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Biochemical and Functional Aspects of Gonadal Biosynthesis of Steroid Hormones in Teleost Fishes

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Differences between gonadal steroidogenesis and corticosteroid biosynthesis are less marked in teleosts than in mammals. Enzymes which are normally adrenocortical in mammals, such as steroid 11β-and 21-hydroxylases have in fish a gonadal location. Hence, secretory products and intermediates may include, besides progestogens, androgens and estrogens, also 11-oxygenated and 21-hydroxylated steroids, though never both at the same time. A combinatory model of steroid molecular evolution is proposed to account for this heterogeneity in vertebrate steroid patterns. The model implies that taxonomic differences in steroid secretions may have arisen for mutations not only in the structural genes coding for steroid-biosynthetic enzymes but also in the regulatory genes determining their expression among steroidogenic endocrines.

In teleosts, the targets which have evolved a responsiveness to gonadal steroids may be classified into three groups: intragonadal, peripheral and pheromonal. This reflects not only a progressive elongation of the communication channel but also the adoption of different mechanisms of action. In the gonads, the control over meiotic maturation seems to be a primordial steroid function performed through post-transcriptional induction of protein synthesis, probably without intervention of intracellular receptors. Increased biosynthesis of maturational steroids at the time of ovulation has been observed in the sea bass. In addition to their well-known role as blood-borne agents aimed at somatic targets, fish gonadal steroids may be excreted in the environment as pheromones to mediate sexual communication. Preliminary experiments indicate that, in *Gobius jozo*, etiocholanolone glucuronide can attract the ripe female and trigger egg release.

Introduction

Aim of this article is to discuss selected topics of interest concerning the biochemistry and physiology of sexual hormones in teleosts. The most specialized aspects of fish gonadal steroidogenesis evidenced by previous reports, including ours own, will be dealt with to support a combinatory model of steroid molecular evolution in vertebrates.

Adaptations of biosynthetic mechanisms to provide steroids for the intragonadal control of gametogenesis and for pheromonal communication will be considered

in an effort to encompass the real range of steroid-target interactions in fishes. Preliminary experimental results are presented which suggest that, in *Gobius jozo*, testicular androgen conjugates may act as attractants and sexual stimulants on the ripe female.

Unfortunately, editorial limits of space do not consent either an exhaustive coverage of pertinent papers or detailed description of experimental procedures. Despite these deficiencies, we hope that this presentation may prove provocative enough to draw further work or debate on the treated subjects.

The Evolution of Steroid Hormones in Fish as compared to other Vertebrates

Comparative studies by steroid biochemists clearly indicate that differences between gonadal steroidogenesis and corticosteroid biosynthesis are less marked in fishes than in mammals. Enzymes which are normally adrenocortical in mammals, such as steroid 11β-and 21-hydroxylases, have in teleosts a gonadal location. Hence, secretory products and intermediates may include, besides progestogens, androgens and estrogens, also 11-oxygenated and 21-hydroxylated steroids.

In males, 11-oxygenated androgens are the major testicular hormones and may be synthesized by either 11β-hydroxylation of androstenedione and testosterone (*cf.* Ozon 1972, Colombo & Colombo Belvedere 1975) or side-chain splitting of 21-deoxycortisol (Colombo & Colombo Belvedere 1977, Colombo et al. 1978). The latter could derive from either 11β-hydroxyprogesterone, first identified in *Coris julis* (Reinboth 1974), or 17α-hydroxyprogesterone. Oxidation by the enzyme 11β-hydroxysteroid dehydrogenase may provide the 11-keto analogs of 11β-hydroxylated C_{21} and C_{19} compounds. So far, a pool of seven 11-oxygenated steroids has been obtained from the testes of different teleosts : 11β-hydroxyprogesterone, 11-ketoprogesterone (Colombo et al. 1978), 21-deoxycortisol, 11β-hydroxyandrostenedione, adrenosterone, 11β-hydroxytestosterone and 11-ketotestosterone (*cf.* Colombo & Colombo Belvedere 1975).

Formation of 11-oxygenated androgens has been observed also in the ovaries of seven marine and freshwater species, although rather disparate interpretations have been given on its functional significance (*cf.* Colombo & Colombo Belvedere 1975, Kristofferson et al. 1976). A possible heterogeneity in fish steroid patterns is suggested by the ovarian occurrence of steroid 21-, and not 11β-hydroxylase activity in another group of seven marine species (*cf.* Colombo & Colombo Belvedere 1975, 1977, Colombo et al. 1978). In this case, the resulting products are two 11-deoxycorticosteroids, i.e. 11-deoxycorticosterone and 11-deoxycortisol, the yields of which are often very high.

Though first reported in teleosts, gonadal biosynthesis of 11β-and 21-hydroxylated steroids seems to be rather common among vertebrates with the exception of mammals. The enzyme 11β-hydroxylase has been detected in newt (Lupo Di Prisco 1971) and bird ovaries (Colombo & Colombo Belvedere 1978) whereas 21-hydroxylase activity has been found in the ovaries of a cyclostome (Hirose et al. 1975), urodeles (Colombo et al. 1977), reptiles (*cf.* Colombo & Colombo Belvedere 1975) and in elasmobranch testis (Simpson et al. 1964).

Thus, it is evident that, unlike other low molecular weight hormones, gonadal steroids have not maintained a chemical uniformity throughout the vertebrates. The same holds true for the adrenal steroidogenic tissue which secretes 17-deoxycorticosteroids in certain classes or orders and mainly 17α-hydroxycorticosteroids in others (*cf.* Idler & Truscott 1972).

Any evolutionary model meant to account for this variability among vertebrate steroid hormones must consider that any informational change in their chemical structure is protein-mediated. While the phylogenetic history of polypeptide and protein hormones has progressed through the processes of gene mutation and duplication causing amino acid substitutions and molecular divergence (De Haen & Neurath 1976), steroids could be modified only indirectly through changes in the composition of the enzymatic sets involved in their biosynthesis. Differences in set composition may originate not only from variations in the genetic codification of the constituent enzymes, shifting their substrate affinity or favoring enzyme multiplicity, but also from alterations of gene expression rearranging enzyme tissue distribution and activity level. Since comparative enzymology of steroid-transforming enzymes is still scarcely developed (Sandor et al. 1972) and research on isoenzymatic forms is restricted to mammals (Hudson et al. 1976, Orta-Flores et al. 1976), it is at present difficult to evaluate the impact of protein evolution on vertebrate steroidogenesis.

On the other hand, even assuming that steroid-biosynthetic enzymes were coded by stereotyped structural genes, some of the present taxonomic differences in steroid secretions could still be explained with mutations in the regulatory program which determines the strategy of expression of such genes among steroidogenic endocrines. This mechanism is suggested, for instance, by the evolutionary logic in the assortment of steroid 11β-, 17α- and 21-hydroxylases, which are crucial for steroid hormone biosynthesis, in vertebrate gonads and adrenals (table 1).

Reports on steroid hormones from cyclostomes to mammals (*cf.* Idler & Truscott 1972, Sandor 1972, Ozon 1972) support the view that these three enzymes were already coded in the genome of vertebrates since their appearance and were maintained thereafter. Vertebrates are likely to have inherited rather differentiated steroidogenic capabilities from their invertebrate ancestors as suggested by the finding of the above steroid hydroxylases also in the gonads of several invertebrates (De Loncamp et al. 1974, Colombo & Belvedere 1976, Teshima & Kanazawa, 1970).

Table 1 *Distribution of steroid 11β-, 17α-and 21-hydroxylases in adrenocortical and gonadal tissues of vertebrates*

Endocrine	11β-OH-ase	17α-OH-ase	21-OH-ase
Adrenal cortex	Obligatory	Facultative	Obligatory
Gonads	Facultative but incompatible with 21-OH-ase	Obligatory	Facultative but incompatible with 11β-OH-ase

Discussing the ubiquitous presence of steroids in the biosphere, Sandor et al. (1975) have postulated that some components of the steroid-synthetic apparatus might be of primordial origin and possibly antecedent to the emergence of eucaryotes. Such biological antiquity coupled with the complexity of the biochemical mechanisms underlying steroid hydroxylations, oxidative cleavage of steroid side-chains and other reactions should have restricted the chances of drastically innovative changes in the structure of steroid-metabolizing enzymes during the span of vertebrate phylogenesis.

Conversely, the great number of possible independent enzymatic interventions on the steroid nucleus might have favored a combinatory rather than a divergent type of steroid hormone evolution. Table 1 shows that enzyme associations within glands are really more conservative than could be expected according to combinatory statistics. The genetic program for steroid 17α-hydroxylase, for instance, is always expressed in

the mature gonads of vertebrates as are those for 11β-and 21-hydroxylases in the adrenal.

Since the facultative distribution of these enzymes is arranged symmetrically between gonads and adrenal (which allows most variants with least overlapping of terminal products), vertebrates may have two patterns of glucocorticosteroidogenesis and three of gonadal hormone biosynthesis. In this respect, the main difference between a mammal like the rat and teleost fish is that in the former gonadal and cortical steroidogenic patterns do not include facultative enzymatic activities whereas in the latter they do.

Interestingly, codification for steroid 11β- and 21-hydroxylases may become derepressed in the gonads of man during tumorigenesis, probably for a loss of control over gene expression (*cf.* Colombo & Colombo Belvedere 1975).

Although other steroid-biosynthetic hydroxylases are less known comparatively, the scheme of their tissue affinities can sometimes be surmised. Thus, in vertebrates, steroid 18-hydroxylase is likely to occur only in the adrenal while 19-hydroxylase seems obligatory in the ovary but facultative in the testis and adrenal (*cf.* Idler & Truscott 1972, Sandor 1972, Ozon 1972). For many other enzymes, however, which centrally or peripherally contribute to profiles of circulating steroids or to their mechanism of action (King & Mainwaring 1974), available information is insufficient for phylogenetic considerations.

Steroid Targets of Gametogenic Elements in Teleosts

A constant feature of the vertebrate gonads is the close association between steroidogenic and gametogenic elements. Since they develop from different and noncontiguous embryological matrices, their definitive anatomical proximity might answer a need of functional coordination. This is suggested particularly by studies on teleosts and amphibians, where gonadal steroids have been found to exert both morphogenetic and integrative actions on the germinal cells.

Treatment with adequate doses of heterologous sex hormones can reverse phenotypically the sexual differentiation of fish gonocytes (*cf.* Yamamoto 1969, Takahashi 1975a,b). The delimitation of a critical period of hormone effectiveness (Hackmann & Reinboth 1974), the retention of fertility in the inverted adult and a certain steroid specificity in sexual orientation (*cf.* Yamamoto 1969) are evidence in favor of an inductive rather than a pharmacological effect. In the functional gonad, steroid secretion seems to be not only harmonious with the gametogenic cycle but also involved in its regulation, at least in certain vertebrate groups. A well-studied process under steroid control is the resumption of meiotic maturation in the postvitellogenic oocytes of fish and amphibians. Although the source of maturational steroids in the Indian catfish has been located in the interrenal (Sundararaj & Goswami 1977), in other species the most likely producers appear to be the follicular cells (Hirose 1972, Jalabert et al. 1973, Redshaw, 1972).

To reinitiate the arrested meiosis, these cells should transduce a systemic gonadotropic stimulus into a local steroid signal accepted by the oocyte. 11-Deoxycorticosteroids are the most potent maturational agents in certain teleosts (van Ree et al. 1977, Goswami & Sundararaj 1974, Hirose 1976) and amphibians (Morril & Bloch 1977), whereas progesterone and 20β-dihydroprogestogens are more effective in others (Jalabert 1976, Brachet 1974).

Interestingly, the ovarian biosynthesis of 11-deoxycorticosteroids in the sea bass increased strongly at the time of ovulation (Colombo et al. 1978) as did the plasma concentration of 11-deoxycorticosterone in

female *Tilapia aurea* at the beginning of spawning (Katz & Eckstein 1974).

A striking feature in the promotion of meiotic maturation by steroids is that their mechanism of action does not seem to involve a modulation of gene transcription as in somatic steroid targets (O'Malley et al. 1976). Both in teleosts and amphibians, steroid-induced maturation is blocked by puromycin or cycloheximide but not by actinomicin D or mitomicin C, indicating that, although protein synthesis is obligatory, DNA-dependent RNA synthesis is not necessary (Jalabert 1976, Sundararaj & Goswami 1977; *cf.* Redshaw 1972).

Moreover, steroid-receptor interaction does not appear to occur within the cytoplasm but at or near the oocyte surface (Dennis & Ecker 1971) and is less specific than usually observed since a variety of nonestrogenic steroids may trigger a meiotic response (Jalabert et al. 1973, Goswami & Sundararaj 1974, Morril & Bloch 1977).

It seems that when steroids intervene as local mediators on gametic elements their mechanism of action is less specialized than when they operate as systemic hormones on somatic targets. Possibly, the control over meiotic maturation was a primary function of gonadal steroids which evolved before their endocrine role on sexual and reproductive organs.

That spermatogenesis like, ovogenesis, is steroid-dependent is suggested by the fact that meiotic stages in cultured fish testis were maintained and stimulated in the presence of testosterone but disappeared in its absence (Remacle 1976). Unfortunately, the molecular dynamics of this influence is still unknown.

Also in mammals testosterone is apparently needed for the completion of meiosis during spermatogenesis (Hansson et al. 1974). On the other hand, the situation in the mammalian ovary is peculiar because the meiosis-inducing action of LH on oocytes does not seem to be mediated by a steroid (Leiberman et al. 1976).

Gonadal Steroid Pheromones in Teleosts

Social communication in certain teleost species is largely based on the exchange of pheromonal messages which serve to regulate territoriality, hierarchy, schooling, alarming, sexual activity and parental behaviour (*cf.* Pfeiffer 1974). The origin and chemistry of fish pheromones are practically unknown, but different workers have suggested that gonadal secretions may provide signals for sexual recognition and attraction in some fishes.

A substance contained in the ovarian fluid of the gravid female, *Bathygobius soporator*, can elicit courtship in the male (Tavolga 1956). In *Lebistes reticulatus*, the male becomes hyperactive when exposed to a substance released by the female and presumptively identified as an estrogen (Amouriq 1965).

Recently, we have proposed that the mesorchial glandular mass of the testis of the black goby, *Gobius jozo*, might be a source of pheromonal steroids (Colombo et al. 1977). Histoenzymological tests for hydroxysteroid dehydrogenases and ultrastructural observations (Colombo & Burighel 1974) have shown that this structure is specialized in steroid biosynthesis and represents an unusually large testicular enrichment in Leydig cells. A similar testicular organization has been described also in *Gobius paganellus* (Stanley et al. 1965).

In both species, this impressive steroidogenic apparatus appears to satisfy the need of producing huge amounts of steroids in a conjugated form (Colombo et al. 1970, Colombo et al. 1977). In *G. jozo*, a major compound, conjugated as both "glucuronide" (hydrolyzed by β-glucuronidase) and "sulfate" (solvolyzed), was identified as etiocholanolone (5β-androstan-3α-ol-17-one) for the formation of which the following biosynthetic

sequence was suggested : pregnenolone—→ 17α-hydroxypregnenolone —→ dehydroepiandrosterone —→ androst-4-ene-3,17-dione—→5β-androstan-3, 17-dione—→etiocholanolone—→conjugated etiocholanolone (Colombo et al. 1977).

Since steroid conjugation usually abolishes hormonal activity and facilitates excretion, a massive testicular synthesis of water-soluble conjugates is likely to reflect an adaptation for steroid export in a surrounding aqueous medium.

The black goby is known to be a territorial species in which the male prepares a rudimental nest where to attract the female for mating (Mozzi 1968). Therefore, we considered worthwhile to test preliminarily whether etiocholanolone glucuronide could act as a pheromonal cue for the female during her search after the male.

Twenty females of *G. jozo* were captured by angling in July, at the peak of their breeding season, in the Venetian lagoon near Chioggia. All females had a distended belly but 15 were in a postvitellogenic condition (mean standard length of 8 cm ± 0.6 SD and mean body weight of 13 g ± 1.7 SD), while 5 emitted eggs on stripping (8.1 cm± 0.5 SD and 12.5 g ± 1.2 SD). After an acclimation of 1–2 days in the laboratory, specimens were tested in a glass tank (68 × 68 × 35h cm) enclosed by polystyrene panels on all sides and containing about 150 l of sea water. The tank bottom was cross-lined into 25 identical numbered squares and four nests, obtained from neckless brown glass bottles halved longitudinally, were positioned crosswise against the middle of the tank walls (figure 1). Nests were oriented with the entrance towards the tank centre and the closed bottom against the tank wall. Inside one of the nests was accomodated the tip of a 50 cm-long polyvinyl chloride cannula connected to a 50-ml glass syringe, the piston of which was moved by a Sage Instruments pump, mod. 355. The syringe was filled with a solution of 1 μg/ml of etiocholanolone glucuronide (Sigma, St. Louis, MO.) in Millipore filtered sea water (the steroid was omitted in the controls) and the pump was set to give a flux of 1ml/min for 50 min.

Females were introduced singly into the tank and their movements were watched through a peep-hole on the cover panel by an observer. The numbers of the squares visited by the female in the tank were recorded on tape in real time. Data were quantified by scoring each square in the matrix with a dot for every 15 sec spent continuously by the female in that square (figure 1). The time schedule of each female in the tank was as follows : (*a*) unwatched conditioning (1hr); (*b*) watched conditioning (10-15 min before the pump start); (*c*) latent period (10 min after the pump start); (*d*) testing (40 min till the pump stop); (*e*) testing watched at intervals (2 hr after the pump stop). Scores were given from period *b* through *c* and *d*.

Females in postvitellogenic condition gave a negative response, that is their exploratory behaviour in the tank was not appreciably influenced by the steroid (figure 1A). Controls were also negative.

On the other hand, all the females discharging eggs on stripping concentrated their movements, after the latent period, on the nest from which the steroid was diffusing (figure 1 B–D). They showed an evident interest in that area by coming in and out of the nest, by bordering it all around head-on and by resting on it for long periods. Finally, 3 females laid and glued some eggs on the nest, one after 43 min and two after 2 hr from the pump start.

These preliminary results indicate that conjugated etiocholanolone may act as a sexual pheromone on the female of *G. jozo* but only when she is ready to lay her eggs. At this stage, this substance is likely to attract the female towards the male and also

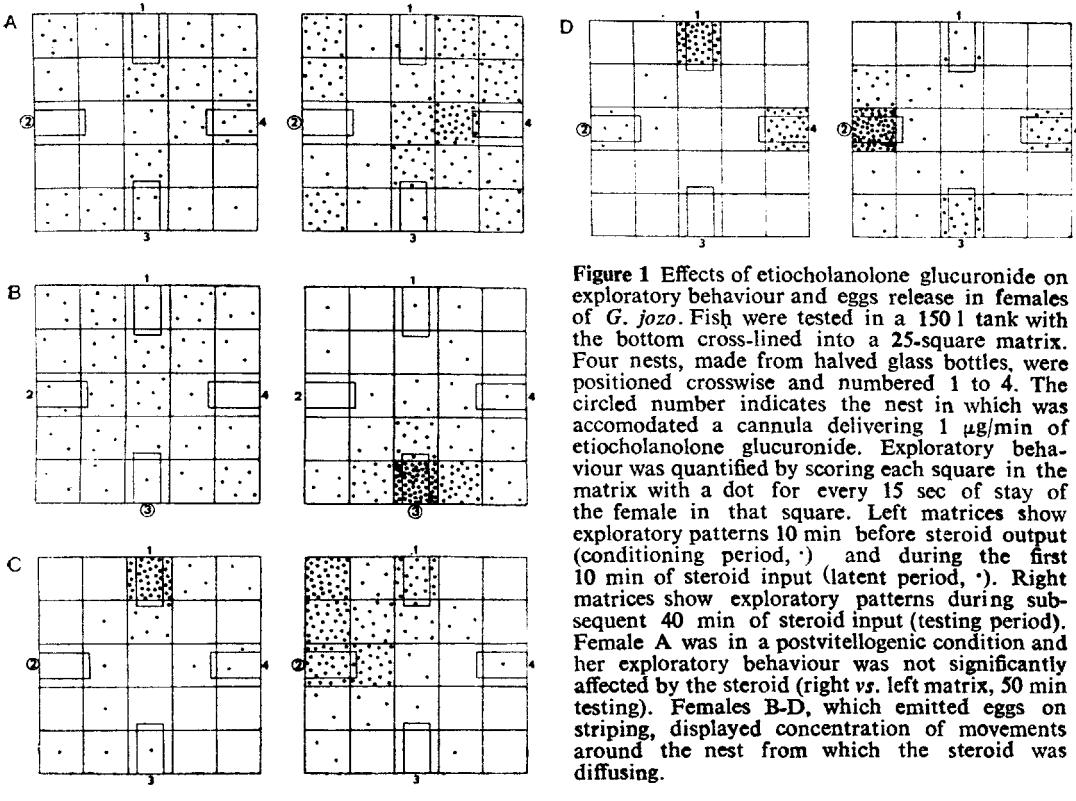

Figure 1 Effects of etiocholanolone glucuronide on exploratory behaviour and eggs release in females of *G. jozo*. Fish were tested in a 150 l tank with the bottom cross-lined into a 25-square matrix. Four nests, made from halved glass bottles, were positioned crosswise and numbered 1 to 4. The circled number indicates the nest in which was accomodated a cannula delivering 1 μg/min of etiocholanolone glucuronide. Exploratory behaviour was quantified by scoring each square in the matrix with a dot for every 15 sec of stay of the female in that square. Left matrices show exploratory patterns 10 min before steroid output (conditioning period, ·) and during the first 10 min of steroid input (latent period, ·). Right matrices show exploratory patterns during subsequent 40 min of steroid input (testing period). Female A was in a postvitellogenic condition and her exploratory behaviour was not significantly affected by the steroid (right *vs.* left matrix, 50 min testing). Females B-D, which emitted eggs on striping, displayed concentration of movements around the nest from which the steroid was diffusing.

to trigger egg laying after a longer exposure. Thus, a testicular steroid is presumably employed by the male to entice the female into mating and to coordinate gametic transfer.

This situation is reminiscent of that described in pigs where a testicular steroid, 5α-androst-16-en-3-one, is transferred from the boar's saliva to the sow to evoke in the female the immobilization reflex for copulation (Claus 1975).

In conclusion, teleosts display a very versatile use of gonadal steroids in the integration of reproductive processes. From intragonadal targets, steroid control has been extended apparently to peripheral sexual organs and brain centres and further adapted to mediate sexual communication. The complexity of the regulatory circuits channelled through the gonads of fish is certainly not less impressive than that of land-living vertebrates and its appreciation should be indeed a good start for future research.

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