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**Advancing Puberty in the Black Carp
(*Mylopharyngodon piceus*) and the Striped
Bass (*Morone saxatilis*)**

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(*MORONE SAXATILIS*)**

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**Advancing puberty in the black carp (*Mylopharyngodon piceus*)
and the striped bass (*Morone saxatilis*)**

Abstract

The black carp (bc)GtH II β cDNA was amplified and isolated, cloned and sequenced. Comparison of the bcGtH II β deduced a.a. sequence with that of GtH II β from other teleosts revealed high homology to cyprinid species and a lower homology to salmonid or perciform fish. The gene coding for the bcGtH II β was isolated and sequenced. Three bc recombinant phages which hybridized to the goldfish GtH I β cDNA probe were isolated and are currently being characterized.

The region coding for the mature GtH II β was expressed in a bacterial expression vector resulting in the production of a recombinant protein. *In vitro* folding resulted in a protein only 1.3% of which displaced the native common carp GtH II in a RIA. Therefore, the common carp GtH RIA was utilized for the physiological studies at the current phase of the project.

Two non-functional sites were identified along the brain-pituitary gonadal axis in the immature black carp. The pituitary is refractory to GnRH stimulation due to a block proximal to the activation of PKA and PKC, probably at the level of GnRH receptors. The gonads, although capable of producing steroids, are refractory to gonadotropic stimulation but do respond to cAMP agonists, indicating a block at the GtH receptor level. Attempts to advance puberty in 2 and 3 y old black carp showed that testosterone (T) stimulates GtH synthesis in the pituitary and increases its sensitivity to GnRH. A 2 month treatment combining T+GnRH increased the circulating GtH level in 3 y old fish. Addition of domperidone to such a treatment facilitated both the accumulation of GtH in the pituitary and its response to GnRH.

The cDNA of striped bass GtH α , I β and II β subunits were amplified, isolated, cloned and sequenced, and their deduced a.a. sequences were compared with those of other teleosts. A ribonuclease protection assay was developed for a sensitive and simultaneous determination of all GtH subunits, and of β actin mRNAs of the striped bass. GnRH stimulated dramatically the expression of the α and GtH II β subunits but the level of GtH I β mRNA increased only moderately. These findings suggest that GtH-II, considered in salmonids to be involved only in final stages of gametogenesis, can be induced by GnRH to a higher extent than GtH-I in juvenile striped bass.

The native GtH II of the striped bass was isolated and purified, and an ELISA for its determination was developed. The production of all recombinant striped bass GtH subunits is in progress using the insect cell (Sf9) culture and the BAC-TO-BAC baculovirus expression system. A recombinant GtH II β subunit has been produced already, and its similarity to the native subunit was confirmed. The yield of the recombinant glycoprotein can reach 3.5 μ g/ml after 3 days culture.

All male striped bass reach puberty after 3 y. However, precocious puberty was discovered in 1 and 2 y old males. Females become vitellogenic during their 4th year. In immature 2 y old females, T treatment elevates the pituitary GtH II content while GnRH only potentiates the effect. However, in males GnRH and not T affects GtH accumulation in the pituitary. Neither GnRH, nor T treatment resulted in gonadal growth in 2 y old striped bass, indicating that either the accumulated GtH II was not released, or if released, the gonads were refractory to GtH stimulation, similar to the situation in the immature black carp.

In 3 y old female striped bass, 150 day GnRH α treatment resulted in an increase in GSI, while T treatment, with or without GnRH α , resulted in a decrease in oocyte diameter, similar to the effect seen in the black carp. Further attempts to advance puberty in both fish species should take into account the positive effect of T on pituitary GtH and its negative effect on ovarian growth.

Objectives of the Original Research Proposal

1. To isolate and clone cDNA and/or genomic DNA sequences coding for GnRH forms in the black carp and the striped bass. These sequences will be used as probes to examine the expression of these genes during the various phases of puberty, and in response to ovarian steroids.
2. To isolate and clone cDNA and/or genomic DNA sequences coding for the β subunit of GtH I and GtH II from the black carp and the striped bass. These sequences will be introduced into expression vectors and the coded proteins produced will serve for establishing the respective radioimmunoassays for the two types of GtH in each fish species.
3. To analyze parameters associated with puberty in fish at various phases of sexual maturation. This static study will span GnRH gene expression and content in the brain and pituitary; pituitary content and circulating levels of GtH I and GtH II; pituitary content of mRNA specific for β subunits of GtH I and II; histology, immunocytochemistry (ICC) and *in situ* hybridization to mRNA for GnRH in brain and pituitary structures; ICC of GtHs in the pituitary gonadotrophs; ovarian histology combined with circulating levels of testosterone (T) and estradiol (E_2).
4. To conduct dynamic challenge experiments in fish at various stages of sexual maturation in order to test the functional state of the endocrine components of the axis during the process of puberty. These tests will encompass the release of GtH I and II *in vivo* and *in vitro* from the pituitary in response to GnRH, and the secretion of T and E_2 from the ovary in response to gonadotropins.
5. To analyze the parameters detailed in objectives 3 and 4 in three year old black carp and 2 y old striped bass chronically treated with either ovarian steroids alone, or in combination with GnRH given in slow-release devices.
6. To formulate the hormonal treatment by which the puberty of the black carp and the striped bass can be advanced.

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General Introduction

Puberty is defined as the process leading to the development of the gonads to a stage at which the animal can reproduce for the first time. It is distinct from the process of gonadal recrudescence which occurs seasonally in sexually mature animals.

It is well established that puberty in fish, as in other vertebrates, is dependent on age and body size. In a given population, fast-growing fish reach sexual maturation earlier than their siblings. Rearing at low density and adequate feeding were found to advance puberty of carp (Sehgal and Toor, 1993). However, this is not always the case, for example, catfish reared at high temperature and poor feeding reached puberty at a size lower than normal (Richter et al., 1982).

Female black carp reared under optimal routine conditions in Israeli fish farming reach sexual maturity at the age of 6+ years (males mature one year earlier) which is similar to the puberty age of feral fish in their natural habitat in China (Hepher and Pruginin, 1981). The striped bass reach puberty at the age of 5 years and body weight (bw) of 10-15 kg. At the age of 5 years, black carp reach a bw 7-8 kg but their ovaries are still vestigial and dormant. It is unlikely that at such body mass the fish is "unable" to spare the resources required for the development of the ovary. Other cyprinids such as the silver carp or grass carp develop their gonads at a much smaller size. It is most probable that under optimal rearing conditions, age and size required for puberty is under genetic control which developed during the evolution of the species.

Whatever may be the ultimate or the proximate factors controlling fish maturity, the actual process of gonadal development is channeled through neuronal and endocrine cascades along the brain-pituitary-gonadal axis (B-P-G axis). These involve the brain, especially the hypothalamus and its stimulatory and inhibitory neurohormones, the pituitary and its gonadotropic hormones and the gonads and their sex steroids.

The endocrine background of fish reproduction

The brain and hypothalamic hormones

Environmental and social cues perceived by sense organs and transmitted to the central nervous system are translated into humoral agents secreted by the hypothalamus through its nerve ramifications in the pituitary, thus regulating the secretion of gonadotropins (GtHs). The best known agents of the hypothalamus are gonadotropin-releasing hormone (GnRH) which stimulates the release, and dopamine (DA) which acts as an antagonist (reviewed by Peter et al., 1986; 1991). Dopaminergic inhibition is paramount in cyprinids (Lin et al., 1988), consequently, routine spawning induction in carp species by the hypothalamic approach often combines the peptide with DA antagonists (reviewed by Yaron, 1995). However, dopaminergic inhibition in the striped bass is only marginal (Zohar et al., unpublished). Other brain agents, such as norepinephrine, serotonin, melatonin and GABA may have a modulatory effect on GtH release (reviewed by Kah et al., 1993). In addition, regulatory amino acids localized in GnRH and GtH producing cells have been implicated in many neural functions including the modulation of the B-P-G axis (Magliulo-Cepriano and Schreibman, 1993).

Until recently, the brain of most teleosts was considered to contain two forms of GnRH: a form specific to fish, the salmon GnRH (sGnRH), and the chicken GnRH II (cGnRH II) which is apparently present in all non-mammalian vertebrates (Sherwood and Coe, 1991; Sherwood et al., 1993). The distribution of these forms in the brain, and their ontogeny in salmonids (Okuzawa et al., 1995; Suzuki et al., 1992) suggest that cGnRH II is probably not involved in the regulation of gametogenesis, although it binds to the same GnRH receptors in the pituitary (Habibi and Peter, 1991) and is a very potent effector of GtH release (reviewed by Chang et al., 1993).

Ongoing collaboration partly supported by BARD has led to the discovery, by the Baltimore and the Eilat teams, that the brain of the striped bass (Gothilf et al., 1995a) and seabream (Powell et al., 1994; Gothilf et al., 1995b; 1996) contains three forms of GnRH: sGnRH, cGnRH II and a novel form, hitherto unknown, referred to as seabream GnRH (sbGnRH). sbGnRH is 500-1,000 times more abundant than other GnRH forms in the pituitary of sexually mature striped bass (Gothilf et al., 1995a) and other farmed marine fish (Powell et al., 1994). It stimulates GtH release (Zohar et al., 1995a) and synthesis (Elizur, Zmora, Rosenfeld and Zohar, unpublished) in the seabream. These results strongly indicate that in striped bass, and probably in other fish, sbGnRH, a form overlooked so far, is the major endogenous regulator of GtH secretion.

All studied forms of GnRH bind to the same class of high affinity - low capacity receptors on the plasma membrane of the gonadotropic cells in the goldfish (Habibi et al., 1987, 1989) and the seabream (Pagelson and Zohar, 1992). Surprisingly, sGnRH acts also on the somatotropes to stimulate the release of growth hormone in goldfish (Marchant et al., 1989) and tilapia (Melamed et al., 1995).

Superactive analogs of GnRH (GnRHa) are commercially available and are used in routine spawning induction in a variety of commercial fish. To avoid the stress due to repeated handling in fish unresponsive to a single injection of GnRHa, controlled release delivery systems for GnRH have been developed. These devices are highly efficient in spawning manipulation in a variety of species (reviewed by Zohar, 1989, 1996; Zohar et al., 1994, 1995b, 1996; Mylonas et al., 1995, 1996).

The stimulatory effect of GnRH on GtH release in fish is mediated through a cascade of intracellular events spanning the influx of Ca^{2+} , phosphoinositide turnover, the formation of diacylglycerol and inositol-trisphosphate, mobilization of Ca^{2+} from intracellular sources, activation of protein kinase C (PKC) and the formation of arachidonic acid and its active metabolites (Jamaluddin et al., 1989; Levavi-Sivan and Yaron, 1989; van Asselt et al., 1989; Chang et al., 1993; Mikolajczyk et al., 1990). There is evidence that cAMP also takes part in this mediation as a parallel or interconnected transduction system (Yaron and Levavi-Sivan, 1990; Levavi-Sivan and Yaron, 1991, 1992, 1995).

Gonadal steroids affect GtH secretion in several ways. In immature trout or eel, testosterone (T) and estradiol (E_2) have a positive feedback effect by increasing GtH

content in the pituitary (Crim et al., 1981; Gielen et al., 1982; Dufour et al., 1983, 1989; Montero et al., 1995). In adult fish, the opposite effect is observed, namely, the classical negative feedback mechanism whereby excess of T or E₂ reduces GtH release (Goos, 1987).

Pituitary gonadotropins

A single gonadotropin was first isolated from the pituitary of the common carp which was believed to perform all functions attributed to such a hormone. It is a glycoprotein heterodimer composed of non-covalently linked subunits and α and β similar to the general structure of glycoprotein hormones in other vertebrates (Burzawa-Gerard, 1982). The duality of gonadotropin forms (GtH I and GtH II) was established by Kawauchi and his co-workers in salmon (Suzuki et al., 1988a,b; Itoh et al., 1988; Swanson, 1991; Swanson et al., 1991) and has since been found in a range of fish species: bonito (Kawauchi et al., 1991); killifish (Lin et al., 1992); gilthead seabream (Elizur et al., 1995a,b) and tilapia (Rosenfeld et al., in press; Melamed et al., in press). Both GtHs are quite similar in their biological activity as assessed by stimulating ovarian E₂ secretion. However, GtH II is more potent in stimulating the secretion of the maturation-inducing progestogen 17 α , 20 β -dihydroxy-4-pregnen-3-one (17,20-P) from postvitellogenic follicles (Suzuki et al., 1988c; Swanson et al., 1991). Immunocytochemical (ICC) studies in several fish showed that the appearance of GtH I and GtH II coincides with specific developmental stages of the reproductive cycle (Schreibman et al., 1990). GtH I, but not GtH II, was found to stimulate E₂ secretion from ovarian follicles of trout during early and mid vitellogenesis (Sumpter et al., 1991) and to stimulate the incorporation of vitellogenin into the oocytes (Tyler et al., 1991).

In line with the concept of GtH duality in fish, two types of GtH β subunits and a single α subunit were isolated from pituitaries of common carp. The biological activities of the two GtHs were similar, with GtH II showing higher potency (Van Der Kraak et al., 1992). However, only a single gonadotropin (GtH II) was purified from the silver carp (*Hypophthalmichthys molitrix*) and the bighead carp (*H. nobilis*) pituitary and the nucleotide sequences of their α and β subunits were established (Chang Y.S. et al., 1990; Huang et al., 1990). The amino acid sequence of the β chain

in the two species of this genus shows a great similarity to that of the common carp. The difference in subunit α is by only 2 residues, and that in subunit β by only 4 residues.

Recently, through the advances in molecular biology, the duality of gonadotropins was confirmed in the goldfish, and the cDNA encoding the GtH I β subunit was cloned and shown to be expressed at low levels in fish at various stages of gonadal development (Yoshiura et al., 1995).

Gonadal steroids

In salmonids, throughout the vitellogenic phase GtH I stimulates the conversion of cholesterol by the follicular thecal cells into testosterone via pregnenolone, 17 α OH-P and androstenedione. Testosterone is aromatized by the granulosa cells to E₂ (Nagahama, 1983). This gonadotropic effect is mediated by cAMP in salmonids (Yoshikuni and Nagahama, 1991) as well as in tilapia (Bogomolnaya and Yaron, 1984). When final oocyte maturation is approaching, a shift occurs in the steroidogenic pattern: the production of E₂ recedes concomitantly with an increase in the secretion of the maturation inducing progestogen, 17 α , 20 β -dihydroxy-4-pregnen-3-one (17,20-P). The decrease in E₂ in carp is only for a few hours during final oocyte maturation which is concomitant with the surge of 17, 20-P (Yaron and Levavi-Zermonsky, 1986; Drori et al., 1994).

It is well established that the GtH effect on the testis is mediated as in the ovary by cAMP. GtH stimulates the formation of T and its conversion to the main fish androgen 11-ketotestosterone (11-KT) both of which are secreted from Leydig cells. 11-KT activates Sertoli cells to stimulate premitotic spermatogonia and the complete process of spermatogenesis in the fish. During spermiation, there is an increase in 17,20-P which is formed by the sperm cells (reviewed by Yoshikuni and Nagahama, 1991).

Advancing puberty

In the Japanese eel, 75 or 105 day treatments with T implants resulted in a dramatic increase in the pituitary GtH content and oocyte development up to the preovulatory stage. However, no increase in the circulating level of GtH could be noted (Lin et al., 1991). In the European eel primed with estradiol, GnRH treatment combined with a

dopamine antagonist resulted in an augmented release of GtH and ovarian development (Dufour et al., 1988). Advancing puberty by hormonal manipulations has not been attempted in neither in cyprinids nor in perciform fish.

Hypothesis and objective

The hypothesis underlying the research is that the onset of puberty in the black carp and the striped bass is delayed by the non-functional state of certain components (blocked sites) along the brain-pituitary-gonadal axis. The blocks are lifted when the fish reach normal puberty. The rationale of the research is that a systematic study of the endocrine cascade in the immature fish, by both static and dynamic and molecular approaches, will reveal these sites and allow the formulation of a hormonal treatment which may activate these non-functional components and advance puberty of the fish.

The overall objective of the proposed research was to study the endocrine background of puberty in two teleost species representing two major lineages of the teleost fishes (Cypriniformes and Perciformes) and share a similar occurrence of late puberty.

An important step towards such a study was to isolate the DNA sequences coding for the β subunits of GtH I and GtH II from the black carp and the striped bass which would enable the use of these sequences as probes to study the steady state levels of their mRNA under hormonal manipulations. This would also enable the production recombinant β GtH subunits, the generation of antisera against these peptides and the development of specific radioimmunoassays.

Chapter 1:

GtH β subunits of the black carp (*Mylopharyngodon piceus*); isolation of cDNA and genomic sequences and production of recombinant GtH II β protein.

Introduction

The aim of this part of the project was to firstly isolate the DNA sequences coding for the β subunits of GtH I and GtH II from the black carp and use these sequences as probes to study the steady-state levels of their mRNA under hormonal manipulations. The second aim was to produce recombinant β GtH subunits, generate antibodies (ab) against these peptides and develop GtH RIAs using these recombinant peptides and their antisera.

Experimental procedures

Fish and isolation of RNA

Black carp were obtained from Kibbutz Ha'Maapil hatchery where fish are reared in earthen ponds with a supply of pelleted feed (24% protein). RNA was isolated from freshly excised pituitaries by a scaled down guanidinium thiocyanate method (Chomczynski and Sacchi, 1987)

cDNA preparation and tailing

cDNA was synthesized using 1 μ g of total RNA isolated from a single pituitary and AMV reverse transcriptase (Promega, Madison, WI, USA). An oligo dT-adaptor primer was used in the 3' RACE and a gene specific primer in the 5' RACE (Frohman, 1990). The cDNA obtained was sufficient for 100 PCR amplifications. cDNA was tailed with Terminal Deoxy Transferase using dATP following the specifications of the manufacturer (Promega) prior to use in 5' RACE.

PCR amplification

The 1-5 β -GtH II PCR primer was designed according to the first 5 amino acids of the common carp mature GtH II β peptide (Chang Y.S. *et al.*, 1988). The BC1 and BC2

primers were designed according to the bcGtH IIB cDNA sequence. (Table 1.1). PCR amplifications were carried out in 25 µl using 1 unit Taq polymerase (Promega), buffer (Promega), 1.5 mM MgCl₂, nucleotides (0.2 mM final concentration, for each nucleotide), 12.5 pmole of each primer, 2 µl of cDNA and a drop of mineral oil. Cycling parameters were: 3 min denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C and 1 min extension at 72°C (last extension step was for 10 min).

Cloning and sequencing of PCR products

PCR products were separated on 1.2% agarose gels and the regions containing the expected size fragment were sliced and purified using glassmilk Geneclean II kit, (Bio 101, Calif. USA). The products were ligated into the pGEMT plasmid (Promega). Ligations, transformations and plasmid preparations were according to Sambrook et al. (1989).

Double stranded plasmid DNA was sequenced using the Sequenase version II kit (USB, Ohio),(Sanger et al., 1977). Universal T7 and T3 sequencing primers as well as gene specific primers gave the complete sequence of both DNA strands. PCR products of two independent PCR amplification reactions were cloned and sequenced to avoid potential errors introduced during the PCR amplification.

Cloning of genomic DNA

A black carp genomic DNA library was produced using the lambda Zap II phage cloning vector (Stratagene). The genomic DNA was digested with the restriction enzyme EcoRI and then fractionated on NaCl gradients. Fractions were collected and those corresponding to DNA fragments of 3-6 kb were dialyzed, ethanol precipitated, and ligated to EcoRI digested phage arms. The ligation mix was packaged *in-vitro* and plated. The recombinant phages were screened three times with the homologous bc GtH IIB cDNA probe or with heterologous seabream or goldfish GtH IB cDNA probes.

Protein expression

The cDNA sequence encoding for the black carp mature β -GtH II protein was engineered into the pET11a expression vector (Novagene) and the recombinant protein was produced according to the manufacturer's recommendations.

Western blot

The recombinant protein was run on a 17% SDS polyacrylamide gel electrophoresis as described by Sambrook et al. (1989). Western blot was carried out according to Towbin et al. (1979) using the seabream GtH antibody (Zohar et al., 1990a) in 1:5,000 dilution. The Hoffer minigel and Mighty Small transphor tank unit were used.

Refolding of the recombinant protein

The recombinant protein was denatured in 8 M urea, 50 mM DTT and refolded according to two procedures. The first, followed exactly as described in Stern et al. (1993). The second was a modification of the procedure described in Huth et al. (1994). Specifically, the two HPLC elutions were omitted and the refolding was carried out immediately on the urea denatured samples. The refolding conditions tested were incubation of the denatured protein in the presence of 0, 0.5, 1, 1.5 and 2 M urea for 1 min and 1, 2, 3 and 4 hours. The folding was stopped by addition of 100 mM sodium iodoacetate and 50 mM Tris-HCl, pH 8.7 followed by dialysis of the samples in PBS at 4°C to remove the urea. After the dialysis, the samples were lyophilized and resuspended in PBS for gel and RIA analysis.

Radioimmunoassay (RIA)

The RIA was carried out employing the double antibody method using purified common carp GtH obtained from Dr. B. Breton (Rennes). Common carp GtH antibody was developed by Dr. V. Lichtenberg, (Hamburg). The RIA was performed as described in Levavi-Zermonsky and Yaron, (1986) and Yaron and Levavi-Zermonsky (1986) except that the secondary antibody was donkey anti rabbit (Amerlex, Amersham, UK).

Results

PCR amplification of GtH II β

Pituitary RNAs, prepared from black carp 1,3 and 8 year old were used as templates for cDNAs synthesis. The one and three year old fish showed no gonadal development while the eight year old fish was a female with fully developed oocytes. The cDNA encoding for bcGtH II β subunit was successfully PCR amplified only from the fish aged three and eight years.

3' RACE PCR was used to obtain the sequences encoding for the mature bcGtH II β subunit. A degenerate primer, designed according to the first five amino acids of the common carp mature GtH II β , (primer 1; Table 1.1), was used together with the universal adapter primer (primer 5; Table 1.1) to amplify GtH II β cDNA. The PCR product was sequenced from amino acid 1 in the mature peptide to the poly A tail, including the 3' untranslated region.

#	primer nucleotide sequence		direction on cDNA	designation of primer
1	5'GCATATGTCTT(AT)(CT)CTTC CACC(AC)TG 3'		5' - 3'	1-5 CARP
2	5'AGTTTCGTACTCTGTGG 3'		3' - 5'	BC2
3	5'ATGACAGGCTAGTACAC 3'		3' - 5'	BC1
4	5'GACTCGAGTCGACATCGATT TTTTTTTTTTTTTTTTT 3'	OLIGO dT/ ADAPTER	3' - 5' 5' - 3'	
5	5'GACTCGAGTCGACATCG 3'	UNIVERSAL ADAPTER	3' - 5' 5' - 3'	

Table 1.1. PCR primers

5' RACE was used in order to obtain the 5' untranslated as well as the signal peptide coding regions. GtH II β primer 2, (Table 1.1), which derived from the 3' untranslated sequence of the bcGtH II β cDNA, was used as a gene specific primer in the 5' RACE cDNA synthesis. The resulting cDNA was A tailed and then PCR amplified using the universal oligo dT / adaptor primer (Primer 4; Table 1.1) together with GtH II β primer

(primer 3; Table 1.1), which was designed according to the deduced last two amino acids of the bcGtH II β cDNA and the following 9 bp.

The products from the 3' and 5' RACE PCR were cloned and sequenced. The nucleotide sequence and deduced amino acid sequence of the bcGtH II β cDNA were determined and are shown in Fig. 1.1.

1	CTC	GAG	TGC	ACA	TCG	ATG	TTT	GTT	TCT	TTT	TTT	TTT	36
37	CCA	GTG	TAT	CGC	GCG	TTT	CTA	GAA	TCC	CTT	ATC	CCT	72
73	CAC	TTA	TCC	TCC	GCC	TGC	TTC	CCA	GGG	TTT	CCT	CTC	108
109	ACG	CCG	GAC	AGG	AGC	GCT	CAC	CGT	TCC	TCA	CCT	CTT	144
145	TGA	CGG	CCT	TTA	TAA	ATG	CTC	TGT	GAC	TGA	ACA	CAT	180
181	CTA	ACA	GCC	TGC	TGA	GCA	ACG	GGG	ACA	CCT	GTC	AAG	216
217	ATG	CTA	GTT	GTT	CGA	AAC	ATC	CTC	CTT	CTC	TTA	TTC	252
	M	L	V	V	R	N	I	L	L	L	L	F	
253	TGT	TAT	GTT	GTT	CTA	CTA	GTA	TTT	GCT	CAA	AGC	<u>TCT</u>	288
	C	L	V	V	L	L	V	F	A	Q	S	S	
289	<u>TTT</u>	<u>CTT</u>	<u>CCA</u>	<u>CCA</u>	TGT	GAG	CCA	GTT	AAT	GAG	ACT	GTC	324
	F	L	P	P	C	E	P	V	N	E	T	V	
325	GCG	GTG	GAA	AAA	GAG	GGC	TGT	CCA	AAA	TGT	CTG	GTG	360
	A	V	E	K	E	G	C	P	K	C	L	V	
361	TTT	CAG	ACC	ACC	ATC	TGC	AGT	GGC	CAC	TGC	CTA	ACA	396
	F	Q	T	T	I	C	S	G	H	C	L	T	
397	AAG	GAG	CCT	GTA	TAC	AAG	AGC	CCA	TTT	TCC	ACT	GTC	432
	K	E	P	V	Y	K	S	P	F	S	T	V	
433	TAC	CAA	CAC	GTG	TGC	ACT	TAC	CGG	GAC	GTC	CGC	TAC	468
	Y	Q	H	V	C	T	Y	R	D	V	R	Y	
469	GAG	ACG	GTC	CGC	TTG	CCA	GAC	TGT	CCC	CCC	GGG	GTG	504
	E	T	V	R	L	P	D	C	P	P	G	V	
505	GAC	CCC	CAT	ATC	ACC	TAC	CCG	GTG	GCT	CTC	AGC	TGC	540
	D	P	H	I	T	Y	P	V	A	L	S	C	
541	GAC	TGC	AGC	CTC	TGC	ACC	ATG	GAC	ACG	TCC	GAC	TGT	576
	D	C	S	L	C	T	M	D	T	S	D	C	
577	ACC	ATC	GAA	AGC	CTG	CAG	CCT	GAT	TAC	TGC	ATG	TCT	612
	T	I	E	S	L	Q	P	D	Y	C	M	S	
613	CAG	AGA	GAG	GAT	TTC	CCT	<u>GTG</u>	<u>TAC</u>	<u>TAG</u>	<u>CCT</u>	GTC	ATC	648
	Q	R	E	D	F	P	V	Y	STOP				
649	AAA	<u>CCA</u>	<u>CAG</u>	<u>AGT</u>	<u>ACG</u>	AAA	<u>CT</u>						666

Fig. 1.1. The complete nucleotide sequence and deduced amino acid sequence of the GtH II β cDNA from the black carp. The position of PCR primers are underlined.

Homology to other β -GtH subunits

The bcGtH II β deduced amino acid sequence was compared to that of β -GtH II subunits from a number of teleosts. The position of all 12 half cystines are totally conserved among the examined species, (Fig. 1.2). The homology between β -GtH II deduced amino acid sequence of the black carp and that of the silver carp is 100%, 99% with the bighead carp and grass carp, 96% with the common carp, 77% , 76% and 65% with the masu salmon, chum salmon and gilthead seabream, respectively and 34% with the chum salmon GtH I β (Table 1.2).

PCR amplification of GtH I β cDNA sequences

The cDNA prepared from pituitary RNA the three black carp females was subjected to PCR amplification using a range of primers designed according to conserved windows of homology between GtH I β sequences from a number of teleosts. These primers were used successfully to amplify the GtH I β cDNA from the seabream and the striped bass. In spite of numerous attempts, no PCR product was obtained from any of the black carp cDNAs.

BC	S	F	L	P	P	C	E	P	V	N	E	T	V	A	V	E	K	E	G	C	P	K	C	L	V	F	
CC	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	
SC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BHC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
GC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CSII	-	L	M	Q	-	-	Q	P	I	-	Q	-	-	S	L	-	-	-	-	-	-	T	-	-	-	I	
MS	-	L	M	Q	-	-	Q	-	I	-	Q	-	-	S	L	-	-	-	-	-	-	T	-	-	-	I	
SB	F	Q	-	-	-	-	Q	-	I	-	Q	-	-	S	L	-	-	-	-	-	-	-	-	H	P	V	
CSI	E	C	R	Y	G	-	R	L	N	-	M	-	I	I	-	-	R	-	D	-	H	G	S	I	T	I	
BC	Q	T	T	I	C	S	G	H	C	L	T	K	E	P	V	Y	K	S	P	F	S	T	V	Y	Q	H	
CC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BHC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
GC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CSII	-	-	P	-	-	-	-	-	-	V	-	-	-	-	-	F	-	-	-	-	-	-	-	-	-	-	
MS	-	-	P	-	-	-	-	-	-	I	-	-	-	-	-	F	R	-	-	-	-	-	-	-	-	-	
SB	E	-	-	-	-	-	-	-	-	I	-	-	D	-	-	M	-	T	R	Y	X	X	-	-	-	-	
CSI	X	-	-	X	-	A	-	L	-	E	-	T	D	L	N	-	E	-	T	W	L	P	R	S	-	G	
BC	V	C	T	Y	R	C	V	R	Y	E	T	V	R	L	P	D	C	P	P	G	V	D	P	H	I	T	
CC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BHC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
GC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CSII	-	-	-	-	-	-	-	-	-	-	K	I	-	-	-	-	-	-	W	-	-	-	-	-	V	-	
MS	-	-	-	-	-	-	-	-	-	-	M	I	-	-	-	-	-	-	W	-	-	-	-	-	V	-	
SB	-	-	-	-	-	-	L	H	-	K	-	F	E	-	-	-	-	-	-	-	-	-	-	-	T	V	
CSI	-	-	N	F	K	E	W	S	-	-	V	K	Y	-	E	G	-	-	S	-	-	E	-	F	F	X	
BC	Y	P	V	A	L	S	C	D	C	S	L	C	T	M	D	T	S	D	C	T	I	E	S	L	Q	P	
CC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BHC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
GC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CSII	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	
MS	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	
SB	-	-	-	-	V	-	-	S	-	G	-	-	A	-	-	-	-	-	-	-	F	-	-	-	-	-	
CSI	I	-	-	-	K	-	-	-	-	I	K	-	K	T	-	N	T	-	-	D	R	I	-	M	A	T	
BC	D	Y	C	M	S	Q	R	E	D	F	P	V	Y														
CC	-	F	-	-	-	-	-	-	-	-	-	-	L														
SC	-	-	-	-	-	-	-	-	-	-	-	-	-														
BHC	-	F	-	-	-	-	-	-	-	-	-	-	-														
GC	-	F	-	-	-	-	-	-	-	-	-	-	-														
CSII	-	F	-	I	T	-	-	V	L	T	D	G	D	M	W												
MS	-	F	-	I	T	-	-	V	L	T	D	G	D	M	W												
SB	N	F	-	-	N	D	I	P	F	Y	Y																
CSI	-	S	-	I	V	N	P	L	E	M																	

Fig 1.2. Comparison of the amino acid sequences of the bcGtH II β and other teleost β GtH subunits. CC = common carp GtH β (Chang et al. 1988), SC = silver carp GtH β (Chang et al. 1990), BHC = bighead carp GtH β (Lo et al., 1991), GC = grass carp GtH β (Lo et al., 1991), CS II = chum salmon GtH II β (Sekine et al., 1989), MS = masu salmon GtH II β (Kato et al., 1993), SB = seabream GtH II β (Elizur et al., 1995a), CS I = chum salmon GtH I β (Sekine et al., 1989).

Species	% homology
BC - CC	96.5
BC - SC	100
BC - BHC	99.1
BC - GC	99.1
BC - CS II	76.1
BC - MS	77
BC - SB	65.5
BC - CS I	34.5

Table 1.2. Percent homology between the mature black carp GtH II β and other teleosts GtH β s. Abbreviations and sources are as Fig. 1.2

The isolation of bcGtH IIB genomic DNA sequences

A bc genomic DNA library was prepared and screened with the bc GtH IIB cDNA as a probe. Two recombinant phage have turned out to be positively hybridizing after three rounds of purification. The genomic sequences were "rescued" from the phage and were found to contain an insert of about 5 kb. The insert was sequenced using gene specific primers and was found to contain a 99 bp intron located three amino acids downstream from the fifth cystein (Fig. 1.3). We are currently in the process of completing the determination of the full genomic sequence.

1	AAA AAG AGG GCT GTC CAA AAT GTC TGG TGT TTC AGA CCA CCA	42
	K E G C P K C L V F Q T T I	
43	TCT GCA GTG GCC ACT GCC TAA CAA AGG TCG TAC TTC CGT TTT	84
	C S G H C L T K INTRON.....	
85	ATT TGC ATG GAA ACC ATT TTT AGT ttg cat taa tat tat tat	126
	.....	
127	aca agc atc aat att tcc agt ggt gac ata aag tca gtg tat	168
	.....	
169	atc ttg ctt gta gGA GCC TGT ATA CAA GAG CCC ATT TTC CAC	210
	.....E P V Y K S P F S T	
211	TGT CTA CCA ACA CGT GTG CAC TTA CCG GGA CGT CCG CTA CGA	252
	V Y Q H V C T Y R D V R Y E	
253	GAC GGT CCG GTT GCC AAG ACT GTC/gap/TGC ACC ATG GAC AGG	294
	T V R L L P D C 20aa C T M D R	
295	TCC GAC TGT ACC ATC GAA AGC CTG CAG CCT GAT TAC TGC ATG	336
	S D C T I E S L Q P D Y C M	
337	TCT CAG AGA GAG GAT TTC CCT GTG TAC TAG CCT GTA TCA AAC	378
	S Q R E D F P V Y STOP	
379	CAC AGA GTA CGA CAA ACT CTG CCA CTC TAA ATC AGA TAA TCT	420
	.	
421	CAC ATA GAT GTA TAT AAT AAA AAG TAC ATA CTT ATA CTT AGT	462
463	TT	464

Fig 1.3. The partial nucleotide sequence and deduced amino acid sequence of the gene coding for bcGtH IIB. The sequence of the intron is presented in lower case.

The isolation of bc GtH IB genomic DNA sequences

The bc genomic DNA library was initially screened with a seabream GtH IB cDNA probe, however, no positively hybridizing phage were obtained. In June 1996 we received a goldfish GtH IB cDNA clone from Dr. Makito Kobayashi, Tokyo

University, Japan. We have used this cDNA as a probe on Southern blots of bc genomic DNA and obtained a strong hybridization signal. The goldfish cDNA sequence was then used as a probe to screen the bc genomic DNA library. Six positively hybridizing phages were identified. Of these, three were positive on the third round of hybridization, and those three are currently undergoing the rescue procedure to a phagemid. As the goldfish cDNA probe gave a strong hybridization signal with a DNA from a number of different carp species, (Kobayashi, pers. comm), we are confident that we have finally identified the recombinant phages containing the bc GtH I β genomic sequence.

The expression of the recombinant bcGtH I β protein

The region coding for the mature bcGtH I β peptide was cloned into the pET11a expression vector. Expression of the recombinant protein was analyzed by a denaturing polyacrylamide gel electrophoresis (PAGE). As demonstrated in Fig. 1.4a, the putative bcGtH I β peptide migrates as a dominant 22 kd protein. Furthermore, Western blot analysis showed that this 22 kd protein immunoreacted with seabream GtH II antiserum (Fig. 1.4b), thus confirming its identification as the recombinant bcGtH I β protein.

Fig. 1.4. The recombinant bcGtH I β protein. a) SDS PAGE of the denatured recombinant GtH I β protein. On the left is the pre-stained SDS PAGE low molecular weight standards. b) Western blot of the denatured recombinant bcGtH I β protein reacted with seabream GtH antibody.

RIA of the refolded bcGtH IIB recombinant protein

The refolding of the recombinant protein was carried out using two different procedures; the first according to Stern et al. (1993), where the refolding is carried out through stage dialysis of the denatured protein, and the second according to Huth et al. (1994) in which the denatured protein is exposed to reducing conditions for various periods of time before dialysis. The refolded proteins were tested for their ability to displace the native common carp GtH in RIA.

Using an antiserum raised against common carp GtH, (Ribeiro et al. 1983), increasing concentrations of black carp pituitary extract resulted in displacement of the labeled carp GtH in a curve parallel to that of the standard cGtH (Fig. 1.5; see also Fig. 2.1). This would suggest immunological similarity between the GtH present in the black carp pituitary and the common carp GtH, justifying the use of the RIA system for the examination of the recombinant peptide.

Using this antiserum, we tested the ability of the bcGtH IIB recombinant proteins to displace the native common carp GtH. The recombinant protein refolded according to Stern et al. (1993) did not significantly displace the standard carp GtH (data not shown). On the other hand, refolding of the recombinant protein according to Huth et al., (1994) led to a significant displacement of the common carp native GtH. Calculation of the actual amounts which reacted in the RIA showed that only 1.3% of the refolded protein was responsible for this displacement (Fig. 1.6).

Fig. 1.5. Validation of the use of common carp GtH RIA for the black carp GtH. Displacement of standard cGtH (Breton, Rennes) by black carp pituitary extract in serial dilution.

Fig. 1.6. Displacement curve of the common carp GtH standard by increasing concentrations of the refolded recombinant bcGtH II β . (Refolding conditions: 4 h in 2M urea).

Discussion

In this report we describe the isolation and sequencing of the black carp GtH II β cDNA and genomic sequences, the identification of recombinant phage hybridizing to the goldfish GtH II β cDNA sequence and the expression of the recombinant β subunit using a bacterial expression system.

The method used for the isolation of the bcGtH II β cDNA was RACE PCR. The advantage of this approach is that we were able to isolate the full length coding region, as well as the 5' and 3' UT regions, from a single pituitary without the need of constructing a pituitary cDNA library, a task which in the *Fundulus heteroclitus* for example, consumed 3,000 pituitaries (Lin et al., 1992). In species such as the black carp, where individual fish, particularly at the reproductive stage, are both scarce and expensive, the RACE PCR approach may be the only feasible way for obtaining such sequences.

The deduced amino acid sequence of the bcGtH II β contains 23 a.a of a signal peptide which is very similar to that of the silver carp (3 a.a differences); however, it shows marked differences when compared to the sequence of the signal peptide of common carp, salmon and seabream.

The deduced mature peptide coding region contains 117 a.a. and shows 100% homology to that of the silver carp, 99% homology to that of the bighead and grass carp and 96% homology to the sequence of the common carp. The homology to GtH II β of non-cyprinid teleosts is much lower and ranges between 77% (masu salmon) to 65% (gilthead seabream). Some homology to the GtH I β from the chum salmon was detected (34%); however the regions showing this homology were very short and scattered.

The cDNA sequences encoding the bcGtH I β could not be amplified by PCR. We have tried using a range of primers, some degenerate, that were designed according to the short windows of homology that exist among GtH I β sequences from other teleosts and cDNA prepared from females at three different stages of development. This lack of success apparently occurred because of the high rate of divergence found in teleosts GtH I β sequences in general. Last year, after two years of failed attempts on our part, the GtH I β cDNA from the goldfish was cloned. In June 1996 we obtained this clone and have confirmed, by genomic Southern blot hybridizations, that DNA sequences highly homologous to the goldfish probe are present in the black carp and therefore, it is most likely that the duality of gonadotropins, at least in the evolutionary sense, exists also in the black carp.

The isolation of genomic bcGtH I β and II β is partially complete. The GtH II β gene was isolated, however, the determination of its nucleotide sequence is not yet complete. We have been able to establish that an intron, probably intron 2, is located at the conserved position of three a.a. downstream from the fifth cysteine, at a.a.38. This intron is relatively short (99 bp) compared with 154 bp in the common carp, 174 bp in the salmon and 1,326 bp in the tilapia. The intron has a high A/T content of 77% and its sequence is not conserved.

The GtH I β genomic sequence is at the final stage of isolation. Recombinant phages hybridizing to the goldfish GtH I β cDNA sequence were isolated and have already undergone three rounds of phage purification and are now being rescued as a phagmid.

We expect to confirm shortly, by DNA sequencing, that we have indeed isolated the gene coding for the GtH I β subunit from the black carp.

The protein expression study was initiated in order to produce a recombinant bcGtH I β peptide for the generation of specific antiserum to be used later in GtH II RIA. The classical purification approach of the native hormone could not be employed in the case of the black carp because of the scarcity of sexually mature fish, i.e. those likely to express high levels of GtH II. The β -subunit of the native GtH II is a glycoprotein with 12 cysteine residues which form 6 interchain disulfide bonds, similar to other glycoprotein hormones. In spite of these specific features, a biologically active recombinant gonadotropin, β hCG, was successfully produced using the pET11a bacterial expression system, confirming that the correct tertiary structure of the subunit can be obtained (Huth et al., 1994). This was achieved by *in vitro* refolding of the recombinant protein, which in the bacterial system did not undergo any post-translational modifications characterized for eukaryotic cells. In the present work we did not attempt to obtain a biologically active protein but a recombinant protein which would be immunologically similar to the native subunit, to be used in an immunoassay. We have tried two methods of *in vitro* refolding. The first method, described by Stern et al. (1993), had been used successfully for the refolding of eIF-4a protein, however, it did not produce any bcGtH I β recognized in the common carp RIA. Yet a recombinant protein, refolded using the modified Huth et al. (1994) procedure, displaced the common carp native GtH in a RIA. Out of the total refolded protein, the proportion of immunologically recognizable protein appears to be rather low (1.3%). However, a number of factors may have caused this figure to appear lower than it actually is. It should be emphasized that the RIA in which the recombinant protein was tested used the native cGtH, comprising both the α and β subunits while the recombinant protein consists of the β subunit only. Furthermore, the RIA system utilized is a heterologous system. Therefore, the actual similarity of the refolded recombinant protein to the native subunit is probably higher than measured.

In summary, the black carp (bc) GtH I β cDNA was amplified and isolated using RACE PCR. The cDNA was cloned, sequenced and found to be 679 bp. Comparison of the bcGtH I β deduced amino acid sequence with that of GtH I β from other teleosts

revealed 100% homology to the sequence of the silver carp, 99% to the grass and bighead carp, 96% to the common carp, 76% to the chum salmon and 65% to the gilthead seabream. The gene coding for the bcGtH II β was isolated and sequenced. It was found to contain a 99 bp intron at the conserved position of 3 a.a downstream from the fifth cysteine. Three bc recombinant phage which hybridized to the goldfish GtH II β cDNA probe were isolated and are currently being characterized.

The region coding for the mature GtH II β peptide was expressed in the bacterial expression vector pET11a resulting in the production of recombinant protein. Two different procedures for *in vitro* folding of the recombinant protein were attempted. Only 1.3% of the refolded recombinant protein displaced the native common carp GtH II in a radioimmunoassay.

Chapter 2:

The Brain-Hypophyseal-Gonadal Axis in Immature Black Carp and Trials in Advancing its Puberty

Introduction

The black carp, *Mylopharyngodon piceus* is a large cyprinid fish originating from the large rivers in the monsoon regions of China. It feeds preferentially, but not exclusively, on freshwater snails and mussels (Hepher and Pruginin, 1981). It is an edible fish of high quality, but is of specific importance due to its ability to control mollusk populations in rivers, lakes and ponds (Shelton et al., 1995). Black carp introduced into reservoirs in Israel efficiently reduced the nuisance caused by snail populations clogging filters, pumps and even water flowmeters (Leventer, 1979; Leventer and Teltsch, 1990). Another aspect of the uncontrolled snail populations is their association with water flukes causing severe diseases such as schistosomiasis. In addition, the black carp is used in certain Israeli polyculture systems to alleviate the problem of other digenean trematods infecting fish.

The black carp reaches puberty at an exceedingly late age (females at 6-7 y and males one year earlier). Keeping a broodstock for many years before they spawn for the first time is rather risky and expensive. Currently, the supply of fry can not meet the demand in Israel and abroad (especially Egypt) and fry are sold at prices comparable to those of ornamental fish. Advancing puberty in this fish may alleviate the problem. In addition, its late puberty makes the black carp an excellent model for studying the endocrine background of puberty in fish and other vertebrates.

This part of the report describes the state of the endocrine elements in the B-P-G axis of fish at the age of 2 and 3 years, the *in vivo* and *in vitro* dynamic challenge experiments designed to define non-functional components (blocks) along the axis, and summarizes the trials performed to advance the fish puberty.

Materials and methods

In vivo challenge experiments

In June of each of the project years, fish were transferred from the ponds into holding circular tanks (6 m³) for acclimation. They were then divided into three groups

of 12 fish each. Fish were weighed to the nearest 100 g and marked with a pit tag for individual identification. In order to examine the competence of the intact pituitary to respond to GnRH, one group was injected with Dagin containing 10 µg/kg sGnRH_a ([D-Arg⁶, Pro⁹-NET]sGnRH) and 20 mg/kg of the dopamine receptor antagonist, metoclopramide. Another group was injected calibrated common carp pituitary extract (containing 350 µg cGtH/kg) to examine the competence of the gonad to secrete sex steroids in response to GtH. A third group was injected saline and served as a control. Blood samples were taken from the caudal vessels before injection and 4, 8, 12 and 24 h thereafter. Before each treatment or sampling, the fish were anaesthetized with ethyl aminobenzoate (0.15 mg/l). Plasma samples were kept at -20°C till assayed for cGtH, E₂ and T.

In vitro challenge experiments

These experiments were performed on the control fish of the *in vivo* experiments after one or two weeks rest. Challenge experiments were carried out on primary culture of pituitary cells in order to test the response of the pituitary cells to GnRH or agents activating components of the GnRH signal transduction. In order to examine the steroidogenic response to GtH stimulation, incubation experiments were performed in which gonadal (and fat-pad) tissue fragments were challenged with gonadotropic preparations or the GtH second messenger, cAMP.

Culture of dispersed pituitary cells

The pituitaries were collected aseptically into modified medium 199 and were trypsinized for 40 min at room temperature with trypsin-EDTA solution. The process was terminated by addition of 20% fetal calf serum (FCS). The cells were counted and their viability was determined by trypan blue exclusion. Aliquots of the dispersed cells were taken for determination of their cGtH content, and the rest were plated at 0.25×10^6 cells/well on 24 multiwell plates in medium 199 containing 10% FCS. The cells were cultured for 2 days at 28°C under an atmosphere of 5% CO₂ before challenging with GnRH or activators of elements in the GnRH transduction cascades. Other details were as in Levavi-Sivan and Yaron (1992).

cGtH determination

cGtH level was determined in plasma samples, whole pituitary extracts or pituitary cell extracts by RIA using the common carp GtH (cGtH) prepared by B. Breton (Rennes) or by E. Burzawa-Gerard (Paris) as a standard. The use of cGtH for measuring the GtH II of the black carp is justified by the parallel dilution curves obtained from black carp pituitary extract and plasma and that of the cGtH standard. (Fig.2.1). In addition, the amino acid sequence, as deduced from the black carp GtH II β cDNA, was found to have 96% homology with that of the common carp (see Chapter 1). Other details of the RIA are as in Levavi-Zermansky and Yaron (1986), Yaron and Levavi-Zermansky (1986) and Drori et al. (1994). The sensitivity of the assay is 0.8 ng/tube. The intra- and inter-assay coefficients of variation are 4.1% and 20%, respectively.

Fig. 2.1. Cross-reactivity between the standard common carp GtH (ir-cGtH) and black carp pituitary extract (BPE) and plasma in serial dilution.

Estradiol (E₂) determination

Estradiol was determined by double antibody RIA using estradiol 17 β (Ikapharm, Israel) as unlabelled standard; ¹²⁵I-6-BSA-oxime-estradiol-17 β (Rotem, Israel) as a tracer, and our own highly specific anti-estradiol serum generated in rabbits (Bogomolnaya and Yaron, 1984). These components and the dried diethyl-ether extract of the plasma or medium were dissolved in the RIA buffer containing 0.04 M EDTA, 0.04 M Tris, 15.5 μ M NaN₃ and 1% gelatin. The second antibody was donkey

anti rabbit IgG coupled with magnetizable compound (Amerlex, Amersham). The sensitivity of the assay is 2.9 pg/ml

Testosterone determination

A double-antibody radioimmunoassay was established in a protocol similar to that for E₂ using standard testosterone (Fluka), ¹²⁵I- testosterone (Rotem, Israel) and a testosterone antiserum kindly given by Dr. R. Schulz, Utrecht.

It should be noted that the use of the iodinated steroids of this source did not yield constant results, and the variations between assays was over one order of magnitude. Therefore, the values of steroid determination are only valid for comparing results within individual experiments.

RNA extraction and quantitation of GTH II β mRNA

The pituitaries were sonicated in 6 M Urea, 3 M LiCl, 0.2 mg/ml heparin and 50 mM sodium acetate (pH 5.5) with the addition of 10% Sarcosyl (1/50 v/v). The mixture was incubated overnight at 4°C. The homogenate was centrifuged and the pellet was dissolved in DEPC double distilled water. RNA was extracted twice with a phenol-chloroform mixture (1:1), and was precipitated overnight at -20°C with absolute ethanol/ 8 M LiCl (50/1 v/v). After additional centrifugation, the pellet was redissolved in water. The RNA was loaded on a nylon membrane (GeneScreen Plus, NEN, USA) using a slot-blot apparatus (Schleicher & Schuell, Germany). The black carp GtH II β cDNA probe (see Chapter 1) was labeled with the random hexanucleotides primers method (Biological Industries, Beth-Haemek, Israel). After prehybridization, the labeled cDNA was added and left overnight at 65°C. Quantification of mRNA was made by a phosphoimager (Bio-Imaging Analyzer BAS, Fuji, Japan) equipped with the TINA program. The results of each blot were corrected for recovery using *Sparus aurata* β actin cDNA to determine β actin mRNA in the same sample.

Development and characterization of delivery systems for GnRH and steroids.

Polymer-based, biodegradable delivery systems were developed for the sustained administration of GnRH superactive analogs and testosterone. The GnRH α delivery system was made up of the co-polymer of fatty acid dimer and sebacic acid. It

released the GnRH into treated striped bass for a period of 8-10 weeks (Mylonas et al., 1995). The testosterone delivery system was made up of the copolymer polylactic-polyglycolic acid. The release of testosterone from this system and its physiologic effect lasted for 4-8 weeks in both the striped bass (Holland et al., 1995) and the black carp (Gur et al., 1995).

Statistical analysis

The significance of differences among group means was examined by ANOVA followed by the Least Significant Differences Test (LSD; Blossom) or the Bonferroni t-test (Instat Graph Pad). Results are presented as mean $\pm$ s.e.m. Means marked by the same letter do not differ significantly from each other.

Results

Gonadal histology of 2 y old black carp

Histological examination of the rudimentary gonads have shown the presence of well-differentiated oocytes up to 73 μ m in diameter or primary spermatogonia arranged in cysts (Plate 1) This would indicate that although the gonadal tissue of 2 y old black carp is very rudimentary, it has already passed the stage of sexual differentiation.

Gonadal development in prepubertal black carp

The gonadosomatic index of male black carp has not changed during their third and fourth years. However, in females a considerable increase in ovarian relative weight was noticed during the second half of their 3rd year (Fig. 2.2). This increase in the GSI is also reflected in the diameter of the largest oocytes encountered in the ovary.(Fig. 2.3). it should be noted, however, that only a small proportion of the oocytes showed such an increase. In these oocytes cortical alveoli made their initial appearance. The GSI of three 4 y old females examined in June 1996 showed a further increase reaching over 0.3%. The GSI in 3 y old males did not change from the previous year (Fig. 2.2). However, in a single 4 y male examined in June 1996 the GSI was comparable to that of the females (not shown).

Fig. 2.2. Gonadosomatic index (% of bw) in 2, 3 and 4 y old black carp. Mean±s.e.m. (n=5-6). Means marked with the same letter do not differ significantly from each other ($p>0.05$). Means were analyzed separately for each sex.

Fig. 2.3 Maximal oocyte diameter in 2 and 3 y old black carp. The largest oocyte encountered in ovarian sections was determined in each fish and averaged for each date Mean±s.e.m. (n=5-6). Means marked with the same letter do not differ significantly from each other ($p>0.05$).

Plate 1. Gonads of 2 y old black carp. Stain: Mallory-Azan (facing page).

1a: whole testis

Picture 1: Section of 2+ y old black carp testis stained with Hematoxylin-Eosin.

1b: Spermatogonia (SP) and Leydig's cells (LC).

Picture 2: Section of 2+ y old black carp ovary stained with Hematoxylin-Eosin.

PGC, primordial germ cells; OOC, primary oocyte; OL, ovigerous lamella

Picture 3: Section of 3+ y old black carp ovary stained with Hematoxylin-Eosin.

V, cortical alveoli.

Identification of Blocked Sites in the Hypothalamic-Pituitary Axis

In vivo challenge experiments in fish of 2 and 3 y old

The aim of this experiment was to examine whether immature 2 or 3 y old fish respond to stimulation by Dagin (a combination of GnRH with a dopamine receptor antagonist) by elevating GtH levels in the circulation. In both experiments cGtH levels in fish injected with Dagin did not differ from the control fish throughout the 24 h experiment (Figs. 2.4, 2.5). This would indicate that although the pituitary of 2 or 3 y old black carp contains certain amount of GtH, the gland is unresponsive to the releasing hormone. This was in spite of the fact that the acute inhibitory effect of the endogenous dopamine was curtailed by the dopamine inhibitor metoclopramide, at a dose of 20 mg/kg which is effective in other carp species (Drori et al., 1994).

Fig. 2.4. *In vivo* challenge experiment in 2 y old black carp. cGtH levels in fish injected with Dagin (10 μ g sGnRH α + 20 mg metoclopramide).

Fig. 2.5. *In vivo* challenge experiment in 3 y old black carp. cGtH levels in fish injected with Dagin (10 μ g sGnRH α + 20 mg metoclopramide).

In vitro challenge experiment

The following experiment was carried out to define the site at which the response of the pituitary to GnRH is blocked. Pituitary cells of 2+ y old black carp were dispersed and cultured for 2 days, and then challenged for 2 h with sGnRH or cGnRH II (1 pM-1 μ M), with the activator of adenylyl cyclase, forskolin (10 μ M) or the activator of protein kinase C (TPA; 12.5 nM). A slight but significant increase (43%) in the release of GtH occurred in response to the highest dose of cGnRH II. However, a dramatic release of the gonadotropin occurred when components of the GnRH signal transduction were activated externally by forskolin or TPA (Fig. 2.6). The results of this experiment indicated that the response to GnRH in the 2 y old black carp is blocked along the transduction cascades at a site upstream of the formation of cAMP, and upstream of the activation of PKC, probably at the level of the GnRH receptors.

Fig. 2.6. *In vitro* challenge experiment. cGtH release from cultured pituitary cells of 2 y old black carp in response to increasing concentrations of sGnRH, cGnRH II, to forskolin (10 μ M) or to TPA (12.5 nM). Mean $\pm$ s.e.m. (n=6 wells).

Effect of testosterone *in vitro* on GtH release

The experiment was designed to examine the possibility that testosterone (T) can facilitate the response of the pituitary cells of immature black carp to GnRH stimulation. Pituitary cells of 2 y old black carp were dispersed and cultured for 2 days in the presence of T (1 nM-100 nM) and then challenged for 2 h with sGnRH or cGnRH II both at 100 nM. cGtH release increased in the cells exposed to T and was further augmented when challenged with the GnRHs. In this experiment sGnRH was at least as effective as cGnRH II (Fig. 2.7).

Fig. 2.7. cGTH release from cultured pituitary cells exposed for 2 days to graded concentrations of testosterone (T) and then challenged for 2 h with either sGnRH or cGnRH II (100 nM). Mean $\pm$ s.e.m. (n=4 wells). Means marked with the same letter do not differ significantly from each other ($p>0.05$).

Facilitation of the response to GnRH by T, E₂ and 11-ketotestosterone (11-KT)

As aromatase is known to be present in the pituitary of fish, it was important to ascertain whether the facilitation seen above results from T action as an androgen, or is due to its aromatization to E₂. Pituitary cells were cultured for 2 days in the presence of either T, E₂ or the non-aromatizable androgen 11-KT (all at 100 nM) and then challenged with sGnRH for 2 h.

cGTH release in response to the GnRH was higher in cells exposed to E₂ than in cells exposed to T. Cells exposed to 11-KT did not show any significant increase in GtH release when challenged with GnRH. (Fig. 2.8) indicating that the effect of T on GtH release in the pituitary of 2 y old black carp results from its aromatization to E₂.

Fig. 2.8. cGTH release from cultured pituitary cells of 3 y old black carp exposes *in vitro* for 2 days to testosterone (T), estradiol-17 β (E₂) or 11 ketotestosterone (11-KT) and then challenged with sGnRH or cGnRH II for 2 h. Mean $\pm$ s.e.m. (n=7 wells). Means marked with the same letter do not differ significantly from each other ($p>0.05$).

Identification of Blocked Sites Along the Pituitary- Gonadal Axis

Estradiol secretion from the ovary of mature fish in response to cGtH

The experiment was conducted in order to examine the response of ovarian tissue of mature black carp to gonadotropic stimulation to establish a baseline for comparison to immature gonads. Fragments of the ovary were incubated for 4 h in a shaker bath at 26-28°C with 4 changes of the neat medium. They were then exposed for 4 h to IBMX only (0.1 mM) or to IBMX and calibrated carp pituitary extract containing 0.1, 0.5, 1, 2 or 4 µg cGtH/ml. The medium was collected and the secreted estradiol was determined by RIA.

Estradiol secretion from the fragments of the mature ovary, although containing a considerable number of atretic follicles, increased in response to cGtH stimulation reaching a four fold increase at the highest cGtH dose (Fig. 2.9).

Fig. 2.9. Estradiol-17 β secretion from ovarian fragments of mature black carp ovary in response to graded doses of cGtH (calibrated carp pituitary extract) in the presence of 0.1 mM IBMX. Mean $\pm$ s.e.m., n=5. Means designated by the same letter do not differ significantly ($p > 0.05$). The ovary contained a considerable number of atretic follicles.

Estradiol secretion by the gonadal tissue and gonadal fat pad in immature fish

Segments of the rudimentary gonads or adipose tissues were preincubated for 4 h, and then exposed for 3 h to either IBMX alone (0.1 mM), or in combination with carp pituitary extract (CPE) calibrated to contain 0.1-4 µg cGtH/ml or with dbcAMP (0.3-3 mM). E₂ secretion from gonadal segments was stimulated in a dose-dependent manner by dbcAMP (0.3 - 3 mM). However, the secretion of the estrogen remained at the basal level when exposed to CPE containing cGtH (Fig. 2.10).

Fig. 2.10. Estradiol secretion from gonadal segments of 2 y old black carp in response to CPE calibrated to contain cGTH at the concentrations marked (a)) or to dbcAMP (b) all in the presence of 0.1 mM IBMX. Mean $\pm$ s.e.m., $n=4$.

While preparing the tissue for incubation, it was evident that a fat pad is firmly attached to the rudimentary gonad which was difficult to separate. It was suspected that part of the secreted steroid originates from this fat pad. In the next experiment, carefully cleaned gonadal tissue was incubated in parallel with the fat pad. In order to ascertain that the steroidogenic capacity is unique to this fat-pad, visceral adipose tissue from around the gut was also examined in parallel wells.

Estradiol secretion from the gonadal fat-pad was stimulated by either IBMX alone (0.1 mM), or in combination with dbcAMP (0.3 and 1 mM) or hCG (1 and 10 iu/ml). Although the secretion in response to these agonists was always higher than the basal secretion, the response to the lower concentration of dbcAMP, or to hCG at both concentrations did not exceed that in response to IBMX alone (Fig. 2.11). The stimulated secretion of estradiol from the fat pad was always higher than from the rudimentary gonad. However, none of the agonists used were able to stimulate the secretion of E_2 from the visceral fat.

Fig. 2.11. Estradiol secretion from the gonadal tissue, gonadal fat pad and from visceral adipose tissue (visceral fat) in response to IBMX alone (0.1 mM) or in combination with dbcAMP (dbc) or hCG. Mean $\pm$ S.E.M., $n=3$. Means designated by the same letter do not differ significantly; comparison was performed among tissues in the same treatment. Asterisk marks a significant difference from the IBMX treatment.

Advancing puberty in the black carp by sustained testosterone treatment

in vivo testosterone treatment

Encouraged by the possibility of enhancing GtH release by testosterone as established by the *in vitro* experiment, 2 y old fish (1.5-3.2 kg bw; 11-12 fish in each group) were injected once with T given in a slow-release device at 0, 0.8, 1.6 or 3.2 mg/kg. Blood was sampled before injection and 4, 7, 14, 21 and 28-32 days thereafter. At the end of the experiment, fish were analyzed, each group on a successive day. The pituitary cells of 5-6 fish were pooled for culture and for cGtH content and 5 pituitaries were taken for quantification of GtH II β mRNA. The gonads were taken for incubation and for histological examination.

Circulating testosterone levels: Plasma testosterone levels increased gradually in a dose-dependent manner till day 14 and then gradually declined reaching the initial level at the end of the experiment (Fig. 2.12).

Fig. 2.12. Circulating levels of T in 2 y old black carp injected once with slow-releasing microspheres containing testosterone. n=11.

cGtH content in pituitary cells: at the end of the experiment, cGtH content in pituitary cells of fish injected with T-containing microspheres increased in correlation with the dose reaching maximal levels in fish injected 1.6 mg T/ kg (Fig. 2.13).

Fig. 2.13. cGtH content in 0.25×10^6 dispersed pituitary cells of 2 y old black carp injected once with T-containing microspheres. Pituitary cells of 4-5 fish of each group were pooled and cGtH was determined in triplicate in each of 4 aliquots of each pool. Mean $\pm$ s.e.m. (n=4). Means marked with the same letter do not differ significantly ($p > 0.05$).

Black carp GtH IIB mRNA and GtH Basal and TPA-stimulated release : The steady-state levels of bcGtH IIB mRNA, as measured by slot-blot hybridization, increased in the pituitaries of fish injected with T microspheres (Fig 2.14). Due to technical error, the level of the mRNA of fish injected with the highest dose of T could not be determined. The basal and TPA-stimulated cGtH release from cultured pituitary cells showed an increase in T-treated fish dependent on the dose. (Fig. 2.15)

Fig. 2.14. GtH IIB mRNA steady-state level in 2 y old black carp injected once with T-containing microspheres. Mean±s.e.m. (n=4). Means marked with the same letter do not differ significantly ($p>0.05$).

Fig. 2.15. Basal and TPA-stimulated cGtH release from 2 y old black carp injected with T-containing microspheres. Mean±s.e.m. (n=5-6 wells). Means marked with the same letter (basal- lower case, TPA - upper case) do not differ significantly ($p>0.05$).

The increase in GtH IIB mRNA, cGtH content in the pituitary cells and the elevated basal and TPA-stimulated release indicate that one month treatment with testosterone resulted in an increase in synthesis of the hormone in the pituitary.

cGtH release in response to GnRH: The pituitary cells of T-treated fish showed an increased sensitivity in culture to sGnRH (Fig.2.16) and cGnRH II (not shown). The sensitivity to the peptide increased in parallel with the dose of T administered to the fish and with the dose of the GnRH given *in vitro*. This is best demonstrated in the pituitary cells of fish given the lowest dose of T. (It should be noted that due to technical fault during the preparation of the culture, pituitary cells of the fish injected with 1.6 mg/kg T did not respond at all to the GnRH).

Fig. 2.16. sGnRH stimulated release of cGtH from cultured pituitary cells of 2 y old black carp injected with T-containing microspheres. Mean $\pm$ s.e.m. (n=5-6 wells). Means marked with the same letter do not differ significantly ($p>0.05$).

Circulating cGtH: in spite of the increase in the content of GtH in the pituitary of the T-treated fish and the augmented response to GnRH in cultured pituitary cells, the level of the gonadotropin was not elevated throughout the experiment (except in single group at one sampling point (Fig. 2.17)).

Fig. 2.17. Plasma cGtH levels in 2 y old black carp during the 4 week treatment with T-containing microspheres. Mean $\pm$ s.e.m. (n=11). Means marked with asterisk differ significantly from the level of the control at the same sampling point ($p<0.05$).

It should be emphasized that the results obtained in culture were achieved with dispersed pituitary cells in which the connection with hypothalamic nerve terminals is

disrupted. In intact fish, these cells are in close contact with nerve terminals secreting neurohormones, like dopamine, which can inhibit GtH release despite its high content in the pituitary. In order to achieve a treatment which will facilitate the release and elevate the circulating GtH level, a more advanced experiment was conducted in which fish were treated for 10 weeks with a combination of T, GnRH and a dopamine receptor antagonist. For this experiment, 3 y old black carp were used. Untreated fish at that age do not respond to acute treatment of GnRH α and a dopamine antagonist (see Fig. 2.5).

Advancing puberty by hormonal manipulation combining testosterone, a super-active GnRH analog and domperidone

Seventy five 3 y old fish (2.5-4.0 kg bw) were divided into 5 groups and treated as in Table 2.1. The experiment lasted for 10+ weeks during which blood was sampled every 2 weeks.

Table 2.1: Details of treatment of 3 y old black carp.

Group	GnRH*	Testosterone**	Domperidone***
Control	-	-	-
T	-	2 mg/kg	-
GnRH	2 x 150 µg/kg	-	-
T+Gn	2 x 150 µg/kg	2 mg/kg	-
T+Gn+DOM	2 x 150 µg/kg	2 mg/kg	4 x 5 mg/kg

* [D-Ala⁶ Pro⁹ NEt]-mGnRH given once monthly in a slow-release device as microspheres

** Testosterone given once in a slow-release device as microspheres

*** The dopamine receptor antagonist was given in mixture with saline containing 1% metabisulfite on alternate weeks.

At the end of the experiment, each group of fish was examined separately at a 2-3 day intervals. Fish were anaesthetized and weighed, blood was sampled and the pituitaries and gonads were excised. Two pituitaries with brain attached were fixed in Bouin's fluid and were processed for histological and immunocytochemical (ICC) analysis. Four pituitaries were used for RNA extraction and slot-blot hybridization to determine the steady-state level of the bcGtH II β mRNA, the cells of remaining pituitaries were dispersed and used for quantification of the cGtH content and for *in vitro* challenge experiments. The gonads and the adjoining fat-pads were taken for histology and for incubation with gonadotropin.

Gonads were stained with hematoxylin and eosin or with Masson's trichrome stain. The eight brain and pituitary samples (two from each group except the controls) were stained with Masson's trichrome stain and were subjected to the following antisera (Table 2) for ICC study using the avidin-biotin method (Vectastain, ABC Elite):

Table 2.2: Dilutions and Sources of the Antisera Used in This Study

Antiserum against	Source	Dilution
Chicken GnRH II	H.J.Th. Goos	1:1,000
Mammalian GnRH (20-3)	H.J.Th. Goos	1:500
Lamprey I GnRH	S. Sower	1:10,000
Salmon GnRH	Y. Zohar	1:800
Coho salmon GtH I & II	P. Swanson	1:500 & 1:1,000
Tilapia GH	S. Holtzman	1:30,000
Dopamine (glutaraldehyde conjugate)	Chemicon Corp.	1:200
Dopamine D ₂ Receptor	Research & Diagnostic antibodies Inc.	1:200

Histology and ICC of brain and pituitaries: Little or no GnRH immunoreactivity was detected in the brain or pituitary gland when tested with any of the antisera listed in Table 2.2. Control animals (tissues derived from *Xiphophorus*) displayed typical immunoreactive responses.

Immunoreactivity to anti-GtH I was not detected in any of the samples. Immunoreactivity to anti-GtH II was generally low. It was less intense in fish treated with T+ GnRH + Dom compared to those injected with only T or GnRH. There may be a marginal increase in the immunoreactivity in GnRH- and T+GnRH- treated fish compared to fish given T alone.

No immunoreactive DA was found in any of the samples although a typical immunoreactive response was detected in brains and pituitaries tissues derived from *Xiphophorus*. Immunoreactivity to anti-dopamine D₂ type receptor was detected in the GtH zone of the pituitary gland. The immunoreactivity appears as fine granules, often in clusters, in a distinctive distribution and outside of the gonadotrophs. There was no apparent difference in immunoreactivity in any of the experimental groups.

The immunoreactive response to anti-Tilapia GH appeared to be diminished in intensity in T + GnRH + Dom treated fish as compared to the other groups.

Far above, gonadotrophs were most numerous and displayed the most prominent cytological signs of active synthesis and active release of gonadotropin in the fish treated with T + GnRH + Dom. These cells were large and contained few granules in their cytoplasm. Their nuclei were similarly enlarged and the nucleoli were prominent and stained bright red.

Plate 2 (facing page)

Figs 1 - 3. A black carp treated with T + GnRH + D.

Fig. 1. Section of pituitary gland stained with Masson trichrome. The wide band across the middle of the gland is occupied by GtH and GH producing cells.

Fig. 2. Immunoreactivity to GTH is seen in low intensity but in many cells. Compare with Fig. 1 which is an adjacent section.

Fig. 3. Note large pale GTH cells with red staining GH cells. GTH cells show signs of hyperactivity (see text for discussion). Higher magnification of Fig. 1.

Figs 4 & 5. Pituitary gland from a fish treated with T only.

Fig. 4. Low levels of immunoreactive GtH localized in relatively few cells.

Fig. 5. Pale cells are gonadotrophs surrounded by GH cells (red). Compare with Fig. 3 (same magnification) and note smaller size of cells and little indication of cellular activity.

Plate 2

1

2

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4

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Plate 3

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7

8

cGtH content in pituitary cells: pituitary cells of 5 fish from each group were dispersed for challenge experiment in culture. cGtH was measured in 20-28 aliquots of the cell suspension from each group, each aliquot containing 250,000 cells. cGtH content in cells taken from GnRH-treated fish did not differ from that in the controls. However, cGtH content in cells taken from fish treated with in combination containing T was about fifteen fold higher than in the controls, while in fish treated with the combination of T+Gn+Dom the content was more than thirty fold higher (Fig 2.18).

Fig. 2.18. cGtH content in dispersed pituitary cells of black carp treated with T or GnRH alone or together, or combined with domperidone Mean±s.e.m., n=20-28 wells. Means marked with the same letter do not differ significantly ($p>0.05$).

Plate 3

Figs. 6 - 8. Pituitary gland from a fish treated with T + GnRH

Fig. 7 shows the distribution of immunoreactive GtH in Fig. 6 which is stained with Masson's trichrome.

Fig. 8. Masson's stained section showing the limited number of GtH cells. Their cytological profile is more like the T-treated animal. Compare with Fig. 3 and Fig. 5.

bcGtH II β mRNA: RNA was extracted from 5 pituitaries in each treatment group and hybridized with the cDNA probe of bcGtH II β . In all fish treated with T in any combination, the level of the transcript was significantly higher than in the control fish. The level of the transcript in the GnRH-injected fish did not differ from that in the controls (Fig. 2.19).

Fig. 2.19. Steady-state levels of the black carp GtH II β mRNA in pituitaries of 3 y old black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n as marked. Means marked with an asterisk differ significantly from the control ($p<0.05$)

Basal and TPA-stimulated cGtH release from cultured pituitary cells: Pituitary cells of 5 fish from each group were dispersed, pooled and cultured for 2 days. Cells were then challenged for 2 h with TPA (12.5 nM) and the medium was collected from these and unstimulated wells (basal release). Basal cGtH release from cells of the GnRH-treated fish did not differ from that of the controls. The basal release from cells of T-treated fish with or without GnRH was four-fold higher than in the controls or GnRH-treated. However, cells of fish treated with the combination of T+Gn+Dom showed a much higher increase (9-10 fold; Fig. 2.20). A similar pattern was seen after exposure to TPA where the release was considerably higher.

Fig. 2.20. Basal and TPA stimulated cGtH release from cultured pituitary cells of 3 y old black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n as marked. Means marked with the same letter (basal- lower case, TPA - upper case) do not differ significantly ($p>0.05$).

cGtH release from cultured pituitary cells in response to sGnRH and cGnRH II: cGtH release from cultured pituitary cells prepared as above and challenged with sGnRH or cGnRH II at increasing concentrations (0.01-1 μ M) showed a pattern similar to that of the basal release. The release from cells of GnRH-treated fish was similar to that of the controls, it was higher in cells taken from T-treated fish, with or without GnRH, while the highest release was noted in cells taken from fish treated with the combination T+Gn+DOM (Fig. 2.21). The magnitude of the response to sGnRH and cGnRH II was similar (not shown).

Fig. 2.21. cGtH release in response to sGnRH from cultured pituitary cells of 3 y old black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n as marked. Means marked with the same letter do not differ significantly ($p>0.05$).

Circulating GtH: Initially, all fish treated with testosterone in any combination showed an increase in the circulating level of cGtH above the levels of the control. From the 8th week on, the combination of GnRH with T resulted in cGtH levels higher than the T alone. The effect of domperidone addition could be noted in the later part of the experiment but the levels were not significantly different from the combination T+GnRH (Fig. 2.22).

Fig. 2.22. Circulating cGtH level in 3 y old black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n=8-12. Means marked with asterisk differ significantly from the controls at the same sampling point ($p<0.05$).

Effect of the combined treatment on gonadal development: In the control and GnRH-treated fish, the diameter of the largest oocytes encountered in ovarian sections was significantly higher than in fish of the other groups (Fig. 2.23). It would appear that T treatment in any combination resulted in inhibited growth of the ovarian follicles.

Fig. 2.23. Oocyte diameter in 3 y old female black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n as marked. Means marked with the same letter do not differ significantly ($p>0.05$).

Secretion of estradiol from ovarian fragments: Ovarian fragments of fish from each treatment group were incubated separately in 6 replicates for 4 h in the neat medium. The fragments were then challenged for 3 h with hCG (10 iu/ml) in the presence of 0.1 mM IBMX. Estradiol secretion was measured in the medium and calculated as the ratio to the basal secretion. When applicable, the ratio was averaged for each treatment group. The ratio of estradiol secretion from ovarian fragments of fish treated with any combination containing T was significantly higher than from those of fish treated with GnRH alone or from control fish (Fig. 2.24).

Fig. 2.24. Estradiol secretion in response to hCG (ratio to basal secretion) from ovarian fragments of 3 y old female black carp treated with T or GnRH alone or together, or combined with domperidone. Mean $\pm$ s.e.m., n as marked. Means marked with the same letter do not differ significantly ($p>0.05$).

Discussion

Ontogeny of the gonads

The gonadosomatic index (GSI) in males did not change during the 3rd year. In females, however, there was a small but significant growth of the ovary which was reflected both in GSI and in the histological examination demonstrating an increase in oocyte diameter and increase in number of oocyte exhibiting cortical alveoli. In their 4th year, the GSI increased considerably reaching 0.3% of body weight. These results are in line with the observations on the incremental ovarian growth which occurs every summer in common carp in central Europe till reaching puberty at the age of 5 years (Horváth, 1986).

The hypothalamic-hypophyseal axis in the immature black carp

The dose of Dagin employed in the *in vivo* challenge experiment (10 μ g/kg GnRHa and 20 mg/kg metoclopramide) is sufficient to increase the plasma cGtH in adult common carp to a level which will result in final oocyte maturation and ovulation (Drori et al., 1994). However, this treatment did not elicit any increase in cGTH in 2 or 3 y old black carp. This would indicate that the pituitary of these immature fish is

refractory to GnRH stimulation although the dopaminergic inhibition was acutely abolished by metoclopramide.

The lack of GnRH effect in the immature fish is further supported by the inability of sGnRH to stimulate GtH release from the culture of the dispersed pituitary cells of these fish although their contact with hypothalamic nerve fibres was disrupted. However, a significant increase in cGtH release occurred in response to the protein kinase C (PKC) activator, TPA, or to the stimulation of adenylyl cyclase by forskolin. Activation of PKC is an essential component of the GnRH signal transduction in all vertebrates studied so far, including goldfish (Chang et al., 1991, 1993, 1994; Jobin et al., 1993, 1996) and tilapia (Levavi-Sivan and Yaron, 1989, 1993). Elevation of cAMP was found to increase GtH release in goldfish (Chang et al., 1992) and its involvement in GnRH signal transduction has been established in tilapia (Levavi-Sivan and Yaron 1995) and *Channa punctatus* (Mukhopadhyay et al., 1995). The results of this experiment suggest that the gonadotrophs of the black carp are capable of producing cGTH (probably GtH II type) and that certain components of the GnRH transduction cascade already exist in the pituitary of the black carp 4 years before the puberty age. Furthermore, the results suggest that in the immature black carp, a block exists in the transduction cascade upstream to the activation of PKC and PKA, presumably at the level of the GnRH receptors.

The two-day presence of T in the culture medium rendered the pituitary cells of immature black carp responsive to both sGnRH and cGnRH II. This provides evidence that T has a direct effect on the pituitary cells probably by increasing the number of GnRH receptors. However, the fact that estradiol was more effective than T, while the non-aromatizable androgen 11-KT was ineffective, indicates that the effect of testosterone is due to its conversion into estradiol.

The hypophyseal-gonadal axis in the immature black carp

The fact that the rudimentary gonad did not respond to gonadotropic stimulation but did respond to dbcAMP or IBMX indicates that the enzymes involved in steroidogenesis are present in the rudimentary gonad of the immature black carp and can be activated by elevating cAMP levels. This would mean that the irresponsiveness of the immature gonadal tissue to gonadotropic stimulation, is due to a block upstream to the formation of cAMP, probably at the GtH receptor level.

The fat-pad showed a similar but much stronger response to these agonists, whereas the capacity of the visceral fat to produce estradiol was barely noticeable and could not be stimulated by cAMP agonists. It is assumed that the gonadal fat-pad, probably derived from the mesoderm of the genital ridge, as in amphibians (Heuttner, 1958) relates in its origin to the endocrine cells of the gonads, and is thus capable of producing steroids. The fat pad adjoining the testis of the newt *Triturus cristatus* was also found to contain sex steroids (Chieffi et al., 1967). Due to its large mass in relation to the rudimentary gonad, this fat-pad should be considered to constitute a unique and major source of sex steroids during the process of puberty. The exact embryonic source of this fat-pad, its relationship to the gonads and its steroidogenic function awaits further research.

Trials in advancing puberty in the black carp

The long term treatment of 2+ y old black carp with testosterone corroborates the findings *in vitro* about the responsiveness to GnRH. The fact that in the treated fish, there was an increase in the steady state level of the mRNA encoding the GtH II β subunit as well as higher content of cGtH in the pituitary cells, indicates that the steroid has an additional effect, namely, the stimulation of GtH synthesis

These results are in agreement with previous findings in juvenile rainbow trout in which administration of aromatizable androgens had a stimulatory effect on the pituitary gonadotropin which was abated by an aromatase inhibitor (Crim and Evans, 1979, 1983; Crim et al., 1981). Also in the female Japanese eel, chronic T treatment elevated pituitary gonadotropin accompanied by progressive ovarian growth and vitellogenesis (Lin et al., 1991). This situation may differ in immature *Xiphophorus maculatus* in which both T and the non-aromatizable androgen 11-KT increased the number and activity of the gonadotrophs (Schreibman et al., 1986). A sexual dimorphism in the activity of gonadal steroids was found in the European eel, whereby GtH pituitary content in males was elevated by either E₂ or T, while only E₂ was effective in females (Dufour et al., 1983; Montero et al., 1995). In T-treated black carp, the GtH II β mRNA level increased dramatically depending on the dose of the androgen while in the European eel, T and E₂ have a differential action on mRNA encoding GtH subunits (Qurat et al., 1991). The specific and differential effects of

gonadal steroids on the expression of α and the two β subunits of the black carp await the construction of the respective cDNA probes which is still in progress.

Although the content of cGtH increased in the pituitary of T-treated black carp, and the response to GnRH was augmented in the culture of their pituitary cells, the cGtH level in the plasma did not rise at all throughout the experiment. This apparent discrepancy could be explained by the possible dopaminergic inhibition in the immature fish. Indeed, immunocytochemical study revealed the presence of dopamine D₂ type receptors around the gonadotrophs of the black carp. Dopamine inhibition was probably augmented by the high T levels in the intact pituitary, as reported in the goldfish (Omeljaniuk et al., 1989; Trudeau et al., 1993) but was absent in the dispersed pituitary cells devoid of any contact with nerve endings.

The possible scenario described above required an additional experiment in which testosterone treatment of immature black carp was combined with sGnRH α to promote GtH release while abating the possible dopaminergic inhibition by domperidone, a dopamine D₂ receptor antagonist.

The results of this experiments showed that T, in any combination, had a significant stimulatory effect on the amount of cGtH in the pituitary, on the bcGtH IIB mRNA, on the basal and TPA-stimulated release and on the response *in vitro* to GnRH. These results confirm the findings of the previous trial carried out on 2 y old black carp with testosterone alone and allow the conclusion that T has twofold effect on the pituitary of the immature black carp: the stimulation of GtH synthesis and the increase of the pituitary sensitivity to GnRH stimulation, probably by stimulating the formation of GnRH receptors. However, these conclusions do not exclude the possibility that testosterone also has an effect on hypothalamic or hypophyseal GnRH content or release as demonstrated in the European eel (Montero et al., 1995). Furthermore, T may also have an effect on other hypothalamic neurohormones capable of modulating GtH release (Trudeau et al., 1993).

Domperidone, when given together with T and GnRH augmented considerably all the parameters achieved with the combination T+GnRH, except the bcGtH IIB mRNA which remained elevated to the same extent as in the pituitaries of T-treated fish. Retrospectively, the design of the experiment was incomplete. Two more groups should have been included in the experiment if more fish had been available, namely:

DOM alone and DOM+GnRH. Nevertheless, we may assume that long-term repression of dopaminergic inhibition facilitated the stimulatory effect of testosterone on pituitary cGTH content and the release in response to GnRH *in vitro*. It is possible that a long-term treatment with this DA receptor antagonist increased the binding of GnRH to the pituitary cells as reported in the African catfish (de Leeuw et al., 1988). Preliminary experiments in which cultured pituitary cells of 4 y old black carp were exposed to increasing concentration of domperidone, showed a significant and a dose-dependent increase in cGtH release which was not altered by a concomitant exposure to sGnRH (not shown). This would point to a possible direct effect of DOM on the pituitary cells not necessarily involving GnRH receptors.

An experimental approach similar to that designed for the black carp (Yaron et al., 1995) was taken for studying the block along the hypothalamo-hypophyseal axis in the immature freshwater eel (Montero et al., 1996). *In vivo* estradiol pretreatment was followed by an increased accumulation of GtH in the pituitary cells of the eel while only a small fraction of the GtH was released. Pituitary cells taken from E₂-treated fish responded in culture to both TPA and the native GnRH forms indicating that the GnRH transduction cascade distal to PKC activation is present in the immature eel pretreated with E₂. Unfortunately, A parallel experiment using TPA in eels untreated with estradiol was not included in this report leaving unsolved the question of whether the immature eel pituitary possesses this part of the cascade, as shown in the present study. Perfused pituitaries of E₂-pretreated eels did not release GtH in response to depolarization of the cell membranes by K⁺ unless this was accompanied by GnRHa and inhibition of catecholamine synthesis. It was suggested that in the immature eel gonadotrophs, the voltage sensitive calcium channels are non-functional due to a strong inhibitory effect of dopamine (Montero et al., 1996).

It would appear that GnRH had no effect on the maximal diameter of the oocytes while testosterone had an inhibitory effect on the growth of ovarian follicles. Fish treated with the steroid, in any combination, had oocytes with maximal diameter smaller than the controls or GnRH-treated fish. However, the *in vitro* secretion of estradiol, in response to hCG, was higher in T-treated fish. This apparent contradiction could have been explained by the lower proportion of steroidogenically-inert mass in the smaller ovaries of the T-treated fish. However, estradiol output was calculated as

the ratio to the basal secretion, therefore the mass of the tissue or its composition is accounted for in the results.

In conclusion, two non-functional sites along the B-P-G axis were identified in the immature black carp. The pituitary is refractory to GnRH stimulation due to a block proximal to the activation of PKA and PKC, probably at the level of GnRH receptors. The gonads, although containing the enzymatic machinery to produce steroids, are refractory to gonadotropic stimulation indicating a block at the GtH receptor level. Attempts at advancing puberty showed that testosterone, and its conversion to estradiol, is of cardinal importance in stimulating GtH synthesis in the pituitary, and in increasing the sensitivity of the pituitary to GnRH. A long-term treatment combining T+GnRH can result in an increase in the circulating GtH in the immature fish. Domperidone given in such a prolonged treatment may facilitate the accumulation of GtH in the pituitary by reducing dopaminergic inhibition. It would appear that the dose of testosterone should be reduced in order to minimize its inhibitory effect on ovarian growth.

Chapter 3:

Studies on Striped Bass Gonadotropins

Introduction

Recognition of the importance of the recently established GtH-I in the process of puberty in salmonids led to intensive efforts to purify this hormone from fish pituitaries. However, in most cases, including that of the striped bass (present project) these efforts did not yield large enough quantities of hormone to enable the development of immunoassays. Therefore, it became clear that the molecular approach is the one to be adopted for producing recombinant GtH I. Our studies thus focused on cloning the cDNAs of striped bass GtHs, and on producing the recombinant hormones in an expression system that is able to faithfully produce glycoproteins and thus similar to the native GtHs, namely the baculovirus expression system. The recombinant hormone will be used to develop immunoassays to measure GtH levels. If successful, this novel approach would represent a major breakthrough in the field of fish reproductive endocrinology, since it would alleviate the need to harvest hundreds or thousands of pituitaries for hormonal purifications. The availability of the GtH cDNAs would also allow studies on the ontogeny of GtH I & GtH II gene expression and its regulation. We report here the results of our research on the striped bass GtHs, using the molecular approach.

Isolation and cloning of cDNA sequences coding for striped bass GtH-I and GtH-II subunits

Two striped bass pituitary glands were pooled prior to the RNA extraction. One gland was obtained from a female fish during early stages of vitellogenesis, whereas the other was taken from a female during the final stages of vitellogenesis. We presumed that this would increase our chances of obtaining both GtH I and GtH II mRNAs. Total pituitary RNA was extracted using a scale-down of the one-step guanidinium /phenol/chloroform method. First strand cDNA was synthesized by AMV

reverse transcriptase (Promega, U.S.A) primed with a poly(t) adaptor (gactcgagtcgacatcgat17), according to the manufacturer's protocol.

To design synthetic oligonucleotides for 3' RACE PCR amplification, all the known sequences of teleost GtH subunits were aligned. Highly conserved regions were identified and used as templates for degenerate primers. Some of the oligos contained a restriction site at their 5' ends for directional cloning. For each subunit, a set of nested forward and reverse primers were designed. The primers were synthesized at the UMAB Biopolymer Laboratory on an Oligo 1000 Synthesizer (Beckman, U.S.A). For each gonadotropin subunit, the first PCR amplification was carried out with the 5' primer and the reverse adaptor primer (gactcgagtcgacatcga).

For GtH-I β , the first round of PCR was carried out with the 'GtH I' and 'adaptor' primers. The second amplification with 'GtH I' and nested '54-51' resulted in a 189 bp fragment. This fragment was ligated to a pBlueScript SK. The identity of the fragment was confirmed by nucleic acid sequencing. This fragment was used to pull out a 539 bp sequence from a striped bass pituitary cDNA library (kindly donated by Dr. T.T. Chen). The cDNA consists of 96 bp of 5' UTR, 360 bp of coding region and 68 bp of 3' UTR, followed by a poly(A) sequence. The coding region encodes a 15 amino acid (a.a) signal peptide and a 105 a.a mature GtH I β . A putative N-linked glycosylation site is found at position 12. The deduced amino acid sequence for the mature GtH I β cDNA is shown in Fig. 3.1.

For GtH-II β , The first round of PCR was carried out with the '11-15' and 'adaptor' primers. The second amplification with nested '18-22' and the 'adaptor' primers resulted in a 422 bp fragment. The identity of this fragment was also confirmed by nucleic acid sequencing. This fragment was used to pull out a 607 bp sequence from the cDNA library. The cDNA consists of 9 bp of 5' UTR, 444 bp of coding region and 137 bp of 3' UTR, followed by a poly(A) sequence. The coding region encodes a 33 a.a signal peptide and a 115 a.a mature GtH II β . A putative N-linked glycosylation site is found at position 10. The deduced amino acid sequence of the mature GtH II β cDNA is shown in Fig. 3.1.

```

GtH-I   1  GQGC SFGCHPTNISIQVESCGLTEVIYTTICEGQCYHEDLVYISHYERPE 50
          |           |   |   |           |||| |   |   |
GtH-II  1  FQLPPCQLINQTVSLEKEGCPKCHPVETTICSGHCITKDPVIKIPFSNVY 50

GtH-I   51  QRICT.GDWSYEVKHIKGCPVG....VTYPVARNCECTTCNTENTGCGRF 95
          |  ||  |  |           || |   |||||  |  |  |
GtH-II  51  QHVCTYRDLHYKTFELPDCPPGVDPTVTYPVAQSCHCGRCAMDTSDCTF. 99

GtH-I   96  PEDIP SCLSF*           105
          |
GtH-II 100  .ESLQPNFCMNDIPFYY* 115

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Figure 3.1: Comparison of the deduced amino acid sequences of striped bass mature GtH-I β and GtH-II β . Asterisks indicate a stop codon, dots represent gaps introduced to maximize homology. The vertical lines indicate identical residues.

There is only 30% homology between the cloned cDNAs. Striped bass GtH I β shows high homology with those of other teleost species, and a phylogenetic analysis clusters striped bass GtH I β with all other known teleost GtH I β . The same applies for GtH II β . These tests confirm that we have cloned the two striped bass gonadotropins β subunits.

Quantification of GtH-I and GtH-II mRNA

In order to measure both GtH protein and mRNA steady-state levels, we had to develop a sensitive, quantitative and reproducible methods. For the mRNA levels, we are presently developing an RNAase protection assay for the α GtH and for the two β subunits. In the meantime, we used Northern hybridization to start measuring subunit mRNA levels in striped bass pituitaries. We first studied the effect of GnRH α on GtH gene expression in juvenile striped bass.

Methods: To study the effect of GnRH on GtH subunit gene expression in immature fish we injected 5 juvenile striped bass with a GnRH analog ([D-Ala⁶,Pro⁹Net]-LHRH = GnRH α). This GnRH α was shown to be highly potent in final oocyte maturation and spermiation stimulating sexual maturation in the striped bass. The fish

were 1 year old and their average bw was 59 ± 5 g. They were injected with a dose of 20 ng GnRHa/g bw, dissolved in 0.5 μ l of 0.9% NaCl /g bw. The control group was injected with saline. The fish were sacrificed 9 h post-treatment and their pituitaries were immediately excised and frozen in liquid nitrogen until storage at -70°C . For the extraction of total RNA, all 5 pituitaries from each group were pooled.

Analysis by Northern hybridization: Total pituitary RNA was extracted using a scaled-down version of the one-step guanidinium/phenol/chloroform method, resulting in about 4 μ g total RNA. Total RNA was then electrophoresed on a 1% agarose/2.2 M formaldehyde gel and transferred to a MagnaGraph nylon membrane by upward capillary transfer. The probe which was the full length GtH cDNA for each subunit, was labeled with ^{32}P by a Prime-it II random priming kit to a specific activity greater than 10^9 cpm/ μ g. For standardization of the signals, the membranes were stripped and hybridized to a β -actin probe. Hybridizations were carried out with radiolabeled probes (10 ng/ml hybridization solution) in a formamide solution (50% formamide, 5xSSPE, 0.1% SDS, 5xDenhardt's and 100 μ g/mL denatured and sonicated calf thymus DNA) for 16 hours at 42°C . At the end of the incubation the filters were washed with 2xSSC/0.5% SDS for 30 minutes at 65°C . The filters were then autoradiographed on X-ray film for 48 hours, and the signals were quantified using a Model 620 video densitometer. The results for each subunit mRNA levels were corrected for recovery by dividing by the β -actin mRNA level in the same sample.

Results and discussion: The α -subunit, GtH-I β and -II β mRNAs exhibited a specific single high mobility band at the bottom of the gel (Fig. 3.2). In the control group, expression of the α subunit and GtH-II β could be detected, while the expression of GtH-I β was just above the detection limit. GnRHa administration stimulated a 10 and 5-fold increase in the expression levels of the α subunit and GtH-II β , respectively, but only a 2-fold increase in the level of GtH-I β mRNA.

Fig. 3.2. GnRHa effect on the expression of the glycoprotein α -subunit, BGtH-I and BGtH-II in striped bass pituitaries analyzed by Northern hybridization. Five juvenile striped bass were injected with 20 ng/g bw GnRHa and sacrificed 9 h later. The control group was injected with saline only. Total pituitary RNA was separated by electrophoresis on a 1% agarose/2.2 M formaldehyde gel. The RNA fragments were transferred to a nylon membrane for hybridization with ^{32} P-labeled striped bass glycoprotein α -subunit (lanes 1 and 2), GtH-I β (lanes 3 and 4), GtH-II β (lanes 5 and 6) and rainbow trout β -actin (lanes 7 and 8) cDNAs. The bands were detected by autoradiography for 48 h. (+)-GnRHa treated, (-)- controls.

Our finding that GtH-II β gene expression in juvenile striped bass is more sensitive to GnRHa stimulation than GtH-I β (5 vs. 2-fold increase) indicates that the genes for GtH-I β and -II β are regulated differentially, further emphasizing the functional duality of fish gonadotropins suggested by other studies. Furthermore, it demonstrates that the pituitary of the immature striped bass is responsive to GnRHa stimulation.

Development of ribonuclease protection assays for striped bass GtH I β and GtH II β mRNAs.

The original proposal suggested that the levels of the GtH subunit gene expression will be measured by dot blot hybridization. The recent advances in the development of a ribonuclease protection assays (RPA) has made this technique much more robust and we have decided to adopt this method for the analysis of the GtH mRNA levels in striped bass pituitaries. RPA has several advantages over solid support hybridization, including the ability to measure simultaneously and with high sensitivity the levels of multiple mRNAs (GtH-I, GtH-II and β -actin).

To compensate for RNA extraction efficiencies and pituitary size we used the mRNA levels of β -actin as a reference. To this end we have cloned the striped bass β -actin cDNA and verified its identity by sequencing. We have used β -actin universal PCR primers to amplify and clone a fragment of striped bass β -actin. These primers are directed towards highly conserved regions in the β -actin sequence and can be used to amplify the β -actin cDNA from any species. The PCR reaction yielded a 843 bp fragment, which is of the expected size. The nucleotide sequence and the deduced amino acid sequence are given in Fig. 3.3.. The identity of the sequence was confirmed by comparing our sequence data to the GenEMBL database: the closest sequence was the common carp β -actin (98% identity).

```
1  TNWDDMEKIW HHTFYNELRV APEEHPVLLT EAPLNPKANR EKMTQIMFET
51  FNTFAMYVAI QAALSLYASG RTTGIVMDSG DGVTHTVPIY EGYALPHAIL
101 RLDLAGRDLT DYLMKILTER GYSFTTTAER EIVRDIKEKL CYVALDFEQE
151 MGTAASSSSL EKSYPEPDGQ VITIGNERFR CPEALFQPSF LGMESCGIHE
201 TTYNSIMKCD VDIRKDLYAN TVLSGGTTMY PGIADGMQKE ITALAPSTMK
251 IKIIPPERK YSVWIGGSIL ASLSTFQQMW I
```

Fig. 3.3: The deduced amino acid sequence of striped bass β -actin (fragment).

At present we are establishing protocols for the ribonuclease protection assay to be used in the simultaneous quantification of GtH subunits in single striped bass pituitaries. When established, we will quantify the mRNAs of GtH I β and II β in all pituitary samples we collected during the studies on the ontogeny of striped bass brain-pituitary-gonadal axis and its hormonal regulation.

Production of recombinant striped bass gonadotropin using a baculovirus expression system.

In fish, as in other vertebrates, gonadotropins (GtH) are key hormones in the initiation and control of gonadal development, and their study is essential for the understanding of reproduction. The presence of two gonadotropins (GtH-I and GtH-II) is now well established in fish. These hormones are glycoproteins composed of two subunits: a common α subunit and a unique β subunit which confers biological and immunological specificity to the hormone. Although the presence of GtH-I and GtH-II has been established in the striped bass (Hassin *et al.*, 1995), little is known about their role in the reproduction of the fish. This is due mainly to the lack of specific assays for these two hormones. For this reason, our laboratory has focused on the development of specific assays to measure striped bass (sb) GtH-I and GtH-II. sbGtH-II has been purified and an ELISA (Mañanós *et al.*, 1996) and a RIA (Blaise *et al.*, 1996) were validated for its measurement. To date, all attempts to purify the native sbGtH-I have been unsuccessful. This has led us to try an alternative way for the production of GtH-I by using a molecular approach. The baculovirus expression system, an eukaryotic system, was used to produce GtH-I β , -II β and - α subunits. This system enables post-transcriptional modifications (O'Reilly *et al.*, 1992), which are essential for the immunogenicity and biological activity of these glycoproteins. This system was already successfully used for the production of human gonadotropins and carp GtH α subunit.

The system chosen was the BAC-TO-BAC baculovirus expression system (GibcoBRL). This system is a rapid and efficient system for generating recombinant baculovirus. It uses a site-specific transposition to insert the clone into a bacmid propagated in *E. coli*. The recombinant virus is easily isolated avoiding multiple rounds of plaque purification and thereby greatly accelerating the process.

The cDNA coding for the sbGtH -I β , -II β and - α have been cloned and inserted in pGEM in our laboratory (Hassin *et al.*, 1995). The clones were inserted in a pFastbac

vector, transposed in a baculovirus shuttle vector (bacmid) and propagated in *E. coli*. The recombinant bacmids were used for transfection in insect cells, *Spodoptera frugiperda* (Sf9) cells, with Cellfectin reagent. A plaque assay was used to select the virus. Several passages with the infected virus cells were conducted to increase the titer of the virus. The expression of the recombinant sbGtH-II β in the cells was verified by Western blot, using a specific antibody against the native sbGtH-II β (Fig. 3.4). This demonstrated that the size of the recombinant hormone (estimated at 20 Kd) is slightly smaller than that of the native hormone (estimated at 22 Kd), probably because of differences in the carbohydrate composition of the product. A kinetic assay was performed to check the variance in the expression of the sbGtH-II β (Fig. 3.5).

Fig. 3.4. Western blot of purified native sbGtH-II β (nGtH-II β , lane 1) and of cell extract infected with baculovirus containing sbGtH-II β clone (rGtH-II β , lane 2). The antibody used was specific for the native sbGtH-II β .

Fig. 3.5. Kinetics of GtH-II β release into the culture media of Sf9 cells infected with the baculovirus containing the GtH-II β clone. The quantity of sbGtH-II β present in the cell media was measured by ELISA, using the antibody produced against the native sbGtH-II β .

The quantity of hormone in the cell media, measured by ELISA, increased as early as 12 hours post-infection (0.3 μ g/ml) and was maximal around 3 days post-infection (3.5 μ g/ml). Serial dilution of the media containing the recombinant sbGtH-II β and of the native sbGtH-II β resulted in the same displacement curve in the ELISA indicating immunological similarities between the two hormones. This work with the GtH-II β has allowed us to determine the best conditions for expression, which was used for the expression of the GtH α and GtH I β subunits.

After the above-mentioned characterization, large scale production of the three hormone subunits ensued. The subunits were purified from the cell media, using the same protocol used for the purification of the native sbGtH-II β . After an ethanol extraction, the glycoproteins in the media were separated by size using low pressure gel chromatography. The size range corresponding to the sbGtH-II β (as indicated by Western blot) and the range estimated for sbGtH-I β from the deduced amino acid

sequence of the cDNA, was further separated by reverse phase HPLC. A single peak was observed for the two subunits, at a retention time of 41 minutes for the GtH-II β and 45.4 minutes for the GtH-I β . Identification of the recombinant sbGtH-II β and GtH-I β will be confirmed by SDS-PAGE and N-terminal sequencing.

These hormones will be used to generate antibodies and specific assays will be developed for the measurement of striped bass GtH-I and GtH-II. These assays, and principally the GtH-I assay will provide a better understanding of the importance and the specific role of these hormones during the reproductive cycle and principally at the onset of puberty.

It is our belief that we have made a significant progress in the area of producing recombinant faithfully expressed striped bass GtH I and II. This is the first time these fish hormones are faithfully produced using the molecular approach. This approach will alleviate the need to collect thousands of pituitaries to purify fish gonadotropins, and will enable a simple production of the problematic GtH I for the studies of its endocrine functions.

Chapter 4:

Hormonal Regulation of the Onset of Puberty in Striped Bass, *Morone saxatilis*

Introduction

The striped bass, *Morone saxatilis* is an anadromous teleost indigenous to the Atlantic coast of North America. Like many teleosts from temperate waters, the striped bass has an annual reproductive cycle with group-synchronous ovarian development (Specker et al., 1987). Although most male striped bass reach maturity in their third year, females can mature anywhere from 3- to 8 y of age. The major part of oocyte growth (vitellogenesis) in the mid-Atlantic region occurs between November and May and, depending on temperature, spawning can take place anytime from March until June. In wild female striped bass, the plasma levels of the two major female reproductive steroids, 17 β -estradiol (E₂) and testosterone (T) are low in previtellogenic fish with oocytes in either primary growth or early secondary growth stages (lipid droplet accumulation; Berlinsky and Specker, 1991). The levels of both steroids increase during vitellogenesis until the final oocyte maturation (FOM). After ovulation, the levels decrease and return gradually to the levels in previtellogenic fish (Berlinsky and Specker, 1991). In captive striped bass, steroid hormone levels follow a pattern similar to that reported in the wild. E₂ levels in captive fish increase gradually during vitellogenesis, but peak levels are not reached until just prior to FOM (Woods and Sullivan, 1993).

In captive male striped bass, the levels of the two major androgens, T and 11-ketotestosterone (11-KT) increase upon the onset of spermatogenesis in late September and a further increase occurs at the initiation of spermiation in late February or early March (Woods and Sullivan, 1993).

Our major objective was to study the ontogeny of gonadal development and reproductive hormones in the striped bass *Morone saxatilis* during the first four years of life, before and throughout the onset of puberty. Also, to study interactions along the hypothalamus-pituitary-gonadal axis leading to the initiation of sexual maturity.

Ontogeny of the brain-pituitary axis

Twenty striped bass from two age groups were sampled routinely for 2 years. Fish from spring 1991 and 1993 spawnings were sampled monthly starting in December 1993. Fork length and body weight were recorded and blood was collected from each individual for the determination of GtH I, GtH II, and the gonadal steroids testosterone (T), 11-ketotestosterone (11-KT, in males only) and 17 β -estradiol (E₂, in females only). Gonads were removed, weighed for the calculation of the gonadosomatic index (GSI), and tissue samples were fixed in 4% formaldehyde: 1% glutaraldehyde for histology. Pituitaries and brains were collected from each sacrificed individual, immediately frozen in liquid nitrogen (pituitaries) or on dry ice (brains) for the determination of GnRH gene expression (brains), GtH gene expression (pituitaries) and GnRH and GtH contents (pituitaries). The samples were stored at -80°C until further analysis.

Males

In January 1996, the last sampling was completed. GSI of immature and mature male striped bass are presented in Figs. 4.1 and 4.2, respectively. In December 1993, all 6-month-old fish had immature gonads, however, in February 1994, when body weight was only 51 $\pm$ 8 g (mean $\pm$ s.e.m.), a single individual was found with distinct developing testes. In April, 5 males were found, two of which were spermiating (GSI= 4.8 $\pm$ 0.5 %). One and two-year-old fish with developing testis, or having extractable milt, are referred to as precocious or mature males. The distinction between precocious and immature individuals can be made during the natural reproductive season for striped bass (December to June). All males reached maturity in their 3rd year and were not sampled thereafter.

In May 1994, enough plasma could be collected from individual fish to determine individual T levels and from September 1994 onwards, also 11-KT levels could be determined. Monthly profiles of T and 11-KT are shown in Figs. 4.1 and 4.2 for immature and mature males, respectively. In immature males, T and 11-KT levels remain relatively low (below 1 ng/ml) throughout the first 2 years. In mature males,

however, both T and 11-KT levels show seasonal changes which coincide with increases and decreases in GSI. During the 2nd and 3rd years, T and 11-KT reach peak values of 1.1 ± 0.2 and 2.7 ± 0.6 ng T/ml, and 2.2 ± 0.5 and 3.5 ± 0.8 ng 11-KT/ml, respectively.

In the first two years, approximately 45% of all males were precocious. Mean ($\pm$ s.e.m.) spermatozoid count of 1-year-old precocious males was $92.3 \pm 1.8 \times 10^9$ spermatozoa/ml, which is slightly higher than that of mature, 3 year old males ($77.0 \pm 5.6 \times 10^9$ spermatozoa/ml). This would indicate that precocious striped bass males may be functionally reproducing individuals although it is yet unknown whether these males, as the precocious parr of Atlantic salmon, are able to participate in actual spawning with the much larger females, and whether they are really able to fertilize the spawned eggs. The existence of precocious males is well documented in other teleost species, but has not been described yet in the striped bass.

Fig. 4.1 Gonadosomatic index (%) (GSI) and plasma T and 11-KT levels (pg/ml) of immature, 1- and 2-year-old, male striped bass. nd: non-detectable.

Fig. 4. 2. (GSI- %) and plasma T and 11-KT levels of mature, 1- to 3-year-old, male striped bass.

Females

During the first 2 years, all females were previtellogenic, some showing cortical alveoli in the oocytes, and although there was a slight increase in gonad weight, mean GSI remained low (Fig. 4.3). From February to June 1994 (3rd year), the percentage of females reaching the vitellogenic stage increased from 0 to 69%. As a result of the onset of vitellogenesis, oocyte diameter increased from 200 μm (November 1993) to 400-600 μm (April 1994) and GSI increased concurrently. Although GSI increased during this time, it remained relatively low compared to 4-year-old females. From April to July, a high degree of atresia was noted in ovaries from vitellogenic, 3-year-old fish. These females never reached sufficiently advanced stages to be suitable for spawning induction. During the 4th year, GSI increased further ($\text{GSI} = 4.0 \pm 0.3\%$) and all females reached advanced stages of vitellogenesis in April 1995. At this time, oocyte diameters ranged from 600 to 700 μm .

Fig. 4. 3. GSI (%) and plasma T and E₂ levels in 1- to 4 y old female striped bass. During the 3rd year, some females remained in previtellogenic stage (pre.vtg) but plasma steroid levels were not significantly different between the two groups ($P>0.05$).

During the first 2 years, plasma T and E₂ levels were below the minimal detection levels of the radioimmunoassays used (50 pg/ml for T-assay and 10 pg/ml for the E₂-assay), and are therefore not shown in Figure 3. During the 3rd year, plasma T and E₂ levels were not significantly different ($P>0.05$) between previtellogenic and vitellogenic females, and therefore no further separation was made in the steroid levels between these groups. The T levels in females were about 0.2 ng/ml and did not show any seasonal fluctuations. Also E₂ did not show strong seasonal fluctuations but the levels increased slightly to 0.14 ± 0.06 ng/ml during the 4th year. It is still unclear why E₂ levels were low during the 4th year while GSI increases considerably and all females reached advanced stages of vitellogenesis during that year.

Ontogeny of the responsiveness of the brain-pituitary-gonadal axis to hormonal regulation

Effect of GnRH and testosterone on the brain-pituitary-gonadal axis

Two-year-old striped bass: In December 1994, 280 striped bass of the 1993 spring spawning, about 150 g bw were selected. The sex of these immature fish could not be determined by biopsy, thus the sex-ratio of the selected group is unknown, however, spermiating precocious males were excluded. The fish were divided into 4 groups of 70 fish each and kept in 90-gallon tanks. Fish from each group were divided over 2 tanks. Group 1 was injected microspheres containing [D-Ala⁶,Pro⁹]-LHRH (GnRHa). These microspheres were shown to release GnRHa for at least 8 weeks. Group 2 was injected a T-microsphere-preparation which would cause elevated plasma T levels for at least 50 days. Group 3 was treated with both the GnRHa and T preparations, and group 4 received blank microspheres and served as a control. The GnRHa and T preparations were administered intra-muscularly at a dose of 300 µg GnRHa and 4 mg T per kg bw.

In February 1995, 50 days after the initiation of the experiment, 30 fish from each group were bled and sacrificed. Brains, pituitaries and gonads were collected as described above. The remaining fish were reinjected with the microspheres every 50 days until May 9, 1995 (150 days of treatment), when all fish were sacrificed. GSI and pituitary GtH-II content are shown in Fig. 4.4 for the 50 day sampling, and in Fig. 4.5 for the 150 day sampling.

No differences in gonadal morphology could be observed in the females among treatments throughout the study: T (in the non-T-treated groups) and E₂ levels were below the detection levels of the respective RIAs. Pituitary GtH-II levels in all treatment groups were higher after 150 days of treatment than after 50 days. At both sampling times, T treatment and T+GnRHa treatment resulted in a significant increase in pituitary GtH-II content ($p < 0.05$; Figs. 4.4 and 4.5). This confirms the finding that T stimulates GtH-II accumulation in the pituitary of immature fish, while GnRHa having a possible potentiating effect.

Fig. 4.4. GSI and pituitary GtH-II content of two-year-old striped bass after 50 days of treatment with either GnRH α , testosterone or a combination of both. Mean $\pm$ s.e.m. Means with different letters are significantly different ($P < 0.05$).

Fig. 4.5. GSI and pituitary GtH-II content of two-year-old striped bass after 150 days of treatment with either GnRH α , testosterone or a combination of both. Mean $\pm$ s.e.m. Means with different letters are significantly different ($P < 0.05$).

Precocious males were present in all groups. In immature males, GnRHa treatment resulted in an increase in GSI as compared with the control after 150 days. However, testicular morphology did not seem to be affected. On May 9th, GSI was reduced significantly in all precocious males ($p < 0.05$), indicating that at this time the gonads of these males were already regressing. GnRHa and the combined treatment had an inhibitory effect on GSI at that time. None of the treatments seem to affect the levels of plasma 11-KT. However, spermiating males had a significantly higher level of 11-KT than immature males (1.68 ± 0.18 ng/ml vs. 0.41 ± 0.07 ng/ml; $P < 0.01$). Also plasma T levels were significantly elevated in spermiating males (0.8 ± 0.1 ng/ml) vs. immature males (0.1 ± 0.1 ng/ml; $P < 0.01$) either in the control or in GnRH treated groups (non-T-treated groups). Similar to the situation in females, pituitary GtH-II levels were higher after 150 days of treatment than after 50 days with the exception of immature males in the control group. Both GnRHa treatment and the combined T+GnRHa treatment resulted in significant elevation in pituitary GtH-II content ($P < 0.05$) after 150 days. (Fig. 4.5). These results indicate that in males, GnRHa and not T has a stimulatory effect on pituitary GtH-II levels.

3-year-old female striped bass: Starting in November 1995, a similar experiment was performed using 3-year-old female striped bass of 620 ± 9 g bw. At this age, sex could be determined by gonadal biopsy, and since all males would have reached natural maturity during their 3rd year, only females were selected. Each group consisted of 26 fish, 10 of which were to be sacrificed after 50 days (December 18, 1995). The remaining fish were reinjected twice every 50 days, and were sacrificed after 150 days. (March 28, 1996). Blood, brains, pituitaries and gonads were collected as previously described, and oocyte diameters of the 10 largest oocytes were measured and averaged for each fish.

Fig. 4. 6. GSI of three-year-old female striped bass after 50 days (A) or 150 days (B) of treatment with either GnRHa, testosterone or a combination of both. Mean $\pm$ s.e.m. Means with different letters are significantly different ($P < 0.05$).

Fig. 4.7. Diameter of the 10 largest oocytes in three-year-old female striped bass after 50 days (A) or 150 days (B) of treatment with either GnRHa, testosterone or a combination of both. Mean $\pm$ s.e.m. Means with different letters are significantly different ($P < 0.05$).

After 50 days of treatment, GSI was significantly reduced in fish treated with T alone or in combination with GnRHa (Fig. 4.6A). Also oocyte diameter in these two treatments was significantly lower than in the control or GnRHa-treated groups ($P < 0.05$; Fig. 4.7). After 150 days of treatment, GnRHa had a stimulatory effect on GSI, and although T alone, or in combination with GnRHa, did not affect the GSI (Fig. 4.6B), it did result in a significantly lower oocyte diameter ($p < 0.05$; Fig. 4.7B). GnRHa treatment seems to partially compensate for the inhibitory effect of T on

oocyte diameter, so that mean oocyte diameter in fish treated with the combination of GnRHa+T was not significantly different from that of the control group ($P<0.05$).

Discussion

Ontogeny of the brain-pituitary-gonad axis

Our studies show that male striped bass can reach sexual maturity during their first year. This is one year earlier than previously reported for striped bass in the mid-Atlantic regions (Jones et al., 1977). In the first two years, approximately 45% of all males were precocious, while the remaining males reached maturity during their third year. Plasma levels of both T and 11-KT closely followed the annual changes in GSI indicating that both steroids are involved in the regulation of testicular maturation. This is in agreement with a previous study which shows a simultaneous increase of both T and 11-KT during spermatogenesis with peak levels being attained upon the onset of spermiation (Woods and Sullivan, 1993). Maximal GSI increased yearly during the first 3 years and maximum T and 11-KT levels increased concomitantly. This would suggest a positive correlation between testicular size and steroid production due to an increase in steroid producing cells and/or an increased steroid producing capacity of the cells. Immature males had low levels of both T and 11-KT, which did not seem to correlate with low GSI values ($<0.5\%$) during the first two years.

At our facility, 69% of the female striped bass entered vitellogenesis in their third year. These females reached maximal oocyte diameters of 400-600 μm and mean GSI of approximately 2% in April, while immature females had small oocytes (150-200 μm) and a low GSI (approximately 0.5%) at this time. Although high oocyte diameters were reached, none of the females reached sufficient advanced stages suitable for spawning induction, instead they underwent massive atresia. Several teleost species, including the striped bass, have been shown to initiate secondary growth (incorporation of lipid droplets, cortical alveoli and yolk proteins derived from vitellogenin) at least one year before the expected time of spawning (Berlinsky et al.,

1995; Chapman et al., 1987). Although reabsorption of the secondary growth oocytes has been suggested, this phenomenon of preovulatory atresia in young fish remains unclear and requires further research.

During the fourth year, all females became vitellogenic and oocyte diameters of 600-700 μm were reached in April. Subsequently, mean GSI increased to approximately 4% and E_2 and T levels increased slightly compared to 3-year-old vitellogenic females. Although fairly advanced stages of vitellogenesis were reached, circulating levels of these steroids remained low throughout the 3rd and 4th year. It is possible that the reproductive axis of these young fish was not "mature" enough yet, resulting in inadequate levels of some reproduction-related hormones, and therefore, preventing full oocyte maturation. Inadequate levels of reproductive hormones, including sex steroids, is thought to underlie the process of ovarian atresia (reviewed by Guraya, 1986). Nevertheless, it should be emphasized that also in 5-year old mature fish, circulating levels of E_2 and T remained low during the process of vitellogenesis (below 1.0 ng/ml and 0.4 ng/ml, respectively) and peak values are reached only prior to final oocyte maturation (Woods and Sullivan, 1993). Also Mylonas (1996) reported low E_2 and T levels (0.2 and 0.1 ng/ml, respectively) in post-vitellogenic striped bass with mean oocyte diameter of 760 μm . Obviously, low circulating levels of these steroids during vitellogenesis are not uncommon in female striped bass, however E_2 levels probably do need to reach a critical level to ensure proper growth of the oocytes and completion of vitellogenesis.

Ontogeny of the responsiveness of the brain-pituitary-gonadal axis to hormonal regulation

In two y old females, no differences in gonadal morphology could be observed among treatments throughout the study, and T (in the non-T-treated groups) and E_2 levels were below the detection levels. This suggests that none of the hormone therapies resulted in an advancement of sexual maturation at this age. However, in both the 50 day and the 150 day samplings, T and the combined treatment significantly increased pituitary GtH-II content. This confirms the finding that T

stimulates GtH-II accumulation in the pituitary of immature fish (Swanson and Dickhoff, 1988), with GnRHa having a possible potentiating effect. Pituitary GtH-II levels in all treatments were higher after 150 days of treatment than after 50 days. As the levels were also higher in the control group, this increase could have been due to seasonal factors, either external or internal, since the 150 day sampling took place towards the end of the reproductive season, and changes in pituitary responsiveness could have been occurred.

Also in males, testicular morphology did not seem to be affected by the treatments, but a small effect was seen on GSI. After 150 days of treatment, GSI was reduced significantly in all precocious males indicating that at this time, the testes were already regressing. GnRHa and the combined treatment had an inhibitory effect on GSI at that time. In immature males, GnRHa treatment resulted in an increased GSI after 150 days as compared to the controls. Regardless of treatment, spermiating males had a significant higher level of 11-KT and T than immature males. This supports the previous observation that both androgens are involved in testicular maturation. Similar to the situation in females, pituitary GtH-II levels were higher after 150 days than after 50 days, with the exception of immature males in the control group. Both GnRHa and the combined treatment elevated pituitary GtH-II levels significantly after 150 days. This observation indicates that in males, GnRHa and not T has a stimulatory effect on pituitary GtH-II levels. However, it is not clear yet whether this is a true sex-specific response or whether it is related to stage of maturity of the fish, since the reproductive axis in males could have been more mature than in females at this age.

Although pituitary GtH-II content could be elevated in both sexes by hormonal manipulation, no gonadal maturity could be induced. This could mean that either the accumulated pituitary GtH II is not released into the circulation or even when released, the immature gonad of the 2 y old fish is not responsive to the GtH stimulation. Future studies on both GtH release and gonadal responsiveness will have to clarify this point.

After 50 day treatment, both GSI and oocyte diameter were significantly reduced in fish treated with T alone or in combination with GnRHa. After 150 days treatment, GnRHa had a stimulatory effect on GSI, and although T alone or in combination with GnRHa did not effect GSI, it did result in a significantly lower oocyte diameter. Since pituitary and plasma hormone levels of these fish have not been measured yet, it is still unknown whether this inhibitory effect of T is a direct effect on the ovary and/or an indirect effect mediated indirectly by GtH.

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Title: Advancing puberty in the black carp (*Mylopharyngodon piceus*) and the striped bass (*Morone saxatilis*)

Description of The Cooperation

The Brooklyn team carried out the histological and immunocytochemical studies on black carp material sent from Tel-Aviv. Based on the immunocytochemical findings that cGnRH II is prevalent in the immature black carp pituitary, the Tel Aviv team modified the original *in vitro* challenge experiment to examine the effect of this peptide on GtH release in cultured pituitary cells.

The delivery systems for the sustained administration of GnRHa and testosterone was developed and tested by the Baltimore team. These systems were used for manipulating the onset of puberty in black carp by the Tel-Aviv and in the striped bass by the Baltimore team.

cDNA probes prepared by the Eilat team were used for quantification of the black carp GtH II β mRNA.

The production of the recombinant GtH II β peptide, its refolding and binding studies to cGTH antiserum, were done jointly by the Eilat and Tel-Aviv teams.

The striped bass GtH II β antiserum developed by the Baltimore team is being used by the Eilat team for the analysis of the expression of the recombinant GtH II β protein.

The determination of the various GnRH forms of the black carp is carried out by the Baltimore team.

In June 1994, M.P.S. visited Israel and took part in the first *in vivo* challenge experiment. In summer 1995, Y.Z. visited Israel to work in Eilat and Tel Aviv and to help in the preparation of the annual report. In July 1995 the Israeli PIs (A.E. and Z.Y.) visited (unpaid by BARD) the laboratories of the US partners to discuss the results achieved and to plan the next phase of the project. In March 1996 Z.Y. visited the two laboratories in the US to examine and discuss the results achieved there and to compare them to the work done by the Israeli teams. Y.Z. is currently (October 1996) in Israel to participate in the preparation of the final report.

All partners are interconnected by Email enabling almost daily contacts.

These are only examples of the close contacts and mutual interdependence among the four teams taking part in the project, and illustrate the necessity of such cooperation for carrying out the work plan of each laboratory.

Title: Advancing puberty in the black carp (*Mylopharyngodon piceus*) and the striped bass (*Morone saxatilis*)

Evaluation of the research achievements with respect to the original research proposal and its objectives

Objective 1 was carried out in the striped bass. The presence of three GnRH forms has been demonstrated in the brain and pituitary. Only the sbGnRH form was identified in the pituitary indicating its relevance to the control of reproduction. All three GnRH genes have been cloned from the striped bass genomic library and characterized. A ribonuclease protection assay is being developed for studying the gene expression of the three GnRH forms. Immunoassays for determination of the three GnRHs are currently optimized. The cloning of the black carp GnRH genes was initiated

Objective 2 was carried out in the black carp and the striped bass. The cDNAs of the three GtH subunits (α , IB and IIB) were isolated and cloned in the striped bass, and the production of the recombinant subunits is underway. In the black carp, a genomic library was constructed to enable future studies on the genes encoding the subunits and their regulation. The gene and the cDNA encoding the GtH IIB subunit were isolated. The GtH IIB cDNA was utilized as a probe to determine the steady-state level of its mRNA under hormonal manipulations in the two species. The isolation and cloning of the subunit IB of the black carp is still in progress.

Objective 3 was performed in the two fish species. The GnRH gene expression and content was done only in the striped bass, but samples of both species were secured for future determination. The circulating levels of GtH II was measured in both fish at various ages. Histology and ICC studies of the brain and pituitary were performed mainly in the black carp. Gonadal histology and steroid determinations at various ages were carried out in both species.

Dynamic challenge experiments *in vivo* (objective 4) were carried out in both species. The *in vitro* challenge experiments carried out in the black carp identified two sites along the hypothalamo-pituitary-gonadal axis which are non-functional in the immature fish. One was defined at the GnRH receptor level in the pituitary and the other at the GtH receptor level in the gonads. Testosterone (T) treatment was found to reduce the block at the pituitary level.

An unexpected source of sex steroids was discovered in the fat-pad attached to the rudimentary gonad of the black carp. The embryonic origin of this tissue is still to be clarified, however, due to its large mass as compared to the gonad, and its response to stimulation, it may play an important role in the process of maturation.

Encouraged by these results, long-term experiments were carried out in both species whereby fish were treated with T alone or in combination with a superactive GnRH analog given in slow release devices. The results achieved indicated again the importance of T in stimulating GtH II formation in the pituitary and its response to GnRH. However, this treatment proved only partly effective in elevating the circulating level of the gonadotropin. In both species T was found to reduce ovarian growth.

An additional experiment, which deviates from the original plans, was performed. In this experiment, 3 y old immature black carp were treated with testosterone and GnRHa as above, and the endogenous dopaminergic inhibition was curtailed by adding the dopamine D₂ type receptor antagonist, domperidone. The results show that reducing the inhibition of DA may be an important factor in advancing puberty, at least in the black carp. A similar treatment protocol combining T+GnRHa+domperidone has proven also effective in advancing and synchronizing ovarian maturation in grey mullet (*Mugil cephalus*) to a stage at which a controllable spawning induction could be achieved successfully.

Definite formulation of hormonal manipulation to advance puberty in the striped bass and the black carp seems to be premature at this stage. However, this phase of the project has yielded already a great number of experimental tools of DNA technology, and endocrine information on the process of puberty in fish. These tools are indispensable for further research on the subject. BARD support of the project for additional three years is greatly appreciated.

Conclusions

A breakthrough in the field of fish endocrinology was achieved in the project by the production of recombinant GtH subunits using DNA technology. This will enable the establishment of immunoassays for the various subunits of fish gonadotropins, hormones which occupy a central position in the control of reproduction. Furthermore, this development will provide the necessary tools to study the differential roles of the two GtHs in the process of fish puberty.

The physiological and histological observations and experiments have been carried out to the present stage using suboptimal tools available at the time, but the samples collected are stored to be analyzed using the new tools when ready. Nevertheless, the *in vivo* and *in vitro* dynamic challenge experiments provided valuable information on the endocrine status of the immature black carp and the striped bass which represent important lineages of teleost fish, the Cypriniformes and the Perciformes.

Devices for slow release of GnRHa and testosterone were developed and characterized for use in the two species under various temperature regimes.

The *in vitro* challenge experiments carried out in the black carp identified two sites along the hypothalamo-pituitary-gonadal axis which are non-functional in the immature fish. One was defined at the GnRH receptor level in the pituitary, and the other at the GtH receptor level in the gonads. Testosterone (T) treatment was found to circumvent the block at the pituitary level in both fish.

An unexpected source of sex steroids was discovered in the fat-pad attached to the rudimentary gonad of the black carp. The embryonic origin of this tissue is still to be clarified, however, due to its large mass as compared to the gonad, and its response to stimulation, it may play an important role in the process of maturation.

Long-term experiments were carried out in both species whereby fish were treated with T alone or in combination with a superactive GnRH analog given in slow release

devices. The results achieved indicated again the importance of T in stimulating GtH II formation in the pituitary and its response to GnRH. However, this treatment proved only partly effective in elevating the circulating level of the gonadotropin. In both species T was found to have an inhibitory effect on oocyte development. This would indicate that future T treatments should be more subtle.

Reducing the inhibition of DA may be an important factor in advancing puberty, at least in the black carp. A similar treatment protocol has proven also effective in advancing and synchronizing of ovarian maturation in the grey mullet (*Mugil cephalus*) to a stage at which a controllable spawning induction could be achieved successfully.

Definite formulation of hormonal manipulation to advance puberty in the striped bass and the black carp seems to be premature at this stage. However, this phase of the project has yielded already a great number of experimental tools of DNA technology and endocrine information on the process of puberty in fish which are indispensable for further research on this subject.

List of Publications Resulting From Research Project IS-2149-92

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