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Comparative Aspects of Steroid Metabolism

STEROID GLUCURONIDES AS MALE PHEROMONES IN THE REPRODUCTION OF THE AFRICAN CATFISH *CLARIAS GARIEPINUS*—A BRIEF REVIEW

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Summary—After ovulation, female African catfish are strongly attracted by the odor of male conspecifics. This attraction depends on the presence of the seminal vesicle, a part of the male reproductive organs. Removal of the seminal vesicle illustrates this fact. A low dose of seminal vesicle fluid, added to the water, appears to be highly attractive for catfish which have ovulated. Fractionation of the fluid and testing of the different fractions shows that steroid glucuronides could be responsible for the attraction. These steroid glucuronides can be identified with gas chromatographic-mass spectrometric analysis. A mixture of glucuronides, prepared to resemble the composition of the seminal vesicle fluid, evokes a dose-dependent attraction. The most potent odorant, observed by measuring electrical responses from the olfactory epithelium and from the olfactory tract appears to be 3α,17α-dihydroxy-5β-pregnan-20-one-3α-glucuronide.

PHEROMONES IN FISH

A general introduction

Fish and other aquatic animals are surrounded by seas of chemicals which have an important impact on their physiology and behavior. Detection of compounds released by predators, food and conspecifics are essential for survival, growth and reproduction. The range of soluble biochemical products which may serve as chemical signals in the aquatic environment are more extensive than in animals relying on airborne volatile compounds [1]. Therefore, it's not surprising that chemical communication is widespread in fish.

Pheromones, or intraspecific chemicals, have been defined by Karlson and Lüscher [2] as substances that are secreted to the outside by an individual and received by one or more individuals of the same species. Here they initiate a specific reaction, for instance a defined behavior or a developmental process. These pheromones are known to be involved in a wide range of processes such as general social attraction, species and individual recognition, parent–young interactions, alarm and fright reactions, migration (homing in salmon) and reproduction [1, 3–5]. Reproductive or sex pheromones affect a variety of responses, including stimulation of spawning readiness, sexual attraction and elicitation of courtship and spawning behavior [1, 6–10]. They have been categorized as releaser and primer pheromones, reflecting a difference in response time of the processes they stimulate. "Releasers" directly trigger a rapid response in the pheromone recipient, whereas "primers" alter the physiological status after a delay of hours or days. In most cases they do this through an action on the endocrine system [1]. The aquatic medium is characterized by a slow transmission and therefore a slow fade-out of the pheromone signal. It is tempting to suppose that as a consequence of these features some of these water-borne pheromones have multiple functions and may evoke both primer and releaser effects.

Releaser pheromones that evoke sexual attraction have been described in various species of fish. It is, however, not always clear whether such an attraction response is due to a real reproductive pheromone, or represents a general social attraction [9]. For instance, it has

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been claimed that in several Ictaluriid catfish species [11–15], rainbow trout, *Salmo gairdneri* [16], zebrafish, *Brachydanio rerio* [17] skin, mucus, urophysis and urine contain pheromones which evoke attraction of conspecifics. In these cases, however, there is no indication that the pheromone is related to reproduction. In experiments with other oviparous fish, the response to pheromones appears to be restricted to a sexual context.

Female sexual pheromones

In many species female releaser pheromonal activity has been found. It appears that holding water from ripe or ovulated females was more effective in attracting males or in inducing male reproductive behavior than water from non-ripe or unovulated females. This has also been found in several species of Belontiid [18–20]; ayu, *Plecoglossus altivelis* [21]; the cichlid fish *Haplochromis burtoni* [22]; goldfish, *Carassius auratus* [23, 24]; frill fin goby, *Bathygobius soporator* [25]; loach, *Misgurnus anguillicaudatus* [24]; rainbow trout [27]; pond smelt, *Hypomessus olidus* [28]; zebrafish [29] and in sea lamprey, *Petromyzon marinus* [30]. In the viviparous guppy, *Poecilia reticulata* [31, 33] and black molly, *Mollienesia sphenops* [34] a sex pheromone causes attraction of males and sexual behavior only shortly after parturition when females show maximal receptivity for mating. In most of these studies, the ovary was found to be the source of the sex pheromones.

A female primer pheromone was also hypothesized to be secreted by the ovary [9, 10, 35–37]. In the goldfish it is released prior to ovulation and stimulates milt production and gonadotropin (GTH) release in male goldfish.

Male sexual pheromones

Male attractants that function only in a sexual context are described in five Belontiid species [19], in the blenny, *Blennius pavo* [38]; the black goby, *Gobius jozo* [6, 39]; and in the sea lamprey [30]. In the black goby, sea lamprey and in some Belontiid species the response to the pheromone increased, or was only observed, during the period between ovulation and oviposition. The pheromonal production site is not uniform between species. In the blenny, it was found [38] that the attractant is secreted by the appendices of the anal fin spines and is probably under the direct influence of GTH from the pituitary. Colombo [6, 39] suggested that the attractive substance of the black goby is synthesized in the mesorchial gland, which is part of the testis. In the sea lamprey, Teeter [30] demonstrated the importance of the urogenital tract as a site of pheromone production, milt alone being ineffective. However, milt from ripe Pacific herring, *Clupea harengus pallasi*, induced mass spawning activity [40].

Primer pheromonal activity has also been described. The testes were considered to be the origin of a primer pheromone evoking oocyte maturation and ovulation in zebrafish [41]. Further, the existence of a male primer pheromone, inducing ovarian growth in pubertal African catfish by enhancing vitellogenesis, was observed by Van Weerd [42].

Identity of the sexual pheromones

Recently, there has been rapid progress in the elucidation of the chemical identity of pheromones. It appears that steroid hormones or their metabolites are responsible for the attraction of female black goby [6, 39], male zebrafish [29] and male guppies [33]. They also exert a primer effect, evoking ovulation in female zebrafish [41], GTH release and spermiation in male goldfish [9, 10, 35, 37]. In the goldfish and guppy these responses were obtained by administration of $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one and estradiol-17β, respectively. Most steroids, however, are apolar, poorly soluble in water and therefore they only have a limited transmission range. On the other hand, steroid conjugates are excellently soluble in water and therefore ideal to function as pheromones. Moreover, because of their gonadal origin they may provide information about the reproductive status of the fish. Glucuronidation has been found to be a common mechanism in the inactivation of steroid hormones in vertebrates, including fish [43]. These "inactive" steroids, however, were shown to elicit sexual attraction in zebrafish [7, 29], black goby, guppy and goldfish [6, 39] and ovulation in zebrafish [41].

SEXUAL PHEROMONES OF THE MALE AFRICAN CATFISH, *CLARIAS GARIEPINUS*

Culture and reproduction of the African catfish

Research on the effects of pheromones on reproduction has for practical reasons largely been confined to small species of fish of no aquacultural significance. However, the use of pheromones may be a new approach in the manipulation of spawning in fish culture [44]. Practical application of pheromones in fisheries has been reported by Timms and Kleerekoper [14]. Mississippi fishermen placed cages with ripe female channel catfish (*Ictalurus punctatus*) in the river to catch large numbers of males. The pheromone in the milt of Pacific herring is thought to be suitable for spawn-on-kelp operations in which impounded herring are induced to spawn on previously harvested kelp [40]. Similar strategies may be applicable in fish culture.

For several reasons, the African catfish, *Clarias gariepinus*, (*C. lazera*, [45]; *Cypriniformes*, *Clariidae*), is a very promising fish species for aquaculture [44, 46]. It has one major drawback: artificial reproduction to obtain fry, requires specialized facilities and skilled hands. Under laboratory and fish farm conditions, the catfish reach maturity at 6–9 months of age. From this moment, postvitellogenic eggs and ripe sperm cells are present but final egg maturation, ovulation and spermiation do not take place spontaneously. The reason for this lack of spontaneous reproduction in captivity is an insufficient preovulatory release of GTH by the pituitary [47]. Egg maturation and ovulation can be induced by hypophyzation with exogenous GTH [48] or by an endogenous GTH surge elicited by the administration of a mixture of luteinizing hormone-releasing hormone analogue (LHRHa) and dopamine antagonists such as pimozide, domperidon, sulpiride [47] or injection with the steroid hormones 17α-hydroxyprogesterone, 17α,20β-dihydroxy-4-pregnen-3-one [49] or 11-deoxycorticosteron-acetate [50]. Although these hormone-injection techniques are very effective in evoking ovulation, they have the disadvantage of being too complicated for untrained fish farmers. With the use of pheromones it might be possible to stimulate ovulation and, spawning in African catfish, simply by adding the substances to the water, as has been demonstrated in the angelfish, *Pterophyllum scalare* [57] and the zebrafish [41, 52]. In male goldfish it was shown that pheromones indeed cause a GTH surge [35, 36].

The prenuptial and spawning behavior of the African catfish has been studied under natural conditions [53–55], and in aquaria and ponds [56]. All these studies mention the importance of a stimulus associated with flood water or a rise in water level at the onset of a spawning run. Different hypotheses have been put forward with respect to the nature of the environmental cues leading to mass spawning. Van der Waal [56] considered a substance called petrichor, dissolved in flood waters that have run over dry ground as the final stimulus. Bruton [53], however, was of the opinion that spawning is due to a combination of physical and chemical factors such as changes in water chemistry, pH, temperature, clarity, flow velocity, flooding of plants and access to suitable spawning sites. Whatever the nature of the cue may be, it leads to aggregation of catfish in groups. In these groups, males show a sequence of prenuptial aggressive interactions and only the victors form pairs with females [53]. These pairs swim to the spawning sites in shallow water, on the edge of a lake or of a pool, to proceed with courtship and spawning. During courtship, males and females butt and chase one another. The male exhibits shivering motions over its body and frequently bumps and nudges against the female abdomen, while the female butts the male near the urogenital papillae. Before actual spawning, the female is followed closely by the male. Then the male gets ahead of the female and adopts a U-shape position around the head of the female for several seconds after which the male ejaculates sperm and the female releases a cloud of eggs. The eggs are distributed in all directions by a few vigorous beats with the females tail. This courtship and spawning behavior, including resting intervals, lasts one or two hours. Bruton [53] observed that pairs usually mate once or twice before they were disturbed and separated by conspecifics. He also saw catfish beating their tails vigorously when swimming past an egg deposition site after the original pair had left, and suggested that this might be due to a pheromone released during mating. Indeed, it is likely that coordination of these reproductive activities, which mostly take place during the night and in turbid water, is mediated by a variety of inter-sexual communication systems in which chemical communication plays a dominant role.

Based on the earlier mentioned experiments with zebrafish [29, 41] and black goby, guppy and goldfish [6, 39], it was hypothesized that in nature, steroid conjugates, produced by the gonads of African catfish of both sexes, play an important role as pheromones in reproduction. Utilization of such substances in catfish farming might mean abandoning hormonal injections to obtain fry. If so, this treatment would make artificial reproduction even more simple. Moreover, steroidal attractants may be useful in fisheries.

Source and nature of male sexual pheromones

Under laboratory conditions, female catfish which have ovulated through hormone treatment attract a males attention by butting and chasing it, a behavioral pattern which induces the male to follow the female.

To test pheromonal activities, attraction tests were performed with female catfish placed in a U-shaped two-choice maze [57]. A conspecific was placed in both ends of the aquarium behind a perforated partition. An unovulated female allowed to choose between water containing a male or a female conspecific did not show an obvious preference to either of them. After hormonally induced ovulation, however, females spent more time at the side where the male fish was kept. Olfactory stimuli are essential for this behavior since bilateral lesion of the olfactory tracts abolished the attraction response. When confronted with a male fish from which the seminal vesicle was removed and an intact male fish, female fish spent more time on the side of the latter after ovulation. Removal of the testes made males more attractive for ovulated females. Castration evokes an enlargement of the seminal vesicle, as previously described in the Indian catfish, *Heteropneustes fossilis* [58]. These results indicate that shortly after ovulation female African catfish are attracted by male conspecifics, and that the male pheromone originates from the seminal vesicle.

The seminal vesicle appears to be a strongly active secretory gland [59, 60]. It consists of lobes with numerous tubules, lined with epithelial cells that secrete a fluid, the seminal vesicle fluid, containing acid polysaccharides, acid, neutral and basic proteins, and phospholipids. This fluid, which is excreted via the sperm ducts, immobilizes the sperm cells and, after ejaculation, prolongs the period of sperm activity. Ultra-structural and enzyme-histochemical studies indicate an active steroid synthesis in interstitial cells and glucuronic acid synthesis in interstitial and epithelial cells.

Additional experiments [61] confirm that the seminal vesicle synthesizes an attractant. Seminal vesicle fluid elicits a dose-dependent attraction when added to the aquarium water of ovulated catfish. On the other hand, a high dose results in an avoidance response. Similar responses with high doses of attractants were observed in zebrafish [17], Ictaluriid catfish [12, 13] and Arctic charr, *Salvelinus alpinus* [62]. After fractionation of the fluid, the steroid conjugate fraction appeared to contain the attractant. Other constituents of the fluid such as polysaccharides, proteins, phospholipids and steroids failed to attract the females. After a treatment with β-glucuronidase, the steroid conjugate fraction lost its attractive effect. It can be concluded that the attractant consists of one or more steroid glucuronides.

Steroid glucuronide biosynthesis in the seminal vesicles

The steroidogenesis in the seminal vesicles of the African catfish was extensively studied [63–66]. Steroid metabolism was studied *in vitro* by homogenate and tissue incubations with [^{3}H]pregnenolone and [^{3}H]androstenedione as precursors. The metabolic pathway follows the 5-ene/4-ene route from pregnenolone via progesterone or 17α-hydroxypregnenolone to 17α-hydroxyprogesterone and androstenedione and then towards testosterone, 11β-hydroxyandrostenedione and 5β-reduced C19-steroids. Also a bypath from 17α-hydroxyprogesterone to 17α-hydroxy-5β-pregnan-3,20-dione and 3α, 17α-dihydroxy-5β-pregnane-20-one could be demonstrated. Apart from these compounds bioconversion to several steroid glucuronides has been found (Table 1). Moreover, with gas chromatography-mass spectrometry (GC-MS) the presence of these steroid glucuronides, mentioned in Table 1, could be demonstrated in the seminal fluid [67, 68]. A quantitative study [61] by GC-MS of the steroid glucuronides in seminal vesicle fluid from castrated males with enlarged seminal vesicles showed that most of the steroid glucuronides levels varied between 2×10^{-8} and 4×10^{-8}. However, the levels of testosterone glucuronide and etiocholanolone glucuronide (both/9×10^{-8}M) were considerably higher (Table 1).

By testing these glucuronides, it appeared that a synthetic mixture, prepared according to the GC-MS analysis of the fluid, induces a dose-dependent attraction of ovulated female catfish. It can therefore be concluded that the attractant

Table 1. Steroid glucuronide levels in the seminal vesicle fluid of the male African catfish

Steroidglucuronide	Concentration ($\times 10^{-8}$M)
3α,17α-Dihydroxy-5β-pregnan-20-one-Gl	2.2 ± 0.2
Etiocholanolone-Gl	8.6 ± 2.8
Testosterone-Gl	9.0 ± 2.0
3α,17β-Dihydroxy-5β-Androstane-Gl[a]	2.4 ± 1.9
3α,17β-Dihydroxy-5β-Androstan-11-one-Gl[a]	2.4 ± 0.3
3α,17β-Dihydroxy-5β-Androstan-17-one-Gl[a]	4.2 ± 1.0
3α,17β-Dihydroxy-5β-Androstane-Gl[a]	2.4 ± 1.1

[a]The position of the glucuronic group is unknown.

in the seminal vesicle fluid of the male African catfish consists of one or more steroid glucuronides.

Steroid glucuronides as olfactory stimuli

The distinction between olfaction and taste in fish is difficult since olfactory and gustatory stimuli both occur in the aqueous medium. A procedure to distinguish between taste and smell mediated responses is to study whether blocking olfactory input eliminates the response. Blocking of the gustatory input, however, is far more difficult [4]. With a few exceptions, for instance those regarding the nipping contact of male black molly, with female conspecifics [34], and those regarding the stimulation of the sexual activity of male three-spined stickleback, *Gasterosteus aculeatus* [69], all studies on pheromone detection, including our own studies on attraction inducing pheromones in the African catfish, demonstrate the importance of the olfactory system. Several behavioral and electrophysiological studies (for reviews see [3, 4] indicate that olfaction is more sensitive than taste, permitting food and pheromones to be detected at a distance, whereas gustation functions primarily as a contact sense.

To determine the possible role of steroid glucuronides from the seminal vesicle as olfactory stimuli for female African catfish, electrical responses were obtained from the olfactory epithelium by underwater electro-olfactography (EOG) recording [70]. The EOG reflects multi-unit receptor cell activity, responsible for the initiation of the olfactory neural impulses. The multi-unit electro-physiological recordings from the olfactory epithelium show that seminal vesicle fluid is an extremely potent odorant in female African catfish. The steroid glucuronides share in the olfactory response to seminal vesicle fluid. Their stimulatory capacity appeared to be strongly dependent on their molecular structure, especially the location of the glucuronic acid group. From the steroid glucuronides tested, those with glucuronic acid on the 3α-position were the most potent odorants; 3α,17α-hydroxy-5β-pregnane-20-one-3α-glucuronide has the lowest detection threshold (10^{-11} M). When the glucuronic acid is located at the 17β-position, steroid glucuronides were less potent as odorants; detection thresholds were about 10^{-7} M. Those with glucuronic acid at the 3β-position had thresholds between 10^{-6} and 10^{-7} M. Considering these thresholds in relation to their concentrations in seminal vesicle fluid [68], it is unlikely that steroids glucuronidated at the 3β-position play an important role as pheromones.

Olfactory pathways in the brain

The African catfish has pedunculated olfactory bulbs, i.e. the bulbs are connected to the telencephalon by long olfactory tracts. The olfactory tracts can be subdivided into medial (MOT) and lateral (LOT) olfactory tracts. Attraction tests with female African catfish with bilaterally transsected LOT or MOT showed that the MOT and not the LOT is essential for the pheromonal response [71]. In addition, the neural connections of these tracts with regions in the brain were traced with horseradish peroxidase. It was shown that the area dorsalis telencephali contains numerous neurons from which processes terminate in the olfactory bulb. This area may be involved in the regulation of the responsiveness to pheromones. High densities of terminals from bulbar mitral cells in the posterior pars ventralis and pars supracommissuralis of the area ventralis telencephali (pVv-Vs), nucleus preopticus periventricularis (NPP), nucleus preopticus and nucleus recessus posterioris indicate a function of these areas in the perception of pheromonal stimuli. In goldfish [72] and himé salmon (kokanee), *Oncorhynchus nerka* [73] neuroethological experiments have demonstrated the importance of the pVv-Vs and NPP regions in the regulation of sexual behavior. The olfactory fibers that end in the preoptic region may represent a pathway via which pheromones evoke GTH release and ovulation, as this preoptic region contains perikarya of GnRH neurons with axons running towards the proximal pars distalis of the pituitary [74] and contacting the gonadotropic cells [75].

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