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The sexuality of cultured hermaphroditic fish species : analysis of morphological and endocrinological features in a protogynous hermaphrodite, *Epinephelus microdon*, as a basis for further research to control reproduction in the grouper

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Abstract — *The polynesian grouper,* **Epinephelus microdon**, *was chosen as an experimental model in order to investigate about factors controlling sexuality in groupers.*

232 fish were captured in the wild and sacrificed, 124 of these fish were analyzed for sex steroids. Sex was determined by histological observation. Female and male fish were respectively found mostly in the smaller and the larger weight range whereas fish with intersexual gonads were observed in a large intermediate range (0.5 to 1.9 kg). However, some large females could also be observed. The maximum proportion of mature fish was observed in may 86, but interannual variations could occur. Plasma and gonadal concentrations of testosterone, 11β-hydroxyandrostenedione and estradiol exhibited some variations depending on sex and maturity stage. The most striking result is the similarity between the endocrine steroid pattern of intersexual individuals and immature females, characterized by a high ratio 11β-hydroxyandrostenedione/11-ketotestosterone.

50 fish captured in the wild and individually identified were maintained in captivity during one year and submitted to periodic blood samplings and gonadal surgical biopsies. Protogynous sex-inversion was observed in 3 cases, whereas 3 cases of sex-reversion (from male into female) and 2 cases of inversion immediately followed by reversion were also observed. Stable females and males exhibited sex-specific plasma profiles of estradiol and 11ketotestosterone. The occurence and the type of sexual change is hypothesized to be related to the initial sex ratio in tanks and to be partly regulated by social factors.

INTRODUCTION

Various cultured fish species of potential interest for aquaculture are successive hermaphrodites. Some are protandric, such as the seabass or barramundi, *Lates calcarifer* (Moore, 1979; Davis, 1982) and the sea bream *Sparus aurata* (D'Ancona, 1941, 1949, Zohar et *al.*, 1978), some others are protogynous, such as the grouper *Epinephelus tauvina* (Tan and Tan, 1974). This kind of sexuality raises several problems for broodstocks management, concerning the prediction of natural sex inversion (age and/or size) and the artificial control of sex. Steroid treatments have been used successfully in several gonochoric species in order to control sex in fish culture (reviewed by Hunter and Donaldson, 1983), but they appear to pose problem in amphisexual fish. According to Reinboth (1987), there has been no example that a steroid could promote sex-inversion from male to female in a protandric species. Although some success has been obtained in protogynous species, many problems still remain in order to optimize treatments. In groupers for example, sex-inversion has been attempted through the empirical administration of androgens. The results were negative with testoterone implants in *Thalasoma bifasciatum* (Kramer et *al.*, 1988) but they may appear encouraging with synthetic androgens in the food, since spermiating males can be obtained after oral application of methyltestosterone during several months in *E. tauvina* (Chen et *al.*, 1977), in *Mycteroperca microlepis* (Roberts and Schlieder, 1983) and in the blue-spotted grouper, *E. fario* (Kuo et *al.*, 1988). However, artificially sex-inversed males can spontaneouly reverse into females after cessation of the androgenic treatment (Chen, 1979; Roberts and Schlieder, 1983), or fertilization attempts using the sexually reversed males can be unsuccessful (Kuo et *al.*, 1988). Such drawbacks are not really surprising, taking into account past conflicting experiments performed in various ambisexual fish (see Reinboth, 1970). So far, only few physiological proofs have been supporting the hypothesis by Yamamoto (1969), according to which the natural inducers of gonadal differentiation in teleosts would be steroids (Van den Hurk and Slof, 1981; Nakamura and Nagahama, 1985; Baroiller et *al.*, 1988). As underlined by Reinboth (1988), steroid hormones are still supposed to play a major role in sex-inversion, but a cause and effect relationship has not yet been established. Obviously, more studies are required in order to characterize possible specific steroid fluctuations announcing or accompanying sex-inversion at both endocrinological and gonadal levels.

However, experimentation in the above-mentionned cultured species may encounter limitations due to the large size of the fish. Therefore, a smaller sized polynesian species, *E. microdon*, available in the wild (Tuamotu archipelago), was chosen as an experimental model. The first step, reported here, was to describe some general morphological and endocrinological features of each sexual phase in fish either sacrificed just after capture or maintained in tanks and submitted to periodic sampling of blood and gonad by surgical biopsy. In the latter animals, several inversions (from female to male) and reversions (from male to female) were observed during captivity. A further detailed individual endocrinological and histological analysis of these phenomenons will be published separately.

MATERIAL AND METHODS

Groupers from the species *Epinephelus microdon* were caught with line in Tikehau atoll (Tuamotu archipelago), kept for 1 to 5 days within a net cage in the lagoon, then transported by boat (22 hours) within small oxygenated sea water tanks (100 kg fish/m^3) to the Pacific Oceanic Center (C.O.P.) in Tahiti (French Polynesia) where they were either sacrificed or placed in rearing tanks (30 m^3) for further experiments.

Each sacrificed fish was killed by a knock on the head. A 5 ml blood sample was taken from a caudal vessel using a syringe previously rinced with a solution of sodium heparinate (1000 UI/ml in NaCl 0.9 %), cooled at 4°C and centrifuged (10 mn at 3000 g). The supernatant plasma was kept frozen (-20°C) untill subsequent steroid assays. Various body measurements were taken : weight (whole or eviscerated body, gonad, liver), standard length, lengths between the nose and the operculum, the dorsal and the pelvian fins, and the body maximal perimeter. A piece of gonad in median position was put into Bouin-Hollande fixative and further processed for histological examination. Rest of the gonads was kept frozen (-20°C) for steroid assays.

Fifty fish captured between February and May 1986 were individually tagged and kept for at least one year in tanks supplied with sea water from the lagoon, in order to follow possible sex changes in different situations of initial sex ratio. They were fed with a mixture of 50 % fish meal and 50 % crushed fresh fish (bonito). Every two months, these fish were anaesthetized (phenoxy-ethanol, 0.06 % in sea water) and submitted to the following operations :

— body external measurements and blood sampling (2 ml) for plasma preparation in the same way as for sacrificed fish,

— surgical sampling (biopsy) of a small piece of gonad (2-5 mm^3), processed for further histological observation. The piece of gonad was taken alternatively in each gonad after a short incision (2-4 cm) in the abdominal wall. Stitches were removed one week after operation.

After each operation the fish were treated during one hour in a solution of formalin (100 ppm) and malachite green (3 ppm) in sea water.

— On the first sampling time, a rapid and approximative determination of the gonadal sex was performed on a squash of fresh gonadal tissue taken by aspiration with a catheter introduced through the genital papillae and observed under microscope at a low magnification ($\times$ 20). On the basis of this rough sex-determination, the fish were distributed into 3 tanks, in order to reach 3 different « expected » sex ratios (50, 100 and 25 % females, respectively).

— On the final sampling time (usually after more than one year), the fish were sacrificed, following the same protocol as for wild animals.

Fixed gonad samples were embedded in paraffin, sectionned 4 μm thick, and colored with Regaud hematoxylin, orange G and anilin blue (Gabe, 1968).

Frozen samples for steroid assays were processed as follows :

— plasma samples were extracted twice in 50/50 cyclohexane/ethyl acetate,

— the whole gonad (when weighing less than 2 g) or a 2 g sample was first homogenized in 1 ml saline solution (NaCl, 0.9 %), before addition of recovery tracers (tritiated estradiol and testosterone). After addition of 4 ml ethanol, the sample was homogenized again, then submitted (twice during 15 s.) to ultra-sonic sounds (Branson sonifier B-12, 20000 Hz), and centrifuged 15 min. at 3000 g. After recovery of the supernatant, the pellet was extracted once more in aqueous ethanol (80 %), re-centrifugated, and the two supernatants were pooled. This ethanolic preparation was partially evaporated, then extracted 3 times with 5 ml dichloromethane. The last organic extract was dried and solubilized in 0.2 ml ethanol. Steroids were separated by chromatography on sepha-dex column (0.5 cm diameter, 14 cm height) using dichloromethane/methanol 95/5 eluent. Elution volumes are recovered in the following succession :

— fraction 1 (1 ml) : discarded,

— fraction 2 (0.8 ml) : androstenedione,

— fraction 3 (1.2 ml) : testosterone (T), 11-ketotestosterone (11KT), 11βOH-androstenedione (11βOH δ 4),

— fraction 4 (1.4 ml) : estrone (E1),

— fraction 5 (3.1 ml) : estradiol-17β (E2).

Before assay, fractions 3 and 5 from gonadal samples extracts and plasma samples were recovered respectively with 0.9, 0.5, and 0.8 ml phosphate buffer (0.01 M, pH 7.25) with 0.1 % gelatine. Then, steroid assays were performed as described by Fostier et al. (1978, 1982). 11βhy-droxy-androstenedione antibody was a gift from Prof. R. Reinboth (Mainz Univ.)

RESULTS

Wild fish

Sex determination in immature or intersexual fish was performed on histological sections using classical criteria already described in the grouper (Bruslé and Bruslé, 1975; Abu-Hakima, 1988). Moreover, in order to establish possible relationships between endocrinological and histologi-cal observations, we established a simplified scale of gonadal maturity (table 1).

The weight repartition of each sex in the whole sampled population is shown in fig. 1 (no fish weighing less than 0.3 Kg could be caught by our technic of capture). Most female fish were found in the range between 0.3 and 1.7 Kg, but some large female were also observed. Male fish occured in the range 0.5-2.3 Kg, and intersexual fish between 0.5 and 1.9 Kg.

The maturity stages of sampled groupers revealed very heterogeneous at any sampling time. Nevertheless, the proportion of mature animals (stage 4) in the successive samples exhibited a significant variation throug-hout the year, with a maximum in May 86 (Fig. 2). However the proportion

of mature fish appeared significantly different in February 86 and in February 87, showing in addition, that some interannual variations could occur.

The mean plasma concentrations of 11ketotestosterone, testosterone and estradiol are presented in Fig. 3. For each sex, data were grouped

Tab. 1. — Scale of gonadal maturity in the grouper *Epinephelus microdon*

| Sex	Index	Histological criterion (germinal cells)
male	1	Only spermatogonia
	2	from spermatogonia to spermatocytes
	3	from gonia to spermatids (some spermatozoa)
	4	mostly spermatozoa (spermiation)
	5	general atresia
female	1	oogonia and basophilic oocytes
	2	previtellogenetic oocytes
	3	early vitellogenesis (yolk < 50 % ovarian volume)
	4	**late vitellogenesis (yolk > 50 % ovarian volume)**
	5	**general atresia**
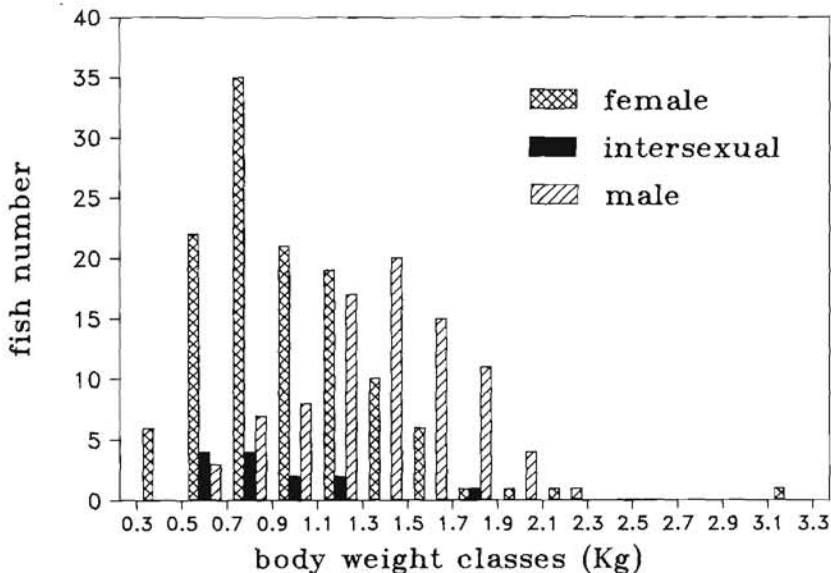
Figure 1. — Weight repartition of each sex type in the whole sampled population of wild *Epinephelus microdon*.
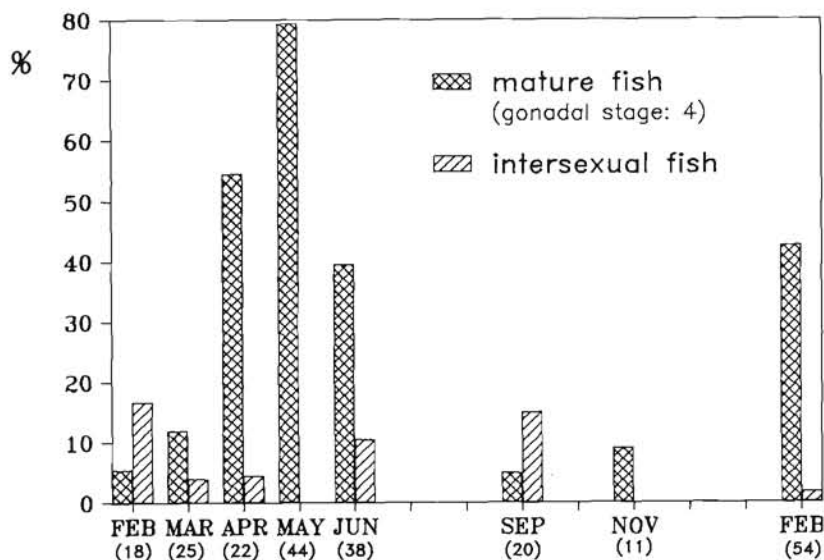
Figure 2. — Percentages of mature fish (males and females at gonadal stage 4) and fish with intersexual gonads in the various samples of wild *E. microdon*. Numbers between brackets represent the total number of fish captures in each sample.
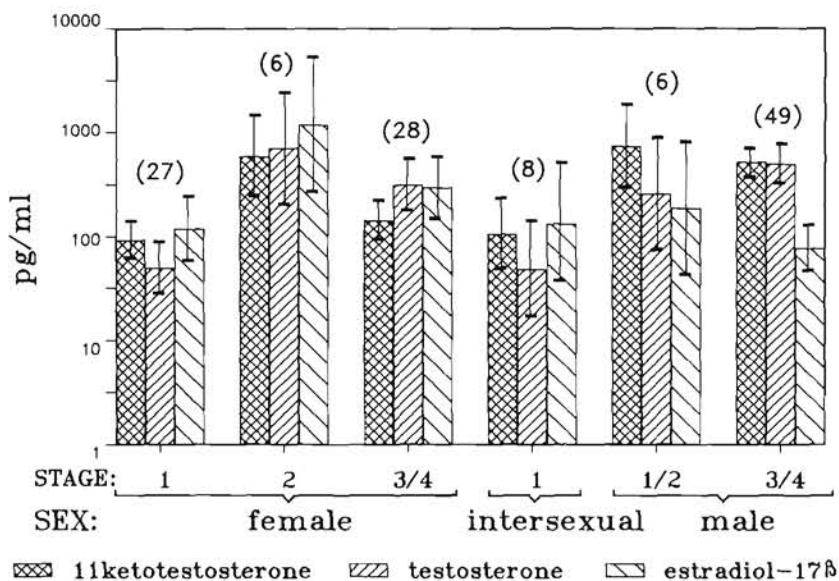

Figure 3. — Mean concentrations of 11-ketotestosterone, testosterone and estradiol in the plasma of female, intersexual and male fish at various stage of gonadal maturity. Vertical bars represent the confidence limits of the mean at the probability level p = 95 %. Numbers between brackets represent the total number of fish at each stage for each sex type.

together according to maturity stages and independently of sampling times. Moreover data concerning successive maturity stages were also grouped together when not significantly different (vitellogenetic female, stages 3 and 4; males stages 1 and 2, 3 and 4). Plasma concentrations of the three steroids assayed are the lowest and very similar in both immature females and intersexual males. Interestingly, all categories of fish exhibit noticeable levels of E2, including males and intersexual fish. Only previtellogenetic females (stage 2) present E2 levels significantly higher than other fish categories. As a whole, differences between sexes appear very small at the plasmatic level, with a tendancy for an inversion in the ratio E2/androgens between females and males.
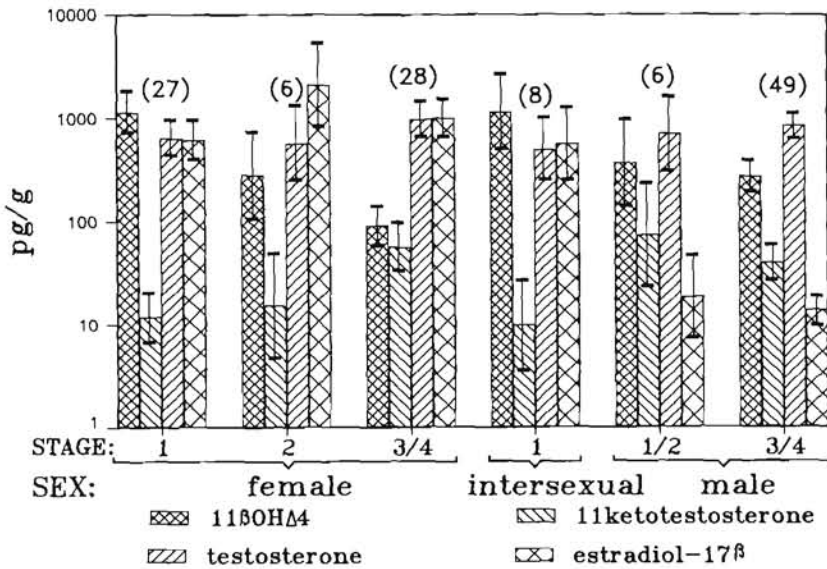

Figure 4. — Mean concentrations of 11β-hydoxyandrostenedione, 11ketotestosterone, testosterone and estradiol in the gonads of female, intersexual and male fish at various stage of gonadal maturity. Vertical bars and numbers between brackets like in fig. 3.

The mean gonadal concentrations of 11βOH∆4, testosterone, and 11ketotestosterone are shown in Fig. 4. Data are grouped together as above. The most striking features are :

— the mean concentrations of each steroid assayed look very similar in immature females and in intersexual fish, with high levels of 11β OH β 4, testosterone and estradiol, and low levels of 11ketotestosterone,

— although present at much lower concentrations than in females, E2 is also still present at noticeable levels in male gonads.

Captive fish

After histological control, it appeared that some of the rough sex determinations performed on a squash from a gonadal sample taken by

aspiration through a catheter, were wrong. Therefore, the actual sex ratio at the beginning of the experiment is somewhat different from the sex ratio expected initially in the different tanks (Fig. 5). However, the final sex ratio observed when the fish were sacrificed after one year, at the end of the experiment, showed interesting changes compared to the actual initial values (the assumption about the reality of these changes, some of which were quite unexpected, was based on the histological observation of the successive samples of gonads taken by surgical biopsy every two months on each individual, and of the final sampling of the whole gonad, at the end of the experiment) :

— in tank Nr 1, where males were in large excess, 3 reversions (change from males into females) occured, leading to a final sex ratio close to 50 %/50 %,

— in tank Nr 2, where the sex-ratio between males and females was initially equilibrated, no changes were observed in the final sex ratio. However, it appeared from the histological observation of successive individual gonadal samples, that one inversion and one reversion had occured during the experiment,

— in tank Nr 3, in which females were in excess, 2 inversions and one reversion occured.

The different kinds of sexual evolution during the experiment are summarized in Fig. 6.

	expected	actual	final	
1	♂ 8 (50%) ♀ 8 (50%)	♂ 12 (75%) ♀ 4 (25%)	♂ 9 (56%) ♀ 7 (44%)	3 reversions
2	♂ 0 (0%) ♀ 18 (100%)	♂ 8 (44%) ♀ 10 (56%)	♂ 8 (44%) ♀ 10 (56%)	1 inversion 1 reversion
3	♂ 4 25%) ♀ 12 (75%)	♂ 4 (25%) ♀ 12 (75%)	♂ 5 (31%) ♀ 11 (69%)	2 inversions 1 reversion

sex−ratio

TANK NUMBER

Figure 5. — Evolution of sex ratio in captive groupers *E. microdon* placed during 14 months in 3 tanks. The « expected » sex ratio was an initial estimation obtained by the observation of squashes from fresh gonadal samples taken with a supple catheter at the beginning of the experiment. The « actual » sex ratio was obtained after histological observation of the first gonadal sample, at the beginning of the experiment. The « final » sex ratio was obtained by histological observation of the gonads of sacrificed fish, at the end of the experiment. The occurence of so-called « inversions » and « reversions » was established by the histological observation of successive gonadal samples from each fish.
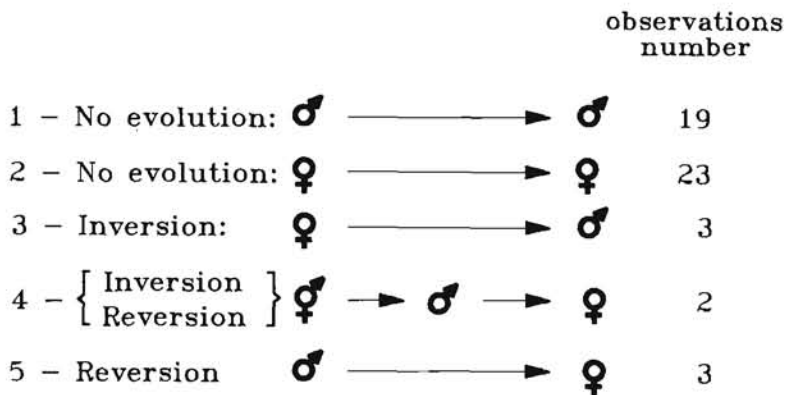
observations
number

1 – No evolution: ♂ ─────────► ♂   19

2 – No evolution: ♀ ─────────► ♀   23

3 – Inversion: ♀ ─────────► ♂   3

4 – { Inversion
      Reversion } ⚥ ─► ♂ ─► ♀   2

5 – Reversion   ♂ ─────────► ♀   3

Figure 6. — Schema of the different kinds of sexual evolution observed in captive groupers during the experiment.

The mean evolution of plasma levels of 11ketotestosterone, testosterone and estradiol in « stable » females and males (exhibiting a stable gonadal sex-phenotype throughout the experiment) are respectively shown in Fig. 7 and 8. Besides the evolution of these steroid profiles throughout the year, partially linked to the evolution of gonadal maturity, the most striking feature lies in the differences between males and females concerning the ratio between estradiol and 11ketotestosterone. Whereas females are characterized by high levels of estradiol and low levels of
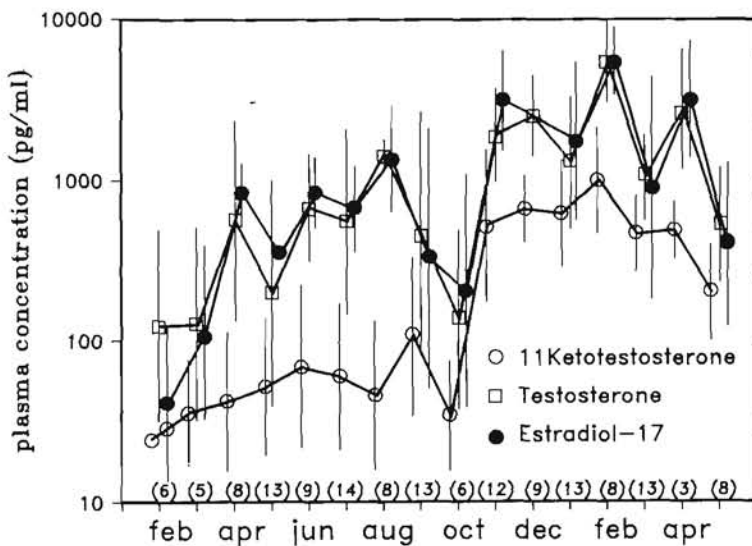
Figure 7. — Evolution of the mean concentrations of 11-ketotestosterone, testosterone and estradiol in the plasma of sexually stable captive females. Vertical bars represent the standard deviation of the mean. Numbers between brackets represent the number of sampled fish at each sampling time.

11ketotestosterone, males are characterized by an opposite situation. Testosterone levels keep in the same order of magnitude in both sexes.
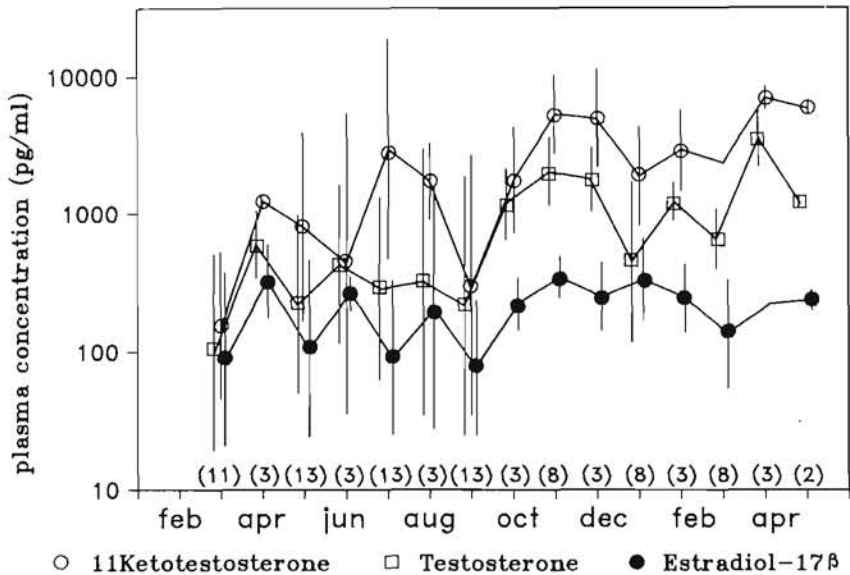

Figure 8. — Same as fig. 7 in sexually stable males.

DISCUSSION

Reproduction in the polynesian grouper, *Epinephelus microdon,* is associated with migratory and gathering phenomenons (for example in the pass of Tikehau atoll, as observed in May 86), like in other grouper species (Johannes, 1978, Colin et *al.,* 1987, Smith, 1972). In May, our samples contained only such fish, thus confirming the seasonal character of the phenomenons. But they were also probably biased to the detriment of immature or intersexual animals. Preferential spawning seasons have been mentionned in other groupers (Moe, 1969, Bruslé, 1976, Johannes, 1978, Bouain, 1980, Chen et *al.,* 1980, Moore and Labisky 1984, Loubens, 1980). However, fully mature *Epinephelus microdon* were caught all year long, which is in agreement with the data of Loubens (1980). Moreover, the seasonnal frequency of mature fish may exhibit interannual variations, as shown by the comparison of the proportion of mature fish in February 86 and in February 87.

The proterogynous character of a species may be deduced from the analysis of the size structure of each sex within the whole population (Sadovy and Shapiro, 1987). In groupers, secondary sexual characters are generally lacking (with possible exceptions such as in *Centropistes striatus,* according to Lavenda, 1949), thus often requiring a direct or even an histological observation of the gonads. Such analysis of the size structure of the population have been performed by considering the repartition of

weight (Bruslé and Bruslé, 1975 a,b, 1976; Barnabé, 1974), body length (Moe, 1969; Bouain, 1980; Bruslé and Bruslé, 1975 a,b, 1976; Colin et al., 1987; Thompson and Munro, 1978), and age (Moe, 1969; Chen et al., 1980; McErlean and Smith, 1964; Moore and Labisky, 1984). The wide overlapping between size criteria of each sex show a great variability in the time of sex-inversion as already underlined by Bruslé (1982), Thompson and Munro (1978) and Moe (1969). In The case of *Epinephelus microdon*, we tried to use various morphometric measurements, but we could not find any criterion better than weight, in order to discriminate between sexes.

Finally, we observed the occurrence of very large females of *Epinephelus microdon*. This phenomenon was also observed in other grouper species, and it was suggested that such large females could have escaped to the process of sex inversion which would not be a general rule. Without eliminating such a possibility, our own results showing that reversions from male to female can occur in captivity in *E. microdon* also suggest that the same kind of sex reversion could occur in the wild.

According to our results, and from an endocrinological point of view, sexual inversion in *Epinephelus microdon* looks like a return to the immature female stage, before the development of male gametogenesis. However, this hypothesis would require to be verified in the case of other steroids than those assayed in the present study. For example, 5-reduced androgens might be important in the steroid biosynthesis pathways of protogynous species (*Monopterus albus* : Yeung et al., 1985; *Coris julis* : Reinboth and Becker, 1984; *Spicara maena* : Reinboth, 1979).

The comparison between the various sexual stages shows no qualitative difference. Such an ubiquity of androgens and estradiol has already be mentionned in other ambisexual fish (Yeung and Chan, 1987 a,b; Shapiro et al., in press). Besides, the levels range found in our data like in others, appear very low compared with those reported in gonochoric species such as salmonids for instance (Fostier et al., 1983).

Considering our results on the gonadal concentrations of steroids (Fig. 4), the intersexual state appears to be characterized by a decrease of 11-ketotestosterone and a rise of 11β-hydroxy-androstenedione. A possible role for 11β-hydroxylation in the male sexual determination has already been suggested in gonochoric species (Van den Hurk and Slof, 1981; Baroiller et al., 1988). Most endocrinological studies on the sexual steroids in ambisexual fish have been dealing with gonadal steroidogenesis *in vitro* (Reinboth, 1979; Chan and Yeung, 1983; Fostier et al., 1983), and only few data are available about plasma levels (Idler et al., 1976; Chan et al., 1975; Yeung and Chan, 1987a,b; Shapiro et al., in press); Moreover, no measurements were performed so far, to our knowledge, in the gonadal tissue itself. Most of our own assays in sacrificed *Epinephelus microdon* were carried out both on plasma and gonadal samples (Fig. 3 and 4). The steroid concentrations measured in each case are not stricly comparable since plasma levels are not only determined by the gonadal synthesis. Plasma levels are regulated by the gonadal secretion rate, the synthesis and metabolism of other peripheral steroids, and the metabolic clearance rate. Nevertheless, a similar evolution as a function of maturity stages can be observed both in gonads and in

plasma in the case of estradiol, which also appears as the best discriminating steroid between males and females. Besides, a similarity between the steroid patterns of intersexual fish and immature females can be observed either at the plasma or at the gonadal level. From our data, the rise in 11β-hydroxy-androstenedione level could be related to a decrease in 11β-oxydoreductase activity leading to 11-ketotestosterone. Such a fact, in addition to the further decrease in estradiol levels in males (due to a lowered aromatase activity ?), may be important from a practical point of view. The stimulation of a particular physiological endocrine pattern in order to promote sexual inversion may require more cues than increasing the level of only one specific steroid. For that purpose, the use of specific steroidogenesis blockers may provide useful tools, in addition to the administration of exogenous hormones, in order to modulate the ratio between various hormones.

Our data on the evolution of sex ratio in captive fish, showing several occurences of protogynous sex inversion, comfirm the ambisexual character of the polynesian grouper, *Epinephelus microdon*. This species can therefore be considered as protogynous like other grouper species in which sufficient data are available, taking also into account the weight repartition of sampled wild fish among which no male could be found below a certain weight. However, our data also bring the proofs that reversions from male to female are possible and occur as frequently as protogynous inversions, in the conditions of captivity. To our knowledge, this is the first time such a data is observed in any grouper species. Considering the evolution of sex ratio in the different tanks as a function of the initial ratio, it must be noticed that the sexual changes which occured contributed to some readjustment towards a ratio close to 1 male/1 female, when the initial ratio was unbalanced. Although these changes cannot be considered as significant, considering the number of inversions and reversions, the total number of fish, and the absence of repetitions in the experiment, they bring some support to the hypothesis that social factors could participate in the determinism of sexual changes in the grouper as well as in other serranids (see Shapiro, 1986, for review).

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