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POSSIBLE WAYS OF THE ARTIFICIAL INDUCTION OF SEXUAL MATURATION AND REPRODUCTION OF THE EUROPEAN EEL (*ANGUILLA ANGUILLA* L.).

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1. INTRODUCTION

Eel belong to the catadromous fish species, which means that after living in freshwater mature individuals migrate to sea (Sargasso Sea) to spawn. Since no one has been able to grow European eel larvae to glass eel size yet, we can not speak about eel breeding in the narrow sense of the term just only eel rearing. The annual production of eel farms in Europe is based on the capture of glass eels entering into rivers. Abundance of the European eel has dropped drastically from the 1980's due to overfishing of glass eels and adults, persistent pollutants, changes of ocean warming (Lobón-Cerviá, 1999; Pedersen and Dieperink, 2000; Dekker, 2000; Robinet and Feunteun, 2002; Knights, 2003). In the last decades, a new swim-bladder parasite *Anguillicola crassus* of the Japanese eel – has appeared in Europe infecting the European eel. It caused severe and well-published fish mortality in Lake Balaton in 1991 (Molnár et al., 1991). This infection can lead to a serious handicap for the infected fish reducing their swimming speed (Sprengel and Lüchtenberg, 1991). It may also decrease their reproduction fitness in a natural environment.

Due to their restricted availability, the prices of juvenile and adult eel are among the highest of farmed fishes (Tesch, 1991). For instance Japan has not exported frozen cultured eels since 1968, because the local demand has increased with a corresponding increase in price (Usui, 1991). In 1996, when the Balaton Fishing Company caught 500 tons of eel, it formed 70% of their gross income (István Szabó personal communication).

The economic and environmental importance of eel production by artificial propagation is evident.

Japanese scientists are in the forefront of the artificial propagation of eel. Since Yamamoto and Yamautchy (1974) obtained fertilised eggs and larvae from Japanese eel by hormone administration, many researchers have succeeded

in obtaining eel larvae. Tanaka et al. (2003) were the first who managed to raise Japanese eel larvae to glass eel size. There are only two scientific groups – Belorussian (Bezdenezhnykh and Prokhorchik, 1984; Prokhorchik, 1986; Prokhorchik et al., 1987) and Danish (Pedersen 2003 and 2004), which successfully produced hatched European eel larvae but these larvae died within 4 days. The general aim of research of this species is to improve the method for safe fertilisation technology including the timing of artificial ovulation, egg incubation and larvae hatching because these quantities are inconstant at this moment.

Although, Hungary has been in the forefront in developing technologies for the efficient propagation of several farmed fish such as carp (*Cyprinus carpio)*, pike-perch (*Sander lucioperca*), pike (*Esox lucius*) and catfish (*Silurus glanis*) (Horváth et al., 1984), propagation experiments for eel have not been carried out yet.

1.1. BIBLIOGRAPHICAL SUMMARY OF EEL

The word *anguilla* originated from the Latin *anguis* = snakelike phase (Medvegyné, 1983). Taxonomically the European eel belongs to; phylum *Chordata*; subphylum *Vertebrata;* section *Gnathostomata;* branch *Pisces;* class *Osteichthyes;* subclass *Actinopterygii;* band *Neopterygii;* divisio *Teleostei*; superorder *Elopomorpha;* order *Anguilliformes;* suborder *Anguilloidei;* family *Anguillidae;* genus *Anguilla* and species *Anguilla anguilla* L. (Györe, 1995). According to Berinkey (1966) the major taxonomic keys of eel are: fin formulas, D (dorsal fin) 245-275, A (anal fin) 176-249(215), P (pectoral fin) 15-21, V (ventral or pelvic fin) lacks, C (caudal fin) 7-12, number of vertebrates 111-119 (114-116).

By using molecular phylogeny Aoyoma and Tsukamoto (1997) assumed, that an ancestral eel (*Anguilla celebensis*) lived in the western Pacific Ocean during Eocene or earlier, which dispersed westward, probably by larval transport in the global circum-equatorial current through the northern edge of the Tethys Sea. This group split into two species: *A. anguilla* and American eels (*A. rostrata*), which entered the Atlantic Ocean, and a second group, which dispersed southwards and split into the east African species and Australian species.

1.2. DESCRIPTION OF MORPHOLOGY OF EEL AT DIFFERENT DEVELOPMENTAL STAGES

The *A. anguilla* undergoes many developmental changes during its life cycle. Its shape and colour depends on its habitat.

1.2.1. LARVAL STAGES

Eel has pelagic eggs, the size diameter of *A. anguilla* is 0.81-1.2 mm at ovulation (Boëtius and Boëtius, 1980; Pedersen, 2003 and 2004). The larva is called *leptocephalus* and it lives only in the ocean. It is completely different from the later postmetamorphic and continental stages regarding both body shape and colour (Tesch, 1991). Deelder (1970) divided the larval life of eel into two parts, embryonic and larval phase. The larval phase has been further subdivided into pro-larval and post larval phase. Eel at different developmental stages are shown in Figure 1.

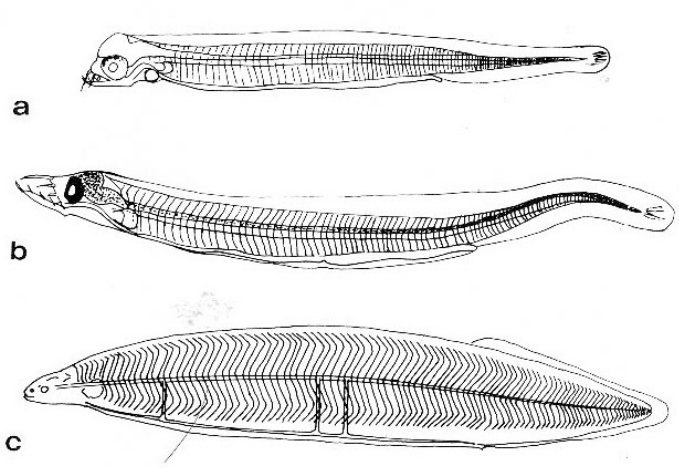

Figure 1. Developmental phases of eel larvae a; prealarvae; b: postlarvae; c: postlarvae of the leptocephalus (after Jespersen, 1942 cit. Tesch, 1991).

There is no information about the first embryonic phase of eels living under natural conditions. Prokhorcik (1986) and Pedersen (2003 and 2004) described the stages of early ontogeny of *A. anguilla* in artificial conditions. In the pro-larval phase the yolk sacs of the larvae contain an oil globule (Schmidt 1923). The larvae are characterised by their thin, longish and transparent bodies

and needle-like teeth. Prokhorchik et al. (1987) described the pro-larval development up to 4 day-old fry under artificial conditions. The *leptocephalus* development of eel fry in the post-larval phase was described by Schmidt (1923). The main feature of this phase is that the larva has a willow leaf-like shape. Their natural feeding behaviour is unknown (Larsen and Dufour, 1993). Earlier it was assumed that eel larvae drifted with the help of the Gulf Stream and the journey took three years (Schmidt, 1923). Based on age determination of glass eels, Lecompte Finiger (1994) established that *leptocephali* from the Sargasso Sea arrive at the European coast at the age of 180-290 days, which indicates a faster travelling speed than that of the stream. Consequently, eel larvae must swim actively during their journey.

1.2.2. GLASS EEL AND ELVER

When larvae arrive at the continental shelf, they undergo their first metamorphosis and become eel-like in shape. They are still transparent and - accordingly - they are called glass eel. During the metamorphosis they stop eating and shrink in all dimensions while the larval teeth are replaced by adult teeth and the digestive tract is reorganised (Larsen and Dufour, 1993). Cicotti et al. (1993) described the process of pigmentation and found that it takes from four to eight weeks, but it can not be determined exactly when it finishes. The most typical feature of the elver is its swimming against the stream (Gönczy and Tahy, 1985).

1.2.3. YELLOW EEL

This stage is characterised by dull body pigment, usually with a yellow-green tinge, and no sheen. The upper parts are grey, brownish, greenish or yellowish while the lower parts are dull white or grey (Usui, 1991). While the head is

small and flattened on top, the mouth is rather big and stretches as far as the eyes. Several rows of tiny and sharp teeth are found in the jaws. Its gill gaps are tight and covered by relatively small flaps (Harka, 1997). Depending on the different habitats and feeding habits, there are two head forms: wide-headed and narrow-headed eels. However, intermediate forms may also occur in the same water system (Györe, 1995). The proportion of time spent in freshwater and ocean habitats can be determined by using the ratio of stroncium and calcium in the otholiths of eels captured in seawater. Applying this method, Tsukamoto et al. (1998) found that there are some freshwater eels, which are not catadromous, and it is the sea population that primarily contributes to the reproduction of the species.

1.2.4. SILVER EEL

The silvering process is the second metamorphosis in an eel's life cycle. Various morphological and physiological modifications take place during this phase.

The most conspicuous external change is in the colour of the skin. A silvery tint begins to develop on the side of the body and it gradually spreads ventrally. Until the silver tint completely covers the surface of the abdomen, the eel is referred to as being "half silver". Dorsally and dorsolaterally, the eel becomes darker in colour. The pectoral fin becomes black. Changes in the colour are probably adaptations related to the transition from early benthic to the later pelagic habits. Another adaptation to the later pelagic or mesopelagic habits in the deep sea is the enlargement of the eyes (Tesch, 1991). Several studies reported physiological and morphological features that indicate sexual maturation induced by hormone application. Eye size is correlated with body length and gonad development (Pankhurst, 1982a; Pankhurst and Lythgoe, 1983) and the development of the cephalic lateral line (Pérez et al., 2000).

According to a structural study of the eyes, the total number of rods increases markedly while the density of the photoreceptors remains approximately constant during maturation (Pankhurst, 1982a). Cell number in the inner nuclear layer and ganglion cell layer decrease by 50 % (Pankhurst and Lythgoe, 1983). There is a decrease in the concentration of mucous cells from the anterior regions to the posterior regions, while the epidermis was thickest in the median parts of the body. Besides these common characteristics, sexual dimorphism is also evident: in silver eel the epidermis is thicker than in immature yellow eels while female silver eels have thicker epidermis and the greater concentration of superficial mucous cells (Saglio et al., 1998). There is a process of atrophy in the olfactory organs during maturation. The density of mucous cells in the olfactory lamellae decreases (Pankhurst and Lythgoe, 1983). The dermis increased in thickness with both size and sexual development. Hormone treated eels show a loss of mucous cells accompanied by degeneration of the epidermis. Scale areas increase from 50% in sexually immature adults to 145% in sexually maturing eels (Pankhurst, 1982b). Silver phase of Japanese eel (*A. japonica)* have more developed *rete mirable*, gas gland and submucosa than yellow eels and the development of swim bladder components increases with sexual maturity only in the early maturation process (Yamada et al., 2001).

According to Gönczy and Tahy (1985) there are three kinds of silver eel phases:

Actively feeding silver eel

This is an intermediate developmental phase between the yellow and the typical silver eel. The eye size is still small and the shape of the pectoral fins is spoon-like.

Adult pre-migrating form

The most characteristic signs of this phase are that the eye size reaches about 10 mm and the pectoral fin takes a lance-like shape.

Silver eel migrating to spawning site

Eel do not grow in this phase and stops feeding. According to several observations it seems remarkable that most of the eels living in Hungarian waters keep feeding actively and they probably stop feeding only after arriving at delta areas.

The fat content of eel continuously increases from 5-15% (yellow eel) to 25-28% (silver eel) (Usui, 1991). It is hypothesized, that eel stop feeding in the silvering stage, and the mobilisation of the energy stores is activated during migration and maturation. The main fuel sources in this process are lipids - 80% of the total energy demand (Boëtius and Boëtius, 1985) - mainly in the form of triacylglycerols of the depots in the white muscles (Lewander et al., 1974; Dave et al., 1975). Furthermore, fat is also accumulated in the liver during silvering (Lewander et al., 1974). According to the findings of Boëtius and Boëtius (1980) crude fat content of the body has to exceed a value of 20%, as silvering and the start of migration may be strongly connected to a minimum level of fat deposit. Larsson et al. (1990) assumed that one triggering factor for the spawning migration was to reach a minimum bodyweight of 350 g, which usually means 11-year old fish. However, Svedäng et al. (1996) who studied Swedish *A. anguilla* populations, did not find any evidence either for the hypothesis that there is a critical age and size when eel enter the silvery stage or that size and age at maturity are positively related. The age at the onset of maturity in female *A. anguilla* (Svedäng and Wickstörm, 1997) and in both sexes of *A. japonica* (Tzeng et al., 2000) were inversely related to the growth rate, so the reproductive tactic of eel is to become sexually mature at the earliest possible age. This theory is supported by several publications where 3-year old farm eels were used in the artificial induction of sexual maturation or propagation successfully (Tanaka et al., 1995; Ohta et al., 1996; Ijiri et al., 1998; Sato et al., 2000). Svedäng and Wirkstöm (1997) reported lower body fat content in female silver eel in Sweden, suggesting a more flexible maturation

process than it was hypothesised earlier. This means short nutrition periods during early maturation to ensure the possibility of survival and spawning. Ginekken and Thillart (2000) reported that, according to their calculation, about 60 % of the fat reserves is sufficient for migrating as well as for gonad development.

Size and weight of eel at different stages are summarised in Table I.

Table I. Summary of the minimum and maximum sizes of three developmental stages of the A. anguilla (Dekker et al., 1998).

Dimension			Minimum	Maximum
Glass eel		cm	5.4	9.2
Yellow eel		cm	6.9	133
		g	0.31	6599
Silver eel	Male	cm	21.2	44.4
		g	21	148
	Female	cm	26.4	101
		g	31	2137

1.3. CHRONOLOGY OF RESEARCH ON REPRODUCTIVE BIOLOGY OF EEL

For long centuries theories on the reproductive biology of *A. anguilla* was based on guesses and assumptions. Although, a great number of important discoveries have been made, the "eel mystery" has not been solved in its entirety yet.
Some of the most important discoveries in eel research and reproductive biology are briefly listed in the following historical summary (Müller, 1975):

350 BC. Aristotle described the migration of adult eel to the sea and assumed that juveniles swim up the rivers.

1777. Mondini described the frilly (curtain-like) organs as gonads.

1864. Redi observed eel migrating to the sea to mate and the young fish returning.

1856. Kaup coined the name *Leptocephalus brevirostris* for a newly discovered fish species that he failed to recognise as eel larvae.

1874. Syrski described the lobular organs as testes.

1896. Grassi and Calandruccio observed the metamorphosis of *leptocephalus* for the first time.

Since the first *Leptocephalus* larvae were found in the Messina Strait, it was believed that the spawning ground was in the Mediterranean Sea. Schmidt (1923) disputed this theory because larvae also occurred along the coast of Western Europe. He assumed that the spawning place might be situated in the Atlantic Ocean. After 22 years of research Schmidt (1923) outlined the spawning ground in the Sargasso Sea by calculations based on data of sea currents and larvae size. However, no one has been able to catch either an adult specimen or egg in the above-mentioned area (Larsen and Dufour, 1993). Migration and orientational factors are unknown, but there are several theories about them. It is assumed that the water temperature and olfactory (Westin, 1990 and 1998) as well as water discharge and moon phase (Vøllestad et al., 1994) help eel to orient near European coastal waters.

Fricke and Tsukamoto (1997) assumed that eels spawn in one place within Sargasso Sea. Wirth and Bernatchez (2001 and 2003) reconsidered the panmixia paradigm of *A. anguilla* investigating mitochondrial DNA of glass eels from different place of European coasts. It was stated that there may be three models for spawning time and place. According to the first model, there is a temporal delay between the arrivals of adult eels from different latitudes at the common breeding site. In the second model, more than one spawning site is used by some populations and different currents carry the leptocephali back to

their parents' original freshwater habitat. Third, there is only one shared spawning area where assortative mating occurs and larval homing to parents' habitat takes place using an unknown mechanism.

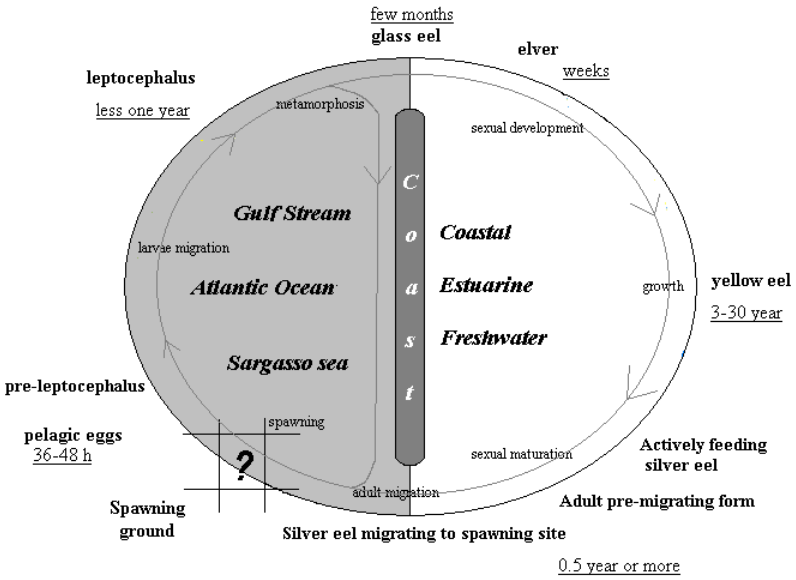

Figure 2. Life cycle of *A. anguilla* (Based on works of Lecompte-Finiger, 1994; Gönczy and Tahy, 1985; Tsukamoto et al., 1998).

1.4. EEL DISTRIBUTION

The *A. anguilla* is found in most European water systems, partly due to natural immigration and partly as a result of stocking. Its habitat stretches form the faraway Pechora River in the North all the way to the Western and Southern European coasts. Practically there is no river in Europe where eel do not migrate to. Apart from Europe, *A. anguilla* can be found along the Atlantic coast of Morocco and in smaller numbers in rivers of North Africa and the Middle East, which flow into the Mediterranean Sea. Most of *A. anguilla* eel larvae from the Sargasso Sea arrive at the Atlantic coast of France. Immigration of eel into the

Black Sea via the Bosporus Strait is minimal (Pintér, 1980a and 1989). There are several reports about the decline in *A. anguilla* due to several causes. The stock is now ten times smaller than it was in the 1980s (Feunteun, 2002). The causes of this decline are unknown but they are separated into two main types:

- Oceanic habitat destruction. A possible effect of long-term oceanic and climate changes (Knights, 2003; Feunteun, 2002; Wirth and Bernatchez, 2003).
- Freshwater habitat destruction for freshwater migration (Feunteun, 2002), overfishing (Lobón-Cerviá, 1999; Pedersen and Dieperink, 2000; Feunteun, 2002; Knights, 2003) *Anguillicola crassus* swimbblader parasite infection (Sprengel and Lüchtenberg, 1991; Ashworth and Balanc, 1997; Feunteun, 2002), accumulation of different types of chemical pollutants in lipid storages, which cause sublethal toxicity during the migration (Robinet and Feunteun, 2002; Feunteun, 2002), habitat loss, water quality (Feunteun, 2002).

1.4.1. EEL IN HUNGARY

Eel has been known in Hungary for a long time. Marsigli (Marsilius) described this species in the Lower Danube in Hungary in 1726 (cit. Vutskits, 1915; Mika, 1936; Vásárhelyi, 1962; Gönczy and Tahy, 1985). At that time eel occurred sporadicaly in the Hungarian water systems. According to Hermann (1887) and Unger (1916) eel was common in the River Poprad while it only occurred sporadically in the Danube (Landgraf, 1888; Répássy, 1902; Vutskits, 1915; Unger, 1916; Mika, 1936; Sterbetz, 1957). The presence of eel in other water systems was observed by Sterbetz (1957; 1959 and 1960). In order to increase the numbers of eel in Hungary, the issue of the introduction of eel stocks in the Danube and later in Lake Balaton was raised (Herman, 1887; Répássy, 1902;

Vutskits, 1915; Károly, 1928). The first elvers were released into the Danube in 1862 (Répássy, 1902) and into the Hungarian part of the Danube in 1887 (Unger, 1916). Vásárhelyi (1962) reported other introductions mainly to Lake Velencei and Fertő and to other waters. The introduction of eel was started without any practical experience, and was carried out solely on the basis of foreign results (Gönczy and Tahy, 1985). Pintér (1984) published a selected bibliography about the *A. anguilla* in the Hungarian fishery literature.

1.4.2. EEL IN LAKE BALATON

The first introduction into Lake Balaton took place in 1890 (Vásárhelyi, 1962), when István Hunyadi stocked 20000 elvers (Gönczy and Tahy, 1985). After a long break it was professor Schäperclaus, a fish biologist of European reputation from the German Democratic Republic (GDR), who proposed the restarting of the introduction of eel into Lake Balaton. Miklós Ribiánszky and Dániel Nagy investigated the results of eel introduction in the GDR just after professor Schäperclaus´s suggestion and the first introduction was carried out in 1961 (Horváth, 1971). Annual introductions took place from 1962 to 1987 with several millions of individuals. There was no introduction in 1985 and between 1988 and 1990. The last stocking was in 1991. Further introduction has been prohibited. From 1961 to 1991, 79 803 000 elvers were stocked into Lake Balaton (Virág, 1997). Ribiánszky (1965; 1968 and 1969), Tölg (1962 and 1963), Gönczy (1978), Pósvai (1988) and Gönczy and Tölg (1997) studied the economic potentials arising from the introduction of this fish species in our fisheries. An eel-trap was built at the start of the Sió canal at Siófok to recapture sexually mature, migratory eel (Elek, 1970). There are some publications about the feeding habit of eel in Lake Balaton (Szitó and Búz, 1975; Bíró, 1976). Eel is an intensive food utilizer due to their omnivorous feeding behaviour, and in

Lake Balaton it grows rapidly even as compared to other parts of Europe (Bíró, 1974).

1.5. SWIMBLADDER PARASITE (*ANGUILLICOLA CRASSUS)* AND ITS EFFECT ON EEL POPULATION IN LAKE BALATON

These nematodes belong to the *Anguillicola* live in the eel's swimbladders. Five species of the *Anguillicola genus* are known so far. Two of them originate from Eastern Asia while New Zealand, Australia and South Africa also have their own *Anguillicola* species. The parasite, whose original host is the *A. japonica* feeds on blood and causes no visible damage in the fish. However, the *A. anguilla* shows definite damage to 1 to 3 worms / swimbladders contrary to the *A. japonica* that has, an *A. anguilla* may be infected by 5-30 worms simultaneously. Infection in the *A. anguilla* leads to pathological changes in the swimbblader, it causes loss of appetite, reduced viability and emaciation. This parasite spreads extremely quickly in Europe, eel stock of most countries had become infested by 1990 (Csaba et al., 1991). The first specimen of the *Anguillicola crassus* were found in Lake Balaton's eel in September 1990 (Székely et al., 1991; Csaba et al., 1991). Adult worms are located in the lumen of the swimbladder, while third and fourth larval stages inhabit the wall of the organ. Adult worms feed on host blood (Békesi et al., 1997), Molnár (1993 and 1995), Molnár et al. (1991 and 1994) Molnár and Csaba (1991), Molnár and Moravec (1994) documented that natural infection by *A. crassus* caused mass mortality in Lake Balaton during 1991-1992. Csaba and Láng (1992), Szakolczai (1992), Szegletes and Nemcsók (1992) hypothesised that the role of this parasite in the eel mortality was important but it was not exclusive. Other causes such as "red disease" *Aeromonas punctata* (Pénzes, 1992) or exogenous toxicity (Gönczy, 1992) could also contribute to the serious fish destruction.

1.6. FISH GONADS AND GAMETES

Generally, fishes have one pair of bilateral gonads suspended from the dorsal portion of the body cavity, the mesovarium in females and mesochium in males (Hibiya, 1982).

Several histological studies were published on the development and early differentiation of gonads in the *A. anguilla* (Bieniarz et al., 1981; Colombo et al., 1984; Colombo and Grandi, 1995 and 1996) and finally it was Colombo and Grandi (1995 and 1996) who managed to reveal the unusual process of sexual differentiation in this species. Eel gonad development correlates with body size rather than with age from the earliest stages. Final sex differentiation occurs starting a juvenile hermaphroditic stage of a gonad which has the general histological features of an early testis but contains primordial germ cells: oocytes as well as spermatogonia. The development of testis can be influenced by various environmental factors, such as great population density and the presence of brackish water (Figure 3.)

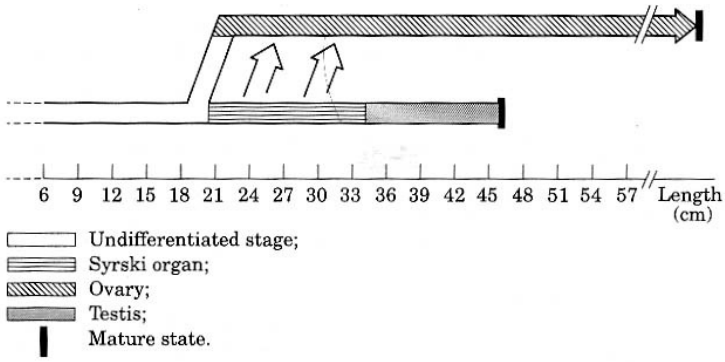

Figure 3. Diagram of the development and differentiation of eel gonads in relation to body size. The ovaries can be differentiated from undifferentiated gonads or from Syrski organs (large arrows) (Colombo and Grandi, 1996).

1.6.1.OVARY

The structure of eel ovary is one of the simplest among fishes; the ovary hangs down like a curtain and after the ovulated eggs are released into the body cavity, it is discharged from the body. This type of ovary is called gymnovarian (Hibiya, 1982).

Although the gonads are twins, their structures are asynchronous. In the case of a ca. 30 centimetre female the right gonad stretches approximately one centimetre farther anteriorly than the left one, while the left gonad spreads over the genital area 2 centimetres farther posteriorly than the right one. The left ovary is heavier by 2-3%,longer and contains more oocytes than the right one (Tesch, 1983).

Oogenesis starts with the proliferation of the oogonium on the ovarian lamella. The oogonium at the early stage is a large cell with a large nucleus while at the following stage, after multiplication, the cell becomes considerably smaller. The oogonium after the multiplication stage develops into the primary oocyte. The size of the cell is not so different but the chromosomes at first appear thread-like and are distributed throughout the nucleus (leptotene stage). The chromosomes then assemble at one side of the nucleus adjacent to the nuclear membrane localises at the opposite end of the nucleus (zygotene stage). Next, the primary oocyte enters the pachytene stage in which the nucleolus moves to the central part of the nucleus and the bivalents to the edge of the nucleus: at the same time, the nucleus increases in size to become the germinal vesicle. The many nucleoli become smaller, move to the periphery of the nucleus and arrange themselves in order on the inner side of the nuclear membrane. Furthermore, the follicle cells surrounding the oocyte become clearly distinguishable (diplotene stage). The follicle cells constitute multiple layers in Elasmobranchia and Amniota but exist as a single layer in bony fishes.

With the accumulation of yolk substances, i.e. vitellogenesis becoming morphologically up to this time, becomes acidophilic, and the growth of the oocyte proceeds. The essential yolk substances are three kinds, i.e., yolk vesicles, yolk globules and oil droplets. The time of appearance of the above three kinds of yolk substances differs among species, but the yolk vesicles always appear earlier than the yolk globules. When the accumulation of yolk substances becomes conspicuous, hyperplasia of follicle cells is recognised, and the squamous theca cells line up in two layers to form an outer and inner *theca* membrane outside the follicle cell layer. Moreover, radial striation also becomes clear and this stratum is called the *zona radiata*. The thickness of the egg membrane decreases just before the complete maturation. With the completion of vitellogenesis, movement of the germinal vesicle, fusion of yolk globules and grouping of oil droplets occur and, especially in marine fishes the egg diameter increases sharply. Marked increase in body weight occurs due to water absorption. The degree of fusion yolk globules differs among species. After the movement of the germinal vesicle to an animal pole, the first meiotic division occurs and the first polar body is released. Subsequently, the second meiotic division starts and an egg in which division arrested at the metaphase is ovulated. The ovary just after spawning is composed of many postovulatory follicles, immature oocytes and mature eggs left unspawned, etc. the follicle cells in the postovulatory follicles, hypertrophy and multiply, showing phagocytozis (Hibiya, 1982). Eel oogenesis corresponds with the process described above. Lokman and Young (1998) divided the eel oocyte developmental stages into 10 phases based on the results of earlier works. I, oogonium; II, previtellogenic oocyte (oocytes in chromatin nucleolus or perinucleolus stage, with strongly basophilic cytoplasm and no or few oil droplets); III, cortical alveolus stage (oocytes with oil droplets in cytoplasm, but not strongly basophilic cytoplasm); IV, early vitellogenic ooccyte (containing

peripheral yolk granules); V, mid-vitelogenic oocyte (containing peripheral yolk granules and central yolk platelets); VI, late vitellogenic oocyte (beginning of peripheral yolk fusion); VII, migratory nucleolus stage oocyte; VIII, ovulated egg; IX, post-ovulatory follicle; and X, atretic follicle.

1.6.2. EGGS

Fishes produce yolky eggs, particularly those producing eggs that are oviposited onto/into substrates (Tyler and Sumpter, 1996). The eggs, which are ready to ovulate, contain large amount of yolk (*polilecithalis* egg) in the greatest number of fish species but some fish (*Acipenser, Dipneusti, Amiidae)* have less amount of yolk (*mesolecithalis egg*). Yolk content disperses irregularly, yolk material clusters around one pole of the egg (*telolecithalis* egg). Eggs can be divided into a yolk-free area (animal pole) and a vegatative pole, which contains a large amount of yolk materials (Kiss, 2000).

The size of eggs is varying from 0.3 mm (*Cymatogaster aggregatus*, Percichthydae) to 8 cm diameter (*Latimeria chalumnae*, Latimeriidae). Fish spawning pelagic eggs - for instance *A. anguilla* - generally produce smaller eggs than substrate-spawning species do (Tyler and Sumpter, 1996). The egg size of the *A. anguilla* is about 1 mm (0.93-1.4 mm: Fontaine et al., 1964; 1.05±0.15 mm: Boëtius and Boëtius 1980; 0.95-1.07 mm: Anonymus, 1983; 0.897±0.1077 mm: Bezdenezhnykh and Prokhorchik, 1984; 1.1-1.2 mm : – swollen eggs - Prokhorchik et al., 1987; 1.2±0.06 mm: Amin, 1997; 0.811±0.1 – 0.955±0.017 mm: Pedersen, 2003; 0.829±0.046 – 0.924±0.058 mm: Pedersen, 2004). The absolute fecundity is varying 0.7-2.6 $\times$ 10^6 (Boëtius and Boëtius, 1980), 1.48 $\times$ 10^6 (Amin, 1997). The freshly stripped eggs show great variations in transparency. Clear eggs can be stripped with opaque specimens (Boëtius and Boëtius, 1980; Bezdenezhnykh and Prokhorchik, 1984; Amin, 1997; Pedersen, 2003 and 2004). Boëtius and Boëtius (1980) described three types of eggs

according to their oil globule content; eggs with several globules, eggs with few globules and eggs with one globule only. Pedersen (2003) divided the egg developmental phase into four maturational stages: stage 1- small, black non transparent cells; stage 2- larger eggs with a dark - grey cytoplasm containing numerous, small dark oil droplets; stage 3- the greyish cytoplasm and the oil droplets became more transparent, the oil droplets increased in diameter and decreased in number; stage 4- eggs at the migratory nucleus stage, cytoplasm as well as oil droplets become highly transparent. If final maturation is not correctly timed, the oocytes may overripen.

1.6.3.TESTIS

The testicular structure in teleosts is variable from species to species, although two types, lobular and tubular can be identified according to the differentiation of the germinal tissue. The eel testis is of the lobular type, which is typical for most teleosts (Nagahama, 1983). The lobular type testis is composed of numerous lobules that are separated from each other by a thin layer of fibrous connective tissue. Within the lobules the primary spermatogonia undergo numerous mitotic divisions to produce cysts containing several spermatogonal cells. During maturation, all of the germ cells within one cyst are at approximately the same stage of development. As spermatogenesis, and then spermiogenesis proceed, the cysts expand and eventually rupture, liberating sperm into the lobular lumen which continues in the sperm duct (Nagahama, 1983).

As in oogenesis, spermatogonia undergo proliferation, growth and maturation and division. The spermatogonium at the early stage is a large oval cell with one large round nucleolus. At the latter stage of proliferation it is small and round. After the proliferation stage the spermatogonium develops into the

primary spermatocyte. As the oocyte goes through the leptotene into the zygotene stage, the chromosomes assemble at one end of the nucleus. They then spread throughout the nucleus, and after a while the nuclear membrane disappears. Each chromosome becomes thick and short and lines up with the others. The primary spermatocyte develops into the secondary spermatocyte by maturation (or meiotic) division and then undergoes a second maturation division to develop into a spermatid. The spermatid then develops into a spermatozoon with a head, middle piece and long tail. Sertoli cells (or cyst cells), interstitial cells, and Leydig cells, are present in testis. Sertoli cells exist with the germinal cells and are believed to play a role in the supplying of nutrients. Leydig cells exist in the connective tissue of testis and secrete sex steroids (Hibiya, 1982).

The gonads of silver eels living in freshwater are immature and the sexual maturation of animals in captivity never occurs (Dufour et al., 1988). Similarly to the females, there are also a lot of histological studies on the hormonal induced spermiogenesis of *A. anguilla* males (Boëtius and Boëtius, 1967; Colombo et al., 1987; Leloup-Hâtey et al., 1985; Khan et al., 1987).

1.6.4. ULTRASTRUCTURE OF FISH SPERMATOZOA

A great number of studies have been published on the ultrastructure of eel spermatozoa. They have revealed strange spermatozoa ultrastructure including their crescent-shaped nucleus, rootlet originated from neck region, flagellum of the 9+0 pattern and pseudoflagellum extending from the proximal centriole (Billard and Ginsburg, 1973; Todd, 1976; Gibbons et al., 1983; Wooley, 1997; Wooley, 1998; Okamura et al., 2000). The sperm of the *A. anguilla* possesses a large oblong head, which is attached eccentrically by one end to the basal end of the flagellum. The axis of the head is approximately perpendicular to that of the

flagellum, thereby forming an elbow-shaped region where the two structures join. The head is characteristically shaped like a long narrow spoon (Gibbons et al., 1983). The rootlet projects from the neck close to the base of the flagellum (Okamura et al., 2000). From the proximal centriole two sets of filaments arise, each containing three subfibriles. One group of five filaments extends anteriorly along the inner concave side of the head. A group of four modified filaments pass along the outer, convex, side (Todd, 1976). The construction of the flagellum is very simple; between the duplets the outer dyneim arms, the radial spokes and the central structure are missing (Wooley, 1998). The structure of the inner dyneim arms are similar to those of other fishes and other vertebrates (Wooley, 1997).

Table II. Comparison of the morphometric measurements of the spermatozoa of A. anguilla. Abbreviations: TEM - transmission electronmicroscopy, SEM - scanning electronmicroscopy, N/A – not assayed.

Head length (μm)	Head width (μm)	Flagellum length (μm)	Rootlet length (μm)	Mitochondrion diameter (μm)	Method	Source
ca. 9	N/A	ca. 30	N/A	N/A	TEM& SEM	Meske, 1973
6-9	N/A	ca. 35	N/A	N/A	TEM& SEM	Gibbons et al., 1983
5.4(±0.4)	1(±0.2)	25(±5.5)	1.2(±0.2)	0.8(±0.3)	SEM	Okamura et al., 2000

1.7. HORMONAL CONTROL OF OOCYTE GROWTH

According to the comprehensive study of Tyler and Sumpter (1996) several hormones have been implicated in regulating oocyte growth in fish: they include gonadotropin (GTH), thyroxide, triiodothyronine, growth hormone, insulin and insulin-like growth factors (IGFs), but generally, there has been a lack of

significant progress in gaining a clear understanding of their precise roles. Some of these hormones probably act indirectly: for example growth hormone, which can modulate the rate of body growth and in turn modulate female fecundity and egg size. Growth hormone, however, may also act synergistically with GTH in regulating steroidogenesis, possibly through IGFs. This review focuses on hormones that have been shown or are believed to have a direct effect on oocyte growth.

In fish there are two GTHs, termed GTH I and GTH II, which are believed to be similar to FSH and LH, respectively. The data associated with establishing the role of GTH II are clear: it plays a central role in bringing about ovulation of the mature oocyte. The functions in oocyte growth are associated with GTH I, however, they are less clear. Studies have indicated that GTH I plays a role in oocyte growth during vitellogenesis. Ovarian steroidogenesis is controlled by gonadotropin, and both GTH I and GTH II can stimulate steroid secretion in cultured follicles. However, physiologically, GTH II is undetectable in the plasma until very close to ovulation, whereas plasma levels of GTH I are elevated during vitellogenesis. Therefore it is likely that GTH I controls the ovarian production of oestradiol-17β and stimulates the hepatic production of vitellogenin (VTG) and egg shell proteins. *In vitro* studies have shown that GTH I aphysiological concentrations may exchange the rate of uptake of VTG into vitellogenic oocytes in salmoids. Stimulation of VTG uptake into early to mid stages of vitellogenic development, but is ineffective in this capacity later in development.

The release of GTH is controlled by a stimulating and an inhibiting neuroendocrine regulation. The stimulating regulation is the gonadotropin releasing hormone (GnRH), which is produced in the hypothalamus. The GnRH producing cells are in connection directly with the GTH cells in the hypophysis. The release of GnRH is induced by the stimuli of environmental spawning

conditions and pheromones. Dopamine influences the inhibition of spontaneous or GnRH stimulated GTH release. In the pituitary the nerving of the GTH releasing cells is direct and the dopaminerg inhibition is attained through D-2 receptors. Dopamine directly affects the inhibition of the release of GnRH as well (Szabó, 1998).

1.8. METHODS OF LARGE SCALE FISH PROPAGATION

According to Woynárovich (1994) there are five methods by which the induction of ovulation of fish can be achieved.

1. Suitable spawning conditions should be provided.

For instance, the induction of spawning of carps in 'Dubics ponds' or using of nests for some catfish breeders are examples of this method.

2. Deleting the dopamine barriers by using a dopaminerg compound

It is sufficient to stop the effects of the gonadotropin releasing inhibiting factor by using pimozid or domperidone, so the hormonal cascade processes can be initiated in several fish species. In other fish species the domperidone treatment is necessary for the artificial induction of propagation.

3. ***Application of synthetic gonadotrop releasing hormone analogues***

In several fish species, it is necessary to use domperidone combined together with GnRH-analogues due to the action of those compounds In the practice mother fish receive their doses in two portions (0.8-1 and 5-10 µg, respectively). When only one dose is used 6-10 µg is applied. Overdosing can induce antagonistic effects, which can even kill the mother fish.

4. Application of pituitary

According to this method an exogenous pituitary extract is injected into the mother fish which is ready to spawn. The foreign pituitary extract induces the final maturation or spawning.

5. Application of MIS (maturation inducing steroid)

According to many experiments ovulation and spawning can be induced with MIS but exact dosing is not easy.

1.9. ARTIFICIAL INDUCTION OF SEXUAL MATURATION IN *A. ANGUILLA* MALES

In contrast to other fish species, where only the final part of maturation needs stimulation by different types of hormones during artificial propagation, in the case of eel the whole process of gametogenesis has to be induced.

Similarly to other fish species, eel males can be matured more easily than females by hormonal administration. Boucher was the first, who managed to induce maturation in *A. anguilla* males in 1935 by applying hormones (Tesch, 1983). Several different hormonal extracts, such as carp pituitary, hCG, prolanin, gestyl, ambion pregnil, tireotroph hormone, spleen extracts, were administered to induce full sexual maturation in males (Tesch, 1983).

Fontaine (1936) treated males with a preparation from the urine of a pregnant woman and managed to get sperm release.

Between 1958 and 1964 Boëtius and Boëtius (1967) carried out several experiments to describe a suitable standard method for the efficient induction of full sexual maturation of males. The application of several different human chorion gonadotropin (hCG) combinations (1000 IU/week, 250 IU/0.5 week, 250 IU/0.3 week, 100 IU/week, 100 IU/3 week until maturity) led to full sexual maturation, and the duration of the maturation periods did not differ significantly from that of the standard injected controls (250 IU hCG weekly intramuscularly, at a water temperature of 14±2°C). Hypophysectomized, standard injected silver eels reached the experimental maturation as easily as their standard-injected controls with intact pituitary. The maturation period

decreased with increasing salinity, but not much when the wide intervals between the chosen salinities are taken into consideration. Influence of light upon experimental maturation was found to be negligible. Experimental maturity can be induced from 13°C (and probably a few degrees lower, but under 8°C, no eel reached sexual maturation) up to 25.5°C, the process of maturation can not be completed at higher temperatures. Mathematical analysis of the temperature / maturation period data indicated an optimum temperature of about 20°C. A relationship between the changes of eye size and growing testes (characterised by seven developmental stages) was described. The authors observed that many males often survived hormonally induced maturation which questioned the accented view, that eel die after spawning.

Meske (1973) treated males with Solcosplen (a protein free extract of fresh calves "spleen"), Synahorin (a mixture of gonadotropic hormone of the anterior lobe of the hypophysis and of the placenta of warm-blooded animals, in the ratio of 1:9) and Cyren B (a synthetic oestrogen, diethylstilbo-estrolddipropionate). The males were treated according to the following scheme:

-one group was injected with 0.2 ml Solcosplen / fish at intervals of two weeks,

-second group was injected with 0.2 ml Solcosplen plus 50 IU Synachorin / fish at intervals of two weeks,

-third group was injected with 50 IU Synahorin plus 0.25 mg Cyren B / fish at intervals of two weeks.

Only the Solcosplen and Synahorin mixture brought positive results in all male eels. All treated males in the second group released sperm.

Dollerup and Graver (1985) injected hCG 500 IU on days 0 and 7. After spermiation the eels were given food (from day 118) and started to eat and grow. Later two further sexual maturations were induced (injections on days 179 and

186, and on days 400 and 407). They obtained 91% mature *A. anguilla* males in the first maturing cycle and 83% and 70% in following ones, respectively.

Bieniarz and Epler (1977) carried out experiments with wild males. Seven group were formed based on the rearing conditions (artificial seawater and freshwater) and weekly hormone administrations (100 IU hCG/fish, testosterone proprionate 5 mg/fish, desoxycorticosterone 5 mg/fish and control injecteing with saline solution. Neither testosterone nor desoxycorticosterone injections had any influence on the testis. Sperm was stripped from freshwater and seawater males at the sixth injection by using weekly injections of hCG, but the freshwater stock died two weeks after spermiation. The spermatozoa of the specimens kept in the artificial seawater was of higher quality than that from freshwater-reared ones.

Leloup-Hâtey et al. (1985) injected males with 0.6 mg carp pituitary 3 times a week steroidogenesis for five weeks in freshwater at 22°C to investigate on the testicular. For the fifth week, gonado somatic index (GSI=gonad weight/bodyweight with the gonads × 100) increased from 0.066±0.004 % to 2.181±0.036 % and the testicular tissue contained numerous spermatozoa.

Khan et al. (1987) reported on the sexual maturation cycle in *A. anguilla* males (intact and Hypophysectomized) in freshwater. The stock was injected once with a dosage of 250 IU hCG/individual and full sexual maturation was achieved 3 months later.

Colombo et al. (1987) described the complete testicular maturation of eel. They induced the males with a single injection of hCG (1000IU/fish) and obtained full sexual maturation after 4 weeks in freshwater at 24°C.

Pérez et al. (2000) worked on farmed eel in seawater and applied hCG; 1.5 IU/body weight g weekly. Spermiation started on the fourth week of treatment and generally continued for at least 10 weeks, until the fish died. The percentage of spermiating males reached 83.3% on the fifth week of treatment

and was maintained at 100% from eight to tenth week of treatment. Milt volume increased progressively from the beginning of spermiation. The greatest motility of sperm was detected during the ninth week of treatment. Sperm density was increased significantly 6 hours after hormone injection and decreased later. In contrast, highest sperm mobility and motility were observed 24 hours after hCG injection, suggesting that this is the best time to obtain good quality sperm.

In males a single injection may be sufficient. Using different dosages of weekly hCG treatments can shorten the maturation time and prolong the spermiation period.

1.10. ARTIFICIAL INDUCTION OF SEXUAL MATURATION OF FEMALES

Fontaine et al. (1964) were the first who induced full sexual maturation and ovulation of *A. anguilla* females. This result was obtained by repeated carp pituitary injections firstly, then dezoxy-corticosterone-acetate administration three month later. Only one from ten females ovulated spontaneously in this way. In the following years the focus point of research on artificial induction of sexual maturation of eel moved to Japan. The Japanese scientists were the first who managed to induce full sexual maturation and ovulation in *A. japonica* and hatched vigorous larvae. Table III. shows the summarised results of eel maturation and propagation in different kind of eel species. Parallel with attempts to induce sexual maturation in silver eels by hormone treatment, attempts were made to change the environment in a way that would permit sexual maturation to occur spontaneously or would induce maturation. Fontaine et al. tried to induce sexual maturation by using gradual submersion of caged females to a depth of 450 m for 3 months in the Mediterranean Sea in 1985. GSI

increased slightly (from 1.6 to 2.2), but pituitary gonadotropin content increased 27 times (cit. Larsen and Dufour, 1993).

Table III. Summarised data of experiments of the artificial induction of sexual maturation of eel

Experi-mental stock	Hormone administration			Result	Source
	Maturation	Ovulation	Matu-ration time		
A. japonica					
-	Four types of pituitary		3 months (10. injec.)	spawning	Hibiya, 1970 (cit. Tóth, 1973)
-	40 IU Synahorin (mammals gonadotropin), 0.2 mg synthetic oestrogen (dietyl-stylbestrol) and 30 IU tocoferol (E vitamin) / individuals at intervals 5-10 days		3 months	final maturation phase	Nose, 1971 (cit. Tesch 1983)
Silver eel	4 Chum salmon or 8 pink salmon pituitaries / body weight kg +1000 IU hCG two fishes at weekly administration		9-12 weeks	One female spawn (GSI=8.7%), others in final maturation phase (GSI from 40 to 72%)	Yamamoto et al., (1974)
Silver eel	Protocol of Yamamoto et al. (1974) was applied		Data were not given	Fertilised eggs, larvae hatching	Yamamoto and Yamauchi, (1974)
Silver eel	Protocol of Yamamoto et al. (1974) was applied		Data were not given	Fertilized eggs Larvae rearing for 14 days	Yamauchi et al., (1976)
Silver eel	75 µg T[1] / bw g		75 days	GSI= about 10%	Lin et al., (1991)
	75 µg T+ 0.5 µg LHRH-A[2]				
	75 µg T + 75 µg DOM[3]/ bw[4]				
	50 µg T / bw g		105 days	GSI = about 20% GSI max=39.8%	
	50 µg T+0.5 µg LHRH-A/ bw g				
Silver eel	Commercial gonadotropin, puberogen, DES-Na and vitamin E for 6 occasions (different dosages and combinations) Once a week	salmon or carp pituitary extract + LH-RH analogue and vitamin E or puberon (different dosages and combinations) Once a week	12-14 weeks	Hatchable eggs Spawning behaviour	Satoh et al., (1992)

[1] T - 17α methyltestosterone
[2] LHRH-A - lutein-hormone-releasing-hormone analogue
[3] DOM - domderidone (doamine receptor antagonist)
[4] bw – body weight

Experi-mental stock	Hormone administration			Result	Source
	Maturation	Ovulation	Matu-ration time		
A. japonica					
Feminised cultivated eel	20 mg pituitary powder / fish once a week	17α H[5] DHP[6]	9-15 weeks	Neither MIS hormones induce germinal breakdown of oocytes at tertiary yolk stage. Oocytes over 800µg in diameter more sensitive to the steroids	Kagawa et al., (1995)
Cultured eel	Salmon pituitary (dose is not given)	DHP (dose is not given)	-	Larvae rearing for 18 days	Tanaka et al., (1995)
Protocol of Tanaka et al. (1995) was applied				Fertilised eggs Larvae rearing for 13 days	Kurokawa et al., (1995)
-	20 µg Salmon pituitary / bw g once a week	-	Over 25 weeks	Vitellogenic growth proceed in about 50% responders while in others oocytes ceased development at the early vitellogenic stage	Ijiri et al., (1995)
Feminised cultured eel	20 mg Salmon pituitary / bw kg once a week	2µg DHP / bw g	8-17 weeks	Fertilised eggs Described the Ohta protocol to induce full sexual maturation and induction of ovulation	Ohta et al. (1996) Ohta et al. (1997a)
Cultured eel	1 µg / bw g Water-in-oil-water emulsion/ fish	LG emulsion[7]	10-11 weeks	GSI=33.6-47.7% Migratory nucleus stage oocytes (mnso)	Sato et al., (1997)
		FIA emulsion[8]		GSI =37.2-55.9% 7.-9. week mnso then overripened	
Cultured eel	20 mg Salmon pituitary / bw kg once a week	2µg DHP / bw g	10 weeks	Fertilised eggs Hatching (used milt and incubated testicular milt)	Ohta et al., (1997)b

[5] 17αH - 17-hydroxyprogesterone
[6] DHP - 17α20β dihydroxy-4-pregnen-3 one
[7] LG emulsion - water-in-oil-in-water type emulsion using lipophilised gelatin
[8] FIA emulsion - water-oin-oil emulsion prepared with Freund's incomplete adjuvant

Experimental stock	Hormone administration			Result		Source
	Maturation	Ovulation	Maturation time			
A. japonica						
Cultured eel / Acclimation for seawater: 1 week	20 mg Salmon pituitary/fish once a week	2µg DHP / bw g	8-16 week	Fertilised eggs Hatched larvae		Kagawa et al., (1998)
Cultured eel / Acclimation for seawater: 3 months			7-12 week			
Cultured eel	20 mg SP[9]/ bw kg once a week	-	12-23 (18.6)	Rate to be induced to mature	28%	Ijiri et al., (1998)
Feminised eel	20 mg SP/ bw kg once a week	1µg DHP / bw g	11-24 (16.6)		64%	
	40 mg SP/ bw kg once a week		8-16 (10.9)		71%	
Silver eel	20 mg SP/ bw kg once a week		8-11 (10.2)		100%	
Cultured eel	LG emulsion 2mg /ml /bw g once a week	LG emulsion containing sGTH[10] and 17αH 2 mg/fish	9-14 weeks	Fertilised eggs Larvae hatching (survival days: 1-7)		Sato et al., (2000)
	LG emulsion 2mg /ml /bw g biweekly		12-14 weeks	Fertilised eggs No larvae hatching		
Silver eel	LG emulsion 4mg /ml /bw g Once a week		5-9 weeks	Fertilised eggs Larvae hatching (survival days: 8-12)		
	LG emulsion 4mg /ml /bw g biweekly		8-10 weeks	Fertilised eggs Larvae hatching (survival days: 2-4)		
Protocol of Ohta (1997) was applied				Fertilised eggs Larvae rearing for 100 days		Tanaka et al., (2001)
Protocol Ohta et al (1996) was applied				Fertilised eggs Larvae rearing for 40 days		Okamura et al., (2002)
Protocol of Ohta et al (1996, 1997) was applied				Fertilization with cryopreserved sperm hatching		Tanaka et al., (2002)
Protocol of Ohta et al (1996, 1997) was applied				Fertilised eggs Larvae rearing for 40 days		Pedersen et al., (2003)
Feminised eels	20 mg Salmon pituitary / individual once a week	2µg DHP / bw g	8-10 weeks	Fertilized eggs (31.5-71.2%) Hatching (13.3-67.5%)		Seoka et al .,(2003)

[9] SP – salmon pituitary
[10] sGtH – salmon gonadotropin

Experimental stock		Hormone administration			Result	Source Maturation
		Maturation	Ovulation	Maturation time		
Attempts on the artificial induction of sexual maturation in other eel species						
A. rostrata	Yellow	1 mg Carp pituitary/ fish at every 3-5 days		90-104 days	GSI=1.5-3.3%	Edel, (1975)
	Silver eel			31-130 days	GSI=1.8-44.8% One female egg releasing	
		0.8 mg LH[11]/fish twice a week		35 days	GSI=2.6-4.2%	
A. rostrata	Silver eel	1 mg Salmon and Carp pituitary /bodyweight kg and 500 IU hCG[12] / animal	F2α[13]+500IU hCG a day later 1-2 mg DHP / fish	39 days	No ovulation	Sorensen and Winn, (1984)
			2 mg DHP / fish	39 days	Fertilised eggs Survived to the gastrula stage	
			0.25 mg LHRH, 12 h later 0.25 mg LHRH+2.5mg pimozid / fish	39 days	No ovulation	
A. dieffenbachii	Silver eel	5 mg Chinook salmon pituitary / body weight kg	-	53 days	Reached the migratory nucleolus stage	Lokman and Young, (1998)
A. australis	Silver eel	5 mg Chinook salmon pituitary / body weight kg	17-hydroxyprogesterone	40-120 days	Fertilization and hatching Larvae survived up to 5 days	Lokman and Young, (2000)
A. dieffenbachii				30-120 days	Fertilised eggs without embryonic development	

[11] LH – lutein hormone
[12] hCG – human chorion gonadotropin
[13] F2α – prostaglandin F2α

Experi-mental stock	Hormonal administration			result	Source
	maturation	ovulation	Matu-ration time		
A. anguilla (trials for induction of sexual maturation by pituitary extracts)					
-	2 mg pituitary / bw 100 g once a week	Dezoxy-cortikosterone -acetate	Three months	One female from ten ovulated spontaneously	Fontaine et al., (1964)
Silver eel	0.2 ml spleen extract / fish	At intervals of two weeks	12 weeks	None of them effects on gonad development	Meske, (1973)
	0.2 ml spleen extract+50IU Synahorin[14] / fish				
	50 IU Synahorin + 0.25 mg Cyren B[15] /fish				
Silver eel	Several dosages and combinations of: Carp pituitary, hCG, FSH[16], Oe[17], DL-α-Tocopherol.		20-100 days	GSI=2-65%	Boëtius and Boëtius, (1980) (Pintér, 1980b)
	The most effective combination: 15 mg Carp pituitary + 500IU hCG / fish Twice a week		40-80 days	Fertilised eggs Development up to gastrula stage	
-	Carp pituitary (dose is not given)		-	Spawning or egg releasing was not observed. Fertilization attempts were unsuccessful Oocytes sizes varied 0.95-1.07 mm	Hilge, 1983 (cit. Anonimus, 1983)
-	Carp pituitary in increasing doses (doses not given)		5-6 months	Stripped eggs	Bezdenezhnykh and Prokhorchik, (1984)
-	Carp and Eel pituitary (doses not given)		5-6 months	Fertilised eggs Embryo development Larvae rearing up to 4 days	Prokhorchik (1986) Prokhorchik et al., (1987)
-	Carp pituitary		-	Spawned female could be fed again and induced a second spawning almost four year later	LeBelle and Fontaine, (1987)
Silver eel	15mg Pacific salmon pituitary + 500 IU hCG / fish twice a week		7-38 days	GSI = 2.6-9.2%	Boëtius et al., (1991)
	Different dosages of Pacific salmon pituitary and hCG combinations / day		42-68 days	GSI = 12.7-53.5%	

[14] Synahorin - a mixture of gonadotropic hormone of the anterior lobe of the hypophysis and of the placenta of warm-blooded animals, in the ratio of 1:9
[15] Cyren B - a synthetic oestrogen, diethylstilbo-estrolddipropionate
[16] FSH – folliculus stimulating hormone
[17] Oe - oestrogen

Experi-mental stock	Hormonal administration			result	Source
	maturation	ovulation	Matu-ration time		
A. anguilla (trials for induction of sexual maturation by pituitary extracts)					
Silver eel	Carp pituitary once a week (dose is not given)		3 months	Advanced phase of maturation without egg releasing	Fricke and Kaese, (1995)
Yellow eel	0.25g Carp pituitary / bw kg three times a week		6-8 weeks	Control GSI=0.44% Treated GSI=0.95% (Average values)	Peyon et al., (1997)
Silver eel				Control GSI=1.45% Treated GSI=5.48% (Average values)	
Silver eel	500IU hCG twice a week		8 weeks	No ovarian development	Amin, (1997)
	4mg CP[18] or 4mg CP+500IU hCG once a week		80 days		
	8mg CP twice a week		78 days	Nearly ripe ovary conditions	
	12mg CP+ 500IU hCG		47-72 days	GSI=12%	
	12 mg CP +500IU hCG twice a week		40-47 days	GSI=38.8% (25% ripeness)	
	8 mg CP +500IU hCG twice a week		70 days	GSI=68.4% Spawning behaviour observed without spawning. Fertilisation test were unsuccessful	
Silver eel	18.2 mg salmon pituitary powder / fish once a week	2 µg DHP / bw g	16-25 weeks	Final oocyte maturation	Pedersen, (2003)
	36.4 mg salmon pituitary / fish twice a week	2 µg DHP / bw g	7.5-11.5 weeks	Viable egg. Embryo Development. Two surviving embryos hatched and lived 2 days	
Cultured eel	Milligram pituitary powder/week: 13.65-27.3	1 mg DHP / fish	11-29 weeks	Embryo development	Pedersen, (2004)
	13.65-18.2			Hatching	
	18.2			Embryo development	
	13.65			Embryo development	

[18] CP – Carp pituitary

Experimental stock	Hormonal administration			result	Source
	maturation	ovulation	Maturation time		
Artificial hybridisation *A. japonica* × *A. anguilla*					
Silver female *A. japonica* Farm male *A. japonica* and *A. anguilla*	Protocol of Ohta et al (1997) was applied Hybrid (*A. japonica* × *A. anguilla*) Control (*A. japonica* × *A. japonica*)			hybrid: Fertilisation rate 78±11.3% Survived up to 30 days control: Fertilisation rate 75±7.1% Survived up to 34 days	Okamura et al., (2004)

Experimental stock		Hormonal administration			result			Source
		maturation		Maturation time				
A. anguilla (trials for induction of sexual maturation by other type of hormones)								
Freshwater eel		17β-estradiol	3.1µg / bw g	11 inj. for 24 days	Pituitary GTH content	NS	GSI values were not significant to the control (saline injected)	Dufour et al., (1983)
		progesterone				Sig. higher		
		Testosterone				NS		
		cortisol				Sig. higher		
		17β-estradiol	2.5µg / bw g	11 inj. for 24 days		Sig. higher		
		progesterone				NS		
		Testosterone				Sig. higher		
		cortisol				NS		
Silver eel pretreated estradiol 17β	freshwater	GnRH-A		5 daily inj., started after E_2[19] treatment	GSI, pituitary GTH, Plasma GTH were not significantly differ from control (only E_2-treated group)			Dufour et al., (1988) (Larsen and Dufour, 1993)
		Pimozid						
		Pimozid and GnRH-A						
		GnRH-A		13 inj for 28 days				
		Pimozid						
		Pimozid and GnRH-A			significant increasing GTH level and GSI=2.62±0.15% control GSI =1.93±0.15%			
		Pimozid and GnRH-A		31 inj. for 14 weeks	GSI=4.56%			
	seawater	GnRH-A		15 inj. for 48 days	GSI, pituitary GTH, Plasma GTH were not significantly differ from control			
		Pimozid						
		L-α-Methyl-DOPA						
		Pimozid and GnRH-A			significant increasing GTH level and GSI=3.03±0.25% control GSI (E_2-treated group) =1.8±0.06%			
		L-α-Methyl-DOPA and GnRH-A			significant increasing GTH level and GSI=2.8±0.15%			

[19] E_2 - oestrogen

Experi-mental stock		Hormonal administration			result		Source
		maturation		Maturation time			
A. anguilla (trials for induction of sexual maturation by other type hormones)							
Silver eel	freshwater	GnRH-A	GnRH-A 0.1µg/g, Pimozid 10µg/g, Domperidone 5µg/g	Two months two weekly injections	GSI and plasma GTH were not significantly differ from control		Dufour et al., (1991) (Larsen and Dufour, 1993)
		Pimozid					
		GnRH-A and Pimozid			significant increasing GTH level and GSI		
	seawater	GnRH-A		One months two weekly injections	GSI and plasma GTH were not significantly differ from control		
		Pimozid					
		GnRH-A and Pimozid			significant increasing GTH level		
		GnRH-A		Three months two weekly injections	GSI and plasma GTH were not significantly differ from control		
		Domperidone					
		GnRH-A and Domperidone			significant increasing GTH level		
Yellow eel		Testosterone	2 µg / bw g /twice a week	Three months	GTH-II cellular content: All treatments were significantly differ from the control (saline injected)l	GSI values were not significant to the control	Huang et al., (2001)
		Cortisol					
		Testosterone + cortisol					
		progesterone					
		Testosterone + progesterone					

1.10.1. EXPERIMENTAL STOCKS

During the maturation experiments, different kinds of experimental stocks were used based on their developmental stages and place. At the beginning silver eel was applied for artificial maturation tests either in *A. japonica* (Yamamoto et al., 1974; Yamamoto and Yamautchi, 1974; Yamautchi et al., 1976) or in *A. anguilla* (Meske, 1973; Boëtius and Boëtius, 1980). Japanese scientist prefer cultured eel nowadays (Sato et al., 1997; Ohta et al., 1996 and 1997a; Kagawa et al., 1998) because the farmed eel can be kept more easily, the origin and the state of health can be controlled better. The sex of eel is influenced by environmental factors and these factors favour male determination in intensive farm conditions. Prevention from this situation experimental stock were feminised at glass eel stage. They were feminised as glass eel by feeding them on commercial diet containing estradiol-17β at a concentration of 10 mg/kg for 4-5 months and raised for a further 20-30 months in a freshwater pond on normal commercial feeding (Kagawa et al., 1995; Ohta et al., 1996; Seoka et al., 2003). Ijiri et al. (1998) compared the rate of development of follicles during the artificial maturation in three groups: follicles of silver eel developed the fastest and reached the final maturation stage after, on average after 10 weeks of treatment. The oocytes of feminised eels and cultivated eel developed more slowly, requiring approximately 17 and 19 weeks of treatment, respectively. All silver eel were induced to the final maturation phase, while feminised and cultivated eel reached final maturity in only 86% and 29%. Feminised eel were also subject to a high dose treatment of salmon pituitary extract (40μg / body weight g). High dose treated feminised eel were induced to the final maturation phase in a shorter time (11 weeks) and with greater efficiency (71%) than low dose treated feminised eel. Kagawa et al. (1998) examined the effects of the rearing period in seawater on induced maturation by hormonal treatments. The

oocytes of female eel kept in seawater for 3 months completed vitellogenesis by a significantly smaller number of injections of salmon pituitary extracts than those of females kept in seawater for only one week. Fertility and hatching rates of the two experimental groups did not significantly differ. Histological studies showed that oocytes of females kept in seawater for 3 months became large and attained at the primary yolk globule stage, whereas oocytes of the females kept in seawater for one week were at the oil drop stage. These results indicate that long term rearing in seawater induces vitellogenesis in the females, resulting in the shortened period of completion of vitellogenesis by injection of salmon pituitary extract.

Silver females were used for artificial propagation trials in the *A. anguilla* (Boëtius and Boëtius, 1980; Amin, 1997, Pedersen, 2003), where considerable result could be obtained. V. Hilge managed to reach full sexual maturation in females, which were reared in sea and freshwater. Since ovulation could not be induced the question has remained open whether the freshwater rearing is handicap factor for artificial induction of sexual maturation (Anonym., 1983).

The initial body weight of experimental *A. anguilla* females varied: 654 g (ovulated female): Fontaine et al. 1964; 775.1±222.6 g (GSI<40% and egg stripping): Boëtius and Boëtius, 1980; 402 g (this fish survived the mature phase and managed to obtain a second mature phase in 4 years): Le Belle and Fontaine 1987; 400-900 g: Amin 1997; 795-890 mm (‘ready to spawn’): Fricke and Kaese 1995; 623-837 g: Pedersen 2003; 577-790 g: Pedersen, 2004).

1.10.2. HORMONES AND PROTOCOLS

There are two different types of hormones for artificial induction of sexual maturation according to published results:

1. Different kinds of pituitary extracts (salmon, carp, eel) and mixtures of pituitary and other hormone types (puberogen, Des-NA, vitamin E, LH-RH analogues)

2. Synthetic hormones and compounds of hypothalamus (GnRH-analogues, GnRH-A + dopamine receptor antagonists) or gonadal hormones (cortisol, testosterone, oestrogen, progesterone)

The pituitary extracts or mixtures of pituitary and other types hormone are effective for the induction of sexual maturation by using repeated administration. The maturation times depend on the type of the applied hormones, the dose of hormones, the temperature of the water, the origin of the experimental stock. The number of ovulated females and ovulated eggs are low with poor fertility and hatchability by using pituitary treatment (Yamamoto et al., 1974; Edel, 1975; Boëtius and Boëtius, 1980; Satoh et al., 1992). Lin et al. (1991) managed to stimulate ovary development up to prespawning stage by using of serial implantation of testosterone pellets. Nagahama (1987) reported that the 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is an effective oocyte maturation inducing hormone, Richter et al. (1985) managed to induce ovulation in African catfish (*Clarias gariepinus*) by using 17α-hydroxiprogesterone without previous gonadotropin treatment. Kagawa et al. (1995) reported about that various progesterone molecules (17α-progesterone, DHP) are possible effective hormones for artificial induction of ovulation in eel as well. It is well known that body weight of female teleosts shows rapid increase prior to ovulation. This increase is directly related to the hydration of the pre-ovulatory oocytes. In the *A. japonica*, the change in female body weight is also reliable indicator of the last phase of ovarian maturation (Yamamoto et al., 1974; Satoh et al., 1992). Microscopic observation of ovarian oocytes indicated the general relationship between the body weight index (body weight/body weight at the first pituitary injection) and the diameter of the pre-ovulatory oocytes. Neither

17α-progesterone nor DHP induced germinal vesicle breakdown (GVBD) of oocytes ranging 600-700 µm, but oocytes at a migratory nucleus stage (700-800 µm) underwent GVBD in response to the steroids. Oocytes over 800 µm became more sensitive to maturation inducing steroids (Kagawa et al., 1995). At this time the body weight is 110-120% (Ohta et al., 1997a). These data indicate that an increase in body weight to about 110 % together with an oocyte diameter are reliable signs that the final treatment for induction of the last phase of final maturation and ovulation can be started by using DHP. Based on this information Ohta et al. (1996; 1997a,b) improved this method and described a protocol for the induction of final maturation and ovulation of the *A. japonica* (Figure 4.). Vitellogenesis was induced in feminised farm eel by using weekly injection of salmon pituitary. Those females, which exceeded 110% body weight indexes, were injected with pituitary extract as a priming dose two days after the weekly injections. On the next day following the priming dose eels were given DHP injection. The temperature of the tank was then raised to 22.5°C from 20°C. There are great differences between the weights of the stripped eggs (300-500 g) and sperm (1-3 g). Therefore, Ohta et al. (1997b) used artificial seminal plasma to dilute the milt, which correspond - in ionic constituents - to the seminal plasma and pH of the milt of artificially matured eel. The ovulation occurred within 23 hours after the final treatment (fertilisation rate 84.6%). It is possible that the time of ovulation of the eel is entrained by a circadian rhythm because most females ovulated early morning. Amin (1997) and Pedersen (2003 and 2004) used this method and managed to induce ovulation in *A. anguilla* females successfully.

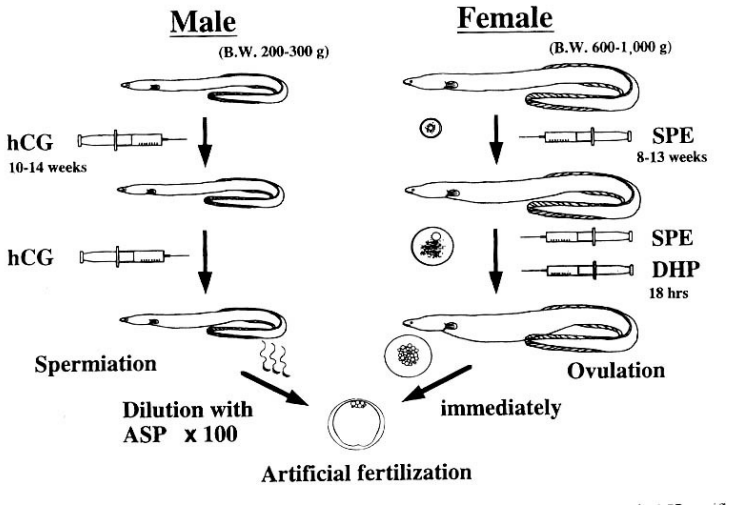

Figure 4. Summary of the artificial induction of maturation and fertilisation in the *A. japonica* (ASP→ artificial seminal plasma; DHP→ 17,20 –dihydroxy-4-pregnen-3-one; hCG→ human chorion gonadotropin; SPE→ salmon pituitary extract (Ohta et al., 1997).

A novel method (water-in-oil-in-water type emulsion using lipophilised gelatine and cotton-seed oil, containing salmon GTH and steroids) was developed and used to induce full sexual maturation and ovulation successfully (Sato et al., 1997).

Different kinds of gonadoliberin agonists or combinations of gonadoliberin agonists and dopamine receptor antagonist treatments are used efficiently to induce ovulation and sperm release in artificial propagation of fish. In the *A. anguilla*, the steroid hormones cause an increase in GTH level in plasma and pituitary cells but these are not effective to induce full sexual maturation (Dufour et al. 1983; 1988 and 1991; Huang et al., 2001). Similarly to the Japanese trials, carp-pituitary treatment may be a tool to get ripens *A. anguilla* eggs, but the efficiency of the method is far from the practical needs as

reported by several authors (Fontain et al., 1964; Boëtius and Boëtius, 1980). Prokhorchik (1986) and Prokhorchik et al. (1987) used eel and carp pituitary extracts for the induction of sexual maturation and ovulation but percentage of females ovulated and the hatching rate were not mentioned. The best results to induce ovulation and obtain fertilizable eggs, were attained by using 17α20β-dihidroxi-4-pregnen-3-one or 17α-hydroxiprogesterone by using Ohta protocol (Amin, 1997; Pedersen, 2003 and 2004).

1.11. FERTILISATION

Fertilisation tests of eel were carried out by using the dry fertilisation method. The freshly stripped eggs were mixed with freshly stripped sperm before seawater was added to them. Eggs were kept in Petri dishes, aquaria, jars or tanks where the water was changed or flown through (*A. japonica:* Yamautchi et al., 1976; Ohta et al., 1996 and 1997a,b; Sato et al., 2000*;* Tanaka et al., 2002; Seoka et al., 2003; *A. anguilla:* Boëtius and Boëtius, 1980; Amin, 1997; Pedersen, 2003 and 2004; *A. rostrata:* Sorensen and Winn, 1984; *A. australis:* Lokman and Young, 2000).

Ohta et al. (1996; 1997a) investigated the fertility and hatching rate of the ovulated eggs of the *A. japonica*. Ovulated eggs were stripped in 3 hour intervals. Eggs showed a marked decrease in fertility and hatchability 6 or 9 hours after the ovulation. To obtain good quality eggs, the artificial fertilization must be carried out immediately after ovulation. The results showed that females ovulating 15 hours after a DHP injection had better quality eggs than females ovulating at a later time, 18 or 21h after the injection. The low motility of sperm was not a major cause of the poor fertility rates because high fertility rates (81.6%, 89.6%) could be attained by using low percent motility (29.7-

40.3%) sperm as well. The hatchability of eggs showed great individual differences (0-47.6%) (Ohta et al., 1996). Ohta et al. (1997b) used artificial seminal plasma to dilute the milt and testicular spermatozoa for fertilisation as well. The motility of testicular spermatozoa was only 2.6%. It accrued 63.3% motility occurred when it was incubated in K30 solution (consisting 30 mM KCl, 134.5 mM NaCl, 1.3 mM $CaCl_2$, 1.6 mM $MgCl_2$, 20 mM $NaHCO_3$, and buffered with 20 mM TAPS-NaOH at pH 8.1) for 4 hours. There were no significant differences between diluted milt and incubated milt (fertility about 30%, hatchability about 10%), while the testicular milt showed significantly lower values (fertility about 5% and hatchability 2% respectively). Tanaka et al. (2002) used cryopreserved sperm for fertilization The hatchability of eggs fertilised with cryopreserved sperm was generally low (2.4-22.5%) compared to fresh sperm (11.6-32.5%).

Pedersen (2003) fertilised *A. anguilla* eggs with fresh and stored sperm (at 5°C for up to 12 hours) and used artificial (Tropic Marine) seawater. Boëtius and Boëtius (1980) studied freshly stripped eggs. Those sank in 31ppt salinity**.** Prokhorchik et al. (1987) fertilised eggs were on the surface at 35ppt. The fertilisable eggs swim on the surface or a few millimetres to centimetres below it at 30 ppt salinity (Pedersen, 2003). Pedersen (2004) described several suitable protocols for propagation of the *A. anguilla.* The best protocol (hatching) was to use 13.6 mg salmon pituitary extract followed 22 hours and 40 minutes later by DHP treatment when the oocytes had reached the migratory stage (763-930μm). The second best protocol (embryos developed to the hatching stage) a weekly salmon pituitary extract dose of 13.6 mg followed by a priming pituitary dose of 1.8 mg 2 days later, 11 hours 40 minutes after which DHP was injected.

1.12. LARVAE REARING ATTEMPTS

1.12.1. *A. JAPONICA*

The first successful fertilisation and hatching result was published by Yamamoto and Yamauchi (1974). Hatched larvae survived for about 5 days.

Yamauchi et al. (1976) described the larvae development from hatching to the 14 day-old-larvae.

Mochioka et al. (1993) kept alive and fed premetamorphosing *leptocephalus* larvae *of Murina cinereus* and *Conger myriaster* by bit pieces out of a lump of squid paste. The paste is not their natural food, but nevertheless a possible food item for rearing eel from eggs.

Tanaka et al. (1995) were the first who managed to observe eel larvae ingesting rotifers. On the thirteenth day after hatching, a larva caught from the bottom of the rearing tank was found to have retained rotifers in its digestive tract (S-type rotifers *Brachionus rotundiformis*). In spite of the lot of effort, the natural diet of pre-leptocephalus eel larvae remains unknown.

Kurokowa et al. (1995) described the development of the digestive organs during the 13 days after hatching. The larvae were fed rotifers (*Brachionus plicatilis*), which were cultured with algae *(Nannochloropsis oculata*) and yeast at 25°C from 8 days post-hatch.

Tanaka et al. (2001) managed to feed captive-bred eel larvae by slurry-type diet made from shark egg powder. The larvae survived for 100 days in 0.5-2% and raised to 22.8mm in total length in aquaria.

Okamura et al. (2002) reported about the ontogeny of the lateral line system in leptocephali of the *A. japonica* revealing the existence of three morphologically different types of lateral line organs.

Pedersen et al. (2003) was the first who described the triptic enzyme

activities in larval eel. They managed to investigate the digestive response and rates of growth until 36th days after hatching.

Tanaka et al. (2003) were the first who managed to rear larvae, which were obtained by artificial propagation, to glass eel stage. Although *preleptocephalus* larvae can be fed on slurry-type diet made from shark egg yolk (Tanaka et al., 2001) but these larvae cannot be raised to the following stage. The diet was improved by supplements of krill hydrolysate, soybean peptide, vitamins and minerals. Larvae fed on this new diet have grown to 50 to 60 mm in total length, and have begun to metamorphose into glass eels approximately 250 days after hatching.

1.12.2. *A. ANGUILLA*

Only Belarusian (Bezdenezhnykh and Prokhorchik, 1984; Prokhorchik et al. 1987) and Danish (Pedersen, 2003 and 2004) researchers managed to observe the hatching of the *A. anguilla* larvae so far. Prokhorchik et al. (1987) reported that the hatching started after about 47 h with mass hatching taking place after 50-60 h, and was completed 110 h after fertilisation. At this time the newly hatched larvae were 2.5-2.7mm. Pedersen (2003) reported that the surviving embryos had a delayed hatching on day 5 after fertilisation, and the bodies of the newly hatched larvae were rolled up, probably due to the prolonged period within the egg. Prokhorchik (1986) reported on the postembrionic development of the larvae of the *A. anguilla* up to 4 days. The larvae were probably poor because the newly hatched larvae were on the bottom with the back down. Newly hatched larvae of the *A. japonica* are up in the water column, they are only found on the bottom when dead or dying (Pedersen, 2003). Vigorous larvae could be obtained, and kept alive for one day (Pedersen, 2004).

1.13. THE CRYOPRESERVATION OF EEL SPERM

The method of cryopreservation of fish sperm can be described briefly according to Horváth and Urbányi's (1999) article as follows:

- Collection of sperm by using stripping or pressing of removed testes.
- Microscopic classification. In this step the quality of the sperm is investigated, characteristics are monitored such as motility, intensity and time of spermatozoa motility, semen density and consistence. Then those sperm samples are selected so that they are suitable for cryopreservation. The most important quality of sperm is motility.
- Preparation of the freezing medium. The freezing medium has to fulfil the following requirements: it must be generally physiological for the spermatozoa and protect them from the harmful effects that occur during cryopreservation. The freezing medium consists of two ingredients: the extender and the cryoprotectant. The extender is usually a salt or sugar solution (or a mixture of these) with a good buffer capacity. The extenders are usually similar to the semen of the applied fish species. Cryoprotectants prevent the cells from the harmful effects of freezing. There are two important types of cryoprotectants. Some of them penetrate the cells (dimethyl sulphoxide (DMSO or Me_2SO), acetamid, dimethyl acetamid, glycerine, methanol, ethylene glycol and propylene glycol). These are usually liquids with small molecular weight that help cells to lose their water content and stabilise their membranes and proteins. However, it is still unknown how cryoprotectants exactly work. Although most of them are strong poisons at room temperature, they are not harmful to cells at a low temperature. The other group contains so-called external cryoprotectants, which do not penetrate the cells but stabilise the structure of the cell-membranes (sugars and polymers). External cryoprotectants can only be used together with penetrating ones while penetrating

cryoprotectants can be used alone too.

- Dilution. Sperm can be diluted using the freezing medium in different ratios depending mostly on sperm density. After diluting there is a necessary waiting period (i.e. equilibration). During this period the cryoprotectant is absorbed into the cells and the osmotic pressure equalises between the intra- and extra-cellular spaces.
- Freezing. The freezing itself can be done using either dry ice or vapour of liquid nitrogen. For using dry ice the most widespread method is the so-called pellet-method. Small holes are made on the surface of a solid block of carbon dioxide (surface cavities) and the diluted sperm is dropped into them. The advantage of this method is that it is very simple, but its disadvantage is that the size of the pellets varies and the sperm needs some defrost material. According to the so-called 'straw method', which is the most widespread one, the sperm is suctioned up into straws immediately after dilution. The samples can be frozen in blocks of dry ice or using liquid nitrogen vapour. There is a simple method when the liquid nitrogen is poured into styrofoam boxes and styrofoam frames are placed on the surface of the liquid nitrogen. Straws are placed onto the frames for some minutes, then they are plunged directly into the liquid nitrogen. After freezing, the samples are transferred into storage containers where they can be kept for months or years, during which individual samples are thawed for motility monitoring. There is a more expensive but more reliable method when computerised or programmed freezers control the freezing speed. Thawing takes place in a suitable hot water bath in several seconds. It is important to avoid the processes of re-crystallinisation (post-thawn speed).
- Microscopical investigation. This step is similar to the above mentioned.
- Fertilisation test.

Hungarian scientists are in the forefront in cryopreservation (Magyary et al., 1996a,b) and in developing methods, how to froze samples in the vapour of

liquid nitrogen in an insulated styrofoam box (Horváth and Urbányi, 2000; Horváth et al., 2003).

The induction of sexual maturation in captivity is a long process promoted by weekly hormone injections. The synchronisation of ovulation and spermiation is not easy but the cryopreservation of the sperm would make the synchronization of spermiation and ovulation unnecessary. This technique has been successfully used in the case of more than 200 fish species (Billard et al., 1995). The preservation of *A. japonica* sperm has also been accomplished. Ohta and Izawa (1996) reported short-term cold storage of sperm of *A. japonica*. Different concentrations of Na^+/K^+ ratios of diluted sperm were investigated for spermatozoa motility during a 28-day storage at 3 °C. According to the best results spermatozoa showed 35.5-48.8% motility after 21 days of storage, and 22.1-26.2% motility after 28 days. Tanaka et al. (2002) were the first to publish and apply a practical way of cryopreservation of eel sperm. Cryopreserved sperm containing 76.2 mM NaHCO3, 137mM NaCl, 1.4% Soya lecithin and 10% dimethyl sulphoxide was used for fertilizing eggs, which hatched successfully afterwards (hatchability ranged from 20.7 to 69.1%). Asturiano et al. (2003) tested different media and protocols for *A. anguilla* cryopreservation. The best results were obtained with media, which had been developed by Tanaka et al. (2002). Motility was 32.22±9.37% post-thawing. The second best result was obtained with *A. anguilla* seminal plasma (NaCl 125, $NaHCO_3$ 20, KCL 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5) solutions plus DMSO and phosphatidylcholine, with 1:5 and 1:100 dilutions, respectively (24.17±9.73%).

2. CONCLUSIONS FROM THE LITERATURE

Since Yamamoto and Yamautchy (1974) obtained fertilised eggs and larvae from *A. japonica* by hormone administration, many researchers have succeeded in obtaining eel larvae. Tanaka et al. (2003) were the first who managed to raise *A. japonica* larvae to glass eel size. Although, many trials have been carried out to mature the *A. anguilla* and to propagate them, the Belarusian experiment (Bezdenezhnykh and Prokhorchik, 1984; Prokhorchik, 1986; Prokhorchik et al., 1987) was the only successful one for a long time. They managed to describe the development of the larvae up to the fourth day after the hatching when the larvae died. Pedersen (2003 and 2004) also obtained fertilised eggs and hatched eel larvae, but those lived only for 2 days.

Based on the literature, the following statements can be made.

- Males can be reared in either fresh or in seawater (artificial and natural salinity), on the other hand females should be kept exclusively in seawater during maturation experiments.
- In laboratory experiments, males survived hormone treatments and repeated spermiation could be induced after the feeding period in seawater rearing.
- Although from the point of view of handling animals, farmed or cultivated feminised females are more appropriate for reproduction experiments, wild silver females can be more easily induced to mature.
- In males, a single injection of hormones may be sufficient. Using different dosages of hCG treatments weekly, the maturation time can be shortened and the spermiation period prolonged.
- Repeated hypophysis hormone administrations are suitable for induction of the sexual maturation of female eel.
- The artificial induction of sexual maturation by using GnRH or steroid

analogues did not lead to success in *A. anguilla* females.

- Dopamine receptor antagonists or vitamins itself were not appropriate to induce maturation but these ingredients can decrease the maturing period or make the artificial induction.
- There are several methods for the induction of ovulation in *A. anguilla*. Pituitary extract induces ovulation, but the quality, fertility and hatching rates of obtained eggs are lower than those of other fish species. Japanese scientists used 17α20β-dihydroxy-4-pregnen-3-one and 17α-hydroxyprogesterone for induction of ovulation of eel. This synthetic hormone can be an effective tool for making egg-stripping safe.
- While there is a method to rear *A. japonica* larvae to glass eel size in case of *A. anguilla*, no one has been able to get feeding larvae so far.
- There are methods for successful cryopreservation of sperm both of *A. japonica* and *A. anguilla*.

3. AIMS

The objectives of my research were:

1. Induction of sexual maturation of *A. anguilla* females by using different types of hormones.
2. To investigate whether any factor effects the artificial maturation such as the *Anguillicola crassus* infection or artificial laboratory conditions (artificial seawater, which was made of table salt).
3. To examine whether freshwater rearing is influencing the development of the spermatozoa.
4. To attempt the cryopreservation of eel sperm for further fertilisation trials using a freezing extender developed for common carp sperm.
5. To investigate if computer tomography is a suitable tool to follow different physiological processes during maturation. This work also aimed to locate the fat, metabolised during the starvation period of sexual maturation i.e. obtaining information about the changes of intramuscular and abdominal fat content as well as to follow the gonad development during maturation.

4. SUMMARY OF THE MATERIALS AND METHODS

The experimental trials, investigations and analysis were carried out in the fish laboratory of Georgikon Agricultural Faculty of Veszprém University in Keszthely (Chapter One, Two, Three and Four), Kaposvár University (Chapter Five) and Szent István University (Chapter Three and Four).

4.1. ORIGIN OF THE EXPERIMENTAL STOCKS

Females used in the experiments originated from Lake Balaton caught by electric fishing (Chapter One and Two), from cultured eel in Köröm eel farm (Chapter Three, Four and Five), and from a lake in Greece, Ioannina (Chapter Three). All individuals were selected by conditional signs such as health condition, form, appearance. The first step was to prevent the experimental stocks from different kinds of infections. Therefore, females and males were treated by 0.05% EMS (formalin and malachite green) solution bath for 10 minutes. In order to prevent the *pseudodactylogyrosis,* experimental stocks were treated by Vermox®, as mebendazol **(**Székely and Molnár, 1988**).** Females were adapted to seawater for 5-7 days. The "artificial sea water" was made by using commercial table salt (Sale Marino Fino, CIS®). The water was changed when the water quality required. Males were kept in standing tap-water (Chapter Three, Four and Five) with inner sponge filtration (Chapter Three and Four) or outer filtration and recirculation (Chapter Five). Females were kept in closed plastic tunnels (80 cm long, 20 cm diameters), which had been perforated (diameters 2cm- one row 12 holes, 10 rows/tunnel) to ensure water flow, oxygen application and to protect females from each other's aggression. Keeping eel

individual plastic tunnels the identification of the individuals as well. The photoperiod was close to the natural rhythm in all experimental cycles.

4.2. ANAESTHETISATION

Before handling females were anaesthetised in a plastic tank (approx. 40 litres) by clove oil *Syzygium aromaticum* (10 drops / 10 litres water). Two tanks were used. One of them contained the anaesthetic water and the other just fresh aerated water. Treated females and males were rinsed in this clear aerated water in order to remove the residual of anaesthetics form their gills. If their swimming seemed to be normal again they were transferred back to the experimental tanks.

4.3. HORMONES AND HORMONE TREATMENT

Carp pituitaries were offered by the Balaton Fishing Company (Chapter One) and were bought from a Polish company (average weight per piece ranged from 1.9 to 3.5 mg (Chapter One, Two). Silver carp pituitaries were also given by the Balaton Fishing Company (Chapter One).

Beside pituitary glands clean hormone products were also used. Dopamine receptor antagonist was used in form of MOTILIUM (RENEAL Co.®)

Two types of synthetic hormones were used to induce maturation and promote ovulation:

- OVURELIN (D-Phe^{6}-GnRH-Ea, RENEAL, Co. ®)
- OVOPEL (10 μg D-Ala^{6},Pro^{9}NE and 10 μg metoclopramid as dopamine receptor antagonist / pellet, INTERFISH Ltd ® Hungary).

Just before the hormone administration, the calculated hormone portions were pulverised in a mortar and dissolved either in fish-physiological solution (0.65%

NaCl solution – Chapter One) or human physiological solution (Cloratum-RENEAL®-Chapter Two, Three, Four, Five). The dosages were sucked up into 1 ml syringe (OMNICAR®). If two hormones were applied at the same time they were injected as a mixture from a syringe. The injection was given intraperitonealy with approximately 5 cm before the genital area.

4.4. HISTOLOGICAL PREPARATIONS

For histological investigations, 0.5-2 cm^3 samples of gonads were cut out and put in 5-8% formalin solution in order to fix them for days or months. All histological preparations of gonads were made at the Department of Pathology of the Keszthely Hospital. Embedding was performed by an automatic machine (Shandon citadel 2000). The machine immersed samples in 12 different tanks containing formalin, tap water, DI-surface active detergents, Bio-Clear and Paraffin for one hour in each. At the end of the programme samples were removed from the machine and put in a tray where heated paraffin was poured onto the samples. After the paraffin was cooled down to room temperature 5-7 µm slides were cut from the blocks by a microtome (Microm HM400). The slides were placed into another automatic machine (Pictomat-II), that deparaffined and stained the preparates (Bio-clear, DI surface active detergents, distilled water, haematoxylin, tap water, eosin, DI surface active detergents, DI surface active detergents and xilol, respectively). Finally histological preparations were covered with covering glass by gluing of pertex (medile GmBH). The preparations were investigated using an Olympus B061 microscope and photos were taken by FUJI FinePixer 2800 digital camera (Chapter One, Two, Three, Four, Five).

Statistical analysis used in the chapters are explained where they were used.

4.5. THE METHOD AND IMAGE CREATION OF COMPUTER TOMOGRAPHY

The following chapter was written with the help of a PhD dissertation (Romváry, 1996). The principle of this method can be seen in Figure 5.

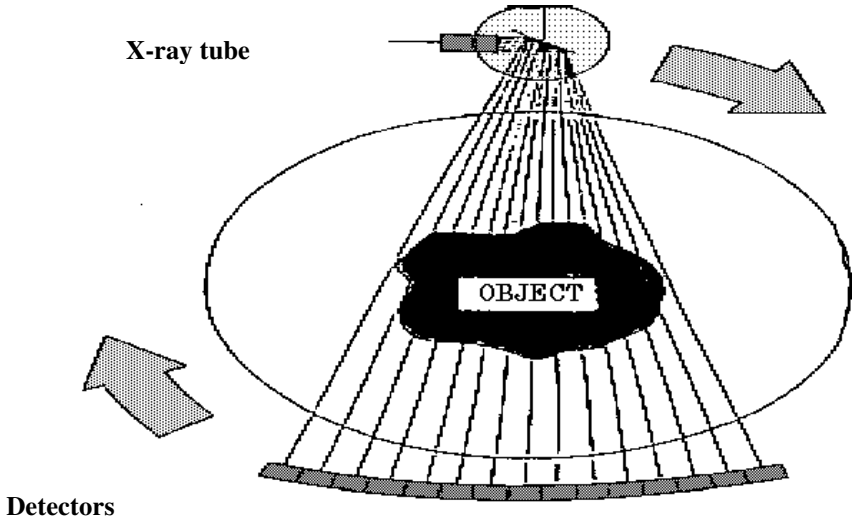

Figure 5. Three-dimension image creation (Romváry, 1996).

The figure demonstrates the operating principle a third-generation CT, which can also be found at the Kaposvár University. At the top of the figure we can see an X-ray tube, which emits X rays of variable widths, while on the opposite side below there is a detector field. During the operation of the equipment these two units turn round the examined object or animal in 360 degrees in a synchronised way. During its motion (arrows indicate the direction) the X-ray tube emits an X-ray impulse of 1-2 ms at each selected rotation angle. The rays are picked up by the detector field, which is always opposite the tube. While recording the width of the cross section plane can be modified within limits. An internal

and/or the plane of the ‘gentry’ or investigating gap can also be slanted (see Fig. 6).

At each turning angle the computer of the CT assigns the values of ray intensity arriving at the certain detectors to a numeric value. The CT of Kaposvár University contains 512 detectors. For instance, the product of the number of detectors and the impulses (360) provides the 184320 elemental data in case of an X-ray emission at every degree. The tomogram (CT-image, scan) is created from these data and the values of the rotating position belonging to them are created by the computer of the equipment. This is a 256 × 256 matrix, each element (65,536 pieces) of which is characterised by the X-ray absorbing ability of a particular elemental square (pixel) of the cross-section area.

Besides the above-mentioned technical details, the theoretical principle of the application of CT is based on the fact that different types of tissues have different X-ray absorption capacities. Based on the density values, the tissues can be separated by using the Hounsfield scale, which was named after its inventor. In a CT-image where the greyness scale (2^{12}=4096) for each 12-bit pixel ranges between -1,000 (no absorption) and +3,095 (total absorption) the generally accepted 0 value represents the density value of water.

The relationship between the values of ray absorption and the density is represented by the following expression:

$$HU = (\mu - \mu_{water} / \mu_{water}) \times 100$$

where μ is the linear ray-absorption coefficient of a unit of volume value

Since density values higher than + 1,000 are practically not found in living organisms, the scale usually ranges from –1000 to +1000 for simplicity.

The first human examinations were started at the beginning of 70’s. After a

couple of years, at the beginning of 80's it was proved in Norway that the different types of tissues of livestock have different characteristics and do not overlap on the Hounsfield scale (HU). For instance, the density of fat tissue is between –200 and –20 and that of the muscle tissue ranges from +20 to +200 on the scale. The following figure 6. represents the whole scale and the absorption ranges of different tissues.

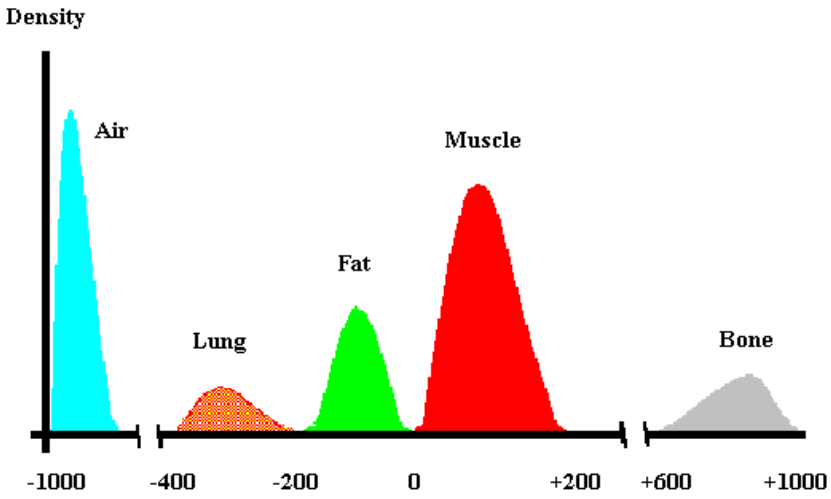

Figure 6. X-ray absorbing values of different organs (Romváry, 1996).

The modern tomography can represent half-percent differences between pixels. If the presentations of a great number of values, which are received in this way, are not modified on the greyness scale, useless images would appear on the screen. It is caused by the fact that the human eye is able to make a distinction between only a few dozen of grey shades. The solution is the so-called window technique, with the help of which a range of important for the investigation can be expected from all the primary information stored by the computer.

4.6. SUPPLEMANTARY MATERIALS AND METHODS FOR THE CHAPTER ONE

Since Chapter One does not contain materials and methods in details, I present it as shortly. Eels were selected from commercial electro fishing catch in Lake Balaton in September 2000. Our stock was adapted to seawater (30ppt salinity, 20±1.5°C, 1m^3 tank – with water filtration) and was kept without feeding. Four experimental groups were established:

- a non-injected group (average body weight (BW) = 459.7±13.4 g, n=3) control group 1
- a group injected with 0.65% NaCl solution (1 ml / fish) (BW = 432.3±40.5 g, n=3) control group 2
- a group injected with silver carp pituitary (5 mg / fish) (BW = 439.0±47.4 g, n=4) SP group and - a group injected with carp pituitary (10 mg / fish) and dopamine receptor antagonist (2 mg/fish) mixture. (BW = 532.3±150.8 g, n=4) CP group.

Females, after anaesthesia by clove oil, were treated by abdominal injections twice a week. The first injection was given on 23 October. The groups 1.-3. were killed at the 23rd injection. Different external and internal signs of sexual maturity were recorded:

I index according to Pankhurst (1983a) was calculated. Pieces of gonads were fixed in 8% formalin, 7 μm thick histological preparations were stained with haematoxylin-eosin. Developmental stages of oocytes were determined according to Lokman and Young (1998). Except for one specimen, the 4th experimental group was injected with a mixture of one pellet OVOPEL (GnRH-A + dopamine receptor antagonist) and a double dosage of carp pituitary (20mg/fish) for the induction of ovulation. The number of *Anguillicola crassus* (swimbladder parasite) in the ovulated females was determined.

5. CHAPTERS

Chapter One:

Müller, T., Binder, T., Tóth, A., Bercsényi, M. (2001). Induced maturation and successful eggs release in the European eel (*Anguilla anguilla*). Ramowy plan seminarium „Wylęgarnia 2001", 14-15/March, Golysz, (Wylęgarnia 2001-2002 Wydawnicto Instytutu Rybactwa, Olsztyn-Kortowo, pp. 103-104).

Chapter Two:

Müller, T., Váradi, B., Horn, P., Bercsényi, M. (2003). Effects of various hormones on the sexual maturity of European eel (*Anguilla anguilla* L.) females from farm and lakes. Acta Biologica Hungarica, 54 (3-4) 313-322.

Chapter Three:

Müller, T., Urbányi, B., Váradi, B., Binder, T., Horn, P., Bercsényi, M., Horváth, Á. (2004). Cryopreservation of sperm of farmed European eel *Anguilla anguilla.* Journal of The World Aquaculture Society 35(2), 225-231.

Chapter Four:

Müller, T., Baska, F., Váradi, B., Horn. P., Bercsényi, M. (2005). Testis histology in artificially maturated European eel (*Anguilla anguilla* L.) at the end of sexual maturation and spermatozoa ultrastructure in freshwater rearing. Acta Biologica Hungarica 56 (1), 169-172.

Chapter Five:

Müller, T., Romvári, R., Bercsényi, M., Hancz, Cs., Molnár, T., Szabó, A., Horn, P. (2004). Following the artificially induced eel maturation process by means of *in vivo* CT scanning. Journal of The World Aquaculture Society 35(2), 217-224.

5.1. CHAPTER ONE

Induced maturation and successful egg release in the European eel (*Anguilla anguilla* L.)

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Despite numerous attempts, successful artificial propagation of the European eel has rarely been reported. In our effort to develop a technology for the induction of gonadal development, final maturation and spawning, we have applied a course of different hormonal treatments in eel females. Experimental stock was selected by colour, size and general condition from fish collected by electric fishing in lake Balaton during September 2000. Fish were adapted to seawater gradually ($1m^3$ tank, 3% salinity, 20±1,5°C). Eels were kept without feeding. Four experimental groups were established: a non-injected control group, a group injected with fish physiological saline solution, a group injected with a mixture of carp pituitary and dopamine antagonist and a group injected with silver carp pituitary. Female eels were treated by abdominal injection twice a week after anaesthesia by clove oil. Experimental groups except for those injected with carp pituitary were sacrificed after the 23rd injection. External appearance (body colour, shape and colour of pectoral fin, eye size, change in body weight) and gonad development (oocyte stages) were monitored. The experimental group injected with carp pituitary and dopamine antagonists was used for induction of ovulation and stripping. Our results demonstrated a weak but distinct correlation between the appearance of external parameters of maturation and the final maturation of the ovary. Interestingly, non-injected eels showed better external parameters than the group injected with saline solution. The appearance of external parameters of maturation and gonad development of females injected with silver carp pituitary,

carp pituitary and dopamine antagonist far exceeded that of the other treatment groups. Hormone treated eels increased in body weight after the 15th injection due to water hydration of oocytes. Induction of ovulation was attempted after the 21-25th treatment by injection of increased dose of pituitary and addition of Ovopel (synthetic mixture of GnRH analogues and Dopamine antagonists). Stripping of mature eggs was successful as demonstrated by normal activation of eggs in water. Normal development of perivitelline space by swelling was recorded. Initial egg development was observed as demonstrated by the fusion of oil droplets in the yolk and formation of animal pole cytoplasm. Stripping of eggs has been recorded by video camera. All of our experimental stock was infected with *Anguillicola crassus*, hence the infection is not a barrier factor of the artificial induction of maturation.

SUPPLEMENTARY INFORMATION TO THE RESULTS

Table IV. Treatments and their effects on various parameters of the eels

No	Treatment	No of injections	Life day	Body weight changes (%)	Eye index	GSI (%)	Oocyte develop-mental stages
1	Negative control	-	81	-13.7	8.7	1.02	IV
2		-	81	-17.4	5.9	0.54	III, IV
3		-	81	-13.9	6.5	0.9	III, IV
4	Positive control	23	81	-8.8	5.9	1.2	II, III
5		23	81	-12.7	4.4	0.7	III, IV
6		23	81	-2.4	5.2	1.3	III, IV
7	Silver carp pituitary treatment	23	81	-3	8.7	4.1	IV, V
8		23	81	0	7.2	4.3	V
9		23	81	+1.6	10.6	2.7	V
10		23	81	+1	6.5	12.5	V
11	Carp pituitary + Dopamine receptor antagonist treatment	19	65	-3.9	11.9	21.1	V, VI
12		21	67	+7.4	12.8	37	Stripped egg
13		22	71	+5	11.1	39.9	Stripped egg
14		25	83	+28.2	15.0	51.3	Spontaneous egg release / stripped egg

Our results demonstrated weak correlations between the appearance of exterior parameters of maturation and final maturation of the ovary. Interestingly the non-injected eel had more advanced external parameters then the group injected with 0,65% NaCl solution. The external parameters of maturation and gonad development of females injected with Silver carp and carp pituitary extracts, exceeded those of the other groups, except for body colour. The body weight of the hormone-treated eels increased due to hydration of oocytes (Table IV.).

We were able to strip matured eggs at two occasion. These eggs showed normal activities after putting them into water, such as the development of perivitellic space and the fusion of oil drops. Ovulated females were infected by A*nguillicola crassus* (Table IV.). In the case of one female spontaneous ovulation and egg release started before stripping.

The quality of eggs was characterised by morphological parameters since at the time of stripping we had no ripen males. Eggs were stripped by gently pressuring on abdomen. Majority of the eggs contained a large number of small

oil drops, which gathered around the centre of the eggs. Some eggs had less amount (6-10) oil drops, which were larger in diameters than the ones with more oil drops. Oil drops fused with each other into a large one (0.6 mm) several hours after water activation. At this time the perivittelinar space and the animal pole appeared. The diameters of the freshly ovulated egg varied between 0.9-1.1mm and after the water activation these increased to 1.2 - 1.4 mm. We estimated that about 50 % of the eggs showed the signs of "ready to fertilise" eggs as described in the studies mentioned above. GSI values of ovulated females were 42.75±7.56 % (n=3). 12.84±8.04 % of the whole GSI remained the body cavity. They contained the following oocyte stages:

I = 9.87±1.08 %;

II= 37.42±1.24 %;

III - IV = 17.26±3.58 %;

V-VI-VII = 30.5±1.24 %;

VIII = 4.92±0.73 %.

The oocytes of ovulated females were in different developmental stages. Based on these results our opinion is that eels have asynchron gonads.

The presence of asynchron gonads may perhaps be due to two facts:

- Female eels spawn periodically in the nature.
- The artificial induction of sexual maturation by using different types of pituitary extracts has to be improved in order to obtain better results.

Anguillicola crassus infection

68, 6 and 77(!) specimens of nematodes were found in the 12^{th}, 13^{th}, 14^{th} females of wall and lumen of swimbladder, respectively. The walls of the swimbladders were thicker and dotted with tiny blood drops.

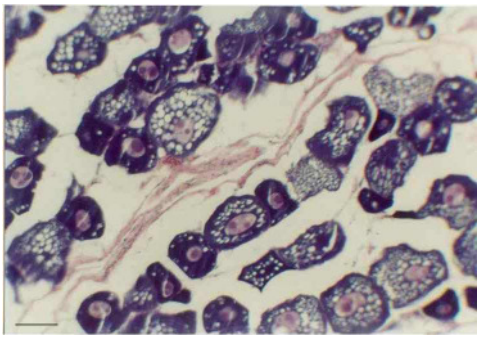

Fig. 7/a.

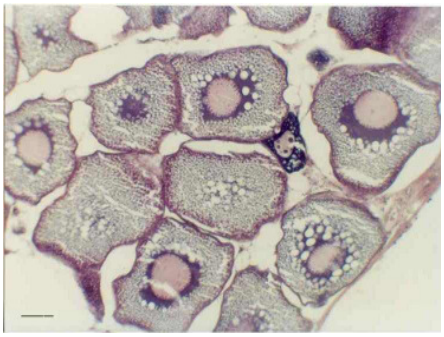

Fig. 7/b.

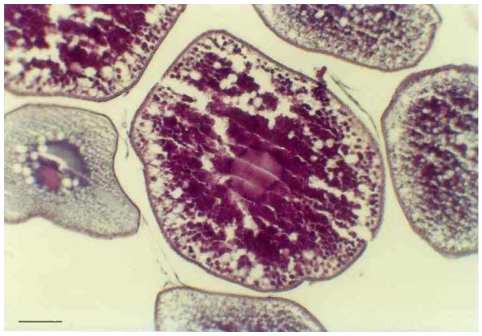

Fig. 7/c.

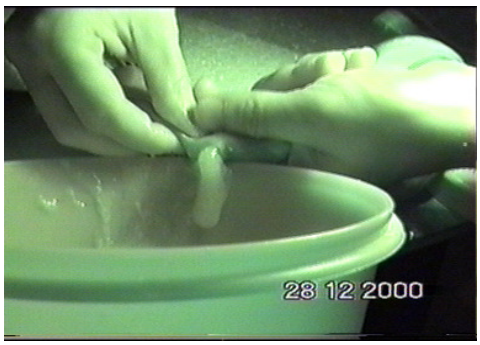

Fig. 8.

Fig. 9.

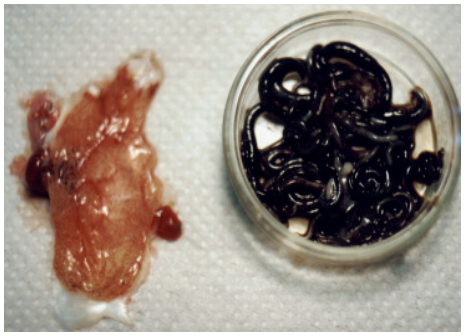

Fig. 10.

Fig.7. Histological pictures of oocyte developmental phases. Scale bar: 100. Fig.7/a. 1st control (GSI=1.02%); Fig.7/b. 10th SCP (GSI=12.5%); Fig.7/c. 11th CPD (GSI=21.1%); Fig.8. Egg stripping from 12th female; Fig.9. Native photo of the stripped eggs after the water activation (12th female), Fig. 10. Swimbladder and *A. crassus* from 14th fish (77 nematodes).

5.2. CHAPTER TWO

Acta Biologica Hungarica 54 (3–4), pp. 313–322 (2003)

EFFECTS OF VARIOUS HORMONES ON THE SEXUAL MATURITY OF EUROPEAN EEL (*ANGUILLA ANGUILLA* L.) FEMALES FROM FARM AND LAKES

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Previously described and alternative methods of the induction of sexual maturation in the European eel were investigated. Weekly administrations of a gonadoliberin agonist (GnRH-A=D-Phe^6-GnRH-Ea) did not induce statistically significant effect on the gonads of treated eels in none of the dosages used (0.1 μg and 10 μg/fish). Carp pituitary extract and carp pituitary extract together with a dopamine antagonist caused considerable external changes (increase in eye size) and significant gonadal development in two treatment groups: wild and cultivated stocks. The induction of the ovulation by double amount of CP and gonadoliberin agonist with dopamine antagonist mixture was not successful in a wild stock. Fertilisation of stripped eggs of farm eel was attempted unsuccessfully in, due to low egg quality. An advanced phase of the sexual maturation process could be induced in specimen infected by *Anguillicola crassus* indicating, that nematode infection is not a limiting factor in the artificial propagation of the European eel.

Keywords: Eel – artificial maturation – *Anguillicola crassus* – hormonal treatment

INTRODUCTION

Breeding of European eel is still an unresolved problem as no reports have been published about the rearing of eel larva up to glass eel size in captivity. All of the eels found in waters of Europe originate from natural spawning. At the beginning of the migration of freshwater eels their gonads are immature, and sexual maturation will never occur in animals kept in captivity [3]. The maturation process can be induced by different kind of exogenous hormonal treatments. There are several reports about the artificial propagation of Japanese eel *A. japonica* [16, 17, 18, 20, 25, 29, 30, 31, 32], American eel *A. rostrata* [26], New Zealand freshwater eel *A. australis* and *A. dieffenbachii* [12], but reports about artificial induction of sexual maturation in European eel are rare [1, 2, 5]. Successful fertilisation was achieved in only a few occasions [21, 22], while the longest larval rearing lasted only for four days.

In the last decades a new swim-bladder parasite *A. crassus*, – a parasite of the Japanese eel – has appeared in Europe infecting the European eel. It caused severe fish mortality in Lake Balaton in 1991 [13]. This infection can lead to a serious hand-

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icap for the infected fish reducing their swimming speed [27]. It may decrease the reproduction fitness in natural environment. Our investigation suggests that the infection of *A. crassus* does not hinder the artificially induced sexual maturation of female European eels [14]. The goal of the present study was to investigate the possible ways of artificial induction of sexual maturation of female European eels.

MATERIALS AND METHODS

Four experiments were carried out in the fish reproduction laboratory of the Georgikon Faculty of Agriculture, University of Veszprém in Keszthely. The eels used in the experiments were collected from Lake Balaton (Hungary), Lake Ioannina (Greece) and an eel farm, in Köröm (Hungary). Before starting the hormone treatments experimental fish stocks were disinfected by malachite green-formaldehyde bath and were adopted to seawater conditions during a five day period (reaching 30‰ salinity). In order to prevent *pseudodactylogyrosis* the experimental stocks were treated by mebendazole (Vermox®, Richter Gedeon Co.) [28]. In the first and second experiments females were kept in a 1000 l tank connected with a 50 l filtertank while in the third and fourth experiments a 400 l fish tank was applied also with the same filtertank. No feeding was applied during the experiments. Females were treated with abdominal injections after anaesthesia by clove oil *(Syzygium aromaticum)*. The animals were kept in a photoperiod close to natural seasonal rhythm. Experimental arrangement is summarised in Table 1.

First experiment

Eels were selected from a catch by commercial electric fishing in Lake Balaton in June 2001. Three experimental groups were established:

– a non-injected control group, 3 individuals, $w = 383.7 \pm 75.5$ g (Control)

– a group injected with 0.1 µg ovurelin/fish (D-Phe^6-GnRH-Ea, Reanal, Co.) twice a week, 3 individuals, $w = 402.3 \pm 32.4$ g (GnRH-A-0.1)

– a group injected with 10 µg ovurelin/fish twice a week, 4 individuals, $\acute{w} = 393.6 \pm 97.7$ g (GnRH-A-10).

The injections were carried out in an 81 day period. Water temperature was maintained at 21 ± 0.5 °C.

Second experiment

Five females, selected from a 3-year-old stock $w = 487.4 \pm 39.6$ g, were transported from the eel farm in Köröm (Hungary) in November 2001. Fish were injected once a week with 15 mg powdered carp pituitary in 0.65% NaCl solution/kg body weight, (CP-Farm). Water temperature was maintained at 20.3 ± 1.2 °C. For induction of

ovulation 20 mg carp pituitary/kg body weight + half pellet OVOPEL per fish (10 μg D-Ala^6,Pro^{9}NE GnRH/a and 10 μg metoclopramid as dopamine antagonist, Interfish Ltd. Hungary) were used. In order to obtain sperm 5 males were prepared for spermiation by weekly injections of 250 IU human chorion gonadotropin/fish (Richter Gedeon Co.). In a fertilisation test a mixture of sperm from all five males was added to stripped eel eggs.

Third experiment

Five female eels ($w = 1626.6 \pm 217.9$) were imported from Ioannina (Greece) in March 2002. They were injected by 15 mg carp pituitary/kg body weight twice a week (CP-Greece). Two individuals died on the 34th and the 45th days due to unidentified reasons. For inducing ovulation the same hormones were used as in the second experiment. Water temperature ranged between 21.5–24 °C.

Fourth experiment

Five females ($w = 488.4 \pm 144.9$) were selected from a catch from Lake Balaton in August 2002. Each female was treated by 15 mg carp pituitary/kg body weight and 2 mg motilium (dopamine antagonist Janssen Pharmaceutical Co.) twice a week (CP-Bal). Water temperature was maintained at 17.3 ± 0.8 °C.

At the end of the experimental cycles eels were killed by decapitation and several external and internal signs of sexual maturity were examined: I index [19]: $\{(A+B/4)^2 \pi/L\}$ where A: horizontal eye diameter, B: vertical eye diameter, L: body length. GSI was measured (gonado-somatic index) = gonad weight/body weight including the gonads ×100. Pieces of gonads were fixed in 8% formaline, and 5–7 μm thick histological slices were stained with haematotoxylin-eosin. The oocytes, showing clear nucleoli were characterised according to Hibiya [6] and Nagahama [15]. Classification of the oocytes from the oogonium stage (I) through to migratory nucleolus stage (VII) was carried out as described by Lokman and Young [11]. The histological observations were carried out by using an Olympus B061 microscope and images were taken by a Fuji FinePix 2800 digital camera.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Relationship between the GSI and eye indexes was investigated by correlation and regression analysis. Eye indexes of control group was compared to the eye indexes of treated eels using a two-sample *t*-test. Differences were considered significant for $p < 0.05$.

RESULTS

Eye index

Eye indexes of the two groups injected with gonadoliberin agonist (GnRH-A-0.1 7.83 ± 2.24; GnRH-A-10 7.68 ± 3.26) were lower than that of the control group (11.27 ± 2.40), but the difference was not significant. The eye index values of the CP treated females were higher (CP-Farm 13.1 ± 1.70; CP-Greece 15.4 ± 2.09; CP-Bal 13.89 ± 1.08) than in the control group, but the difference was not significant.

Table 1
Individual data of the experimental fish

No.	Treatment	Number of treatments	Length of life in the experiment (day)	GSI (%)	Oocyte developmental stages	Number of *A. crassus*
1	control	–	81	0.79	II, II/III	3
2		–	81	0.86	II, II/III	2
3		–	81	0.86	II, III	0
4	GnRH-A-0.1	23	81	0.9	II, II/III	5
5		23	81	0.92	II, II/III	3
6		23	81	0.83	II	2
7	GnRH-A-10	23	81	1.1	II, III	1
8		23	81	1.22	II, III	0
9		23	81	0.33	II	0
10		23	81	0.86	II, III	5
11		23	81	0.41	II, III	6
12	CP-Farm	17	119	12.20	IV, V	
13		17	119	17.80	IV, V, VI	
14		18	129	4.4	III, IV	
15		19	136	NA*	stripped egg	
16		22	157	21.30	IV, V, VI	
17	CP-Greece	21	67	32.60	IV, V, VI, VII, preovulated egg	
18		23	75	22.30	IV, V, VI, VII, preovulated egg	
19		23	75	23.60	IV, V, VI, VII, preovulated egg	
20	CP-Bal	28	101	11.78	IV, V	5
21		30	109	15.99	IV, V, VI	0
22		32	113	16.29	IV, V, VI	9
23		33	115	4.24	III, IV	2
24		33	116	12.46	IV, V	3

* NA = not assayed

Induced gonadal development

The two different dosages of gonadotropin analogues were not able to induce remarkable increase of ovaries in the treated groups as indicated by GSI values (control 0.84 ± 0.04; GnRH-A-0.1 0.88 ± 0.05; GnRH-A-10 0.78 ± 0.40). The hormonal treatment with carp pituitary proved to be effective (CP-Farm 13.93 ± 7.37; CP-Greece 26.17 ± 5.61; CP-Bal 12.15 ± 4.87). All individuals of the CP-Greece group reached the final maturational phase but the induction of the ovulation was not successful. The treated females died within 12 hours after the final treatment. At this time small pieces of their ovaries were extruded from the genital hole.

In only one occasion a female of CP-Farm group successfully ovulated. 12 hours after the last injection, eggs were stripped by a gentle pressure on the abdomen. The stripped eggs were placed in seawater (37‰ salinity) where only some eggs (cca. 5%) showed the signs of ripening (perivitelline space and normal size of animal pole appearing). Fertilisation tests were not successful.

Histological study

All typical stages of the oogenesis from previtellogenic oocytes to mature eggs were found in the ovaries of treated groups (Table 1). Ovaries of the untreated females contained predominantly previtellogenic oocytes (81±16.97 μm) with basophilic cytoplasm and no or only a few oil droplets (Fig. 1/a). Less amount of oocytes belonged to the categories between the perivitellogenic and cortical alveolus stages (108 ± 16.41 μm) where the oil drops start to fill the cytoplasm. The histological picture of GnRH-A-0.1 injected ovaries were similar to the control while the ovaries of GnRH-A-10 injected females consisted of oocytes predominantly from the cortical alveolus stage (154 ± 27.88 μm) (Fig. 1/b). In the case of CP groups, there were all stages of oocytes found from the early vitellogenic phase to maturated eggs. The rate of the various stages depended on the GSI value of the fish. The early vitellogenic oocytes are characterised by large and small peripherial yolk granules (349 ± 54.72 μm) (Fig. 1/c). In the oocytes in midvitellogenic phase (436 ± 47.37 μm) (Figs 1/d, e) the granules filled out the cytoplasm The following stage detected was the last stage of vitellogenesis (526 ± 54.66 μm), where the yolk granules fused with each other to form a single mass of yolk. There were some large lipid droplets in the ooplasm (Fig. 1/g). There were many cells found in the migratory nucleolus stage (567.5 ± 45.72 μm) (Fig. 1/f). Some pre-ovulated eggs were also found that contained some smaller lipid droplets (559 ± 42.16 μm). All fish from the CP-Greece group reached full sexual maturity indicated by the lipid fusion in oocytes (Fig. 1/h, Fig. 2).

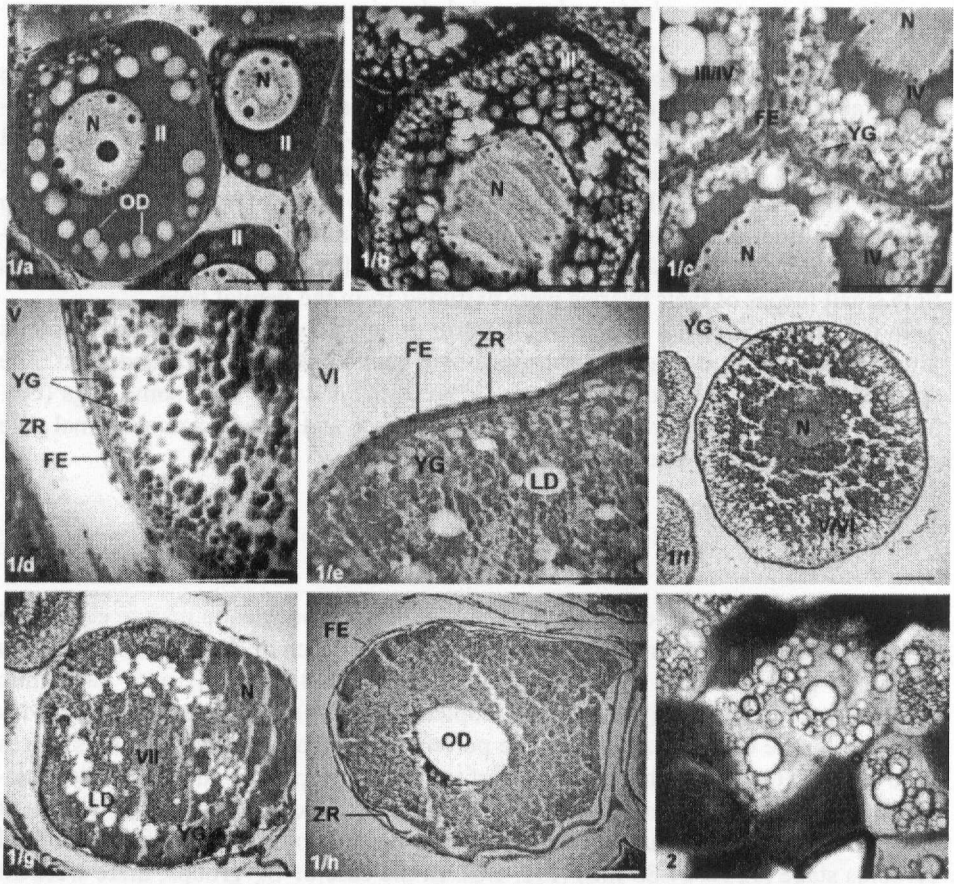

Fig. 1. Histological pictures of oocyte developmental phases. Scale bar: Figs 1/a–e 50 μm, 1/f–h 100 μm. Fig. 1/a. 2nd control (GSI = 0.79%), Fig. 1/b. 8th GnRH-A-10 (GSI = 1.22%), Fig. 1/c. 14th CP-Farm (GSI = 4.4%), Fig. 1/d. 20th CP-Bal (GSI = 11.78%), Fig. 1/e. 13rd CP-Farm (GSI=17.8%), Fig. 1/f. 16th CP-Bal (GSI = 32.6%), Fig. 1/g. 17th CP-Greece (GSI = 21.3%), Fig. 1/h. 18th CP-Greece (GSI = 22.3%)

Fig 2. Native photo of piece of ovary from 19th CP-Greece fish between two glass-slides

Abbreviations: FE follicular epithelium, G granulosa cell, LD lipid droplet, N nucleus, OD oildrop, YG yolk granule, ZR zona radiata, II previtellogenic oocyte, III stages III oocyte, IV stages IV oocyte, V stages V oocyte, VI stages VI oocyte, VII stages VII oocyte

Correlation between the GSI and the eye size

There was no significant relationship between the GSI and eye size neither in the GnRH-A injected nor in the control group. There was a weak correlation between the GSI and the eye size index in the CP groups ($r^2 = 0.51$, $P < 0.05$).

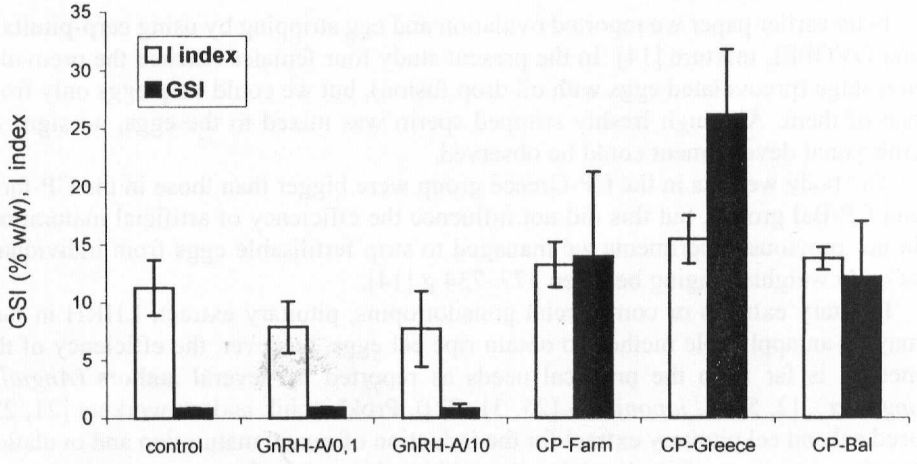

Fig. 3. Summarised diagram of the GSI and eye data among the trials

Anguillicola crassus infection

The swim-bladder walls of fish coming from Balaton lake were found thicker then the normal state, showing the effect of *Anguillicola infection* in all individuals, while eels from the Köröm farm and from Greece (Ioannina lake) showed intact and normal swim bladders. In the swim-bladders of Balaton eels there were nematodes from larval to adult stage. The number of nematodes found in one fish ranged between 1 and 9 (see Table 1). Characteristically, infected fish displayed swim-bladders, dotted with tiny blood drops.

DISCUSSION

Different kinds of gonadoliberin agonists are used efficiently to induce ovulation and sperm release in artificial propagation of farmed fish species. In contrast, in the present work long-term treatment (81 days) of two doses of OVURELIN did not induce sexual maturation in European eel females. The oocytes in the OVURELIN treated group remained in the previtellogenic and cortical alveoli phase, similarly to the control. Dufour and co-workers [3, 4] found, that estradiol-17β (E_2)-pretreated females injected by an other form of GnRH-A (des-Gly^{10},(D-Ala^6)-LHRH ethylamide, SIGMA), or dopamine antagonists (pimozide or domperidome) alone were unable to increase GTH concentration in blood, or to increase GSI. GnRH-A and pimozide or domperidome together caused a significant increase in plasma GTH level and the fish reached max. 4.56% GSI by the end of the 78 days experiment. The histological analysis of the oocytes indicated that the vitellogenesis process started.

In an earlier paper we reported ovulation and egg stripping by using carp-pituitary and OVOPEL mixture [14]. In the present study four females reached the preovulation stage (preovulated eggs with oil drop fusion), but we could strip eggs only from one of them. Although freshly stripped sperm was mixed to the eggs, no signs of embryonal development could be observed.

The body weights in the CP-Greece group were bigger than those in the CP-farm and CP-Bal groups, but this did not influence the efficiency of artificial maturation. In our previous experiments we managed to strip fertilisable eggs from individuals of body weights ranging between 377–734 g [14].

Pituitary extracts or commercial gonadotropins, pituitary extract, LHRH in turn may be an applicable method to obtain ripe eel eggs, however, the efficiency of the method is far from the practical needs as reported by several authors (*Anguilla anguilla* – [2, 5]; *A. japonica* – [25, 31, 32]). Prokhorchik and co-workers [21, 22] used eel and eel pituitary extract for the induction of sexual maturation and ovulation but the percentage of ovulated females and hatching rate of eggs were not reported. Best results to induce ovulation and obtain fertilizable eggs, were obtained by using 17α-20β-dihydroxi-pregnen-3-one or 17α-hydroxyprogesterone (*A. rostrata* – [26]; *A. dieffenbachii* and *A. australis* [*12*]; *A. japonica* [7, 9, 10, 16, 17, 18, 20, 23, 24, 30].

The GSI values of fully maturated *A. anguilla* females, ranged from 26.17 ± 5.61% (present study), 31.5% [5], over 40% [2], 42.7 ± 7.56% [14] to 68.4% [1].

Our observations that farmed European eels are suitable for the artificial maturation process are in accordance with results on Japanese eel [7, 8, 9, 10, 16, 17, 24]. The speed of induced ovary maturation was highest in silver eels, on average after 10 weeks of treatment, while feminised and cultivated eels developed slower, reaching sexual maturity only in about 17 to 19 weeks respectively. There were considerable differences in silver, feminised and cultivated eels regarding the percentage of fish reaching the maturation phase (100% silver, 64% feminised and 29% cultivated eels) [8].

In this study, sexual maturation was induced in *Anguillicola crassus* infected European eel females. With our preliminary result [14] we provide support to the theory that though the nematode infections can cause problems in the migration of eels to the Sargasso Sea, but it may not limit hormonally induced artificial propagation.

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5.3. CHAPTER THREE

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Cryopreservation of Sperm of Farmed European Eel *Anguilla anguilla*

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Abstract.—Sexual maturation and sperm release were induced in farmed European eels *Anguilla anguilla* kept exclusively in fresh water by using two dosages of human chorion gonadotropin (100 International Unit (IU)–Group one and 250 IU/individual per week–Group two). Sperm release took over 13 wk in both groups. The quality of sperm was investigated on the eighth, ninth, and tenth wk. The average cell densities were $0.94 \pm 0.4 \times 10^{10}$ (Group one) and $0.93 \pm 0.6 \times 10^{10}$ (Group two) spermatozoa/mL. The estimated motility of eel sperm was 33, 55, and 49% on the eighth, ninth, and tenth wk of treatment, respectively. The estimated average motility of samples selected for cryopreservation was 73 ± 10%, while the post-thaw motility of cryopreserved samples was 36 ± 11%. The extender originally developed for common carp sperm crypreservation together with methanol as cryoprotectant was found suitable for the cryopreservation of European eel sperm.

All European eels *Anguilla anguilla* in Europe originate from natural spawning. Eel farms raise only glass-eels that were caught from natural waters. The price of juveniles and adult eels is one of the highest among farmed fishes. The economic and environmental importance of producing eel by artificial propagation is evident. While several authors reported on successful egg or sperm stripping (Fontaine et al. 1964; Boëtius and Boëtius 1967; Meske 1973; Billard and Ginsburg 1973; Bieniarz and Epler 1977; Dollerup and Graver 1985; Leloup-Hâtey et al. 1985; Khan et al. 1987; Pérez et al. 2000; Müller et al. 2001) and fertilization (Boëtius and Boëtius 1980; Prokhorchik 1986; Prokhorchik et al. 1987) in this species, all attempts to rear the larvae for more than 1 wk were unsuccessful so far. Low percentages (1–2%) of Japanese eel, *A. japonica* larvae were successfully reared for over 100 d (Tanaka et al. 2001). Induction of sexual maturation in captivity is a long process promoted by weekly hor-

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mone injections. Synchronization of ovulation and spermiation is not easy. Sperm cryopreservation would make the synchronization of spermiation and ovulation unnecessary. This technique has been developed for over 200 species of fishes (Billard et al. 1995) and has been found suitable for preservation of genetic resources of endangered species (Piironen 1993; Tiersch et al. 2000) as well as for general breeding purposes in cultured species (Kurokura et al. 1984; Tiersch et al. 1994; Magyary et al. 1996; Horváth and Urbányi 2000). Due to the lack of reproduction technology no genetic improvement has so far been achieved in eel. Systematic genetic improvement can be expected in domesticated stocks by using traditional and biotechnological methods.

Preservation of Japanese eel *A. japonica* sperm has been accomplished. Ohta and Izawa (1996) reported on a short-term cold storage, and Tanaka et al. (2002) described a suitable method for the cryopreservation of sperm in Japanese eel.

The objective of our work was to induce complete sexual maturation in eel males as well as to attempt cryopreservation of eel sperm for further fertilization trials using a freezing extender developed for common carp sperm.

Materials and Methods

Fish and Hormonal Treatment

Twelve eel males (140.8 ± 30.9 g) were transferred from the Köröm Eel Farm to the laboratory of Veszprém University, Georgikon Faculty of Agriculture in December 2001. The males were divided into two groups (six individuals each) and stocked in two 160-L volume aquaria, both supplied with the same recirculating fresh water. Water temperature was maintained at 20 ± 1.5 C, and the photoperiod was close to natural, about 9–10 h of light a day. According to Ohta et al. (1996) and Pérez et al. (2000), fish were not fed during the experiment.

The fishes were anesthetized by clove oil *Syzygium aromaticum* (10 drops in 10-L water) before treatments and milt collections.

Hormone treatment was administered as follows:

- Fish in Group one (six individuals) were given 100 International Unit (IU) of human chorion gonadotropin (hCG) (Richter Gedeon Ltd. Hungary)
- Fish in Group two (six individuals) were given 250-IU hCG.

The calculated dosage of hormone was injected intraperitoneally each week. First injection took place on 15 December 2001 while the last one (19th) was administered on 20 April 2002. At the weekly administrations, the total body weight of males was measured. Cryopreservation of sperm was performed three times: following the eighth, ninth, and tenth injections.

Sperm Volume

Sperm was collected 24 h following the injections. The genital area was dried with a soft towel, and the sperm was collected with a gentle pressure on the abdomen into 2-mL sterile dry syringes having 0.1-mL marks. The volume of sperm collected into the syringes was recorded, and the sperm was subjected to motility analysis.

Concentration of Spermatozoa

Sperm was diluted in a 1:500 or 1:1,000 ratio (depending on the density) with freshwater and the number of spermatozoa was determined by counting in a Bürker chamber three times (at the eighth, ninth, and tenth injections).

Estimation of Sperm Motility

Eel sperm was diluted at a 1:99 ratio in modified Kurokura solution (Magyary et al. 1996) containing 360-mg NaCl, 1,000-mg KCl, 22-mg $CaCl_2$, 8-mg $MgCl_2$, and 20-mg $NaHCO_3$ in 100-mL water which was developed for the cryopreservation of common carp sperm. Then 19 μL of artificial seawater (3.5% NaCl solution) was dropped

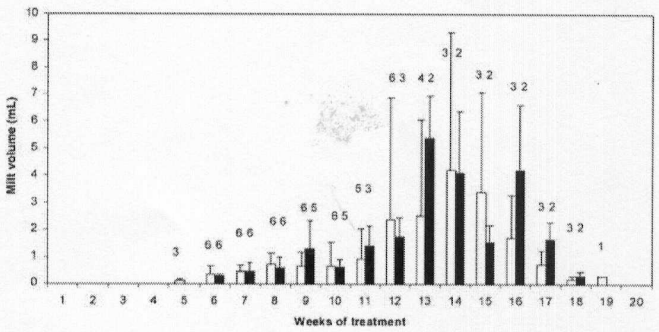

FIGURE 1. *Production of sperm during the experimental period. The mean ± SD of the milt volume in the Group one (black column), Group two (white column). Numbers above the columns are the number of spermiating males.*

onto a glass slide and 1 μL of diluted sperm was mixed with the seawater. The motility of the sperm sample was estimated at 400× magnification using a Zeiss Laboval microscope. Motility of stripped sperm samples was estimated each experimental week.

In case of cryopreserved sperm 1 μL of thawed sperm was mixed with 199 μL of artificial seawater in an Eppendorf tube to achieve the correct dilution ratio. For pipetting we used HTL-LM2 pipette with 1.3% accuracy. Then an aliquot of this mixture was immediately transferred onto a glass slide and the motility was estimated as described above.

Cryopreservation of Sperm

Sperm samples showing motility higher than 50% were chosen for cryopreservation. A freezing diluent was prepared in a test tube composed of 3.2 mL of modified Kurokura solution and 400 μL of methanol, and finally 400 μL of sperm was added to it. Thus sperm was diluted in a 1:9 ratio and the final concentration of cryoprotectant was 10%. Sperm was drawn up to 250-μL straws immediately after dilution. Samples were frozen in the vapor of liquid nitrogen in an insulated styrofoam box. Liquid nitrogen was poured into the box and styrofoam frames were placed onto the surface of liquid nitrogen. The height of frames was 4 cm. Straws were placed onto the frames for 3 min, then they were plunged directly into liquid nitrogen. After freezing samples were transferred into storage dewars and kept for 3 mo when individual samples were thawed for motility estimation. Thawing took place in a 40-C water bath for 5 sec. Motility was estimated according to the method described above.

Statistical Analysis

Motility of sperm samples collected each experimental week were compared using one-way ANOVA with Dunnett's post test. Motility of frozen thawed sperm was compared to the motility of samples chosen for fertilization using a two-sample *t*-test. Spermatozoa numbers of the two groups at the eighth, ninth, and tenth wk were compared by using a two-sample *t* test. All statistical analyses were performed with the help of GrapPad InStat statistical software, version 3.00 for Windows 95 (GraphPad Software Inc., San Diego, California, USA).

Results

Sperm Volume, Concentration of Spermatozoa and Weight Changes of Fish

Three individuals (50%) of the second group started spermiation following the fifth treatment. After the sixth administration, all fish of both groups produced milt (Fig. 1). Milt volume increased continuously in both groups until the 13th wk but in

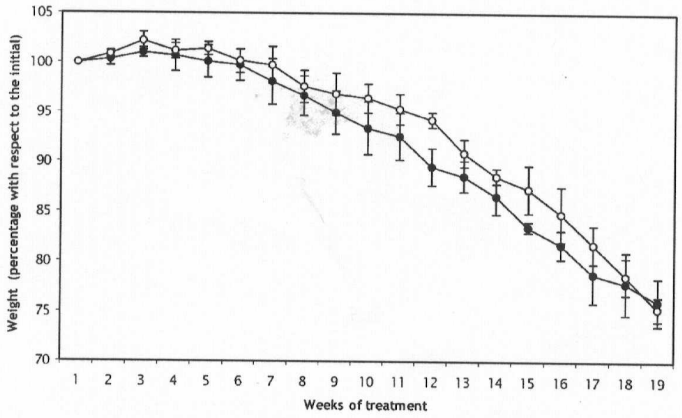

FIGURE 2. *Changes of body weight of eels during the experiment. Group one (white circle), Group two (black circle).*

the first group a slight decrease was observed from the 14th to 15th injections. There was a decrease in milt production from the 16th injection until the end of the experiment in both groups. The average milt production during the experiment was 1.3 ± 1.5 mL per individual ($N_{stripped}$ = 46) in Group one and 1.2 ± 2.2 mL per individual ($N_{str.}$ = 64) in Group two. Extremely high volumes were stripped in some occasions, over 5 mL from the 12th to the 16th wk in both groups (5.9, 6.5 mL in Group one and 10.1, 11.4 mL in Group two). The body weight of the males increased up to the fourth to fifth wk then it decreased gradually to the end of experiment. The average weight loss was 25% during the maturation process (Fig. 2).

The average number of spermatozoa in Group one was 0.94 ± 0.4 × 10^{10} cell/mL, while this value was 0.93 ± 0.6 × 10^{10} spermatozoa/mL in Group two at the three experimental trials. There was no significant difference in number of spermatozoa either between both groups or among the 3 wk.

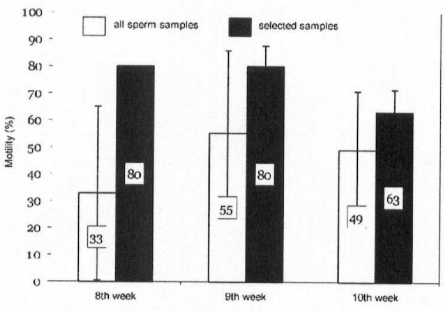

FIGURE 3. *Motility of all sperm samples and of those that were chosen for cryopreservation trials on the eighth (N_a = 12 for all and N_s = 3 for the selected samples), ninth (N_a = 11 and N_s = 6) and tenth (N_a = 10 and N_s = 6) wk of treatment. All data are given as least square means ± SD.*

Fish Mortality

Three males died in Group one (one at the ninth and two males at the 12th hormonal administration) and two individuals in Group two (at the 12th and 19th treatment). The reason of death was not clarified.

Sperm Motility Before Cryopreservation

Eel sperm was immotile in the modified Kurokura solution. Average motility of eel sperm (activated in artificial seawater) was 33 ± 32, 55 ± 31, and 49 ± 22% on the eighth, ninth, and tenth wk of treatment, respectively (Fig. 3). Very high individual fluctuations in the quality of sperm were

observed each week, e.g., the minimum and maximum values were 0 and 80%, 2 and 90%, 10 and 70% on the consecutive weeks, respectively. No statistically significant difference ($P > 0.05$) was found between the motility of sperm stripped on the eighth, ninth, or tenth wk of treatment. On the eighth wk, three, and on the ninth and tenth wk, six samples each showing the highest motility were chosen for cryopreservation.

Sperm Motility after Thawing

Average motility of the samples selected for deep freezing was 73 ± 10%, significantly differing ($P < 0.0001$) from the average of the post-thaw motility, 36 ± 11%.

Discussion

In the present study we confirmed that full sexual maturation and spermiation can be successfully induced in fresh water in European farmed eels using two dosages of hCG.

Boëtius and Boëtius (1967) reported that the maturation period increases with the decrease in salinity. Bieniarz and Epler (1977) stripped sperm from freshwater males by using weekly injections of 100-IU hCG per individual (six times) but their stock died 2 wk after spermiation. Khan et al. (1987) also reported the completion of sexual maturation cycle in European eel males in freshwater. They injected their stock once with a dosage of 250-IU hCG per individual, and full sexual maturation was achieved 3 mo later. We achieved full sexual maturation in 50% of the first group following the fifth wk and 100% spermiation in both groups after the sixth wk. Group two, 250-IU hCG per individual, produced sperm continuously for over 15 wk, while the first group (100-IU hCG per individual) produced sperm continuously for only 13 wk. Pérez et al. (2000) worked on farmed eel in seawater and applied hCG (1.5 IU per g of bodyweight) weekly reaching spermiation at the sixth injection.

Pérez et al. (2000) reported that the control and hCG-treated individuals showed phenomena of maturation (enlargement of the eye, black pectoral fin, well developed cephalic lateral line), and some control males even produced sperm. It was reported that control and treated animals were held in a recirculating system so pheromones could affect control males. The gonads of silver eels in fresh water are immature; sexual maturation of animals kept in captivity never occurs (Dufour et al. 1988). In this present study the experimental stock was kept in freshwater, thus sexual maturation took place upon hormonal effect. Ohta et al. (1996) used saline control (injection of 0.6% NaCl solution) for long-term maturation (14th weekly injection), and the testes of control males remained immature.

There were large differences in the milt volume between individuals. We have little data to explain this phenomenon. Bieniarz and Epler (1977) reported a range of 0.1–2.8 mL per individual in the first three strippings in fresh water, while Pérez et al. (2000) had about 2 mL until the ninth wk and about 3 mL per 100-g bodyweight in the final period of the experiment. Ohta et al. (1996) reported that milt weight per fish gradually increased with the weekly injections (250-IU and 750-IU hCG) in Japanese eel. After the 11th injection, the quantity of milt produced became constant (about 1–2 g) until after the 13th injection, and reached 3.98 ± 1.48 after the 14th injection in the 250-IU group. At this time they finished the observation. The largest milt production was 10.49 g after the 11th injection from one male of the 750-IU hCG group. The results of the present study are similar to those of Ohta et al. (1996), but we had peaks both in the 100 and 250-IU hCG treated groups, and our observations lasted for 19 wk.

The average spermatozoa concentrations of the two groups were $0.94 \pm 0.4 \times 10^{10}$/mL (Group one) and $0.93 \pm 0.6 \times 10^{10}$/mL (Group two) which are close to the values reported by Bieniarz and Epler (1977),

whose average was 0.99 $\times 10^{10}$/mL at the eighth injection.

Both treated groups lost approximately 25% of their weight during the 19 wk of the trial. These results differ considerably from those of Pérez et al. (2000); in their experiment treated males lost 35% of their initial weight after 15 wk of treatment.

The most suitable time to obtain sperm with the highest motility proved to be the ninth wk (Pérez et al. 2000) and 24 h after injection when using weekly injections (Ohta et al. 1997; Pérez et al. 2000). According to our observations there were no significant differences in the motility of sperm samples collected on the eighth, ninth, and tenth wk of treatment. High fluctuations in sperm motility were observed among samples collected each experimental week, thus the selection of best samples for cryopreservation on the basis of estimation or measurement of initial motility is extremely important.

Motility of cryopreserved sperm after 3 mo of storage was lower and significantly different from that of the fresh samples selected for cryopreservation. This corresponds with the observations of Tanaka et al. (2002) on the Japanese eel who found that there was a significant difference between frozen sperm samples stored in liquid nitrogen and the control group of fresh sperm. Lower motility values do not necessarily mean unsuccessful fertilization. In salmonids post-thaw motility rarely exceeds ~20–30%, yet this sperm is suitable for fertilization. Observations on Japanese eel showed that the hatching rate of eggs fertilized with cryopreserved sperm increased with the increase of volume of sperm used (Tanaka et al. 2002).

According to our results, the extender originally developed for the cryopreservation of common carp sperm seems suitable for the freezing of eel sperm together with methanol as cryoprotectant. The extender has been used successfully for the cryopreservation of common carp semen (Magyary et al. 1996) as well as for other cyprinid species (Horváth and Urbányi 2001). Methanol was also found to be a suitable cryoprotectant for eel sperm. Although so far only dimethyl sulfoxide (DMSO) was used for the cryopreservation of eel sperm (Tanaka et al. 2002), methanol was found to be superior to DMSO in a number of cell types, including fish spermatozoa (Harvey et al. 1982). The use of 0.25-mL straws further facilitates the simplification and standardization of the technology as 0.5 and 0.25-mL straws are widely used in the bovine industry.

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5.4. CHAPTER FOUR

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THE TESTIS HISTOLOGY OF ARTIFICIALLY MATURATED EUROPEAN EEL (*ANGUILLA ANGUILLA* L.) AT THE END OF SEXUAL MATURATION, AND SPERMATOZOA ULTRASTRUCTURE IN FRESHWATER REARING

SHORT COMMUNICATION

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The artificial induction of sexual maturation of European eel males was carried out by using weekly hCG administrations. Histological pictures showed that the testis tissues developed and regressed naturally and no pathological changes took place under the conditions of artificial rearing in freshwater. According to light and electron microscopic investigations the morphology and motility of the spermatozoa of males kept in freshwater proved to be similar to those in seawater. The authors suppose that freshwater rearing of males is not a barrier factor in the artificial propagation of European eels.

Keywords: Sexual maturation – European eel – spermatozoa

Based on early observation it was supposed that eels die after spawning. In laboratory experiments males survived hormone treatments [1] and repeated spermiations could be induced when the males were fed between the treatment periods [3]. Previous results clearly revealed the unique ultrastructure of eel spermatozoa, which include a crescent-shaped nucleus, a rootlet attached to the neck region, a flagellum of the 9+0 pattern, and a pseudo-flagellum extending from the proximal centriole [4, 6]. The goals of the present study were to look into the testis histology in this critical period and to clarify whether the salinity of the rearing water influences the spermatozoal microstructure. Three groups (w = 101 ± 30.9 g), each consisting of 3 males were formed and reared in freshwater. The first group served as control (group one), the second was given 250 IU human chorion gonadotropin (hCG)/fish/week for 8 weeks (group two) and the third group was given 250 IU hCG/fish/week for 19 weeks (group three). The males of group one were killed at the beginning of the experiment, group two on the 9th week and group three on the 27th week, two

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Abbreviations: *dc* distal centriole, *dS* degenerated spermatozoa and spermatids, *Ct* connective tissue, *E* erythrocytes, *f* flagellum, *L* lumina, *m* mitochondrium, *n* nucleus, *pc* proximal centriole, *r* rootlet, *S* spermatozoa, *Sg* spermatogonium, *Sc1* primary spermatocyte, *Sc2* secondary spermatocyte, *St* spermatid

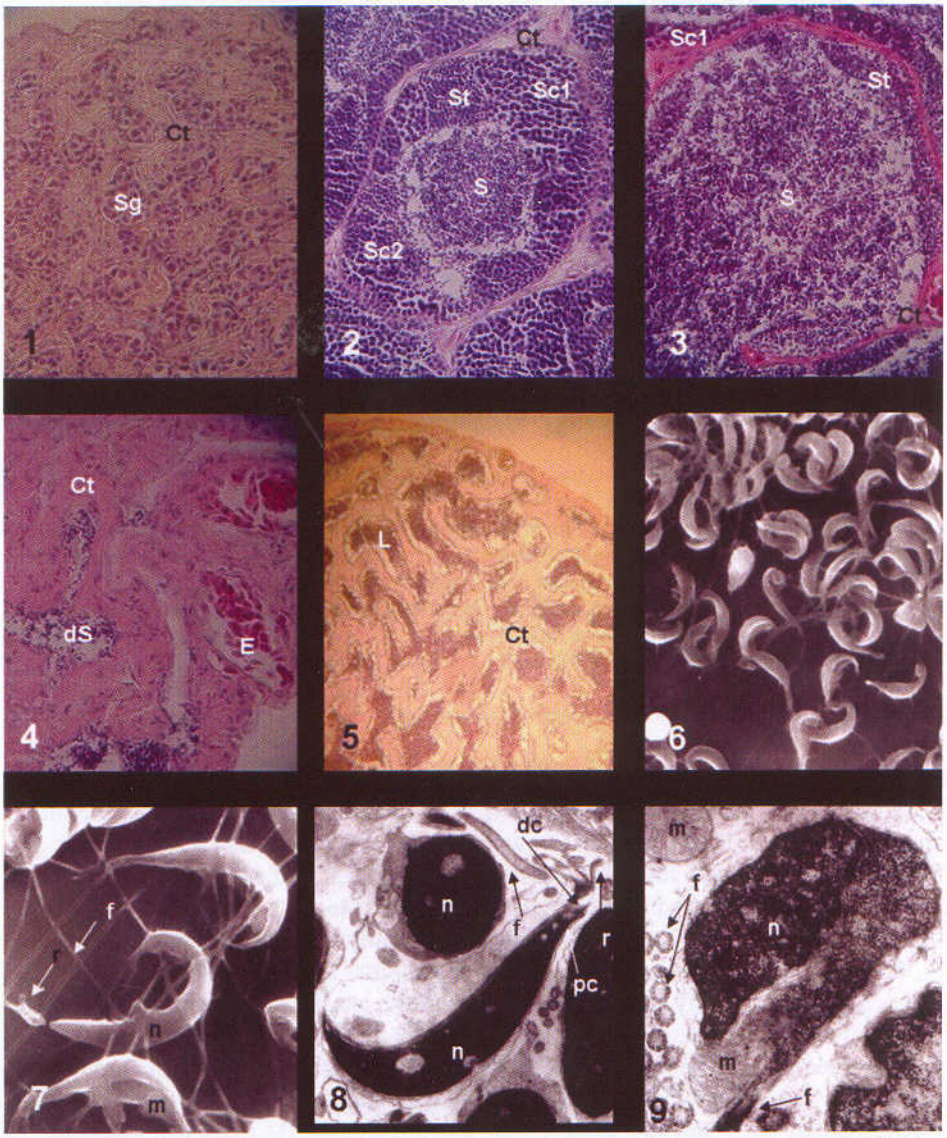

Fig. 1. Testis cross-section of untreated male. Spermatogonic tissue with many spermatogonia. The trabecula of the testis interstitium composed mainly by elastic and collagenic connective tissue elements (GSI = 0.07%), ×400. *Fig. 2.* Cross-section of a fully mature testis (GSI = 3.15%), ×400. *Fig. 3.* Matured spermyocytes in the lumina of the testes parenchyma surrounded with connective tissue (yellow) and smooth muscle (red) cell elements (7th fish, GSI = 8.11%), ×400. *Fig. 4.* Testis cross-section of 8th fish (GSI = 0.64%), ×400. *Fig. 5.* The tissue of the testis after release of the sperm. The trabecula of the testis interstitium composed by collagenic connective tissue elements (9th fish, GSI = 0.53%, Van-Gieson), ×100. *Fig. 6.* Scanning electron micrograph of mature eel spermiocytes. SEM, ×1300. *Fig. 7.* Scanning electron micrograph of mature eel spermiocytes. SEM, ×5300. *Fig. 8.* Longitudinal section of a mature spermiocyte. The head is intensive electron dense. TEM, ×5200. *Fig. 9.* Cross-section of the head of an eel spermyocyte with the chromatin and a part of the tubular mitochondrium. TEM, ×13 200

months after their last hormone treatment. Pieces of testes were fixed in 4% formaline, embedded in paraffin and 5 μm thick slices were stained with heamatoxylin-eosin for light microscopic observations. For the electron microscopic investigations small pieces of testes were fixed in Na-cacodylat buffered 2.5% glutardialdehyde, postfixed in buffered 1% osmium tetraoxide, embedded in Durcupan ACM. Ultrathin sections were made and examined with a JEOL 100 S electron microscope. The sperm obtained from the male eels was examined both in transmission and scanning electron microscopy.

The motility of the sperm sample was estimated at ×400 magnification using an Olympus CO11 microscope. One drop of artificial seawater (3.5% NaCl solution) was dropped onto a glass slide and ~1 μl sperm was mixed with it.

The investigated features of the testes of control (immature GSI (gonad weight/body weight × 100) = 0.07 ± 0.01%) and treated (midmature GSI = = 3.54 ± 0.47%) groups were similar as it was described earlier [2, 5] (Figs 1, 2). In group three 179 days after the first injection and 45 days after the last one the testes showed two different histological stages. On the wall of tubules of the 7th fish (GSI = 8.11%) there were some clusters of spermatocytes and spermatids and the tubules were full of spermatozoa. A large amount of blood vessels appeared in the connective tissue which is rich in smooth muscle elements. The 8th fish (GSI = 0.64%) and the 9th one (GSI = 0.53%) possessed thicker connective tissue than the number 7. They had smaller tubules, which contained only a small amount of spermatids and spermatozoa showing degenerated forms. The number of their erythrocytes increased compared to the 7th fish, which showed macroscopically visible hyperaemia at the necropsy. Apart from the spermatozoa there were Leydig and/or other somatic cells within the connective tissue. The various cell types are not easy to identify by common histological techniques [2]. In the testes of the hormonally treated eels an increased amount of smooth muscle could be detected by Van Gieson's staining. The ultrastructure of the mature spermiocytes showed no differences in comparison to naturally matured cells described in the literature (Figs 6–9, Table 1) [6]. The cytoplasm of the mature spermatozoa contained one large tubular mitochondrium at the apical part of the cell. The chromatin of the cells was highly electron dense, showing no fine structure, except a certain granulation.

The histological pictures showed that the testis tissues regressed naturally and no pathological changes took place under the conditions of artificial rearing either in freshwater (present study) or in seawater [1]. Males reared in freshwater can produce

Table 1
Data about the spermatozoa (*n = 50)

Head length (μm)	Head width (μm)	Flagellum length (μm)	Rootleth length (μm)	Mitochondrion diameter (μm)	Sources
5.4 (±0.4)	1 (±0.2)	25 (±5.5)	1.2 (±0.2)	0.8 (±0.3)	Okamura et al., 2000
5.6 (±0.5)	0.9 (±0.3)	26 (±0.7)	1.2 (±0.3)	0.8 (±0.4)	Present study*

morphologically the same spermatozoa as "seawater males". The spermatozoa could be activated (10–90% motility) in artificial seawater. This is promising with regard to the multiple use of males. Since there is no significant difference between the morphology and motility of freshwater and seawater spermatozoa, the authors assume that the freshwater rearing of males is no limiting factor in the artificial propagation of European eels.

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5.5. CHAPTER FIVE

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Following the Artificially Induced Eel Maturation Process by Means of In Vivo CT Scanning

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Abstract

Artificially induced maturation of 10 male eels *Anguilla anguilla* L. weighing 170 ± 30.3 g was followed for 6 wk with in vivo CT scanning in a repeated manner. Since testis tissue has a well defined X-ray density value, the volumetric development could be described. Parallel with this process, a strong decrease of the abdominal fat volume was measured and depicted with the applied surface smoothing method. As an affect of the hCG treatment (250 IU/week per eel) at the third wk, the testis could clearly be recognized on the images. From that time the average testis volume values of the 10 eels were 4,975, 6,845, 11,583 and 14,553 mm³ respectively. At the sixth wk, the relative testis volume in the abdomen was 25%. In the fillet a characteristic shift (from −10 to −20 Hounsfield units) of the density histogram towards the fat interval was detected. The average density value of the fillet was found to be zero; this was supported furthermore by the histological analysis of the fillet (fat to muscle rate 0.35–0.40). On the basis of the above findings, the applied CT scanning method was proven to be suitable to follow in vivo the maturation process of male eels.

European eel *Anguilla anguilla* belongs to the catadromous fish species. After a long-term freshwater cycle, it migrates to the Sargasso Sea to spawn (Schmidt 1923). It is hypothesized that eels do not take up feed from the silvering stage (mature form); during migration and maturation the mobilization of the energy stores is activated. The main fuels in this process are lipids (about 80% of the total energy demand) (Boëtius and Boëtius 1985) in first line triacylglycerols from intramuscular sources (Lewander et al. 1974; Dave et al. 1975). Furthermore fat is also accumulated in the liver during silvering (Lewander et al. 1974). According to Boëtius and Boëtius (1980), the crude fat content of the body has to exceed the value of 20%, as silvering and the start of migration may strongly be connected to a minimum level of the fat depots (Larsson et al. 1990). Svedäng and Wirkström (1997) reported lower body fat content in female silver eels in Sweden, indicating a more flexible maturation process than was hypothesized earlier. This suggests short nutrition periods during the early maturation, ensuring the possibility of survival and spawning. Ginekken and Thillart (2000) reported that according to their calculation about 60% of the fat reserves of

migrating silver eel females can be converted into gonads by the end of their journey to the Sargasso Sea.

However, there is a lack of information about the exact localization of the fat mobilized during maturing. Body fat content and GSI (gonadosomatic index) measurements are mostly performed post-mortem. Therefore, non-invasive methods, ensuring the possibility of in vivo measurements are of great importance. One of these methods is computerized tomography (CT), a possibility for in vivo examinations, applied first by Skjervold et al. (1981) in animal breeding. This method was also applied by Love and Lewbart (1997) and Bakal et al. (1998) in the field of pet fish radiographic diagnostics to identify and describe pathological alterations. However, CT was rarely used in fish culture related studies. Gjerde (1987) applied CT as an effective tool in the family selection program of rainbow trout. Rye (1991) applied a similar methodological approach to predict carcass composition of Atlantic salmon. Einen et al. (1998) investigated the effect of starvation prior to slaughter onto the fat depots of Atlantic salmon by means of CT. Romvári et al. (2002) applied CT imaging to determine the fillet composition of four freshwater fish species.

Although the freshwater eel gonads are immature, and sexual maturation does not occur in animals kept in captivity (Dufour et al. 1988), the maturation process in European eel males can be induced by exogenous hormonal treatments (Boëtius and Boëtius 1967, 1980; Meske 1973; Bieniarz and Epler 1977; Dollerup and Graver 1985; Pérez et al. 2000).

The goal of the current investigation was to develop a specific methodology for testis imaging of male eels, which is applicable for further volumetric measurements in vivo. This may provide important additive information to the problem of artificial propagation of eels, which is currently unsolved. Furthermore, the location of the fat, metabolised during the starvation period of sexual maturation, was also aimed to define, i.e., to obtain information about the changes of intramuscular and abdominal fat content during the maturation.

Materials and Methods

Experimental Animals and Handling

Ten male eels *Anguilla anguilla L.* (average body weight 170 ± 30.3 g, average body length 44.7 ± 2.3 cm, selected from a 3-yr-old farm eel stock) were transported from an eel farm at Köröm to the fish laboratory of the Kaposvár University, Hungary, in March 2002. Fish were kept in circulated fresh water in a 150-L volume tank partitioned into 10 subunits. Fish were not fed during the trial. In order to prevent *pseudodactylogyrosis,* experimental stock was treated by mebendazole (Vermox®, Richter Gedeon Co., Székely and Molnár 1987). The temperature was held at 22 ± 1.5 C. Eels were injected weekly intraperitoneally with 250 IU (International Unit) human chorionic gonadotropin (hCG, Richter Gedeon Co.) (Ohta et al. 1996) soluted in 0.3-mL 0.9% NaCl/fish for 6 wk. Before the treatments the eels were anaesthetized by clove oil *Syzygium aromaticum* (10 drops in 10-L water).

CT Scanning, Image Processing, and Analysis

Two d after the weekly repeated hCG administrations, the eels were scanned in vivo by means of a Siemens Somatom Plus S40 spiral CT scanner at the Institute of Diagnostic Imaging and Radiation Therapy of the Kaposvár University. After narcosis (clove oil) eels were fixed in a plastic holder covered with a wet towel in a prone position. High resolution CT scans were taken from the animals using a zoom factor of 8.8, with 5-mm slice thickness, and no gap between the following slices. First the topogram was taken, being highly similar to a conventional X-ray image. In this, the location of the cross-sectional images (tomograms) was defined to cover the abdominal region (from the end of the operculum

till the beginning of the caudal fin) totally, including the testes. Depending on the size of the eels, 20–24 cross sectional images covered the above-mentioned region.

The basis of the image post-processing method was the fact that CT images consist of pixels (512 × 512 in a single cross-sectional image), and each pixel represents a defined Hounsfield unit (HU) value. Therefore from each image it was possible to collect the number of pixels falling into the HU value of interest. As between the consecutive images there was no gap. A pixel also represents a voxel, a basic volumetric unit. In the present approach the extreme density values (e.g., bone and air) were excluded from the evaluation and only those between −150 and +100 on the Hounsfield scale were taken into account, a range fully including fat (from HU −150 to HU −10) and muscle (from HU +10 to HU +100). This HU range was reduced by summarizing the pixel frequency data of each consecutive 5 HU values; this resulted altogether in 50 so-called "Hounsfield variables" (HUv). Consequently, those between 1 and 28 represent fat, while those above, i.e., the range of HUv 32–50, characterize muscle tissue. This type of data reduction is widely used in medical imaging, and it was also successfully applied in the quantitative body composition measurement of fish (Romvári et al. 2002).

In order to establish changes separately in the different body regions, the abdomen (including the testes and the abdominal fat) and the fillet were also analyzed as different regions of interest (ROI), which is, in practice, a demarcated area in the cross-sectional image (Fig. 1).

Data obtained were used for further statistical and plot procedures and for computing the frequency of HU variables falling into each interval in one scan or in scans taken in serial manner. To the depiction of histograms gathered from serial scans, 3D surfaces were constructed on the basis of the HU variable histograms of the consecutive images, applying the negative exponential interpolation smoothing method, to illustrate the alterations according to the anatomic locations.

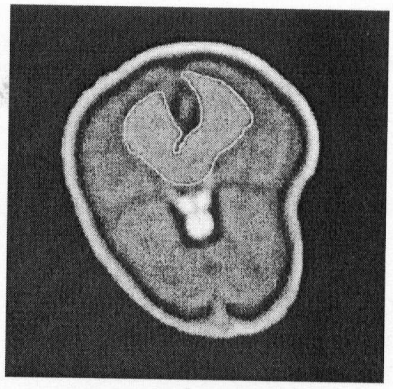

FIGURE 1. *Cross-sectional CT image by the abdominal region, marking the ROI of the testis.*

Histological Analysis

To determine the location of fat-tissues within the body, one additional fish was sacrificed after the sixth treatment; pieces of its body were fixed in 8% formaline. Histological preparations (5–7 μm thick) were stained with heamatoxylin-eosin. The fat and muscle topography was classified according to Yokote (1982) and Pankhurst (1982). Three experimental males were selected randomly, anaesthetised eels were sacrificed by decapitation at the sixth wk, and pieces of testes were fixed in 8% formaline. Cytological features of testes were determined according to Hibiya (1985). Microscopic visual observations were archived by a FUJI FinePix 2800 camera applied to an Olympus B061 microscope.

Results

Spermitation

At the sixth wk of treatment all hCG-induced males produced milt. Stripped spermatozoa could be activated by artificial seawater (salinity 35 g/L). In the histological preparations of the testes, all cell stages of spermiogenesis could be found. Irrespec-

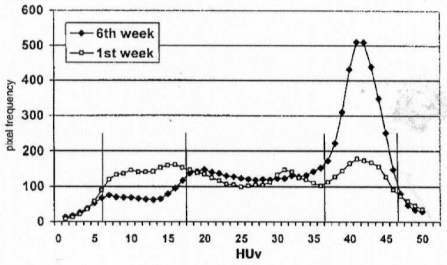

FIGURE 2. *HUv distribution histograms of the total abdominal region at the first and sixth wk (characteristic fat volume: HUv 5–17; testis: HUv 37–47).*

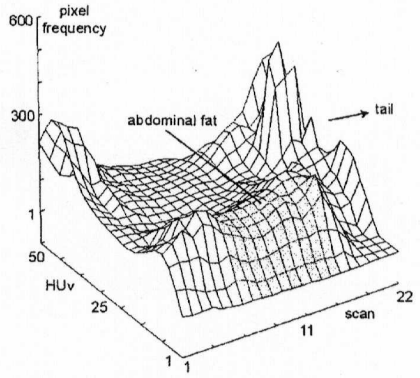

FIGURE 3. *3D histogram of the abdominal region composition at the first wk (Serial number of the scans: X-axis; HU variables: Y-axis: frequency of pixel density values: Z-axis).*

tive of the developmental stages of germ cells, the summarized HUv values were constant and represented the testes tissue during the maturation phases.

Body Composition Changes

Changes in the abdominal region are plotted in Fig. 2, where the average density values are given in the first and sixth wk. These histograms were constructed from the density data of the 10 examined eels to indicate the mainly interesting HUv intervals. This was also the basis of the further 3D histograms.

A characteristic fat volume decrease (between the HU variables 5 and 17) and the presence of the testes can be clearly seen (37–47 HUv) comparing the histograms obtained from the first and sixth wk. However, within the HUv range of 18 and 36 no particular alteration was measured.

The image-taking procedure was performed weekly; therefore, the time-course of the testis development could also be determined. To describe the time-dependent improvement of the testes the linear curve fitting method was used. When summarizing the pixels falling into the specified HUvs between weeks 3 and 6, a linear fitting resulted an r square value of 0.98 from the average data of the 10 eels in the four time points, showing a strong and continuous testis development. During the follow-up study, mature testes were clearly recognized on the scans after the third wk only.

The average volume values of the testes of the 10 eels were 4,975 ± 1,432, 6,845 ± 3,103, 11,583 ± 4,680 and 14,553 ± 4,630 mm^3, at the third, fourth, fifth and sixth wk, respectively. The characteristic testis HUvs were declared to fall between 37 and 47.

However, when demonstrating the abdominal composition with the negative exponential smoothing method (Figs. 3, 4, and 5), the reciprocal changes of the abdominal fat and the growing testes represented the two major processes. The serial number of the scans is marked on the X-

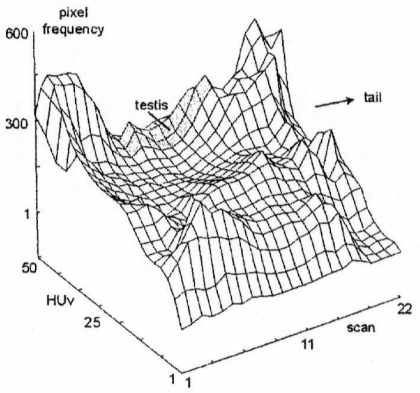

FIGURE 4. *3D histogram of the abdominal region composition at the fourth wk.*

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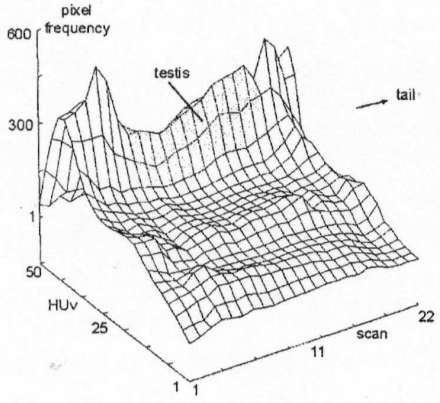

FIGURE 5. *3D histogram of the abdominal region composition at the sixth wk.*

axis, the HU variables on the Y-axis (numbering from 1 to 50, after reducing by 5 from −150 to +100), and the frequency of pixel density values were plotted on the Z-axis.

In Fig. 3 (1st wk) the abdominal region is shown. In this interval fat depots are marked (HUv 5–17). Before the eighth scan of the beginning of this region cranially, the liver, and following the 16th scan, the fillet is shown. In Fig. 4, representing the fourth wk an intermediate state demonstrates the characteristic tendency, namely the volumetric development of the testis, as marked in the image. The last imaging state (Fig. 5, sixth wk) shows a notable fat volume decrease (HUv 5–17) and the parallel improvement of the testis tissue. The process described above was characteristically located in the ROI of the abdomen, and not in the fillet.

In contrast, the further analysis of the fillet resulted in changes of its fat content. In Fig. 6 it can be seen that the total amount of pixels representing the fat in the fillet (sum HUv 1–28/sum HUv 1–50 in the ROI of the fillet) showed a significant increase during the treatment, based on individual data of the fish.

This alteration was also detected in the shift of the peak of the fillet density histo-

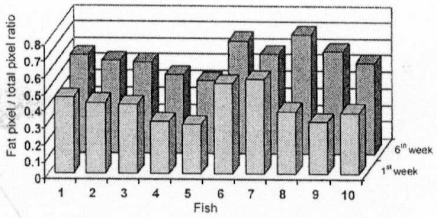

FIGURE 6. *The relative fat content of the fillet at the first and at the sixth wk of treatment in the 10 individual eels investigated.*

gram (Fig. 7). This shift can be described, when taking the HU variable comprising the maximum number of pixels into account; in this regard a movement toward the fat interval was shown, ranging between −2 and −4 HU variables (between −10 and −20 HU values).

However, when handling only the fillet as a region of interest, an unusual phenomenon was seen during the trial, namely the average density of the ROI was found to be zero. As on the Hounsfield scale, fluid water has the zero Hu value, the structure of the fillet was analyzed in detail. Myomers of the eel skeletal muscles are surrounded by myocomma, representing connective tissue. As the CT resolution (i.e., the size of a single pixel) is 0.96 × 0.96 mm, structures smaller than this size are not visualized and computed as imaginary units. The

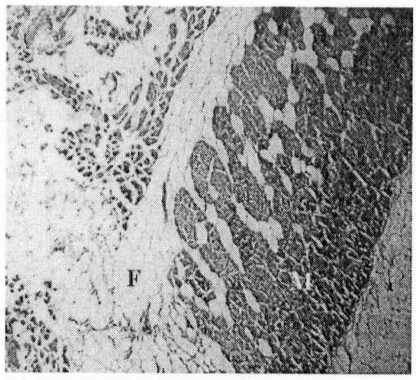

FIGURE 7. *Fillet HUv distribution histograms in the first and sixth wk of the maturation.*

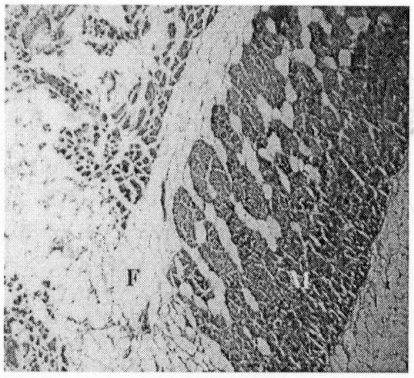

FIGURE 8. *Transverse fillet section at the sixth wk (Scale 100 μm; M: muscle, F: fat).*

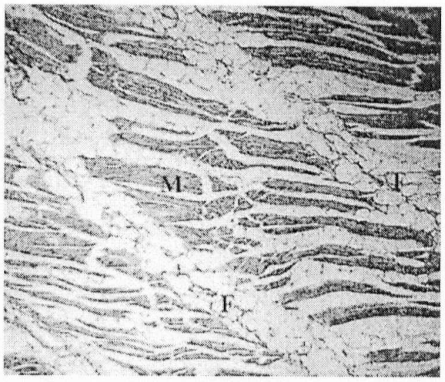

FIGURE 9. *Longitudinal section of fillet (M: muscle, F: fat).*

cause of the zero average density value, therefore, may be the special morphology of the eel skeletal muscles, comprising typical muscle areas with thin connective tissue layers.

These results were furthermore supported by the histological analysis based on sample taking from the right upper spine area of the fillet in the abdominal part. According to the section processing the muscle tissue results in red while the fat is white color (Fig. 8, 9). Both in the cross and the longitudinal sections 35–40% of the total pixels fall into the fat interval, which supports the average zero HU value of the fillet.

Discussion

Based on the above findings, both the CT scanning method and the applied anaesthesia repeated weekly were proven to be suitable to follow the maturation of male eels. At the sixth treatment all males were successfully induced for spermiating, which agrees with previous reports. Bieniarz and Epler (1977) stripped sperm from freshwater males by using weekly injections of 100-IU hCG per individual (six times). Pérez et al. (2000) worked on farmed eel in seawater and applied 1.5-IU hCG per g of body weight weekly, reaching spermiation at the sixth injection. Accordingly after the sixth scanning event, vital spermia were obtained from all fish in the current study.

The characteristic Hounsfield value interval representing the testis could be defined from the scans; however, the time-course measurement of the testis development could be performed only in the trial state when the testis reached a size to be recognized on the scans, after the third wk onwards. However, the abdominal fat depletion could be followed already from the first wk on.

Regarding the abdominal fat metabolism during the treatment period, it is hypothesized that the energy demands of the development of the testes and the concomitant spermiogenesis were, at least in part, covered from the depletion of the deposited fat. During the maturation process the eels did not take up feed; therefore, the energy demands could be met only from an endogenous source, which concerns mainly the fats (Boëtius and Boëtius 1985). Moreover, according to Boëtius and Boëtius (1967), there is a strong relationship between the testis size and the phase of maturation.

A possible explanation for the fat increase shown in the fillet can be in part the special keeping condition, as eels were kept in freshwater tanks without the possibility to swim during the trial period. Ginekken and Thillart (2000) found that eels' fat

"consumption" rates were about twice higher in swimming phase than in rest. Therefore, it might be possible that eels mobilized fat (e.g., from the abdominal depot) but the rate of fat oxidation in the muscles was lower, resulting in an increase of the fillet fat content. Eels use not only the tail fin but nearly the total body for the undulatory fish swimming (Müller et al. 2001; Ellerby et al. 2001). It was also published (D'Août et al. 2001) that the specific morphology of muscle and the possible power output does not vary along the eel body. Ellerby et al. (2001) reported that the silver-phase slow muscle generate more power than yellow-phase slow muscle which may be due to its ability to exert larger pressures.

Furthermore, the deposition, as well as the distributional alteration of the muscles of different fiber types, may also have serious effects on the fillet fat content. Based on histological results, Pankhurst (1982) found that the increasing proportion of red muscles (with high lipolytic activity) was attributable to an accumulation of lipid in the muscle during the artificial maturation of female eels. Mobilization of the lipid store would appear to occur via the red muscle compartment.

In summary, it could be demonstrated that eel muscles contain a relative high amount of fat with such a special distribution (i.e., fat cells between muscle fibers) and structure that fall below the resolution of the CT scanner. However, changes in the fillet fat content could be characterized by means of CT, and a strong increase was described during the treatment of the current study between laboratory conditions.

Acknowledgments

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6. DISCUSSION

The observations on the artificial maturation of *A. anguilla* female show that there are a lot of problems one has to face if willing to work in this field (Boëtius and Boëtius 1980, Boëtius et al., 1991; Amin, 1997; Pedersen, 2003 and 2004).

One of these problems is the selection of the initial experimental stock. It can be stated that the eel population of Lake Balaton is rather old. The 11-22 year old fish weigh only 362±165.8 g and the ratio of the sexually mature eel is low (9%) relative to their ages (manuscript on this is in preparation). Over this long time, persistent pollutants can build up in fat stores, which can be harmful during the experimental period. The nearly 50% infection rate by *Anguillicola crassus* is another aggravating factor for maturation experiments. Sexual maturation was induced by several times in the Balaton eel, but egg stripping could be obtained only at one occasion. We can support the theory that the nematode infections can cause problems during the migration (mechanical and physiological problems) to the Sargasso Sea but it is not a barrier factor of the artificial propagation. As we showed above, the females from Lake Balaton are not the best stock for long-term maturation experiments. Farm eels can be handled more easily and in a different way than wild stocks. All farm eel – used in our experiments - were free from *A. crassus* but all of them were infected by *dactylogyrosis*. Three experimental cycles had to be stopped due to this lethal infection (not published data). Finally Mebendasole treatment helped to overcome this problem as suggested by Székely and Molnár (1987). Unfortunately, at this moment there are not any eel farms in Hungary. We managed to induce the maturation of farm eel easier than that of the Balaton ones, but egg stripping occurred at only one occasion here, too. Greek eel

seemed the most suitable stock for experiments. They were large enough and free of *A. crassus*.

Experimental stock was kept in artificial seawater under natural circadian rhythm of light. Females were individually separated. They were kept closed in tubes where they could not swim. This prevented their aggressive behaviour and also made their identification easier. Though this keeping method should be improved because it did not hinder the maturation of fish.

During our 4-year series of experiments on female eel we tried to apply various hormones but only carp pituitary with or without dopamine receptor antagonists proved to be an effective hormone for artificial induction of sexual maturation. The induction of ovulation by applying a double dose of carp pituitary and GnRH-A mixture gave ambiguous results. At one occasion (Chapter One) we managed to strip fertilisable eggs, which showed the signs of ripening, as well as the appearance of perivitellinic space and the formation of animal pole after water activation (Boëtius and Boëtius, 1980; Amin, 1997; Pedersen, 2003 and 2004). In other experiments the same hormonal administration was unsuccessful (Chapter Two – Greek eels) and caused mortality. The exact reason for this effect is unknown. In our experiment (Chapter One) silver carp pituitary successfully induced late vitellogenic phase in the oocytes. Repeated GnRH-A administrations in doses of 0.1 and 10μg did not induce remarkable ovarian development. This result is in accordance with the other publications (Dufour et al., 1983; 1991) and results of one of my earlier reports (Müller, 1999).

The work with males – similarly to other species was easier. In rivers and lakes the ratio of males seems to be very low. For instance only one male was found in Lake Balaton out of over 400 individuals during 2001-2003 (not published data). This may come both from the unique sex determination of the eel, which is influenced by environmental factors, and the fact that the smaller

males are easier preys for larger fish or cormorans (*Phalacrocorax carbo*). In spite of this, the male's ratio in farms is high, over 80%.

We could successfully induce full sexual maturation and long term spermiation of farmed European eel males in freshwater. These experiments using hCG injections lead to similar results as other's experiments in seawater (Boëtius and Boëtius, 1967; Bieniarz and Epler, 1977; Khan et al., 1987; Pérez et al., 2000). Unfortunately, it is not declared, that fresh water rearing could replace the use of salt water, because the lack of fertilization tests.

Histological pictures showed that testis tissues - after a long spermiation period - redeveloped natural deformations. This is promising in regard of the multiple use of males. Males reared in freshwater produced morphologically the same spermatozoa as reported in seawater (Billard and Ginsburg, 1973; Todd, 1977; Gibbons et al., 1983; Okamura et al., 2000). We could activate the spermatozoa (10-90% motility) in artificial seawater (3.5 ‰ salinity). Since there is no significant difference between the morphology and motility of the freshwater and seawater spermatozoa, it is supposed that freshwater rearing of males is not a barrier factor for the artificial propagation of the *A. anguilla*. In order to verify the above statement it would be important to use this sperm for fertilisation tests.

Our experiments on the cryopreservation of eel sperm show that the extender originally developed for the common carp sperm (modified Kurokura extender) is suitable for freezing eel sperm together with methanol as cryoprotectant. Although, prior to our experiments only dimethyl sulfoxide (DMSO) was used for the cryopreservation of *A. japonica* sperm (Tanaka et al., 2002) and DMSO and phosphatidylcholine were used in *A. anguilla* (Asturiano et al., 2003), methanol was found to be superior to DMSO in a number of cell types, including fish spermatozoa (Harvay et al., 1982). It would be important to

remark that we started the cryopreservation pro-experiments in May 2001, so we had not known anything about the Japanese and European trials in this field.

We found that the CT scanning method is suitable to follow the maturation of male eel *in vivo*. Regarding the metabolism of abdominal fat during the maturation period it is hypothetised that the energy demand of the development of the testes and the concomitant spermiogenesis was at least in part, covered by the depletion of the deposited fat. During the maturation process the eel did not feed, therefore their energy demand could only be supplied from an endogenous source, which may concern mainly the fat (Boëtius and Boëtius, 1985). Possible explanation for the fat increase shown in the fillet can in part be the special keeping condition, as eel were kept in freshwater tanks without swimming during the trial period. Therefore, it might be possible that the eel mobilised fat (e.g. from the abdominal depot) but the rate of fat oxidation in the muscles was lower, resulting in an increase of the fillet fat content. This fact is also supported by Müller et al. (2001) and Ellerby et al. (2001), describing the undulatory fish swimming, using not only the tail fin but nearly the total body. Ellerby et al. (2001) reported that the silver-phase slow muscle generate more power than yellow-phase slow muscle which may be due to its ability to exert larger pressures. It was also published (D'Août et al., 2001) that the specific morphology of muscle and the possible power output does not vary along the eel body. Furthermore, not only the deposition, but also the distributional alteration of the muscles of different fiber types may also have serious effects on the fillet fat content. Pankhurst (1982c) found, that the increase of muscle lipids was partly attributable to the higher relative amount of red (i.e. oxidative, with high lipolytic activity) muscles developing during the maturation of female eel. It could be demonstrated that eel muscles contain a relative high amount of fat with such a special distribution (i.e. fat cells between muscle fibres) and structure that falls below the resolution of the CT scanner.

However, changes in the fillet fat content could be characterised by the means of CT, and a strong increase was described during the treatment of the current study, in laboratory conditions.

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7. PROPOSALS

There is no technology for the artificial propagation of European eel. There is little information about the feeding habit of larvae and all this information originated exclusively from wild-caught fry, so practical proposals can be made only in the future.

I suggest collecting fish for maturation experiments from the catches during September and November because the ratio of silver eel increases at this time. Silver eel have well developed gonads and the maturation time can be shorter. There is not a critical age when eel enter the silver stage and neither the size nor the age are directly related to maturity in a relatively old population such as the Lake Balaton stock. However, the age at the onset of maturity in eel was inversely related to the growth rate. The start of migration to the spawning place depends principally on the body weight and not on the age. The previous four years' results showed that catching fish suitable for the artificial induction of sexual maturation became more difficult in Lake Balaton year by year.

Based on our results, the silver carp pituitary is also on effective hormone extract for maturation. I suggest using longer periods to hormonal treatments with 5mg/fish/treatment or to use a double dose (10 mg/fish weekly administration to reach the pre-ovulated stage. There is a huge amount of silver carp caught in Lake Balaton annually and its pituitary is larger (7-10 mg / fish) than that of the carp.

To induce ovulation I propose the use of Ohta protocol (17-hydroxyprogesterone or 1720β dihydroxy-4-pregnen-3-one as a final injection. Based on results both in *A. japonica* and *A. anguilla* this MIS hormone induces ovulation with great safety.

According to our result, which is also in accordance with my earlier findings and other publications GnRH-A or GnRH-A with dopamine receptor antagonist mixture are not recommendable to induce sexual maturation.

I suggest comparing different types of extenders (Artificial Japanese and European eel seminal plasma, modified Kurokura, glucose etc.) and cryoprotectants (DMSO, ethanol) and combinations of these to describe a uniform procedure for eel sperm cryopreservation.

I suggest the use of computer tomography to select the experimental stock for artificial induction of sexual maturation. If females have not got enough fat resources at the beginning of the experiment they can not be applied for a long term maturation experiments. Low amount of fat indicates a the high probability of mortality during the starvation period and consequently causes an unsuccessful experiment.

8. NEW SCIENFITIC RESULTS

- Based on my research I can conclude that the artificial induction of sexual maturation in both sexes of the European eel is possible under artificial conditions. For instance, commercial table salt is suitable to simulate seawater salinity.
- The application of double amounts of the mixture of carp pituitary, GnRH-A and dopamine receptor antagonist products gave contradictory results regarding the inducing of ovulation.
- Silver carp pituitary is effective to induce an advanced phase of sexual maturation in females.
- According to our results the extender originally developed for the cryopreservation of common carp sperm together with methanol as cryoprotectant seems suitable for the deep-freezing of eel sperm.
- The applied CT scanning method is proven to be suitable to follow the maturation processes *in vivo* (gonad development, fat mobilisation from abdomen to filet, etc.) in males.

9. ÖSSZEFOGLALÁS

A európai angolna a katadrom halak csoportjába tartozik, amely azt jelenti, hogy egy hosszantartó édesvizi életszakasz után a tengerbe vándorolnak szaporodni. Az angolna tenyésztéséről a szó szoros értelmében nem beszélhetünk, csak felneveléséről, hiszen még senkinek sem sikerült európai angolna lárvát mesterséges szaporítást követően felnevelni. Az európai angolna farmok ivadékszükségletüket természetes vízi fogásból nyerik. Azonban a vízszennyezés, az *Anguillicola crassus* fertőzöttség, valamint a kontinentális vizekbe bevándorló ivadék és az elvándorló ezüst angolnák túlhalászata miatt a világ angolna állományai csökkennek, melynek mértéke az európai angolna esetében oly súlyos, hogy ez a faj önerőből, segítség nélkül valószínűleg nem képes fennmaradni!

A disszertációm témája az angolna mesterséges szaporítás lehetőségének vizsgálata volt, figyelembevéve annak hazai vizeinkben végbemenő reprodukciós sajátosságait. Laboratóriumi kísérletek a Veszprémi Egyetem Georgikon Mezőgazdaság-tudományi Kar és a Kaposvári Egyetem hal laboratóriumaiban míg kiegészítő vizsgálatok a Szent István Egyetem Halgazdálkodási valamint a Kórbonctani és Igazságügyi Állatorvostani Tanszékén folytak.

Kísérleteimet vadon befogott, vagy halgazdaságban nevelt, valamint úszóhólyag féreggel (*Anguillicola crassus*) fertőzött, vagy attól mentes állományokon végeztem. Az ivarérleléshez többféle hormont és azok kombinációit használtam fel. Eredményeimet az alábbiakban foglalom össze.

- Sikerült ikrát nyernem mesterséges tengervíz felhasználása mellett és *A. crassus* fertőzőttség ellenére is.

- Megállapítottam, hogy ovuláció kiváltására alkalmazott dupla mennyiségű pontyhipofízis és az OVOPEL (GnRH-A + dopamin receptor antagónista) nem minden esetben eredményezett sikeres ikranyerést.
- A busa hipofízis eredményes ivarérlelő hatású.
- OVURELIN (GnRH-A) 0,1 és 10 µg / hal dózisban a 81 napos kísérlet alatt nem váltott ki petefészekfejlődést.
- Készítettem egy hisztológiai térképet a petefészek fejlődésről.
- Megállapítottam, hogy édesvízben ivarérlelt hímeket hosszú idejű spermatermelésre lehet késztetni heti hCG kezeléssel.
- Édesvízben, hasonlóan a sósvízi kísérletekhez az angolnák túlélik a hosszantartó hormonkezelést, ami megelőlegezi a nagy genetikai értékkel rendelkező hímek több ciklusú felhasználásának lehetőségét.
- Édesvízben tartott hímek spermájának a morfológiai és fiziológiai paraméterei (motilitás, mennyiség, jellemző alaktani paraméterek) hasonlóak a sósvízben tartott halak spermájának paramétereihez.
- A pontysperma mélyhűtésének technológiája eredményesen alkalmazható az angolna sperma mélyhűtésére.
- Hisztológiai térképet készítettem a here szöveti fejlődéséről, illetve visszafejlődéséről.
- Vizsgálataim alapján a computer tomograph hatékony eszköz a hormonálisan indukált ivarérlelés során bekövetkező változások (elsősorban a gonádfejlődés, illetve a zsír - a hasüregből a filé izomszövetei közé történő - mobilizációjának) nyomonkövetésére.

10. SUMMARY

Eel belong to the catadromous fish species. This means that after a long-term freshwater living they migrate to spawn to the Sargasso sea. Since no one has been able to bring up European eel larvae to glass eel size till now, we cannot speak about eel breading in the narrow sense of the term, only eel rearing. Eel farms in Europe base their annual production on the capture of glass eels entering rivers and raise them to market size. All the eel populations around the world have been decreasing since the 1970s. This is due to a lot of factors, as water pollution, overfishing of glass and adult eels *Anguillicola crassus* nematode infection etc. It is probable that the European eels are not able to sustain their own stocks without human help.

The subject of my dissertation was to investigate the possibility of the artificial propagation of European eel.

The experiments were carried out at the Department of Zoology of Veszprém University and also at the Department of Fish and Companion Animal and Institute of Diagnostic Imaging and Radiation Therapy of Kaposvár University. Complementary experiments were made at the Department of Fish Culture and Department of Pathology and Forensic Veterinary Medicine of Szent István University.

I carried out different maturation experiments with eel using different types of hormones. The fish originated from a farm or the wild, were free or infected by *Anguillicola crassus*. The results are summarised as follows:

- Based on my research I can conclude that the induction of sexual maturation in both sexes of European eel is possible under artificial conditions. Ovulated eggs could be obtained in spite of the facts that

artificial sea water was used and the experimental fish were infected by Anguillicola crassus.

- The application of double amounts of the mixture of carp pituitary, GnRH-A and dopamine receptor antagonist hormones gave contradictory results regarding the induction of ovulation.
- Silver carp pituitary is effective to induce an advanced phase of sexual maturation in eels.
- GnRH-A in doses of 0.1 and 10μg / fish did not induce a remarkable ovarian development.
- A histological map was assembled following the developmental stages from previtellogenic oocytes to preovulated eggs.
- Freshwater reared males can be induced for a long-term sperm production by using weekly hCG administration.
- Freshwater reared can survive the hormonal induction of sexual maturation similarly to seawater reared ones. This is promising in regard of multiple use of males, having high genetic value.
- “Freshwater males” produce morphologically the same spermatozoa as "seawater males". The spermatozoa could be activated in artificial seawater. Since there is no significant difference regarding the morphology and motility between freshwater and seawater spermatozoa, it can be assumed that freshwater rearing of males is not a barrier factor of the artificial propagation of European eel.
- A histological map was assembled following the developmental stages from immature testis’s tissues through mature to regressed form.
- According to our results the extender originally developed for the cryopreservation of common carp sperm seems suitable for the deep freezing of eel sperm using methanol as cryoprotectant.

- The applied CT scanning method proved suitable to follow the maturation process *in vivo* (gonad development, fat mobilisation) in males.

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12. PUBLICATIONS IN CONNECTION OF THE THESIS

12.1. PAPERS

Müller, T., Binder, T., Váradi, B., Bercsényi, M. (2001): Az európai angolna (*Anguilla anguilla* L.) hormonálisan indukált ivarérlelése és sikeres ikranyerése (*Artificial induction of sexual maturation and successful egg release*). Halászat 94 (3), 115-118. (in Hungarian with English summary).

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12.2. PROCEEDINGS

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Müller, T. (2000): Az európai angolna (*Anguilla anguilla*) hormonálisan indukált ivarérlelésének hagyományos és alternatív utjai. Diploma munka (PATE Georgikon MgTud-i Kar, Keszthely), (Kézirat).

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12.3. POPULARIZING ARTICLES

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13. PUBLICATIONS OUT OF TOPIC OF THESIS

13.1 PAPERS

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Kucska, B., Binder, T., Bódis, M., **Müller, T**. (2002): Ragadozó halak-csuka, sügér és menyhal -mesterséges tápon történő nevelése. VIII. Ifjúsági Tudományos Fórum. Keszthely, március 29. CD kiadvány pp.1-5

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14. CURRICULUM VITAE

Surname: Müller *First name*: Tamás
Birth date: 2/04/74 *Nationality:* Hungarian

Position title: 2003-2004 Assistant lecturer
2005- Professor's assistant

Education:

Institute and location	Degree	Year	Field of study
Újhelyi Imre Agricultural Secondary School, Szentlőrinc	GSCE	1993	
Újhelyi Imre Agricultural School, Szentlőrinc	Technician degree	1994	animal husbandry and health
Veszprém University, Georgikon Faculty of Agriculture, Keszthely	M.Sc.	2000	agricultural engineer
Kaposvár University, Faculty of Animal Science, Kaposvár	Ph.D. student	2000-2003	fisheries science

Knowledge of languages:
Hungarian (mother toung)
English (certification-intermediate level C in 2003)
German (certification-base level A in 2003)

Teaching experience:
Zoology (Veszprém University)
Fish culture (Veszprém University, Mosonmagyaróvár University)

Principal research fields:
-Artificial induction of sexual maturation of European eel
-Artificial propagation of Volga perch (*Sander volgense*)
-Hybridisation among the Percids
-Growing carnivorous fishes pike, pikeperch, Volga pikeperch, on pellet

Number of scientific and professional publications:
Scientific articles in journals: 11 (i.f.: 5.541)
Scientific articles in conference publications: 15
Scientific oral and poster presentations: 8
Popularizing articles: 1

Professional experience (in abroad):

1999, United Kingdom (University College of London, Genetic Laboratory): to train the fish facilities (3 weeks)

2000, Germany: to take part in an artificial propagation of catfish (2 days)

2001, China (Hubei Province Fisheries Res. Institute and Beijing Fisheries Cooperation): to study the traditional and modern fishery methods in pond culture (10 days)

2002-2004, France (CEMAGREF, Bordeaux and Brest University, Brest): to work on eel reproduction biology and eel silvering within EELREP project 5th framework (4 times, sum. 5 weeks)

2003, Germany (Genetic Institute of Karlsruhe): to study genetical methods in studying fish larvae development (3 days)

Professional experience(in Hungary):

1994-2000, Balaton Fisheries Company (fishery in natural waters, eelfarm Hévíz): practical training (several times, sum. 7 months)

2000, (SzIE University, Gödöllő) practical training (1 day)

2003, Attala (Interfish Ltd): artificial propagation of tench *Tinca tinca* (1 day)

Supervision activity:

6 theses for Faculty of Student Scientist Conference (four times 2nd, two times 3rd places)

3 theses for National of Student Scientist Conference (one 2nd, one 3rd places)

4 diploma (two at Veszprém University, one at Kaposvár University, one at SzIE University)