Proc. Indian natn. Sci. Acad. B 45 No. 5, pp 421–428 (1979)

On Steroidogenic Pathways in Ambisexual Fishes*

RUDOLF REINBOTH
Institut fur Zoologie, Johannes-Gutenberg-Universitat, Saarstrasse 21, D 6500 Mainz F R G

In vitro incubations with labelled precursors (testosterone and progesterone) have been carried out with gonadal tissues from various ambisexual teleosts during different physiological stages: a simultaneous hermaphrodite (*Serranus cabrilla*), protogynous (*Coris julis*, *Spicara maena*) and protandric (*Pagellus acarne*) species were used. After extraction and purification by chromatography on paper and thin-layer (Kieselgel) metabolites were characterized by their mobility, after derivative formation (acetylation, oxidation, reduction) and crystallization to constant specific activity. A list of 26 metabolites which could be characterized so far is given. Data are presented which indicate correlations between biological events related to sex-inversion and peculiarities of steroid biosynthesis. But the pattern of metabolite formation varies greatly between incubations from one and the same species. Our data suggest caution towards conclusions that are derived from single experiments which take into account a fairly limited number of well-known steroids only.

Introduction

A pioneering study on the relationship between steroids and spontaneous sex inversion was provided by Chan and Phillips (1969) on the protogynous ricefield eel *Monopterus albus*. The authors came to the conclusion that in the female phase estrogens predominate and that during the intersexual stage a hormonal shift from estrogen to androgen occurs which is concomitant with the structural transformation of the gonad. Later studies *in vivo* on levels of sex steroids in peripheral plasma revealed corresponding hormonal patterns (Chan et al. 1975). For the protandric sea bream, *Sparus auratus*, Colombo et al. (1972) claimed that gonadal steroids do not differ from those which are known—so far—from gonochoristic species but they emphasized that during sex inversion gonadal steroid biosynthesis is probably at a minimum. On the same species, Eckstein et al. (1978) reported, as their main result, that (mammalian) gonadotropin inhibits the production of testosterone (from androstenedione as the precursor) in testicular tissue prior to the transformation of functional males to females. Idler et al. (1976) reported surprising amounts of 11β-OH-testosterone and a lack of 11-ketotestosterone in blood or plasma of several ambisexual species. d' Istria et al. (1973) observed testosterone but neither estrone nor estradiol

*With support by the Deutsche Forschungsgemeinschaft

by radioimmunoassay in plasma from the hermaphroditic *Serranus cabrilla.*

It is hard to see any guiding line which might interconnect these data for a better understanding of the strange phenomenon of teleost ambisexuality in relation to steroid metabolism. Studies in the author's laboratory on several species which represent different types of ambisexuality (Reinboth 975a) do not improve the rather confusing picture.

Recent Investigations

In the past we continued to analyze steroid metabolites from the same species which were used in the previous study (Reinboth 1975). *Spicara maena* which belongs to the emmelichthyids was included as another protogynous type (*cf.* Reinboth 1962).

Table 1 lists all the steroids for which crystallization to constant specific activity was achieved—according to the standards established by Axelrod et al. (1965)—as the final step of identification with the technical equipment being at our disposal. There are a few other fully saturated steroids which have been characterized by their chromatographic mobility and the chromatographic behaviour of derivatives. The confirmation of their identity by the crystallization procedure is a matter of time. The number of samples indicated in table 1 is rather fortuitous. Therefore, it must be emphasized that none of the compounds has been recognized as being specific for either a species or the sex-type of gonadal tissue. This statement, however, does not preclude the possibility (or even likelihood) that some of the substances may be correlated to peculiar biological conditions when the quantitative study is carried further than at present.

To the best of my knowledge the entire literature on steroid metabolites from gonadal tissues of teleosts does not show a similar variety as presented in table 1. In spite of a fairly intensive search we have not been able to get indications for the occurrence of 20β-reduction and 21-hydroxylation. The lack of saturated C_{19} and C_{21}-steroids with a functional group at C-11 in our list must be attributed to difficulties in obtaining all the isomers which have to be taken into consideration as authentic cold substances on the commercial market. There are good reasons to assume their presence from the results of oxidations. In one case we oxidized a steroid with acetylable HO-group(s ?) to 5α-pregnane-3,11,20-trione and achieved its crystallization to constant specific activity after having added 10 mg of the authentic steroid as carrier.

Whereas we have been able to characterize nearly all metabolites which show up in appreciable amounts when testosterone is used as the precursor, the situation remains very unsatisfactory in the case of progesterone. This applies mainly to the large amount of polar material (partly more than 50% of total radioactivity recovered in the neutral fraction). Although we do not have difficulties in separating the 3, 11, 17-androstanetriones and 3, 11, 20-pregnane-triones the results of oxidations remain confusing. After a systematic investigation of different oxidation procedures applied to a large number of pure C_{21}-steroids we are unable to share the opinion of Brooks and Norymberski (1952) that oxidation of C_{21}-steroids should be a useful tool in order to determine the nature of their side chain. At the moment we only tend to say that oxidation by the CrO_3-pyridine-complex (Poos et al. 1953) seems to be the safest procedure in order to avoid major destructions.

It appears that steroids with functional groups other than at C-3, 11, 17 and 20 might be introduced into the molecule by gonadal tissue. But this point awaits clarification by more sophisticated analytical methods of organic chemists. A joint study with such experts is in progress. With some

Table 1 *List of metabolites which have been crystallized to constant specific activity*

Compound	Precursor	Number of Samples	Species (Sex)
5α-Androstane-3α, 17β-diol	Testosterone	1	*Spicara maena* (♀)
5α-Androstane-3β, 17β-diol	,,	1	*Serranus cabrilla* (♂)[1]
5α-Androstane-3, 17-dione	,.	3	*Serranus cabrilla* (♂), *Spicara maena* (♀), *Pagellus acarne* (→ ♀)[2]
5β-Androstane-3, 17-dione	,,	1	*Serranus cabrilla* (♀)
5β-Androstane-3α, 17β-diol	,,	7	*Coris julis* (*pr.*♂)[3], *Pagellus acarne* (♂) *Spicara maena* (♂, ♀), *Serranus cabrilla* (♀)
5β-Androstane-3β, 17β-diol	,,	3	*Serranus cabrilla* (♀)
5α-Androstane-3α-ol-17-one	Progesterone, Testosterone	2	*Spicara maena* (♀)
5β-Androstane-3α-ol-17-one	Testosterone	3	*Pagellus acarne* (→ ♀), *Serranus cabrilla*(♀)
5α-Androstane-17β-ol 3-one	,,	1	*Serranus cabrilla* (♂)
5β-Androstane-17β-ol-3-one	,,	4	*Coris julis* (*pr.*♂)[3], *Serranus cabrilla* (♀)
5β-Pregnane-3α, 20α-diol	Progesterone	2	*Serranus cabrilla* (♂, ♀)
5α-Pregnane-3, 20-dione	,,	2	*Coris julis* (♀), *Serranus cabrilla* (♂)
5β-Pregnane-3, 20-dione	,,	2	*Serranus cabrilla* (♂)
5α-Pregnane-3β-ol-20-one	,,	2	*Coris julis* (♀), *Serranus cabrilla* (♂)
5β-Pregnane-3α-ol-20-one	,,	3	*Pagellus acarne* (♂→[4]), *Serranus cabrilla* (♂), *Spicara maena* (♂)
5β-Pregnane-3β-ol-20-one	,,	1	*Serranus cabrilla* (♀)
5β-Pregnane-3α, 17α, 20α-triol	,,	(2)[5]	*Serranus cabrilla* (♀)
5β-Pregnane 17α-ol-3, 20-dione	,,	5	*Serranus cabrilla* (♂, ♀), *Spicara maena* (♂, ♀)
4-Androstene-11β, 17β-diol-3-one	Testosterone	9[6]	*Coris julis* (♀), *Pagellus acarne* (♂), *Serranus cabrilla* (♂, ♀), *Spicara maena* (♂)
4-Androstene-3, 17-dione	Progesterone, Testosterone	11	*Coris julis* (*pr*, ♂), *Pagellus acarne* (→ ♀), *Serranus cabrilla* (♂, ♀), *Spicara maena* (♂, ♀)
4-Androstene-11β-ol-3, 17-dione	Testosterone	1	*Serranus cabrilla* (♂)
4-Androstene-17β-ol-3, 11-dione	,,	6[6]	*Coris julis* (→ ♀, *sec.* ♂)[8], *Pagellus acarne* (♂), *Spicara maena* (♀), *Serranus cabrilla* (♂, ♀)
4-Pregnene-11β, 17α-diol-3, 20-dione	Progesterone	2	*Serranus cabrilla* (♂, ♀)
4-Pregnene-11β-ol-3, 20-dione	,,	6	*Coris julis* (→ ♀, *pr.*♂). *Serranus cabrilla* (♂, ♀), *Spicara maena*(♀)
4-Pregnene-17α-ol-3, 20-dione	,,	4	*Coris julis* (*pr.*♂), *Pagellus acarne* (♀), *Spicara maena* (♂), *Serranus cabrilla* (♂)
1,3,5(10) Estratriene-3, 17β-diol	,,	1	*Spicara meana* (♀)

1) ♂ =ovarian+testicular tissue pooled; 2) → ♀ ovarian tissue from inverting specimen(s);
3) pr.♂ =primary male; 4) ♂→ testicular tissue from inverting specimen(s); 5) 2 crystallizations from the same incubation; 6) 1 crystallization as acetate; 7) 1 crystallization as acetate, 1 as adrenosterone after oxidation; 8) sec. ♂ =secondary male.

reservation we are inclined to say that under the experimental conditions that we have used C-17, 20-lyase-activity appears to be low. It is there, however, as proved by the fairly regular occurrence of minor amounts of 4-androstenedione. But in all instances we failed completely to trace C_{19}-steroids with functional groups at C-11. Likewise testosterone has never been found by us.

When we take a look at the metabolic activities of gonadal tissues from various species at different stages of their sexual life-history, several aspects start to emerge more clearly in spite of an uncomfortable variability among different incubations from material which seems to be alike biologically. Comments must be restricted to a few points only.

(A) Pagellus acarne

The protandric sea bream *Pagellus acarne* is particularly interesting due to the simultaneous presence of both testicular and ovarian tissue before the final stage of a functional female is reached. Both germinal tissues can be separated from each other mechanically and incubated as either "ovary" or "testis".

Contrary to the opinion by Colombo et al. (1972) on the related *Sparus auratus* (*cf.* comments by Reinboth 1975a) the metabolic activity of both gonadal tissues from inverting specimens of *Pagellus acarne* (collected in March and April—spawning season: July–September) is high. Surprisingly, the regressing testicular tissue is clearly more active in the metabolic transformation of testosterone than progressing ovarian tissue from the same specimens. The lowest amount of original substrate (less than 10% of total radioactivity recovered) was observed in regressing testis. About 50% of the metabolites were present as androstanedioles (mainly 5β-A-3α, 17β-diol). The total amount of 11β-OH testosterone and 11-ketotestosterone together was considerably larger than that of testosterone. But comparing the relative amounts of 11β-hydroxy and 11-ketotestosterone from two different incubations the ratio of both steroids was different. In one case, 11β-OH testosterone exceeded 11-ketotestosterone by far (*ca.* 3 : 1), in the other sample both steroids were present in similar amounts.

Incubations from the ovarian part of inverting specimens yielded a different pattern. Whereas in regressing testis no steroids less polar than testosterone could be found, such steroids (4-androstenedione, 5β-androstane-3α-ol, 17-one and 5α-androstanedione) were present in significant quantities.

With progesterone as precursor for the same biological material we also observed that similar quantities of regressing testicular tissue (about 0·5 g/incubation) left about 10% of the original substrate at the most. 5β-P-dione outweighed 5α-P-dione by far (about 2 : 1). Ovarian tissue from inverting specimens incubated with progesterone has not yet been extracted.

When ovarian tissues of functional females and testicular tissues of functional males (both of them from specimens of the same catch) are incubated, the metabolic transformation of the substrate seems to be larger by the male part—irrespective of the season (spring or late autumn).

(B) Spicara maena

In the protogynous *Spicara maena* (spawning season during late spring) I observed considerable differences between males and females and variations in relation to the season. In both sexes the metabolic activity is higher in spring than in autumn (judged by the quantities of remaining testosterone as substrate). The total amount of 11β-OH testosterone is always larger than that of 11-ketotestosterone. Both steroids are produced in larger quantities by testicular than by ovarian tissue, but it seems puzzling that their share among the metabolites is lower

in spring than in autumn. Less polar steroids (e.g. 4-androstenedione, androstanolones and androstanediones) represent a much larger proportion (35% or more of total radioactivity recovered) in spring than in autumn.

The saturated diketones merit a particular remark. In quiescent females the fraction of the androstanedione(s?) was about 3% or less. In incubations from maturing animals I observed a doubling or even tripling of this amount. In females 5α-androstanedione was the only isomer that could be found. In males the androstanedione-fraction likewise was much lower in autumn than in spring. But whereas in autumn 5β-androstenedione was found to be the only isomer, 5α- and 5β-androstanedione occurred at an approximate ratio of 1:1 in spring.

When progesterone was used as precursor in this species with maturing tissue 5α-pregnane-dion prevailed clearly in males whereas in females the 5α-isomer was the only one that could be found. 5β-pregnanedione was absent or hardly detectable.

(C) *Coris julis*

In the protogynous *Coris julis* testicular tissue becomes visible only after sex inversion has started by degenerative regression of the ovarian element (Reinboth 1962, 1975b). In addition there is a clear difference between primary and secondary males (Reinboth 1962, 1970, 1975b). Some results of our incubations may serve to illustrate problems that should caution everybody against hazardous conclusions from single incubations. Figure 1 shows the results from the first chromatographic run of the neutral fraction on paper in Bush B_3: (*a*) primary males, (*b*) secondary males, (*c*) females, (*d*) inverting specimens. Whereas *a–c* derive from animals of pooled catches within a few days in June (incubations done the same day nearly simultaneously), *d* stems (necessarily) from a later period in the year.

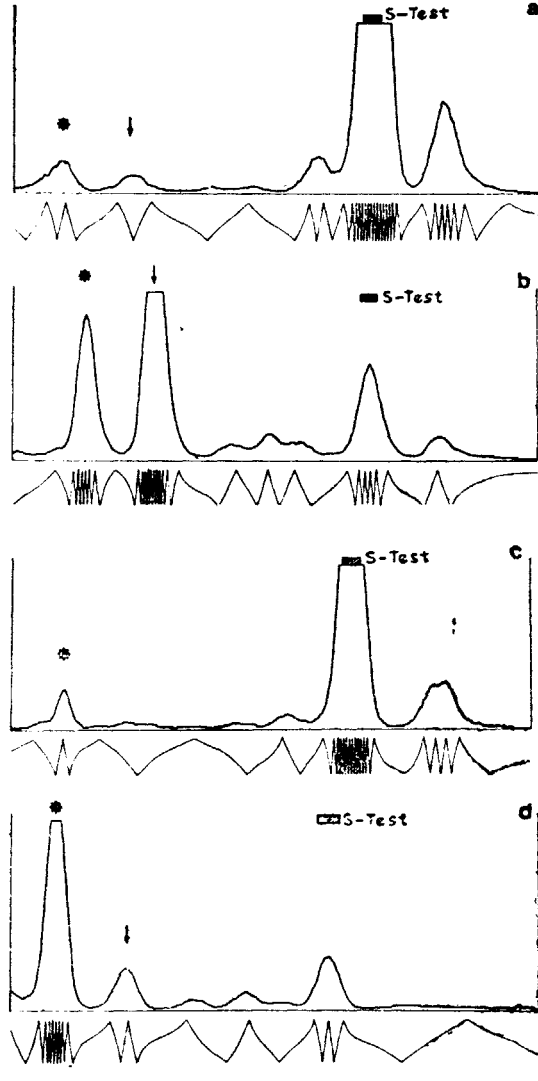

Figure 1 Scannograms from the first chromatographic run on paper (Bush B_3) of the neutral fraction of incubation media from the protogynous *Coris julis* (testosterone as precursor)

a, primary males; **b**, secondary males; **c**, females; **d**, inverting specimens (**a–c**: June 16; **d**: September, 12)

*=11β-OH-testosterone sed ↓ 11-ketotestosterone

The prominent peaks near the starting lines represent mainly 11β-OH-and 11-keto-testosterone respectively. It seems to be

obvious that the testicular tissue of secondary males is the most active sample and that the metabolic activity of ovarian tissue (being in the process of advanced vitellogenesis at this time of the year) is surprisingly low. The production of 11β-OH-testosterone in inverting animals is remarkably high. Yet, these patterns of metabolic activity could not be reproduced in a corresponding set of experiments carried out under the same experimental conditions in another year with a biological material which I consider(ed?) to be alike. I discovered that much less testosterone was metabolized by inverting specimens than in the case which is illustrated here. And the same holds true for the activity of gonads from secondary males. The only conclusions that one might draw at present are: (1) testes from secondary males metabolize testosterone more actively than testes of primary males, and (2) the metabolic activity of ovarian tissue generally appears to be relatively low.

With regard to the prevalence of 5α- over 5β-reduction I must refer at present to results from incubations when progesterone was used as the precursor. For example: 5β-pregnane-3,20-dione was the major isomer in both types of males and in inverting specimens the gonads of which had been incubated the same day (August 13). But in (functional) females incubated simultaneously 5α-pregnane-dione turned out to be the only saturated 3,20-dioxo-steroid.

(*D*) *Serranus cabrilla*

The survey of gonadal steroid metabolism in ambisexual species would remain incomplete without a few words on simultaneous hermaphrodites for which *Serranus cabrilla* is taken as an example. After qualitative pilot studies by myself, my coworker M Latz started to quantify the metabolites (expressed as per cent of total radioactivity recovered) which were isolated from the incubation media. Although our analytical procedures differed considerably in many details, the overall-picture of our results is in good agreement. Latz analyzed altogether 8 incubations (4♂, 4♀ tissues) whereas I worked on 4 incubations (2 of each sex from the same period within the animals' reproductive cycle). A few salient results are reported below; details will be reported elsewhere.

For the first time in our laboratory Latz paid attention to the occurrence of conjugated steroids (cf. Latz 1978). He observed that close to 30% of the precursor's radioactivity may become incorporated into conjugates (mainly sulfates). Ovarian tissue produces more conjugates than the testicular component and the quantity of a particular conjugated steroid may be larger than the corresponding free substance (e.g. 5β-A-3α, 17β-diol and 5β-A-17β-ol-3-one). In *Serranus*, 5β-reduction predominates by far in both sexes. One example may serve as illustration. From an incubation of ovarian tissue 39.2% of the total radioactivity which could be recovered was identical with the original substrate testosterone. The two 5β-androstanedioles representing 22.7%, 15.4% were 5β-androstane-(3α or 17β)-ol-(17 or 3)-one, and 2.3% could be identified as 5β-androstanedione. Likewise, when progesterone was taken as precursor, 5β-reduction was the most common metabolic pathway. Comparing *Serranus* with sex-inverting species a remarkable difference is the fact that production of C_{19}-steroids with a functional group at C-11 occurs at a much lower rate in ovarian than in testicular tissue from the same animals. And for ovarian tissue it seems to be a peculiar feature that 11-ketotestosterone outweighs 11β-hydroxytestosterone which—in a few cases—could not be found at all. But the same steroid is always formed by testicular tissue in large amounts. The ratio 11β-OH-T/11-keto-T was found to vary between 3 : 1 and 13 :1 .

(E) C_{21}-Δ^4-steroids

In table 1, 17α-OH-progesterone, 11β-OH-progesterone and 21-desoxycortisol are listed as Δ^4-steroids. The quantity of the latter compound is always low and might have been lost (at least in some cases) during purification procedures. The presence or absence of the other two steroids does not yet reveal any distinct patterns. In 6 incubations from *Serranus* (3 testicular, 3 ovarian), 17α-OH-P was always produced by the male component whereas the same steroid from ovarian tissue was found only once in autumn (quiescent period). With regard to 11β-OH-progesterone the *in vitro* biosynthesis could be observed in all species that were examined and in *Coris* and *Serranus* its production by both germinal tissues could be established safely (*cf.* table 1).

(F) Phenolic steroids

While work on purification of the phenolic steroids is not yet complete, it appears that aromatization is low (less than 1%) in all cases and sufficient amounts of estradiol-17β which can be used for crystallization are available only from ovarian tissue which is either mature or close to maturity.

Conclusions

The foregoing data present more problems than answers to questions. But our results should support the opinion that it is rather useless to confine one's attention to a very small number of metabolites only while establishing a relationship between biological conditions and patterns of steroid metabolism. The value of *in vitro* studies versus investigations *in vivo* is a matter of dispute. As long as our knowledge on physiological effects of certain gonadal steroids in teleosts remains virtually unknown, the significance of scattered data from single experiments is highly questionable. The important results by Chan & Phillips (1969) notwithstanding one has to be cautious towards comparisons between facts which are well established for mammals but hardly substantiated in teleosts. This point is supported by the findings of Chan et al. (1975) that exogenous androgens are ineffective in the protogynous *Monopterus* "in causing the destruction of developing oocytes and in enhancing the quiescent male gonia into spermatogenetic activities".

The recent study by Eckstein et al. (1978) raises immediately the question as to what reasons justify attributing to testosterone a particular role in the process of sex inversion when we see that this steroid is metabolized to a large degree by gonadal tissues of ambisexual teleosts. Steroids with a functional group at C-11 should not be neglected (Reinboth 1978) The recent paper by Ungar et al. (1977) is an important hint to watch out for species specific peculiarities and that (pharmacological?) effects of certain steroids may not correspond with steroidogenic capacities of tissues from the same species.

My own data emphasize the necessity to check more precisely the interdependence of biological parameters with biochemical events. For mammals there is little doubt that 5α-reduction of testosterone to 5α-DHT plays a significant role for the understanding of physiological events related to the influence of C_{19}-steroids (*cf.* Wilson 1975). If similar mechanisms are applicable to teleosts we cannot escape the tiresome task of registering the metabolic activities of teleosts tissues on a broad scale before we learn to evaluate which events may be negligible as purely catabolic processes.

References

Axelrod L R, Matthijssen Ch, Goldzieher J W and Pulliam J E 1965 Definitive identification of microquantities of radioactive steroids by recrystallization to constant specific activity; *Acta endocr. Suppl.* **99** 77

Brooks C J W and Norymberski J K 1952 Characterization of the corticosteroid side-chain; *Chem. Ind.* 804–805

Chan S T H and Phillips J G 1969 The biosynthesis of Steroids by the gonads of the ricefield eel *Monopterus albus* at various phases during natural sex reversal; *Gen. Comp. Endocr.* **12** 619–636

——, Wai-sum O and Hui S W B 1975 The gonadal and adrenohypophysial functions on natural sex reversal; in *Intersexuality in the Animal Kingdom*, pp. 201-221 ed Reinboth R Heidelberg : Springer Verlag

Colombo L, Del Conte E and Clemenze P 1972 Steroid biosynthesis *in vitro* by the gonads of *Sparus auratus* L. (Teleostei) at different stages during natural sex reversal; *Gen. Comp. Endocr.* **19** 26–36

Eckstein B, Abraham M and Zohar Y 1978 Production of steroid hormones by male and female gonads of *Sparus aurata* (Teleostei, Sparidae); *Comp. Biochem. Physiol.* **60B** 93–97

Idler D R, Reinboth R, Walsh J M and Truscott B 1976 A comparison of 11-hydroxytestosterone and 11-ketotestosterone in blood of ambisexual and gonochoristic teleosts; *Gen. Comp. Endocr.* **30** 517–521

d'Istria M, Valentino A and Guasco R 1973 Dosaggio radioimmunologico di ormoni sessuali nel plasma di *Serranus cabrilla* durante il ciclo riproduttivo; *Atti Soc. Peloritana, Sc. Fis. Mat. Nat.* 241–244–III/IV

Latz M 1978 *In vitro Untersuchungen über den Steroidmetabolismus während des ovariellen Zyklus bei Haplochromis burtoni* (*Günther*), (Cichlidae, Teleostei). Ph. D. Thesis, University Mainz

Poos G I, Arth G E, Beyler R E and Sarett L H 1953 Approaches to the total synthesis of adrenal steroids V; *J. Am. Chem. Soc.* **75** 422–429

Reinboth R 1962 Morphologische und funktionelle Zweigeschlechtlichkeit bei marinen Teleostiern (Serranidae, Sparidae, Centracanthidae, Labridae); *Zool. Jb. Physiol.* **69** 405–480

—— 1970 Intersexuality in fishes; *Mem. Soc. Endocr.* **18** 515–543

—— 1975a *In vitro* studies on steroid metabolism of testicular tissue in ambisexual teleost fish; *J. Ster. Biochem.* **6** 341–344

—— 1975b Spontaneous and hormone-induced sex-inversion in wrasses (Labridae); *Pubbl. Staz. Zool. Napoli* **39** Suppl. 550–573

—— 1978 Bioassay for androgenic effects of various C_{19}-steroids in juvenile cichlid fish; *Gen. Comp. Endocr.* **34** Abstract no. 60

Ungar F, Gunville R, Sundararaj B I and Goswami S V 1977 Formation of 3α-Hydroxy-5β-pregnan-20-one in the ovaries of catfish, *Heteropneustes fossilis* (Bloch); *Gen. Comp. Endocr.* **31** 53–59

Wilson J D 1975 Metabolism of testicular androgens; in *Handbook of Physiology* Sect. 7: Endocrinology, Vol. V: Male reproductive system; pp 491–508; eds R O Greep, E B Astwood (Washington: American Physiological Society)