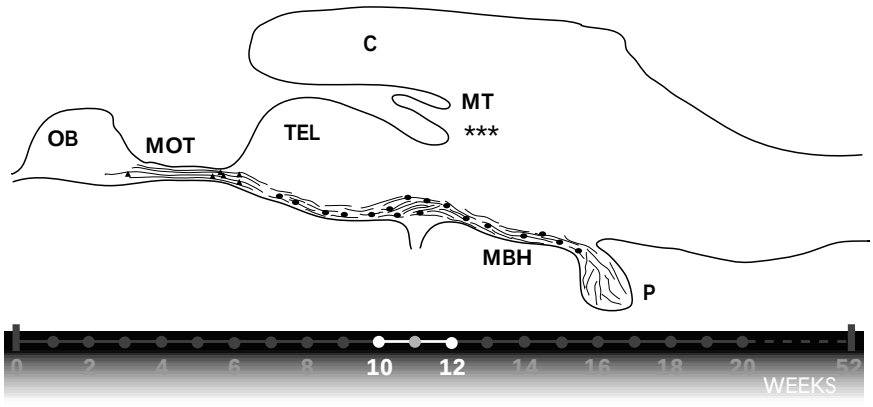
Chapter 5

# Gonadal steroids and the maturation of the species specific gonadotropin-releasing hormone system in brain and pituitary of the male African catfish (*Clarias gariepinus*)

**E.A.Dubois, S.Slob, M.A.Zandbergen, J.Peute, H.J.Th.Goos**

Comp. Biochem. Physiol 2001 in press



# Gonadal steroids and the maturation of the species specific gonadotropin-releasing hormone system in brain and pituitary of the male African catfish *(Clarias gariepinus)*


## Abstract

The effect of testosterone (T), 11-ketotestosterone (KT) and estradiol ($E_2$) on the development of the catfish gonadotropin-releasing hormone system (cfGnRH) of male African catfish (*Clarias gariepinus*), at the onset of puberty (between 10 and 12 weeks post hatching [ph]) was investigated. The cfGnRH neurons, located in the ventral forebrain, were visualized by immunofluorescence and their numbers were determined and the amounts of cfGnRH-associated peptide (cfGAP) in the pituitary were measured by RIA. Steroid treatments did not significantly alter the numbers of immunoreactive GnRH neurons. However, T and $E_2$ caused an increase in the amount of GnRH, demonstrated by the intensity of the immunostaining of GnRH neurons and fibers in the brain and the amount of cfGAP in the pituitary. Treatment with KT, the main circulating androgen in adult male catfish, did neither change the number of cfGnRH neurons, nor elevated the cfGnRH content in the pituitary. In previous experiments with younger, prepubertal fish (2-6 weeks ph), T caused an elevation of the number of cfGnRH neurons to the same level as present in pubertal fish of 12-14 weeks. We conclude that the onset of puberty in the male African catfish coincides with the completion of the - steroid dependent - structural maturation of the cfGnRH system in the brain. T and/or $E_2$, however, still are able to exert a positive influence on the amounts of cfGnRH during the later stages of pubertal development, thus still playing a role in the control of the cfGnRH system.


## Introduction

To date 15 different forms of gonadotropin releasing hormone (GnRH) have been identified (Powell et al. 1996b; Carolsfeld et al. 2000; Yoo et al. 2000; Okubo et al. 2000a; Montaner et al. 2000). In most species two forms are expressed in the brain, but it was recently shown that a third GnRH exists in modern teleost species (Amano et al. 1997; Parhar et al. 1998; Fernald and White 1999). The two (or three) forms not only differ in amino acid sequence,

but also in embryonic origin, localization and function. The species specific GnRH (also indicated as GnRH1; Fernald and White 1999) is localized in the ventral forebrain/hypothalamus; its most obvious function is to control hypophyseal gonadotropin release. The neurons that produce this GnRH originate from the olfactory placode (King and Millar 1992; Sherwood et al. 1993; Parhar 1999; Fernald and White 1999). GnRH2 is the highly conserved chickenGnRH-II (cGnRH-II), present in all vertebrates and localized in large neurons in the midbrain; its function is still under debate (King and Millar 1992; Fernald and White 1999). The third form (GnRH3), as found in modern teleost species, is expressed in the terminal nerve, olfactory bulbs and rostral telencephalon (Parhar 1999; Fernald and White 1999).

The functional development of the GnRH1 system in immature teleosts is under stimulatory control of gonadal steroids, especially testosterone (T). This has been demonstrated for the masu salmon (*Oncorhynchus masou*; Amano et al. 1994), rainbow trout (*Oncorhynchus mykiss*; Goos et al. 1986; Breton and Sambroni 1996), platyfish (*Xiphophorus maculatus*; Schreibman et al. 1986) and African catfish (*Clarias gariepinus*; Dubois et al. 1998). In the European eel (*Anguilla anguilla*), estradiol ($E_2$) alone or in combination with T was equally effective (Montero et al. 1995).

In the African catfish two different forms of GnRH have been identified: catfish GnRH (cfGnRH = GnRH1) and cGnRH-II (Bogerd et al. 1994). CatfishGnRH is distributed over the entire ventral forebrain i.e., from olfactory bulb till pituitary, whereas cGnRH-II was exclusively found in the midbrain tegmentum (Zandbergen et al. 1995). We previously investigated the effects of T and 11β-hydroxyandrostenedione (OHA) on the development of the cfGnRH system in the brain of prepubertal catfish (2-6 weeks of age, gonads still undifferentiated; Dubois et al. 1998). T treatment resulted both in an increase in number of cfGnRH neurons and the intensity of the immunostaining, whereas OHA had a weak positive effect on the cell size only.

The period of pubertal development in the male African catfish spans the time between the first meiotic division in the testes (between 10-12 weeks of age) and the completion of the first wave of spermatogenesis (20-24 weeks of age) and covers the functional development of brain-pituitary-gonad axis.

In a previous study we demonstrated that the cfGnRH system in the brain hardly develops any further during the process of puberty (Dubois et al. 2000). However, we have shown that the prepubertal development of the cfGnRH system is accelerated by T treatment (Dubois et al. 1998). Therefore, we hypothesized that the cfGnRH neurons are already structurally differentiated at the onset of puberty. In the present study, we investigated whether gonadal steroid hormones still have an effect on the number of cfGnRH neurons and the cfGnRH content in the pituitary at the onset of pubertal development. To that end we studied the effects of three different steroid hormones on the

cfGnRH system of the male African catfish at the onset of puberty by determining the numbers, size and immunoreactivity of cfGnRH neurons, and by quantifying the cfGnRH levels in the pituitary by radioimmunoassay.

## Materials and methods

African catfish were raised in the aquarium of the Research Group for Comparative Endocrinology by artificial fertilization. At the age of 10 weeks males received silastic pellet implants (by making a small incision in the abdominal cavity) containing 30μg/g body weight of T, 11-ketotestosterone (KT), $E_2$ or no steroid (control), respectively. After 2 weeks blood samples were taken. The fish were killed by decapitation and brain, pituitary and testes were sampled. Per experimental group 5 brains and attached pituitaries were fixed for immunocytochemistry as described earlier (Dubois et al. 1998). In brief, the tissues were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, cryoprotected in graded sucrose and frozen. After sectioning, the highly specific anti-cfGAP (Zandbergen et al. 1995) against cfGnRH-associated peptide was applied to stain the cfGnRH neurons. The numbers of cfGnRH-ir neurons were counted in every alternate section and their size was measured (Dubois et al. 1998). Furthermore, the differences in staining intensity were established in a double-blind test. The remaining pituitaries (n=15-20) were processed for cfGAP determination by RIA. This assay has been developed previously (Dubois et al. 2000) for two reasons. Antisera against GAP are more specific compared to GnRH-antisera. Moreover, cfGnRH does not contain a tyrosine residue and is therefore hard to use as label in an iodine-based RIA. Testicular histology was studied as described before (Dubois et al. 2000) in order to determine the stage of spermatogenesis. Stage I indicates the presence of spermatogonia in the testes; stage II is characterized by spermatogonia and spermatocytes in the testes; in stage III also spermatids are present; stage IV, all stages of spermatogenesis. The plasma of each group was analyzed, in order to check the effect of the implantation on steroid plasma levels (Schulz et al. 1994). All data were analyzed by one-way ANOVA followed by Fisher's least significant difference test ($p<0.05$) and their results are given as means ± SEM.

## Results

All steroid implantations caused increased plasma levels (Fig. 1). The T implantation (Fig.1a) resulted in a 16-fold increase of the T levels in the plasma. The level of $E_2$ was 7 times higher in the $E_2$-treated group than in the control fish (Fig.1b) and the KT levels had increased 20-fold after KT treatment (Fig.1c).

Histological analysis of the testes showed that spermatogenesis was advanced in fish treated with 11KT (all in stage III), while 40% of the controls, T- and $E_2$-treated fish were in stage I and 60% in stage II.

The steroid treatments neither affected the total number of cfGnRH neurons in the brain (Fig.2) nor the number per specific brain region (telencephalon, suprachiasmatic area and medial basal hypothalamus; data not shown). Similarly, no change in the size of the cfGnRH neurons was observed after any of the steroid treatments.

The intensity of the immunostaining in the T-treated group, however, was stronger than in all other groups; the $E_2$ and KT groups were not affected in this respect. In Fig. 3 the suprachiasmatic area of a control fish (Fig. 3A) and a T-treated fish (Fig. 3B) are depicted, the latter showing both higher density of cfGnRH fibers and a more intense immunofluorescence of the individual fibers and neurons.

The quantification of cfGnRH in the pituitary revealed a significant increase after T (347 (±37pg GAP/mg protein) and $E_2$ (278 ±29pg GAP/mg protein) treatment versus controls (204 ±26pg GAP/mg protein); treatment with KT had no effect (171 ±14pg, Fig.4).

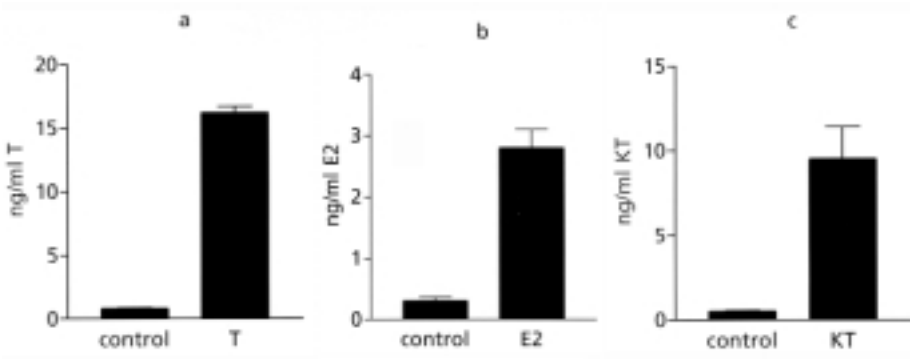



**Fig. 1** Plasma levels of T (a), $E_2$ (b), and KT (c) in male African catfish of 12 weeks of age after a two-week treatment with the respective steroid (right) and the control group (left). The data are plotted as mean ±SEM ng steroid/ml plasma; n=15-20.

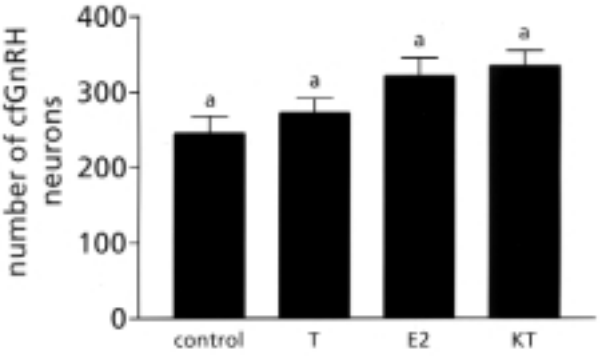



**Fig. 2** Total number of cfGnRH neurons in the brain of African catfish after two weeks of steroid treatment. Mean ±SEM; n=5. No significant differences after Fisher's least significant difference test. Groups sharing the same letter are not significantly different.

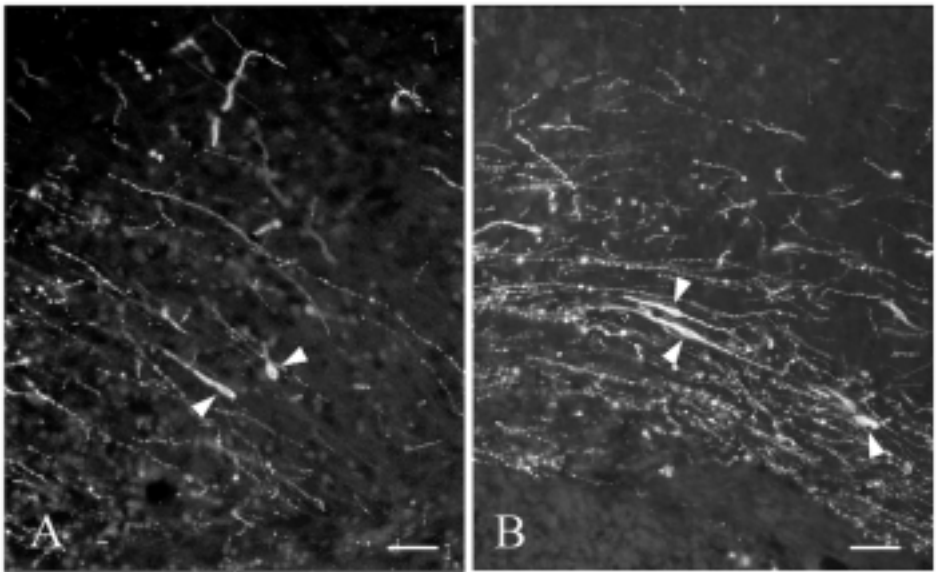



**Fig. 3** Images obtained by immunofluorescence microscopy. CfGnRH neurons and fibers in the suprachiasmatic area of control fish (A) and T treated fish (B) of 12 weeks of age. The arrows point at the cfGnRH cell bodies, scalebar = 50 μm.

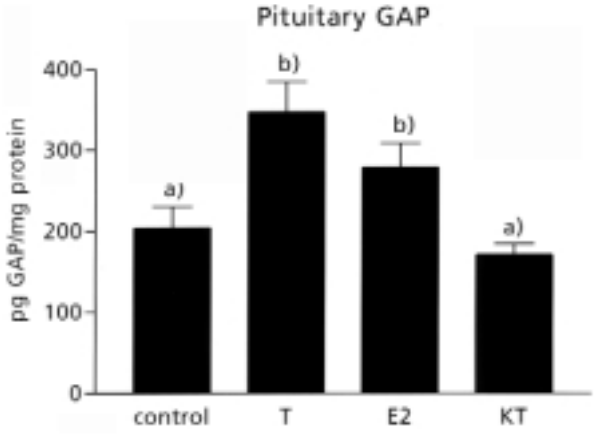



**Fig. 4** CatfishGnRH content in the pituitary after two weeks of steroid treatment in male African catfish. The data are plotted as mean ±SEM pg cfGAP per mg protein; n=15-20. Groups with the same letter are not significantly different.

## Discussion

After the steroid implantations, the plasma levels of resp. T, $E_2$ and 11KT were all significantly elevated. Although the induced steroid levels are high as compared to the control values, they are within the physiological range of the more advanced developmental stages (20-40 week-old adolescent male catfish; Schulz et al. 1994). On the testicular level, 11KT advanced the stage of spermatogenesis, whereas T and $E_2$ had no effect on the spermatogenesis. These

findings correspond with earlier studies in the African catfish (Cavaco et al. 1998b).

Exogenous gonadal steroids accelerate the maturation of the brain-pituitary-gonad axis, as shown in many teleost species (Nagahama 1994; Xiong et al. 1994; Amano et al. 1997). Sexual maturation in the male African catfish starts with the differentiation of the testis at six weeks of age (Hurk et al. 1989). The first appearance of spermatocytes in the testes around week 12 marks the onset of puberty (Schulz et al. 1999). At the level of the brain, cfGnRH was first observed in the ventral telencephalon at two weeks of age (Dubois et al. 1998). Ten weeks later, at the onset of puberty, the perikarya and fibers of the cfGnRH system are present in the brain from olfactory bulb till pituitary. The formation of the cfGnRH fiber network around the gonadotrophs in the pituitary, however, develops later during puberty between 10 and 20 weeks of age (Dubois et al. 2000).

In pubertal African catfish, pituitary and testes are susceptible to different native steroid hormones. Testicular development is stimulated by members of the 11-oxygenated androgens: 11β-hydroxyandrostenedione (OHA) and 11-ketotestosterone (KT) (Cavaco et al. 1998b). OHA is the main testicular product, which is converted in the liver into KT, the main androgen in the plasma (Cavaco et al. 1997). Another group of steroids e.g. aromatizable androgens like T and androstenedione, as well as $E_2$, were shown to have their domain of action on the gonadotrophs in the pituitary (Cavaco et al. 1998a; Cavaco et al. 1998b). Thus, these two groups of steroids act in different domains of the BPG-axis.

The question which type of steroid plays a dominant role in the maturation of the cfGnRH system is still under debate. In three successive studies, we have investigated the effects of gonadal steroids on the maturation of the cfGnRH system in brain and pituitary during three developmental periods: in prepubertal fish. Fish were studied from 2 till 6 weeks of age (Dubois et al. 1998), at the onset of puberty between 10 and 12 weeks of age (the present study) and during later puberty, up to 17 weeks (Dubois et al. 2000). As parameters, the number of cfGnRH neurons in the brain, the content of cfGnRH in the pituitary and the overall immunostaining intensity were used.

The 11-oxygenated steroids, powerful in stimulating the testicular development, appeared not to be involved in the maturation of the cfGnRH system in any stage of pubertal development. Testosterone, however, has a stimulating effect on cfGnRH immunoreactivity in brain and pituitary in all three age groups. The size of the cfGnRH neurons could be altered by steroid treatment in prepubertal catfish, but could not be influenced anymore at the onset of puberty. In contrast to the African catfish, the GnRH cell size in the adult cichlid (*Haplochromis burtoni*) can be regulated by castration or steroid replacement (Soma et al. 1996).

Also the increase in the *number* of cfGnRH neurons due to T was only observed in the prepubertal fish, i.e. between 2 and 6 weeks of age (Dubois et al. 1998). At later stages of pubertal development, the number of neurons is no longer susceptible to changes in T levels, which indicates that already at the onset of puberty the program of cfGnRH neuron recruitment is completed.

The content of cfGnRH in the pituitary increased after T or $E_2$ treatment in fish at the onset of puberty. These levels equal the amounts of cfGnRH as measured in the non-stimulated pituitary at the age of 14 weeks (stage 2-3 of spermatogenesis; Dubois et al. 2000). In late pubertal fish (17 weeks old; Dubois et al. 2000), the cfGnRH content in the pituitary can still be increased by T. The stimulatory effect of T or $E_2$ on the content of GnRH was also observed in the trout (Goos et al. 1986; Breton and Sambroni 1996), eel (Montero et al. 1995), and platyfish (Schreibman et al. 1986).

Thus, the structure of the cfGnRH system is morphologically differentiated at this stage, whereas the amount of cfGnRH peptide within the neurons and fibers is still under control of steroid hormones.

Whether T has a direct effect on the GnRH neurons is not known. The presence of androgen receptors in the goldfish brain has been reported (Gelinas and Callard 1997), but not (yet) in the brain of African catfish. An indirect effect of T, via aromatization to $E_2$, is also feasible. At least aromatase is present in regions of the catfish brain, including the preoptic area and basal hypothalamus (Timmers et al. 1987), where numerous GnRH neurons are located. Likewise, the maturation of the gonadotrophs in the pituitary is dependent on the aromatization of T to $E_2$ (Rebers et al. 2000b).

The overall increase in GnRH staining intensity and content after T treatment can be explained in two ways: (1) T stimulates the synthesis of cfGnRH or (2) this steroid inhibits the release of cfGnRH, thus causing an accumulation. Results of other studies in teleosts provide evidence for increase in GnRH numbers (Amano et al. 1994) and content (Schreibman et al. 1986; Goos et al. 1986; Montero et al. 1995; Breton and Sambroni 1996), but not for a direct increase in GnRH synthesis after T treatment. In the African catfish, there is some evidence for an inhibition of the GnRH release after the elevation of T plasma levels rather than increase synthesis, since T treatment resulted in a decrease in LH plasma levels (Cavaco et al. 1998a).

Summarizing, we conclude that the structural development of the cfGnRH neurons system in the ventral forebrain of the African catfish is under stimulatory control of T only until the onset of puberty. During puberty, however, T and/or $E_2$ still influence the amounts of cfGnRH within both perikarya and fibers, thus indicating their role in the control of the cfGnRH system.