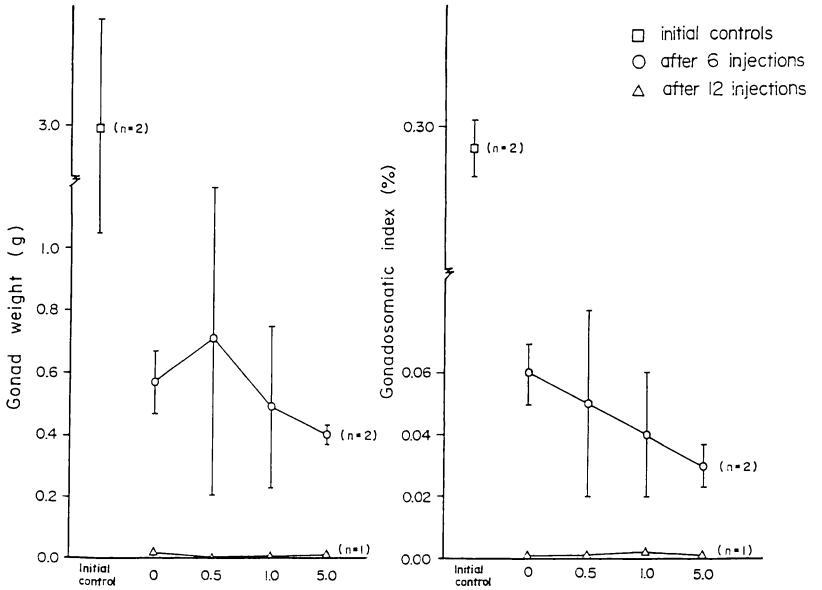

Fig. 3. Gonad weight and gonadosomatic index values of juvenile grouper sampled as initial controls and after three and six months of treatment with various doses of 17α-methyltestosterone (MT).

No milt or gonadal tissues were obtained by gentle pressure on the abdomen or after cannulation of fish that were not sacrificed. Body coloration of fish did not change following sex inversion.

Compared with the initial controls, GW and GSI values of all experimental fish decreased markedly with consecutive sampling (Fig. 3).

Discussion

Sex inversion in juvenile *Epinephelus suillus* from immature females to mature males was induced by 15-day injections of MT. Spermatogenesis was initiated in fish treated with the two lower MT doses, whereas fish given the highest dose had numerous spermatozoa. In contrast to fish in other treatment groups, the induction of spermatogenesis in fish injected with 5 mg MT kg^{-1} BW indicated that prolonged treatment of a high dose of MT was effective in accelerating and maintaining spermatogenesis. However, a dose lower than 5 mg MT kg^{-1} BW was recommended for inducing spermiation since fish given the highest dose did not release milt. High doses of MT probably caused malformation, or even agenesis of the sperm duct system (unknown reviewer, pers. comm.). Milt discharge was never observed in fish treated with 5 mg MT kg^{-1} BW, even though gonads of fish in this group showed the most advanced stage of inversion from female to male after six injections. Long-term administration of high dosages of MT has also been reported to inhibit spermiation in immature, gonochoristic milkfish (Lee et al., 1986).

MT has successfully induced spermiation in several grouper species. In this study, accumulated doses of 3 and 5 mg MT kg^{-1} BW induced spermatogenesis and spermiation in 2-year old *E. suillus*. Spermatogenesis and spermiation in 2-year old *E. fario* and 3-year old *E. tauvina* were reported after fish received an accumulated dose of 70 and 160 mg MT kg^{-1} BW (Kuo et al., 1988) and 80 and 145 mg per fish (Chen et al., 1977), respectively.

In addition to the accumulated dose, the different modes of steroid administration to recipient fish may have contributed to some of the differences observed among several grouper species studied. MT was administered through the diet in both *E. tauvina* and *E. fario* and by intramuscular injections in *E. suillus*. In the two former studies, the actual dose ingested by each fish was probably much less than the computed dose, since feed blocks containing MT may not have been totally consumed and the steroid could have leached into the surrounding water to some extent. Therefore, the occurrence of spermiation in *E. suillus* at an accumulated dose which was 5 and 32 times

lower than in *E. tauvina* and *E. fario*, respectively, suggests that administration of MT every 15 days by injection is a more economical way to effect sex inversion.

Stimulation or inhibition of spermatogenesis by MT may have also been affected by the age and/or size of the fish (Borg, 1981; Andersson et al., 1988). Compared to *E. suillus* (1.2 kg), *E. fario* (1.69 kg) and *E. tauvina* (5–6 kg) were larger and capable of undergoing female maturation at 2 and 3 years respectively, before the onset of sex change from female to male. There is no available data on the age of first maturation in female *E. suillus*. However, the results of the present study indicated that the 2-year old fish had not matured as females, and that sex change to mature male occurred prior to female maturation.

Factors other than MT administration were probably also involved in triggering the onset of sex change in juvenile *E. suillus*. For instance, the presence of transitional gonads in all fish sampled (controls as well as experimentals) after six injections suggests that the onset of natural sex inversion is related to age. Stromal cells and primary oocytes appeared earlier than spermatogenic cells. In protogynous hermaphrodites *Brachydanio rerio*, *Macropodus opercularis* (Chan and Yeung, 1983), *Coris julis* (Bruslé, 1987) and *Thalassoma duperrey* (Nakamura et al., 1989), stromal cell proliferation and degeneration of primary oocytes, the first cytological signs of sex inversion, were observed before the proliferation of spermatogonia and formation of testicular lobules. Oogonia and spermatogonia appeared to be similar under both light and electron microscopy (Reinboth, 1982; Bruslé, 1987). However, most of the bipotential gonial cells in *E. suillus* were presumed to have differentiated into active spermatogonia not only because *E. suillus* was undergoing sex change, but also because of their abundance along the inner ridges of the gonadal lamellae (Chan and Yeung, 1983). The presence of primary oocytes and spermatogenic cells suggested that onset of sex change in *E. suillus* is at 2 years of age.

Regardless of treatment, spermatogenesis in fish with a minimum BW of 1.2 kg (Table 1) and the presence of milt in fish weighing at least 1.5 kg (Table 2) indicated that the size is important for sex change in *E. suillus*. Spermatogenesis and spermiation were observed in fish which were 71% to 150% larger than the smallest fish sampled. In some gregarious protogynous species such as coral reef fishes, sex inversion is believed to be socially controlled. Removal or absence of a male from a group causes the largest immature female to change sex (Reinboth, 1980; Warner, 1988). However, social control of sex inversion has not been reported in groupers and needs further investigation.

All testes examined showed signs of having been secondarily derived, as in other serranids and wrasses (Hourigan and Kelley, 1985; Sadovy and Shapiro, 1987; Shapiro, 1987). Thus, *E. suillus* is monandric (Chan and Yeung, 1983). To date, primary or initial males in groupers have not been reported. However, a residual lumen, remnant oocytes and peripheral sperm sinuses were absent in the testis of a reef goby *Coryphopterus personatus*, a protogynous hermaphrodite found in the Caribbean (Cole and Robertson, 1988).

Since all groupers that are sequential hermaphrodites are protogynous, the presence of primary oocytes and spermatogenic cells in juvenile *E. suillus* indicates the existence of juvenile hermaphroditism or intersexuality in this species, as suspected by other workers (Shapiro, 1987). Males collected were smaller than most immature females, hence it was hypothesized that such male groupers had not passed through a functional adult female phase but had developed through a juvenile phase, which possessed ovarian structures, as reported in other gonochoristic fishes (Takahashi and Shimizu, 1983; Cole and Robertson, 1988; Matsuyama et al., 1988). Juvenile intersexuality in *E. suillus* probably explains why sex change in groupers occurs all year round, rather than being limited to the immediate postspawning period (Shapiro, 1987). Juvenile hermaphroditism has also been reported in *Oncorhynchus mykiss*, *Macropodus concolor*, *M. opercularis*, *B. rerio* (Chan and Yeung, 1983) and *Chrysophrys auratus* (Francis and Pankhurst, 1988). This is in contrast with other fishes which undergo a functional female phase before becoming males (Chan and Yeung, 1983; Yeh et al., 1986; Abu-Hakima, 1987; Kuo et al., 1988).

A decrease in the gonad weight and GSI in all fish sampled during sex inversion has also been reported in *C. julis* (Bruslé, 1987), *E. fario* (Kuo et al., 1988) and *T. duperrey* (Nakamura et al., 1989). This decrease probably represented inversion from female to male due to the marked decrease in size of the primary oocytes during their degeneration.

There were indications that development of gametic cells proceeds anteriorly in the gonads of *E. suillus* during sex inversion. The middle and poste-

rior regions of the gonads in some fish examined were more developed than the anterior regions. Similar directional differentiation in the transforming gonad has also been reported for *Coris julis* (Bruslé, 1987) and *Chrysophrys auratus* (Francis and Pankhurst, 1988).

In summary, induction of sex inversion in juvenile *E. suillus* by MT was probably synergistic with age and size. Spermatogenesis and spermiation occurred only in larger fish at 2 years, regardless of treatment. However, spermatogenesis was accelerated and maintained by prolonged treatment with high doses of MT ($5\,mg\,kg^{-1}$ BW), even in smaller-sized fish. The reproductive biology of *E. suillus*, especially its sexual patterns and reproductive cycles, needs to be investigated further so that the influence of factors inducing sex change may become clearer.

Acknowledgments

We thank the staff of the Fish Breeding and Microtechnique Services Laboratory for technical assistance, Isidro Tendencia for the photomicrographs, Thomas F. Hourigan for fruitful discussions, and Clarissa L. Marte and Gerald F. Quinitio for reviewing the manuscript.

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17α-メチルテストステロン注射によるハタ科魚類の一種 *Epinephelus suillus* 幼魚における性転換の誘導

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Epinephelus suillus の2歳の幼魚（平均体重1.2kg）を各8–9尾の4群に分け，0.5–5.0mg/kg 体重の 17α-メチルテストステロン (MT) を15日毎に筋肉内に注射した．15日毎に生殖腺生検と腹部搾出試験を行ない，6および12回の注射の後に魚を屠殺した．実験開始時の対照魚は，一次卵母細胞より成る未熟な卵巣を有していた．MTの6回注射後には，調べたすべての魚の生殖腺に体細胞と生殖原細胞の増殖が観察され，最小体重 1.2kg の魚の生殖腺には，一次卵母細胞退行と造精細胞の存在が認められた．0.5および1.0mgのMTを投与された大型魚（体重1.5kg）は，MTの積算投与量がそれぞれ5および12mgに達した後に放精をみせた．しかし，同じ処理郡の小型魚の生殖腺は，6回注射後 (0.7–1.0kg) および12回注射後 (0.6–1.3kg) にも一次卵母細胞と生殖原細胞を有するにすぎなかった．対照的に，5mgのMTで処理されたすべての魚は，6回注射後（体重 1.2–1.6kg）および12回注射後（体重0.8kg）には活発な精子形成を営む精巣を有していた．*E. suillus* 幼魚における雌から雄への性転換の誘導は，おそらく年齢および体重と相乗的になされるらしい．