BLUE GOURAMI

(TRICHOGASTER TRICHOPTERUS)

MODEL FOR LABYRINTH FISH

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CHAPTER 1

INTRODUCTION

1.1 LABYRINTH FISH

The suborder Labyrinthici, which belongs to the order Perciformes, is characterized by the presence of a chamber, or labyrinth, above the gills for the retention of air for breathing (Fig. 1.1).

The 16 known genera contain about 80 species, distributed throughout most of southern Asia, India, and central Africa (Forselius, 1957; Hodges et al., 1964; Vierke, 1988). However, the systematic characteristics of Labyrinthici are not in agreement and many synonymies are used. According to Vierke (1988), taxonomists classify the labyrinth fishes into four families: Anabantidae (Genera: *Sandelia, Ctenopoma, Anabans*), Belontiidae (*Trichopsis, Trichogaster, Sphaerichthys, Pseudosphromeus, Parosphromeus, Malpulutta, Hlostoma, Ctenops, Collisa, Betta, Belontia*), Osphromenide (Genus *Osphronemus*), and Helostomatidae (Genus *Helostoma*). Most are tropical fish distributed throughout southern Asia, India, and central Africa.

In Labyrinthici, an air-filled breathing cavity known as the labyrinth is located above the gills under the operculum, on top of the head behind the eyes (Fig. 1.1). The labyrinth is a circular spot of very wrinkled tissue, which offers more surface area for oxygen intake. This cavity supplements the breathing function, since it is well suited to gaseous interchange. Because of these accessory organs, anabantidae

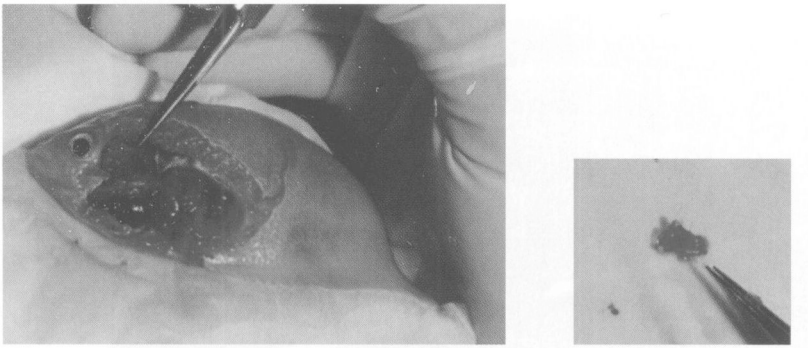


Fig.1.1. The labyrinth organ in the fish (Prof. G. Degani).

fishes can even survive in water with very little oxygen content. One of the very common habitats is rice fields (Fig. 1.2).

Most form "bubble" nests. The males often become territorial and very protective of the nest. As the female prepares to lay her eggs, the male wraps himself around her, catches the eggs in his mouth and spits the eggs onto the nest. The bubbles give the fry the oxygen they need for their first stages of development.

Forselius (1957) reviewed the behavior, systematics, distribution, and the biology of the anabantidae species. The blue gourami in particular shows a very complex social and spawning behavior, and because of this it has become a common subject for ethological studies. Miller (1964) described in detail the social and spawning behavior of the blue gourami, though the first paper containing a description of the reproductive behavior is that of Hodges and Behre. The literature contains other studies on the same subject (Cheal and Davies, 1974; Miller and Robinson, 1974; Tooker and Miller, 1980; McKinnon and Lilley, 1986).

1.2 BLUE GOURAMI

The blue gourami (*Trichogaster trichopterus*) or the three-spot gourami is native to Malaysia and Thailand. The blue gourami gets its name due to its basic blue coloring. The gourami does have a touch of silver on its belly and even a slash of green on the tips of its gill covers and fins. This native blue gourami also has two large black spots on its sides, with the eye making up the third spot (hence the name 3-spot).

The blue gourami can grow to a maximum size of about six inches in a 30–40 gallon tank.

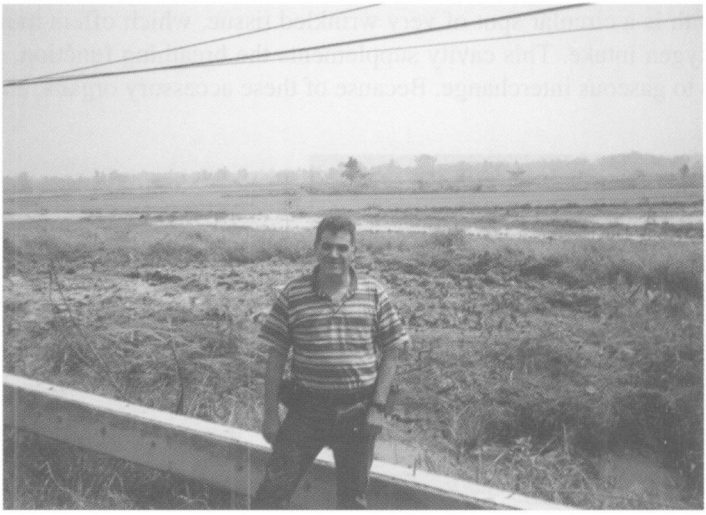


Fig. 1.2. Rice paddies, habitat of many labyrinth fish.

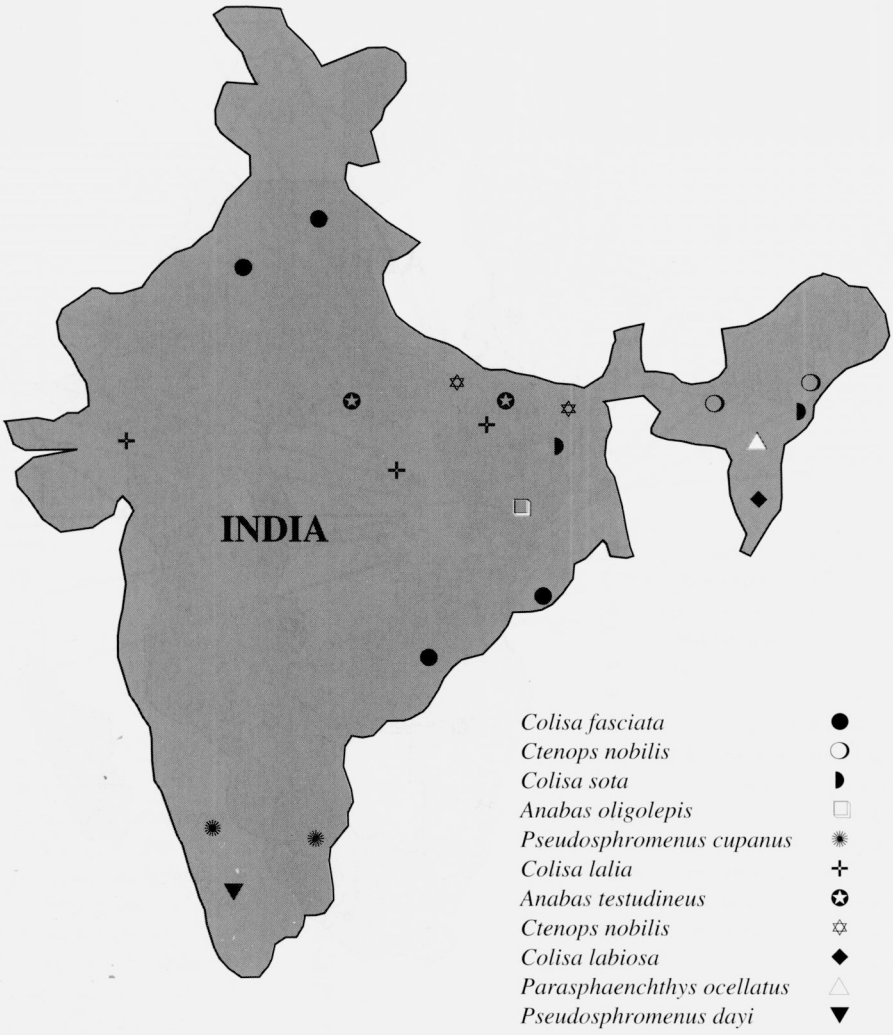


Fig. 1.3. Distribution of labyrinth fish in India (Vierke, 1988).

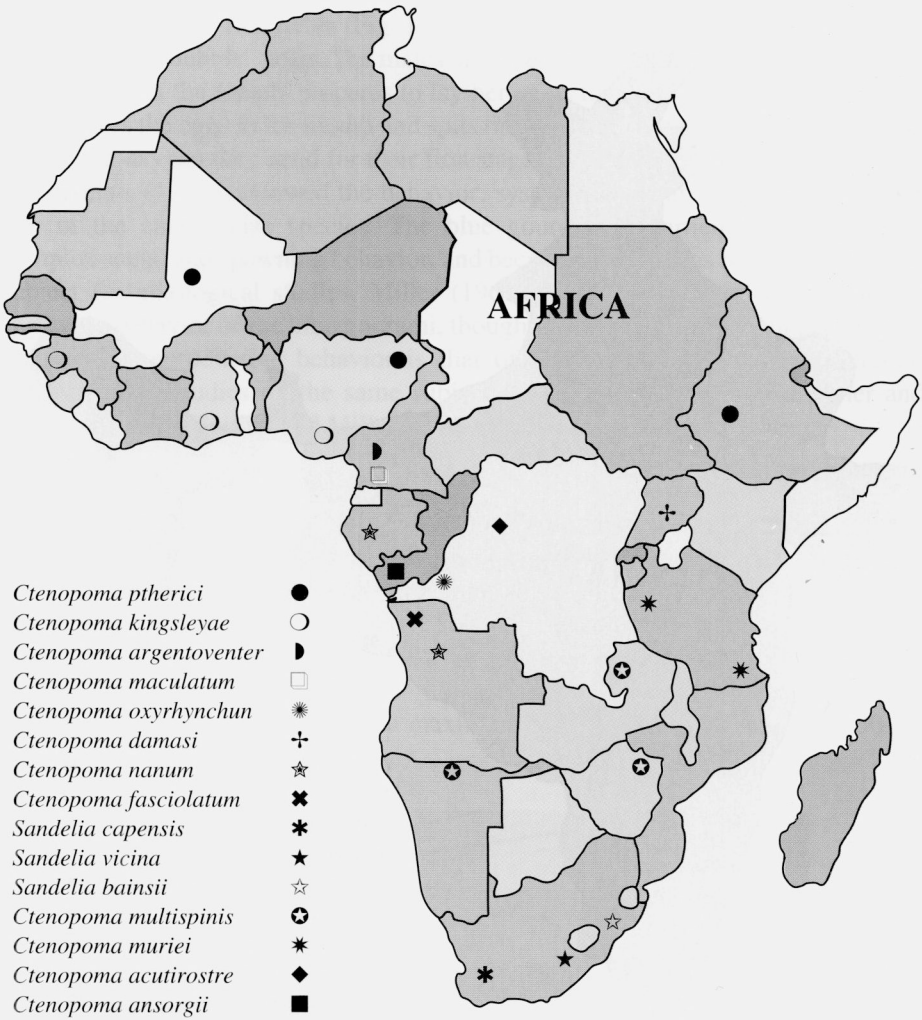
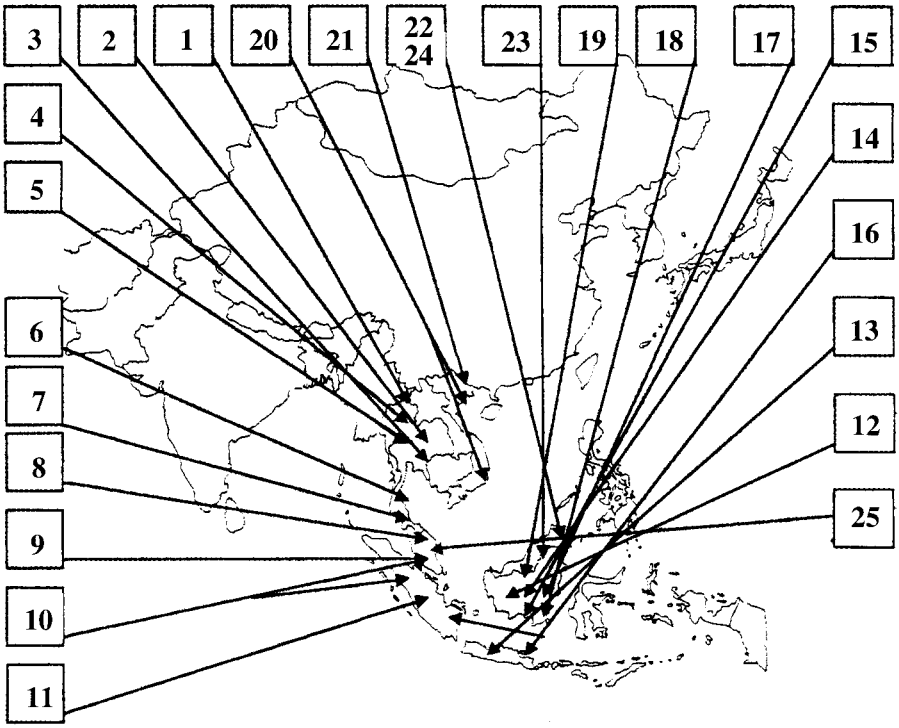


Fig. 1.4. Distribution of labyrinth fish in Africa (Vierke, 1988).



Batta imbellis	6	*Parasphromenus deissneri*	25
Batta splendens	5	*Trichogaster leeri*	9
Trichogaster trichopterus	4	*Trchoosis vittattus*	10
Trichoposis pumilus	3	*Batta coccna*	11
Trichogaster microlepis	2	*Sphaerichthys osphromenoides*	12
Batta smaragdna	1	*Helostoma temminckii*	13
Macropdus chinesis	24	*Parosphromenus parvulus*	14
Macropdus operculars	20	*Batta edithae*	15
Trichogaster pectoralis	21	*Batta picta*	16
Batta unimculata	22	*Batta anabatoides*	17
Batta macrostoma	23	*Parosphromenus filamentosus*	18
Parosphromenus paludicola	7	*Batta taeniata*	19
Batta pugnax	8		

Fig. 1.5. Distribution of labyrinth fish in SE Asia (Vierke. 1988).

1.3 COLORATION OF BLUE GOURAMI

The blue gourami have many color morphs, and although normally only two are seen in stores, it is quite possible to find the others (Fig. 1.7).

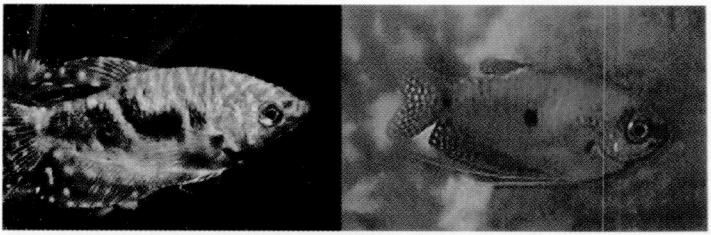
Blue gourami–This is the "normal" color of *T. trichopterus*, closest to the wild form. It is a light powdery blue, normally with an eyespot along the lateral line directly below the dorsal fin, and another on the caudal peduncle.

Gold gourami–This is the other common color morph of *T. trichopterus*. It is orange on top, fading to white below the lateral line, and has more pronounced black stripes. There is usually a less pronounced eyespot on the caudal peduncle and rarely a spot in the middle.

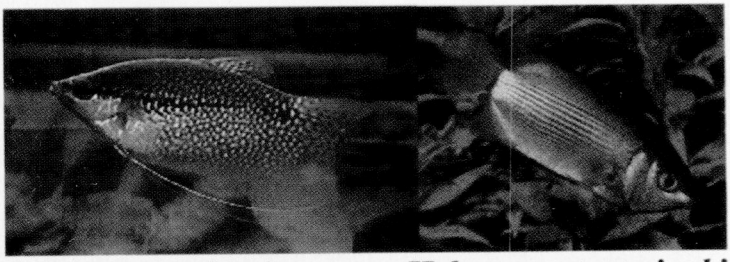
Lavender gourami–This is the result of crossing a blue with a gold. The fish is a brown color, with lavender highlights. It normally has very pronounced black stripes, and usually the three spots like the blue. Fins will show both blue and orange coloration.

Platinum gourami–These seem to result in about 1% of the brood from a pair of lavender gouramis. The fish is nearly albino, with few spots and whitish stripes instead of black. Personally, I think these are quite stunning. They also appear to fetch a slightly higher price at the store.

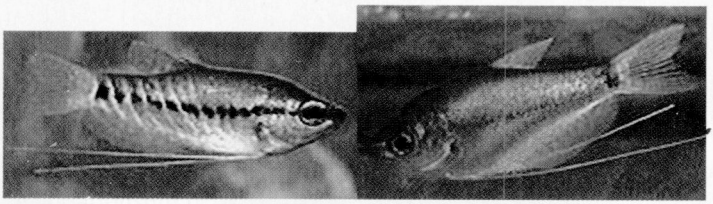
Opaline gourami–This is a variation of the blue gourami. The spots are expanded forming a mottled black pattern toward the rear of the fish.



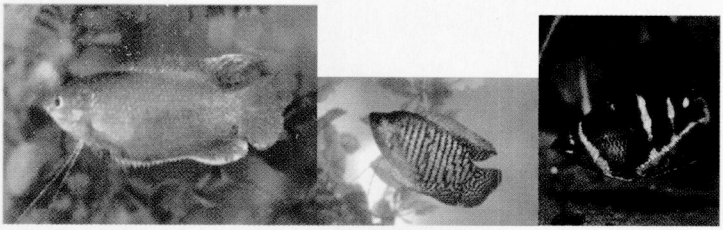
Trichogaster trichopterus *Betta splendens*



Tricogaster leeri *Helostoma temmincki*



Trichogaster pectoralis *Trichogaster microlepis*



Colisa chuna *Colisa lalia* *Sphaerichithys osphromenoides*

Fig. 1.6. Various species belonging to the order Perciformes, suborder *Labyrinthici*, family Belontidae. Some species are economically important, mainly as ornamental fish (JAWA Picture Archive).

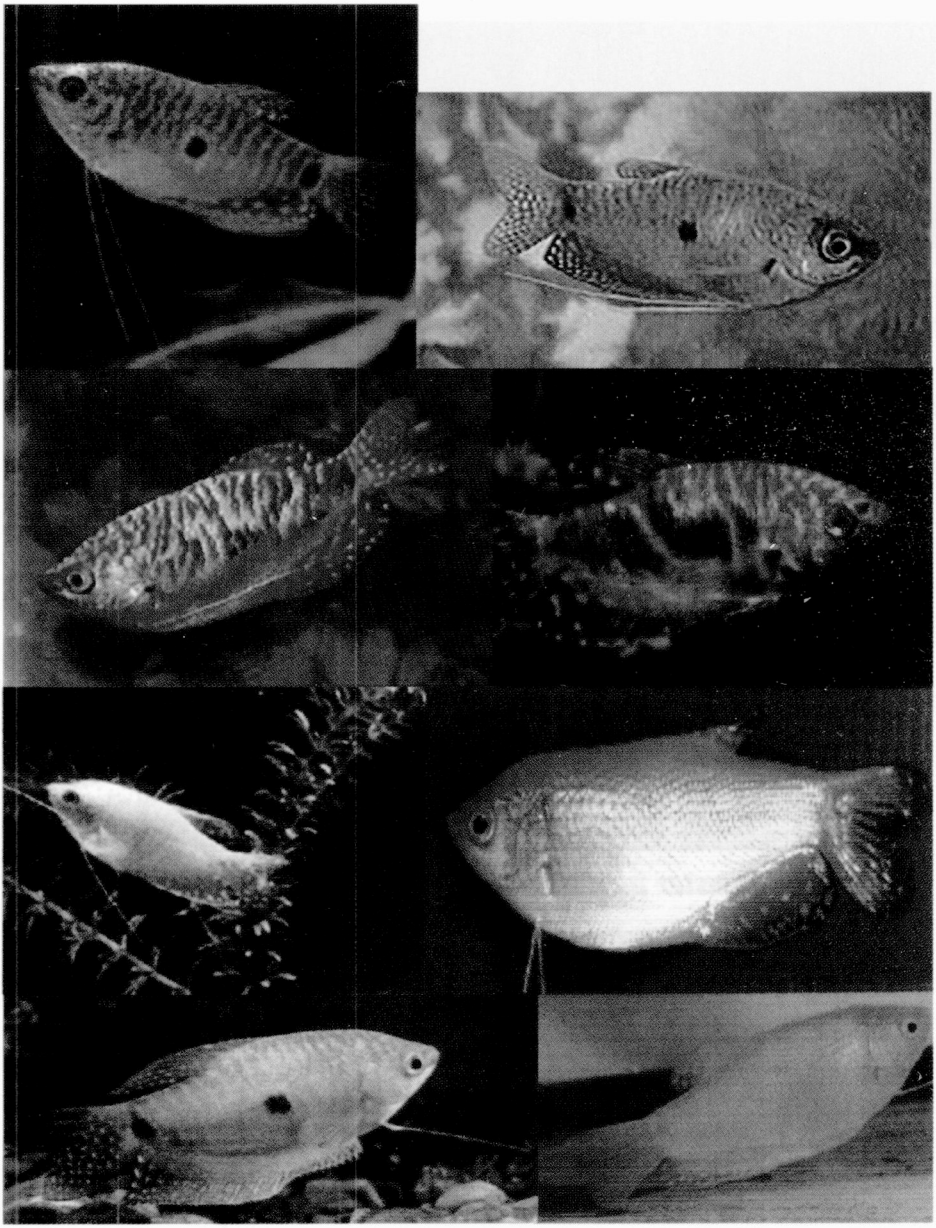


Fig. 1.7. Various strains of blue gourami (*Trichogaster trichopterus*) develop in (JAWA Picture Archive, ROPI Picture, G. Degani).

CHAPTER 2

GENETIC VARIATIONS OF ANABANTIDAE FISH

2.1 INTRODUCTION

Systematics and population genetics are currently investigated, using the means of the electrophoresis on starch (Smithies, 1955) and acrylamide gels (Raymond and Weintraub, 1959) techniques. These methods facilitate the accurate separation of enzymes and other proteins in biological fluids. The use of specific histochemical staining permits study of the activity of individual enzymes on the gels, enabling the genetic variability among the different species, varieties or natural populations to be investigated by enzymatic analysis of allelic variations at specific loci at species and individual levels.

Systematic problems in fish have been solved by comparative analysis of the electrophoretic patterns of the proteins in blood serum proteins, lateral muscle (muscle myogens), and the eye lens (Bonn et al., 1964; Nyman, 1965; Scagnetti and Parisi, 1967). Studies of the intraspecific variation at the biochemical level in teleosts, have found gene-dependent polymorphism in many proteins, such as hemoglobins or transferrins, and tissue enzymes, and have been making them useful as genetic markers to analyze different populations or geographic races, or to examine the relationship between genetic variation and environmental heterogeneity (Utter et al., 1974).

In order to study variations in intraspecific groups, populations, or related species, restriction analysis of DNA was performed by Brown et al, 1979; Hutchinson et al. 1974; Gyllensten et al., 1985; Funkenstein et al., 1990. Fragment pattern analysis of mitochondrial DNA (mtDNA) sequences of genetic divergence among populations of different fish species have been studied extensively (Graves et al., 1984; Arise et al., 1987; Bentzen et al,. 1988; Billington and Herbert, 1988; Grewe and Hebert 1988; Funkenstein et al., 1990).

2.2 GENETIC VARIATION IN LABYRINTH FISH

Degani and Veith (1990) used electrophoresis on starch gel to clarify genetic relationships. In their study, seven members of the subfamily Trichogasternae and three members of the Helostomatidae family were investigated. Emphasis was placed on the suitability of various buffers for electrophoretic separation of specific isozymeisoenzymes, and on the genetic interspecific polymorphism within the suborder of Anabantidae.

The species and varieties (as reviewed by Hoedeman, 1975) which Degani and Veith (1992) used were (numbers in parentheses refer to Figs. 2.1–2.3):

of the subfamily Trichogasternae:

Two varieties of *Trichogaster trichopterus* (Pallas), blue gourami (1)and gold gourami (3), native to Malaysia, Thailand, Burma, Vietnam, and the Indonesian Archipelago, including the islands of Sumatra, Borneo, Java, Madura, and Bali, and a hybrid between the two (2);

T. leeri , pearl gourami (4), native to Sumatra, Thailand, Borneo, and Malaysia;

T. microlepis (Gunther), moonbeam gourami (5), found in Thailand;

C. fasciata (Day), banded gourami (6), found in Bengal and Assam;

Colisa lalia (Hamilton), dwarf gourami (7), native to India;

C. chuna (Day), honey gourami (8);

C. labiosa (Day), thick-lipped gourami, found in Burma (9);

and of the family Helostomatidae:

Helostoma temminicki (Cuvier), kissing gourami (10), native to Sumatra, Java, Borneo, the Malay Peninsula, and Thailand;

Betta splendens (Regan), fighting fish (11), native to Singapore and Thailand;

Macropodus opercularis (Linne), paradise fish (12), found in all kinds of water in China, Korea, and the islands of Taiwan, Hainan, and the Ryukyu group.

Samples of muscle and liver were taken. For outgroup comparison, the St. Peters fish (*Oreochromis aureus*) (13) was used.

Enzymes were separated on appropriate buffers, as previously described by Smithies (1955) and Raymond and Weintraub (1959). The cathodic as well as the anodic parts of the gels were stained simultaneously. The mobility of the isozymeisoenzyme patterns is given in millimeters (Table 2.1).

The suitability of different various buffer systems and enzyme stains in liver and muscle homogenate of *T. trichopterus* and *T. leeri* is shown in Table 2.1.

Buffer B gave an identical patterns for liver and muscle in most of the enzymes. However, an FDP-specific pattern was obtained only in liver homogenates, and an SOD pattern only in the muscle. In the cathodic part of the gel the same isozymeisoenzyme pattern was revealed for liver and muscle in the following enzyme systems: LDH, PGI, SorbDH, ADH, CK, PK, G-3-PDH, GluDH, α-GPDH, and 6-PGDH. This last pattern can be attributed to an enzyme that we called NAD/NADP-Reductase (Red).

Buffer S-2 gave a cathodically migrating band for SOD, which was visible only in the muscle of the pearl gourami.

When **Buffer I** was used, SOD stained both the anodic and the cathodic part of the gel. In muscle and liver homogenates of the blue gourami, GDH, PGI, SOD, CK, PK, FDP GluDH, LDH, PGM, 6-PGDH, G-6-PDH, G-3-PDH, and α-GPDH were all visible. The best stainings were obtained with PGM, SOD and PK. NAD/NADP-

Table 2.1
Enzymes studied, and the suitability of buffer systems, as tested in *Trichogaster trichopterus* and *T. leeri* liver (L) and muscle (M) homogenates

Enzyme	EC*	Abbreviation	Tissue	Buffer
Alcohol dehydrogenase	1.1.1.1	ADH	L	TM
			M	B
Creatine kinase	2.7.3.2	CK	L,M	TM,I,S-2 ,TC
Dipeptidase	3.4.13.11	PEP	L,M	B
			L	TC
β-Esterase	nonspecific	β-Est	L,M	B,TC,TM
			L	S-2
Fructose-1,6-diphosphatase	3.1.3.11	FDP	L	I,B,TC
Glucose dehydrogenase	1.1.1.47	GluDH	M	I,B
Glucose-6-phosphate dehydrogenase	1.1.1.49	G-6-PHD	L	I,B,S-2,TC
			M	S-2
Glutamate dehydrogenase	1.4.1.3	GDH	L	I,B,S-2,TC
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G-3-PDH	L,M	I,B,S-2,TC,TM
α-Glycerophosphate dehydrogenase	1.1.1.8	α-GPDH	L,M	I,B,S-2,TC,TM
Lactate dehydrogenase	1.1.1.27	LDH	L,M	I,B,S-2,TC,TM
Malate dehydrogenase	1.1.1.37	MDH	L,M	B,S-2,TC,TM
Malic enzyme	1.1.1.40	ME	L	I,B,S-2,TM
Phosphoglucomutase	5.4.2.2	PGM	L,M	I,B,S-2,TC,TM
6-Phosphogluconate dehydrogenase	1.1.1.44	6-PGDH	L	I,S-2,TC,TM
Phosphoglucose isomerase	5.3.1.9	PGI	L,M	I,S-2,TC,TM
Pyruvate kinase	2.7.1.40	PK	L,M	I,B,S-2,TC,TM
Sorbitol dehydrogenase	1.1.1.14	SorbDH	L,M	B
Superoxide dismutase	1.15.1.1	SOD	M	I

*Enzyme commission no. gives status of duplicate/single gene activity (Degani and Veith, 1990).

Red stained very well on the cathodic side. In CK of liver homogenates the minor bands fitted exactly with matched the bands of 6-PGDH and PK exactly.

With **Buffer TM** , the bands of the main PK locus appeared sharper than with the other buffer, and MDH and LDH gave the best stains results. Other enzymes were also detected with the TM Buffer (G-3-PDH, α-GPDH, ME, PGM, G-6-PDH, and PGI).

The polymorphisms within the suborder *Labyrinthici* are shown in Figs. 2.1–2.3 and Tables 2.2 and 2.3. For clarification of the mode of inheritance of some enzyme systems it was important to determine the intraspecific polymorphisms. Artificially produced F1-hybrids between two varieties may be even more helpful in solving these problems. Figures 2.1–2.3 show the results for MDH, ME, GDH, α-GPDH, LDH, PEP, SOD, PGDH, and PGM (muscle and liver).

In some samples Malate dehydrogenase (MDH) gave a visible pattern which was visible in some samples consisting of two subunits coded by two gene loci; both loci

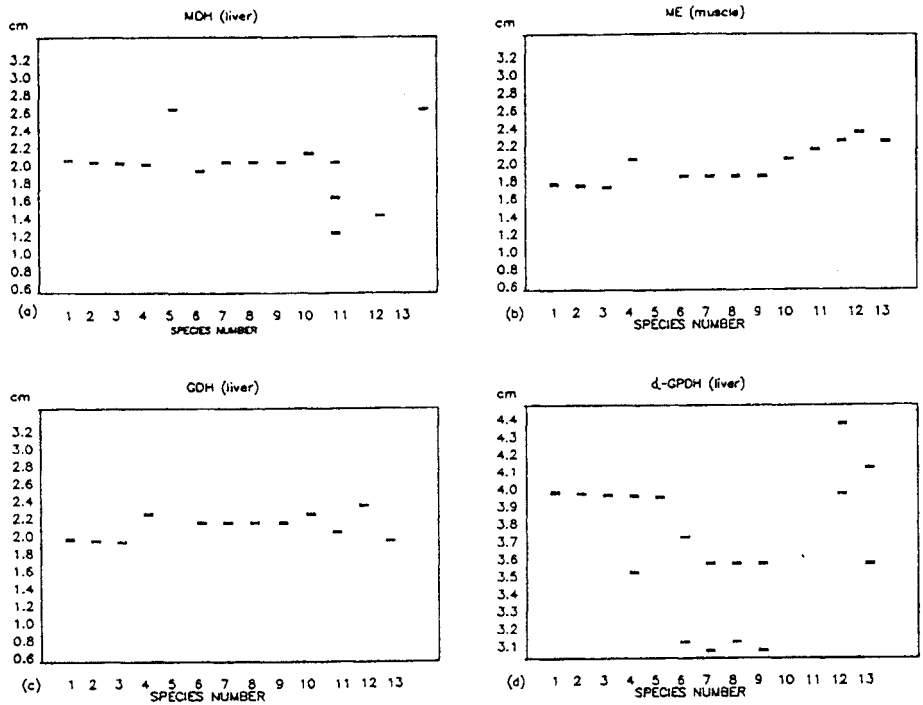


Fig. 2.1. Starch gel electrophoresis patterns, stained for different enzyme systems. 1 = *Trichogaster trichopterus* (blue); 2 = *T. trichopterus* (hybrid blue/gold); 3 = *T. trichopterus* (gold); 4 = *T. leeri*; 5 = *T. microlepis*; 6 = *Colisa fasciata*; 7 = *C. lalia*; 8 = *C. chuna*; 9 = *C. labiosa*; 10 = *Helostoma temminicki*; 11 = *Betta splendens*; 12 = *Macropodus opercularis*; 13 = *Oreochromis aureus* (Degani and Veith, 1990).

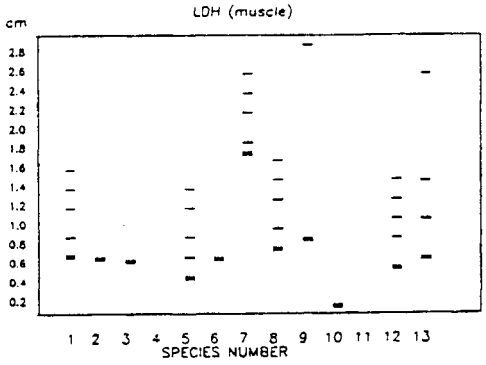


Fig. 2.2. Starch gel electrophoresis pattern of the soluble LDH in different species of Anabantidae fish. 1 = *Trichogaster trichopterus* (blue); 2 = *T. trichopterus* (hybrid blue/gold); 3 = *T. trichopterus* (gold); 4 = *T. leeri*; 5 = *T. microlepis*; 6 = *Colisa fasciata*; 7 = *C. lalia*; 8 = *C. chuna*; 9 = *C. labiosa*; 10 = *Helostoma temminicki*; 11 = *Betta splendens*; 12 = *Macropodus opercularis*; 13 = *Oreochromis aureus* (Degani and Veith, 1990).

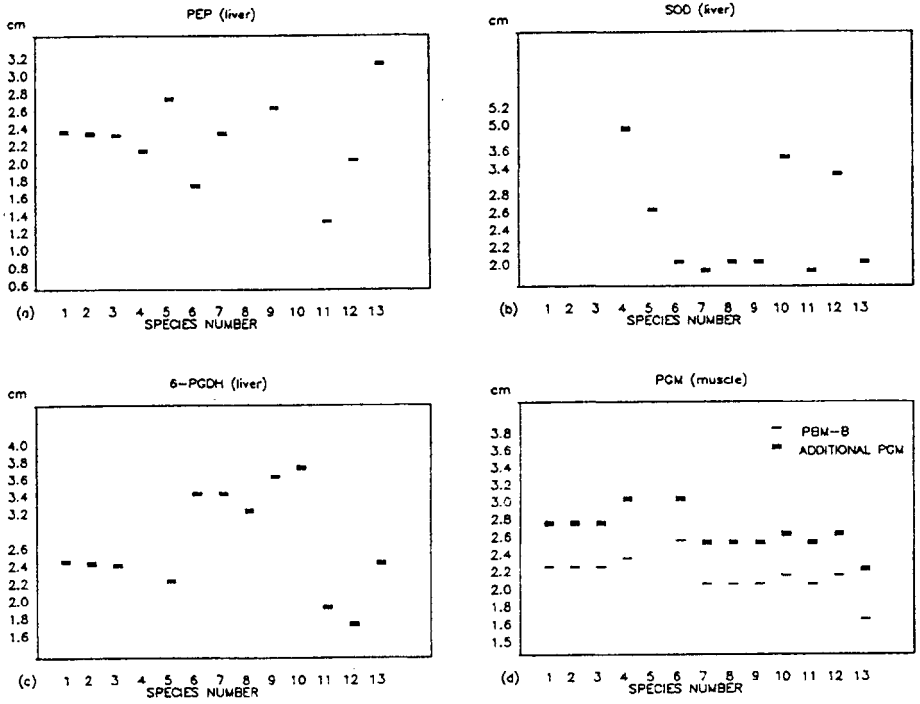


Fig. 2.3. Starch gel electrophoresis patterns, stained for different soluble enzyme systems in different species of Anabantidae fish. 1 = *Trichogaster trichopterus* (blue); 2 = *T. trichopterus* (hybrid blue/gold); 3 = *T. trichopterus* (gold); 4 = *T. leeri*; 5 = *T. microlepis*; 6 = *Colisa fasciata*; 7 = *C. lalia*; 8 = *C. chuna*; 9 = *C. labiosa*; 10 = *Helostoma temminicki*; 11 = *Betta splendens*; 12 = *Macropodus opercularis*; 13 = *Oreochromis aureus* (Degani and Veith, 1990).

were visible in the muscle sample. In liver only the product of the slow-moving locus was visible (Fig. 2.1). The mobility of MDH was found to be the same in *T. trichopterus* and *T. leeri*, but different in *T. microlepis*. The mobility in the genus *Colisa* was quite similar to that of in *Trichogaster* (with the exception of *C. fasciata*). The polymorphism of this enzyme system was very low in both genera, though slightly higher in *Trichogaster*. The mobility of MDH in the species which belong to other families is was different from that in genera *Trichogaster* and *Colisa*.

The malic enzyme (ME) patterns are shown in Fig. 2.1. One locus was detected that is active in the liver. The mobility of this enzyme varied in the genus *Trichogaster*, though not in the genus *Colisa*, and differed on the subfamily level. The polymorphism is was very low in genus *Colisa* (Table 2.2).

For glutamate dehydrogenase (GDH) only one active locus was detected in the liver (Fig. 2.1). This enzyme displayed a mobility which was different among members of the genus *Trichogaster* and similar among those of the genus *Colisa*.

Table 2.2
Polymorphism among different species and varieties (%)

Species	% Polymorphism								
	MDH	ME	GDH	α-PGDH	LDH	PEP	SOD	6-PGDH	PGM
1. *Trichogaster trichopterus* blue	45.4	81.8	81.8	72.7	100	81.8	81.8	81.8	81.8
in genus *Trichogaster*	33.3	33.3	50.0	25.0	100	50.0	50.0	50.0	50.0
2. *T. Trichogaster* blue x gold	45.4	81.1	81.3	72.7	83.3	81.8	81.8	81.8	81.1
in genus *Trichogaster*	33.3	33.3	50.0	25.0	33.3	50.0	50.0	50.0	50.0
3. *T. trichopterus* gold	45.4	81.1	81.8	72.7	7	81.8	83.3	81.8	81.1
in genus *Trichogaster*	33.3	33.0	50.0	25.0	25.0	50.0	50.0	50.0	50.0
4. *T. leeri*	45.4	90.9	100	100	100	100	100	100	100
in genus *Trichogaster*	100	100	100	100	100	100	100	100	100
5. *T. microlepis*	100	100	100	72.7	100	100	100	100	100
in genus *Trichogaster*	100	100	100	25.0	100	100	100	100	100
6. *Colisa fasciata*	100	72.7	72.1	100	81.8	100	81.8	90.9	100
in genus *Colisa*	100	0	0	100	100	100	66.6	66.6	100
7. *C. lalia*	45.4	72.7	72.1	90.9	100	100	90.9	90.9	81.8
in genus *Colisa*	33.3	0	0	66.6	100	100	100	66.6	33.3
8. *C. chuna*	45.4	72.7	72.1	100	100	100	81.8	100	81.8
in genus *Colisa*	33.3	0	0	100	100	100	66.6	100	33.3
9. *C. labiosa*	45.4	72.7	75.0	90.9	100	100	81.8	100	81.8
in genus *Colisa*	33.3	0	0	66.6	100	100	66.6	100	33.0
10. *H. temminicki*	100	90.9	100	100	100	100	100	100	90.9
11. *B. splendens*	100	100	100	100	100	100	100	100	72.7
12. *M. opercularis*	100	100	100	100	100	100	100	100	90.9
13. [Control]									

The GDH mobilities of GDH of *H. temminiki, B. splendens*, and *M. opercularis* were found to be different from one another and there were also differences between them and those of the other species. The polymorphism of this enzyme in the genus *Colisa* was found to be lower than that found in the genus *Trichogaster* (Degani and Veith, 1990)

α-Glycerophosphate dehydrogenase (a-GPDH) has two loci which could not be stained in all the species (Fig. 2.1). These two loci were detected in only one species of the genus *Trichogaster*, *T. leeri*, but in all the species of *Colisa* and in *M. opercularis*. Here the polymorphism was found to be higher in *Colisa* than in *Trichogaster* (Table 2. 2). The mobility of one gene locus product was similar in all members of the genus *Trichogaster* and in all those of genus *Colisa* except in *C. fasciata*, but differed between the genera. However, the second gene product of the second locus, detected in all the species of *Colisa*, did not have identical mobility in all cases.

The lactate dehydrogenase (LDH) has two gene loci for the subunits of this tetrameric enzyme (Fig. 2.2). In most of the species tested, only LDH-A homotetramers were visible. The mobility of LDH-A varied according to species.

Table 2.3
Polymorphism among different species and tissues

Enzyme	Polymorphic for: Tissue	Polymorphic for: *Trichogaster**	Anions	Cathodes	Useful buffer	Useful tissue
ADH	+?	?	B	-	(B)	-
CK	-	+	all	I,S-2	I,S-2	m
β-Est	+	+	all	-	I	l
FDP	?	-	B	-	(B,I)	-
GDH	?	-	all	-	B,I	l
GluDH	+	+	I,B,S-2	-	B,I	l
α-GPDH	+	+	all	-	B,S-2	m+l
G-3-PDH	-	+	all	-	(B,I)	l
G-6-PDH	-	+	all	-	(B,I)	l
HK	N	N	N	N	N	N
LDH	?	+	all	all	TC	m+l
MDH	+	+	B,S-2,TC	I,TC	TC	m+l
ME	+?	?	all	-	B,I	m+l
MPI	N	N	N	N	N	N
PEP	-	+	B,TC	-	B	l
6-PGDH	+	+	all	-	TC,I,B	l
PGI	+	+	all S-2,I	TM,TC	N	-
PGM	-	+	all	TM	B,TC	m+l
PK	+	+	all	TC	I,TC	m+l
SOD	+	+	I,B TM	I,S-2	B	l
SorbDH	bands that appear occasionally—may be nonspecific					
TPI	N	N	N	N	N	N
OR	-	+	B	-	B	l
Red	-	+	-	I,B	B	l

M = muscle; l = liver; N = no activity at all; ? = needs further investigation.
**Trichogaster* means only *T. trichopterus* and *T. leeri* (Degani and Veith, 1990).

Only in this enzyme could a high polymorphism could be detected, among the different several species of *Trichogaster* that were tested (Table 2.2).

Dipeptidase (PEP) displayed a single gene locus, detected in most of the species. It showed differing mobilities on the species level (Fig. 2.3), and a high polymorphism was found. This enzyme could not be stained in *C. chuna* and *H. temminicki*. A pattern identical to that of PEP was found for both superoxide dismutase (SOD) (Fig. 2.3) and 6-phosphogluconate dehydrogenase (6-PGDH) (Fig. 2.3): one gene locus, different differing mobility among various species, and detectability in most of the species.

Phosphoglucomutase (PGM), which is the product of a single gene locus (Fig. 2.3), showed differences in mobility at gene level in several of the species tested. The second, fast-moving band is probably related to a posttranslational modification (protein processing) of the PGM.

Degani and Veith (1990) examined 22 enzyme loci in species belonging to several subfamilies of Anabantidae. Of the 22 enzyme loci which were examined, only three were not detected at all (HK, MPI, and TPI). All the others seem to be suitable for the study of genetic variation. The most suitable enzymes for such a study are: in the liver, β-Est, GDH, α-GPDH, G-3-PDH, PEP, 6-PGDH, and SOD; in the muscle, CK and MDH; and in both liver and muscle, PK, LDH, ME, and PGM. The adaptation of the buffer to the tissue facilitates the detection of enzyme loci, thus permitting the study of the genetic distance between different species belonging to the subfamilies *Trichogasternae* and *Hebostomatidae* as well as the genetic variation among populations from different geographical distributions. In studying the systematic aspect, it was found that within a given genus, at least one of the various alleles in the gene was common to all the tested species. This situation was detected in PGI and MDH, but not in PGM. The difference between the genera *Trichopterus* and *Colisa* was evident in PGI. From the geographical point of view, some species overlapped, while others did not (Hoedeman, 1975). *T. trichopterus*, for example, overlaps all the other species belonging to the same genus. This could be explained on the basis of the relationship among the effects of geography, systematics and genetics of the species tested. In some loci, variations within the genus *Trichogaster* could be found, e.g., PEP and PGM. These loci may be suitable for studying the genetic variations between the species belonging to the genus *Trichogaster*. Hoedeman's study (1975) supports the opinion that *T. trichopterus*, *T. leeri*, and *T. microlepis* are different species from the genetic point of view. Moreover, the genetic variation among the species belonging to the genus *Trichogaster* seems to be greater than that among species of *Colisa*. This hypothesis needs more detailed study, perhaps addressing variations on the DNA level.

Genetic variation has been studied in detail in many species of fish, but little information has been published on the subfamily Anabantidae (reviewed by Kirpichnikov, 1981). In Degani and Veith (1990), MDH of fish tissue, a dimeric enzyme coded by two loci was investigated in muscle homogenate of tested species and showed three different electrophoretic bands. Because only one band was apparently active in the liver of the tested species, the three visible bands in *B. splendens* (Regina and Veith, 1990) probably refer to a heterozygous individual. This finding is in agreement with that reported by Whitt et al. (1973), who described the isoenzymes of MDH in the sun fish species *Lepomis microlopus* and *L. macrochirus*, and in a hybrid of the two. The genetic variation of MDH is well known and this enzyme is one of the most intensively studied; it has been and was reviewed by Kirpichnikov (1981) and Allendorf and Utter (1979).

In the sturgeon *Acipenser guldenstaditi* the spectrum of sMDH is represented by 7–8 bands (Slynko, 1976). In *Clupea harengus* no less than three phenotypes of sMDH were found (Salmenkova and Volokhonskaya, 1973) and in *Sprattus sprattus* two alleles were described (Koval, 1976). Three of four loci of sMDH were detected in rainbow trout and brown trout (Bailey et al., 1970; Allendorf et al., 1977).

In Degani and Veith's study (1990) one locus of ME shows that the mobility of this enzyme varies within the genus *Trichogaster*, though not within the genus *Colisa*, and differs on the subfamily level. This gene locus in fish has a very rare allelic variation, e.g., intraspecies variations have been described in Salmonids and Cyprinids as well as in other fish species (reviewed by Salmenkova and Volokhonskaya, 1973).

Degani and Veith (1990) detected GDH in the liver of Anabantidae in this study in one locus only. The mobility of this enzyme was found to be identical among species of the genus *Colisa*, except for the species *C. labiosa*, but was different among the other Anabantidae species. This enzyme has not been studied intensively in fishes, and little has been published about it.

In α-GPDH Degani and Veith (1990) detected two gene loci in most of the species. The first locus (α-GPDH-A) always stained in all the species. Its mobility was identical at the species level but differed among the genera. The isoenzyme coded by the second locus (α-GPDH-B) seemed to be less active, was not found in all the tested species, and showed variation among the species. This situation was has been reported in other fishes, e.g., salmon and whitefish (see review by Salmenkova and Volokhonskaya, 1973) for the same enzyme. Clayton et al. (1973) described the isoenzymes of a-GPDH in whitefish *Coregonus clupeaformis* and *C. artedi* in great detail. Vuorinen (1984) describes three loci of this enzyme in *Coregonus albula* in muscle, while only two were detected in the liver. All three loci encode for dimeric molecules, and display high polymorphism; and a total of 14 alleles were detected in the various populations tested.

LDH is one of the more thoroughly studied enzymes in fish. It possesses a tetrameric quaternary structure, and in the majority of teleosts three loci have been described. Two of these loci were found in muscle and the third, found solely in fish, is active in the eye retina (Markert et al., 1975). The number of loci of LDH has been determined for many species belonging to different systematic groups and it has been found that this enzyme has from two to five loci, depending on the species (Markert et al., 1975; Kirpichnikov, 1981). In Degani and Veith's study (1990) two locus codes for the subunits were expected, but in most of the tested species only one locus could be detected.

Degani and Veith (1990) detected only one locus of PEP, in the liver; it varied on the species level. It seems likely that this enzyme would be a good marker for systematic study and may be suitable as a marker for Anabantidae.

They also found that 6-PGDH varied among the different various species, except between for *C. fasciata* and *C. lalia*. The mobilities of the isoenzymes belonging to the entire *Colisa* species were identical, except in the case of *C. fasciata*.

These results seem to indicate that in a number of species the high polymorphism in some enzymes can be used as a genetic marker in these species and as an aid in the new field of aquaculture dealing with these systematic groups. Moreover, the low polymorphism that characterized a number of species of the genus *Colisa* could be exploited at a later stage to create new hybrids within the genus.

*2.3 CLONING AND SEQUENCING OF β-GTH-I, β-GTH-II AND GROWTH HORMONE OF BLUE GOURAMI (*TRICHOGASTER TRICHOPTERUS*)*

2.3.1 INTRODUCTION

Oogenesis in teleosts, as in other vertebrates, involves complex interaction along the brain–pituitary–gonad axis. It is generally accepted that the gonadotropin-releasing hormone (GnRH) controls the release of gonadotropin (GtH) from the pituitary gland, which in turn, controls ovarian development (reviews by Millar and King, 1987; Sherwood et al., 1989; Swanson, 1991). Increasing levels of GtH act via the mediation of ovarian steroids, to induce oogenesis from the early growth of primary oocytes to vitellogenesis and through maturation to ovulation.

GtH is a glycoprotein consisting of two distinct subunits, α and β, which are non-covalently bound. In the pituitary of teleosts there are two distinct GtHs (GtH-I and GtH-II), which have been found to share a common α subunit, but differ in their β subunits. The latter is the one which confers the immunoiogical and biological specificity on each hormone. In salmonids, GtH-I and GtH-II cells in the pituitary have been reported (Nozaki et al., 1990a,b; Swanson, 1991) to produced distinct protein expressions at different times stages of the reproductive cycle: GtH-I during oocyte vitellogenesis (Mal et al., 1989) and GtH-II at maturation and ovulation. Most of the physiological studies on teleost GtHs have been conducted on GtH-II, which controls maturation and ovulation. The relation between the functions of GtH-I and GtH-II in fish has not been addressed (Elizur et al., 1996).

Pituitary growth hormone (GH) plays an essential role in the regulation of growth and development, by promoting the division, differentiation, and enlargement of cells (Corin et al., 1990; Copeland and Nair, 1994). The metabolic effects of GH include increased protein synthesis, increased use of fat for energy production, and decreased glucose utilization throughout the body (Copeland and Nair, 1994). GH in fish also influences osmoregulation (Tatsuya and Hirano, 1993; Sakamoto et al., 1993) and reproduction (van Der Kraak et al., 1990; Le Gac et al., 1992). The biological activities of GH have been examined for many fish species (see review by Sakata et al., 1993). During recent years, several GHs have been successfully cloned and expressed in *Escherichia coli* (Goedell et al., 1979 ; Seeburg et al., 1983; Fine et al., 1993; Sakata et al., 1993).

The blue gourami, *Trichogaster trichopterus* Pallas, provides a unique model for the study of the roles of GtH-I, GtH-II and GH in the hormonal control of oogenesis in teleosts, because the stages of its oogenesis can be controlled and examined separately in the laboratory. The blue gourami is multi-spawning, asynchronic and male-dependent (Degani, 1993). The hormone profile of blue gourami during oogenesis has been studied in detail in our laboratory (Degani, 1990; 1993a,b; 1994; Degani and Boker 1992a,b; Degani et al., 1994, 1995), where the cDNAs of the blue gourami β-GtH-I, β-GtH-II (Fig. 2.4), and GH (Fig. 2.5) were cloned and sequenced as genetic markers in order to compare these cDNAs with those of other fish (Goldberg et al., 2000; Jackson et al., 1999).

A.

```
   1  GTCTGTACAGATGTTTAGAGAGTAACAGGCAAAACCTGCAGCAGAGGTTCAACGAGACAA    60

  61  CAGAGATTTACAGGCGTCTGTGCTGCACCCAAAGGATGCAGCTGGTTGTCATGGCAGCAG   120
                                          MetGlnLeuValValMetAlaAlaV

 121  TGTTGGCAGTGGCGGGGGTGGGACAGGGTTGCCGCTTCGGCTGTCATCTAACCAACATCA   180
      alLeuAlaValAlaGlyValGlyGlnGlyCysArgPheGlyCysHisLeuThrAsnIleS

 181  GCTTCCCCGTAGACAGCTGTGGCATCACCGAGTTCATCTACACCACCATATGCGCAGGAC   240
      erPheProValAspSerCysGlyIleThrGluPheIleTyrThrThrIleCysAlaGlyH

 241  ATTGCTACCACGAGGATCCAGTCTACATCGGCCACGATGACTGGGCTGAACAGAAAATCT   300
      isCysTyrHisGluAspProValTyrIleGlyHisAspAspTrpAlaGluGlnLysIleC

 301  GTAACGGGGACTGGACCTACGAAGTAAAACACCTACAAGGATGTCCGGTGGCTGTCACGT   360
      ysAsnGlyAspTrpThrTyrGluValLysHisLeuGlnGlyCysProValAlaValThrT

 361  ACCCTGTTGCCAGAAACTGCGAGTGTACTGCTTGTAATGCAGGAAACACATACTGTGGTC   420
      yrProValAlaArgAsnCysGluCysThrAlaCysAsnAlaGlyAsnThrTyrCysGlyH

 421  ACTTTCATGGATACATACCCAGCTGTCTGTGATTTCAAAGGCACTCATCATTCATCCCAC   480
      isPheHisGlyTyrIleProSerCysLeu

 481  GTTTACTTTGCATCTTTCTCTGATGGTACTAAAATAAACAGATATGTCTTAAAAAAAAAA   540

 541  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  570
```

B.

```
   1  CTGGCTAACCTGCCGCTGACACTAAAGAGGATGATGACTGTAGAAATAAGCAAAGTGTTT    60
                                          MetThrValGluIleSerLysValPhe

  61  GTCCTAATGATGTTAAACCTCTTTCTGGGAGCTTCATCTTCCATTTGGTCTGTGGCTCCA   120
      ValLeuMetMetLeuAsnLeuPheLeuGlyAlaSerSerSerIleTrpSerValAlaPro

 121  GCAGCAGCCTTCCAGCTGCCACCGTGTCAGCTCATCAACCAGACTGTCTCTCTGGAGAAG   180
      AlaAlaAlaPheGlnLeuProProCysGlnLeuIleAsnGlnThrValSerLeuGluLys

 181  GAAGGCTGCCCCAGGTGTCACGCAGTGGAAACGACAATCTGCAGTGGCCACTGCCTCACC   240
      GluGlyCysProArgCysHisAlaValGluThrThrIleCysSerGlyHisCysLeuThr

 241  AAGGACCCAATCATCAAGATACCGTTCAGCAATGTGTACCAGCACGTGTGCACATACCGG   300
      LysAspProIleIleLysIleProPheSerAsnValTyrGlnHisValCysThrTyrArg

 301  GATTTGTTCTATAAGACATTTGAGTTTCCTGACTGTCCTCCTGGTGTGGACCCAGTCGTC   360
      AspLeuPheTyrLysThrPheGluPheProAspCysProProGlyValAspProValVal

 361  ACCTACCCTGTGGCTCTGAGCTGCCACTGCAGCCGTTGTGTGATGGACACATCCGATTGC   420
      ThrTyrProValAlaLeuSerCysHisCysSerArgCysValMetAspThrSerAspCys

 421  ACCTTCGAGAGTCTTCAGCCAGACTTCTGCATGAATGACATACCTTTCTACTACTAGTCT   480
      ThrPheGluSerLeuGlnProAspPheCysMetAsnAspIleProPheTyrTyr

 481  GATGAAATAGCTGCACAGCAAACCAAAAGCAAAAGTAAAATAAATGAATTGTGACAAAAA   540

 541  AAAAAAAAAAAA  552
```

Fig. 2.4. The nucleotide sequences of blue gourami β-GtH-I (A) and β-GtH-II (B). The amino acid sequence of each hormone appears in its three-letter code (Jackson et al., 1995).

```
   1  GTATCAGAAGTGAACCTGAACCTGTATCTGATTTCACAACCGCTATGGACAAAGTCCTGT   60
                                                  MetAspLysValLeuP

  61  TTCTGCTCTTCGTCCTTTCTTTGGGCGTCTCTTCTCAGCCAATCACAGACAGCCAACGTC  120
      heLeuLeuPheValLeuSerLeuGlyValSerSerGlnProIleThrAspSerGlnArgL

 121  TCTTTTCCATCGCTGTTAGCAGAGTCCAACACCTGCACCTGCTCGCCCAGAGACTCTTCA  180
      euPheSerIleAlaValSerArgValGlnHisLeuHisLeuLeuAlaGlnArgLeuPheT

 181  CTGACTTTGAGAGTTCTTTGCAGATTGAAGAGCAGCGTCAGCTCAACAAAATCTTCCTCC  240
      hrAspPheGluSerSerLeuGlnIleGluGluGlnArgGlnLeuAsnLysIlePheLeuG

 241  AGGACTTTTGTAATTCTGATTACATCATCAGTCCCATAGACAAGCACGAGACACAGCGCA  300
      lnAspPheCysAsnSerAspTyrIleIleSerProIleAspLysHisGluThrGlnArgS

 301  GCTCTGTGCTGAAGCTCTCATCAATCTCTTATCGGCTGATTGAGTCCTGGGAGTTCCCCA  360
      erSerValLeuLysLeuSerSerIleSerTyrArgLeuIleGluSerTrpGluPheProS

 361  GCCGCTCTCTATATGGAGGTTCTGCTCAGAGATACCAGATTTCTCCCAAACTGTCAGAGC  420
      erArgSerLeuTyrGlyGlySerAlaGlnArgTyrGlnIleSerProLysLeuSerGluL

 421  TGATGAGAGGCATTCAGCTGCTGATCAAGGCCAATCAGGACGGAGCAGAAATGTTCTCTG  480
      euMetArgGlyIleGlnLeuLeuIleLysAlaAsnGlnAspGlyAlaGluMetPheSerA

 481  ATGGCGTGGTTCCGCAGCTTGCTCCATATGGAAACTACTACCAGAGTCTGGGAGAGGACG  540
      spGlyValValProGlnLeuAlaProTyrGlyAsnTyrTyrGlnSerLeuGlyGluAspG

 541  AGTCGCTCAGGCGCAGCTACGAACTTCTGGCCTGCTTCAAGAAGGACATGCACAAGGTGG  600
      luSerLeuArgArgSerTyrGluLeuLeuAlaCysPheLysLysAspMetHisLysValG

 601  AGACATACCTGACTGTGGCTAAATGCAGACTTTCTCCAGAAGCTAACTGCACTCTGTAGC  660
      luThrTyrLeuThrValAlaLysCysArgLeuSerProGluAlaAsnCysThrLeu

 661  CCCTCGACCTAAATAATAACATAATCATCTGTGTTCTGTAGTCCTGTTCTTTAATGTGTT  720

 721  GACTAGCATTAGCATTAGTTTCTTTCTTGCATTGTCTTTGTTCCAGTTCCAGCACAATGT  780

 781  GATTTCAGACTGCCAGCATATGAAATAAAATTTGTTTGATTCAGAAAAAAAAAAAAAAAA  840

 841  AAAAAAAAAAAAAAAAAAAAA  862
```

Fig. 2.5. The nucleotide sequences of blue gourami GH. The amino acid sequence of each hormone appears in its three-letter code. The putative signal peptide of the growth hormone is underlined (Goldberg et al., 2000).

2.2.2 CLONING AND SEQUENCING OF β-GTH-I, β-GTH-II

A. The nucleotide sequences and the deduced amino acid sequences of the cloned cDNAs

The 5' and the 3' ends of each cDNA were cloned separately but with a short overlap which allowed their proper joining into a single nucleotide sequence.

The sequences of the three cloned cDNAs are shown in Fig. 2.4. The deduced amino acid sequence of each hormone is also shown.

B. Comparison of the amino acid sequences of GtH-I and GtH-II

Figure 2.6 shows the comparison of the amino acid sequences of β-GtH I and β-GtH II. There is only ~30% similarity between them and it is evident that the two polypeptides are very distant from each other. About one-third of the identical

residues are 11 out of 12 cyteines which are probably involved in the formation of 6 disulfide bonds required for proper folding and interaction with the alpha subunit (Goldberg et al., 2000).

C. Comparison of the amino acid sequences of blue gourami b-GtH-I, b-GtH-II to with those of similar polypeptides

The amino acid sequences of the blue gourami β-GtH-I and β-GtH-II were compared with similar fish protein sequences found in the GenBank database.

The comparison of the blue gourami β-GtH-I with similar polypeptides from seven other fish is shown in Fig. 2.6. The blue gourami β-GtH-I shows the closest similarity to its striped-bass counterpart. The two polypeptides share 73% of their residues. The lowest similarity is found between the blue gourami β-GtH-I and that of the baikal omul. Only 55% of their residues are identical. A dendrogram, which graphically represents the relationships among the various β-GtH-I subunits, is shown in Fig. 2.7 (A). A similar situation was found when the amino acid sequences of the blue gourami β-GtH-II were compared with β-GtH-II polypeptides from the same group of fish as in the former comparison. Again, the highest similarity is found with the striped bass β-GtH-II (84% of the residues are identical) and the lowest with the baikal omul β-GtH-II with only 65% of identical residues (Fig. 2.7B). The dendrograrm which represents the relationships among the β-GtH-II polypeptides is shown in Fig. 2.8.

2.3.3 CLONING AND SEQUENCING OF GROWTH HORMONE

COMPARISON OF THE BLUE GOURAMI GROWTH HORMONE WITH THOSE OF SIMILAR FISH

A comparison of the amino acid sequence of the blue gourami growth hormone to similar growth hormones from six different fish is shown in Fig. 2.9. The blue gourami hormone is most similar to that of tilapia, with 82% of their amino acids are identical, and least similar to that of the eel, with 64% identity. The dendrogram which graphically represents the relations among the various growth hormones is shown in Fig. 2.10.

2.3.4. DISCUSSION

In the present study, we have described the cloning and sequencing of three cDNAs coding for two different types of gonadotropin subunits (GtH-I and GtH-II) and growth hormone, from blue gourami (*T. Trichopterus*). This is the first time that cDNAs of these hormones were cloned from a representative of the family Anabantidae.

Blue gourami (*T. trichopterus*) belongs to the order Perciformes and family Anabantidae (synonym Belontiidae). The deduced amino acid sequences of its β-GtH-I, β-GtH-II, and GH were compared with those of similar polypeptides from several other teleosts by using the UPGMA method of cluster analysis, which

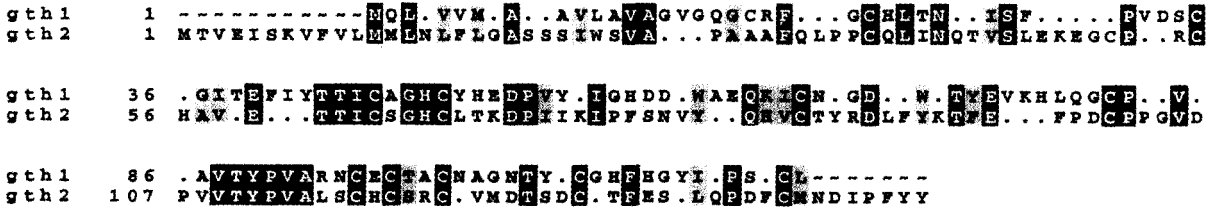


Fig. 2.6. A comparison of the amino acid sequences of β-GtH-I (GtH-I) and β-GtH-II (GtH-II). Identical amino acids are boxed in black, whereas residues with similar physicochemical properties are boxed in gray (Jackson et al., 1999).

Fig. 2.7. Multiple sequence alignment of the blue gourami β-GtH-I (A) and β-GtH-II (B) with similar polypeptides from several other fish. Identical amino acids are boxed in black whereas residues with similar physicochemical properties are boxed in gray (Jackson et al., 1999).

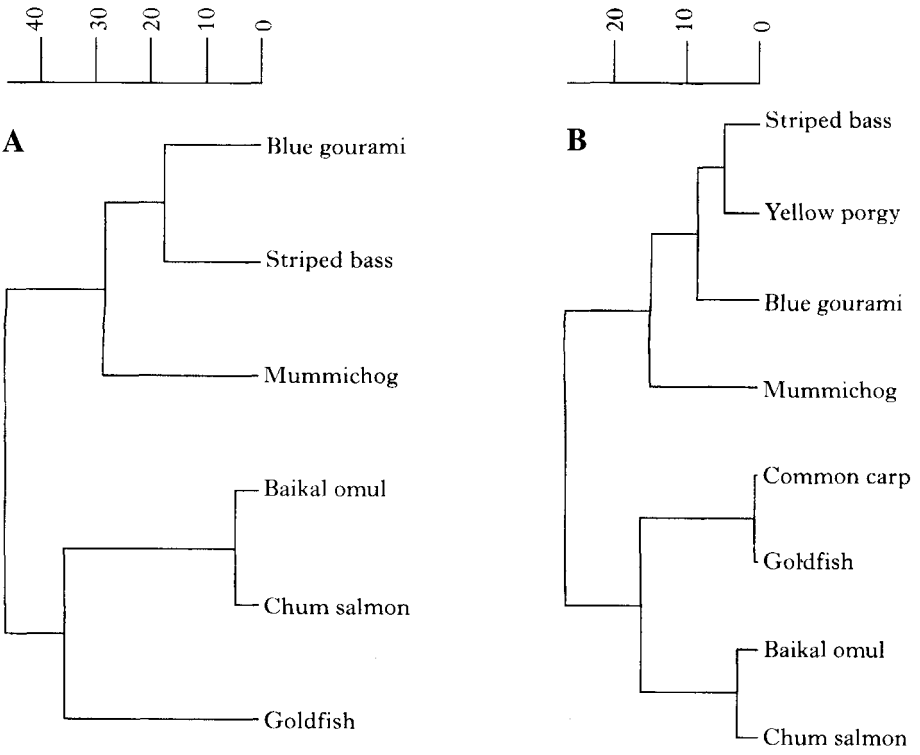


Fig. 2.8. Dendrograms which graphically show the relations among various fish β-GtH-I (A) or β-GtH-II (B). The dendrograms were created by means of the UPGMA method, from similarity matrixes which were produced from the corresponding sequence alignments. The scale bar is a measure of the estimated number of amino acid substitutions per 100 residues which are found when the sequences are compared in a pairwise fashion (Jackson et al., 1999).

creates rooted trees in which a shorter branch length represents greater similarity. The cluster analysis reveals that both β-GtH-I and β-GtH-II of blue gourami were most similar to their striped bass (*Morone saxatilis*) (Hassin et al., 1995) counterparts (Fig. 2.8). Both of these species belong to the order Perciformes, the largest order in teleosts, which includes 251 families. This good agreement between sequence similarity and known systematics is not always found. Goldfish (*Carassius auratus*) and common carp (*Cyprinus auratus*) both belong to the order Cypriniformes and family Cyprinidae, but are very similar only in their β-GtH-IIs whereas their β-GtH-Is show considerable divergence and put them on distant branches of the tree (Fig. 2.8A). It is interesting to note that, in agreement with previous publications (Elizur et al., 1996), our comparison clearly shows that the various amino acid sequences of β-GtH-I have changed more rapidly and show a greater a degree of

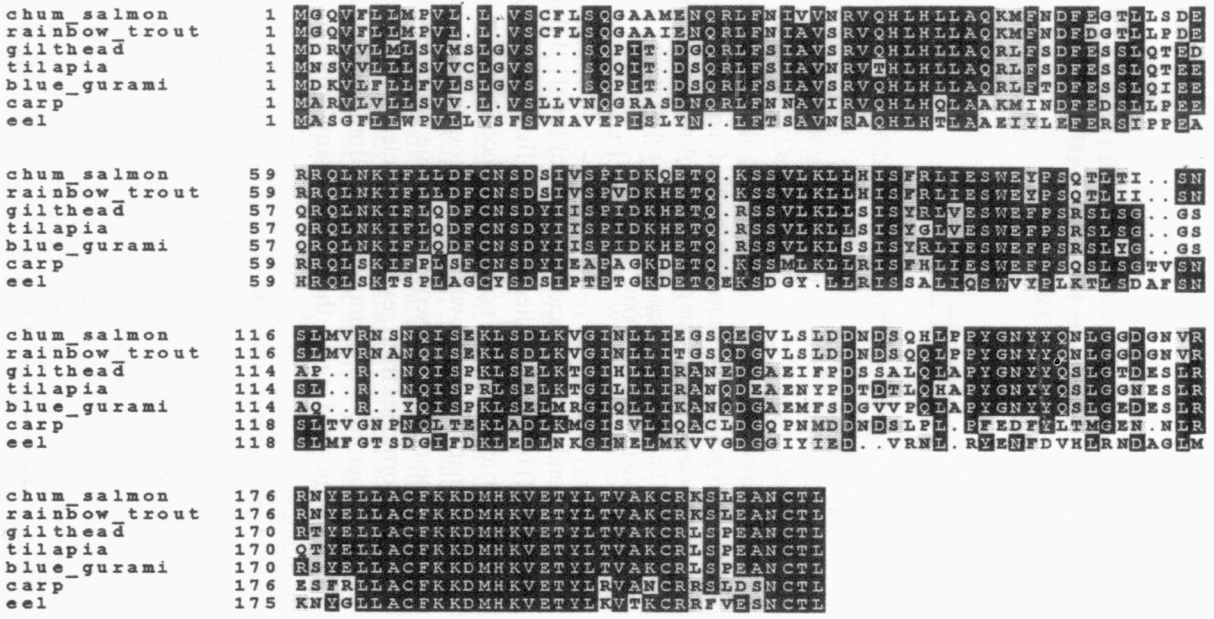


Fig. 2.9. Multiple sequence alignment of the blue gourami growth hormone with six different fish growth hormones (Goldberg et al., 2000).

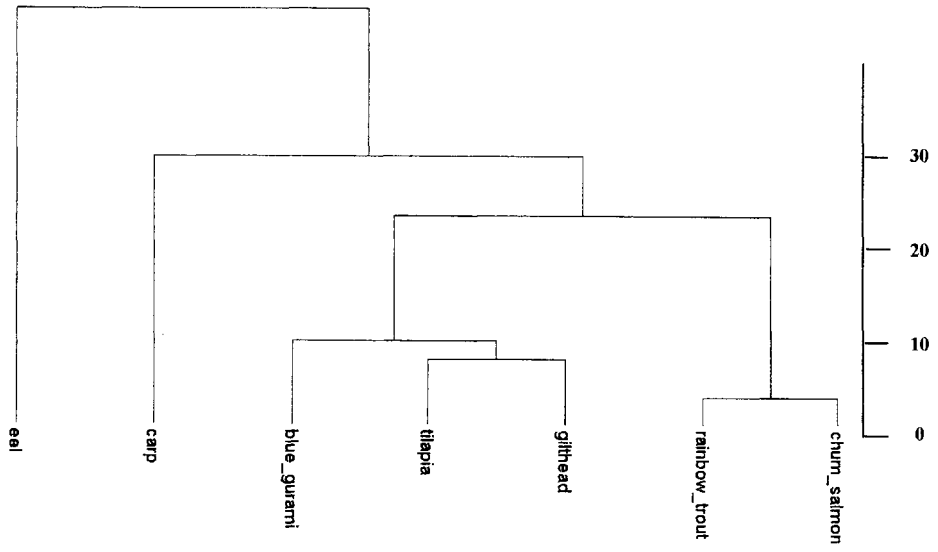


Fig. 2.10. A dendrogram which graphically shows the relations among various fish growth hormones. The dendrograms was created by means of the UPG MA method, and is based on the multiple sequence alignment. The scale bar measures the estimated number of amino acid substitutions per 100 residues (Goldberg et al., 2000).

dissimilarity than the corresponding amino acid sequences of β-GtH-II (Fig. 2.8B).

The growth hormone (GH) has been studied in various fish species, most of them used for human food. As far as we know, no study has been done on fish species belonging to the family Anabantidae to which blue gourami (*T. trichopterus*) belongs. A comparison of the amino acid sequence of blue gourami GH (Fig. 2.9, 2.10) with those of growth hormones from several other teleosts has revealed that it was most similar to the tilapia growth hormone (82%). Both of these fish species belong to the order Perciformes and, again, the sequence similarity is in accordance with prevailing systematics.

It is reasonable to consider that an inference of phylogenetic relations which is based on only one hormone-coding cDNA would have questionable reliability. However, in this study we have presented an analysis of three different polypeptide hormones from blue gourami, each of whichagreed with the classification of this fish. Therefore, the results of the cluster analysis which positioned the blue gourami close to other members of the order Perciformes can be regarded as reliable.

There are many methods of inferring phylogenetic relations from sequence data, each with its advantages and limitations (Swofford et al., 1996). It is possible that the use of a different method would result in somewhat different trees, but we believe that the positioning of blue gourami close to striped bass and tilapia would not change.

CHAPTER 3

THE EFFECT OF DIFFERENT TEMPERATURES, POPULATION DENSITY AND DIET ON THE GROWTH OF LARVAE AND JUVENILES OF LABYRINTH FISH

3.1 INTRODUCTION

Many studies have addressed the nutrition and growth of commercial fish, e.g., *Salmo gairdneri* Richardson, *Cyprinus carpio* L., (Cowey et al., 1985), *Clarias gariepinus* (Degani et al., 1989), *Oreochromis aureus* (Degani et al., 1985) and *Anguilla anguilla* (L.) (Degani et al., 1985, 1986). However, little information is available on the growth and nutrition of tropical ornamental species. In Israel, during the past 10 years, the breeding of ornamental fish has developed as a branch of aquaculture and is aimed at the export markets. Tropical fish such as *Trichogaster. trichopterus*, which are relatively new to aquaculture, are used for hobbies and are marketed all over the world. The breeding of blue gourami under cultured conditions has been described previously (Degani, 1989), and the hormones involved in its reproduction were described in a subsequent study (Degani, 1990).

For the experiments described below, larvae of the dwarf gourami, *Colisa lalia*, were taken from the breeding aquarium 2 days after hatching, for observation of the effects of diet, density, and light regime on survival. Surviving larvae were considered mature when the labyrinth organ had developed and the larva could retain air.

In the first experiment, the highest survival rate was in larvae fed with pond microorganisms, followed, in descending order, by those fed on agar, eggyolk, and yeast. In the second experiment, eggyolk and legume diets produced significantly better results (57–70% survival) than diets of *Tetrahymena* or *Paramecia* (20–30% survival). No significant difference in survival rates was found between high and low stocking density (150 larvae per 5 or 15 l water, respectively). Maintenance under illumination gave significantly higher survival than maintenance in darkness. A long feeding day gave 15% more survival than a short feeding day or a single daily feeding. The addition of vitamin mix to the diet improved survival.

As with other carnivorous fish, the natural diet of *T. trichopterus* comprises various species of invertebrates. The larvae of this species feed on protozoa (infusoria).

The fry (Fig. 3.1) should hatch out after about 24 h and can be fed on a quality

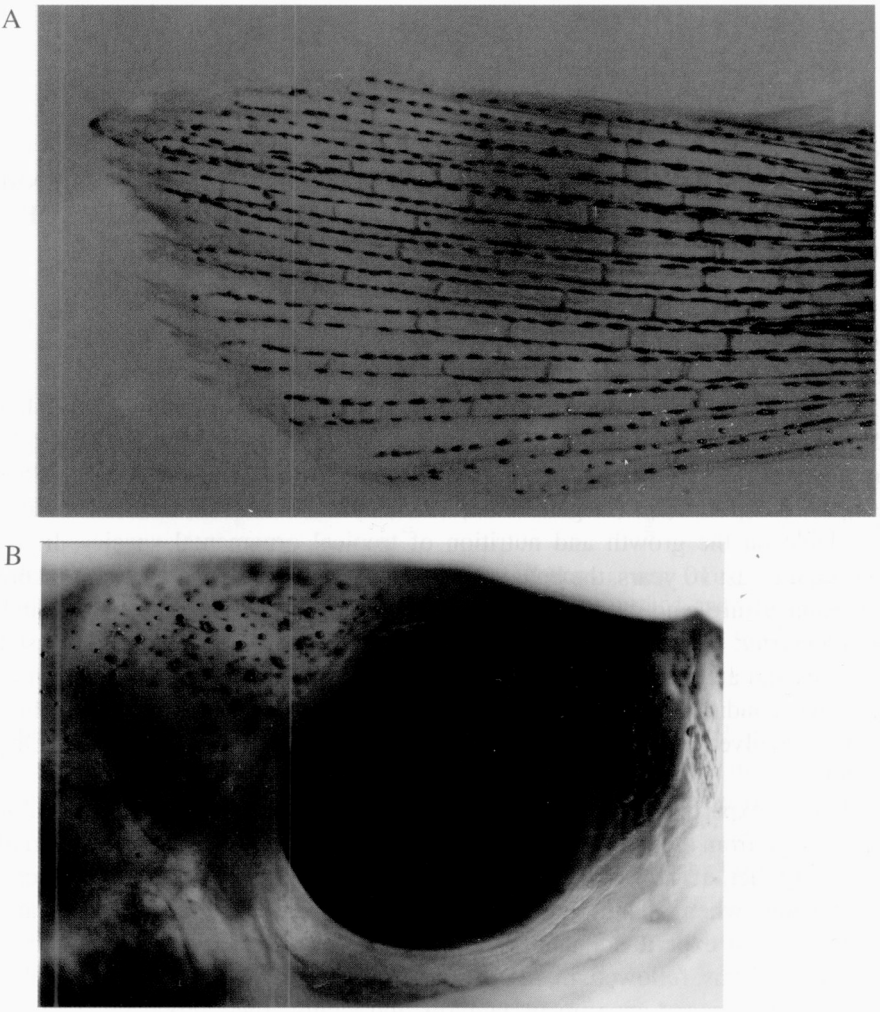


Fig. 3.1. Blue gourami larvae tail (A) and head (B) (× 35).

liquid fry food or infusoria. Small feedings should be carried out several times a day, taking care not to foul the water. After about 10 days, the fry can be fed newly hatched brine shrimp, and partial water changes should be initiated and carried out every few days. The water should still be maintained at about 6 inches (15 cm) in depth and the air above it must remain warm and humid. This is very important to the development of the fry's labyrinth organ, and any chilling can kill the young gouramis.

3.2 DIET AND GROWTH

Degani (1991) performed various experiments to examine the effects of changes of temperature, stocking density, and diets on the growth and body composition of larvae and fingerlings of *T. trichopterus*. The differences among the mean lengths of larvae fed chicken eggyolk, yeast, meal made of shrimp, and yeast mixed with eggyolk were not significant, but there was a significant difference between the mean lengths of larvae fed various species of protozoa (infusoria) and of the group fed the mixture of eggyolk and yeast.

3.2.1 THE EXPERIMENTS

Experiment 1: From two days after hatching, fish larvae were kept at 26 °C in three groups of aquaria measuring 50 × 40 × 30 cm, and each group of three aquaria was fed a different diet: eggyolk, yeast, and yeast + yolk. The lengths of 50 larvae taken at random from each container were measured under the microscope at the start of the experiment, and after 28, 36, 42, and 48 days.

Experiment 2: The larvae were stocked and maintained as for Experiment 1, except that the first group was fed various species of protozoa (infusoria) and a second group was fed eggyolk + yeast.

Experiment 3: As above, with a diet of meal made of shrimp or of eggyolk + yeast.

Experiment 4: The blue gourami fingerlings were kept in small indoor containers (40 × 20 × 20 cm), 10 fish per container, with three identical containers at each temperature (23, 25, and 27 °C), i.e., 30 fish in each temperature group. The fish were fed 5% of their body weight, in two daily portions: at 0700 h (2.5%) and at 1500 h (2.5%). The duration of the experiment was 78 days. The temperature and waterflow systems have been described elsewhere (Degani and Levanon, 1983). Each fish was weighed every three weeks.

Experiment 5: In order to study the effect of population density on the growth of the fish, the fingerlings were stocked at three different densities under the conditions described for Experiment 4; the only difference being the densities: 10, 20, and 30 fish were stocked in the respective groups of containers. The significance of the differences among the experimental groups was estimated by *t*-test and ANOVA.

Experiment 6: Body Composition: In order to study the relation of body composition to body size in blue gourami, fingerlings were kept for 4 months in containers measuring 1 × 1 × 1m at 27 °C. Samples of the fish were taken during the growth period and were frozen (20 °C) and stored for subsequent analysis. Sample preparation was as described in Degani et al., 1986. The fish were cut into slices approx. 0.5 cm thick and a sample (30 g) was homogenized with 30 ml double-distilled water for 45 s (4,500 rpm) in a Polytrom homogenizer.

Table 1
The growth of larvae and juveniles fed with different diets and maintained at different temperatures. Unidentical letters indicate significant ($P < 0.05$; t-test) differences between figures.

Experiment no.		Length of fish (cm) ± SD				
1	Diet	4 days	28 days	35 days	42 days	47 days
	Group A	0.18 ± 0.10a	0.65 ± 0.17b	0.96 ± 0.21d	1.25 ± 0.22e	1.40 ± 0.25f
	Group B	0.17 ± 0.09a	0.60 ± 0.02b	0.85 ± 0.20d	1.20 ± 0.25e	1.35 ± 0.23f
	Group C	0.17 ± 0.01a	0.46 ± 0.21c	0.78 ± 0.21d	1.18 ± 0.20e	1.25 ± 0.22f
	Group D	0.17 ± 0.11a	0.66 ± 0.16b	0.97 ± 0.20d	1.27 ± 0.21e	1.42 ± 0.24f
2	Diet	2 days	15 days	20 days	35 days	
	Group C	0.11 ± 0.09a	0.21 ± 0.15b	0.42 ± 0.17d	0.65 ± 0.20f	
	Group E	0.15 ± 0.10a	0.45 ± 0.20c	0.65 ± 0.21e	1.24 ± 0.25g	
Note: A = eggyolk+yeast; B = yeast; C = eggyolk; D = shrimpmeal; E = infusoria						
3	Temp.	0 days	34 days	62 days	78 days	
	23 °C	0.082 ± 0.03a	0.574 ± 0.35b	1.900 ± 0.40c	3.171 ± 0.50e	
	25 °C	0.070 ± 0.03a	0.607 ± 0.37b	2.285 ± 0.39d	3.760 ± 0.49f	
	27 °C	0.072 ± 0.03a	0.653 ± 0.38b	2.220 ± 0.41d	3.800 ± 0.50f	
4	Density	0 days	26 days	57 days	84 days	
	100/m^3	1.37 ± 0.21a	1.98 ± 0.30d	3.13 ± 0.61g	4.08 ± 0.65k	
	200/m^3	1.35 ± 0.25a	1.73 ± 0.42d	2.17 ± 0.50h	3.23 ± 0.60c	
	300/m^3	1.42 ± 0.22a	1.60 ± 0.40c	1.90 ± 0.61h	2.85 ± 0.30c	
5	Density	0 days	26 days	57 days	84 days	
	100/m^3	2.64 ± 0.61c	3.03 ± 0.71f	3.96 ± 0.75g	5.26 ± 0.81l	
	200/m^3	2.82 ± 0.62c	2.96 ± 0.72f	3.41 ± 0.70i	4.81 ± 0.76m	
	300/m^3	2.86 ± 0.63c	2.86 ± 0.73f	3.10 ± 0.74i	4.54 ± 0.80n	

To determine moisture content, samples of the homogenate were dried overnight in a vacuum oven at 45 °C and a pressure <5 mm Hg. Fat content was determined after extraction in petroleum ether (Park, 1975). Protein content was determined by the Biuret method, after alkaline hydrolysis (Gallagher et al., 1984). Ash was measured by the comparison of sample weights before and after ignition at 600 °C for 5 h (Degani et al., 1986).

3.3 LARVAL GROWTH AND BODY COMPOSITION

The differences among the mean lengths of larvae fed with cooked eggyolk, yeast, shrimpmeal, or yeast + yolk were not statistically significant (Table 1, Fig. 3.2). However, larvae fed with *infusoria* grew significantly larger than those fed with cooked eggyolk + yeast (Table 1, Fig. 3.3 and Fig. 3.4).

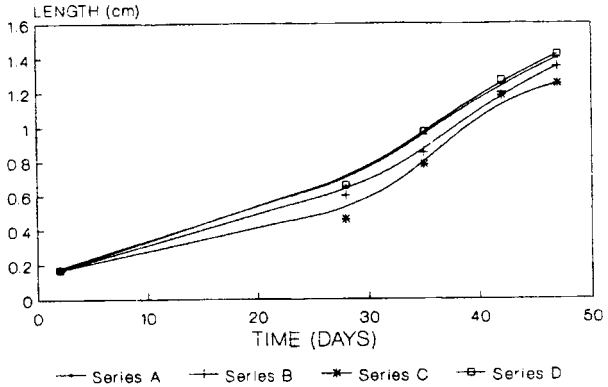


Fig. 3.2. The growth of larvae fed different diets. A = egg yolk + yeast; B = yeast; C = egg yolk (Degani, 1991).

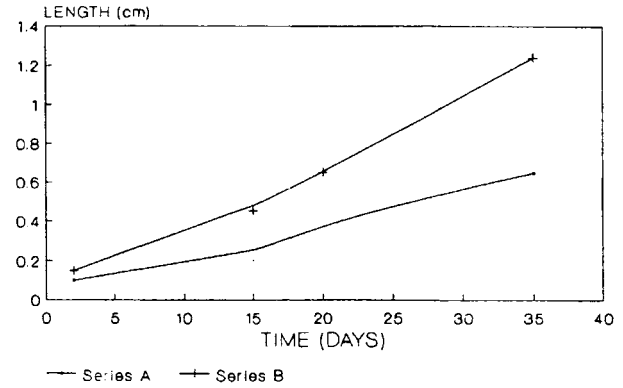


Fig. 3.3. The growth of larvae fed different diets. A = egg yolk + yeast; B = yeast; C = egg yolk; D = shrimpmeal (Degani, 1991).

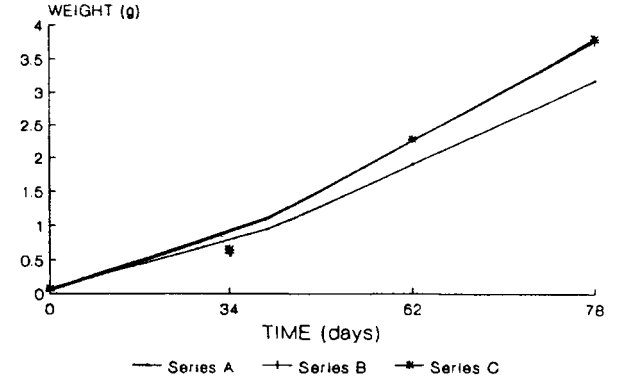


Fig. 3.4. The growth of larvae fed two different diets. A = egg yolk; B = infusoria (Degani, 1991).

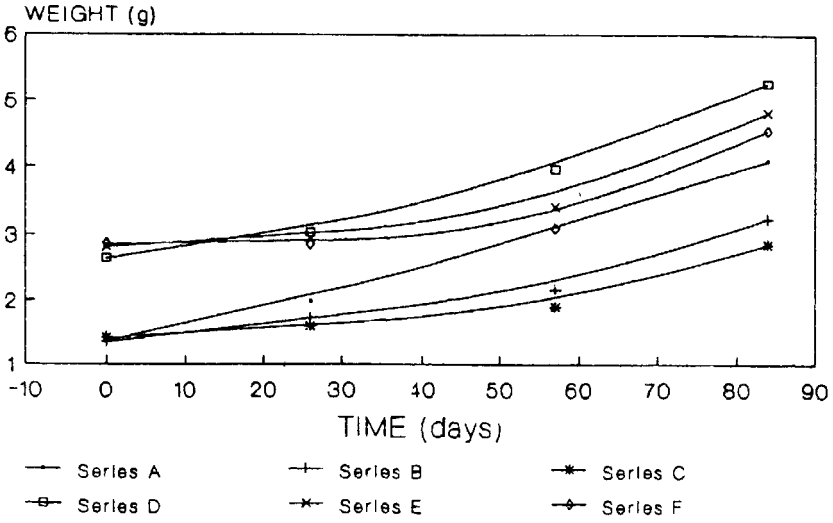


Fig. 3.5. Fingerling growth at various temperatures. A = 23 °C; B = 25 °C; C = 27 °C; D = 100/m^3; E = 200/m^3; F = 300/m^3 (Degani, 1991).

Fish maintained at 25 and at 27 °C grew significantly faster than fish maintained at 23 °C (Table 1, Fig. 3.5), but the mean weights of those kept at 25 and 27 °C were not significantly different.

Increased stocking density significantly decreased growth rate (Table 1, Fig. 3.5).

BODY SIZE AND GROWTH

The protein percentage decreased slightly and that of fat increased slightly during the experimental period. No significant differences were found in the percentages of ash at the various stages of the growth period.

The above results show that 25 or 27 °C ensures better growth than 23 °C, for both larvae and juveniles of the blue gourami. It appears from this experiment that temperature is a very important parameter for the growth of larvae and juveniles, a finding also applicable to other species. In some species, the optimal temperature changes as the fish grows. Hogendoorn et al. (1983) found that the optimal growth temperature for the small African catfish *Clarias gariepinus* of 0.5 g–5.0 g is 30 °C, while for the larger fish (125 g) it is 25 °C. A similar situation prevails in the European eel *Anguilla anguilla*: the optimal temperature for the growth of small eels (0.5 g–5.0 g) is 27 °C and for larger eels (23 g–30 g) it is 23 °C (Degani and Levanon, 1987). Both the African catfish and the European eel live in habitats where water temperature varies during the year, whereas the blue gourami is found in a tropical habitat in which the water temperature is constant (Forselius, 1957). Blue

gourami larvae fed on *infusoria* grew faster than those fed with eggyolk, yeast, shrimpmeal, or yeast + yolk. While we offer no certain explanation of why live food is better than artificial, there are two hypotheses: (1) the composition of live food is better for larval growth; (2) the larvae of blue gourami simply consume more of the infusoria diet.

It was found in this study that increasing the stocking density of blue gourami significantly decreased their rate of growth. Blue gourami is a territorial fish (Forselius, 1957) and its gonadal development and steroid secretion are linked to weight (Degani, unpublished data). Therefore, it is likely that territoriality is an adaptation that ensures space for sufficient growth. The gonadal development and maturation of this species occurs when it attains a weight of 3–5 g, a fact that was established only towards the end of this series of experiments and which, therefore, requires further study. However, it may explain the effect of population density noted here.

The weights of the blue gourami examined in this study ranged from 0.5 to 8.8 g. The percentage of protein increased slightly and that of fat decreased slightly during the growth period. These changes of protein and fat are comparable with those in the African catfish (Degani et al., 1988) but differ from those in the American eel (Gallagher et al., 1984), in which changes in protein, fat, and moisture content were found proportional to body size and were also affected by external parameters, e.g., temperature. The lipid content of goldfish (*Carassius auratus* (L.)) (Knipprath and Mead, 1968) and of brook trout *Salvelinus fontinalis* (Mitchell) has been found to increase with temperature. However, since the blue gourami is a tropical fish, living in water of relatively constant temperature, it is unlikely that temperature can affect its body composition.

CHAPTER 4

REPRODUCTION

4.1 GENERAL

Reproduction in teleost fish involves a very wide and diverse range of processes. Among the over 25,000 known species, we find dioecious and hermaphroditic (including protrandrious, protogynous and synchronous types), oviparous and viviparous species. This wide variety is reflected in the basic anatomy of the gonadal structures and in the hormonal profile during the reproductive cycle. However, with respect to the general endocrinology of gametogenesis and reproduction there is a basic pathway, based on the activity of the hypothalamus–pituitary–gonad axis (Hoar, 1969; de Vlaming, 1974; Schreibman et al., 1990). The final stage prior to reproduction is the production and emission of mature gametes, which is the essential preliminary to reproduction. The development of the gonads is divided into three phases: production of gametes (spermatogenesis and oogenesis), growth and maturation of the gametes (spermiogenesis and oocyte vitellogenesis, which are succeeded by oocyte maturation), and emission of the gametes (spermiation and ovulation).

4.2 SEX IDENTIFICATION

Sexing can be done by observing the dorsal fin of the fish: those of the males are more pointed, those of the females shorter and more rounded (Fig. 4.1). By observing the difference in sexes the mature female body containing eggs can easily be identified.

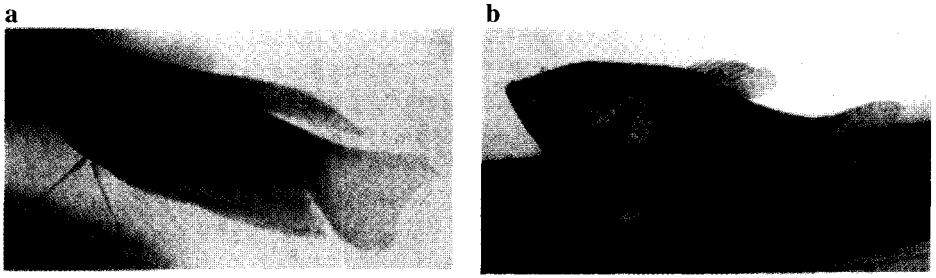


Fig. 4.1. The male (a) and female (b) blue gourami (gold).

4.3 REPRODUCTION OF BLUE GOURAMI

In the natural habitats reproduction occurs all year round but more profusely during heavy rain, when more areas are available for male territory. Descriptions of the blue gourami's sexual behavior (Degani, 1989) are based on studies under artificial conditions.

The onset of the reproductive period is marked by the development of nuptial coloration, the initiation of nest-building behavior in the male, and increasing aggressiveness. During non-reproductive periods, both sexes are generally uniformly pale and do not show aggressive or any kind of territory-defensive behavior. Like many other anabantids, blue gourami deposit their eggs in a floating bubble nest (Fig. 4.2), which is made of bubbles formed by air enveloped in oral mucus. The bubbles are released at the surface, and tend to stick together. The form and composition of the nest vary among species.

A very distinct characteristic of nest building by *T. trichopterus* is the active incorporation of vegetation into the nest. Female nest building is absent or very rare (Miller and Robinson, 1974), and Cheal and Davies (1974) found that the presence of a nest is not a prerequisite for spawning; in many cases they found that the female was able to spawn without the presence of the nest (Figs. 4.2 and 4.3). Nevertheless, in many cases when spawning took place without a nest, the eggs were dispersed over the water surface, and many of them were not fertilized (pers. observ.).

The spawning procedure can be divided into courtship and pre-spawning behavior, spawning (gonadal release and fertilization), and post-spawning behavior, when the male takes care of the young larvae. During the whole process, visual, mechanical and chemical cues are involved. Courtship is characterized by aggressive behavior of the male toward the female: he approaches her and either chases or bites her.

Fig 4.2 Bubble nest of blue gourami.

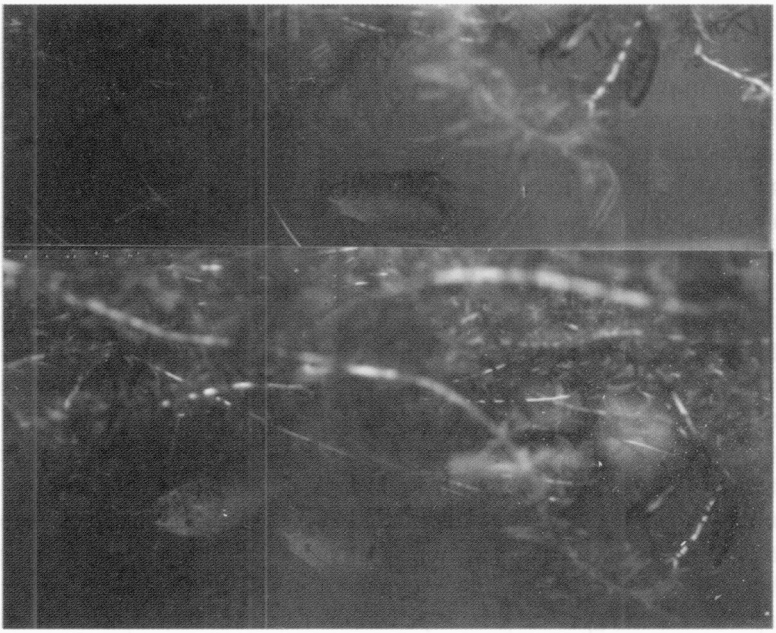


Fig. 4.3. Male blue gourami courtship of female.

Some males are very aggressive, while others ignore the female and spend time taking care of the nest (Miller, 1964). If the female is not ready to spawn, she hides far from the nest and is usually found at the opposite end of the tank. When the female shows signs of readiness, the male begins the so-called lateral spread display, which is characterized by the erection of dorsal and anal fins and spreading of the caudal fin. The body also curves and at maximum intensity it acquires an S shape.

Courtship and spawning behavior are quite difficult to distinguish, but butting or biting by the female seems to have an important signaling function, in limiting male aggression. The male takes a position anterior to the female, who is inclined at 30° to the horizontal with the head higher than the tail. He then moves back and forth stroking the underpart of the female with his dorsal fin. In the last stage of spawning behavior, he swims forward or bends his body in the middle, remains perpendicular to the center of the female's body, and finally flexes into a U-shape into the middle of which the female enters (Fig. 4.4). The male squeezes the body of the female very tightly and both fishes roll over. The roll ceases with the female belly up and the male over her. First, the male emits a cloud of sperm and a few seconds latter the female releases a clutch of eggs, which are immediately fertilized on their way to the surface. The female is then released and both fishes sink to the bottom of the tank. The last stage is repeated several times at each spawning. During post-spawning

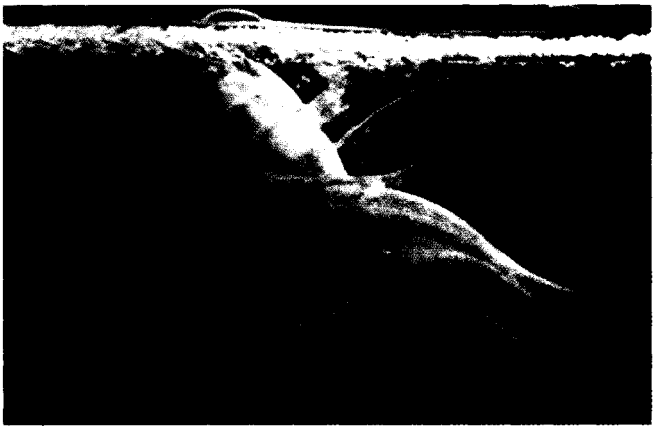


Fig. 4.4. Female spawning the eggs into the nest.

behavior, the male forces the female away from the nest and starts to take care of the floating eggs. He takes them into his mouth and blows them into the nest. In nature, the spent female usually leaves the nest area about this time. The eggs eclode 24 h after fecundation. After eclosion, the male continues to return the larvae into the nest until they reach the free-swimming stage. At each spawning, 500 to 2000 larvae are released. The gourami is an aseasonal breeder and spawning occurs all year round. As in other topical fish, the sexual season shows no correlation with light and temperature, although a peak of reproductive activity in nature may occur after wetter periods, as with *T. pectoralis* (Hails and Abdullah, 1982) (Fig. 4.5).

4.4 OOCYTE DEVELOPMENT IN FISH

Oocytes of non-mammalian vertebrates grow enormously in diameterwhile meiosis is arrested in the first prophase. Vitellogenesis is the process mainly responsible for this tremendous growth. During vitellogenesis a hepatically derived plasma precursor, vitellogenin, is sequestered and selectively taken up from the blood stream by the developing oocyte, which incorporates it and then converts it

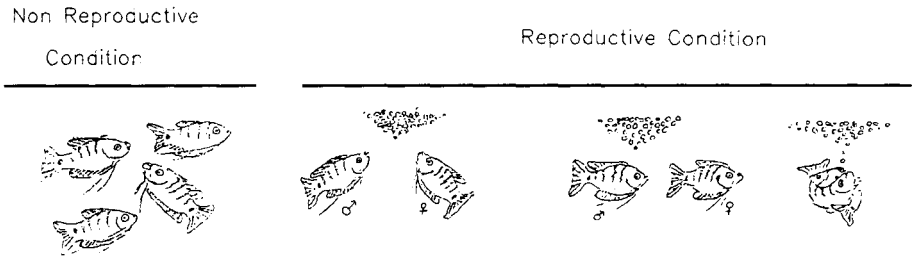


Fig 4.5. The various stages of sexual behavior and spawning of blue gourami (Degani, 1993a).

into yolk protein (Wallace and Selman, 1981; Tyler et al., 1990). This period of primary growth is followed by the process of oocyte maturation, which is a crucial step in the whole process of reproduction. Only after completion of this stage can the oocytes be released and fecundated. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division and subsequent progression to metaphase II (Nagahama, 1994). The first visible event of oocyte maturation is the migration of the nucleus (germinal vesicle migration—GVM) toward the animal pole, and this event is often used as an indicator of the onset of oocyte maturation. After GVM, the germinal vesicle breaks down (GVBD), indicating the end of prophase I. Subsequent events are the condensation of the chromosomes and the extrusion of the first polar body, which marks the end of the first division (Goetz, 1983). In captivity, various species fail to complete gonadal development because their oocytes do not undergo the final maturation stage. Many techniques are commonly used to induce final maturation artificially, in order to facilitate the completion the gonadal cycle (Donaldson and Hunter, 1983).

The maturation process is characterized by intensive morphological and physiological changes in the gonads, pituitary, and brain (Kaul and Rishi, 1986). Cumulative evidence suggests that the oocyte in teleosts is regulated by a series of complementary hormonal actions. The specific actions of those hormones that participate in the maturation process in teleosts is poorly understood, since an appropriate model has not been found to this day. There are many difficulties related to the study of final maturation: in many species it is a seasonal phenomena, and in most it immediately follows vitellogenesis, so that it is impossible to pinpoint the time when it begins. In many species used as models for the study of fish reproduction in captivity, final maturation cannot be induced without hormonal intervention. The blue gourami is an excellent model for the study of the final maturation process because: the blue gourami is an aseasonal breeder, so mature females are available and spawning can take place throughout the year and the whole process, including ovulation and spawning, can be completed in less than 24 h.

4.5 OOGENESIS IN FEMALE BLUE GOURAMI

The ovary of blue gourami is shown in Fig. 4.6. The size of the ovary changes during fish maturation, and in mature females the oocytes fill more then 70% of the female body.

The gonadal development in females blue gourami has been described by Degani and Boker (1992a,b), Degani (1993a,b), and Jackson et al. (1994). In females kept in the absence of males, in a gynaeceum or alone in a reproduction aquarium, the following four oocyte stages have been observed. The growing stages of oocytes in blue gourami can be classified as follows:

Stage 1: Chromatin nucleolus stage. The oocyte measures 30–60 μm in diameter. The cytoplasm is strongly basophilic, with a central nucleus and 2–3 nucleoli per

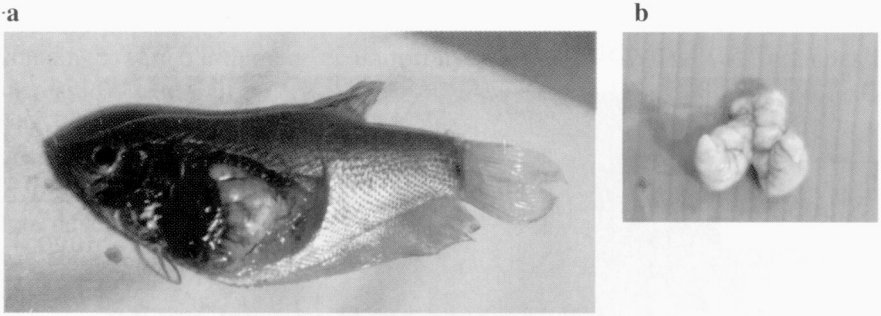


Fig. 4.6 The ovary of blue gourami, in situ (a) and isolated (b).

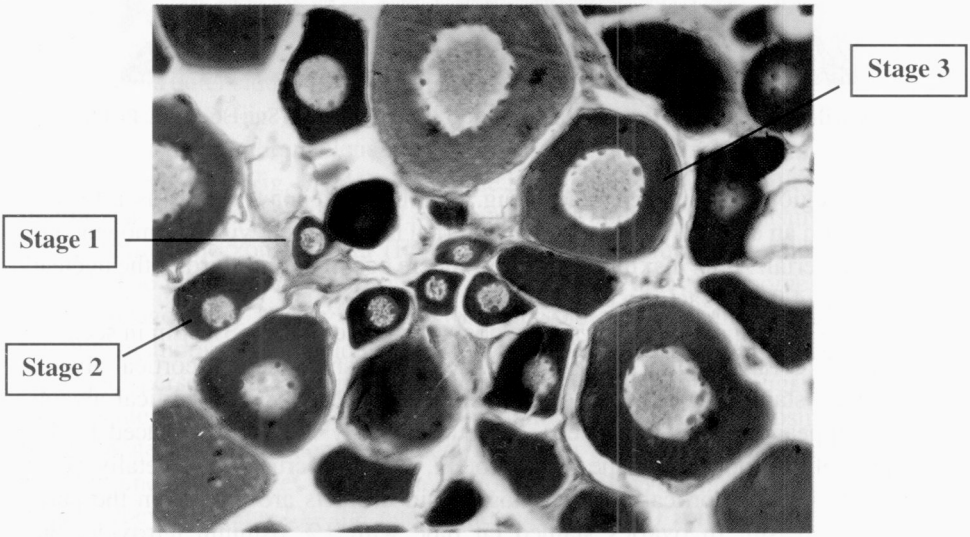


Fig. 4.7. Chromatin nucleolus stage and Perinuclear stage (Prof. G. Degani).

section (Fig. 4.7). The nucleus–cytoplasm ratio (N/C) is 0.5. A single cellular layer surrounds the oocyte (Fig. 4.9.1a).

Stage 2: Perinuclear stage. From the mid- to the late-perinuclear stage, the formation of the vitelline envelope begins. The oocyte diameter is 60 μm (Fig. 4.7), and there is an increase in the absolute diameter of the oocyte and a decrease in the nucleus-cytoplasm ratio. The modal N/C ratio is now 0.45. The perinuclear stage is characterized by the presence of multiple nucleoli at the periphery of the germinal vesicle: five to eight per section. In some oocytes, the nucleus contains an abnormal spherical structure which appears in diverse forms: it may appear as a round acidophilic structure, located in the center or periphery of the nucleus, when an outer

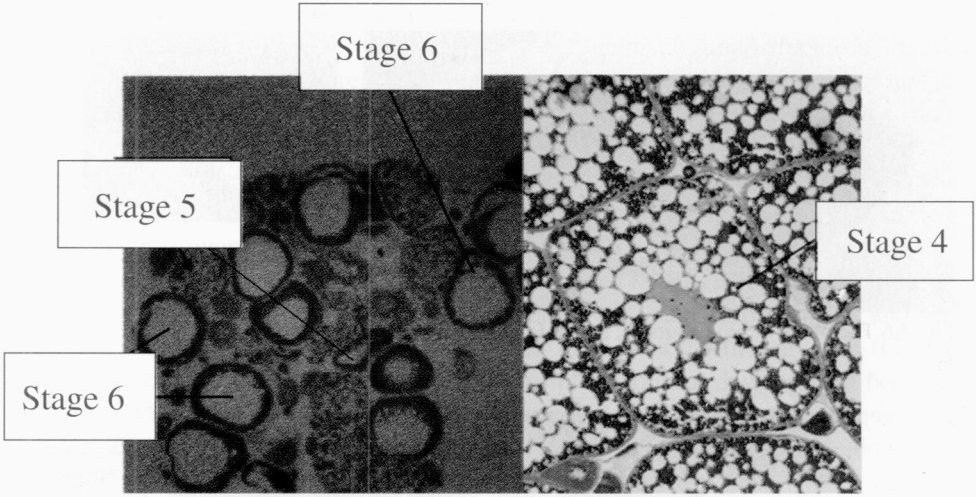


Fig. 4.8. Vitellogenesis (Stage 4), maturation (Stage 5), and ovulation (Stage 6) (Prof. G. Degani).

and inner region can be recognized (Fig. 4.9.1b); or it may appear as a bigger structure with an external capsule and an internal region filled with a homogeneous granular material. At its maximum size, the sphere occupies one-third of the nuclear volume (Fig. 4.9.1a) (Jackson et al., 1994).

Stage 3: Cortical alveolar stage. The oocyte diameter is 150–370 μm in section; the modal N/C ratio is 0.38 (Fig. 4.7). In the cytoplasm, spherical cortical alveoli appear; they stain with Periodic Acid Schiff (PAS) reagent. The cortical alveoli begin to appear around the nucleus, but subsequently they are displaced to the periphery of the oocyte by the yolk protein, which accrues centripetally (Fig. 4.9.2a). During the cortical alveolar stage, lipid droplets are formed in the perinuclear cytoplasm. In ovaries stained en bloc with 1% osmium tetroxide, the droplets stain intensely black. In paraffin-embedded tissue, previously cleared with alcohol and xylene, the lipid droplets appear as empty vacuoles. Surrounding the oocytes there now appear two cellular sheets, the internal granulosa and an external theca layer. Another characteristic of this stage is the formation of nuclear blebs: irregular outgrowths of the nuclear periphery, containing karyoplasma and nucleoli. The nucleus thus displays a distorted appearance. The nuclear blebs disappear during early vitellogenesis (the next stage) and the nucleus assumes a rounded contour (Fig. 4.9.2b) (Jackson et al., 1994).

Stage 4:Vitellogenic stage. Oocyte diameters range between 370 and 500 μm at the beginning of vitellogenesis and reach 500–580 μm toward the end (Fig. 4.8). The modal N/C ratio is 0.19. The first step of this stage, the primary yolk granule stage, is characterized by the first appearance of small, strongly acidophilic granules, which are also positive to protein stains such as bromophenol blue (Fig. 4.9.2c). In the following

step, the so-called secondary yolk granule stage, the nucleus is star-shaped and contains a variable number of uniformly sized nucleoli. The yolk granules have increased in size and are now dispersed homogeneously among the lipid droplets. The PAS-positive granules remain in the periphery of the oocyte, and a well-developed zona radiata is now seen at the oocyte envelope.

In the females transferred into the presence of a male, in addition to oocytes in the four developmental stages described above, the ovaries also contained oocytes that had entered the final maturation stage (stage 5). This stage is characterized by the migration of the nucleus (germinal vesicle), which until now had been at the center of the oocyte, toward the periphery of the cell. In the cytoplasm, a large apical lipid vesicle is formed by the coalescence of the small individual lipid droplets that were formed during the cortical alveolar stage (Fig. 4.8). The nuclear position at the beginning of the migration is at the inner edge of the vitelline belt that surrounds the lipid vesicle (Fig. 4.9.4a). Afterward the nucleus crosses the vitelline belt and remains at its outer side, embedded in a region of a homogeneous cytoplasm without vitelline granules (Fig. 4.9.4b). In oocytes with a peripheral nucleus, the vitelline membrane opposite the nucleus becomes thin, elongates, and invaginates slightly toward the nucleus (Fig. 4.9.4c). Inside the invagination, a large follicular cell (the micropylar cell) appears (Fig. 4.9.4d), which extrude thin cytoplasmic processes that penetrate the zona radiata (Fig. 4.10.5a), thus forming the micropyle (Fig. 4.9.5b). The micropylar cells contain many lisosomes and phagosomes, indicating intense phagosomal activity (Fig. 4.10). After the arrival of the nucleus near the vitelline membrane, the nucleolemma disintegrates and strips of the nuclear envelope lie dispersed in the cytoplasm, which surrounds the nucleus (Figs.4.10.7a,b,c). The disintegrated nuclear envelope is a double membrane, enclosing a cistern approximately 40 nm wide and pierced by nuclear pores. The shape of the nucleus remains unchanged after the disintegration of the karyolemma. The completely mature oocytes (stage 6)—the eggs—measure 575–700 μm and are completely translucent, as a result of the coalescence of the lipid droplets and protein yolk granules.

About 5–10 h after the introduction of a male and a female into the same aquarium, oocytes at final oocyte maturation were observed. In some specimens, the nuclei of several oocytes had already migrated to the oocyte periphery and the nuclear membrane had disintegrated; in others, nuclear migration had barely started. In some females sampled after 20 h, the micropyle was fully formed: it comprises a vestibule continuous with the micropylar canal. In pairs in which the whole spawning cycle had been completed, the mature oocytes had been extruded, and remnants of the follicular envelopes were found in the ovary.

A schematic representation of the oocyte stages in *Trichogaster trichopterus* is presented in Fig. 4.11.

4.6. EFFECT OF MALE BEHAVIOR ON OOCYTE MATURATION

The relationship between male sexual behavior and oocyte maturation in female blue gourami has been described by Degani and Boker (1992a,b), Degani (1993a), and Jackson et al. (1994). In females that were kept in a gynaeceum, a reproductive aquarium without males, oogenesis was arrested at the stage before migration and breakdown. The ovaries of females from the gynaeceum were similar to those of females kept in solitary confinement, and none of their ovaries contained mature oocytes.

The courtship activity of the male started shortly after the introduction of a male-female pair into the aquarium. The male generally swam around the female for 2–3 h before he began to build the nest. In several cases, nest building started a few hours after the male–female pairing;, in other cases, it started one or more days after. When the nest was ready, the male swam in small circles close to the female, trying to drive her under the nest. The process climaxed with the male curving his body around the female while expelling a cloud of spermatozoa, and, almost at the same moment, the female extruded ripe eggs, which were promptly fertilized.

A total of 35 pairs were observed. In 12 cases, the nest building began.3–5 h after male–female contact was established. In four pairs, nest-building activity began 20–30 h after pairing. With eight pairs fertilized eggs appeared in the nest between the fourth and sixth days. In nine cases, no nest-building or spawning activity was observed, and in two cases there was no nest building, but the female spawned. Altogether, oocyte maturation occurred in 24 pairs: mature oocytes were observed in the ovary in nine cases, whereas in 15 cases oocyte development continued until ovulation and oviposition. A model of the effect of male behavior on oocyte maturation is represented in Fig. 4.12.

4.7 BREEDING BLUE GOURAMI IN THE LABORATORY

The breeding of blue gourami in the laboratory or under aquaculture conditions has been described in many publications (e.g., Hodges and Behre, 1953; Forselius, 1957; Miller, 1964; Degani, 1989, 1993a,b).

To achieve a good result in breeding blue gourami, the fish were maintained as a suggestion for procedure in containers measuring $200 \times 20 \times 20$ cm for 6 months, at 26 °C, with 16 h of light and 8 h of darkness (Degani, 1989). They were fed an artificial diet until three weeks before the start of the breeding experiments, when the diet was changed to *Tubifex tubifex* or *Artemia salinita*. Only 0.5–1-year-old fish were taken for this study.

The breeding took place in a 5 gal (20 l) aquarium, with a 0.5–1-inch (1–2 cm) substrate of pea-sized gravel. The tank was covered and illuminated with a 15 W incandescent lamp from 0800 until 2000 daily.

Filtration was by a sponge filter run from an airstone in the corner. The tank was

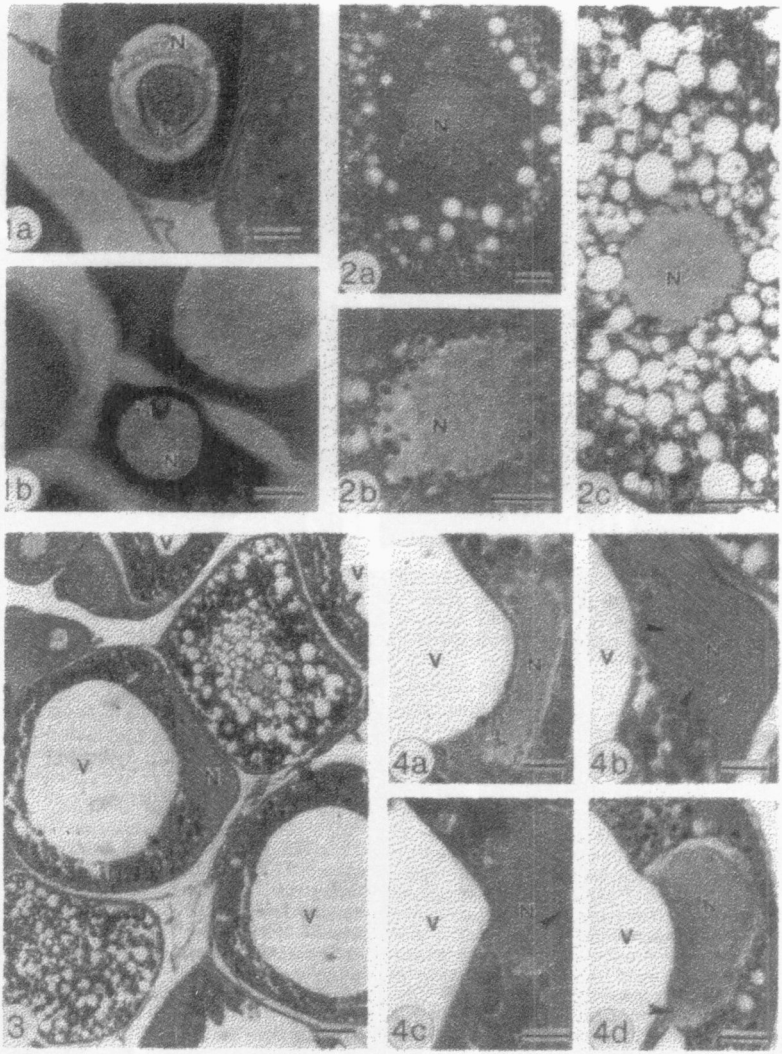


Fig. 4.9. Various stages of oogeneis (Jackson et al. 1994). **1a**: Perinuclear oocyte with an intracellular spherical structure (is). N, nucleus; **1b**: The same as in 1a. Bar = 20 µm. **2a**: Nucleus (N) of a cortical alveolar stage oocyte with irregular outgrowths of the nuclear periphery containing karyoplasm and nucleoli. Bar = 35 µm. **2b**. Similar to 1a at higher magnification. Bar = 30 µm. **2c**: Nucleus (N) of an early vitellogenic oocyte with rounded contours. Bar = 40 µm. 3: Maturating oocytes with apical lipidic vesicles (V). N = nucleus. Bar = 100 m; **4a**: Migrating nucleus (N) of a maturating oocyte at the periphery of the lipid vesicles (V); **4b**: Migrating nucleus surrounded by homogeneous ooplasm near the oocyte envelope. The nuclear envelope is not well defined (arrowhead); **4c**: Opposite the nucleus, the oocyte envelope invaginates slightly (arrowhead); 4d: Remnants of the disintegrating nuclear envelope between the nucleus and the ooplasm (arrowhead). Bar = 50 µm.

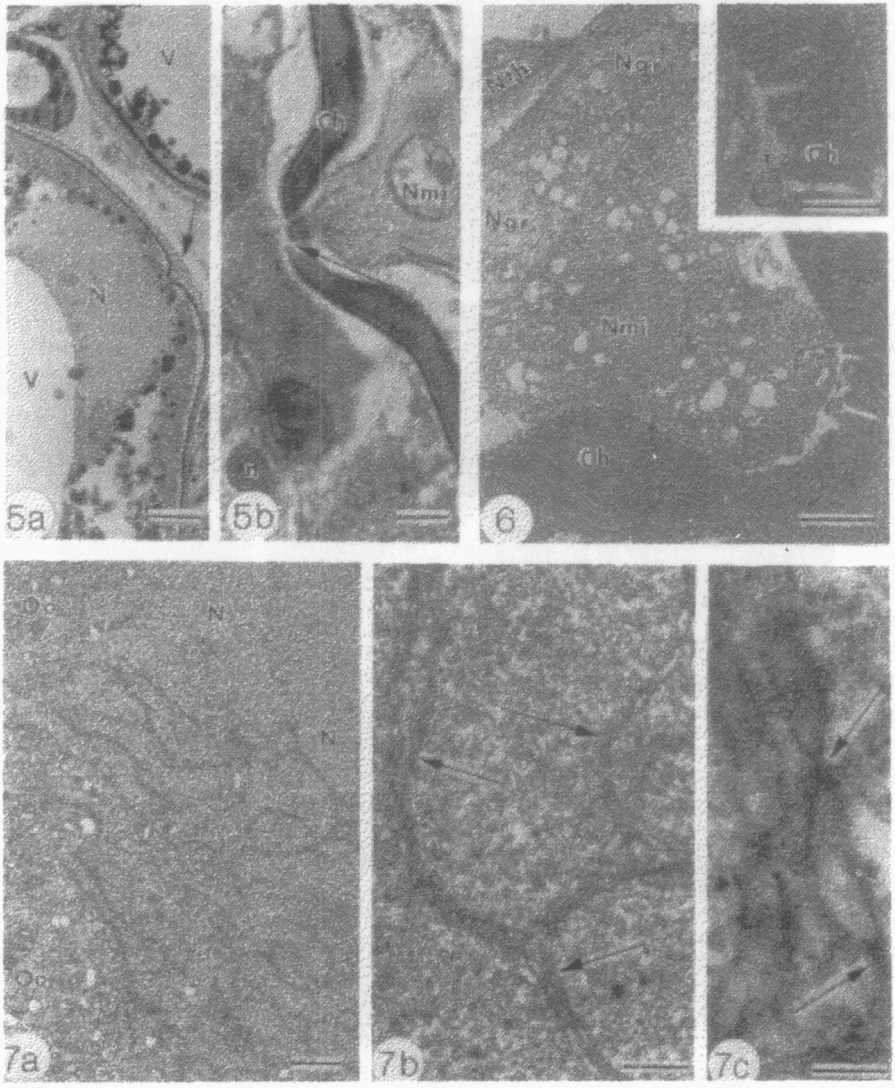


Fig. 4.10. The germinal vesicular migration to the periphery and break done (Jackson et al. 1994). **5a**: Micropyler cell (arrow) inside the invagination of the chorion. Bar = 10 μm; **5b**: As (a) at higher magnification. The micropylar cell blocks the micropyle (arrow) = nucleolus at the nuclear periphery, ch = chorion, Nmi = nucleus of micropylar cell. Bar = 5 μm; **6**: Cytoplasmic processes of the micropylar cell inside the chorion (ch), arrow points to a lisosome. Nmi = 5m. Inset: At a higher magnification. Arrow points to a lisosome. Bar = 2,5 μm; **7a**: Fragments of a disintegrated nuclear envelope at the boundary between nucleus and cytoplasm. Oo = Ooplasm; N = nucleus. Bar = 2 μm; **7b**: As in (a), the nuclear envelope is a double membrane enclosing an interspace pierced by nuclear pores (arrows). Bar 0.5 = μm; **7c**: As in (b), at very high magnification. Bar = 0.1 μm.

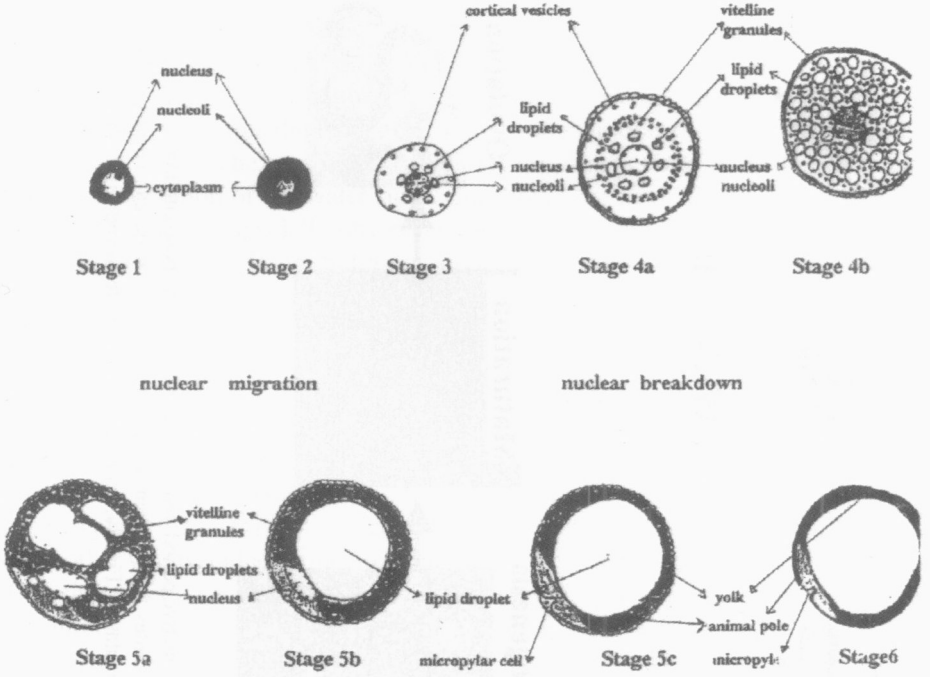


Fig. 4.11. Oocytes stages in *Trichogaster trichopterus*. **Stage 1**: Chromatin nucleolus-stage; **Stage 2**: Perinuclear stage; **Stage 3**: Cortical alveolar stage: Cortical vesicles appear at the periphery of the cell, and lipid droplets begin to appear at the vicinity of the nucleus. **Stage 4**. Vitelloogenesis **Stage 4a**: Dense stainded globules appear in the cytoplasm between the lipid droplets and cortical vesicles, **4b**: Lipid droplets and densely stained globules are dispersed homogeneously in the whole cytoplasm. **Stage 5**: Oocyte final maturation involving, 5a: nuclear migration and beginning of lipid and dense globules condensation; **5b**: End of the nuclear migration and appearance of the micropylar cell at the animal pole; **5c**: Nuclear breakdown; Stage 6: Fully developed oocyte after ovulation when the oocyte leaves the membrane envelope, the micropyle, a thin channel is found at the animal pole (Jackson, 1998).

heavily planted with a large mass of java fern resting on the bottom, and floating water sprite covering about 80% of the surface. The pH was maintained at about 6.0–6.5. The water was kept fairly warm, 81 °F (27 °C).

The male was placed in the breeding tank and left alone for 24–72 h, during which time he may or may not have blown a bubble nest. Only the males which have built nests are ready for breeding.

After this "bachelor" period, the female was introduced into the breeding tank, and the male would be expected to start courting her almost immediately. Courtship takes the form of very conspicuous displays of color and fins (this is very interesting to watch), and also involves what can amount to serious pummeling of the female (Degani, 1993a).

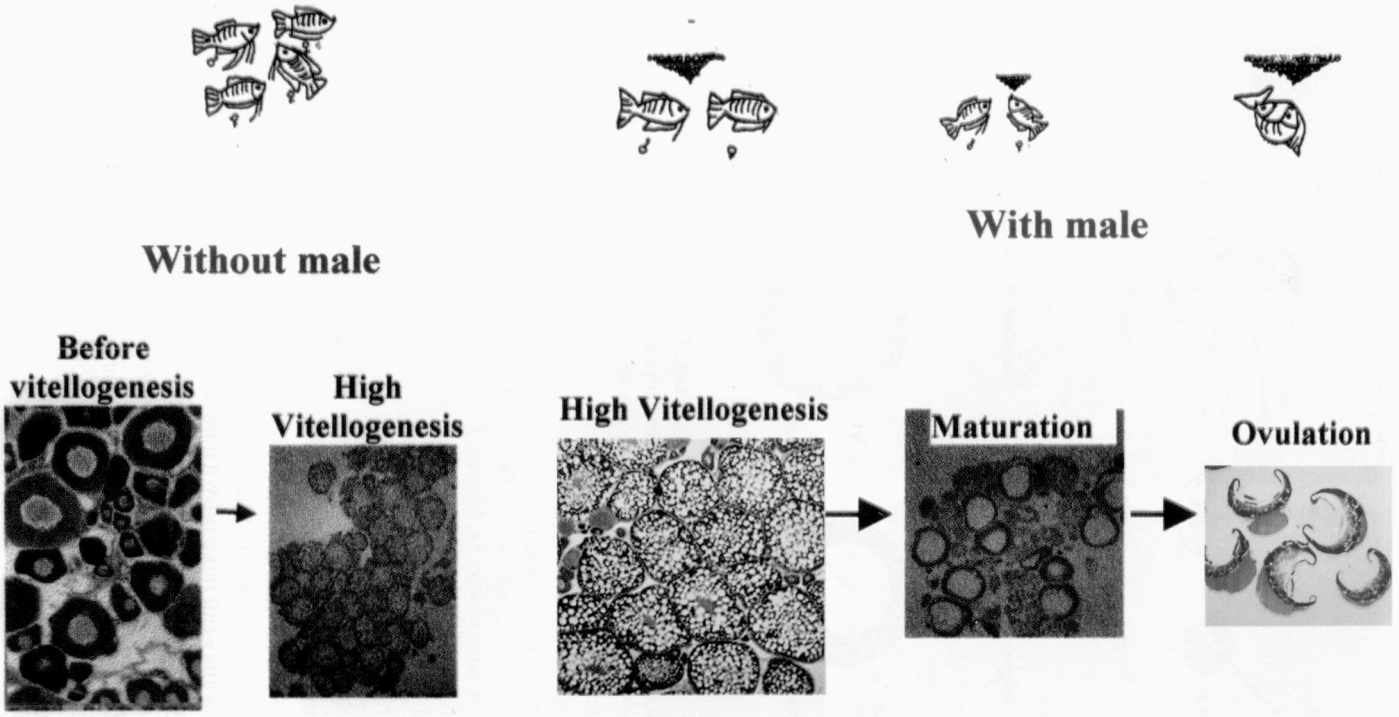


Fig. 4.12. Model of blue gourami reproductive pattern:During the non-reproductive period, females are arrested at the post-vitellogenic stage). Females kept in a big group stay in this condition indefinitely. Only females that are put in the presence of a male can complete the reproductive cycle. Males sexual behavior induces oocyte maturation and ovulation. Finally, explelled oocytes are fertilized before they reach the bubble nest on the surface of the water.

Spawning usually takes place in the small hours of the morning, so anyone wishing to see it should plan on getting up early and should not cause any disturbance around the tank (like people, the fishes don't especially like to be disturbed in the act). The male grips the female in the spawning embrace and literally squeezes the eggs out of her. The eggs float to the surface and are put into the nest by the male. Careful observation of the water surface/nest over the next 72 h should reveal the eggs, and by being especially observant you may be able to see the female looking thinner and well beaten-up, hiding in a corner or behind a plant. The eggs themselves look like tiny oily droplets, each about the size of a pinhead, and it can be especially difficult to recognize them if there is a substantial nest.

4.7 BLUE GOURAMI AS A MODEL FOR STUDYING FISH REPRODUCTION

In nature, the blue gourami, *Trichogaster trichopterus*, breeds year-round. Also under laboratory conditions, females could be induced to reproduce at any time, given the appropriate essential conditions. In this species, the presence and behavior of the male is the factor that induces the adult female to complete the ovarian cycle or, in other words, induces final maturation and ovulation in the female. This makes *T. trichopterus* a very useful model for studying the control of maturation in fish. The influence of male behavior on female readiness has been studied previously by Cheal and Davies (1974), who concluded that in this species the induction of spawning readiness is more susceptible to social environmental variables in females than it is in males. In our study we found that in 24 out of 35 male–female pairs (68.5%) in the first experiment (Degani, 1993a) and in five of 11 pairs (45%) in the second experiment, oocyte maturation was observed in the ovaries, whereas in the females-only aquarium, i.e., the gynaeceum, or in females kept in solitary confinement, the oocytes never passed the vitellogenic stage. The failure of some pairs to produce fertilized eggs may have been due to factors other than the absence of mature oocytes, since no morphological differences were found between the ovaries of such passive females and those of active ones which achieved spawning. In the second experiment, in which fish were killed after 24 h, maturation in additional pairs could have taken place on the second or third day, so the percentage of females that had the potential to reach maturation may be higher than the above figures.

Spontaneous ovulation from fully developed females did not occur; the female spawns only in the presence of a male. When a pair is transferred into an aquarium, the mature female will usually spawn after a period of 24–72 hours. The induction of oocyte maturation by the presence of the male may be due to visual, mechanical, or olfactory stimulation. In *Clarias gariepinus*, the presence of males enhances ovarian growth, both under hatchery conditions and in nature (Henken et al., 1987). In this species, pheromonal steroid glucoronides act upon the olfactory system, which stimulates the nucleus preopticus of the hypothalamus, where the gonadotropic hormone-releasing hormone producing cells are located (Resink, 1988). The emis-

sion of pheromones and induction of gonadal development are modulated by interaction between the sexes (van Weerd and Richter, 1991). Male stimulation of ovarian development is also brought about by tactile cues (van Weerd et al., 1991). In the goldfish, prostaglandin elicits male courtship behavior and female spawning behavior (Sorensen et al., 1987). In the zebrafish, females fail to ovulate when isolated from males, but resume ovulation when exposed to water containing males (Stacey et al., 1987). The influence of male pheromones on oocyte maturation, which is a gonadotropin-dependent process, was investigated by Scott and Canario (1992), who found that 17α,20β-dihydroxy-4-pregnen-3-one induced oocyte final maturation in teleosts, and male pheromones were found in holding water of *T. trichopterus* by Becker et al. (1992). The steroid that induces maturation in the female *T. trichopterus* is 17α,20β-dihydroxy-4-pregnen-3-one (Degani and Boker, 1992a,b). The participation of pheromones in the reproduction of anabantid species has already been established by Mckinnon and Liley (1986), who determined that adult males can discriminate between the presence of ripe and nonripe females and between nonripe females and plain water, through the recognition of female pheromones in the water. It seems that in *T. trichopterus*, visual, chemical, and tactile cues together are essential to induce the onset of final oocyte maturation by the male. In some preliminary experiments, when the parameters were isolated, oocyte maturation in female did not take place. For example, oocytes in final maturation were not detected in ovaries of females that were placed in water in which an active male had built a nest, nor in females that were separated from males by a transparent barrier. The reproductive pattern of *T. trichopterus* is illustrated in Fig. 4.11.

Kobayashi and Yamamoto (1985) divided the sequence of oocyte maturation into four stages determined by the distance of the migrating nucleus from the oocyte periphery, nuclear breakdown, and the extrusion of the first polar body. In *T. trichopterus* oocytes, the sequence of final maturation is divided into five steps (Jackson et al., 1994):

Step 1: The rounded nucleus has already left the central position which it had occupied during the previous stages of oocyte growth and vitellogenesis. The coalescence of lipid droplets has begun, but the central lipid vesicle is not yet fully formed.

Step 2: The slightly concave and elongate nucleus is now located at the outer rim of the lipid vesicle bordering the vitelline globule region.

Step 3: The nucleus that has reached the oocyte periphery is embedded in a mass of clear, homogeneous cytoplasm that had migrated together with the nucleus, and is distinct from the ooplasm containing the vitelline globules. The zona radiata opposite the nucleus invaginates slightly and large micropylar cells occupy the pocket thus formed.

Step 4: Fragments of nuclear membrane appear in the cytoplasm, heralding the first

step in nuclear breakdown. The shape of the nucleus is still distinctive, because of the difference in consistency and hue between the nucleoplasm and ooplasm.
Step 5: Resumption of meiosis and extrusion of the first polar body.

During step 3, the vitelline membrane opposite the nucleus thins out, elongates, and invaginates toward the ooplasm. Inside the invagination, a large follicular cell, the micropylar cell, appears which elongates toward the vitelline membrane, pushing it inward and sending thin cytoplasmic processes into the zona radiata. In the central region of the invagination, thin cavities are observed that cross the breadth of the vitelline membrane, as a prelude to the formation of the micropyle, which identifies the animal pole (Hart, 1990). The fully formed micropyle now contains coalesced cytoplasmic processes inside the zona radiata. The micropylar cell harbors large numbers of lysosomal and phagosomal structures: the cellular organelles which, presumably, are involved in the piercing of the micropyle. Yaron (1971) has suggested that the micropylar cell digests a channel through the chorion, thus forming the micropyle. Other authors suggest that the micropyle is formed concurrently with the differentiation and growth of the chorion during oocyte development (Riehl, 1977; Hart, 1990). The micropylar cell is generally considered to be a very large granulosa cell, with a cytoplasmic process extending into the micropylar canal. The micropylar cell was studied by electron microscope by Riehl (1977), Takano and Ohta (1982), and Kobayashi and Yamamoto (1985), among others. The micropyle consists of a vestibule, which is a large invagination of the vitelline membrane occupied by the bulk of the micropylar cell, and the micropylar canal occupied by the cell processes. After retraction of the micropylar cell during ovulation, the micropyle is open and functional (Kobayashi and Yamamoto, 1985). After fertilization the micropyle closes. The closure of the micropyle inhibits sperm and external pathogens from penetrating into the perivitelline space and seems to be involved in preventing polyspermy and bacterial infection (Yamamoto and Kobayashi, 1992).

There is a functional relationship between nuclear breakdown and the formation of the oocyte micropyle in *T. trichopterus*. Nuclear migration, invagination of the vitelline membrane, and appearance of the micropylar cell are temporally related. As long as females are confined to the gynaeceum these processes are inhibited. Release from the inhibition is triggered by the presence of a male. The remarkable convergence between nuclear migration and micropyle formation raises the question of whether the nucleus migrates haphazardly, with the micropyle forming wherever the nucleus touches the oocyte periphery, or whether the migration path, animal pole, and site of the micropylar cell are determined at an earlier stage. Takashi (1992) studied the development of hair-like structures, the so-called attaching filaments, which are characteristic of the vegetal pole of teleost eggs, and found that the appearance of the vegetal pole occurs prior to the initiation of vitellogenesis, indicating that the animal and vegetal poles of the egg are determinated previously.

It should be emphasized that no invagination of the vitelline membrane or other signs of micropyle formation have ever been observed in the ovaries of females before pairing. The precision of the migration track is noteworthy, since the final location of the nucleus and of the cytoplasm surrounding it are important for the future events of embryonic development (Amanzo and Iyengar, 1990). In some species of lower vertebrates GVBD may commence before GVM has been completed (Brachet et al., 1970), while in others the GVBD starts only after completion of GVM. This is the case in the blue gourami, where intact GV was found at the periphery of the oocyte after GVM had been completed.

The ovaries of *T. trichopterus* are asynchronic, and the oogenetic process conforms to the general pattern described by Nagahama (1983) and reviewed by Wallace et al. (1987). Six different stages were found. During the growth phase of the oocyte (vitellogenic stage), there is an accumulation of cytoplasmic inclusions, of which three different types have been found: yolk vesicles, lipid droplets, and yolk granules. The yolk vesicles, which are the first to appear, react positively to PAS, indicating the presence of mucopolysaccharides. These vesicles are involved in the process of fertilization, when they expose their contents in the perivitelline space, and they are not considered true yolk. The main deposits found in the vitellogenic oocytes are the lipid droplets, whose formation is concomitant with the appearance of the yolk vesicles. These lipid droplets first appear at the periphery, and later occupy most of the cytoplasm. The positive response of the droplets to osmium indicates the presence of unsaturated lipids. The large quantity of lipids in the egg makes it buoyant, enabling it to adhere to the underside of the bubble nest. Sand et al. (1969) determined that 37% of the weight of the ovaries of mature gouramis consists of wax ester. Lipid droplets remain separate until maturation, when they begin to coalesce until a single droplet, located centrally, is found at the end of the process. The high lipid content and buoyancy of the egg may be seen as an adaptation of such species to living in water with a low oxygen content, which necessitates staying on the surface of the water, where more oxygen is available. The last inclusions to appear in the cytoplasm are the yolk granules. These acidophilic granules contain protein and are interspersed among the lipid droplets as individual granules. After the migration of the nucleus, during the process of maturation, they also begin to coalesce, and ultimately become reorganized as a homogeneous mass around the single lipid droplet. Iwamatsu et al. (1992) demonstrated that during final maturation there are changes in the electrophoretic distribution pattern of the yolk proteins, which could be the result of rearrangement of proteins during and after GVM and GVBD.

During the maturation process, fragments of double membranes, enclosing a 20–50-nm-wide perinuclear space and penetrated by nuclear pore complexes, appear in the ooplasm, mainly in the region that surrounds the nucleus. The structure of these membrane fragments is similar to that of the nuclear envelope, which disintegrates

at the end of the prophase of the first meiotic division (Franke et al., 1981; Nigg, 1988). There is no direct evidence that the membrane fragments observed in the ooplasm of *T. trichopterus* originate from its nuclear envelope. Nuclear envelopes and annulate lamellae are very similar in structure, and annulate lamellae have been described in both normal and degenerating oocytes of several fish species (Kessel, 1983, 1992). Kessel (1992) considers the annulate lamellae to be widely distributed in oocytes of all major animal groups. The main difference between annulate lamellae and the nuclear membrane is that the former usually appears as many-layered sheets of stacked membranes, whereas the nuclear envelope is formed of a single layer of double membrane. Moreover, the membrane fragments in the maturing oocytes of *T. trichopterus* appear at the same time as the nuclear membrane disappears from around the nucleus, which can be considered as circumstantial evidence that the membrane fragments of *T. trichopterus* are remnants of the disintegrated karyolemma.

In *T. trichopterus,* however, there are three exceptions to the common model of oogenesis in teleosts:

1. On several occasions in young females, at the end of the perinucleolar stage, nuclei of oocytes have been found to contain spherical or slightly polyhedral structures, consisting of a central granular region surrounded by a capsule. When it first appears inside the nucleus, this structure is similar to a large nucleolus, with cortical and medullar zones clearly delineated. Azevedo (1976) describes intranuclear membranous inclusions at the ultrastructural level in the oocytes of *Xiphophorus helleri.* At the light microscope level, these inclusions appear as small dots near the nuclear membrane; their shape is different from that of the spherical structures found in the oocytes of *T. trichopterus.* Interpretation of the role of the intranuclear structures in *T. trichopterus* is not easy: they may be *special sites of metabolic phenomena (Azevedo, 1976), of pathological events* (Bouteille et al., 1974), or of atresia (Humeau, 1968), or may be intranuclear parasites.
2. During the cortical alveolar stage, the nuclei of the oocytes appear to be distorted, with irregular outgrowths of the nuclear periphery containing karyoplasma and nucleoli, suggesting heightened nucleoli-cytoplasmic exchange. At the vitellogenic stage, the irregularities begin to disappear, and the nucleus returns to its characteristic rounded or elliptical form.
3. Maturing oocytes contain a large apical lipid vesicle, made by the coalescence of lipid droplets that were formed at the cortical alveolar stage. The coalescence of the lipid drops during oocyte maturation is characteristic of those bubble-nest builder species in which the fertilized oocytes float upwards into the nest without the assistance of the parents, because of their lessened specific gravity.

4.9 MALE GONAD DEVELOPMENT

The testes of blue gourami consist of globules enveloped by membranes, very close to which are spermatogenia cells which have no specific forms (Fig. 4.13) (Degani and Jackson, unpublished data). The immature sperm are found very close to the tubule wall and the mature ones in the middle of the tubule. Grier (1981) described the cellular organization of fish testes and spermatogenesis.

In fish, paired testes of about the same size and comprising 1–5% of body weight are located anteriorly in the body cavity, suspended from the dorsal body wall. Spermatogenesis is the process in which the single cell changes through a series of stages: primordial germ cell gonocyte spermatogonium primary spermatocyte spermatid mature spermatozoon (Callard, 1991).

In mature blue gourami males all the stages of spermatogenesis are found, and no differences in spermatogenesis have been found among the various stages of the reproduction cycle in male testes (Degani and Jackson, unpublished data) (Fig. 4.14).

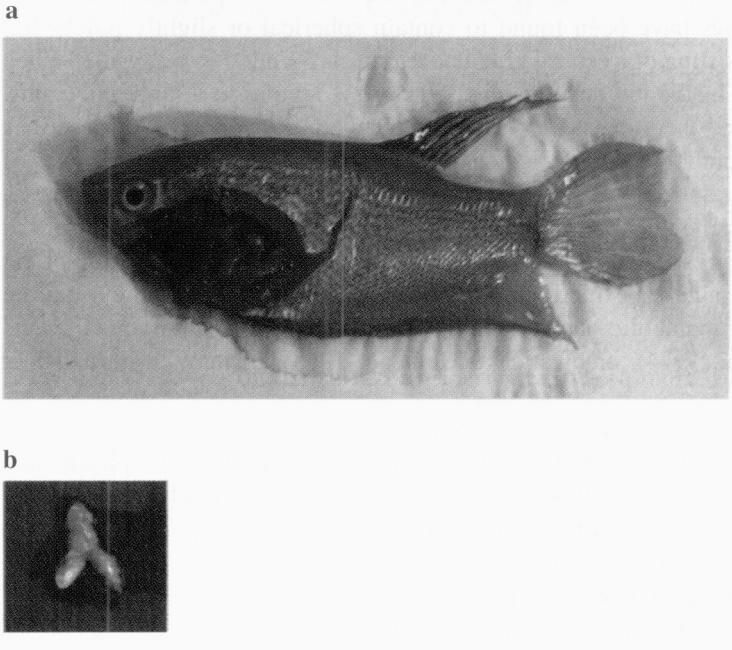


Fig. 4.13. Testes of male blue gourami, in situ (a) and isolated (b).

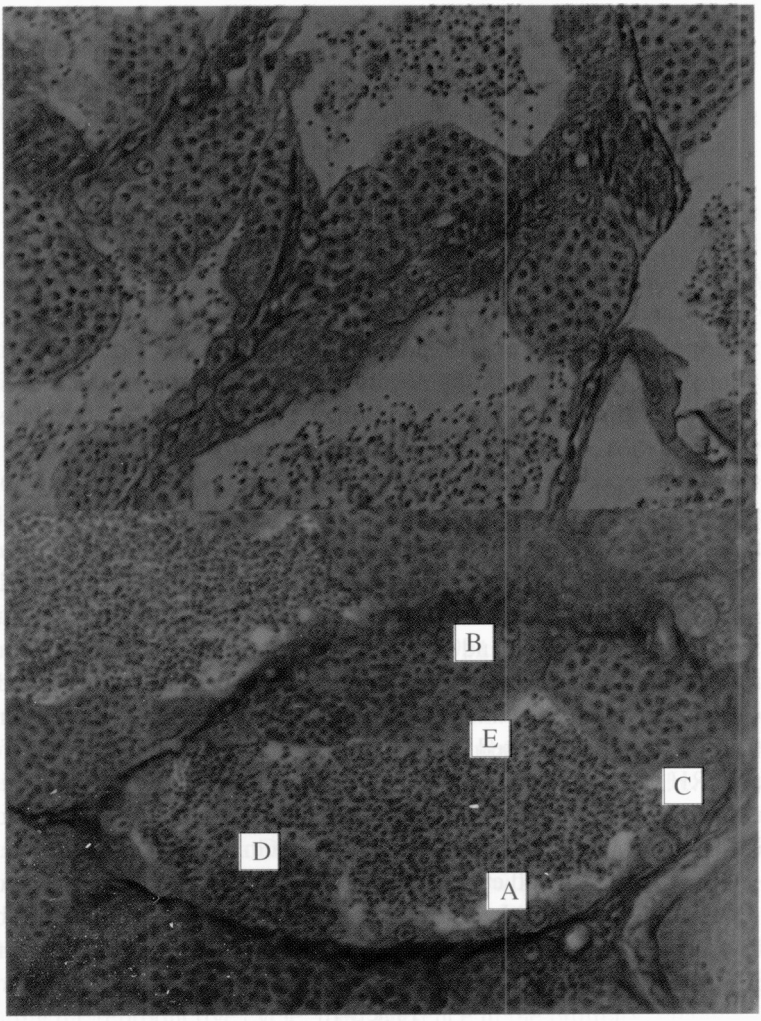


Fig. 4.14. Histology observation of blue gourmi testes. A is spermatogenia, B and C is first and second spermtocit, D is spermatid and E is spermes.

CHAPTER 5

SEX HORMONE IN THE PITUITARY GLAND OF BLUE GOURAMI

5.1 INTRODUCTION

Oogenesis and spermatogenesis in teleosts, as in other vertebrates, involve complex interaction along the brain–pituitary–gonad axis. The gonadotropin-releasing hormone (GnRH) controls the secretion of gonadotropins (GTHs) from the pituitary gland, which in turn control ovarian development. This process occurs under the mediation of the ovarian steroids, from the early growth of primary oocytes to vitellogenesis, and through maturation to ovulation (Sherwood et al., 1989; Swanson, 1991).

Gonadotropin-releasing hormones regulate the reproductive cycle in all vertebrates by stimulating the secretion of GTH from the pituitary gland (King and Millar, 1992a). GnRHs are decapeptides synthesized in the hypothalamus and transferred to the pituitary gland by neurosecretory axons. GnRH was originally isolated from pig (Matsuo et al., 1971) and sheep hypothalami (King and Millar, 1992b). Today, in addition to this GnRH, now called mammalian GnRH (mGnRH), nine others have been isolated and characterized (see Table 1). Two forms of chicken GnRH (cGnRH-I and cGnRH-II) were isolated from the hypothalamus of chickens by King and Millar (1982a,b), and further forms of GnRH have been identified in the brains of fish species: salmon (sGnRH) (Sherwood et al., 1983), lamprey (lGnRH) (Sherwood et al., 1986), catfish (cf GnRH) (Ngamvonchon et al., 1992), dogfish (dgGnRH) (Lovejoy et al., 1994), and striped bass (sbGnRH) (Gothilf et al., 1995a). All these forms are peptides in which residues 1, 2, 4, 9, and

Table 5.1.
Amino acid sequences of known GnRH forms.

	1	2	3	4	5	6	7	8	9	10
Seabream GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
Mammalian GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Chicken GnRH-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
Catfish GnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
Salmon GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
Dogfish GnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2
Chicken GnRH-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
Lamprey GnRH-I	pGlu	His	Trp	Ser	His	Rsp	Trp	Lys	Pro	Gly-NH_2
Lamprey GnRH-III	pGlu	His	Tyr	Ser	Leu	Gku	Trp	Lys	Pro	Gly-NH_2

10 are conserved, while no. 8 is the most variable. In vertebrates, there is ample evidence that more than one form of GnRH may be found in a single species (Sherwood and Coe, 1991; King and Millar, 1992; Sherwood et al., 1993; Sower et al., 1995). Three concurrent forms of GnRH have been detected in several teleosts: the guilthead seabream, *Spaurus aurata* (Powell et al., 1994; Gothilf et al., 1995b; Zohar et al., 1995); the African cichlid, *Haplochromis burtoni* (Powell et al., 1995); the *Lepomis gibbosus* (Powell et al, 1995); the lamprey (Sower et al., 1993); the snoek *Centropomus undecimalis* (Sherwood et al., 1993); and in *Prochilodus lineatus* (Somoza et al., 1994). Five different forms were found in the brain of spotted dogfish, *Scyliorhinus canicula* (D'Antonio et al., 1995).

sGnRH and cGnRH-II are the forms most commonly found in the teleost brain (King and Millar, 1992; Zohar et al., 1995). cGnRH-II is present as a second form of GnRH in all jawed vertebrate classes, with the exception of catfish; cGnRH-I is found only in reptiles and birds, whereas mGnRH has a wide distribution in vertebrates and has been found in some species of primitive bony fish (Sagerwood et al., 1991). cGnRH-II predominates in extrahypothalamic brain regions, while the other forms (sGnRH, mGnRH, or a specific GnRH) are more concentrated in the hypothalamus and reaches the pituitary (Yu et al., 1988; King et al., 1990; Okuzawa et al., 1990). However, in some cases, both forms reach the pituitary where they probably regulate hormone secretion (Yu et al., 1991). Two or more forms of GnRH are present in most species, with cGnRH-II as the most commonly present form (King and Millar, 1997).

The main biological role of GnRH is to stimulate gonadotropin release, but evidence has been found for other physiological roles, some of them related to reproduction:, it may transduce olfactory pheromones in reproductive behavior (Bond and Adelman, 1993), and enhance sexual receptivity (Moss and McCann, 1973). In fish, GnRH stimulates the release of growth hormone as well as GTH (Peter et al., 1990). GnRH occurs in the brain and other locations and may also act as a neurotransmitter, neuromodulator, or local hormone.

A number of characteristics serve to establish the involvement of GnRH peptides in regulating the reproductive cycle: their presence in the pituitary; changes in their levels during gametogenesis (especially during maturation); and their location in hypothalamic nuclei, which is related to the pituitary function.

The distribution of GnRH immunoreactive (ir) neurons varies among the many vertebrate species for which they have been described (for review, see Demski, 1984; Silberman, 1988; Muske, 1993). GnRH ir has been described in such areas as the midbrain, the ventral telencephalon of the terminal nerve (TN) area, the preoptic area (POA) in the midbrain tegmentum (TEG), and the spinal cord. In the dwarf gourami (*Colisa lalia*), a tropical fish also belonging to the Anabantid family, the ir-neurons are located in the TN, POA, and TEG; according to Oka and Ichikawa (1990), TN-GnRH cells project to wide brain areas, while POA-GnRH cells project

mainly to the pituitary. Yamamoto et al. (1995) used antiserum against sGnRH and cGnRH-II in studying the GnRH content in the brain of the dwarf gourami by reverse-phase high-performance liquid chromatography, followed by radioimmunoassay and immunohistochemistry. The TN nucleus reacted strongly against sGnRH and less strongly to cGnRH-II; only sGnRH was found in the preopticus nucleus (NPO), and a strong reaction to cGnRH-II was detected in the midbrain tegmentum. Axons derived from cells found there project mainly to caudal brain regions. In the pituitary, Yamamoto et al. (1995) found a strong positivity to sGnRH and only a small number of fibers which reacted to cGnRH-II. These fibers probably originated from the TEG cells.

Measurements of the expression of the mRNAs of several different GnRHs during the reproductive cycle have shown that different forms of GnRH regulate gametogenesis in different teleost species. In most vertebrates, including a number of fish, only one form of GnRH is believed to be the endogenous gonadotropin releaser (King and Millar, 1992; Powell et al., 1994), though the other forms may also be involved in the reproductive process. In gilthead seabream, a daily spawning fish, the mRNAs of the three different GnRH forms found in the brain reach their highest level 8 h before the spawning, concomitantly with a GTH-II mRNA surge. It was hypothesized that the preovulatory GTH-II secretion and surge are induced by a surge of sbGnRH (the GnRH found in the pituitary), but that the other two forms may also somehow participate in the reproductive process (Gothilf et al., 1995a,b). It has been argued that, in African catfish (*Clarias gariepinus*) and in goldfish (*Carassius auratus*), two hormones are involved in releasing GTH from the pituitary (Peter et al., 1990; Schultz et al., 1993). Salmonids have two GnRH forms in the brain, but in rainbow trout (*Salmo gairdneri*) and masu salmon (*Oncorhynchus masu*), only sGnRH, and not cGnRH-II, has been found in the pituitary during gametogenesis, including final maturation (Okuzawa et al., 1990; Amano et al., 1992).

5.2 THE GENERAL ASPECTS OF THE PITUITARY

The pituitary of the gourami has no distinct stalk; there is a thin layer of neurohypophyseal tissue at the dorsal border of the gland. As in other teleosts, the adenohypophysis (AH) can be divided into rostral pars distalis (RPD), proximal pars distalis (PPD), and pars intermedia (PI). The neurohypophysis (NH) penetrates the AH in the middle of the gland, apparently dividing the PPD into two regions. The neurohypophysis sends branches to the three adenohypophyseal regions, branching out extensively in the PI. The NH can be separated into the anterior neurohypophysis (ANH), functionally related to the RPD and PPD, and the posterior neurohypophysis (PNH), related to the PI. The ANH is found in the dorsal boundary of the gland, and it inserts indentations between the somatotrophs of the PPD and the prolactin and ACTH cells of the RPD. The PNH has a main trunk,

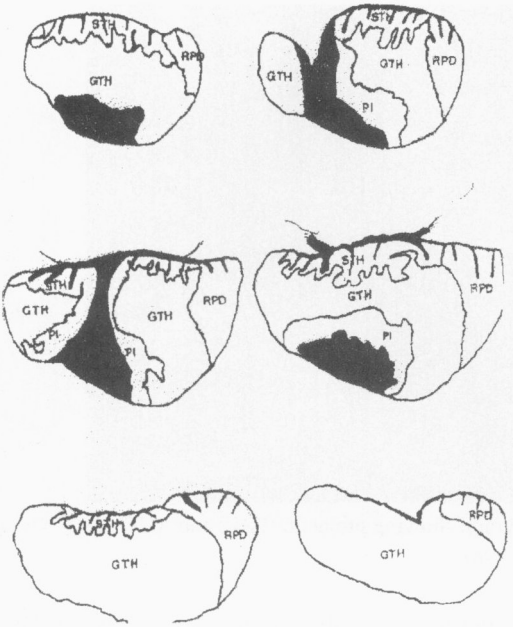


Fig. 5.1. Pituitary of blue gourami: Schematic representation of serial sections: STH = somatotropes (GH). GtH = gonadotropid cells. RPD = rostral pars distalis. PPD = proximal pars distalis. PI = pars intermedia. NH = neurohypophysis (Jackson, 1998).

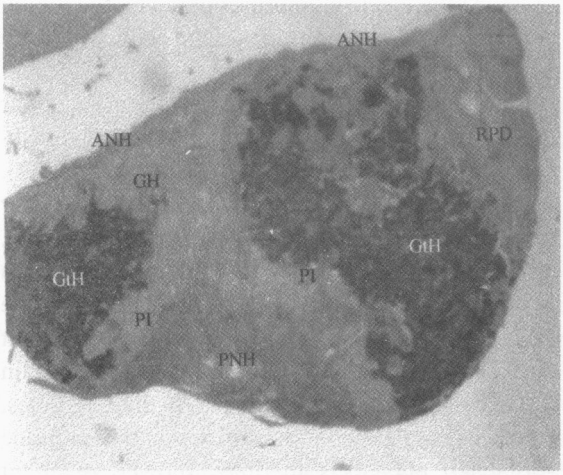


Fig. 5.2. Sagittal section of the gourami pituitary showing the different regions: PI = pars intermedia, RPD = rostral pars distalis, PNH = posterior neurohypophysis, ANH = anterior neurohypophysis and the localization of the 2 cell types of the PPD: GH = growth hormone and GTH = gonadotropic cells. AB-PAS-OG trichrome. (Jackson, 1998).

which penetrates the adenohypophysis in the middle of the gland in close relation to the PI, sending many branches to this adenohypophyseal region. In some places, the PNH is surrounded by one layer of big acidophilic cells (Figs. 5.1 and 5.2).

Sagittal sections, stained with PAS-AB-OG trichrome after oxidation with paracetic acid, revealed a well-defined RPD, which contained two different cell

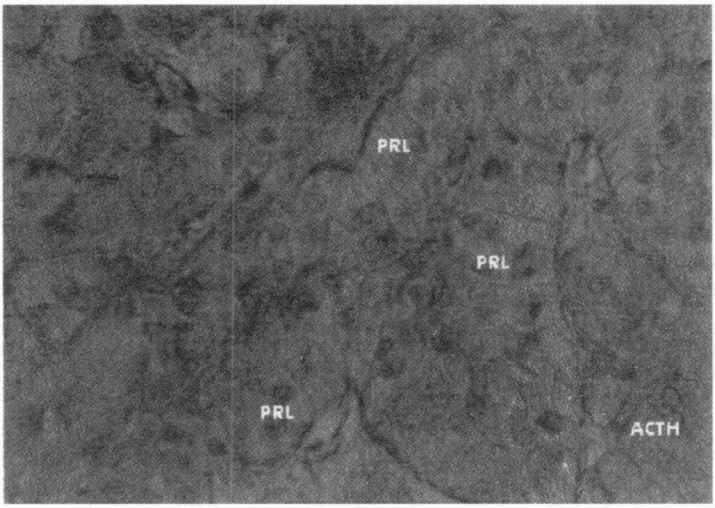


Fig. 5.3. Detail of the RPD of the blue gourami showing prolactin (PRL) and ACTH cells. Orange G-PAS-AB trichrome × 1000 (Jackson, 1998).

types, of which the first and more numerous was stained lightly by Orange-G. These acidophilic cells were elongated and are probably homologous to the prolactin (PRL) cells of other species (Fig. 5.3). At the electron microscopy level, the region of the PRL cells was easily discerned, since prolongations of stellate cells, which are characteristic of this region, were found between the membranes of the adjacent opposed PRL cells (Fig. 5.4). The structure of the PRL cells in the gourami is similar to that of related cells in other teleosts that have been described previously (Abraham et al., 1977; Nagahama et al., 1981; Naito et al., 1983; Rao et al., 1983). The most pronounced characteristic was the presence of a well-developed lamellar endoplasmic reticulum (RE) at the periphery. Electrondense granules were found, usually accumulated at one pole of the cell; a round, slightly peripheral cell nucleus was observed (Fig. 5.5). In the RPD, a second cell type was always found lining the dorsal border of the neurohypophysis. These cells, round in shape, were stained light gray by PAS-AB-OG trichrome (Fig. 5.3), and dark blue by the hematoxylin technique; they are classified as ACTH cells and are characterized by their granules, which show a more electrondense core, separated from the surrounding membrane by an electronlucent hila. The elongated nucleus sometimes showed reentrant features and contained a peripheral nucleolus. The mitochondria were usually dispersed in small groups (Fig. 5.6). ACTH and PRL cells were found closely apposed to the penetrations of the anterior NH in the PPD (Fig. 5.4).

In adult specimens, the major part of the adenohypophysis consists of the PPD and PI. These two regions are closely related, and cells of one of them are sometimes found in the other. In middle sagittal sections, it was observed that the main trunk of

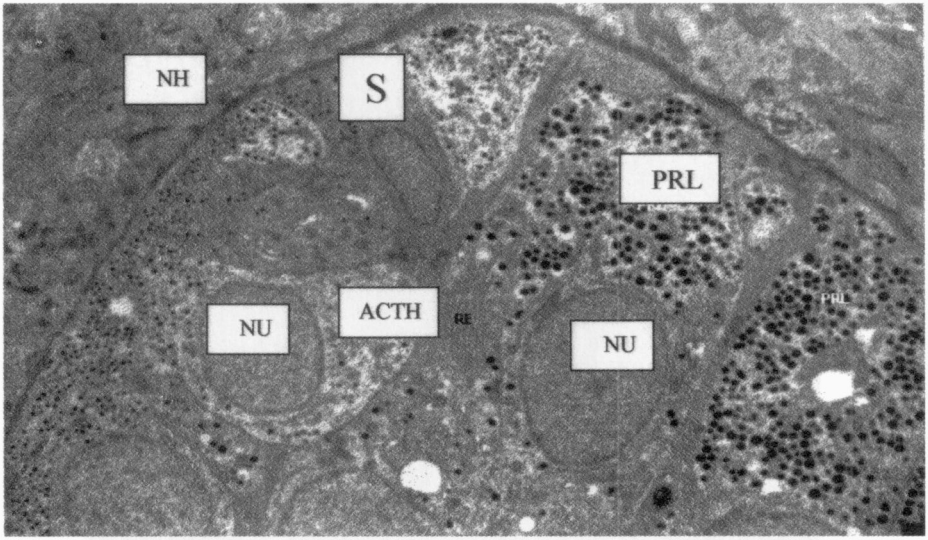


Fig. 5.4. Rostral pars distalis of the blue gourami pituitary. PRL = prolactin cells, ACTH = ACTH cells, NU = nucleus, RE = endoplasmic reticulum, NH = neurohypophysis (Jackson, 1998).

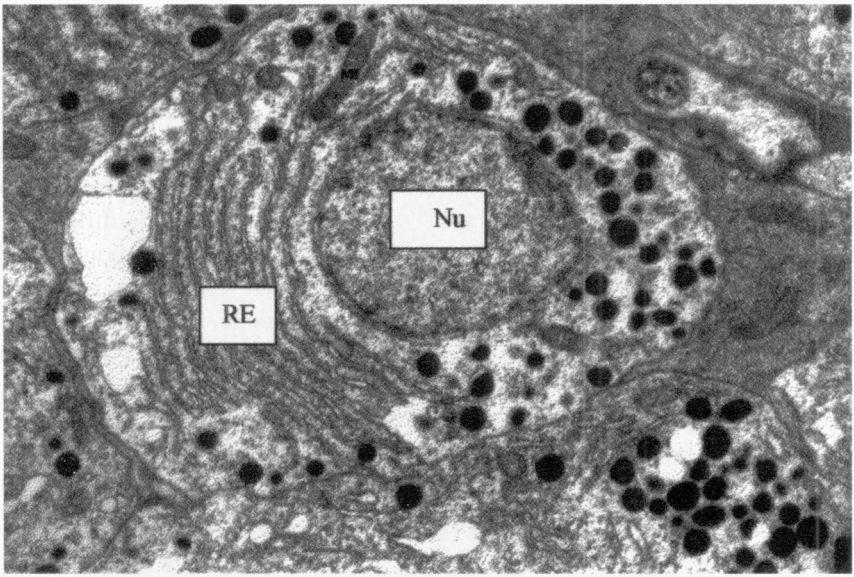


Fig. 5.5. Detail of the prolactin cell containing many secretory granules (arrow). Nu = nucleus, RE = endoplasmic reticulum, mi = mitochondria (Jackson, 1998).

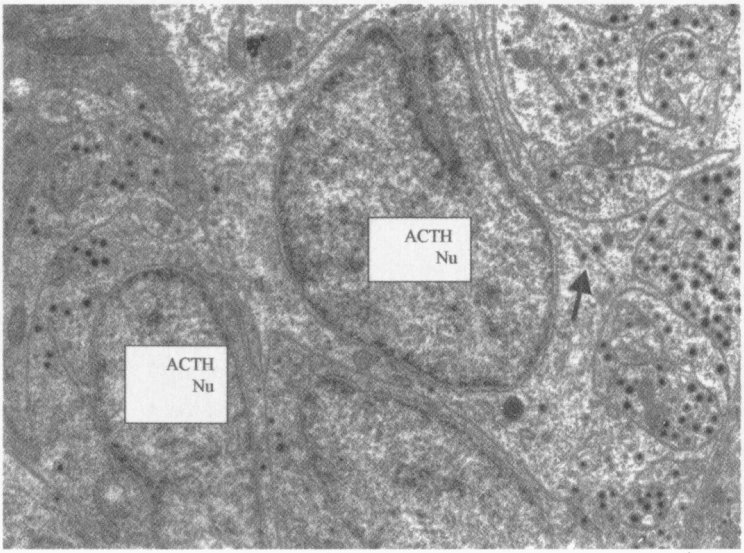


Fig. 5.6. Detail of the ACTH cell containing few secretory granules (arrows), Nucleus of ACTH cell (Nu) (Jackson, 1998).

the NH penetrated the adenohypophysis, apparently dividing the PPD into two regions (Figs. 5.1 and 5.2).

An acidophilic cell, strongly stained by Orange-G, and a basophilic cell, stained dark purple by PAS and AB stains, could be distinguished in the PPD (Fig. 5.7). The first cellular type was found in the dorsal part of the gland, where the neurohypophysis pushes indentations into the adenohypophysis; these cells, also distributed among the basophilic cells (Fig. 5.1), are classified as somatotrophs or growth hormone-producing cells. One layer of acidophilic cells, similar to the somatotrophs, was found bordering the branches of the neurohypophysis. The somatrotophs are round cells with a turgid appearance, containing a peripheral elongated nucleus (Fig. 5.7). Under the electron microscope, these cells are characterized by the presence of electron-dense granules, filling all the cytoplasm, which sometimes contains cytoplasmic inclusions comprising concentric lamellae; the nucleus is usually found at the periphery of the cell. A well-developed Golgi apparatus is also usually observed (Fig. 5.8).

The gonadotrophs form the majority of the PPD; they are scattered in the central and ventral parts, and also in the posterior edge of the gland. Antiserum against carp GTH-I and GTH-II β chain-reacted in the same way with all GTH cells (Figs. 5.9 and 5.10). GTH cells are large, rounded cells with a large, round, usually eccentric nucleus. They are stained purple by AB and PAS stains of PAS-AB-OG trichrome (Fig. 5.7).

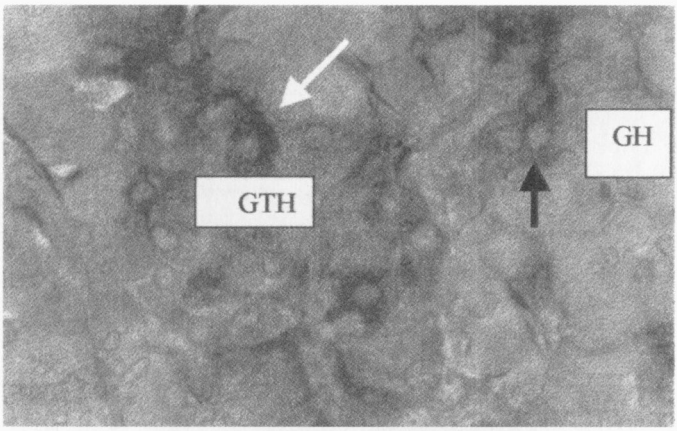


Fig. 5.7. PPD of the gourami showing two different cell types of growth hormone (GH) cell, gonadotropin cells (GTH). Nucleus of GTH cell (black arrow), nucleus of GH cell (white arrow). Orange G-AB-PAS (Jackson, 1998).

At the electron microscopic level, the GTH cells are easily recognized, because they are the only cell type in which globules, as well as the secretory granules, are present in the cytoplasm (Fig. 5.15).

A third cell type was found in the PPD; it was chromophobic and was found in the dorsal part of the gland, close to the GTH cells. Those chromophobes contain an elongated nucleus, which occupies approximately half of the cell volume (Fig. 5.11).

The PI contains two different cell types. The predominant one stains with lead hematoxylin and secretes MSH; in response to AB-PAS-OG trichrome, these cells

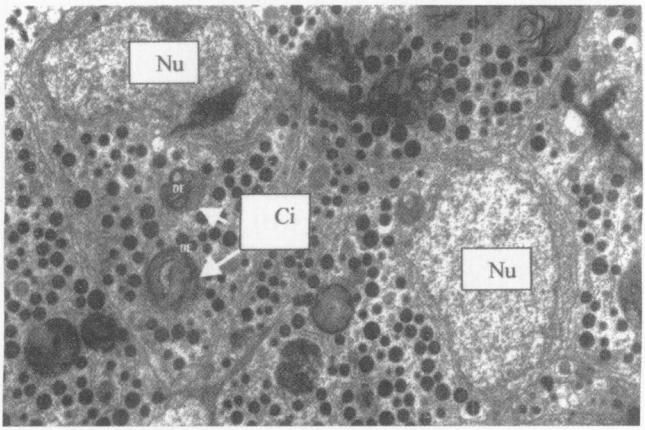


Fig. 5.8. GH cells containing many secretory granules (arrows). NU = nucleus, DE = Debris (Jackson, 1998).

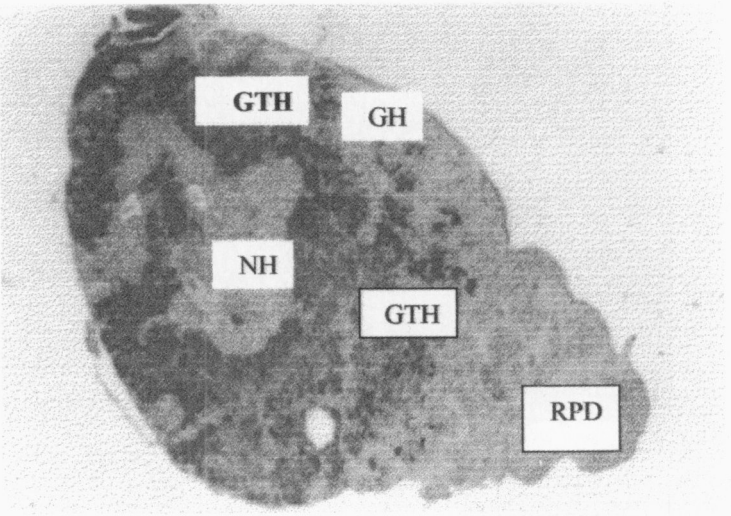


Fig. 5.9. Reaction to anti salmon GTH-I. GTH cells (GTH), Growth hormone cells (GH), Rostral pars distalis (RPD), Neurohypophysis (NH) (Jackson, 1998).

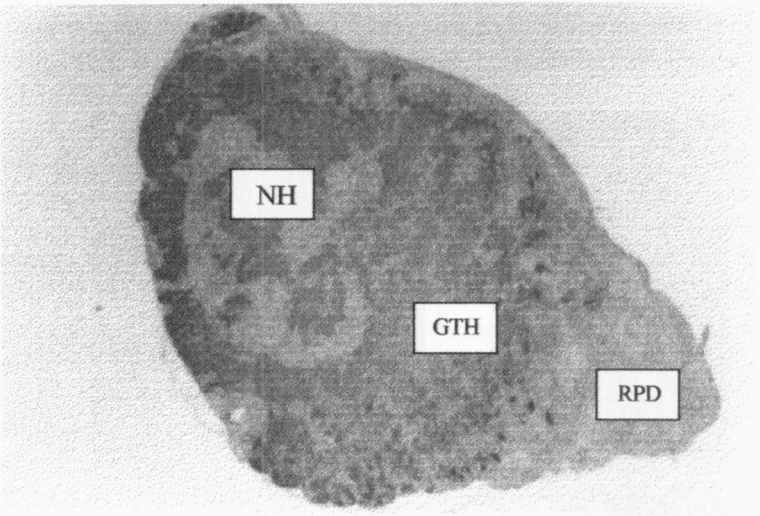


Fig. 5.10. Reaction to anti coho salmon GTH-II. GTH cells (GTH), Rostral pars distalis (RPD), Neurohypophysis (NH) (Jackson, 1998).

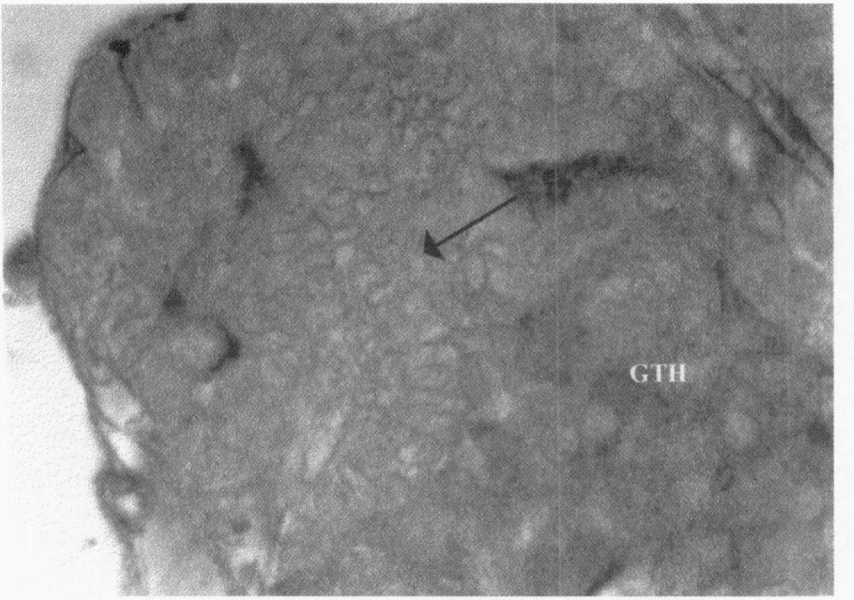


Fig. 5.11. Cell type found at the dorsal part of the PPD (arrow). PAS-AB-OG trichrome × 1000 (Jackson, 1998).

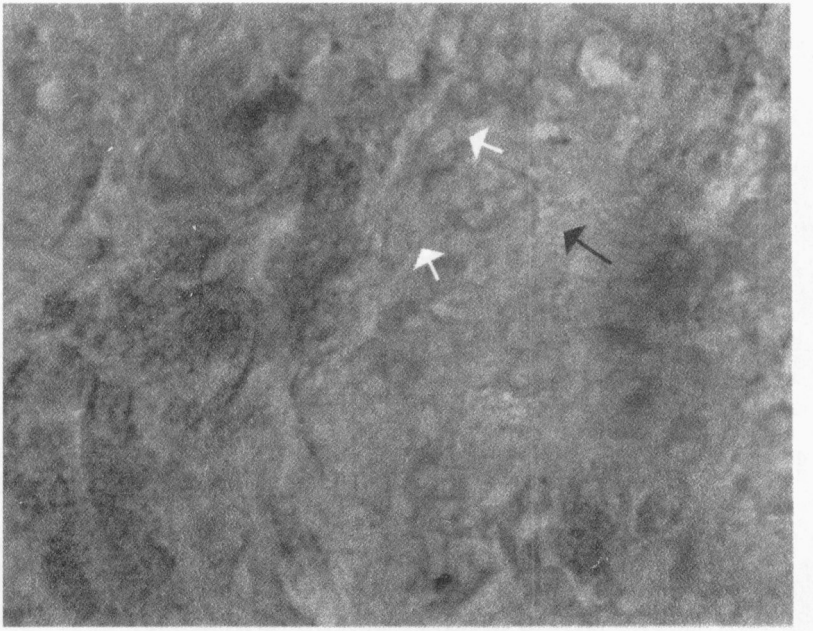


Fig. 5.12. *Pars intermedia* showing the two cellular types PAS cell (white arrow) and MSH cell (black arrow). PAS-AB-ORANGE-G trichrome × 1000 (Jackson, 1998).

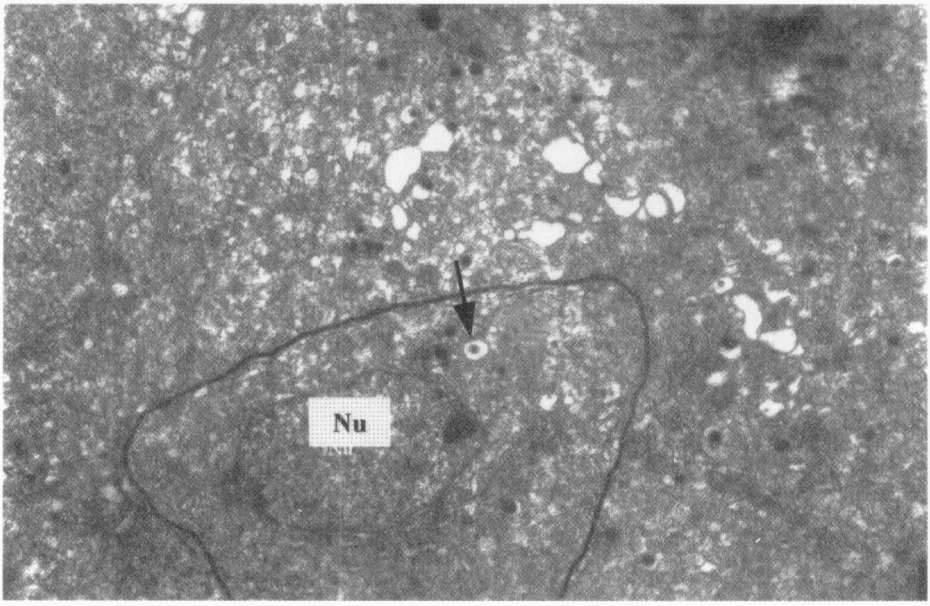


Fig. 5.13. MSH (Pb hematoxylin positive) producing cell showing nucleus (Nu) and very few secretory granules (arrow) (Jackson, 1998).

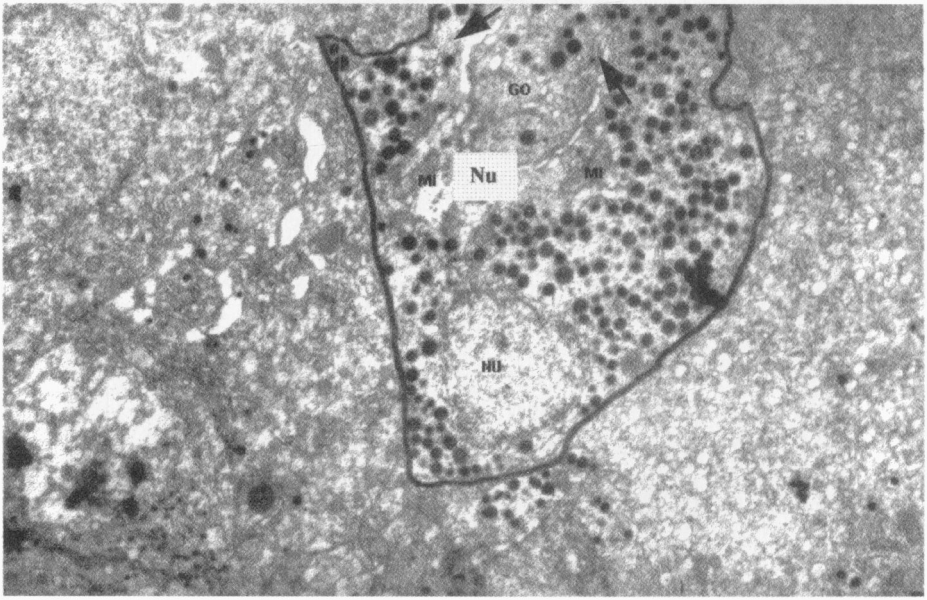


Fig. 5.14. Somatolactin (PAS positive) cell containing many secretory granules (arrow). Nucleus (Nu), Golgi (Go) (Jackson, 1998).

remain chromophobic. The other cell type is positive to PAS and probably corresponds to the somatolactin-secreting cells of other teleosts. The PAS-positive cells are often elongated and have a central round or oval nucleus. Under the electron microscope, they show a small endoplasmic reticulum (RER) and a small Golgi apparatus. The secretory granules are membrane-bound and show a slight variation in electron density. A round nucleus is usually found at the periphery of the cell (Fig. 5.14). The MSH-producing cell type shows a central nucleus with a small nucleolus. A few granules are found in the cytoplasm, which is filled with vesicles containing electrondense material. A small endoplasmic reticulum surrounds the nucleus (Fig. 5.13).

5.3 DYNAMICS OF THE GONADOTROPIC CELLS AND NEUROHYPOPHYSIS DURING THE SPAWNING PROCESS

5.3.1 FEMALES FROM THE GYNAECEUM (NON-REPRODUCTIVE FISH)

In a general view, the region of the GTH cells is well stained by PAS and AB, but more detailed observation shows that the cells in the central part of the gland are stained a lighter purple color with a pink shade, since these cells are stained pink by PAS. The cells found at the periphery are stained blue, since they are less positive to PAS. Under the electron microscope, stained GTH cells are seen to contain many secretory granules and large globules. The globules appeared to be internally homogeneous, and the globules and granules were dispersed homogeneously in the cytoplasm (Figs. 5.15 and 5.16). A second, less common cell type, was found sporadically dispersed among the predominant GTH type I cells; cells of this type were characterized by the presence of small secretory granules and the absence of large globules. Many empty vesicles were found dispersed in the cytoplasm (Fig. 5.16).

Neurosecretory material was found in the ANH, where the NH penetrates the AH, and in interdigitations of the neurohypophysis between the somatotrophic cells, but none related to the RPD was found in it. The PNH was filled with dense granulate neurosecretory material, which was found around PNH blood vessels related to the PI.

5.3.2 FEMALES AFTER PAIRING (REPRODUCTIVE FISH)

In the pituitaries of females that had started the maturation process, a general decrease in the staining of the region of the gonadotropic cells was detected. This decrease was not homogeneous throughout the GTH region. Fewer stained cells and even chromophobic cells predominated at the periphery (Fig. 5.18), whereas in the central portion of the PPD the staining pattern was more uniform. Some GTH cells of reproductive fish showed nuclei that were hypertrophic, compared with those of females that were kept in the gynaeceum (Fig. 5.19). In stained GTH cells of reproductive females the cytoplasmic content was seen to be concentrated at one pole of the cell, and this finding was confirmed at the electron microscope level: a

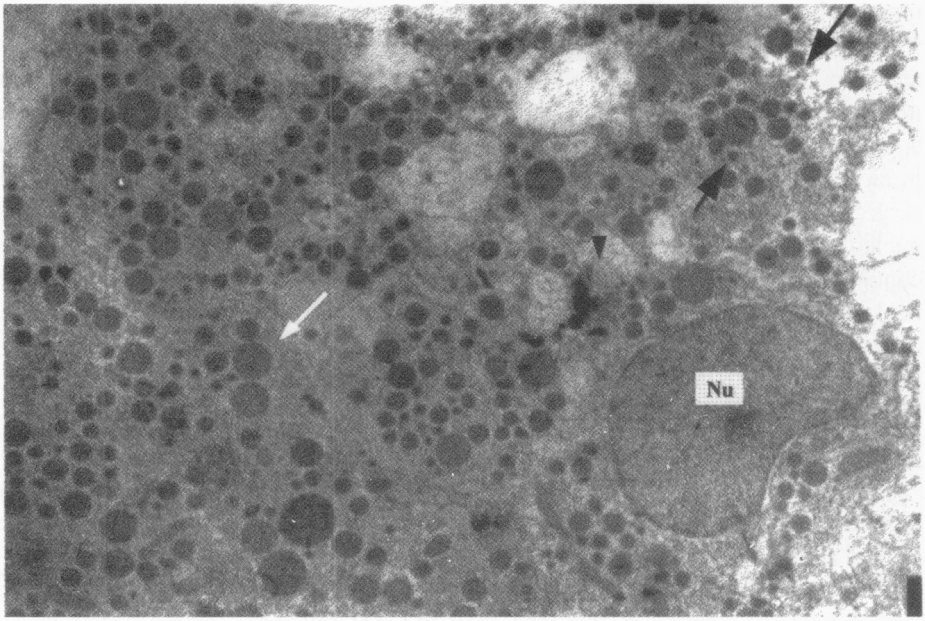


Fig. 5.15. GTH cell of non-reproductive fish showing big globules (white arrow), secretory granules (black arrow), and nucleus (Nu) (Jackson, 1998).

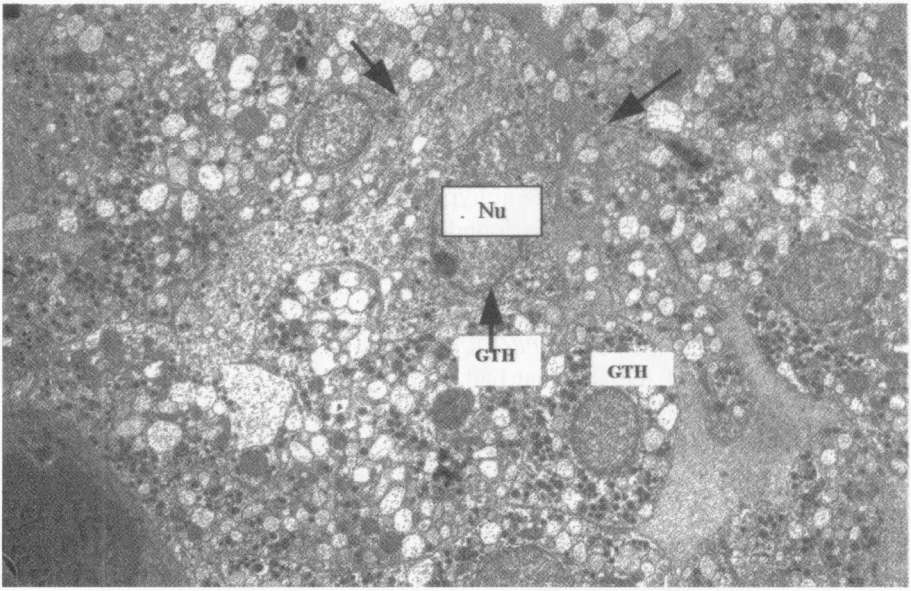


Fig. 5.16. Cell type (arrows) found among the predominant GTH (GTH) cell type in the PPD. Note the difference in the size of the granules. Nucleus (Nu) (Jackson, 1998).

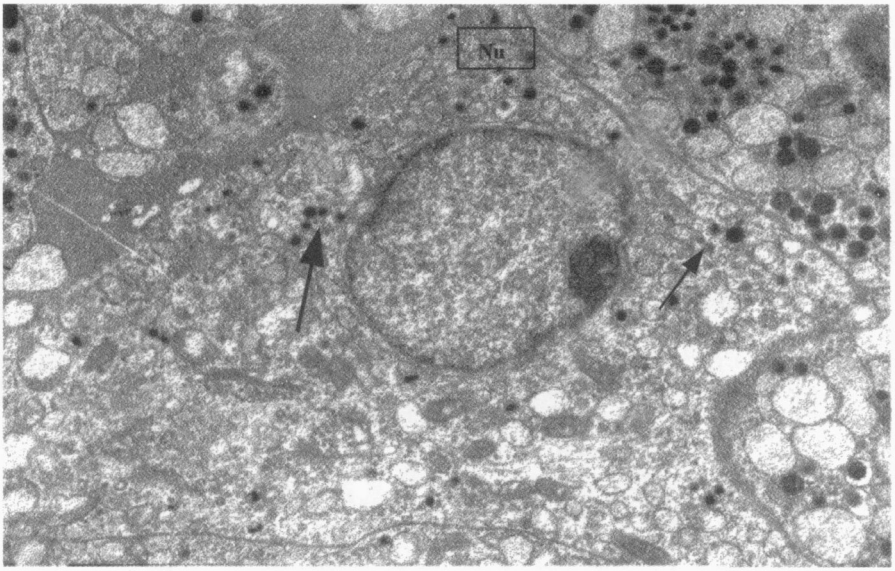


Fig. 5.17. Detail of the same cell type. Note the presence of small secretory granules (arrows) and the absence of the big globules (Jackson, 1998).

polarization of the electrondense granules was found in the GTH cells (Fig. 5.20). In females processed during final oocyte maturation (FOM) or after spawning, a concentration of secretory material was found around adenohypophyseal capillaries (Fig. 5.19).

Under the electron microscope, a decrease in the number of secretory granules in some GTH cells of females processed during FOM was detected (Figs. 5.20, 5.21). Large globules were still found, but they were fewer in number. GTH cells of females after spawning were characterized by the presence of large vacuoles formed by a confluence of cisternae of the endoplasmic reticulum (Fig. 5.22).

In fish processed after larvae were found in the nest (at least 48 h after spawning), the gonadotrophic cells showed a pattern of staining similar to that found in non-reproductive fish (Fig. 5.19).

5.4. THE RELATIONSHIP BETWEEN PITUITARY SEX HORMONE AND REPRODUCTION

Six cell types have been identified in the adenohypophysis of the blue gourami by immunocytochemical andhistochemical methods. The morphology of the pituitary gland and the distribution of the various cell types within the adenohypophysis are similar to the typical teleost arrangement (Ball and Baker, 1969; Holmes and Ball, 1974). The PI containes PAS positive cells and a second cell type which is positive

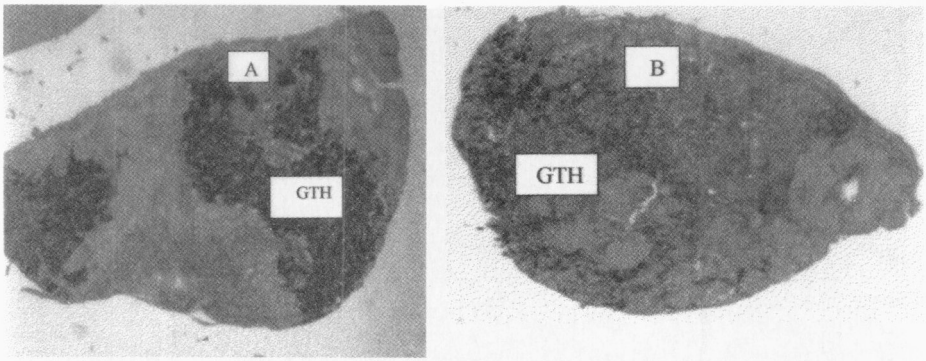


Fig. 5.18. Pituitary of fish before reproduction (A) and after spawning (B), showing the decrease in the staining of the PPD region. AB-PAS-OG trichrome (Jackson, 1998).

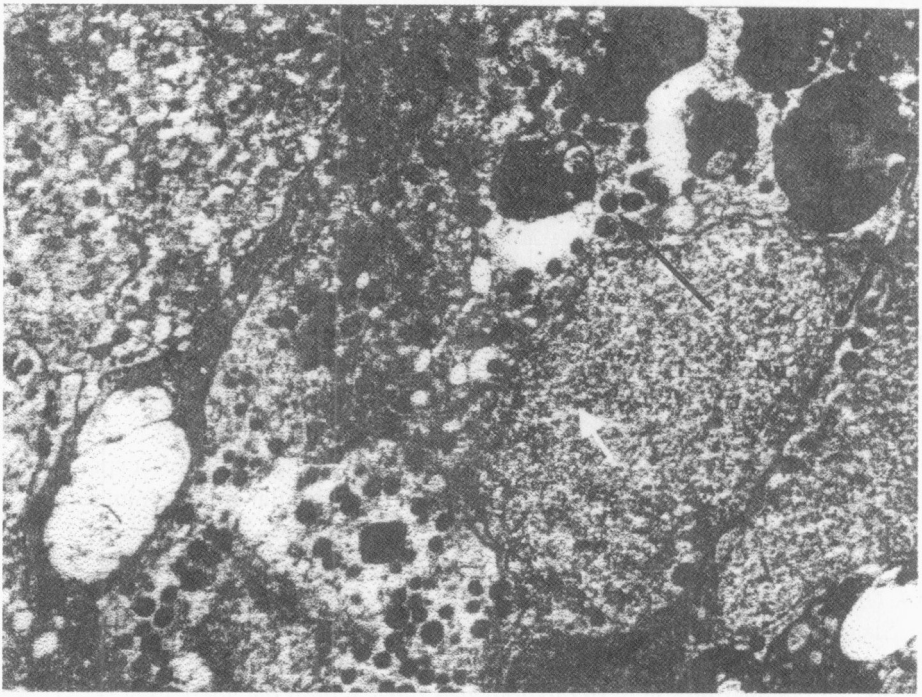


Fig. 5.19. Pituitary of fish killed just after spawning showing the decrease in the staining of the PPD region. AB-PAS-OG trichrome (Jackson, 1998).

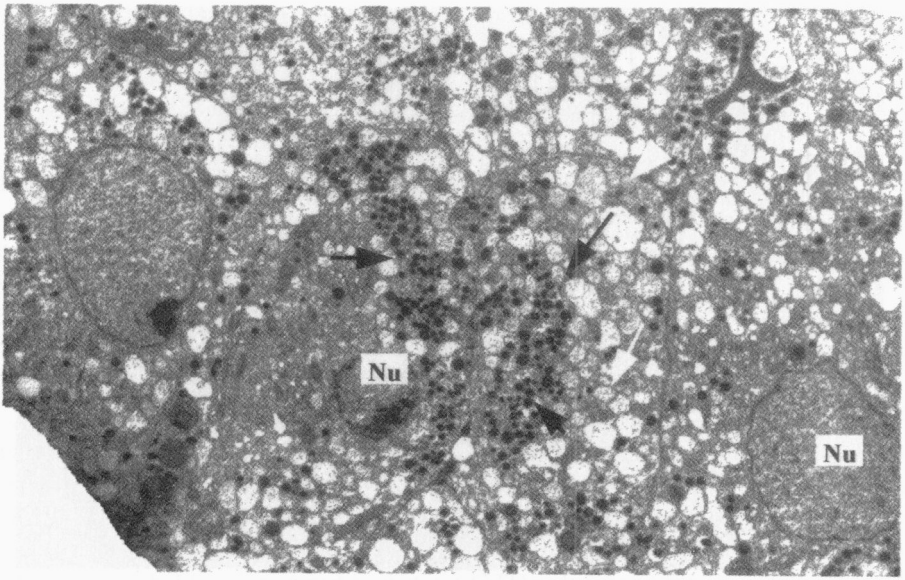


Fig. 5.20. GTH cells of reproductive fish before spawning, showing the concentration of the secretory granules at one pole of the cell (black arrow). Note the presence of many empty vesicles in the cytoplasm (white arrow). Nucleus (Nu) (Jackson, 1998).

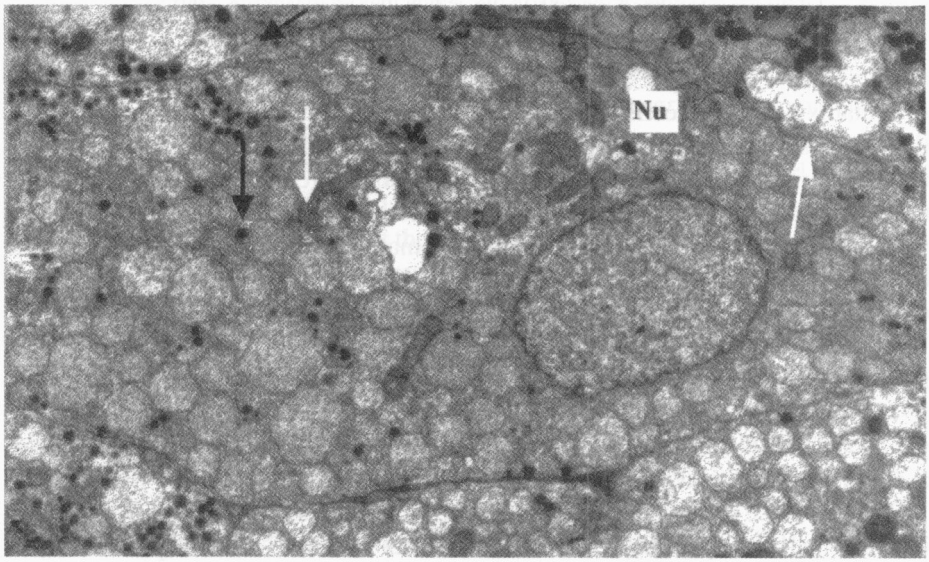


Fig. 5.21. GTH cell of reproductive fish after spawning, showing the decrease in the secretory granules (black arrow) and the increase of empty vesicles (white arrow) (Jackson, 1998).

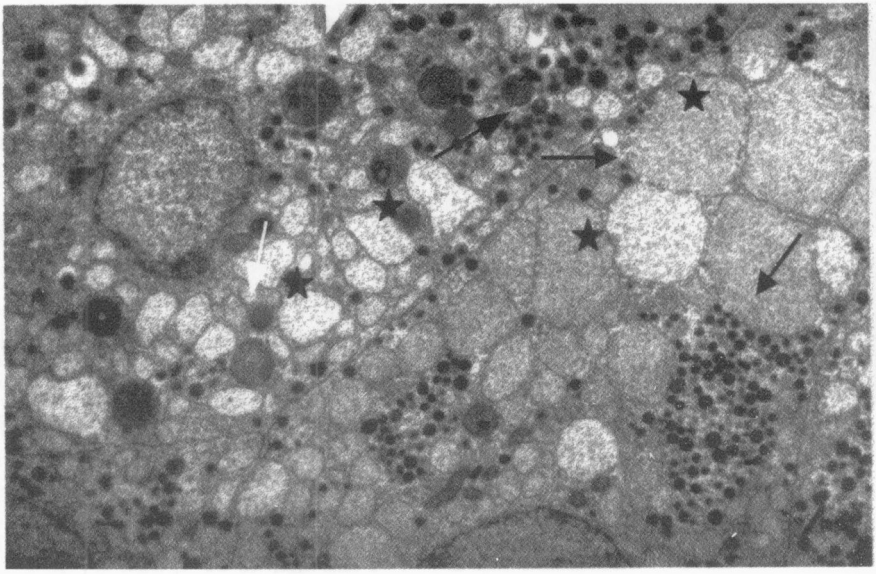


Fig. 5.22. GTH cells of reproductive fish after spawning. On the left, a GTH cell containing many globules (white arrow) together with secretory granules (black arrow) and empty vesicles (★). On the right, a GTH cell showing the big electronlucent regions which are the result of the coalescence of the empty vesicles (★). Secretory granules are concentrated at one pole of the cell (black arrow). Nucleus (Nu). Note the decrease in the number of globules and the concentration of secretory granules at one pole of the cell, in the cell on the right (Jackson, 1998).

to hematoxylin. These cells are the somatolactin- and MSH-producing cells, respectively. In the RPD, prolactin and ACTH cells were identified by staining and topographic characteristics. The PPD contained GH-producing cells (somatotrophs), the acidophilic cells found in the dorsal region, in close association with the branches of the neurohypophysis. Somatotrophs were also found along the external border of the NH, mainly around the main trunk. This unusual localization of the somatotrophs was not described in other perciform species (Jackson, 1998).

Today, it is well accepted that, like the tetrapods, teleosts produce two distinct gonadotropins in different cells of the pituitary (Olivereau, 1976, 1978; Naito et al. 1988, 1991; Nosaki et al., 1990b). However, the localization of the two gonadotrophs in the teleost pituitary is still controversial, and apparently varies according to the species (Nosaki et al., 1990a,b; Schreibman et al., 1990).

The sequence of the genes of two distinct GHT β chains described in Chapter 3 indicate that this species produces two distinct GTHs. Gonadotropic cells were found in the central and ventral parts of the PPD, the same location that has been described for other teleosts (Holmes and Ball, 1974). These GTH cells reacted

positively to the GTH-I and GTH-II anti-coho salmon gonadotropin. It was impossible to differentiate between GTH-I and GTH-II cells by immunocytochemistry alone.

On the basis of location and staining affinities, two different cell types were found in the PPD in addition to the somatotrophs, which may in fact be the two distinct GTH cell types. The first cell type is the typical basophiles of the PPD, which has been described in several teleost species (NEED REF.). These cells are stained by PAS and AB of PAS-AB-Orange-G trichrome or by the Aniline Blue of Mallory trichrome, and show changes in staining intensity dring the reproductive cycle. The second cell type is the chromophobic cells found inclose relation the the ANH in the dorsal part of the gland. These two cell types were defined, in salmon, as GTH-II- and GTH-I-producing cells, respectively. (Nosaki, 1990a).

Three distinct cell types were described in the PPD of the blue gourami at the EM level (Jackson, 1998). The first cell type is characteristically the somatotroph. The second is more abundant and is characterized by the presence of large globules, in addition to the secertory granules. The third cell type has a "foamy" appearance and is characterized bythe presence of dilated vesicles of the RE. The second and third cell bypes probably correspond to distinct GTH cells, and will be called GTH cell Type I and Type II. Corresponding descriptions of distinct GTH cell types have been given in other teleost species, e.g., salmon (Ueda et al., 1984) and rainbow trout (Naito et al., 1995). The nomenclature used by those authors was G (globular) cell for GTH type I, and V (vesicular) cell for GTH type II. It is assumed that the G cell is responsible for the production of GTH-II, while the V cell is the producer of GTH-I.

Type I cells (typical basophiles, G cells) are the ones which showed changes during the reproductive cycle. In females kept without males (non-reproductive), these cells were usually well-stained, showing a dense granulation intheir cytoplasm. At the EM level these cells contained many granules of secretion, large globules, and irregular masses. In pituitaries of femailes that were paired and started FOM, some of these cells showed a decrease in the staining intensity while others remained well-stained. The cells at the ventral edge were the first to show a decrease in staining. At the EM level a decrease in the number of granules and globules of some GTH type I cells was noted, concomitantly with a vacuolization resulting from the increased number of dilated cisternae of the RE. The degranulation in the cell was moderate, and completely degranulated cells were not detected. These data suggest that some GTH was released.

It is clear from the literature tht the secretory granules and large globules in GTH-II producing cells react with the antisera to the β chain of the gonadotropin II, even before the plasmatic surge of this hormone (reviewed by van Oordt and Peute, 1983; Naito et al., 1995). These data suggest that the newly produced GTH-II is stored intracellularly in both the small granules and the large globules prior to the surge of GTH-II in the plasma. Naito et al. (1995) detected biologically active GTH-II in the

scretory granules which reacted with anti α and β GTH-II. The big globules do not have biologically active hormone, since they do not react to antisera against the β chain GTH-II. The function of the globules is still uncertain. Puete et al. (1987) suggested that, in the Aftican catfish, globules participate in the intracellular degradation of GTHJ. Alkaline phosphatase activity has been demonstrated in the globules of this species. In combined morphological, enzymatic, cytochemical and autoradiographic observations, Sharp-Baker et al. (19950 concluded that globules and irregular mases are degradative possible crinophagic structure,s which may develop by fusional events fromsecretaory granules to globues and irregular mass aggregations. Iregular masses were only found in non-reproductive fish in which the previously produced GTH-II is stored and not released.

The most prominent characteristic of GTH cells of FOM and post-spawning fish was the increase in the vacuolar compartment. The increase in the size of the reticular cisternae of the RE is noteworthy at the stage of post-spawning. The big vacuoles obseved are the result of the coalescence of small cisternae of the RE. They are characteristic of post-spawning fish and related to the exhaustion of the RE.

The GTH cells of females killed 2–3 days after spawning showed a staining pattern similar to that of non-reproductive females, indicating that, after release, new hormone was produced and stored in the cells.

The pituitary cytological changes found here indicate that, in non-reproductive females, stored GTH-II is found within the gonadotropes. The presence of irregular masses in GTH cells of some no-reproductive females could indicate that, in some case, stored hormone that was not used was being destroyed. When females were paired, a decrease in the staining intensity, the degranulation, and the formation of large vacuoles were taken as an indication that release was in progress or about to take place. Apparently, this is the moment that GTH cells discharge their secretions into the circulatory system. GTH measurement corroborates the histological findings. The significant increase in plasmatic GTH observed in paired females indicates that GTH-II has been released from the pituitary. In goldfish (Hontela and Peter, 1987) and in rainbow trout (Zohar et al., 1986a,b), it was found that fluctuations in GTH level may be more important in maintaing stimulation of ovarian activity than the long-term progressive change in the concentration of the hormone. This does not seem to be the case in the blue gourami, where a typical GTH surge is fond at the beginning of FOM.

The levels of GTH-II in the pituitary of the females kept with or without males are shown in Fig. 5.23. No significant diffferences between the pituitary GTH-II level in females from the gynaeceum (non-reproductive females) which contained oocytes arrested at the stage of high vitellogenesis (760 ± 96 ng/mg protein) and in paired females that had started FOM (801 ± 80 ng/mg protein).

However, the plasma levels of GTH-II in females in these two different conditions showed a significant difference (Fig. 5.24). The levels of GTH-II in the plasma

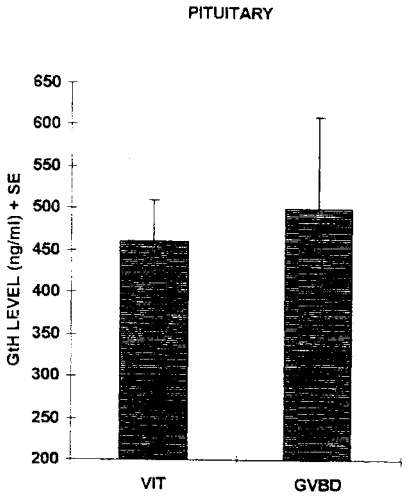


Fig. 5.23. GTH-II level in the pituitary of females kept with (GVBD) and without males (VIT).

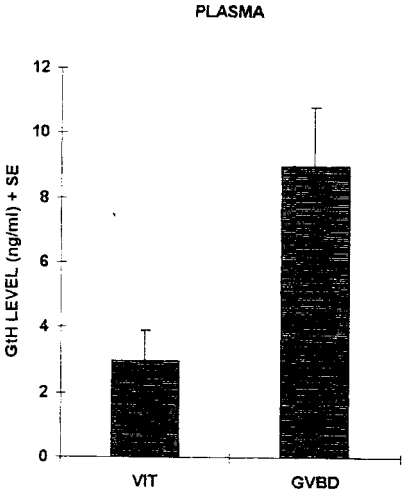


Fig. 5.24. GTH-II levels in the plasma of females kept with (GVBD) and without males (VIT).

were low in non-reproductive females (3.2 ± 1.3 ng/ml), increasing rapidly during oocyte maturation, to reach significantly higher levels (9.3 ± 1.95 ng/ml). In these females, FOM and the concomitant increase in plasma GTH-II levels were induced by the introduction of male specimens into the container (Fig. 5.25).

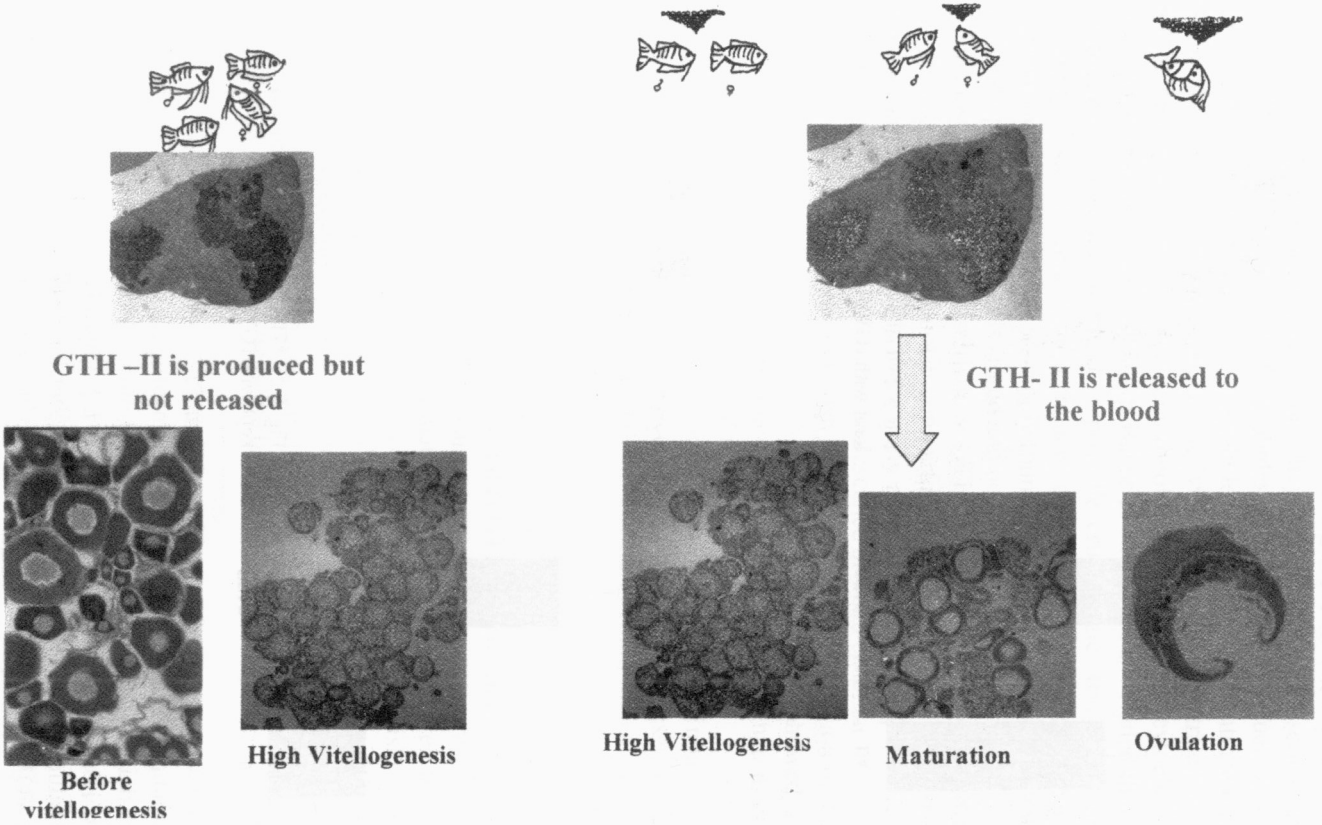


Fig. 5.25. Influence of male on final oocyte maturation. In females kept without males, GTH-II is produced and stored in the pituitary. After transference to male presence, GTH-II is release into the blood, thus causing FOM and ovulation.

CHAPTER 6

HORMONES CONTROL REPRODUCTION IN FEMALE BLUE GOURAMI

6.1 INTRODUCTION

The increasing economic importance of aquarium fish, including the blue gourami, *T. trichopterus*, has encouraged studies of their reproductive physiology. The blue gourami is a multi-spawning, asynchronic, and male- dependent fish (Degani, 1993a,b). Despite difficulties arising from their small size, from a physiological point of view the Anabantidae fish, including the blue gourami, offer the great advantage that the later stages of oogenesis can be controlled. Ovarian development in females starts at three months of age and, in fish maintained in dense populations, and vitellogenesis is completed at five months, at which stage the female is ready to reproduce. However, oocyte maturation and ovulation take place only when the female is isolated with a male. This clear differentiation between the vitellogenic and maturational stages makes this species a good model for the study of hormonal control of oogenesis (Jackson et al., 1994).

Hormonal control of the reproductive cycle of the female blue gourami has been described in detail (Degani, 1990,1993a, 1994; Degani and Boker, 1992a,b). Administration of GnRHa had little or no effect on vitellogenesis, but increased the proportion of oocytes in maturation and ovulation in females that were already in advanced vitellogenesis (Degani et al., 1995). The relationships between sexual behavior, oogenesis, and steroid levels in the gourami, under laboratory conditions, have been described in detail by Degani and Boker (1992a) and Degani (1993). The *in vivo* and *in vitro* effects of GtH administration on oogenesis and steroid levels in the plasma and ovary of blue gourami were also examined in the above studies. It was observed that there are a number of intermediate sub-stages during oogenesis, which show not only morphological differentiation, but also differences in hormone levels. For example, two distinct stages of primary oocyte growth were observed, one with a low gonadosomatic index (GSI), when no effect of administered GtH was detected, and a second with a high GSI, when GtH induces vitellogenesis in the oocytes (Degani, 1994). Nevertheless, studies on oocyte maturation and ovulation in this species have never been correlated with variations on in GtH II levels. In the absence of a suitable assay for GtH II, there was no information on GtH II levels in this species, until the recent development of an enzyme linking immunosorbent assay (ELISA) for measuring GtH II levels in perciform fish, made such an assay available.

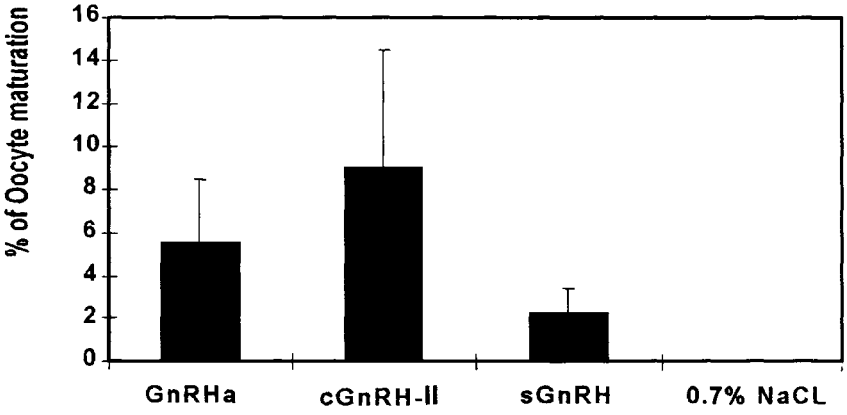


Fig. 6.1. Effects of various GnRHs on oocytes maturation in the female blue gourami (Degani et al., 1997).

6.2 EFFECTS OF VARIOUS GnRHS ON OOCYTE MATURATION IN THE FEMALE BLUE GOURAMI

Degani et al. (1995, 1997) measured the effects on oocyte maturation *in vivo* and on GTH-II secretion *in vitro*, of different various natural GnRHs and a synthetic GnRH. The natural ones were mGnRHa ([D-Ala6, N-Me-deu7]-LHRH), sGnRH [Trp7, Leu8], and cGnRH-II ([His5, Trp7, Try8]); the synthetic GnRH was aGnRH (Fig. 6.1) (Degani et al., 1997).

In primary pituitary cell culture, all forms of GnRHs significantly increased the release of GTH-II into the medium, as compared with controls, in a dose-dependent manner (Figs. 6.2–6.5). The highest levels of induced GTH-II release were obtained from cGnRH-II treatment, compared with sGnRH (81.16 ± 2.7 ng/ml) and GnRHa (65.6 ± 5.5 ng/ml). The differences among the three maximum results were statistically significant. The maximum response to the synthetic form (GnRHa) was obtained after 4 h of treatment, while maximum GTH-II release for the native forms (sGnRH, cGnRH-II) was observed after 8 h (Figs. 6.2–6.5).

In the *in vivo* experiment, we found that injection with GnRHa, cGnRH-II, and sGnRH stimulated the maturation of post-vitellogenic oocytes, whereas no mature oocytes were found in the control group (Fig. 6.1). The percentage of oocytes was significantly higher (d-test; $p < 0.05$) in specimens treated with GnRHa or cGnRH-II than with sGnRH; cGnRH-II showed the strongest effect.

It is well known that GnRH induces the biosynthesis and secretion of GTH in non-mammalian species. The three different forms of GnRH tested caused the transition of oocytes from vitellogenesis to final maturation, and stimulated gonadotropin release from the pituitary cells in a primary cell culture (Figs. 6.3, 6.4, 6.5). The highest GTH-II-releasing activity was found with cGnRH-II, compared with

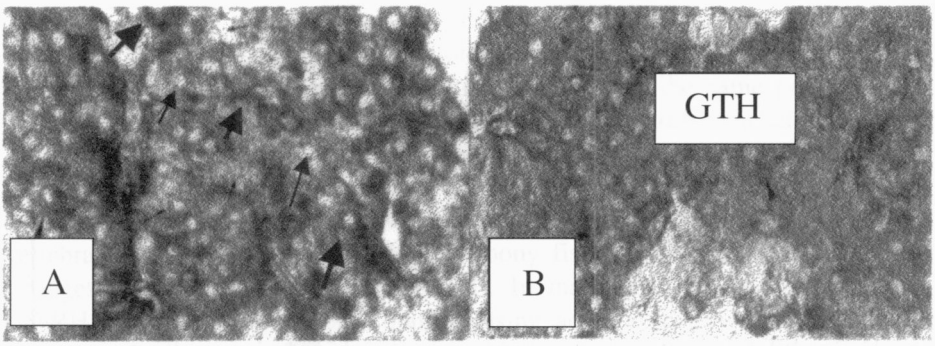


Fig. 6.2. A. Gonadotropic (GTH-II) cells of a fish that received an injection of GnRH and PIM, showing the presence of many chromophobic cells (small arrows) dispersed among strongly stained ones (arrows head). B. Gonadotropic cells after injection of saline (GTH). AB-PAS-OG trichrome, approx. ×1000.

that activity elicited by sGnRH and aGnRH were at levels of 80% and 65%, respectively. cGnRH-II was also the most potent in causing accelerating oocyte maturation.

Zohar et al. (1995) found that in female seabream, cGnRH-II was 7–8 times more potent that sbGnRH (the GnRH found in the pituitary) and twice as potent as sGnRH in inducing the secretion of GTH-II. In this case, the most abundant form of GnRH found in the pituitary was much less potent than cGnRH-II in inducing GTH-II secretion and ovulation. The native form (sbGnRH) may have a shorter half-life than sGnRH and cGnRH-II. In the African catfish, the native cfGnRH was much less potent than cGnRH-II in inducing GTH-II secretion and ovulation, although cfGnRH was present in the pituitary in larger amounts (Ngamvonchon et al.,

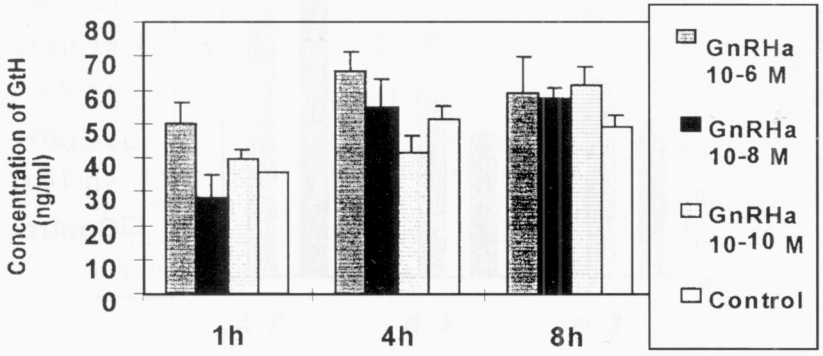


Fig. 6.3. Effect of aGnRH on the release of GTH-II in a primary cell culture of pituitary cells of the blue gourami, *Trichogaster trichopterus* (Degani et al., 1997).

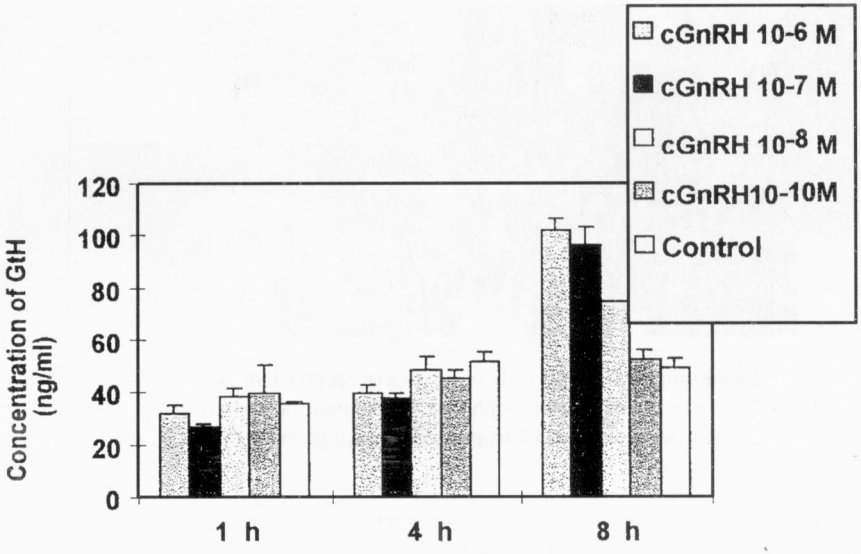


Fig. 6.4. Effects of cGnRH-II on release of GTH-II in primary culture of pituitary cells of the blue gourami, *Trichogaster trichopterus*. (Degani et al., 1997).

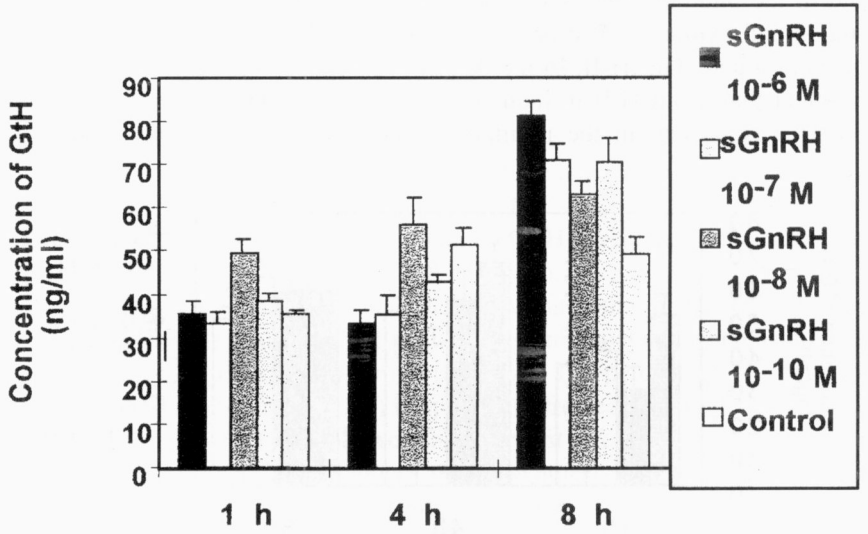


Fig. 6.5. The effect of sGnRH on release of GTH-II of in a primary culture of pituitary cells of the blue gourami, *Trichogaster trichopterus*. (Degani et al., 1997).

1992b). It has been suggested that this is due to cfGnRH having a lower receptor binding affinity than cGnRH-II. In goldfish, cGnRH-II is a stronger GTH-II-releasing agent than sGnRH. Both peptides act through the same population of GnRH receptors, but apparently cGnRH-II may activate a different set of enzymes in metabolic pathways compared to those activated by sGnRH. All types of GnRH stimulate gonadotropin release in all vertebrates, but the specificities of pituitary GnRH receptors differ. In birds, reptiles, bony fish, and cartilaginous fish, all the vertebrate GnRHs have high activity. In bony fish, the GnRH that showed the strongest biological activity is cGnRH-II. In mammals, only mammalian and cGnRH-II exhibit high gonadotropin-releasing activity (reviewed by King and Millar, 1997). It has been hypothesized that, since cGnRH-II is highly conserved, it is likely that its related receptor is also been more conserved than those of other GnRHs. This could explain the high biological activity of this peptide.

The location of the various GnRHs varies with the species. Goldfish have both sGnRH and cGnRH-II fibers in the pituitary (Yu et al., 1988), and catfish *(C. gariepinus)* have cfGnRH and cGnRH-II fibers in the pituitary, but masu salmon (*O. masou*) have only sGnRH in axons that end in the pituitary (Amano et al., 1991). showed that in seabream and striped bass, both of which are perciform species like *T. trichopterus*, only one GnRH (sbGnRH) is the dominant form in the pituitary, and is the one that affects sexual maturation. Yamamoto et al. (1995), using techniques of high-performance liquid chromatography (HPLC) and specific radioimunoassays, isolated and characterized the GnRH forms found in the anabantidae species, *Colisa lalia*. They showed that sGnRH is the native form found in the pituitary, while cGnRH-II is found dispersed in other brain regions. sGnRH is possibly the native form found in the pituitary of the blue gourami, since, to date, no differences in the content of GnRHs among fishes of the same family have been reported. It seems that the sexual behavior of the male gourami causes the female to respond with the secretion of sGnRH from the preoptic area of the brain to the pituitary, resulting in the secretion of GTH-II, which in turn stimulates the secretion of maturation-inducing steroids (Degani and Boker, 1992a).

6.3 EFFECTS OF GnRH ON STEROID PROFILE

The steroid E_2 showed a significant increase ($p < 0.05$) 12 h after intramuscular injection with GnRHa, reaching a maximum after 18 h, and decreasing thereafter (Fig. 6.6). The level of E_2 ranged from 0.5 to 5.0 ng/ml. The level of T also reached its maximum after 18 h, but in this case, there were no significant differences between control and experimental groups, in the variations after injection. The level of T ranged from 0.5 to 2.0 ng/ml. Levels of 17-P in the plasma were lower than those of E_2 and T, ranging from 0.1 to 0.8 ng/ml. The maximum level was again reached 18 h after injection, and was followed by a declining thereafter. The levels of 17,20-P were very low after 12 h, but increased significantly after 18 h and

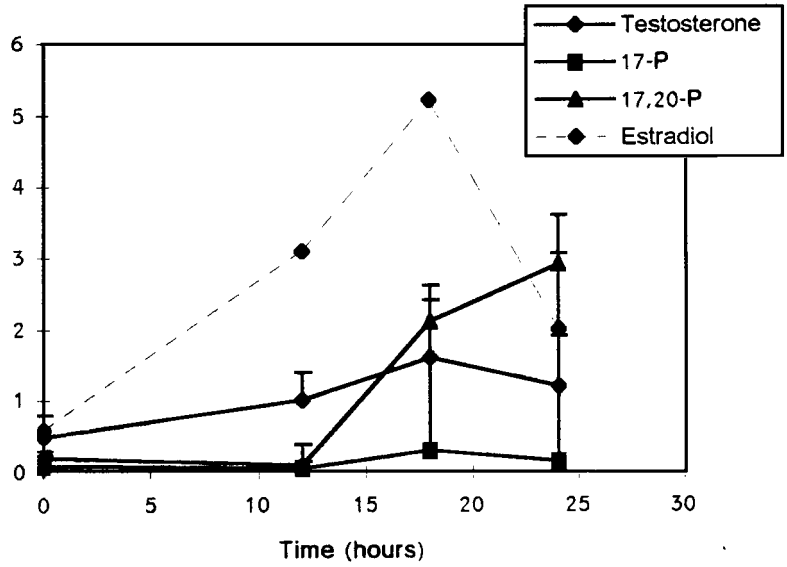


Fig. 6.6. The relationship between GnRH and steroid levels (Degani et al., 1995).

reached their maximum at 24 h. At 12 and 18 h, the release of 17-P and 17,20P showed a significant increase compared with those in the control group.

The present results show that GnRH and Pimozide (PIM), given together or separately, cause oocyte maturation in the post-vitellogenic female *T. trichopterus*. The effects of PIM and GnRHa on maturation in *T. trichopterus* were dose-dependent. Injection of a low dose (200 ng/g) of GnRH, alone or with PIM, caused 20–40% of the females to reach oocyte maturation, while injection of a high dose of GnRH (300 ng/g), alone or together with PIM, caused 80–100% of the females to mature. The most effective treatment was reached by injecting a high dose of GnRH together with PIM: in this case, 40% of the females ovulated. Degani et al. (1995), in a similar experiment in which mature females were injected with a lower dose (100 ng/g BW) of GnRH, alone or together with PIM, in a lower dose (100 ng/g BW), found that mature oocytes were found only in the females that received the injection of GnRH together with PIM.

Gonadotropin secretion in teleosts is known to be stimulated by GnRH and inhibited by dopamine. However, there are marked differences in the relative contributions of GnRH and dopamine to the control of GTH secretion in teleosts. In the cyprinid fishes, such as goldfish or the common carp, dopamine exerts an intense inhibitory control of GTH release; in other teleosts, such as the African catfish, dopamine does not affect GTH release directly, but modulates the effects of endogenous and exogenous GnRH. Finally, there are species such as the atlantic croaker, in which there is an absence of dopaminergic inhibition. It appears that, as in the

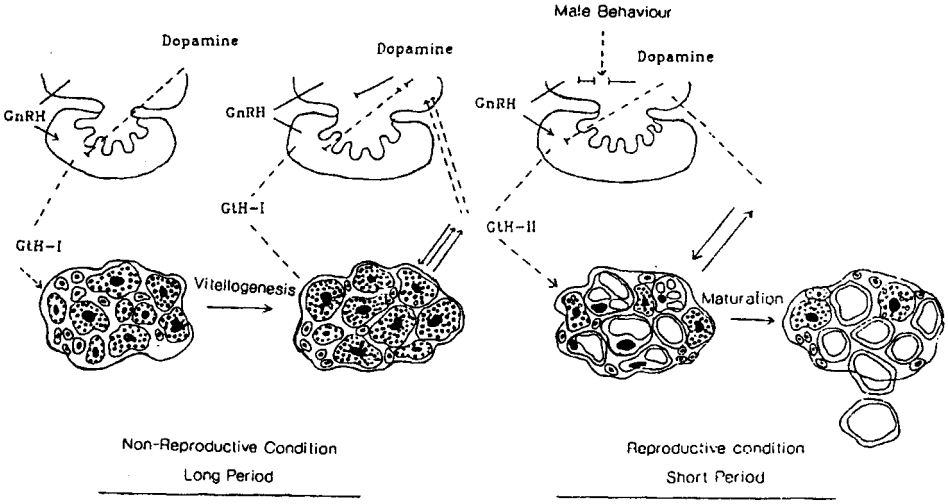


Fig. 6.7. The relationship between GnRH dopamine GtH and oogenesis in blue gourami (Degani, 1993b).

African catfish, the effect of dopamine on GTH release in *T. trichopterus* is modulatory. There is a dopaminergic inhibition of the secretion of gonadotropin, since the dopamine antagonist PIM potentiates the action of the GnRH, and also because 20% of the females in the group injected only with PIM alone reached oocyte maturation. However, females that were injected with aGnRH alone also reached final oocyte maturation (FOM). The relationship between GnRH dopamine GtH and oogenesis in blue gourami was suggested by Degani (1993b) (Fig. 6.7).

The dopaminergic inhibition of gonadotropin release is achieved by a direct action at the pituitary level or at the hypothalamic level (Yu and Peter, 1992).

Our histological examination showed that, in females injected with GnRH together with PIM, the gonadotropic cells were less stained, indicating a greater release of the cytoplasmic content. A similar result was detected in goldfish after injection of PIM and GnRH, but injection of high doses of GnRH alone was also effective in causing gonadotropic release from the pituitary and also in promoting oocyte maturation. This finding reinforces the hypothesis that dopamine modulates GTH release in the blue gourami.

Most of the studies that investigated the effects of GnRH secretion in teleosts have been covered species of the group-synchronic type of ovary development. Billard et al. (1985) reviewed the work performed on Salmonidae and showed that GnRHa causes an increase in the levels of GTH and 17,20-P in the plasma of the male sockeye salmon. In recent years, further work has been done on the effects of GnRH on GTH and steroid secretions in the striped bass.

Degani (1990), in a study of the effects of injection of hGTH in female *T. trichopterus,* found an increase on the plasma level of E_2 during vitellogenesis and maturation, and showed that 17,20-P is the most important steroid involved in the maturation of *T. trichopterus*. The same result was found in the present study after injection of GnRHa and PIM. The implication is that GnRHa causes the secretion of GTH, and this, in turn, regulates the release of the steroids.

In this study (Degani (1990) it was found that the maximum level of E_2 secretion was reached 18 h after injection of GnRHa and PIM, and then declined thereafter. In a later study (Degani, 1993) of steroidal changes in gourami it was found that E_2 varied in a similar pattern during the natural reproductive cycle. Degani (1993) found that the level of E_2 was low during the prebreeding period, increased rapidly during nesting and courtship, decreased during spawning, and then increased once again. A similar pattern of secretion was found in goldfish, *Carassius auratus*. Truscott et al. (1986) found a sharp decrease in the levels of E_2 before ovulation in salmonid fish, and a sharp decrease in the levels of E_2 occurs before ovulation. Sun et al. (1992) found that E_2 peaked 2 h after injection with LHRH analog in the fish ayu, and detected a sharp decrease afterwards. Lower levels of E_2 may be important for the occurrence of ovulation in teleosts (Billard and Peter, 1977). The main action of E_2 is to stimulate the vitellogenesis of oocytes (de Vlaming et al., 1980). In fish such as the blue gourami, with an asynchronic pattern of ovary development, the high level of estradiol found in the post-spawning females could be needed for the beginning of vitellogenesis, to prepare the next batch of mature oocytes.

Testosterone (T) also showed an increase after the injections, although the level of T varied less and decreased more slowly after reaching its peak. This hormone (GnRH) plays an important role in positive feedback regulation of the ovulatory gonadotropin surge in female teleosts. In addition, T also acts as a precursor of E_2, and it was found that, in goldfish, T levels were significally higher before ovulation than after.

The level of 17-P was very low in the plasma and did not show any significant change after injection. The same pattern was found in other systematic groups. The level of this steroid in female gourami during the spawning cycle was very low, but its level rose significantly in the plasma of females during spawning and decreased afterwards.

The level of 17,20-P increased markedly 18 h after injection and remained high. This steroid has been shown to be very effective as a maturation-inducing steroid in most fish (Scott and Canario, 1987), and it seems to be involved in the late stages of final oocyte maturation which includes germinal vesicle migration and lipid droplet coalescence. In an *in vitro* study in which oocytes from mature gourami females which had been injected with hGTH or carp GTH were incubated with 17-P and 17,20-P, it was found that both steroids were capable of inducing nuclear migration and breakdown, 17,20-P being more effective (Degani and Boker, 1992a).

In sum, the plasma levels of E and T were greatest just after the injection and decreased by the time of final oocyte maturation (FOM) and ovulation (18 h after injection), while plasma levels of 17,20-P were high at the stages of FOM and ovulation. These results indicate that the blue gourami conforms to the typical steroidal profile and that 17,20-P is probably involved in FOM in *T. trichopterus* as it is in many teleost species.

6.5 GTH CONTROLS OOGENESIS BY STEROID SECRETION

Figure 6.8 shows the relationship between GSI and vitellogenesis in fish injected with 76 ng/g BW cGtH, and in the control group. At low GSI (GSI <3%) no effect of cGtH on vitellogenesis was detected; up to 4% GSI, cGtH increased the percentage of vitellogenesis; and above 4.5% GSI, no difference was found between the experimental and control groups (Fig. 6.8).

There were no significant differences between the morphological features of the fish injected and those not injected with the hormone. When the percentages of oocytes in fishes of identical weights were compared, however, it was found that although cGtH had no effect on smaller fish (< 3.7 g), in bigger fish (> 4.78 g) it increased vitellogenesis and decreased the percentage of oocytes in endo-vitellogenesis. Degani et al. (1995) suggested a model to described the relationship between GtH and the initial stages of ovary development (Fig. 6.9). Before maturation, the oocyte exhibits only two stages of development: a first stage during which cGtH has no effect on the oocytes of *T. trichopterus,* and a second stage that cGth affected the oocytes. A likely explanation of the results of the present study is that during endovitellogenesis the oocytes develop a receptor to GtH, after which the effect of GtH appears. This hypothesis remains to be proved, however. The question of which hormone controls the potential of oocytes to be affected by GtH, and to reach endovitellogenesis, is at present being studied in our laboratories. The suggested relationship between the hormones and oogenesis in pre-vitellogenesis and vitellogenesis, based on the present and previous studies, is presented in Fig. 6.9.

Degani (1990) used human chorionic gonadotropin (cGtH)) to study the effects of GtH on steroid levels of female blue gourami *in vivo*. The data on changes in steroid levels in both males and females after injection of hCG are shown in Figs. 6.10–6.13.

E_2 was detectable mainly in females, both in the controls and after injection. It was not detected in all the males, and when it was, the level was very low (Fig. 6.10). In females, there was a significant increase in hormone level 10 h after injection with hCG; the level was still high at 15 h and it declined after 20 h.

Peaks of 17-P and 17,20-P concentration were found in females, after 10 and 15 h, respectively. The concentration of 17-P was much lower than that of 17,20-P, while the highest levels, in both cases, were detected after 20 h (Figs. 6.11–6.13). In males, the concentration of 17-P also increased after injection with hCG, though not as

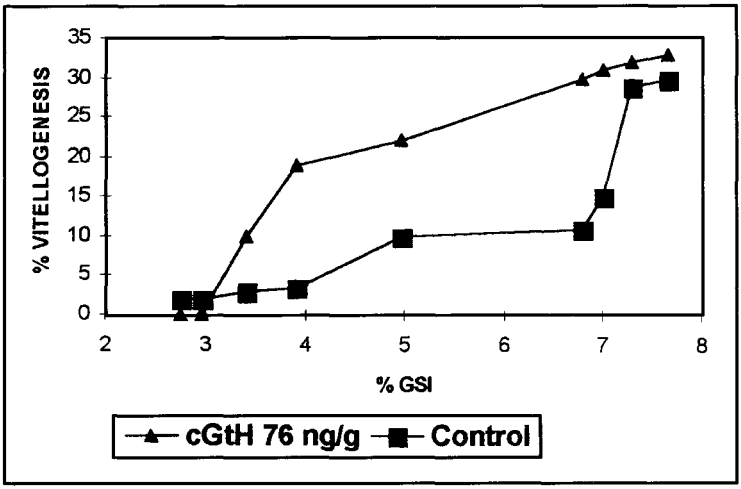


Fig. 6.8. The comparison of percentage vitellogenesis gonado somatic index and GtH (Degani et al., 1995).

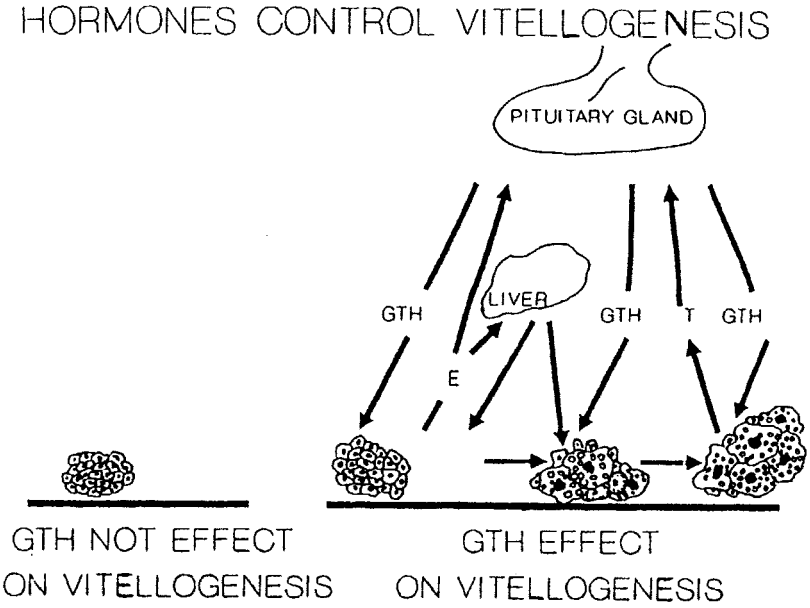


Fig. 6.9. Injections of E_2 and 17,20-P significantly increased the percentage of oocytes in vitellogenesis. No such difference was found as a result of injecting T, nor did it occur in the control groups (Degani et al., 1995).

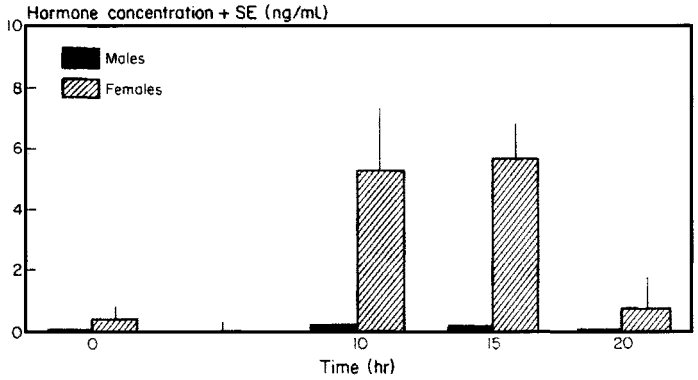


Fig. 6.10. Levels of E_2 in the plasma of male and female *T. trichopterus* after injection with hCG (Degani, 1990).

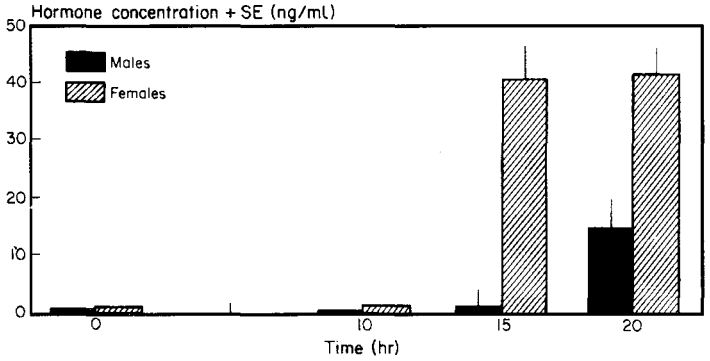


Fig. 6.11. Levels of 17,20-P in the plasma of male and female *T. trichopterus* after injection with hCG (Degani, 1990).

much as in females. A significant difference ($p < 0.05$) between males and females was found at all times of measurement. No great increase was detected in 17,20-P in males, and a significant difference between males and females appeared only after 20 h.

The effects of hCG on T concentrations are shown in Fig. 6.13. The concentration of T increased more rapidly in males than in females, and reached its maximum level in males after 10 h; it then decreased in both sexes. The difference in concentration between males and females only became significant ($p < 0.05$; *t*-test) after 10 h.

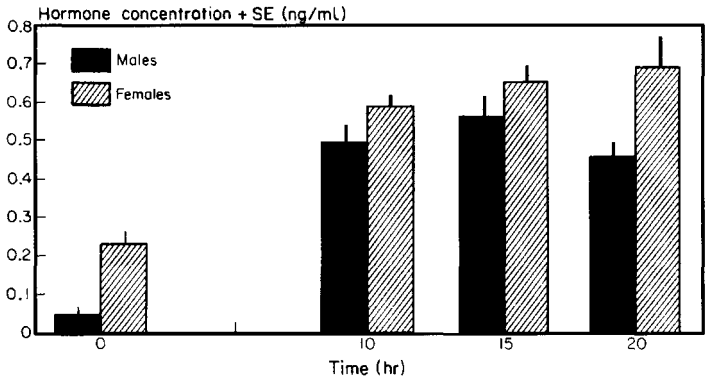


Fig. 6.12. Levels of 17-P in the plasma of male and female *T. trichopterus* after injection with hCG (Degani, 1990).

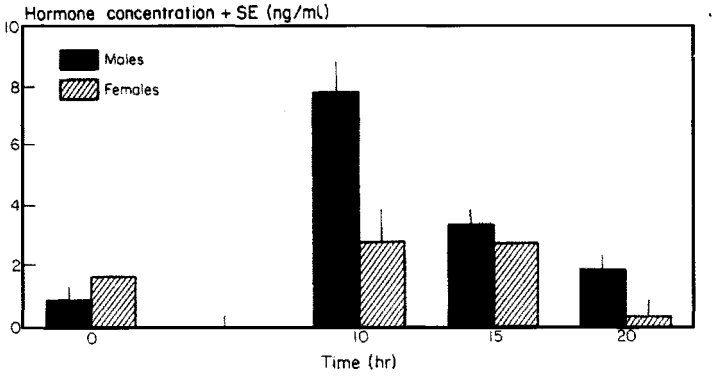


Fig. 6.13. Levels of T in the plasma of male and female *T. trichopterus* after injection with hCG (Degani, 1990).

The levels of P in both males and females increased rapidly for 10 h after injection with hCG, after which point they decreased. A significant difference ($p < 0.05$) between the sexes in the concentration of P was was detected at 10 and 20 h after injection.

Degani and Boker (1992a) examined the effectiveness of 17β-hydroxyprogesterone (17-P), 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P), and human chorionic gonadotropin (hCG) in inducing sensitivity to final maturation of in oocytes in female blue gourami in various *in vitro* treatments: in non-reproductive condition; in reproductive condition together with males; injected with hCG; and

injected with extract of carp gonadotropin (cGtH). Degani and Boker (1992a) showed that the sensitivity of oocytes to hCG or steroid hormones *in vitro* was correlated with the condition of the fish before treatment. While oocytes in the stage of vitellogenesis are found in all mature females, the only oocyte response to hCG or steroid hormones was found in females kept with males or treated with GtH (cGtH) or hCG.

The development of sensitivity to 17,20-P was found *in vitro* in females maintained *in vivo* under the following treatments: with males; after injection with hCG; and after injection with cGtH. Sensitivity to 17,20-P was found only after injection with hCG. The *in vitro* sensitivity to hCG *in vitro* was examined in the ovaries of fish after injection with cGtH. The development of sensitivity to ovulation induction by 17,20-P to induce ovulation was found only in the case of treatment with cGtH. The results obtained in our laboratory (Degani, 1993a; Degani and Boker, 1992a,b) support the hypothesis that the sexual behavior of this species is a very important factor in preparing the female for spawning. During courtship and nest building, the ovary develops sensitivity to hormones, both GtH and steroid. A similar response to injection with GtH was similar to injection by GtH. It is clear that two spaced injections of cGtH have had a greater effect on sensitivity than a single one, and that a long period (48 h) of cohabitation with a male affects the sensitivity of the female to 17,20-P more than a short period. This implies that the presence of the male affects the secretion of GtH in the female, but further study is required to establish this point. Based on these above results and other studies in our laboratories (Degani, 1989, 1990), we suggest that the ovary of the mature female is found through four initial stages. During the building of the nest by the male, the GtH affects the sensitivity of the vitellogenic stages which, in turn, affect the steroid secretions. Male courtship of the female subsequently increases the effect of GtH on the steroids, further affecting the sensitivity of the ovary, to give rise to final ovary maturation and ovulation (Fig. 6.14).

Degani and Boker (1992b) examined the effectiveness of 17-hydroxyprogesterone (17-P), 17, 20-dihydroxy-4-pregnen-3-one (17,20-P), and carp gonadotropin (cGtH) in inducing germinal vesicle breakdown (GVBD) was examined *in vitro* in ovaries of female blue gourami. Induced GVBD was measured at three stages of ovary development: low vitellogenesis (<25%), intermediate vitellogenesis (30%), and high vitellogenesis (>45%). Only a slight influence of treatment with 17-P, 17,20-P, cGtH, or any combination of these hormones was detected in ovaries with a low percentage of vitellogenesis. In the intermediate percentage range, cGtH effected an increase in vitellogenesis, while cGtH in combination with 17-P or 17,20-P, induced a low percentage of GVBD. In ovaries with a high percentage of vitellogenesis, *in vitro* treatment with cGtH had little effect on GVBD but it did increase vitellogenesis. *In vitro* treatment with cGTH and various concentrations of 17-P induced low GVBD. *In vitro* treatment with cGTH and various

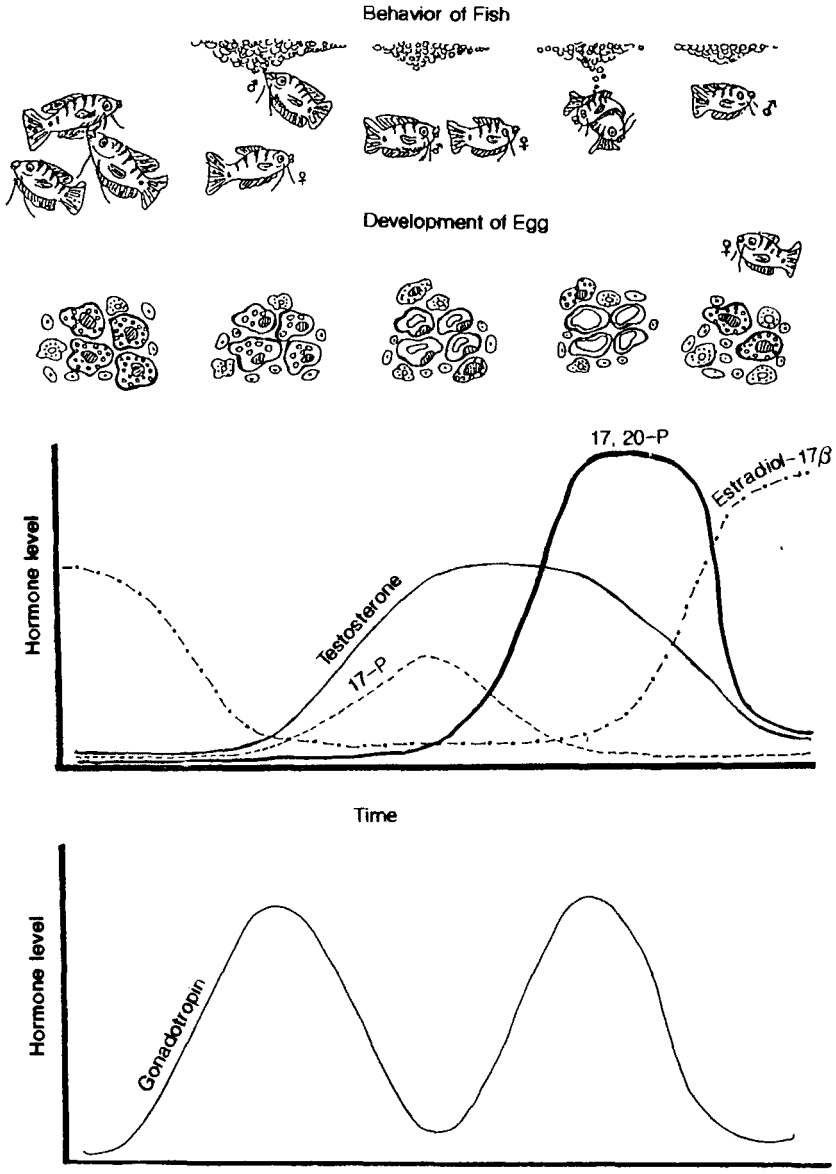


Fig. 6.14. Model of oogenesis—hormone change during sexual activity of *T. trichopterus*. The data on which the model is based are from Cheal et al. (1974) (sexual behavior), Degani (1990) (effect of hCG on steroid secretion), Degani (submitted for publication) (relationship between oogenesis and sexual behavior), and the present study (the relationship between gonadotropin, oogenesis, and steroids) (Degani and Boker, 1992a).

concentrations of 17,20-P induced high GVBD, and the addition of 17-P to those hormones increased the GVBD. The conclusion from these results was that the effect of GtH on the cells of the ovary is to convert the 17-P produced by them to 17,20-P, which is the hormone (17,20-P) that induces maturation (Degani and Boker, 1992a,b) (Fig. 6.15).

6.6 IN VITRO *BIOSYNTHESIS OF STEROIDS IN FEMALE BLUE GOURAMI AT VARIOUS STAGES OF OOGENESIS*

6.6.1 INTRODUCTION

Hormonal changes during the ovulatory cycle in many teleosts have been described elsewhere (for reviews, see Dodd and Sumpter, 1984; Peter, 1981). Nagahama (1987) showed that final oocyte maturation in a number of teleosts is stimulated by maturation-inducing steroids (MIS), produced by the ovary in response to increased levels of gonadotropin (GtH). The most commonly used index of oocyte maturation is the dissolution of the germinal vesicle, or germinal vesicle breakdown (GVBD) (Goetz, 1983; Greely et al., 1986; Selman and Wallace, 1989). Understanding the relationship between oocyte maturation and biosynthesis of steroids is a very important prerequisite for understanding of the mechanism and function of steroids in the process of oogenesis (Nagahama, 1987).

In mature ovaries of many fish species, oocytes of various sizes are randomly distributed (Selman and Wallace, 1989; Wallace and Selman, 1990). The transformation of oogonia to oocytes occurs when oogonia within the germinal ridge enter the early stage of meiotic prophase. During this phase, DNA replication occurs (preleptotene), homologous chromosomes pair and begin to condense (leptotene, zygotene). These pairs subsequently shorten and thicken in oogenesis *per se*. The oogonia and early oocytes are found in nests embedded in the walls of ovigerous lamellae (Selman and Wallace, 1989). A marked increase in oocyte diameter takes place from the chromatin nucleolar stage of primary oocytes to the stages of vitellogenesis. Arrested in late diplotene of the first meiotic prophase, the oocytes then begin an extensive period of growth, concomitant with differentiation of their follicular components; this period is known as the perinucleolus stage. A prominent feature of this stage is the formation of the "yolk nucleus" or "Balbiani body". By the end of the primary growth stage, a multilayered follicle has formed. The first change in cytoplasmic structure observed at the light microscope level is the formation of cortical alveoli, sometimes referred to as "yolk vesicles" or "cortical vesicles" (Selman and Wallace, 1989), a development which can be divided into early, mid-, and late cortical alveolus stages. The stages of vitellogenesis begin when yolk protein constitutes over 80% of the dry weight of the eggs. Vitellogenesis involves the hepatic synthesis and secretion of vitellogenin, its delivery to the oocytes and the formation of yolk bodies, concomitant with vitellogenin, undergoes proteolytic cleavage of vitellogenin into polypeptide.

During oocyte maturation, the germinal vesicle migrates towards the periphery of the oocytes, and the nuclear envelope dissociates. The chromosomes condense and proceed to the first meiotic metaphase, which is followed by the elimination of the first polar body and the remaining chromosomes. The second meiotic metaphase then takes place. In teleosts, once this second arrest has occurred, the oocyte has become mature.

Nagahama (1987) has reviewed the extensive investigations of different hormones in a variety of fish. GtH regulation of gametogenesis is mediated by steroid hormones. 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) has been detected in the plasma of *Oncorhynchus nerka* (Idler et al., 1960; Schmidt and Idler, 1962), and later studies showed that 17,20-P induces oocyte maturation *in vitro* in the ovaries of many fish species. Later, 17α,20β,21-trihydroxy-4-pregnen-3-one was identified as a MIS in *Micropogonias undulatus*, (Trant and Thomas, 1988), and recognized as the major steroid produced *in vitro* in ovaries during final maturation.

In a study of the biosynthesis of 17,20-P in ovaries and testes of *Salmo salar*, Sangalang and Freeman (1988) found that 17α-hydroxyprogesterone (17-P) was a precursor to 17,20-P in these tissues. Lambert and van den Hurk (1982) and Richter et al. (1987) found that a homogenate of postvitellogenic ovary of *Clarias gariepinus* produced mainly dehydro-epiandrosterone and testosterone (T), and a homogenate of postovulatory ovaries produced 17-P and 17,20-P. Schoonen et al. (1987a) collected tissue fragments of ovary of *C. gariepinus* in its natural habitat and found that T was the main end-product before oocyte maturation, together with several 5β-reduced steroids including 5β-pregnane-3a,17a,20a-triol. Following induced ovulation in laboratory-reared *C. gariepinus*, Schoonen et al. (1987b) even observed very polar, 5β-reduced steroids in ovary tissue fragments.

6.5.2 BIOSYNTHESIS OF STEROIDS IN FEMALE BLUE GOURAMI

The process of steroidogenesis during oogenesis has been described in detail for *T. trichopterus* or any other fish of the Anabantidae family described by Degani et al. (1994). The relationship between oogenesis and the biosynthesis of steroids in the ovaries of asynchronic fish, in which the four stages of oocyte development mentioned above occur during all periods of ovary change (albeit in different proportions), is not well understood, whereas an understanding of this relationship might clarify the mechanism of the model of oogenesis in this family.

The various percentages of oocytes at low, intermediate, and high vitellogenesis, and at maturation, in the ovaries in which steroidogenesis was measured after isolation of steroids from the ovary of *T. trichopterus* by TLC is are shown in Table 6.1.

From a comparison of the percentage yields of the various steroids isolated from the ovary of *T. trichopterus* at various stages of vitellogenesis, it appears that the highest yielding steroidone, isolated from the ovary of *T. trichopterus* at various

Table 6.1
Thin Layer Chromatography system used in the purification of steroid metabolites in ovary of *T. trichopterus* at various stages of oogenesis (Degani et al., 1994)

Steroid	Chromatographic sequence
17,20-p	I × 2; II × 2
17-P	I × 2; II × 2; III × 4
T	I × 2; II × 2; III × 4
AS	I × 2; II × 2
11-KT	I × 2; II × 2
5-A-3-ol	I × 2; II × 2; III × 4
5-P-3-17	I × 2; II × 2
E_2	I × 2; II × 2; III × 4

Key: I = toluene/cyclohexane (1/1); II = toluene/ethyl acetate (3/1); III = chloroform/ethanol (92/2); 17,20-P = 17α,20β-dihydroxy-4-pregnen-3-one; 17-P = 17α-hydroxy-progesterone;

stages of vitellogenesis, was 17β-estradiol (E_2). However, the yields of 17,20-P, 5β-pregnane-3a,17a,20a-triol (5-3-ol, 5-P-3-17, 5-A-3-17), and androstenedione (AS), increased rapidly during maturation. The yields of T and 17-P from the ovary were generally low in the ovary, at both vitellogenesis and at maturation.

The ratios of ^{14}C to ^{3}H at various stages of vitellogenesis are shown in Table 6.2. This ratio changed at different stages of oogenesis in all the steroids measured in this study. In the ^{14}C:^{3}H ratio, T increased markedly at Stage V and then decreased again. In 17,20-P, the ratio was very low during vitellogenesis, and increased rapidly at maturation and ovulation. The ratio in 5-P-3-17 changed very little during vitellogenesis, but increased significantly during ovulation. A similar pattern was detected in the behavior of AS. The ratio was very low in AS during vitellogenesis, and increased during maturation and ovulation.

The relationship between the biosynthesis of steroids and oogenesis in the ovary of *T. trichopterus*, on the basis of the results of the present study and those of Degani (1993a,b) and Degani et al. (1994) is summarized in Fig. 6.16.

Three main pathways of steroidogenesis exist during the ovarian phases; they are characterized by the presence of differing proportions of oocyte stages present. The first, from the chromatin nucleolar stage to vitellogenesis, was not examined in the study described here. The second is active when oocytes change during vitellogenesis: the results in our laboratory suggest that this steroidogenetic pathway during vitellogenesis is controlled by GtH, and is not male-dependent. GtH also affects the steroids pathway [P] -> [17-P] -> [AS] -> [T] -> [E_2] in adult females during reproduction (Degani, 1993b). In this case, the secretion of GtH is male-dependent and controls maturation by affecting the synthesis of 17,20-P from 17-P (Degani,

Table 6.2
The ^{14}C:^{3}H steroids ratio in the ovary of *T. trichopterus* at various stages of oogenesis. (initial ^{14}C:^{3}H ratio was 0.12) (Degani et al., 1994).

E_2	C:H	AS	C:H
Stage 4L		1.19	0.00
Stage 4I		0.97	0.40
Stage 4H		0.93	0.60
Stage 5		0.75	4.20
Stage 6		0.72	4.09
T	C:H	11-KT	C:H
Stage 4L		2.00	1.60
Stage 4I		0.00	1.10
Stage 4H		2.00	0
Stage 5		6.40	0.70
Stage 6		2.80	0.80
17-P	C:H	5-A-3-ol	C:H
Stage 4L		2.50	0.00
Stage 4I		1.31	1.50
Stage 4H		0.50	0.00
Stage 5		2.30	0.60
Stage 6		2.00	0.80
17,20-P	C:H	5-P-3-17	C:H
Stage 4L		0.70	4.40
Stage 4I		0.80	2.1
Stage 4H		0.70	0.70
Stage 5		1.18	2.60
Stage 6		1.50	5.00

17,20-P = 17α,20β-dihydroxy-4-pregnen-3-one; 17-P = 17a-hydroxyprogesterone; T = testosterone; AS = androstenedione; 11-KT = 11-ketotestosterone; 5-A-3-ol = 5β-androstane-3β-ol-17-one; E_2 = 17β-estradiol; 5-P-3-17 = 5β-pregnane-3a,17a,20a-triol.

1993a; Degani and Boker, 1992a,b). It is not clear whether these pathways are controlled by two varieties of GtH or by two separate secretions of a single GtH by the pituitary gland of *T. trichopterus*.

6.6.3 SUMMARY OF HORMONE CONTROL STAGES

In summary, the main characteristics of the hormone control of reproduction in the blue grourami, as described in this book and depicted in Fig. 6.17, are as follows.

Only females in high vitellogenesis that were paired with males reached the

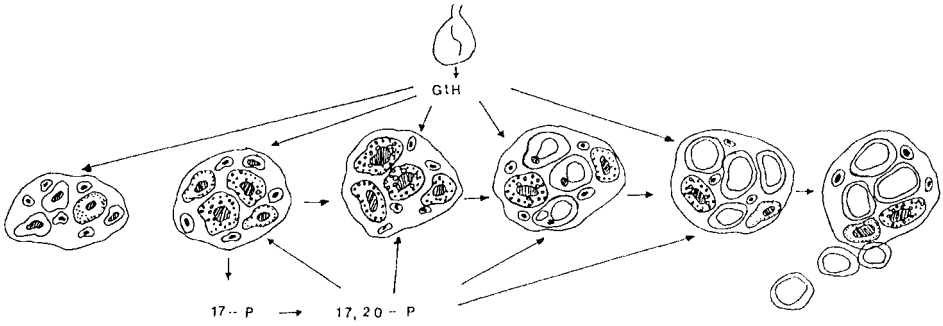


Fig. 6.15. The maturation-inducing effect of cGtH, 17-P and 17,20-P on the ovary (Degani and Boker, 1992b).

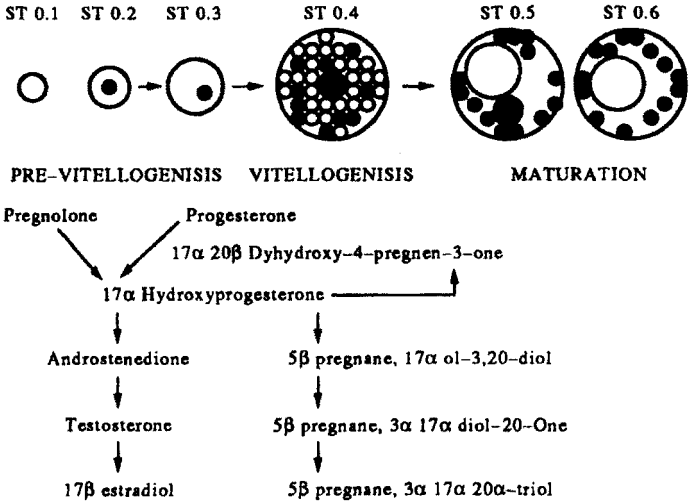


Fig. 6.16. The mean percentage yield of ^{3}H and ^{14}C steroids from incubating ovarian tissue fragments of *T. trichopterus* for various phases of ovarian development: Phase 4L = low vitellogenesis (<25% oocytes in VTG); Phase 4I = intermediate vitellogenesis (25–35% oocytes in VTG); Phase 4H = high vitellogenesis (>35% oocytes in VTG); Phase 5 = appearance of GVBD (some oocytes in GVBD but no ripe eggs); Phase 6 = appearance of ripe eggs. E = 17β-estradiol; T = testosterone; 17-P = 17α-hydroprogesterone; 17,20-P = 17α,20β-dihydroxy-4-pregnen-3-one; AS = androstenedione; 11-KT = 11-ketotestosterone; 5-A-3-ol = 5β-androstane-3β-ol-17-one; 5-P-3-17 = 5β-pregnane-3α,17α,20α-triol (Degani et al., 1994).

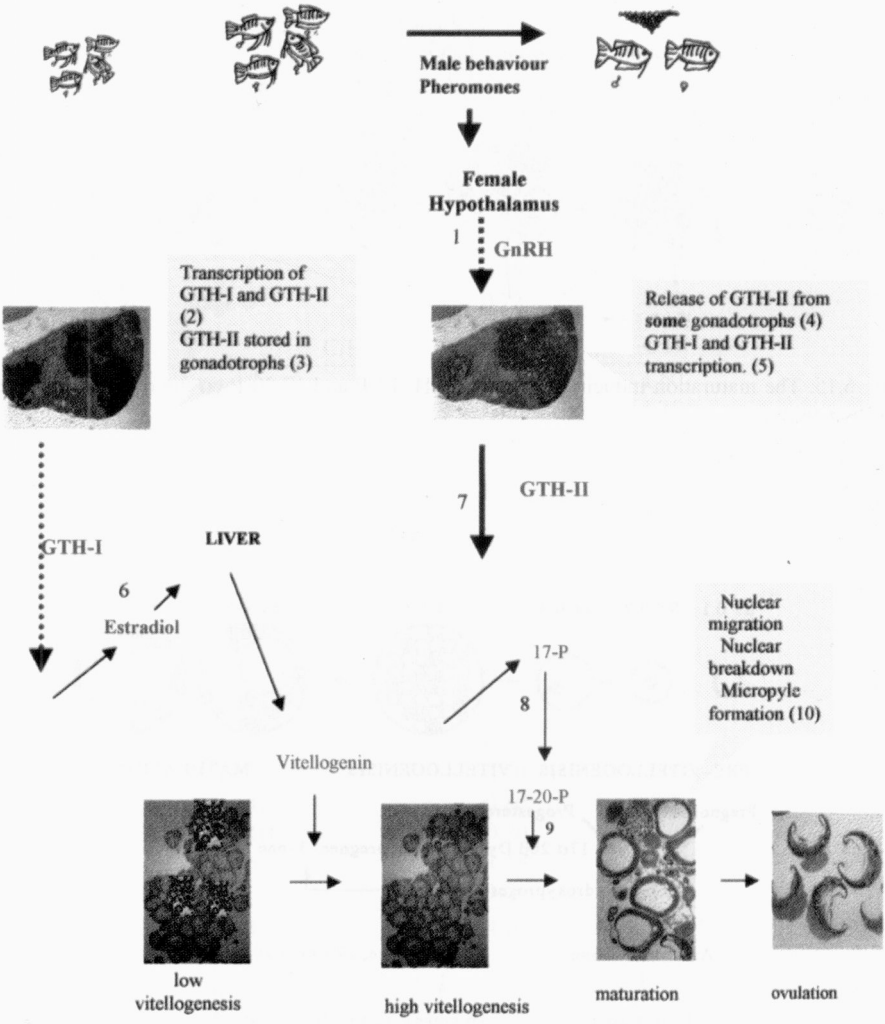


Fig. 6.17. Endocrine control of the reproductive cycle in the blue gourami (*Trichogaster tricheropterus*). During the non-reproductive period, GTH-I is secreted from the pituitary and causes the secretion of estradiol, inducing the liver to produce vitellogenin, which is absorbed by the vitellogenic oocytes. During the reproductive period, and in response to male behavior, GnRH (probably salmon GnRH) is released from the hypothalamus and induces the pituitary to release the stored GTH-II. The latter hormone works on the oocyte membrane layers, causing them to transform the steroid 17-P into 17,20-P, which is the maturation-inducing steroid in the blue gourami. In the presence of this steroid, post-vitellogenic oocytes can reach final maturation and ovulation. The steps of this model have been presented by: Degani (1990, 1994), Degani and Boker (1992a,b), Degani et al. (1994, 1995, 1997), Jackson et al. (1994, 1999).

stages of final oocyte maturation (FOM) and ovulation. Those that were kept in confinement or in a large group never attained FOM and ovulation, and contained oocytes whose development was arrested at the post-vitellogenic oocyte stage. This pattern of male–female interaction, in which the male is essential for the development of the female, is rare among teleosts.

Two different gonadotropins (GTH-I and GTH-II) were cloned from the gourami pituitary. Both showed close similarity to the gonadotropins of the striped bass. The expression of the genes of both gonadotropins was determined by means of RT-PCR. A high concentration of GTH-I mRNA was detected during vitellogenesis, while GTH-II mRNA predominated during FOM. GTH-II was measured in females kept in the absence and in the presence of males: in those transferred to the company of a male, GTH-II was released into the blood, whereas in those kept in the gynaeceum it was stored in the pituitary but not released.

In a second part of the investigation, we studied the effects of GnRH *in vivo* and *in vitro,* in cells of pituitary of mature females. GnRH analog (GnRHa), chicken GnRH-II (cGnRH-II), and salmon GnRH (sGnRH) were all able to elicit gonadotropin release from a primary cell culture of pituitary cells. Of these GnRHs, the most potent was cGnRH-II, and this was also the most effective in eliciting oocyte maturation when injected into females kept in the absence of males.

The effect of the presence of a gonadotropin-inhibiting factor, such as dopamine, was tested by injecting GnRH and/or Pimozide (PIM), a dopamine inhibitor, into mature females. Females injected with GnRH alone were only able to start FOM, but PIM injection potentiated the effect of GnRH, and the females injected with both reached ovulation. The pituitaries of these injected females were subjected to light histology: those from females that had received GnRH and PIM, either separately or in combination, showed fewer stained gonadotropes than those injected with saline, indicating an enhanced release of secretory material.

Finally, we studied the females' steroid profiles during the maturation induced by the injection of aGnRH and PIM. There was a significant increase in the levels of estradiol 12 h after the injection; the levels reached a maximum after 18 h and then decreased. Testosterone and 17-P did not show significant increases after the injection, while the steroid 17,20-P showed an increase in concentration 18 h after the injection.These changes are similar to those that occur in nature during maturation in the presence of a male.

CHAPTER 7

THE RELATIONSHIP BETWEEN GONADOTROPIN AND NEST-BUILDING OF MALE *TRICHOGASTER TRICHOPTERUS* (PALLAS)

7.1 INTRODUCTION

Hormone levels in the plasma of various teleost species have been examined to study the correlation between hormone secretion and reproduction (Kobayashi et al., 1988). The secretion of a gonadotropin-releasing hormone (GnRH) and the subsequent secretion of gonadotropin (GtH) are considered to be responsible for spermatogenesis.

The male blue gourami (*Trichogaster trichopterus)* is territorial and builds a nest of mucus bubbles for the fertilized eggs at the water-air interface. Since territoriality and nest-building are exhibited only by males which are ready to reproduce, this marks a critical time in the reproductive cycle and makes the species a good model for the study of sexual behavior in male fish.

During courtship, when the female is ready to spawn, the male circles and nudges her, then envelops her and squeezes out the eggs, which he then fertilizes. Subsequently, he brings the eggs and fry from the bottom of the water to the nest and tends the brood, retrieving fry that drift from the nest (Miller, 1964).

7.2. RELATIONSHIP BETWEEN MALE SEXUAL BEHAVIOR, GTH AND STEROIDS

Mananos et al. (1997) examined the relationship between male sexual behavior (nest-building) and steroid levels: GtH in the pituitary and plasma, and T in the plasma and testes. Blue gourami (*T. trichopterus*) were maintained in containers, measuring $2 \times 0.5 \times 0.5$ m. Water temperature was controlled at 27 °C and a light regime of 14 h of light and 10 h of darkness was maintained. The fish were kept apart for two weeks by fencing, in small cages ($40 \times 20 \times 20$ cm), before sampling and were fed *ad libitum* with frozen *Artemia salinata*, at 0900 and 1700.

Samples were taken from fish weighing >6 g, 24 h after they began to build nests, to confirm high spermatogenesis and maturity. Control samples were taken from full-sized males that exhibited no nest-building behavior. The levels of gonadotropin (GtH) and the steroids, testosterone (T), progesterone (P), and estradiol-17 (E_2) in the male blue gouramis were measured during nest-building

The pituitary GtH level of nest-builders was significantly higher than that of non-builders (Fig. 7.1); the same pattern was observed in plasma GtH, but the difference

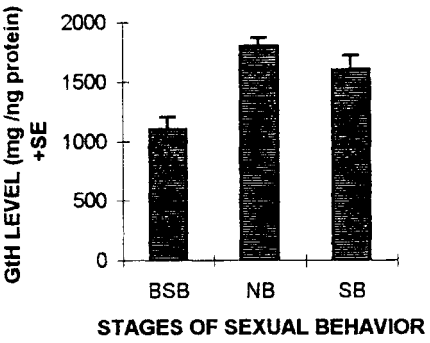


Fig. 7.1. Gonadotropin (GtH) levels in pituitaries of non-building (BSB) and nest-building (NB) male blue gouramis, and in those exhibiting sexual behavior (SB) (Mananos et al., 1997).

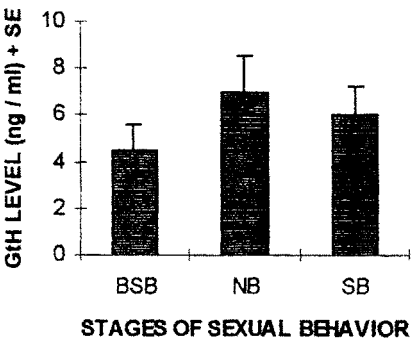


Fig. 7.2. Gonadtropin (GtH) levels in plasma of non-building (BSB) and nest-building (NB) male blue gouramis, and in those exhibiting sexual behavior (SB) (Mananos et al., 1997).

was not statistically significant (Fig. 7.2). In the pituitary, the level of GtH (1159 ng/pituitary) was higher in the nest-building males than in those that were not building nests (647 ng/pituitary). Similarly, the plasma GtH levels were 7 and 5 ng/ml for builders and non-builders, respectively.

The testosterone (T) level in the plasma of nest-builders was significantly higher than that of non-builders (Fig. 7.3), but no significant difference was found between the levels in the testes (Fig. 7.4). The level of T was higher in the plasma of builders (10 ng/ml vs. 6 ng/ml), but there was no significant difference between the levels in the testes. The level of P was higher in the testes of builders than in those of non-builders (4 and 2 ng/ml, respectively; Fig. 7.5), but the difference was not significant; the findings for E_2 were similar, but in the latter case, the levels were very low in both groups.

Mananos et al. (1997) showed a correlation between elevated GtH levels in the pituitary and plasma of *T. trichopterus* and the period of nest-building, an important element in the reproductive behavior. The advantage of studying this species lies in the clear indication of the transition to reproductive activity: the beginning of nest-building activity. In addition, the dependence of sexual activity on territory (Degani, 1993) makes this species a suitable model for study. Few studies have been published to date on the secretion of GtH from the pituitary to the plasma during sexual activity in this systematic group (for review, see Swanson, 1991). There is a rapid rise in the GtH level in the pituitary of the blue gourami when it becomes ready to reproduce, as determined by its maturity and the availability of territory. The effect

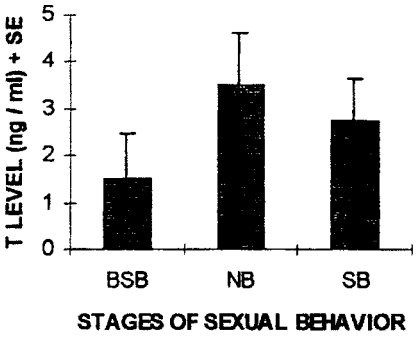


Fig. 7.3. Testosterone (T) levels in plasma of non-building (BSB) and nest-building (NB) male blue gouramis, and in those exhibiting sexual behavior (SB) (Mananos et al., 1997).

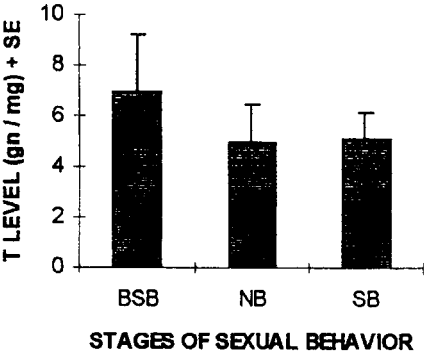


Fig. 7.4. Testosterone (T) levels in testes of non-building (BSB) and nest-building (NB) male blue gouramis, and in those exhibiting sexual behavior (SB) (Mananos et al., 1997).

of secretion of GtH leads, in turn, to secretion of T in the testes, with a marked change in sexual behavior (Degani, 1993).

The process of initiation and continuation of sexual behavior in the blue gourami, hypothesized on the basis of the studies described here and previously, is that when conditions make the male ready to reproduce, as indicated by nest-building, gonadotropin-releasing hormone (GnRH) is synthesized in the hypothalamus of the brain, and secreted into the pituitary; this triggers the synthesis of GtH and its secretion into the plasma, where it leads to the synthesis of T to a high level.

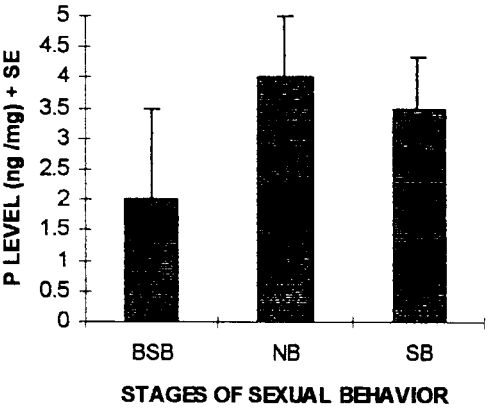


Fig. 7.5. Progesterone (P) levels in testes of non-building (BSB) and nest-building (NB) male blue gouramis, and in those exhibiting sexual behavior (SB) (Mananos et al., 1997).

The E_2 level in the female was found to be high during vitellogenesis, decreasing at the end of this process, at which time 17,20-P increased (Degani, 1990; Degani and Boker, 1992a), whereas the E_2 level remained very low in the male.

Progesterone is the precursor to the synthesis of T in blue gourami (Degani et al., 1994). In the present study, the P level in the testes of the nest-building male rose, but the rise was not significant. It is possible that the synthesis of T also increased, but since it is secreted from the testes, the phenomenon is very hard to monitor. In the female, on the other hand, P is the precursor to synthesis of E_2, which is found at a high level in the reproductive female, but is barely detectable in the male.

CHAPTER 8

PHEROMONES OF BLUE GOURAMI

8.1 INTRODUCTION

Interest in chemical communication among fish has increased greatly in the past decade (for reviews, see Colombo et al., 1982; Liley, 1982; Stacey et al., 1989). In many teleost species, pheromones play important roles at various phases of the reproduction process, e.g., in spermiation in goldfish (*Carassius auratus* L.) (van Weerd and Richter, 1991); oocyte maturation and ovulation in zebra fish (*Brachydanio rerio* Hamilton) (van den Hurk et al., 1987), and in African catfish (*Clarias gariepinus* Burchell) (Resink et al., 1989), and in the synchronization of spermiation and ovulation in goldfish (*Carassius auratus* L.) (Dulka et al., 1987; van Weerd and Richter, 1991).

Several studies have shown that the blue gourami can serve as a model for the examination of chemical communication (Cheal and Davies, 1974; Pollack et al., 1978; Lee and Ingersoll, 1979), little has been published on the effect of male pheromones on the female reproductive system. Degani and Boker (1992a) have shown that male sexual behavior is a very important factor in preparing the female for spawning. During courtship and nest-building by the male ,the ovary develops sensitivity to hormones, both gonadotropin (GtH) and steroids, and Degani (1990) found that injection with GtH elicited a similar response in the female.

The blue gourami is asynchronically multispawning and male-dependent, and maturation and ovulation in the female occur only after courtship by the male. The ovaries contain oocytes at all four prematurational stages of development, both before and after breeding. These stages are: stage 1, the chromatin nucleolar stage; stage 2, the perinucleolar stage; stage 3, the endovitellogenic stage; and stage 4, the exovitellogenic stage (during which yolk granules are deposited and the cortical alveoli develop to fill the entire oocyte) (Degani and Boker, 1992a,b). These stages are described in greater detail below, under "Histological study". Male sexual behavior or injection with GtH have the effect of increasing the percentage of oocytes in the exovitellogenic stage in females where the percentage is low, or causing maturation and ovulation when the percentage of oocytes in exovitellogenesis is already high (Degani and Boker, 1992a). There is no information, however, on whether pheromones produced by the male elicit oogenesis in the female, and this knowledge is important for an understanding of how territorial

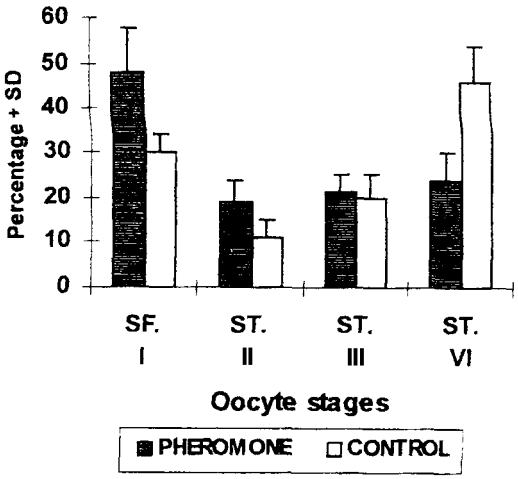


Fig. 8.1 Comparison between the percentages of oocytes in four stages of vitellogenesis in females with initially low vitellogenesis maintained in water containing male pheromones and in control groups (Degani and Schreibman, 1993).

males attract females, and for a fuller understanding of the role of the male in effecting breeding competence in females. However, McKinnon and Liley (1986) have shown that in two species of *Trichogaster*, females secrete a pheromone that affects males.

8.2 THE EFFECT OF PHEROMONES PRODUCED BY THE MALE ON PITUITARY CELLS THAT PRODUCE GTH, AND ON STEROIDOGENESIS, EXOVITELLOGENESIS, AND MATURATION

Degani and Schreibman (1993) studied the effect on female hormones, of pheromone production by males; they exposed female blue gouramis to aquarium water in which males had built nests, and examined gonadotropin cells in their pituitary glands, and steroidogenesis and exovitellogenesis in the ovaries. Thin layer chromatography, using the precursors ^{3}H-pregnenolone and ^{14}C-progesterone, revealed high yields of the steroids 17β-estradiol (E_2), 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P), 5β-pregnane,3a,17a,20β-triol (5β-P-triol) and 11-ketotestosterone (11-KT) in both of the designated experimental groups. In females in which the percentage of oocytes in vitellogenesis (%V) was initially low, this percentage rose significantly in comparison with an unexposed control group (Fig. 8.1); and in females in which %V was initially high, it increased further and, in addition, a significant percentage of oocytes reached maturation. The vitellogenesis percentage (%V)

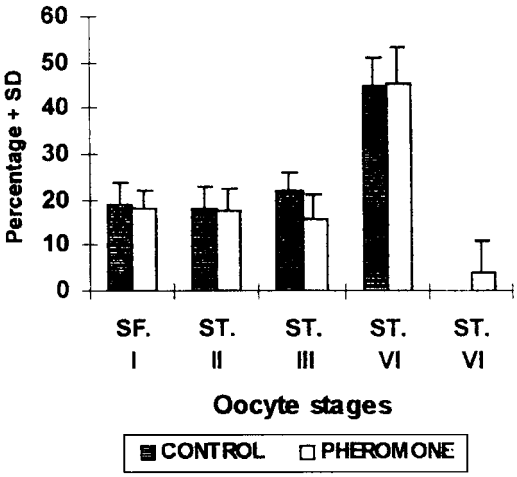


Fig. 8.2 Comparison between the percentages of oocytes in four stages of vitellogenesis in females with initially high vitellogenesis maintained in water containing male pheromones and in control groups (Degani and Schreibman, 1993).

(Stage IV) in females which were maintained in water where males had built nests was significantly higher than that among the control groups (Fig. 8.2).

Steroids isolated from the ovarian samples and detected by TLC are shown in Table 8.2.

Table 8.1
Thin layer chromatography used in the purification of steroid metabolites in the ovary of *Trichogaster trichopterus*, incubated *in vitro* with radioactive precursors (Degani and Schreibman, 1993).

Steroid	Chromatography sequence
17β-estradiol	I × 2; II × 2; III × 4
Testosterone	I × 2; II × 2; III × 4
17,a-hydroxyprogesterone	I × 2; II × 2; III × 4
17α,20β-dihydroxy-4-pregnen-3-one	I × 2; II × 2
11-ketotestosterone	I × 2; II × 2
5β-pregnane-3a,17β,20a-triol	I × 2; II × 2
Androstene	I × 2; II × 2
5β-androstene-3β,17β-diol	I × 2; II × 2; III × 4
5β-androstene-3β-ol-17-one	I × 2; II × 2; III × 4

Key: I = toluene/cyclohexane (1/1); II = toluene ethyl acetate (3/1); III = chloroform/ethanol (98/2).

Table 8.2
Statistical comparison between yields of various steroids in female *Trichogaster trichopterus* maintained in male-holding water with those from females in control group 1 (Degani and Schreibman, 1993).

Hormone	Treatment	%Yield ^{3}H	% Yield ^{14}C
17β-estradiol	Control	6.0 ± 0.5a	0.5 ± 0.07a
	Test	10.08 ± 4.41b	1.1 ± 1.1a
Testosterone	Control	2.1 ± 1.4c	1.2 ± 0.2c
	Test	1.3 ± 0.5c	1.1 ± 0.2c
17,ahydroxyprogesterone	Control	2.6 ± 2.4d	1.7 ± 1.0d
	Test	1.7 ± 0.3d	2.4 ± 2.1d
17a,20β-dihydroxy-4-pregnen-3-one	Control	16.2 ± 12.3e	4.7 ± 1.5e
	Test	17.6 ± 6.9e	11.1 ± 6.1f
11-ketotestosterone	Control	10.3 ± 9.2g	5.6 ± 1.4g
	Test	11.8 ± 9.5g	10.8 ± 9.1g
5β-pregnane-3a,17β,20a-triol	Control	20.1 ± 12.6h	11.5 ± 10.2h
	Test	26.8 ± 14.2h	35.5 ± 10.7i
Androstene	Control	3.8 ± 2.4m	10.8 ± 9.1m
	Test	2.3 ± 0.12m	17.5 ± 6.9m
5β-androstene-3β,17β-diol	Control	1.6 ± 1.5n	1.3 ± 1.2n
	Test	1.2 ± 0.3n	1.5 ± 0.8n
5β-androstene-3β-ol-17-one	Control	0.6 ± 0.4o	0.9 ± 0.8o
	Test	0.5 ± 0.07o	0.6 ± 0.4o

Statistical notation: Different letters attached to test and control yields indicate significant differences ($p < 0.05$) according to Student's *t*-test.

Relatively high yields of steroids were measured by Degani and Schreibman (1993) in E_2 (only ^{3}H marker), 17,20-P (both ^{3}H and ^{14}C), 11-KT (both markers) and 5β-pregnane-3a,17a,20a-triol (5β-P-triol) (both markers). Among these hormones, significant differences (*t*-test) between the test and control groups were found only for the yields of E_2 (^{3}H only), 17,20-P (^{14}C only), and 5β-P-triol (^{14}C only).

The response to anti-GtH by pituitary cells of female *T. trichopterus* is shown in Fig. 8.3. The intensity of the immunoresponse to anti-GtH was lower in the test group than in control group 1, and lowest in control group 2 (Table 8.3).

Trichogaster trichopterus females responded to water in which males had been held, with an increase in the number of oocytes in the stage of exovitellogenesis in those with an initially low %V, and higher yields of E_2 and 17,20-P (^{14}C marker),

Table 8.3
The level of immunoresponse to antiCroaker GtH in pituitary of *T. trichopterus* (Degani and Schreibman, 1993).

Fish No.	Test group (holding water)	Control group 1 (tap water)	Control group 2 (nonreproductive)
1	+	+++	+
2	+	++	+
3	++	+	+
4	++	+++	++
5	+		

Response level: + = low; ++ = intermediate; +++ = high.

compared with controls. These results suggest that male pheromones, in the form of sex steroids, have the effect of preparing the female for spawning. In a previous study (Becker et al., 1992) GC-MS measurements showed that water in which males had built nests contained various steroids and steroid glucuronides, secreted by the male. It was found that such water effected oogenesis in the female and elicited maturation of the oocytes, giving rise to the hypothesis that these steroid glucuronides are sex pheromones. Maturational competence, a necessary precursor to spawning, is marked by an increase in the number of oocytes in exovitellogenesis (Degani and Boker, 1992a) which is effected, according to Degani and Schreibman (1993), by male pheromones. Maturation and ovulation in asynchronic, multispawning fish occur only when a large number of oocytes are in exovitellogenesis. This mechanism is controlled by GtH, which increases the level of 17,20-P, the steroid which directly induces maturation (Degani and Boker, 1992b). This hypothesis is supported by other findings on hormone control of reproduction in these species (Degani, 1990; Degani and Boker, 1992a,b), which show that maturation occurs in the oocytes of the female *T. trichopterus* as a result of either male sexual behavior (nest-building, courtship) or injection with GtH. Degani and Schreibman (1993) found that water in which the male had built a nest brought the oocytes to maturation only in females with a high initial %V.

The effect of pheromones on exovitellogenesis has also been detected in fish which are not asynchronic and multispawning. One of the most extensively studied species is the African catfish (*Clarias gariepinus*), which is an annual spawner. Van Weerd et al. (1988) found that exposure to water in which pubertal males and females had been held stimulated ovarian growth in pubertal vitellogenic females. A later study by the same group showed that exposure to such holding water can boost ovarian development, not only in vitellogenic, but also in previtellogenic females (Van Weerd et al., 1991).

Degani and Schreibman (1993) evaluated the relative amounts of GtH in the gonadotropes by immunohistochemistry. The mechanism of GtH excretion in this

model has up to now been only partly described (Schreibman et al., 1973, 1990); it may be that GtH is secreted at all times, to control vitellogenesis. *T. trichopterus* is multispawning, therefore, oocytes were found at all levels of vitellogenesis, within a single group of fish, as in previous studies (Degani and Boker, 1992a,b), and it appears that the GtH cells in the pituitary glands of different fish in the same experimental group exhibit a correlated variability in their oocyte levels. Further study is required to establish such a relationship, however. According to the findings of Degani and Schreibman (1993), it appears that the pheromones secreted by the males caused the pituitary glands of the females to secrete GtH and that, consequently, the gonadotropes of females maintained in water previously occupied by males evinced a lower immunoresponse than those of females in reproductive condition that were placed in plain tap water. The hypothesis that GtH secretion in females is stimulated by water which has held males would explain the high exovitellogenesis and higher steroidogenesis found in gonads involved in reproduction in the test group.

The effect of male pheromones on female exovitellogenesis and steroidogenesis, elicited by GtH secreted by the pituitary gland of the female, is consistent with the findings of van Weerd et al. (1988, 1991) of a similar reaction in the African catfish, when females were exposed to water that had held pubertal males and females.

The distribution of irGnRH systems in the brain of the dwarf gourami (*Colis lalia*) has been described in detail by Oka and Ichikawa (1990), who found that ganglion cells in the transitional area between the olfactory bulb and the telencephalon, and in the preoptic area contained irGnRG. This distribution is similar to that in other teleosts (Schreibman and Margolis-Nunno, 1987). Yu et al. (1989) described the rise in GtH in goldfish (*Carrassius auratus*); they described in detail the induction of GtH secretion by sex pheromones in the serum that followed the rise in GnRH level in the brain, thus clearly establishing the pathway in this fish. This pathway is equivalent to the proptiinfundibular pathway in other vertebrates, and the GnRH in this pathway is presumed to function as a hypophysiotropic hormone to facilitate the release of gonadotropins from the pituitary. Oka and Ichikawa (1990) proposed that the abundant irGnRH fibers in the ventral telencephalon and the preoptic area might affect some aspects of sexual behavior, since it has been suggested (Degani, 1993) that these are the areas involved in the control of sexual behavior in teleosts. It is our suggestion that the pheromone of the male *T. trichopterus* affects GnRH secretion, causing the secretion of GtH and thereby effecting oogenesis in the female blue gourami.

Finally, although GtH secretion is affected by sight as well as smell (Barfield, 1971), the factor of sight was excluded by Degani and Schreibman (1993) since the female specimens were exposed to holding water only, without the presence of a male or a nest. Other factors have also been found to affect oogenesis in fish, e.g., sexual behavior, as was found in blue gourami by Degani (1993).

CHAPTER 9

THE GTH-I AND GTH-II β SUBUNITS OF THE FEMALES AND MALES BLUE GOURAMI (*TRICHOGASTER TRICHOPTERUS*, PALLAS 1770): EXPRESSION DURING THE DIFFERENT STAGES OF SEXUAL BEHAVIOR

9. 1 INTRODUCTION

GtH is a glycoprotein consisting of two subunits, α and β, which are non-covalently bound. There are two distinct GtHs in the pituitary gland of teleosts: GtH-I and GtH-II. These proteins share a common α subunit, but differ in their β subunits, which confer the immunological and biological specificity to each hormone. In salmonids, GtH-I and GtH-II have been found to differ in their patterns of expression at different stages of the reproductive cycle. GtH-I is expressed during oocyte vitellogenesis and GtH-II is expressed at maturation and ovulation.

Most of the physiological studies of teleost GtHs have been conducted on GtH-II, which controls maturation and ovulation, while the function of GtH-I in fish has not been fully addressed. It is believed that GtH-I regulates gametogenesis, since it promotes the production of 17α estradiol and the incorporation of vitellogenin into the oocytes. GtH-II, on the other hand, is known to be involved in oocyte maturation and ovulation. This hormone is probably responsible for the final oocyte maturation (FOM), since it is more active than GtH-I in stimulating the release of the maturation-inducing factor, 17α,20β,dihydroxy-4-pregnen-3-1 produced by post-vitellogenic oocytes.

The α and β subunits of teleost GtH-I and/or GtH-II are encoded by separate genes. Trinh et al. were the first to clone these genes in teleosts (chinook salmon, *Oncorhynchus tshawytscha*). Since then, sequencing of α and β subunits of both GtH-I and/or GtH-II has been reported for several other fish. β subunits of GtH-I and GtH-II have been cloned and sequenced in chum salmon, *Onchorhynchus keta*, masu salmon, *Onchorhynchus masou*, goldfish *Carassius auratus*, mummichog *Fundulus heteroclitus*, European eel, *Anguilla anguilla*, Baikal omul, *Coregonus auumnalis migratorius*, and gilthead seabream, *Sparus aurata*. The α and β subunits of GtH-I and/or GtH-II have been cloned and sequenced in silver carp, *Hypophthalmichthys molitrix*, chum salmon and Japanese eel, *Anguilla japonica*. The α1 and α2 subunits of GtH have been cloned and sequenced in goldfish and the channel catfish, *Ictalurus punctatus*.

The synthesis and secretion of gonadotropins are regulated by positive and

negative factors that act at the levels of brain, pituitary, and gonads. Most studies that have described the changes in the level of gonadotropins during the reproductive cycle in teleosts relate to the secretion of gonadotropin, but not to their synthesis. The use of a molecular biological approach has made it possible to examine changes in the levels of the mRNA of the gonadotropins during the gonadal cycle. Changes in the expression of GtHs in teleosts, during the gonadal cycle or under the influence of various hormones, have been studied in recent years.

The blue gourami (*Trichogaster trichopterus*) belongs to the family Anabantidae, which contains 16 genera and about 50 species, distributed throughout most of southern Asia, India, and central Africa. This family belongs to the suborder *Labyrinthici*, which is characterized by the presence of an air-filled breathing cavity (the labyrinth), located above the gills under the operculum. The blue gourami is multi-spawning and male-dependent, with an asynchronic ovary development. Its hormone profile during oogenesis has been studied in detail in our laboratory. Since each stage of its gonadal development can be controlled and examined separately in the laboratory, the blue gourami provides a unique model to study the roles of GtH-I and GtH-II in the hormonal control of oogenesis, and the expression of their genes during the gonadal cycle. The aim of Degani's (1994) study was to clone and sequence the cDNAs of the blue gourami β GtH-I and β GtH-II and to quantify the mRNAs of these hormones, in order to understand the dynamics of the synthesis of GtH-I and GtH-II in the female blue gourami during the gonadal cycle. The initiation of messenger RNA (mRNA) synthesis is the first step in the regulation of gene expression and the quantification of mRNA is now commonly used to measure levels of gene expression.

9.2 THE GTH-I AND GTH-II B SUBUNITS OF THE FEMALE BLUE GOURAMI (TRICHOGASTER TRICHOPTERUS, PALLAS 1770): EXPRESSION DURING THE DIFFERENT STAGES OF OOGENESIS

Female blue gouramis (*T. trichopterus*), maintained and bred at MIGAL's Laboratories in northern Israel, were used in Degani's study (Degani et al., 2001). The fish were grown in containers measuring 2 × 2 × 0.5 m, at a temperature of 27 °C, and under a light regime of 12 h L 12 h D. The fish were fed an artificial diet (45% protein, 7% fat) supplemented with live food (*Artemia salina*). The pituitaries were collected from 3-month-old females (3.5 ± 0.6 g) at various stages of gonadal development.

9.3 MEASUREMENT OF B GTH-I AND B GTH-II MRNA LEVELS BY RT-PCR

We measured the relative levels of both β GtH-I and β GtH-II mRNAs by reverse transcriptase (RT) PCR, with 18S rRNA used as an internal standard. The RT-PCR method was employed because the very small size of the blue gourami pituitary gland precluded the use of Northern blotting which requires relatively large amounts

of RNA.18S rRNA was chosen as an internal standard because it is an abundant RNA and its expression is considered stable. It has been observed that the levels of 18S rRNA are more uniform than those of other commonly used internal standards, such as β actin (Ambion Technical Bulletins).

RT-PCR was conducted as described above for 3' RACE-PCR, except that a mixture of random decamers (Biotechnology Services, Weizmann Inst. Science, Rehovot, Israel) was added to the RT reaction, to allow for the synthesis of the cDNA of the 18S rRNA. A separate cDNA pool was prepared from the pituitary of each sampled fish. In addition, small pieces of the ovary of each female were fixed in Bouin fixative (5% glacial acetic acid, 25% formaldehyde, 75% saturated solution of picric acid) and processed for light microscopy in order to determine the stage of ovary development. At least five female blue gouramis from each of the following stages of the reproductive cycle were used in this study: pre-vitellogenesis (immature females), low and high vitellogenesis (mature non-reproductive females), and FOM (mature reproductive females). The procedure was used to induce mature females to reach FOM.

9.3.1 AMPLIFICATION OF THE CDNA OF B GTH-I, BGTH-II AND 18S RRNA

Each amplification of either β GtH-I or β GtH-II cDNA was coupled to an amplification of the internal standard 18S rRNA. In each case, the 18S rRNA cDNA and the hormone cDNA, were amplified in separate tubes, since many attempts to use multiplex PCR had resulted in a considerable interference between the two reactions: amplification of the 18S rRNA cDNA in the same tube as either β GtH-I or β GtH-II cDNA caused a reduction in one or both PCR products. Such interference problems are not uncommon in multiplex PCR (Bercovitch et al., 1997). We carefully calibrated the concentration of each primer pair and the number of cycles, to allow for amplifications which were linearly dependent on the initial concentration of the target cDNA.

β GtH-I and β GtH-II cDNAs were amplified with gene-specific primers (see Table 9.1) at a concentration of 6.25 pmol/reaction. The 18S rRNA cDNA was amplified with specific primers, at a concentration of 5 pmol/reaction. The primers were obtained from Ambion as part of its Quantum RNA kit. The reagents, except for the cDNA and the primers, were prepared as a batch for each set of reactions, to minimize variations. The cycling parameters were the same as those described for the RACE-PCR, but in a smaller volume (50 µl) and with fewer cycles (30).

To quantify the PCR products they (half of the PCR reaction products[?]) were electrophoresed on 2% agarose gel at 120 mA for 45 min. The DNA was then stained with ethidium bromide (0.03 mg in 100 µl distilled water), and imaged with the Eagle Eye II image analysis system (STRATEGENE). The gels were imaged at three different exposure times to avoid the possible saturation of the images. The resulting images were analyzed with the aid of the PCBAS-2 densitometry software (Raytest, GR).

Table 9.1.
cDNA synthesis PCR primers used (Jackson et al., 1999).

Primer name	cDNA target	use	Nucleotide sequence	Degeneracy fold	Direction in relation to the mRNA
P1	GTH-I	3' RACE: PCR	TG(CT)AA(CT)GG(ACGT)GT(ACGT)TGG	×64	5' > 3'
P2	GTH-I	5'RACE:cDNA synthesis	5'GCAGTACACTCGCAGTTTC		3' > 5'
P3	GTH-I	5' RACE: PCR	5'TTACTTCGTAGGTCCAGTCCC		3' > 5'
P4	GTH-I	Complete cDNA cloning	5'GTCTGTACAGATGTTTAGAGAG		5' > 3'
P5	GTH-I	Complete cDNA cloning	5'AACGTGGGATGAATGATGAGTG		3' > 5'
P6	GTH-II	3' RACE: PCR	5'TA(CT)CA(AG)CA(CT)GT(ACGT)TG(CT)AC	×64	5' > 3'
P7	GTH-II	5'RACE: cDNA synthesis	5'CAGTCAGGAAACTCAAATGTC		3' > 5'
P8	GTH-II	5' RACE: PCR	5'TAGAACAAATCCCGGTATGTG		3' > 5'
P9	GTH-II	Complete cDNA cloning	5'CTGGCTAACCTGCCGCTGACAC		5' > 3'
P10	GTH-II	Complete cDNA cloning	5'TTGCTTTTGGTTTGCTGTGCAG		3' > 5'
dT_{17}-adapter	Universal	cDNA synthesis	5' GACTCGAGTCGACATCGA$(T)_{17}$		
Adapter	Universal	RACE PCR	5'GACTCGAGTCGACATCG		

9.4 VARIATIONS IN THE LEVELS OF β GTH-I, β GTH-II AND GROWTH HORMONE MRNAS DURING THE FEMALE REPRODUCTIVE CYCLE

We used the cloned cDNAs to measure variations in the levels of their respective mRNAs in the pituitary gland at various stages of the reproductive cycle. The measurements were done by RT-PCR, as described above. The rationale behind the use of this method was that, under proper conditions, the concentration of a specific amplification product is linearly proportional to that of its corresponding mRNA (Jackson et al., 1997). The amplification of β GtH-I cDNA produced a 306-bp product and that of β GtH-II cDNA a 284-bp product. The amplification of the internal standard (the cDNA of 18S rRNA) generated a 430-bp product. The identity of each PCR product was confirmed by DNA sequencing. Fig. 9.1 shows an agarose gel electrophoretogram of the three PCR products, which were taken from reactions with cDNAs from four different maturation stages.

The variations in the expression of the β GtH-I and β GtH-II genes, at the mRNA level, were evaluated by first measuring the yields of specific PCR products and then dividing them by the yield of the amplified internal standard. This normalization step was intended to eliminate the effects of random changes in the experimental setup. The results are presented in Figs. 9.2 and 9.3. Amplification of cDNA from

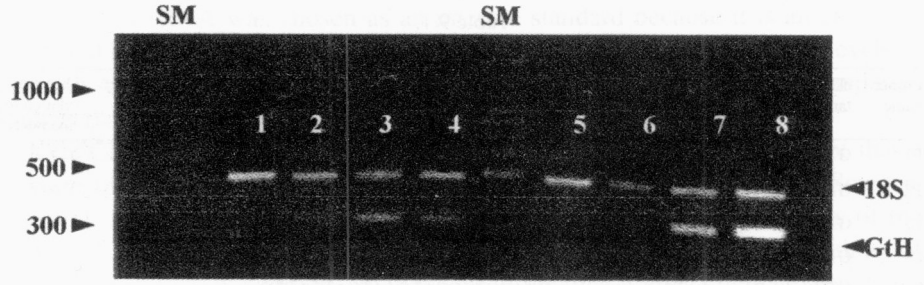


Fig. 9.1. Gel electrophoresis of 18S cDNA together with either GtH-I (lanes 1–4) or GtH-II (lanes 5–80 PCR products. Each lane shows amplification of cDNAs prepared from the pituitary of a single female. Young females (lanes 1–5), pre-vitellogenic females (lanes 2 and 5), high-vitellogenic females (lanes 3 and 7), females at FOM (lanes 4 and 8). (SM= size marker) (Jackson et al., 1999).

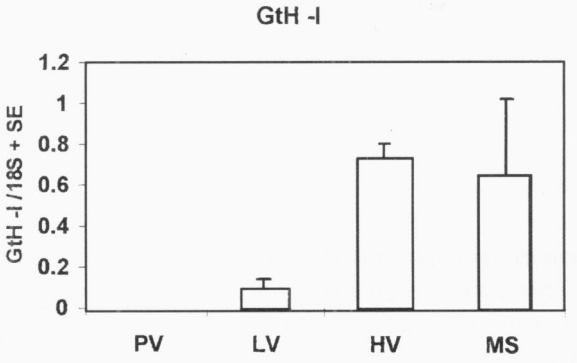


Fig. 9.2. Variations in βGTH-I transcripts levels during gonadal cycle: previtellogenic (PV), low vitellogenesis (LV), high vitellogenesis (HV), maturation (MS) (Jackson et al., 1999).

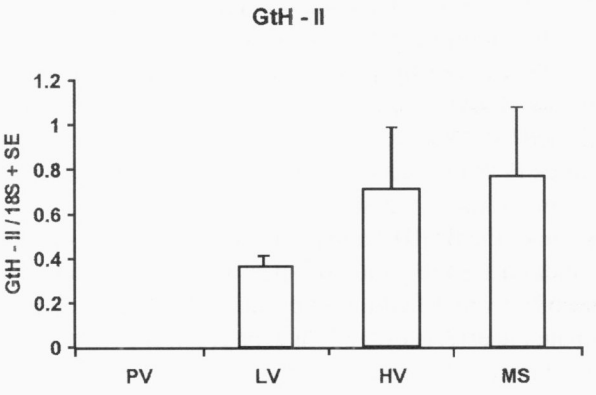


Fig. 9.3. Variations in bGTH-II transcripts levels during gonadal cycle: previtellogenic (PV), low vitellogenesis (LV), high vitellogenesis (HV), maturation (MAT) (Jackson et al., 1999).

pre-vitellogenic fish resulted in almost undetectable levels of β GtH-I and β GtH-II specific products, which indicates very low levels of their transcripts. The expression of β GtH-I and β GtH-II rose at the stage of low vitellogenesis, as manifested by the increased amounts of PCR products. A marked increase in the levels of β GtH-I and β GtH-II expression occurred during the high vitellogenesis stage, and the levels of β GtH-I and β GtH-II transcripts remained high during maturation. There was no significant difference between the levels of β GtH-I and β GtH-II transcripts in high vitellogenesis and in maturation.

The deduced amino acid sequences of β GtH-I, β GtH-II of *T. trichopterus* were compared with those of similar polypeptides from several other teleosts, by multiple sequence alignments and cluster analysis. This analysis revealed that both β GtH-I and β GtH-II of the blue gourami were most similar to their counterparts from the striped bass (*Morone saxatilis*) (Fig. 2.7). Both of these species belong to the order, *Perciformes*, the largest order of teleosts, with 251 families, and therefore this sequence similarity conforms well with known systematics. It is interesting to note that, in agreement with previous findings, our comparison clearly shows that the amino acid sequences of β GtH-I have changed more rapidly and show a lower degree of similarity than the corresponding amino acid sequences of β GtH-II (Fig. 2.7). The rapid divergence of β GtH-I, compared with GtH-II, has already been described in other teleost species.

It would be reasonable to doubt the reliability of an inference of phylogenetic relations based on only one hormone-coding cDNA; however, analyses of two different polypeptide hormones from the blue gourami are presented here, and both are consistent with its classification. The results of the cluster analysis which positioned the blue gourami close to other members of the order *Perciformes* can, therefore, be regarded as reliable.

In Jackson et al., 1997, the temporal differences between the biosyntheses of β GtH-I and β GtH-II during gonadal development were determined by studying the changes in their respective mRNAs. GtH-I transcripts were first detected during low vitellogenesis and an accentuated increase occurred during high vitellogenesis and maturation. The participation of GtH-I in vitellogenesis of teleosts has been reported previously. The presence of GtH-I transcripts in the pituitary glands of low-vitellogenic females was expected since the deposition of vitellogenin had already started in the most developed oocytes. The process of vitellogenesis continues until 30 to 40% of the oocytes are at the post-vitellogenic stage. Increases in the levels of GtH-I transcripts in pre-vitellogenic and vitellogenic teleosts have been described previously.

The level of mRNA of β GtH-I remains high also in reproductive females during the final stage of oocyte maturation. The blue gourami is a multi-spawner with an asynchronic type of ovary development: the same female can spawn four to five times consecutively at 2–3-day intervals (Degani et al., unpublished results). Therefore, vitellogenic oocytes should be found in the ovary of maturating and post-

spawning blue gourami females.

A similar pattern of gene expression has been found for GtH-II. The levels of its β subunit transcript were undetectable in immature females, rose in low-vitellogenic fish and showed a sharp increase during high vitellogenesis. The β GtH-II transcript level remained high at the stage of final oocyte maturation (FOM). An increase in the level of β GtH-II mRNA around maturation and spawning has been described previously, and Degani (1994) observed similar pattern for GtH-II hormone expression in the blue gourami. The beginning of GtH-II expression is in low vitellogenic fish and it is present at high levels in the pituitaries of high-vitellogenic females. However the hormone is released to the blood only during FOM.

It has been suggested that estrogens and androgens stimulate GtH-II synthesis in the pituitary. Dickey et al. (1995) found that injection of immature coho salmon with estradiol caused an increase in the steady state levels of β GtH-II mRNA but not of β GtH-I mRNA. The presence of a steroid response element in the promoter for the β GtH-II subunit gene in salmonids has been described previously. We have found an increase in the level of β GtH-II mRNA in the pituitaries of low-vitellogenic females, which may be the result of the rise in their levels of estradiol. The steady high levels of β GtH-II mRNA in high vitellogenesis and during FOM may be a consequence of the synthesis and release of other regulatory factors, such as GnRH, since at this time there is a decrease in the level of estradiol. GnRH affects GtH biosynthesis and has been shown to cause an increase in the levels of α and β GtH-II mRNAs.

In the blue gourami, both GtH-I and GtH-II RNA levels increase during the advance of the gonadal cycle. This pattern is similar to that found in goldfish and gilthead seabream (*Sparus aurata*). Both are multi-spawners with group-synchronic or asynchronic type of ovary development. This pattern differs from that found in salmonids, where GtH-I is expressed during vitellogenesis and GtH-II during late vitellogenesis and FOM, as was found in rainbow trout, an annual spawner with a synchronous type of ovary. The difference between the patterns of GtH-I and GtH-II gene expression is probably related to the different types of gonadal cycles: multi-spawners vs. annual spawners.

9.5 GENE EXPRESSION OF β GTH-I, β GTH-II AND GROWTH HORMONE IN FEMALE TRICHOGASTER TRICHOPTERUS *(PALLAS 1770) TREATED WITH GNRH ANALOG*

In salmonid species, spermatogenesis is regulated by pituitary GtH-I and GtH-II. Levels of GtH-I rise in the plasma during the early stage of spermatogenesis (spermiogenesis) and decline during spermiation, whereas the level of GtH-II is ery low during spermiogenesis and rises during spermiation (Planas, 1995, Prat, 1996). The salmonids exhibit a difference in sensitivity to GtH-I and GtH-II in the testis: the sensitivity to GtH-II increases towards the last stages of spermatogenesis. (Swanson et al., 1989; Planas and Swanson, 1995) The synthesis and secretion of gonadotropins are regulated by positive and negative factors that act at the levels of brain,

pituitary, and gonads. Most studies describing changes in the levels of gonadotropins during the teleost reproductory cycle are related to the secretion of gonadotropin, but not to its synthesis. Molecular biology techniques have made it possible to examine changes in the levels of the mRNA of the gonadotropins during the gonadal cycle. Changes in the expression of GtHs during the gonadal cycle have been described recently (Meiri, et al., 1995; Weil et al., 1995a,b; Sohn et al., 1999).

Pituitary growth hormone (GH), which is a single chain polypeptide, plays an essential part in the regulation of growth and development, by promoting the division, differentiation, and enlargement of cells (Corin 1990). The GH gene has been cloned from several teleost species. In most teleosts, only one type of GH gene has been cloned, but two GH genes have been found in salmonids and tilapia (see for reviews, see Venkatesh and Brenner, 1997).

The relationship between gonadal development (histological evidence for spermiogenesis and/or spermatogenesis), sexual behavior (nest-building), and mRNA levels of the gonadotropins (β GtH-I and β GtH-II) and growth hormone (GH) in the pituitary of males was investigated (Jackson et al., submitted for publication) Amplification of β GtH-I cDNA showed significantly higher level of mRNA in mature males (whether sexually active or not) than in juveniles. However, following the PCR amplification of β GtH-II cDNA, a significantly higher level of mRNA was found only in the sexually active group than in the sexually inactive group. In this respect, these results suggest that β GtH-I may participate in spermatogenesis, whereas β GtH-II is more involved in spermiogenesis. The level of GH mRNA increased slightly during the process of maturation, but no significant differences were found between the groups studied.

9.5.1 EXPRESSION OF β GTH-I, β GTH-II AND GROWTH HORMONE IN FEMALE TRICHOGASTER TRICHOPTERUS *TREATED WITH GNRH ANALOG*

The importance of gonadotropin releasing hormone (GnRH) as a factor in the release of follicle stimulating hormone (β GtH-I), luteinizing hormone (β GtH-II) and GH is well established. However, its effects on the biosynthesis of those hormones are less clear. In this study, the effect of GnRH analog (GnRHa) on the expression of the β FSH, β LH and growth hormone (GH) was examined in the female blue gourami during advanced vitellogenesis.

The levels of β GtH-I and β GtH-II transcripts in females injected with GnRHa were significantly higher than those of the control ($p < 0.05$) (Fig. 9.4, 9.5). However, no significant difference ($p > 0.05$, t-test) was found between the GH expression of saline (control) and females injected with GnRH. In the ovaries of GnRH-injected fish, some vitellogenic oocytes reached final oocyte maturation, as manifested by nuclear migration and central lipid drop formation (Fig. 9.6). The different effects of GnRH on the transcription of β FSH, β LH, and GH are discussed in relation to other data on teleosts.

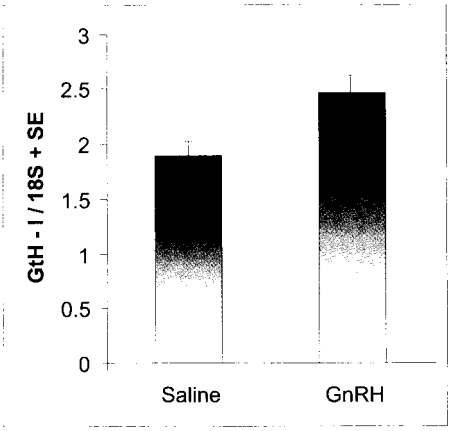


Fig. 9.4. The mRNA levels of GtH-I in female blue gourami injected with aGnRH.

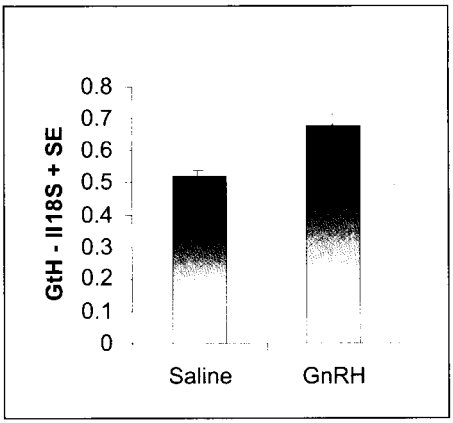


Fig. 9.5. The mRNA levels of GtH-II in female blue gourami injected with aGnRH.

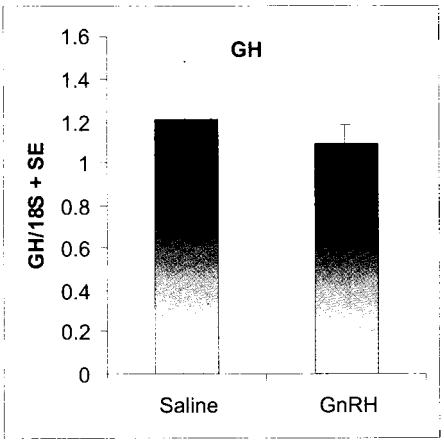


Fig. 9.6. The mRNA levels of growth hormone (GH) in female blue gourami injected with aGnRH.

*9.6 β GTH-I, β GTH-II AND GROWTH HORMONE GENE EXPRESSION IN THE BLUE GOURAMI (*TRICHOGASTER TRICHOPTERUS, *PALLAS 1770) DURING SPERMATOGENESIS AND MALE SEXUAL BEHAVIOR*

The relationship between gonadal development (histological evidence for spermiogenesis and/or spermatogenesis), sexual behavior (nest-building) and mRNA levels of the gonadotropins (β GtH-I and β GtH-II) and growth hormone (GH) in the pituitary of males was investigated (Degani et al., unpublished). Amplification of β GtH-I cDNA showed significantly higher level of mRNA in mature males (whether sexually active or not) than in juveniles. However, following the PCR amplification of β GtH-II cDNA, a significantly higher level of mRNA was found only in the sexually active group. In this respect, these results suggest that β GtH-I may play a role in spermatogenesis, whereas β GtH-II is more involved in spermiogenesis. The level of GH mRNA increased slightly during the process of maturation, but no significant differences were found between the groups studied.

Male blue gourami, bred and maintained at MIGAL Laboratories in northern Israel, were used in this study. The fish were grown in containers measuring $2 \times 2 \times 0.5$ m, at a temperature of 27 °C, and under a light regime of 12L:12D. The fish were fed an artificial diet (45% protein, 7% fat) supplemented by live food (*Artemia salina*). Males and females were maintained separately in groups of about 20 individuals each, in containers of 1 m^3. After two weeks, fish were paired by transferring a male into the presence of a mature female in a small aquarium ($40 \times 20 \times 20$ cm) containing *Elodea*. In most instances, the male started to build a nest on the plant within a few hours.

Fish were sacrificed by injecting an overdose of anesthesia (ethyl amyno benzoate). The body and testes of each specimen were weighed. Testes were taken for histological examination, to determine the stage of gonadal development, and freshly excised pituitaries were processed immediately. Donors of pituitary and testes were: 1) mature males that had started to build a nest after pairing (considered as reproductive or sexually active); 2) mature males that were kept in a group without females, hence did not exhibit nest-building behavior (considered as non-reproductive or non-sexually active); 3) immature males (juveniles).

The pieces of testes were fixed in Bouin and processed until embedding in paraffin. Histological sections of 2–5 mm were obtained by the use of a Reichert-Jung (Austria) microtome. The trichrome of Mallory was used to stain the sections. The levels of β GtH-I, β GtH-II and GH mRNA were measured by reverse transcriptase (RT) PCR, using 18S rRNA as an internal standard. This method was employed because the very small size of the blue gourami pituitary gland precluded the use of Northern blotting, which requires relatively large amounts of RNA. 18S rRNA was used as an internal standard for the measurements, since it is an abundant RNA and its expression is considered stable. It has been observed that the levels of 18S rRNA are more uniform than those of other commonly used internal standards,

such as β actin. The 18S level also proved to be stable for the blue gourami, and did not vary during different reproductory stages.

Total RNA was extracted from each pituitary individually by means of the Rneasy® total RNA kit (QIAGEN, Hilden, GR). In most cases, 40–70 ng/ml of total RNA were obtained from each pituitary.

In order to measure β GtH-I, β GtH-II and GH mRNA levels, the cDNA pool for the 3′ end was synthesized using an AMV RT (Promega). The primer for the synthesis of the 3′ cDNA end consisted of an oligo dT (17 bases), linked to a unique 17 mer adapter and a mixture of random decamers. At least five specimens from each of the following stages of the reproductory cycle were used in this study: pre-spermatogenesis (immature males), males maintained together to inhibit nest-building (non-reproductive males), and mature male nest-builders (mature reproductive males).

9.6.1 VARIATIONS IN THE LEVELS OF β GTH-I, β GTH-II AND GH MRNA DURING THE MALE REPRODUCTIVE CYCLE

Amplification of β GtH-I cDNA produced a 306 bp product; that of β GtH-II cDNA, a 284 bp product; and amplification of the internal standard (the cDNA of 18S rRNA) a 430 bp product. The identity of each PCR products was confirmed by DNA sequencing.

The variations of mRNA levels of β GtH-I are shown in Fig. 9.7. There was no significant difference (t-test; $p < 0.05$) in the β GtH-I between mature fish, whether non-nest builders (mature non-reproductive) or nest builders (mature reproductive). Both levels were significantly higher than in juveniles.

The variations of mRNA levels of β GtH-II are shown in Fig. 9.8. Amplification of cDNA from juveniles and non-reproductive mature fish were low, with no significant difference (t-test; $p > 0.05$) between them. The expression of β GtH-II rose significantly (ANOVA; $p < 0.05$) at the stage of nest-building, as manifested by the increased amount of PCR products.

The product generated by amplification of GH was 733 bp long. The identity of the PCR product was confirmed by DNA sequencing. There were no significant differences between mRNA level of GH (ANOVA or t-test; $p > 0.05$) between juveniles, mature non-reproductive and mature reproductive specimens (Fig. 9.9).

The results of this study indicate that GnRH enhances the expression of β GtH-II and β GtH-I in the blue gourami. The effect is presumably at the level of the transcription. Interpretation of the results suggests that β GtH-I and β GtH-II are differently regulated by GnRH, since the effect on LH is much more pronounced than on FSH. A similar result was obtained by Hassin et al. (1995), after aGnRH injection in striped bass males. Nevertheless, a direct effect of GnRH on β GtH-I transcription, though not on β GtH-II, was demonstrated in a cell culture of maturing salmon. Altogether, these results indicate that the effect of GnRH on the two

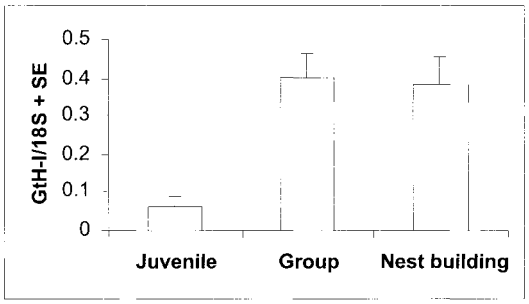


Fig. 9.7. Variations in the β GtH-I transcript levels during the gonadal cycle: Each histogram represents the average of five independent measurements (mean ± SE).

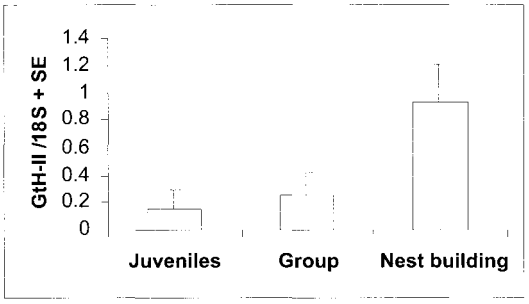


Fig. 9.8. Variations in the β GtH-II transcript levels during the gonadal cycle: Each histogram represents the average of five independent measurements (mean ± SE).

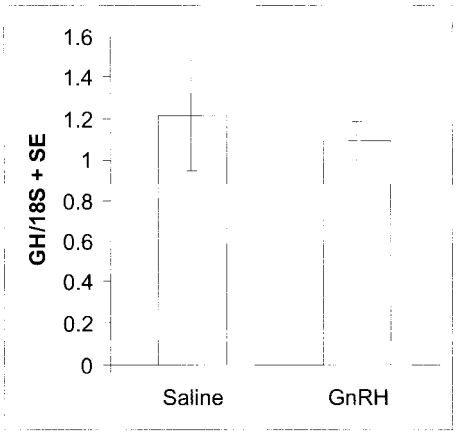


Fig. 9.9. Variations in the GH transcript levels during the gonadal cycle: Each histogram represents the average of five independent measurements (mean ± SE).

gonadotropins depends on the reproductive state of the fish, as previously noted.

The advantage enjoyed by the present study is that in *T. trichopterus* it is possible to study females specifically in advanced vitellogenesis, just before maturation. In the blue gourami, at the end of vitellogenesis, the ovaries contain oocytes arrested at advanced stages of vitellogenesis. Only when males are added to the tank do these oocytes enter FOM. Our explanation, based on the results from several studies performed in our lab, is that during vitellogenesis β GtH-II is produced in the pituitary but not released to the plasma. During this phase β GtH-II and β GtH-I expression is higher than in younger fish. High vitellogenic females transferred to the presence of a male reach FOM. During this period β GtH-II is secreted to the plasma, probably due to GnRH action. The levels of β GtH-I and β GtH-II expression remained high in FOM females and could be a consequence of GnRH action.

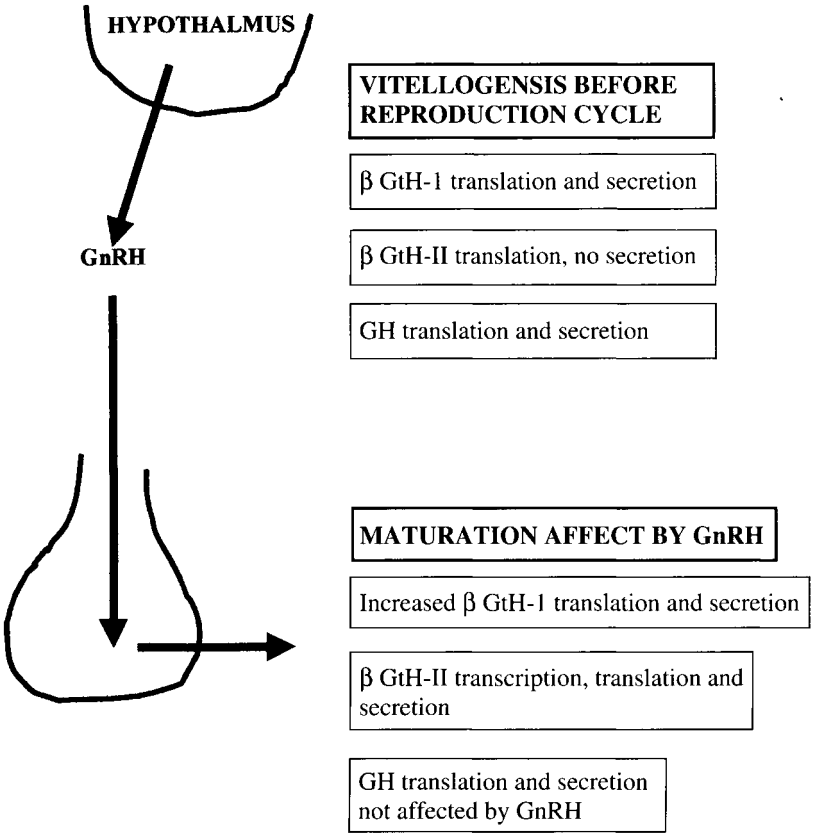


Fig. 9.10. Effect of GnRH on transcription and secretion of β GtH-I, β GtH-II and GH, as demonstrated in the female blue gourami.

The effect of GnRH before FOM is not fully understood, but it is possible that during this phase other factors, in addition to GnRH, are involved in the control of β LH and β FSH expression.

This situation was found not only in females, but also in males during sexual behavior and spermatogenesis (Degani et al., unpublished).

In conclusion we have demonstrated that in female blue gouramis in high vitellogenesis β, β GtH-II, β GtH-I and GH regulation are under the control of GnRH, but in different ways. GnRH affects the transcription of β GtH-II and β GtH-II but not of GH, and affects the release of all the hormones. Further study, investigating the role of other factors in β GtH-I and β GtH-II biosynthesis in high vitellogenic females, could add important information to the overall understanding of the gourami model (Fig. 9.10).

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