2. Biological bases for aquaculture

Aquat. Living Resour., 1996, Vol. 9, **Hors série**, 45-58

Internal and external factors controlling reproduction in the African catfish, *Clarias gariepinus*

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Accepted November 7, 1995.

Goos H. J. Th., C. J. J. Richter. *In*: The biology and culture of catfishes. M. Legendre, J.-P. Proteau eds. *Aquat. Living Resour.*, 1996, Vol. 9, **Hors série**, 45-58.

Abstract

The African catfish, *Clarias gariepinus*, is a highly appreciated species for aquaculture, because of its favourable food conversion, its resistance to diseases, its relatively low requirements for water quality, the possibility for high stocking density and the excellent meat quality. For those reasons even in the Netherlands, there is a modest, but Europe's largest and still expanding, African catfish farming activity.

Although this species grows and matures in captivity, there is no spontaneous reproduction. We could demonstrate that the failure to reproduce resides in the brain-pituiary-gonad axis. Hormones required for oogenesis and spermatogenesis are being produced in sufficient quantities. However, final oocyte maturation, ovulation, spermiation and spawning behaviour do not occur, due to the lack of a gonadotropin surge.

In nature, the prespawning gonadotropin surge is induced by environmental factors such as the water level in the spawning area. Under farming conditions the environmental cues are hard to identify and/or to mimic. In combination with unavoidable stress this causes a blockade of the release of gonadotropin releasing hormone (GnRH). Consequently, gonadotropin surge release fails to occur, which is enforced by an effective hypothalamic dopaminergic inhibition. The gonadrotropin surge induces the conversion of 17 αOH-progesterone into 17 αhydroxy-20 β-dihydroprogesterone, the final maturation inducing substance.

Based on these data, several protocols for artificial propagation could be developed. They include either a treatment with a GnRH analogue in combination with a dopamine receptor antagonist, a treatment with homologous gonadotropin or HCG, or a treatment with 17 αOH-progesterone.

Since a number of years we have used the African catfish as a model for fundamental research on fish reproductive endocrinology. Till now one gonadotropic hormone (GTH) could be demonstrated. Its amino acid composition and sequence was analysed and appeared to be homologous with known forms of the maturational GTH (GTH-II). Specific radioimmuno assays for the complete hormone and its α- and β-subunit respectively, have been developed. cDNAS encoding the subunits have been cloned. They are applied now for Northern blotting and *in situ* hybridization.

GnRHs were fully characterised (a specific catfish-GnRH and chicken-GnRH-II). Specific antibodies against these peptides were raised and the cDNAS encoding the hormone precursor molecules were cloned and used for respectively immunocytochemical localisation and radioimmunoassays, and *in situ* hybridisation. The importance of the two GnRH forms for gonadotropin release was studied. Chicken-GnRH-II appears to be 10 to 100 times more potent than catfish GnRH, probably due to its higher receptor affinity. Catfish GnRH, however, is present in the brain and pituitary about 100 times more than chicken GnRH-II.

Steroid hormone synthesis by ovaries, testis and seminal vescicles was analysed. The sex steroids that play a role in the negative feedback control of gonadotopin release were identified (11-keto-testosterone and testosterone) and their interaction with hypothalamic dopamine metabolism was demonstrated as one of the possible mechanisms of action. Several steroid conjugates from the seminal vesicles were shown to have pheromonal activities, involved in reproductive behaviour. They induce under certain physiological conditions attraction between conspecifics and synchronization of ovulation.

Keywords: Endocrinology, reproduction, catfish, *Clarias gariepinus*.

INTRODUCTION

Recent years have witnessed an increasing interest in the African catfish, *Clarias gariepinus*, for fish farming, not only in African countries but also in countries like The Netherlands. Its favourable food conversion, its resistance to diseases, its relatively low requirements for water quality, the possibility for high stocking densities and the excellent meat quality are the main factors promoting African catfish farming. For these reasons, even in The Netherlands there is a modest but expanding catfish farming industry, now producing some 1 300 tons yearly. Under natural conditions, the annual breading season of this species, as of most Clariidae, is limited to a few months (Bruton, 1979). The internal gonadal maturation rhythm for Clarias species seems to differ from region to region. Spawning starts with the onset of the rainy season and takes place in flooded areas between: (i) July and September (the Nile-delta, Egypt and in the Ubanqui River, Central African Republic), (ii) in March-April in Uganda (lake Victoria), (iii) December and February in Zimbabwe (Mazoe), and (iii) May and August in Israel (Hula-reserve) (for review, see Richter, 1976; Van Den Hurk *et al.*, 1984, 1985).

In captivity, the African catfish usually reaches sexual maturity at 6-9 months of age. From this moment on postvitellogenic eggs and ripe sperm cells are present. However, although oogenesis and spermatogenesis seem to be normal, final ooctyte maturation, ovulation, spermiation and reproductive behaviour do not occur. It is likely that under laboratory and fish-farming conditions the regulation of these processes is incomplete due to unavoidable stress. Natural conditions are hard to mimic on a large scale in a fish farm. In order to overcome this problem and to be able to induce reproduction artificially by means of hormonal treatments, it was essential to investigate the successive steps in the endocrine regulation of reproduction of this species.

In teleosts, as in all other vertebrates, the gonadal functions are regulated by gonadotropic hormones produced by the pituitary gland. The secretion of gonadotropic hromone (GTH) is regulated by neurohormones, mainly of hypothalamic origin and by gonadal hormones. Consequently, the cells producing GTH play a central role in the control of reproduction. Investigations to understand the regulation of these cells led to the development of several effective protocols for artificial propagation of the African catfish. Moreover, these studies contributed to the fundamental knowledge of the hormones of the brain-pituitary-gonadal axis, their chemistry, the control of synthesis and release and their mode of action.

Gonadotropic hormone (GTH)

A glycoprotein with gonadotropic activity has been isolated from the pituitary of male and female African catfish (Goos *et al.*, 1986; Koide *et al.*, 1992). From the amino acid composition and sequence analysis, compared with those of salmon and carp-GTH II-α and GTH II-β it was concluded that it represents the GTH type II or maturational GTH. Its biological activity was tested on the production of cyclic AMP by eel ovarian tissue and on the production of 11 β-hydroxyandrostenedione and 17 α-hydroxy-20 β-dihydroprogesterone by catfish testis *in vitro*. Polyclonal antibodies were raised against the purified β-subunit. Immunocyochemical study showed them to bind specifically to hypophysial gonadotropic cells (Peute *et al.*, 1984) (for morphologica characterisation of gonadotropic cells, see van Oordt and Peute, 1983).

Two molecular forms of GTH have been demonstrated in salmonids: GTH I and GTH II. They show structural homology with respectively mammalian FSH and LH (Suzuki *et al.*, 1988). No functional difference between the two hormones has been demonstrated yet. Although they are equally potent in inducing gonadal steroid hormone production, for several reasons GTH I may be considered to be involved in gametogenesis, while GTH II controls final oocyte maturation and ovulation (Nozaki *et al.*, 1990). To date only one form of GTH has been demonstrated in the African catfish.

Pituitary gonadotropin content and the ultrastructure of the GTH cells as well as gonadal development were followed during an annual cycle in a natural habitat (Hula reserve, Israel). Over a period of one year, male and female specimens were collected monthly. Both the ultrastructural appearance of the gonadotropes and the pituitary gonadotropin content showed cyclical changes in both sexes, which parallelled alterations in the reproductive cycle. The annual reproductive cycle could be divided into three periods. In summer (May until August), during the breeding period, the gonadotropes were large and fully granulated, and pituitary gonadotropin reached maximum levels, even though at least once during that period a gonadotropin surge takes place, leading to oocyte maturation and ovulation, and spermiation. Ovulation and spermiation are not fully compensated by the production of new postvitellogenic oocytes and sperm cells respectively, so that during the breeding period gametogenesis comes to an end. Judging from the 3β-hydroxysteroid dehydrogenase activity, steroid synthesis in the gonads is at its maximum during the breeding period. After spawning, the resting period begins (September-February), with an increasing number of fusion products of secretory granules and globules in the gonadotropes, probably indicating a breakdown of stored hormone. This is followed by the appearance of residual bodies, cell shrinkage and a considerable drop in pituitary gonadotropin content. Gametogenesis remains absent. In late winter and early spring (March-April), during gonadal recrudescence, with full gametogenesis and restoration of sex steroid synthesis, the gonadotropes redeveloped, *i.e.* they increased in size and granulation and at the same time the hypophysial gonadotropin content was augmented

(Peute *et al.*, 1986; Van Den Hurk *et al.*, 1984, 1985). This indicates that induced spawning and artificial propagation of African catfish from this particular region will be possible from March till September.

Thus, feral African catfish have a discontinuous reproductive cycle, regulated by cyclically active gonadotropes. But this is not the case in African catfish raised and constantly kept under favourable husbandry conditions. Throughout the year their pituitaries contain large and densely granulated gonadotropes (Peute *et al.*, 1984), storing large amounts of gonadotropin (De Leeuw *et al.*, 1985 *a*). The gonads of such animals show a continuous cycle with numerous ripe sperm cells and postvitellogenic oocytes at all seasons (Richter and Van Den Hurk, 1982), but without spontaneous spermiation, ooctyte maturation and ovulation (Richter *et al.*, 1987 *b*). The absence of a discontinuity in the annual reproductive cycle is primarily the result of the absence of a prespawning gonadotropin surge and a postspawning regression of the gonadotropes.

Control of gonadotropin release

Gonadotropin releasing hormones

In mammals the release of FSH and LH is stimulated by luteinizing hormone releasing hormone (LHRH). This neuropeptide of hypothalamic origin has been identified as a decapeptide: pyro Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2. In teleost fishes also the release of GTH is under control of a releasing hormone, referred to as gonadotropin releasing hormone (GnRH). GnRH has been demonstrated in crude extracts of hypothalamus and telencephalon of teleosts (for review: see Peter, 1983). From brains of the chum salmon, Sherwood *et al.* (1983) purified GnRH and identified its primary structure as pyro Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH2. This salmon GnRH (sGnRH) differs from mammalian LHRH in only two amino acids, respectively on the seventh and eighth position. In many teleosts, including the African catfish (De Leeuw *et al.*, 1985 *a*) synthetic mammalian LHRH or its superactive analogues stimulate GTH release, both *in vivo* and *in vitro* (for review see Donaldson and Hunter, 1983), indicating an overlap in the biological activity of LHRH and GnRH.

In contrast to mammals in most other vertebrates investigated so far more than one molecular form of GnRH has been demonstrated (for review, see; Schulz *et al.*, 1993 *a*). In the African catfish till now two GnRHs have been found (Sherwood *et al.*, 1989; Bogerd *et al.*, 1992). One of them is identical to chicken GnRH II ([His^5, Trp^7, Tyr^8]GnRH, cGnRH-II), the other one has been identified only in two catfish species so far, the African catfish and the Thai catfish. It will be referred to as catfish GnRH ([His^5, Asn^8]GnRH, cfGnRH) (Bogerd *et al.*, 1992; Ngamvongchon *et al.*, 1992).

Binding of GnRH to specific plasma membrane receptors is the first step leading to gonadotropin secretion (Conn *et al.*, 1981). Receptors for GnRH in the catfish pituitary were characterised using a radioligand prepared from a superactive analogue of sGnRH (D-Arg^6, Pro^9-sGnRH; SGnRHa). The binding of ^{125}I-sGnRHa to pituitary membranes was found to be saturable and displaceable, indicating receptor specific binding. A Scatchard analysis of the saturation data demonstrated the presence of a single class of high-affinity binding sites (Ka = $0.901 \pm 0.06 \times 10^9$ M^{-1}, Bmax = 1678 ± 150 fmol/mg protein). No difference in binding affinity or capacity was found between males and females. Binding affinity and biological activity of a number of mammalian LHRH and sGnRH analogues showed a close correlation between receptor binding and GTH release potency. However, biological degradation may be the primary factor determining the bioactivity of GnRHs in the African catfish (De Leeuw *et al.*, 1988 *a, b*).

In a radio receptor assay, using sGnRHa (D-Arg^6, Trp^7, Leu^8, Pro-NEt) known to have high affinity to catfish pituitary GnRH receptors as radio ligand, displacement by cfGnRH and cGnRH-II respectively was studied. cGnRH-II competed with ^{125}IsGnRHa for pituitary GnRH binding sites, whereas cfGnRH did so only slightly. These data correspond with the GTH releasing potency of the two GnRHs and their respective analogues. *In vitro* studies, using either pituitary fragments or pituitary cell suspensions in a perifusion system or static culture showed effective dosages of 10^{-8} M for native cGnRH-II and 10^{-5} M for cfGnRH. Their synthetic analogues were slightly more potent (Schulz *et al.*, 1993 *a*).

Specific antibodies against cGnRH-II and cfGnRH have been raised. In a radioimmuno assay cross reaction between the two appeared to be less than one percent. Although no specific radioimmuno assay for catfish GnRH could be developed (since this peptide does not contain Tyr, labelling with ^{125}I has not been carried out yet), data based on a cGnRH-II specific and a cfGnRH unspecific assay revealed that cfGnRH may be present in amounts 40-100 fold compared to cGnRH-II. Thus, although cfGnRH has only limited GTH releasing activity, probably due to low receptor affinity, this could be compensated by its high concentrations. Preliminary data have shown that the two GnRHs in the catfish have synergistic actions with respect to GTH release.

Immunocytochemical data about the localisation of GnRH perikarya in the catfish brain have been contradictory. In the first attempt to localise these cells we used an anti-mammalian LHRH antibody. A clear and apparently specific immune reaction was observed in a great number of neurons of the pre-optic nucleus. Later studies, using more specific antibodies, cGnRH-II was localised in the midbrian tegmentum and cfGnRH in the ventromedial hypothalamus. The pre-optic nucleus did not show any

binding with both antibodies. cDNAs, encoding the two GnRH precursor molecules were cloned and used for *in situ* hybridisation. The results of these studies confirmed the immunocytochemical localisation, using the specific antibodies (Bogerd *et al.*, 1994).

Neuro-amines

In goldfish, large lesions in the nucleus lateralis tuberis-region as well as destruction of the pituitary stalk caused a dramatic rise in GTH release (Peter *et al.*, 1978). This points towards the existence of a gonadotropin release-inhibitory factor (GRIF). On the basis of experiments in which lesions were placed in a variety of locations in the diencephalon, Peter and Paulenco (1980) concluded that GRIF originates from the antero-ventral pre-optic region. Chang and Peter (1983) observed a dopaminergic inhibition of the increased GTH release caused by pre-optic lesions and a dopaminergic inhibition of the LHRHa-induced GTH release. They concluded that dopamine (DA) has a GRIF activity; it may act directly by inhibiting spontaneous GTH release and indirectly by suppressing the stimulatory effect of GnRH. A similar mechanism has been demonstrated in a number of other teleost species (carp, *Cyprinus carpio,* Billard *et al.*, 1983; eel, *Anguilla anguilla*, Dufour *et al.*, 1984), including the African catfish (De Leeuw *et al.*, 1985 *a*). In African catfish the effect of the dopamine antagonist pimozide (PIM), a LHRHa and a combination of the two was studied on GTH release *in vivo* as well as *in vitro*. PIM alone increaseed plasma GTH levels in juvenile catfish but was ineffective in adults. LHRa caused an increase in plasma GTH levels but the effect was doubled in females and almost five fold more in males by the combination of LHRHa and PIM. From these experiments it was concluded that dopamine, at least in adults, does not affect GTH release directly, but modulates the effect of endogenous and exogenous GnRH. Likewise, dopamine and its agonist apomorphine inhibited the GnRHa induced GTH release *in vivo* (De Leeuw *et al.*, 1987). The results were confirmed by *in vitro* experiments by De Leeuw *et al.* (1986 *a*). Pituitary fragments and pituitary cell suspensions released GTH spontaneously, which stabilised after a few hours. The dopamine agonist apomorphine caused a decrease in the release from pituitary fragments (which still contain endogenous GnRH), but not from pituitary cell suspensions (free of endogenous GnRH). Apomorphine abolished the GnRHa-induced GTH release from both fragments and cell suspensions. These results were confirmed in a study in which a number of drugs with known anti-dopamine and anti-serotonine properties were tested for their capacity to enhance the GnRH induced GTH release. All anti-dopamine drugs stimulated the GnRH induced GTH release. Non of the anti-serotonine drugs had this effect (Goos *et al.*, 1987).

Van Asselt *et al.* (1988) demonstrated that dopamine acts via the D2 type of receptor to inhibit the GnRH induced GTH release. This followed from the effects of specific D1 and D2 agonistic and antagonistic drugs applied *in vivo* in female African catfish. The D1 antagonist SCH23390 and the D1 agonist SKF38393-A had no effect on the LHRHa-induced GTH release. The D2 antagonists Sulpiride and Domperidone, however, both enhanced the LHRHa induced GTH release. Bromocryptine, a D2 agonist showed its dopamine agonistic effect by inhibiting the LHRHa induced GTH release. Pimozide is considered to be a nonspecific (D1 as well as D2) dopamine receptor antagonist. Its stimulatory effect on GTH release could be abolished by the D2 agonist Bromocryptine, but not by the D1 agonist SKF38393-A. The D2 receptor was further characterised by Van Asselt *et al.* (1990), using [^{3}H]spiperone as radioligand. The binding was saturable and binding characteristics, estimated by Scatchard analysis were: Kd = $3.2 \pm 0.5 \times 10^{-9}$ M and Bmax = 105 ± 5 fmol/mg protein. Specific binding was displaceable with dopamine and with various specific D2 agonists and antagonists. Binding experiments with cell fractions, obtained after centrifugation of dispersed pituitary cells over a Percoll density gradient, showed that most ligand binding was obtained in an enriched gonadotropic cell fraction. This observation indicates that the above mentioned dopamine receptor data account for dopamine receptors on gonadotropic cells.

Interaction between GnRHs and dopamine

Dopamine inhibits the GnRH induced gonadotropin release. *In vitro* studies demonstrated that at least part of the effect of dopamine is directly on the GTH cells. The mechanism of interaction between GnRH and dopamine has not been completely elucidated, but there are indications that more than one mechanism is involved.

De Leeuw *et al.* (1988 *c*; 1989) demonstrated that the binding capacity of pituitary membrane bound GnRH receptors decreased as consequence of dopamine action in catfish as well as in goldfish. Dopamine may also interact with GnRH action at the level of the GnRH post-receptor mechanisms. Therefore some aspects of these mechanisms have been investigated.

The involvement of extracellular Ca^{2+} was investigated. As in mammals, extracellular Ca^{2+} seemed to be obligatory for the GnRH induced GTH release. This was concluded from *in vitro* experiments, in which GTH release was monitored in a perifusion system. The GnRH stimulation of GTH release was strongly inhibited in the absence of Ca^{2+} or in the presence of the Ca^{2+}-channel blocker Nifedipine. Furthermore, the Ca^{2+}-ionophore A23187 mimicked the effect of GnRH, by causing enhanced GTH release. The action of A23187 was dependent on Ca^{2+} (Van Asselt *et al.*, 1989 *a*).

The second messenger cAMP appeared to be involved in GnRH action. A GnRH-like effect could be

induced by the cAMP analogue dibutyryl cAMP (db-cAMP) or by the adenylate cyclase activator Forskolin. In addition, GTH release, induced by the GnRH analogue Buserelin was accompanied with a rise in cAMP concentrations in the incubation medium. In the same study, the dependence of the cAMP synthesis and the effect of extracellular Ca^{2+} was investigated. Db-cAMP and Forskolin were unable to stimulate GTH release in the absence of Ca^{2+}. Buserelin induced GTH release was almost completely inhibited in the absence of Ca^{2+}. The increased production of cAMP, however, remained unchanged. These results indicate that the action of cAMP on GTH release is dependent on Ca^{2+}, whereas cAMP production is not.

For mammals it is generally accepted that dopamine, when bound by D2 receptors, inhibits the enzyme adenylate cyclase. Since the dopamine receptors, involved in the inhibition of the GTH release in the African catfish were characterised as D2 receptors, interference with cAMP production could be one of the mechanisms by which dopamine inhibits GnRH-stimulated GTH release. It was demonstrated that dopamine completely blocks the Forskolin-induced GTH release, whereas Forskolin-induced cAMP levels were not affected by dopamine. Similar results were obtained when GTH release was stimulated with Buserelin. These results indicate that dopamine inhibits GnRH-stimulated GTH release independently of cAMP production or breakdown. This suggests that the site of action of dopamine is located after the GnRH-induced cAMP accumulation. Since it was demonstrated that the actions of GnRH and of cAMP are Ca^{2+}-dependent, calcium mobilisation might be the target of dopamine (Van Asselt *et al.*, 1991).

Gonadal steroid hormones

Gonadal steroid production has been analysed in order to answer three questions: (i) which testicular steroid hormones are involved in the control of gonadotropin secretion? (ii) are the gonads, especially the testis and the seminal vescicles a source for sex pheromones? (iii) which steroid hormones are involved in oocyte maturation and ovulation?

The involvement of gonadal sterois in the control of gonadotropin secretion

Because of the central role of gonadotropin in reproductive processes, the regulation of its release from the gonadotropic cells in the pituitary has been the subject of extensive investigations. It is generally established that gonadal steroid hormones are involved in the regulation of hypophysial gonadotropin secretion. This can be either a positive feedback action of the gonadal steroids to induce the hypothalamus-pituitary system to reach higher activity levels, or a negative feedback action, mainly to stabilise the secretion of GTH. This section will concentrate on the negative feedback in male African catfish.

One of the first observations indicating the existence of such a regulatory mechanism came from Severinghaus (1939), who noticed the appearance of hyperactive cells in the pituitary of the castrated male rat. Later on a correlation was found between the presence of these "castration-cells" and elevated plasma gonadotropin levels. Both could be restored by gonadal steroid replacement.

A number of investigations have shown that a comparable feedback system is present in fish. In the Indian catfish, *Heteropneustus fossilis,* androgens and estrogens have a negative effect on GTH release (Sundararaj and Goswami, 1968). Likewise, anti-estrogens like chlomiphene citrate and tamoxifen appeared to cause a rise in the plasma GTH levels, as was demonstrated in the goldfish (Billard and Peter, 1977). Castration also resulted in increased plasma GTH levels. In the rainbow trout, *Oncorhynchus mykiss* (Billard *et al.,* 1977; Van Putten *et al.,* 1981) and the three spined stickleback, *Gasterosteus aculeatus* (Borg *et al.,* 1985), this was accompanied by a partial degranulation of GTH cells. Billard (1978) and Bommelaer *et al.* (1981) showed that the increase in GTH levels could be prevented by testosterone and estradiol, and to some extent, by 11-ketotestosterone. In the African catfish, De Leeuw *et al.* (1986 *b*) showed that the aromatizable androgens androstenedione and testosterone, but not the 11-oxygenated 11β-hydroxyandrostenedione, had the capacity to decrease castration induced elevated plasma GTH levels. De Leeuw *et al.* (1988 *d*) could demonstrate that castration resulted an increase GnRH binding sites and a higher GnRH sensitivity. Both could be compensated by androgen treatment. As both dopamine and steroid hormones have under certain circumstances a negative effect on GTH release, the action of the two compounds may be related. Evidence for such an interaction was found as early as 1969 by Fuxe and co-workers, who observed that the turnover of dopamine in the median eminence of the rat was decreased after gonadectomy, but restored by treatment with gonadal steroids. Moreover, Knight *et al.* (1981) showed in domestic fowl that concentrations of dopamine in discrete areas of the diencephalon were correlated with changes in plasma luteinizing hormone levels induced by gonadectomy. Testosterone was effective in restoring LH levels and, partially, dopamine concentrations to control values.

We have described and investigated a model to explain the mechanism of action of gonadal steroid hormones in exerting their negative feedback on GTH secretion in connection with the dopaminergic inhibition. It is based on the competitive inhibition by catecholestrogens of the enzyme catechol-O-methyltransferase (COMT), which results in a decreased conversion of dopamine into 3-methoxytyramine (3-MT). With respect to this mechanism a hypothesis was formulated by Lambert *et al.* (1984), described in more detail by De Leeuw *et al.* (1985 *b*), Goos (1987) and Timmers

and Lambert (1988). According to this hypothesis, androgens are aromatised to estrogens by the enzyme aromatase. Subsequently, the estrogens are converted to catecholestrogens by the enzyme 2-hydroxylase, followed by a further metabolization into methoxyestrogens by the enzyme COMT. As COMT is also involved in the inactivation of dopamine by converting it into 3-MT, a regulation of gonadotropin release by aromatizable androgens could be explained by this hypothesis.

All enzymes involved in this hypothetical mechanism, aromatase, 2-hydroxylase and COMT indeed were found in the brain and pituitary of the African catfish (De Leeuw *et al.*, 1985 *b*; Timmers *et al.*, 1987, 1988; Timmers and Lambert, 1988; Van Asselt *et al.*, 1989 *b*). Moreover, Timmers and Lambert (1988) and Van Asselt *et al.* (1989 *b*) showed in enzyme kinetic studies that COMT has a higher affinity for catecholestrogens compared to dopamine, indicating that catecholestrogens may protect dopamine from COMT-induced degradation. Timmers *et al.* (1988) showed that steroids can enter catfish brain, and that subsequently aromatizable androgens are converted into estrogens *in vivo*.

Recently, a series of investigations was carried out to further evaluate this hypothesis.

To determine the gonadal steroids that could be involved in the regulation of GTH secretion the total steroid secretory capacity of the testis of the African catfish was studied *in vitro*, using gas chromatography-mass spectrometry (GC-MS). Incubations were carried out with testicular fragments without adding any exogenous precursor or cofactor. After 24 h of incubation, twenty-two steroids could be identified in the medium. 11β-hydroxyandrostenedione, 11β-hydroxytestosterone, 11-ketotestosterone and 11-ketoandrostenedione were dominating. Besides these 11-oxygenated androgens, lower amounts of testosterone and androstenedione were detected, as well as 5β-reduced pregnanes and 5α- and 5β-reduced androstanes (Vermeulen *et al.*, 1993).

Steroids that play a role in feedback regulation, have to be present in the circulation. Therefore, the steroids that are produced by the testis *in vitro* were identified and quantified in blood plasma before and after castration using GC-MS. Before castration, most of the testicular steroids produced *in vitro* could be detected in the plasma. Quantitatively dominating steroids were testosterone (16.9 $\pm$ 4.3 ng/ml), androstenedione (12.0 $\pm$ 3.9 ng/ml) and 11-ketotestosterone (6.7 $\pm$ 1.8 ng/ml). Levels of all other identified steroids were less than 2.5 ng/ml. After castration, the plasma levels of only the three mentioned steroids decreased dramatically, indicating their testicular origin. (Vermeulen *et al.*, 1994). From these results and those of comparable studies, although limited to androgens, in the sailfin molly, *Poecilia latipinna* (Kime and Groves, 1986), rainbow trout (Schulz and Blüm, 1987), the baltic salmon, *Salmo salar* (Mayer *et al.*, 1990 *a*) and three-spined stickleback (Mayer *et al.*, 1990 *b*), it can be concluded that only a few androgens, aromatizable as well as 11-oxygenated ones, decrease after castration.

Steroids involved in the feedback regulation of the pituitary gonadotropes are supposed to be under gonadotropic control. Fish were treated with two doses of a salmon gonadotropin releasing hormone analogue (sGnRHa). A low dose (0.25 μg/kg body weight), caused an increase in plasma GTH level, comparable to the level after castration. This resulted in an increase of plasma levels of only the three aforementioned steroids, testosterone, androstenedione and 11-ketotestosterone. These results indicate that testosterone, androstenedione and 11-ketotestosterone may be involved in the feedback regulation within the brain-pituitary-gonad axis (Vermeulen *et al.*, 1994).

As the plasma levels of only three testicular steroids decreased after castration, the remaining steroids are possibly produced at extratesticular sites. An alternative source of steroid hormones is the adrenal or interrenal tissue. Studies in vertebrates, including teleosts, revealed that interrenal tissue not only produces corticosteroids but also androgens (Arai *et al.*, 1969; Hyatt *et al.*, 1983; Andò *et al.*, 1989; Balm *et al.*, 1989; Schreck *et al.*, 1989; Bélanger *et al.*, 1990). To elucidate the role of adrenal tissue in the production of "testicular" steroids in the African catfish, the adrenal tissue was localised and *in vitro* incubations with adrenal tissue fragments were carried out. GC-MS analysis of the incubation medium demonstrated that, indeed, most of the "testicular" steroids, that were detectable in the plasma after castration, are produced by the interrenal tissue. Therefore, this can be considered as an extra-testicular source of "gonadal" steroids.

Of the testicular steroids, only testosterone and androstenedione may be involved in the studied feedback model within the brain-pituitary-gonad axis. 11-ketotestosterone, also a canditate for playing a feedback role, was not included, because the hypothetical feedback mechanism requires aromatisation and 11-ketotestosterone is a non-aromatizable steroid. The uptake pattern of tritiated testosterone and androstenedione was studied *in vivo*. [^{3}H]-testosterone and [^{3}H]-androstenedione were injected intravenously in male catfish. After 1.5 h the brains were removed and, after microdissection of the brain, the uptake of steroids in specific, well defined brain nuclei was determined. The highest uptake of steroids was measured in the diencephalic pre-optic area, the *nucleus lateralis tuberis* (NLT), and the *nucleus diffuses lobi inferioris* (NDLI). Although relatively little information exists on the uptake of tritiated steroids in the brain of teleosts, the distribution pattern of [^{3}H]-testosterone in the green sunfish, *Lepomis cyanellis* (Morrell *et al.*, 1975) and the paradise fish, *Macropodis opercularis* (Davis *et al.*, 1977) show striking similarities with the uptake in the catfish brain.

The tritiated androgens injected in catfish were mainly aromatised to estradiol and estrone. Indications that this aromatisation has taken place within the brain nuclei and pituitary and not in peripheral tissue are 1) there presence of only low levels of estrogens in the blood after intravenous injections of the tritiated androgens and 2) conversion into estrogens was also seen after *in vitro* incubation of distinct parts of the brain with the respective tritiated androgens (Vermeulen, pers. comm.).

As the hypothetical model also requires the presence of dopamine, this catecholamine was determined in distinct brain areas using HPLC with electrochemical detection (ECD). High dopamine levels were demonstrated in the tuberal area (NLT), *nucleus recessus lateralis* (NRL) and *nucleus recessus posterioris* (NRP), but low levels in the pre-optic area and the pituitary. The distribution of dopamine-immunoreactivity in the brain of the catfish (Corio *et al.*, 1991) supports the biochemical findings; in the NLT, NRL and NRP high numbers of dopamine-immunoreactive cell bodies were found. Dopamine containing fibres were found in the pre-optic area, which corresponds to relatively low dopamine levels using HPLC-ECD.

Combination of androgen uptake, availability of the enzymes aromatase, 2-hydroxylase and COMT, and the presence of dopamine led to the conclusion that, if the regulation of GTH release occurs by an interaction between aromatizable steroids and dopamine metabolism, the brain regions in which this mechanism might be located are the NLT, NRL and NRP. In spite of the low dopamine content, the pre-optic area and pituitary cannot be excluded because of dopamine fibres innervating these areas.

To get an indication which of the above mentioned brain areas could be involved in the regulation of GTH release by steroid hormones via the dopamine metabolism, the turnover of dopamine was investigated in these brain areas before and after castration, as alterations in dopamine turnover are quantitatively related to dopamine release in mammals (Roffer-Tarlov *et al.*, 1971; Barraclough and Wise, 1982; Dulka *et al.*, 1992). The dopamine turnover was determined by measuring the ratio between dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3-MT), using HPLC-ECD. An effect of castration on dopamine turnover was observed only in the pre-optic area, where a diminished DOPAC/dopamine ratio was found, indicating a decrease in turnover of dopamine.

After establishing the pre-optic ara as the brain area most likely involved in the regulation of GTH secretion according to the postulated hypothesis, the next step was to investigate which of the gonadal steroids has an effect on dopamine turnover in the pre-optic area. Testosterone, implanted in male catfish immediately after castration could maintain the control values of dopamine turnover and GTH plasma levels despite castration.

It must be noted that there is no direct evidence for a role of the enzyme COMT in the regulation of GTH secretion, as 3-MT could not be detected. However, a correlation was shown between increased steroid levels, enhanced dopamine turnover and decreased plasma GTH levels (and vice versa). This provides circumstantial evidence for a role of COMT; high steroid levels could have prevented dopamine from being methylated by COMT. Release of this dopamine, required for the observed effect on GTH release, is reflected by a high conversion rate of dopamine into DOPAC, which was indeed observed.

Schulz *et al.* (1993 *b*) showed that 11-ketotestosterone inhibits GnRH-induced GTH secretion in African catfish. Implantation of 11-ketotestosterone in castrated catfish resulted in a small increase of pre-optic dopamine turnover and a small decrease of plasma GTH levels in castrated animals, but both effects were not significant. However, its effect cannot be explained by the postulated mechanism, as 11-ketotestosterone is not aromatizable.

With the absence of 3-MT and the presence of DOPAC as the only metabolite of dopamine in catfish brain, the regulation model postulated by Khan and Joy (1988) and Manickam and Joy (1989, 1990) for *Claria batrachus* attracts attention. They observed that estradiol is capable of inhibiting monoamine oxidase (MAO), the enzyme that converts dopamine into DOPAC, resulting in higher dopamine levels and a subsequent decrease in pituitary GTH release. In male African catfish, androgens can be converted into estrogens in the brain and pituitary. The estrogens then could inhibit MAO. This, however, would result in a decreased DOPAC/dopamine ratio, which is in contradiction with our observation that the DOPAC/dopamine ratio decreased after castration, and could be restored by replacement of gonadal steroids.

Various regulation mechanisms to explain effects of steroids on GTH release have been investigated in goldfish. According to Callard *et al.* (1990), androgens could exert an effect on GTH secretion via androgen receptors (AR) or estrogen receptors (ER) in the brain, as aromatase, AR and ER were demonstrated in the diencephalic pre-optic area. Furthermore, the aromatase activity showed a strong correlation with reproductive activity. It is not clear whether a regulation of GTH release via AR or ER occur in the African catfish. De Leeuw and coworkers (1986 *b*) showed that estrogens are not capable of decreasing post-castration enhanced plasma GTH levels in the catfish, indicating that ER are not involved. In these experiments, however, it was not excluded that estrogens from the circulation are not able to reach ER in the brain.

Sloley *et al.* (1992) investigated whether control of GTH release in the goldfish could be explained by the hypothesis used in our studies on African catfish. They observed that catecholestrogens do not have an effect on gonadotropin release in golfish.

De Leeuw *et al.* (1987), however, did observe an inhibitory effect of catecholestrogens on GTH release in catfish, indicating species-specific differences in GTH regulation by steroids.

Recently, Trudeau and coworkers (1993) showed an involvement of the amino acids GABA and taurine in the feedback effect of testosterone on pituitary gonadotropic release in goldfish; Although the involvement of amino acids in GTH release has not been studied in catfish yet, it must be noted that testosterone showed a positive effect on GTH secretion in goldfish, while a negative effect is observed in African catfish. Such differences may reflect species specific variation in the control mechanism of GTH secretion. It should be taken in consideration, however, that not only there may exist differences between species, but the feedback control over the GTH secretion may vary during the annual sexual cycle. Most of the experiments on goldfish have been carried out during gonadal recrudescence, when gonadal steroids have a positive, rather than a negative feedback on the hypophysial GTH synthesis and secretion, while our experiments were carried out with fully mature, pre-spermiation male catfish.

Ooctyte maturation and ovulation inducing steroid hormones

In order to study the oocyte maturation and ovulation accompanying steroid synthesis in African catfish, laboratory-reared females with postvitellogenic ovaries were treated with pimozide and LHRHa to stimulate a preovulatory GTH surge. The condition of the ovaries was studied histologically during the next 24 hours following the stimulation, as was the steroidogenic capacity of ovarian tissue by *in vitro* incubation of tissue fragments for 3 hours with [^{3}H]-pregnenolone and [^{3}H]-androstenedione as steroid hormones precursors. As the oocytes entered the stage of germinal vesicle breakdown steroid synthesis shifted from testosterone being the main end product towards the production of 17α, 20β-dihydroxy-4-pregnen-3-one, 5β-pregnane-3α, 17α-diol-20-one, 5βpregnane-3α, 6α, 17α-triol-20-one, 5β-pregnane-3α, 17α, 20β-triol and 5β-pregnane-3α, 6α, 17α, 20β-tetrol. Simultaneously, the production of some C19-steroid glucuronides was enhanced. It could be concluded that the pre-ovulatory GTH surge influences the activity of the enzymes involved in steroidogenesis, leading to a reduced C17-20-lyase and to an enhanced activity of the enzymes 20β-hydroxysteroid dehydrogenease (HSD), 5β-reductase, 3α-HSD, 6α-hydroxylase and UPD-glucuronotransferase. During ovulation the activity of all steroidogenic enzymes, including such key enzymes as 3β-HSD and 17α-hydroxylase, gradually decrease (Schoonen *et al.*, 1989).

Steroids from gonadal origin with pheromonal properties

The role of gonadal steroids is not necessarily restricted to the hormonal one. It has become clear that steroids and their water soluble derivatives may function as pheromones and can act as chemical messengers between conspecifics during the nuptial period. It was hypothesised that the absence of spawning in African catfish, kept under husbandry conditions, is caused by a shortage of suitable pheromones, eliciting spawning behaviour, gonadotropin release, oocyte maturation and ovulation. Two sources have been pointed to with respect to the production of sex pheromones: the gonads and the accessory sex glands (Stacey *et al.*, 1986). During the breeding season Schoonen and Lambert (1986 *a, b*) and Schoonen *et al.* (1987 *a, b, c*) compared the steroid synthesising capacity of testis and seminal vesicles of spawners, *i.e.* fish collected during spawning (Hula Reserve, Israel), and non-spawners, *i.e.* fish not showing breeding behaviour (either from spawning grounds or nearby fish ponds). An important difference between testis and seminal vesicles was the production of steroid glucuronides, which was mainly localised in the seminal vesicles. No real difference between spawners and non-spawners was detected. This indicates that water-soluble steroid glucuronides may be available during the breeding period, not only in feral catfish, spawning or not, but also in fish kept in ponds under non-spawning conditions.

Resink *et al.* (1987 *a, b, c*) compared the capacity to produce highly polar steroids and steroid glucuronides in the testis and seminal vesicles of feral catfish at the end of the resting period, the period of full spermatogenesis and the breeding period. It appeared that the capacity of the testis to convert pregnenolone into 5β-pregnane-3α, 17α, 20α-triol and 5β-pregnane-3α, 17α-diol-20-one increases during the period of full spermatogenesis and the breeding period. Moreover, *in vitro* incubations with androstenedione showed that the capacity of the seminal vesicles to produce testosterone glucuronide increases from the beginning of the period of full spermatogenesis and is followed by in increase in the capacity to produce 5β-androstane-3α, 17α-diol glucuronide during the breeding period. Judging from the presence of the enzymes 3β-HSD and uridine diphosphate dehydrogenase (UDPG), it was concluded that steroid glucuronides can be produced by interstitial cells in the testis of feral and laboratory kept catfish and by interstitial cells in the seminal vesicles of feral animals. A quantitative determination of the two enzymes plus 3α-HSD in prespawning and spawning feral catfish (Resink *et al.*, 1987 *c*) showed an increase in the activity of all three enzymes in the interstitial cells of the seminal vesicles accompanying spawning behaviour. No change was observed in UDPG activity in the epithelial cells. Thus it seems that when it comes to

spawning, steroidogenesis increases both in the testis and in the seminal vesicles, and that the production of steroid glucuronides becomes more pronounced in the seminal vesicles. These enzyme cytochemical data are in agreement with the above mentioned biochemical analysis. Resink *et al.* (1987 *c*) provided evidence that these changes in steroidogenic activities in testis and seminal vesicles are related to an increase in gonadotropin secretion during spawning.

In female feral catfish spawning is also accompanied by a rise is plasma gonadotropin levels. Comparing the capacity of ovarian tissue to metabolise pregnenolone and androstenedione, Schoonen *et al.* (1987 *d*) found that during the breeding period before as well immediately after spawning the ovaries can synthesis estradiol-17β, estrone and several progestines and androgens, including 5β-reduced C21- and C19-steroids. Testosterone and most of the 5β-reduced products were also identified in a conjugated form as steroid glucuronides. In the prespawning ovaries the production of androgens, mainly testosterone prevailed. Shortly after spawning the synthesis of 5β-androstane-3α, 17α, 20β-triol and of most steroid glucuronides, especially testosterone glucuronide and 5β-androstane-3α, 17β-diol glucuronide, had increased. These products are water soluble, and upon excretion might act as pheromones.

Several approaches were followed to investigate whether steroidconjugates act as pheromones.

To study their function in sexual recognition attraction tests have been carried out. It was demonstrated that non-ovulated females do not show clear preference for males or females. However, after ovulation females are attracted by males. Olfactory stimuli are essential for this behaviour since bilateral lesioning of the olfactory tract abolished the attraction response. Extirpation of the seminal vesicles makes males less attractive, whereas compensatory enlargement of the glands by castration increases attractivity of the males. Seminal vesicle fluid elicited a dose-dependent attraction when added to the aquarium water of ovulated females. On the other hand, a high dose induced an avoidance reaction (Resink *et al.*, 1987 *a*).

Seminal vesicle fluid has been fractionated and the fractions were examined in the attraction assay. More than one steroid conjugate was shown to be active. A mixture of synthetic steroid glucuronides, prepared according to the GC-MS analysis of the fluid induced a dose-dependent attraction of ovulated catfish.

Multi-unit electrophysiological recordings from the olfactory epithelium showed that seminal vesicle fluid indeed is an extremely potent odorant in female African catfish and that steroid glucuronides are the effective components. Their stimulatory action appeared to be related to their molecular structure, especially the location of the glucuronic acid residue. Those with glucuronic acid at the 3a-position were the most potent substances; 5β-pregnane-3α, 17β-diol, 20, one-3α-glucuronide has the lowest detection level of all (10^{-11} M). When the glucuronide group is positioned at 17β or 3β, the conjugates were less potent (10^{-6} – 10^{-7} M). With regard to their concentrations in the seminal fluid such threshold doses seemed to be too high to consider these substances as effective pheromones (Resink *et al.*, 1989 *a, b*)

Induction of ovulation with male pheromones could not be demonstrated. However, ovulation could be induced when females were held in the presence of a male and another ovulated female, although visual and tactile stimuli were avoided. Successful ovulation was also obtained when ovarian fluid of an ovulated female was administered as a replacement of the ovulated female. Receptor females with sectioned olfactory tracts, however, did not respond. In addition, it was demonstrated that females exposed to ovarian fluid of ovulated females had increased plasma GTH levels. These responses were limited to the natural breeding period and could only be induced with first generation offspring from feral animals. Later generations only showed a slight increase in GTH levels but no ovulation. The same was observed in experiments beyond the breeding season (Resink *et al.*, 1989 *c*). It is supposed that this pheromonal interaction between females is an important process to synchronise spawning. Indeed, it was observed in the Hula reserve and elsewhere that female catfish do not spawn individually but in groups.

Practical applications

Identification of steroids and steroid glucuronides of gonadal origin and analysis of their pheromonal fucntion may help to overcome the problem of the absence of spontaneous reproduction in African catfish kept under husbandry conditions. In the mean time, endocrinological methods can be used to evoke oocyte maturation and ovulation. As mentioned above, the continuity of the reproductive cycle in African catfish under husbandry conditions is primarily caused by the absence of a prespawning gonadotropin surge. The failure to release a large amount of gonadotropin is not caused by insufficient storage of the hormone in the gonadotropic cells (De Leeuw *et al.*, 1985 *a*). It is more likely that GnRH is not released or is prevented from eliciting its effect. A combination of LHRHa and pimozide induces a sharp rise in gonadotropin release and because of that leads to oocyte maturation and ovulation (De Leeuw *et al.*, 1985 *a*).

This simple method for obtaining viable eggs, however, meets both scientific and practical problems. In the first place the role of pimozide is not certain. It might have served as a dopamine antagonist, but might also have inhibited the action of serotonin. In the second place, pimozide cannot be used in fish farming, because it is not commercially available for this purpose. With that in mind, Goos *et al.* (1987)

have tested a number of potentially psychotropic drugs with variable anti-dopaminergic properties and some drugs with specific anti-serotonergic properties, in combination with LHRHa for their gonadotropin release-inducing capacity. The anti-serotonergic drugs had no effect at all, but two of the anti-dopaminergic drugs appeared to be very potent, and together with LHRHa caused ovulation in all experimental animals. This means that the absence of a pre-ovulatory gonadotropin surge in captive African catfish has to do with a dopaminergic inhibition of the action of the hormone that normally induces a gonadotropin surge. The necessity of combining an anti-dopaminergic drug with a potent LHRH analogue for inducing a strong gonadotropin release, however, indicates that in captive catfish the secretion of GnRH is inhibited by some factor different from dopamine.

Oocyte maturation and ovulation can also be induced by administering exogenous gonadotropin (Eding *et al.,* 1982). Under field conditions injection of a crude homogenate of catfish pituitaries forms a simple and reliable method (Hogendoorn and Vismans, 1980). Pituitary gonadotropin is believed to induce oocyte maturation and ovulation indirectly by stimulating the synthesis of maturational steroids in ovarian follicles (Goetz, 1983). In general, 17α-hydroxy, 20β-dihydroprogesterone is considered to be the main maturation-inducing steroid in teleosts (Goetz, 1983). In this respect, it is of interest that Richter *et al.* (1987 *a*) succeeded in inducing oocyte maturation and ovulation in the African catfish by administering a precursor of the hormone, *i.e.* 17α-hydroxyprogesterone. In the same study the effect of pimozide-LHRHa and 17α-hydroxyprogesterone on oocyte maturation and ovulation, and on the synthesis of various hormones, including 17α-hydroxy, 20β-dihydroprogesterone were compared. It was found that both treatments are equally successful in inducing oocyte maturation and ovulation. Moreover, gonadotropin appeared to stimulate the production of 17α-hydroxyprogesterone rather than the conversion of this hormone to 17α-hydroxy, 20β-dihydroprogesterone.

It is obvious that ovulation induction is of no avail when the ovaries do not contain numerous postvitellogenic oocytes. This means that under natural conditions artificial propagation of African catfish can only be applied successfully during the breeding period and the beginning of the resting period. Bringing mature African catfish from their natural habitat to an indoor hatchery under conditions of optimal food supply, constant water temperature of about 25°C and normal local changes in day length, makes the period of successful artificial propagation increase to 10 or 11 months per year (Richter *et al.,* 1987 *b*). Fish reared from egg to maturity in laboratory tanks under similar feeding and temperature conditions and different photoperiodic regimes showed an uninterrupted ovarian actitity with postvitellogenic ooctytes at all seasons. All year round such fish could be induced to produce large quantities of viable eggs.

It therefore seems that an endogenous rhythm leading to an inherent discontinuous reproductive cycle is normally determined in early life, and can be overcome by proper feeding and by keeping catfish larvae and fingerlings at a constant high temperature. When reaching maturity under such favourable conditions, female African catfish will show an constant production and storage of gonadotropin and a continuous limited release of this pituitary hormone. The low concentration of the hormone in the blood is sufficient for the production and maintenance of postvitellogenic oocytes, but not enough for spontaneous oocyte maturation and ovulation. Such animals can, however, be used for artificial propagation at all seasons, and ensure the production of healthy larvae throughtout the year. This makes the African catfish a promising species for fish farming in its own area of distribution, *i.e.* African and the Near East, as well as in any other place where favourable conditions can be provided at relatively low cost.

Future results of research dealing with the regulation of gonadotropin release and the role of sex pheromones may help to facilitate a spontaneous gonadotropin surge in captive catfish, and may assist in improving culture methods. Last but not least, such investigations will contribute to understanding reproductive endocrinology in teleosts.

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