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Gonadal steroidogenesis and the possible role of steroid glucuronides as sex pheromones in two species of teleosts

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Abstract

In general, female zebrafish, *Brachydanio rerio*, ovulate only in the presence of males. The stimulant must be pheromonal as even male holding water is capable of inducing ovulation. After ovulation the mating phase begins. During this phase the male follows the female and oviposition as well as fertilization takes place. Both the ovulation and the mating are controlled by pheromones synthesized by the gonads. Ovulation can be induced by testicular homogenates. After the lipid material has been extracted from the testicular homogenates, the remaining aqueous phase can still induce ovulation. However, when the aqueous phase is treated with the enzyme β-glucuronidase, it loses the ability to induce ovulation. This is an indication that glucuronides, probably steroid glucuronides, are the compounds responsible.

During the mating phase, ovulated female zebrafish become attractive to males. It was found that, after ovulation, ovarian extracts contain the compounds responsible for attracting males. The attractant consists of a mixture of steroid glucuronides.

After incubation of the gonads with ^{3}H-precursors seven steroid glucuronides have been identified in the testis and five in the ovary.

Under fish culture conditions the African catfish, *Clarias gariepinus*, can produce postvitellogenic oocytes throughout the year. However, in captivity neither males nor females spawn. In female catfish maturation and ovulation can be induced by treatment with gonadotropins. It might be possible that, analogous to the zebrafish, some reproductive processes in the catfish have to be induced by pheromones. It has been demonstrated that pheromonal compounds released by the seminal vesicles are involved in the attraction of female conspecifics. The steroid glucuronide synthesizing capability of the testes and the seminal vesicles of the male catfish are examined, as well as that of the ovary before and after ovulation of the female catfish. Both testes and seminal vesicles appear to be capable of steroid biosynthesis but only the latter synthesizes steroid glucuronides. Six of these conjugates have been isolated and identified. In the female catfish the ovaries are capable of synthesizing seven steroid glucuronides, but only after ovulation.

Introduction

As in most animals, successful reproduction in teleosts depends on behavioral synchrony between mature male and female. In many cases, such synchrony has been shown to be mediated by pheromones (Liley 1982). Such pheromones probably are of gonadal origin. Thus, in mature Pacific herring, *Clupea harengus pallasi*, milt evokes spawning behaviour of males and females (Stacey and

Hourston 1982). Likewise, etiocholanolone glucuronide, a steroid conjugate from the mesorchial gland of *Gobius jozo* appears to have an attractive effect on gravid female conspecifics (Colombo *et al.* 1980, 1982).

The Research Group for Comparative Endocrinology of the Utrecht University is studying reproductive endocrinology of teleosts, including the zebrafish, *Brachydanio rerio*, and the African catfish, *Clarias gariepinus*. Many findings pertaining to the gonads of zebrafish (van den Hurk 1973, 1977; Lambert and van Oordt 1974; van Ree 1976, 1977a, b; van Ree *et al.* 1977; Lambert 1978; Peute *et al.* 1978, 1985; van den Hurk and Lambert 1983) and the African catfish (van den Hurk and Richter 1980; Lambert and van den Hurk 1982; Richter and van den Hurk 1982; van den Hurk and Peute 1985; van den Hurk *et al.* 1985; Schoonen and Lambert 1986a, 1986b) have already been published. Aspects regarding steroid synthesis and the possible role of steroid glucuronides as sex pheromones, included in these publications and from unpublished results, are briefly reviewed in the present paper.

Zebrafish

The zebrafish, a small tropical teleost that can easily be kept in fresh water aquaria, has a reproductive cycle of about five days. Shortly before spawning the animals show a characteristic reproductive behaviour (Fig. 1). When a male and female are brought together in an aquarium, they will for some time explore their new surroundings and each other. During that surveying stage they may show agonistic behaviour. When both are in spawning condition, allied behaviour will follow soon after the fish have become used to their new surroundings. The male will chase the female and will perform nipping activities. The male soon begins swimming very close to the female and pushing her sideways. This leads to oviposition and fertilization of the eggs.

This courtship behaviour will only be shown when the female has ovulated and a female will only ovulate when postvitellogenic oocytes are present in the ovaries and when a male zebrafish is near. It

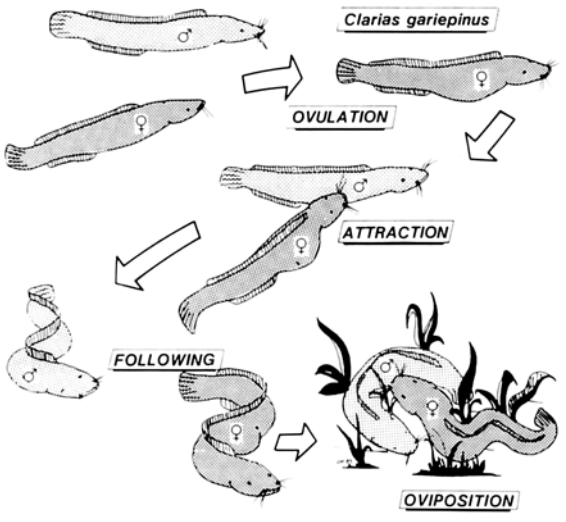

Fig. 1. Schematic representation of some characteristic phases of the reproductive behaviour of the zebrafish, *Brachydanio rerio*.

seems that the female receives a signal from the male, and that the male can perceive whether a female has ovulated. There are indications that these signals are pheromones produced by the testes and ovaries.

Male pheromones

Male holding water, i.e. aquarium water in which male zebrafish have been swimming, will induce ovulation in female conspecifies (Chen and Martinich 1975). In our experiments the testing material was supplied to five animals, each in their own aquarium. All experiments were carried out three times. Male holding water induces ovulation in about 70% of the females. The control activity is less than 5%. Positive results were also obtained by exposing females either to a cell free testis homogenate (± 55%) from males with running ripe sperm or to its water fraction (± 60%), after extraction with dichloromethane. Following a treatment with the enzyme β-glucuronidase (Schoonen and Lambert 1986b) to eliminate possible steroid glucuronides, the ovulation inducing capacity of the waterfraction was virtually eliminated. This seems to point to (steroid) glucuronides as the male pheromone inducing ovulation.

Steroid synthesis in the testis

To identify testicular glucuronides which might function as sex pheromones, steroidogenesis was studied in male zebrafish by incubating small pieces of testicular tissue in Leibovitz-15 medium to which either ^{3}H-pregnenolone and ^{3}H-androstenedione had been added. Pregnenolone incubations provide a general impression of the steroidogenic capacity and androstenedione incubations give an impression of the capability to produce androgens.

After incubation and extraction with dichloromethane, the free steroids were isolated and identified by thin layer chromatography, derivatization and recrystallization to a constant specific activity. The remaining water phase, containing steroid glucuronides, was treated with β-glucuronidase, after which the free steroids could be isolated and identified. The results of these qualitative studies are summarized in Table 1. It remains to be seen whether any of the seven steroid glucuronides synthesized by the testis can function as a pheromone to induce ovulation.

Female pheromones

Since ovulation in female zebrafish is a necessary condition for courtship behaviour, female sex pheromones were looked for in the ovaries, especially shortly after ovulation (van den Hurk and Lambert 1983). Cell free homogenates were prepared from ovaries collected between ovulation and oviposition, 1–2 hours after the onset of oviposition and 3 days after oviposition. Attraction of males by these homogenates was tested as follows. Eight male zebrafish were placed in a 60 L aquarium with a water inlet in the two back corners; one for distilled water, the other for ovarian homogenate diluted with distilled water. One minute after introducing equal amounts of fluid *via* both inlets, the number of males in each half of the aquarium was scored every 10 seconds for 5 minutes. Each test was carried out five times, each time with different animals; thus, 150 observations were made in each experiment. A positive observation was defined by the presence of at least 7 males at the side of the aquarium where the testing material was introduced. When distilled water was introduced *via* both inlets, only 5 positive observations could be made. However, when a diluted homogenate of ovaries, collected three days after oviposition was added to the aquarium water the number of positive observations increased to 16. Diluted homogenates of ovaries collected 1–2 hours after oviposition and between ovulation and oviposition resulted in 54 and 42 positive observations, respectively. This seems to confirm the hypothesis that zebrafish ovaries can produce a pheromone, attracting male conspecifics, especially shortly after ovulation and oviposition. Indeed the most potent homogenate, i.e. the one prepared from ovaries collected shortly after oviposition, did not attract anosmic males.

The homogenate of ovaries collected shortly after oviposition was divided by a dichloromethane extraction into an organic and a water fraction. The latter was subdivided with florisil chromatography into several steroid conjugate fractions such as sulphates, glucuronides and phosphates. The fraction containing steroid glucuronides evoked attractions; 48 positive observations could be observed. Testosterone- and estradiol glucuronide, separately, did not produce attraction. However, a mixture of these two glucuronides led to 27 positive observations. Thus it seems that the ovarian sex attractant is a mixture of steroid glucuronides, possibly including testosterone- and estradiol glucuronide.

Steroid synthesis in the ovaries

As the ovarian attractant might be a mixture of steroid glucuronides, we investigated synthesis of free and conjugated steroids in the zebrafish ovary. Following the same procedure as for the testis, incubations were carried out with postovulatory ovaries, which produce greatest attraction. The results of the analysis are shown in Table 1. Five steroid glucuronides could be demonstrated. Three of them were identical with testicular glucuronides, i.e. 17α-hydroxy-20β-dihydroprogesterone-, testosterone-, and 5α-androstane-3α, 17β-diol glucuronide. The two glucuronides, specific for the

ovaries were estradiol- and 5_{α}-dihydrotestosterone glucuronide.

African catfish

The African catfish, *Clarias gariepinus*, is an excellent fish for pisciculture in African countries (Viveen *et al.* 1985; Richter 1976) and in any surroundings where the water temperature can be kept between about 25–30°C (Hogendoorn 1983). It is known for its hardiness, for its ability to survive when densely stocked in poorly oxygenated water, and for its rapid growth even when fed on waste products of food industries. Under natural conditions the species shows a discontinuous reproductive cycle (van den Hurk *et al.* 1985; Peute *et al.* 1986) with an annual breeding period in early summer or during the rainy season (Bruton 1979). In fish tanks and ponds, however, spawning does not occur; here, oocyte maturation and ovulation have to be induced by treatment with gonadotropins (Eding *et al.* 1982; Lambert and van den Hurk 1982; Richter and van den Hurk 1982).

Breeding under natural circumstances has been described in great detail by Bruton (1979) for a population of *Clarias gariepinus* in Lake Sibaya in South Africa. According to Viveen (unpublished results), breeding is very similar in a population in the Hula nature reserve in Northern Israel, the main difference being that in lake Sibaya spawning usually takes place at night, and in the Hula region during daytime. During the breeding season spawning follows shortly after a rise in the water level and the flooding of the immediate surroundings of the lake in which the catfish live. The initial response to elevated water is aggregation and migration towards the inundated lake shores. Near the spawning grounds the catfish show shoaling behaviour and prenuptial aggression. Probably, ovulation takes place more or less simultaneously in a number of females at this stage (Fig. 2). Under laboratory conditions, female catfish which have been ovulated by hormone treatment show attention for a male by butting and chasing him, behaviours which induce the male to follow the female (Resink, unpublished results). In the wild the female enters the

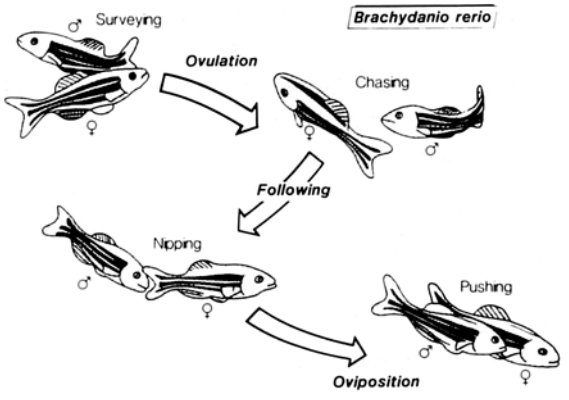

Fig. 2. Schematic representation of some characteristic phases of the reproductive behaviour of the African catfish, *Clarias gariepinus*.

inundated, usually grassy lake shore and the male follows her, taking exactly the same winding trail. At spawning, the male adopts a U-shaped position around the head of the female and in this position sperm release and oviposition occur. By beating her tail, the female mix eggs and sperm and spreads the eggs over the inundated plants in the immediate surroundings.

Coordination of the reproductive activities of male and female African catfish likely is mediated by a variety of intersexual communication systems. Although little is known of chemical communication in this species, there is good evidence that males produce a pheromone which attracts females.

Male pheromones

Given a choice between male and female conspecific, ovulated female catfish are attracted by the male unless the olfactory nerves of the female have been severed (J.W. Resink and R. van den Hurk, unpublished data). Males without seminal vesicles were less attractive, and castrated males with enlarged seminal vesicles were preferred above sham-operated males. Thus, it seems that the male gonads, and especially the caudal part, *i.e.* the seminal vesicles, are producing a sex attractant. Bruton (1979) also points to the possible existence of gonadal pheromones in *Clarias gariepinus* influencing courtship behaviour. Therefore, and in

the light of the results obtained in the zebrafish, it seemed important to identify the steroids, especially the steroid conjugates that can be synthesized by the male and female gonads of the African catfish.

Steroid synthesis in the testes and seminal vesicles

Following the procedure used for the determination of steroid biosynthesis in the zebrafish, small fragments of testes and seminal vesicles from males with running ripe sperm were incubated with ^{3}H-pregnenolone and ^{3}H-androstenedione as precursors.

In the testis ^{3}H-pregnenolone was mainly converted via progesterone and 17α-hydroxyprogesterone into androstenedione, 11β-hydroxyandrostenedione and 11β-hydroxytestosterone (Schoonen and Lambert 1986a). The final products from ^{3}H-androstenedione incubations, i.e. 11β-hydroxyandrostenedione and 11β-hydroxytestosterone confirm these findings. Moreover, two steroid esters were synthesized, one of which, pregnenolone ester, was identified. No steroid glucuronides or other steroid conjugates could be identified in the testes incubations. A summary of the steroidogenic capacity of the testes is given in Table 1.

Histochemical indications of steroid biosynthesis

Table 1. Steroids and steroid glucuronides produced after incubations with Pregnenolone or Androstenedione

		Brachydanio rerio		*Clarias gariepinus*		
	Trivial names	Testis	Ovary	Testis	Sem. ves.	Ovary
5-Pregnen-3β-ol-20-one	Pregnenolone	S	S	S	S	S
5-Pregnen-3β-ol-20-one-ester	Pregnenolone ester	S		S	S	
4-Pregnene-3,20-dione	Progesterone		S	S	S	S
5-Pregnene-3β,17α-diol-20-one	17α-Hydroxypregnenolone	S	S			S
4-Pregnen-17α-ol-3,20-dione	17α-Hydroxyprogesterone	S	S	S	S	S
5-Pregnene-3β,17α,20β-triol		S G				
4-Pregnene-17α,20β-diol-3-one	17α-Hydroxy-20β-dihydro-progesterone	S G	S G			S G
5β-Pregnan-17α-ol-3,20-dione				S	S	S
5β-Pregnane-3α,17α-diol-20-one				S	S G	S G
5β-Pregnane-3α,17α,20α-triol				S	S	
5β-Pregnane-3α,17α,20β-triol						S G
4-Androstene-3,17-dione	Androstenedione	S	S	S	S	S
4-Androsten-17β-ol-3-one	Testosterone	S G	S G		S G	S G
4-Androsten-11β-ol-3,17-dione	11β-Hydroxyandrostenedione	S		S	S	
4-Androstene-3,11,17-trione	11-Ketoandrostenedione	S				
4-Androsten-11β,17β-diol-3-one	11β-Hydroxytestosterone			S	S	
4-Androsten-17β-ol-3,11-dione	11-Ketotestosterone	S		S	S	
5α-Androstane-3,17-dione		S	S			
5α-Androstan-3α-ol-17-one	Androsterone	S G	S			
5α-Androstan-3β-ol-17-one	Epiandrosterone	S G				
5α-Androstan-17β-ol-3-one	5α-Dihydrotestosterone		S G			
5α-Androstane-3α,17β-diol		S G	S G			S
5α-Androstane-3β,17β-diol		S G				
5β-Androstane-3,17-dione					S	
5β-Androstan-3α-ol-17-one	Etiocholanolone				S G	
5β-Androstan-17β-ol-3-one	5β-Dihydrotestosterone				S G	S G
5β-Androstane-3α,17β-diol					S G	S G
5β-Androstane-3β,17β-diol					S G	
1,3,5 (10)-Estratrien-3-ol-17-one	Estrone		S			S
1,3,5 (10)-Estratriene-3,17β-diol	Estradiol-17β		S G			S G

Steroids (S) and steroid glucuronides (G) which could be identified after incubations with ^{3}H-pregnenolone or ^{3}H-androstenedione in the gonads of *Brachydanio rerio* and *Clarias gariepinus*.

by the seminal vesicles (Resink *et al.* 1985) could be confirmed by incubation experiments (Schoonen and Lambert 1986b). Among the steroids synthesized by the seminal vesicles are androgens, 11-oxygenated androgens, 5β-reduced C-19 and C-21 steroids and a pregnenolone ester (Table 1). Furthermore, a potent synthesis of steroid glucuronides could be identified, i.e. 5β-pregnane-3α,17β-diol-20-one-glucuronide, testosterone-glucuronide and four glucuronides of 5β-reduced C-19 steroids (Table 1).

Steroid synthesis in the ovaries

For biochemical studies of the steroidogenic capacity of the ovaries, tissue fragments were treated in the same way as those of the testes and seminal vesicles. These tissue fragments were taken from ovaries collected several hours before and after ovulation induced by HCG treatment (Lambert and van den Hurk 1982) and from ovaries collected at the spawning grounds (Hula nature reserve, Israel) during and after spawning (Schoonen and Lambert in preparation).

The results of the incubations are given in Table 1. Seven steroid glucuronides could be identified. Their synthesis starts shortly before ovulation and rapidly increases after ovulation.

As ovarian steroid conjugates evidently function as sex attractants in zebrafish, they may perform a similar function in the African catfish. It is also possible that they synchronize ovulation in this species. Experiments are being carried out to check these possibilities. In addition to contributing to an understanding of steroidogenesis and pheromone function in fish, such studies may yield results with practical application to reproduction control in aquaculture.

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