GCMS-IDENTIFIED STEROIDS AND STEROID GLUCORONIDES IN GONADS AND HOLDING WATER OF TRICHOGASTER TRICHOPTERUS (ANABANTIDAE, PALLAS 1770)

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Abstract—1. Analysis by gas chromatography-mass spectrometry was performed to identify steroids and steroid glucuronides in gonads of the tropical fish Trichogaster trichopterus and in the water in which the fish were maintained.

2. Full mass spectra of estradiol-17β (E2), testosterone, 17α-hydroxyprogesterone, cholesterol, stigmasterol, 4β-methylcholesterol, estrone, 17α,20β-hydroxy-4-pregnen-3-one (17,20-P) and sitosterol were obtained.

3. The above steroids were detected in both female and male gonads, with the exception of estrone, which was detected only in the male, and 17,20-P, which was detected only in the female.

4. All steroids except 17,20-P were detected in the water in which the fish were maintained.

INTRODUCTION

Chemical communication in fish species has aroused intensive interest in recent years (Stacey, 1989), and such studies have shown that pheromones play an important role in different aspects of the reproduction of teleosts (Colombo et al., 1982; Liley, 1982; Liley and Stacey, 1983; Lambert et al., 1986; Stacey et al., 1986, 1987). These studies examined the connection between pheromones and the reproductive cycle; specifically, Stacey and Sorensen (1986) reported on the aspect of spermation, Resnik et al. (1989) on maturation and ovulation, and van Weerd et al. (1991) on vitellogenesis.

A number of studies concerning chemical communication in Trichogaster trichopterus (Anabantidae, Pallas 1770) from the family Belontiidae indicate that this species may serve as a model for the whole systematic group (Cheal and Davies, 1974; Pollack et al., 1978; Lee and Ingersoll, 1979; McKinnon and Liley, 1987). The species is territorial, and the male constructs a nest of mucus-covered bubbles (Forselius, 1957). Spawning occurs under the nest. The male retrieves the eggs and fry to the nest and subsequently tends them. In early studies, Rossi (1969), Davies and Pilotte (1975) and Ingersoll and Lee (1980) found no response by the male to any possible chemical signal by the female; McKinnon and Liley (1987) have suggested that there is a signal by the female that enables male T. trichopterus and T. pectoralis to differentiate between ripe and unripe females. In the latter study, females were induced to ovulate by an injection of human chorionic gonadotropin (hCG). Degani (1990) showed that such an injection raises the level of estradiol-17β (E2), and later the levels of testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P), hormones which are involved in the onset of vitellogenesis, germinal vesicle breakdown (GVBD) and ovulation. This process is effected not only by hCG (acting as a gonadotropin) but also by the sexual behaviour (Degani and Boker, 1992a) or possibly by pheromones that have not been described in detail.

The purpose of this study was to detect and determine the steroids and glucosteroids in the gonad of male and female T. trichopterus and in the water in which the fish were maintained.

MATERIALS AND METHODS

Specimens

The fish used in this study were gourami, Trichogaster trichopterus, of both sexes, of the third generation bred and grown in the laboratories of Migal, at the age of 5 months. Weights ranged from 8 to 14 g. To bring the fish into reproductive condition, they were kept in containers measuring 1 × 1 × 0.8 m, at a temperature of 27°C and in a light regime of 12L:12D. The fish were fed an artificial trout diet (45% protein, 15% fat) supplemented by live food (Artemia salinata).

The ovary and testis of 5 females and 5 males were examined under different conditions: non-reproductive, maintained in a large group, and 10 that were kept in a separate container: a total of 30 specimens.

When the female was in reproductive condition and the male had built the nest, the female was left with him for a further day and then injected with 600 ng/g carp gonadotropin (GGH) to induce maturation (Degani and Boker, 1992a). Samples of ovary and testis and 11 water were taken, to measure the glucosteroids.

Extraction

The specimens were anaesthetised with phenytoxyethanol, and the ovaries and testes removed. These were cut into small pieces, separately homogenised in distilled water, at a
Table 1. The steroids detected by GCMS from male gonads

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Male o.f.*</th>
<th>Male w.f.†</th>
<th>Female o.f.</th>
<th>Female w.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Testosterone</td>
<td>+</td>
<td>+</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>+</td>
<td>+</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>4-β-Methylcholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>+</td>
<td>+</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>17α,20β-Dihydroxy-4-pregnen-3-one</td>
<td>O</td>
<td>O</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Organic fraction; †water fraction. GC-MS sensitivity = 0.01.

Table 2. The glucosteroids detected by GCMS in the water

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Females</th>
<th>Males</th>
<th>F &amp; M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Estrone</td>
<td>+</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>+</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-β-Methylcholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

extracted with DMC (3 × 5 ml) and the solvent was removed to give the free steroid.

Derivation

Trimethylsilyl (TMS) and oxime-trimethylsilyl derivatives were prepared. The solvent was removed from the samples of steroids by a stream of nitrogen and 2% hydroxyl-
ammonium chloride in pyridine (200 μl) was added. The reaction mixture was heated for 1 hr at 100°C, thereby converting the ketosteroids into the corresponding oxime derivative. The solvent was removed under reduced pressure, and a freshly prepared silylising reagent (100 μl) of N,O-bis(trimethylsilyl)-acetamide (BSA) and trimethylsilyl chloride (TMCS) at a 9:1 ratio was added. The reaction was maintained at 70°C for 1 hr. The volatile compounds were removed under reduced pressure, hexane (2 ml) was added to the mixture to dissolve the silylised steroids, and the polar residue was removed by extraction with acetonitrile (2 x 0.2 ml). External standard methoxycholesterol (MCH) was added, and the solvents removed by a stream of nitrogen. The sample was dissolved in hexane (20 μl) and 8 μl injected into the GCMS.

Capillary gas chromatography–mass spectrometry

A Varian 3400 gas chromatograph mass spectrometer with HP fused silica capillary column (Ultra 1, cross-linked methyl silicone: film thickness, 0.17 μm; 30 m x 0.25 mm i.d.) was used with helium as carrier gas, at a flow rate of 2 ml/min. The injection port temperature was 260°C. The oven temperature was set at 160°C, increased to 190°C, at a rate of 15°C/min, 1 min after injection. After 0.5 min, a second increase, at a rate of 2°C/min, raised the temperature to 280°C. The multiplier detector was set at 1800 V for total ion monitoring, with a scan reach of 100–650 m/z. The mass spectra obtained were non-normalised spectra.
Spectra comparison—similarity index

The spectra of both the steroids derived from the seminal vesicle fluid and the reference steroids were reduced to 10 peaks, selected on the basis of the highest mass, times and the abundance values. The peaks from the reference steroids were stored. The algorithm used to compare the reduced spectra of the unknowns from the seminal vesicle fluid with those from the store was based on the following correlation (or similarity) index equation, given as a standard program in the HP-GCMS.

RESULTS

The steroids and glucosteroids detected in both male and female gonads by GCMS, at a sensitivity of 0.01 ng, and extracted by organic solvent and water, are shown in Tables 1 and 2, and their electron impact mass spectra in Figs 1 and 2.

The organic fraction of ovaries contained a very high concentration of lipids, making the identification of the steroids difficult. Most of the steroids from the gonads were detectable in the water fraction. The steroids E₂, T, 17α-hydroprogesterone (17-P), cholesterol (C), 4β-methylcholesterol (4β-MC), stigmasterol (S), estrone (ES) and 17,20-P were detected in both the organic and the water fractions of the gonads, though 17,20-P was detected only in the female gonad. Glucosteroids detected in the water containers are shown in Table 2. It appears that the difference between the results in Table 1 and Table 2 are due to the concentration of glucosteroids, which is much lower in the aquarium water than in the gonads.

A number of steroid glucoronides detected in the holding water of both males and females in this study have not been mentioned at all in studies of other species, e.g. E₂, 17-P and 4β-MC. On the other hand, a number of glucosteroids have been detected in other species, especially in the African catfish Clarias gariepinus by Schoonen et al. (1987), which were not detected in T. trichopterus in the present study.

DISCUSSION

This study shows that both male and female T. trichopterus secrete steroid glucuronides into the water, and these may function as chemical communicators, a hypothesis supported by previous studies. McKinnon and Liley (1987) show that the holding water of females changes the colour of male T. trichopterus, and the same study shows that the female secretes various glucosteroids. Degani (1990) and Degani and Boker (1991, 1992a) showed that 17,20-P in the female is a maturation-inducing steroid. Schreibman and Degani (unpublished data) found that 17,20-P stimulates the male into building the nest. In the present study, this steroid was found in the water fraction of the female only, whereas most of the steroids were found in both sexes, in the water fraction of the gonads, and in the holding water. The reason why the exception, 17,20-P, was not detected in the holding water is not clear; possibly, its dilution in the water brought its concentration below what is detectable by GCMS.

In goldfish, Carassius auratus, ovarian 17,20-P, which functions hormonally to induce final maturation in the oocytes, is also released into the water to act as a preovulatory primer pheromone that increases male gonadotropin (Sorensen and Stacey, 1987). There are grounds, therefore, for accepting that T. trichopterus may serve as a model from the whole family of Anabantidae.

The present study shows that the male T. trichopterus also secretes glucosteroids into the water. Degani and Schreibman (submitted) found that the water in which the male builds the nest has the effect of increasing the percentage of oocytes in exovielfogenesis in the female, in the same way as an injection of carp gonadotropin (cGtH) (Degani and Boker, 1991, 1992a, b).

17,20-P was detected in the female only when in reproductive condition, or after injection with cGtH. This result accords with previous results, which detected this steroid by high-pressure chromatography (HPLC) after injection with hCG (Degani, 1990). It may be that the absence of 17,20-P in the male can be ascribed to the low response of this steroid in GCMS analysis, and/or to the fact that its oxime diTMS derivative is divided into cis- and trans-configurations.

REFERENCES


