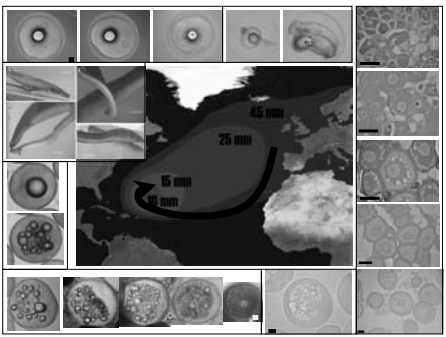

Figure 6 Synthesis of oocyte development and spawning during and after migration in the Sargasso. During silvering, oocytes start to develop up to the lipid vescicle stage. During freshwater migration, lipids are incorporated during an extended lipid vescicle stage. Vitellogenesis probably occurs alerady near or at the spawning grounds. These are indicated by the decreasing size of the leptocephali found by Schmidt (1923). During the last stages of maturation at the spawning grounds, oocytes hydrate and ovulate. Semelparous spawning occurs collective and simultaneous. Fertilised and dividing eggs rise to the surface. Embryos are supposed to hatch around 60 h.a.f. not including possible delay by high pressure as found by Hiroi et al. (2003).

Chapter 8
SUMMARY & RECOMMENDATIONS

Summary

In many parts of the world eel is considered a culinary delicacy already since ancient times. Therefore this species is commercially interesting and target for fisheries and aquaculture. Aquaculture of eel is still completely depending on the wild stocks since breeding in captivity is not successful. The wild stocks have however been declining over the past 25 years to a great extent without any signs of recovery. A wide diversity of factors is assumed to be involved determining the quantity (habitat degradation, restocking, overfishing and migratory obstacles) as well as the quality (pollution, introduction of new diseases) of the spawning stocks. A great need for management and conservation measures exist but knowledge on eel biology is incomplete to provide such tools. The lack of knowledge mainly concerns the mysterious oceanic phase in the amazing life cycle of this catadromous fish species.

The European eel *Anguilla anguilla* is one out of 15 species of freshwater eels that all have an oceanic phase characterised by a long distance spawning migration and a semelparous spawn. European eels are born as leptocephalus larvae in the Sargasso Sea. After a journey of seven to nine months they arive at the continental shelf and display a first metamorphosis into glass eels. As immature yellow eels they reside in the estuaries or migrate upstream the European freshwater rivers where they spend a long feeding stage. After reaching a certain age and size, yellow eels cease feeding and start metamorphosis for a second time: the process of silvering turning them into silver eels.

Silvering is a complex phenomenon linking external and internal modifications. Pankhurst (1982) developed an index on the basis of eye size and bodylength to discriminate between yellow and silver eels. Very recently, Durif (et al., 2005) recognized intermediate phases and suggested a silver index based on eye size, pectoral fin length, bodylength and bodyweight. Still a multidisciplinary discussion continues whether silvering is a true metamorphosis, e.g. a marked and abrupt developmental change in the form or structure of an animal, or a more continuous process correlated to the degree of maturation. We observed that the eyes continue to enlarge during artificially induced maturation in a linear fashion (Fig. 1) indicating a more continuous process.

Only after completion of silvering, silver eels leave the continent between September and November and disappear. They migrate probably at depths between 200 and 600 m depth for 5 to 6 months to the spawning grounds in the Sargasso (reviewed by Tesch & Rohlf, 2003), where spawning is believed to occur in March and April (McCleave et al., 1987; McCleave, 2003). Most certainly the effective genitors are characterised by an excellent swim fitness. Highly efficient energy management is required, not only to fullfill migration, but also to provide the eggs with sufficient lipid stores. When the silver eels leave they are still in a prepubertal condition, while after six months swimming they should be fully mature and ready to spawn.

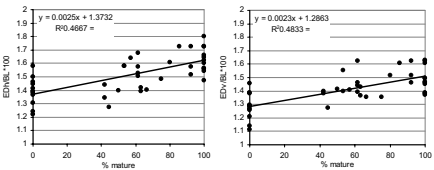

Figure 1 Eye diameter horizontal (a) and vertical (b) of 11 fully matured females with of each 4 paired observations. The time to reach full maturation is taken as 100%. Both eye diameters horizontal and vertical increase linear and in same ratio.

Information about migration, maturation and the interaction between both is lacking. Understanding of natural triggers for maturation could lead to more successful reproduction protocols.

The general aim of this thesis was to establish the energetic requirements for reproductive migration and maturation of European eel and to assess the role of environmental constraints like the influence of the swim-bladder parasite *A. crassus* on the swim performance and the influence of dioxin-like contaminants on embryonic development.

Biomechanical efficiency of anguilliform swimming is considered low. Experimental studies on the swim efficiency of large migratory silver eels are limited. Very recently, our group found that eels swim 4 to 6 times more efficient than non eel-like fish and utilise *c.* 60g fat per kg for migration (van Ginneken et al., 2005b). Performance is likely to vary among silver eels within and between locations, mainly determined by trophic quality. In order to be able to compare the performance of farmed and wild eels from different locations and under different conditions, we developed a swim fitness test in **chapter 2**. Swim trials with 101 female eels weighing 400 – 1500g were performed in 22 Blazka-type swim-tunnels in a climatised room at 18ºC with running fresh or salt water. Speed and endurance swim trials started at 0.5 up to 1 meter per second (m/s) with increments of 0.1 m/s. Since both tests showed similar results, the single day speed test could be used to predict endurance. Eels showed ability to stabilise and maintain metabolic balance. Although they did not swim fast, they swam highly efficient. Eels reached maximum aerobic swim speeds of 0.81 up to 1.24 BL/s body-length per second (BL/s). At optimum swim speeds of 0.58-0.68 m/s or 0.74-1.02 BL/s cost of transport (COT) values were found of 37-50 mg O_2/kg/km which are very low. Energy expenditure during exercise was 20% higher in SW vs FW. Wild silver eels showed lower performance than farmed silver eels. Overall, we can conclude that silver eels can be considered as cruising specialists. If silver eels cruise at optimum swim speeds they would travel for less than 3.5 months to the Sargasso instead of the generally believed 6 months.

Infection with swim-bladder parasite *Anguillicola crassus* is suggested as one of the causes of the collapse of eel populations worldwide. This nematode has been introduced 20 to 30 years ago from Asia and parasitised in short time various eel species in different geographical regions of the world. The effects are energy drain due to its sangivorous activities and mechanical injury of the swim-bladder wall by its migratory activity. These effects are hypothesized to impair spawning migration of European eel. In **chapter 3** we have investigated the effects of infection on swim performance. We hypothesized that parasitic sanguivorous activities reduce swim endurance while the mechanical injury impairs buoyancy control. Eighty eels suffering various degrees of infection have been introduced in the swim-tunnels and subjected to the swim fitness test as developed in chapter 2. Oxygen consumption was measured of large infected silver eels swimming at different speeds allowing to determine swim efficiencies. We found that especially silver eels are targets of infection. Infected eels have lower cruise speeds and higher cost of transport. Eels, that are not infected but contain a swim-bladder damaged by previous infection, show similar effects and even higher cost of transport. Effects thus seem to be associated with swim-bladder disfunction and the resulting loss of neutral buoyancy. This leads to the conclusion that infected eels with damaged swim-bladders have lower success to reach the spawning grounds. Simulated migration trials confirmed fast migration failure (<1,000-km). This study showed that *A. crassus*-infection severely impairs the reproductive potential of eel. Recent studies indicated similar roles for PCB pollution and viruse infection. We can therefore conclude that the downfall of quality (e.g. swim-bladder parasite, EVEX virus, PCBs etc.) of future genitors may well be a major acting force behind the eel's world-wide collapse.

Since European eel *Anguilla anguilla* is one of the most extreme examples of reproductive homing, it is a perfect model to study the poorly understood relation between migration and maturation. In **chapter 4** we investigated this relation. We hypothesized that swimming is involved in metamorphosis (silvering) and release from reproductive inhibition and depressed lipid mobilisation. In this study, we subjected 55 old (>13 years) eels from Lake Balaton (Hungary) to swimming for durations of 1, 2 and 6 weeks. Changes in morphometry and oocyte development were determined to establish the silvering and maturation status. We found that swimming stimulates silvering, shown by enlargement of the eyes already within 2 weeks of swimming. Furthermore, we found that swimming stimulates maturation. Already within 1 week swimming, the gonadal mass increased, oocytes shifted in stage, became larger and large amounts of lipids were incorporated in an extended lipid droplet stage. Synchronisation of oocyte development occurred within and between eels. No indications for vitellogenesis were found. We can conclude that swimming plays a major role in release from reproductive inhibition and mobilisation of lipids to the oocytes. Vitellogenesis and final maturation were not induced and may in the field situation only occur in the surroundings or at the spawning ground itself. Pre-treatment by swimming in protocols to breed eel or other migrant species in captivity may increase sensitivity for hormonal stimulation, fertility and reproductive success.

In **chapter 5**, we artificially matured European eel with the existing protocols for Japanese eel. In Japanese eels, the moment of stimulation of final maturation and ovulation is mainly based on weight increase related to the hydration response of the oocytes, which, in the European eel, is irregular. In contrast to Japanese eel, European eels show wide

individual variability and much slower response to hormonal stimulation. We did not find a difference in response time between Lake Grevelingen and Loire females (Fig. 2) suggesting that this response time is typical for European eels. In this study, the oocyte development of wild European silver eels was followed during final maturation. We describe 7 developmental stages based on 6 parameters: transparency, diameter of the oocyte and position and visibility of the nucleus, and diameter and number of oil droplets. Together, these parameters describe unidirectional changes from immature to over-ripe eggs. The developmental status of the gonads were determined in biopsies from 23 female eels, of which 14 ovulated and were stripped, while 9 gave eggs that could be fertilised. Oocytes matured asynchronously, but this seems to be an artefact, since fertility dropped with every new generation of oocytes. As the timing of ovulation is crucial for fertility of the eggs, our developmental index of oocytes should result in more successful maturation protocols.

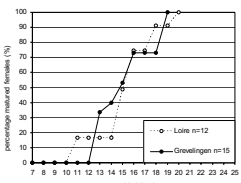

Figure 2 Rate of maturation. Female silver eels from Lake Grevelingen and River Loire were weekly injected with carp pituitary extract. Maturation was determined from regular egg biopsies. Frequency of occurrence of matured females from Lake Grevelingen vs. Loire River. Similar timing and speed of maturation response exists.

During the first three hours post fertilisation (h.p.f.), most eggs in all batches showed meroblastic cleavage up to the eight cell stage. Egg batches of two females resulted in the development of about 1600 embryos at 31-32 h.p.f. (Fig. 3). Embryos of one female (n=100) continued to develop and were found vigorously moving with the pigmented tail at 58-60 h.p.f indicating the onset of hatching. At this time they showed a yolk sac in which the protein part had disappeared and only the fat droplet remained (Fig. 4). Embryonic development continued until 100 h.p.f. when last embryos died. Hatching was not observed.

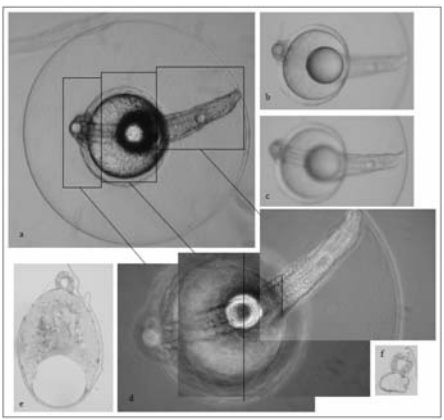

Figure 3 Stretched embryo at 32 h.p.f. photographed with a) phase contrast microscopy and b) and c) with bright field microscopy, d) zoomed-in views showing the developing somites and in e) and f) cross-sections at two locations as illustrated.

Embryos of a second successful female (n=1500) showed serious oedema of the yolksac, a deformed head region and absence of a heartbeat. Such embryonic malformations are typical for PCB-exposed eggs and indicate negative inteference with dioxin-like contaminants. Therefore in **chapter 6** we measured parental levels of dioxin-like contaminants and correlated their distribution to embryonic survival and development. The total dioxin-like toxic potency of the individual gonad batches was determined as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxine) – equivalents (TEQs), using an in vitro reporter gene assay. The observed differences in development and survival showed a significant negative correlation with the TEQ levels in the gonads, already at levels far below the maximal allowable level for fish consumption i.e. 4 ng TEQ/kg fish. The clear inverse relationship between the TEQ-level and the survival period of the fertilised eggs

strongly suggest that the current levels of dioxin-like compounds seriously impair the reproduction of the European eel. The peak of the environmental levels of dioxin-like PCBs and the decline of eel coincide world-wide, further suggest that, in addition to other threats, these contaminants contributed significantly to the current collapse of eel populations.

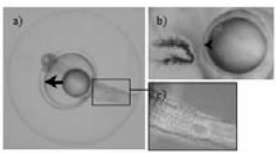

Figure 4 Eel embryos. a) arrows indicate embryonic yolk protein in an embryo at 32 h.p.f. b) yolk protein was absent in an embryo at 60 h.p.f. showing also the pigmented tail. c) detailed view of developed somites and Kupfer organ (phase contrast microscopy).

Few attempts were made to estimate the energetic costs of migration and maturation of European eel. The stored energy of silver eels, mainly as lipids in muscle and under the skin (Fig. 5), should suffice for successful reproduction. In **chapter 7**, we therefore subjected cultured eels and wild large silver eels to simulated migration at different speeds and calculated the cost of transport (COT) from oxygen consumption rates. We found that cultured eels swam at COTs of 34 ± 5 mg O_2/kg/km during 2,173 ± 305 km migration. Wild silver eels swam at higher COTs of 52 ± 12 mg O_2/kg/km during 1,232 ± 172 km migration. COTs were rather constant and similar to values of short term 2h swim tests. Wild silver eels spend 78 ± 4 g fat /kg, or 39% of the fat stores at average fat percentages of 20%, on complete 5,500-km migration. These relatively low values confirm their high swim efficiency. Furthermore, we artificially matured eels from the same batch of wild silver eels by hormonal injections to determine the amount of fat incorporated in the oocytes. We found that eels incorporate 57 ± 22 g fat /kg, or 28% of the fat stores, in the oocytes which is positively related to age. Thus in total 67% of the fat stores is spent on the eel's spawning run (Fig. 6). Fat requirements of average silver eels from high quality trophic habitats are thus not limiting for reproduction.

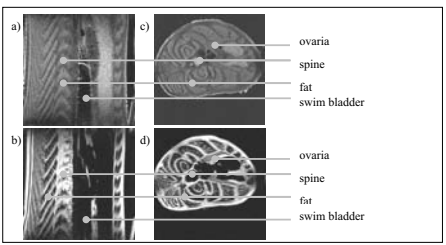

Figure 5 MRI with a 5.6 Tesla magnet (300 MHz) of eel showing where fat is stored with a) water image longitudinal, b) fat image longitudinal, c) water image cross-sectional and d) fat image cross-sectional. (Performed in collaboration with Prof. Dr. Annemie van der Linden, University of Antwerp, Belgium).

From this we can conclude that:

1) Silver eels do not swim fast but highly efficient at all speeds as we found with a developed swim fitness test. Cost of transport is very low and remains constant during migration. Silver eels are therefore cruising specialists. Especially silver eels are target of *A. crassus* infection and show lower cruise speeds and higher cost of transport. The nematodes cause damage on the swim-bladder wall that results in loss of neutral buoyancy and likely migration failure. Swimming releases eel from reproductive inhibition and stimulates mobilisation of lipids to the oocytes.
2) Timing of ovulation may be improved and fertility increased by applying the developed oocyte developmental index. We succeeded in fertilising batches of eggs multiple times and raised embryos up to 4 days after fertilisation. Differences in embryonic development and survival showed negative correlation with dioxin-like contaminant levels in the gonads already at low levels.
3) Fats are mobilised for 39% as fuel and are for 28% incorporated in the oocytes at the same time. Age is a determimant for successful reproduction since a) older eels showed increased capacity to incorporate more fat from the muscle into the oocytes determining higher egg quality, b) older eels are more sensitive for hormonal stimulation, and c) older eels are more susceptible to swimming induced oocyte development.

Recommendations for the protection and restoration of eel stocks

Silvering prepares eels for their oceanic migration indicated by the improved tolerance for seawater transfer and to pressure (Fig. 7). However, no indications were found in this study for improved swim performance of silver eels as compared to yellow eels (chapter 2). Swim exercise however induced silvering and maturation (chapter 4; Fig. 7). Only silver eels were able to complete maturation (Fig. 7). The oldest eels with the highest body weight and condition factor (highest fat percentage) showed highest sensitivity for hormonal stimulation (chapter 6 and 7).

Two strategies may be followed for the protection and restoration of eel stocks: 1) to protect the natural populations especially the silver eels contributing to reproduction, and 2) to stimulate research on artificial reproduction of eels in captivity. Concerning point 1, the quality of the habitat determines the quality of the targeted silver eels (the oldest and fattest). Silver eels should be free from swim-bladder parasite *A. crassus* (this study chapter 3) and viruses like EVEX (van Ginneken et al., 2004, 2005c; Fig. 7) since they impair migration. Furthermore, they should be free from dioxin-like contaminants since these negatively interfere with migratory capacity and have devastating effects on survival and development of offspring (this study chapter 6; Fig. 7). Recommendations therefore involve:

1. Monitoring of the production of the targeted silver eels in each EU-memberstate to estimate the reproductive potential of European eels.
2. Hydrosystems that produce high proportions of the targeted silver eels should be protected.
3. Dioxin-like contamination should be monitored in targeted silver eels of all major hydrosystems and targeted silver eels from areas with low levels should be protected. Actions should be taken to reduce levels.
4. Targeted silver eels from all major hydrosystems should be monitored for viruses and swim-bladder parasites and targeted silver eels from virus free areas with low parasite loads should be protected. Only virus free eel transports should be allowed.
5. Targeted silver eels should be provided migratory passage free from fisheries and obstacles.

Tools for the future of successful artificial reproduction

Success rates in artificial reproduction are still low for the European eel and not only because of disturbing effects of contaminants. The same accounts for Japanese eels although larvae can be bred. Adachi et al. (2003) stated that '*artificially matured Japanese eels exhibit many peculiarities such as variations in yolk accumulation and egg membrane formation, differences in the process of oocyte maturation and serum hormone levels, and other phenomena. These variations seem to indicate abnormality rather than species specificity*'. In artificially matured Japanese eels, vitellogenesis occurs when oocytes measure ≥ 250 µm, yolk globuli accumulate and the chorion thickens. The number of fat droplets still increases during vitellogenesis, which is considered not normal (Adachi et al., 2003). New Zealand longfinned eels are more matured when they leave for their spawning grounds. Their oocytes have more oil droplets, less yolk globuli and a thinner chorion in comparison to oocytes of artificially matured Japanese eels. Adachi et al. (2003) concluded that artificially matured oocytes of *A. japonica* enter vitellogenesis at an earlier stage.

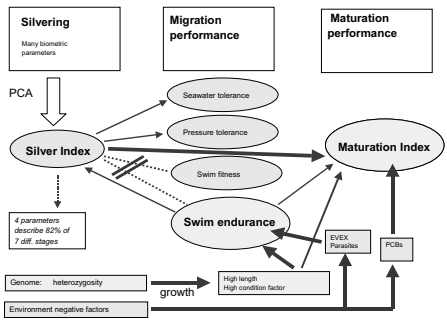

Figure 7 Inter-relationships between the silvering and migration/maturation performance. High silver index correlates with an improved sea water and high pressure tolerance, however, no effect was evident on the swim fitness. High silver index correlates with high maturation index. Large size and high fat content improves maturation as well as swim endurance. Furthermore, it was found that swimming induced silvering and maturation. Negative environmental factors affect the swim endurance and the maturation index. Infections with EVEX/parasites are devastating for swimming eels, while PCBs impair fertility. Taken from the summary and recommendations of the EELREP project.

Table 1 Identification tabel for the reproduction capacity of female European silver eels. Fat content as % of wet weight; Silver stages of female eels are based on silver index scores. Reproduction capacity is indicated from very likely (******) to absent (0). Taken from the summary and recommendations of the EELREP project.

Silver stage	I	II	III	IV	Va	Vb
Body length	--	--	--	--	<70cm	>70cm
Fat content	--	--	--	< 13%	13- 20%	>20%
Reproductive capacity	0	0	0	*	***	******

Providing the offspring with the necessary reserves of fats and proteins is crucial for successful reproduction. The observed differences indicate abnormality of the maturation process.
Thus although the protocol for breeding of eels in captivity is more successful for Japanese eel at this moment, still numerous problems are encountered that can be traced back to the maturation process of the parents. In order to come to a successful breeding protocol, there is a need for knowledge on natural oocyte development, its triggers and its regulation. This study has provided substantial new insight.

I. Selection of eels

This study provided criteria for selection of eels that can be used; Eels should be free from (dioxin-like) contamination. Only European eels from Sardinia and some locations in Ireland fit these criteria nowadays. Trophic habitat quality needs to be high, so silver eels are able to store enough fat reserves. Selected eels should be relatively old (>10 years) since we found increased capacity to incorporate more fat from the muscle into the eggs determining higher egg quality.
Additionaly, in EELREP was showed that correlations existed between body length and silver stage (Durif et al., 2005) with the hormone sensitivity. Large (>70 cm) migratory females (stage S_{FV}) with fat percentages >20% were considered most suitable (Table 1).

II. Increasing maturation sensitivity

This study showed that sensitivity was increased by swimming. Already short term swimming caused 1) release from reproductive inhibition, and 2) mobilisation of fat to the oocytes. Swimming induced incorporation of fats in the oocytes, without inducing vitellogenesis, apparently requires a long swim period. CPE injection induces vitellogenesis almost immediately in oocytes (in European eel after two injections; Palstra et al., unpublished results) that might not be in an appropriate state yet e.g. fat incorporation needs to be finalised. The CPE used in these experiments comes from spawnable carps and other hormones besides LH and FSH may have early effects. Pre-treatment by swimming in protocols to breed eel or other migrant species like salmon (Patterson et al., 2004) in captivity may increase sensitivity for hormonal stimulation, fertility and reproductive success.

III. Improve timing

With the existing Ohta (et al., 1996) protocol, ovulation does not occur spontaneously but needs to be induced. This experimenter needs to make the decision when the time is right and not the eels themselves. In Japanese eels, the moment of stimulation of final maturation and ovulation is mainly based on weight increase related to the hydration response of the oocytes, which, in the European eel, is irregular. The oocyte development index that we developed can be applied instead to improve timing of ovulation and increase fertility. Antoher approach is not to strip but to let eels themselves determine timing of spawning through the action of pheromones by joining matured males and females.

IV. New techniques

One important disadvantage of weekly hormonal induction is the large amount of handling stress. It is well known that stress reduces fertility, so low stress must improve succes. Recent innovations make it possible to circumvent this disadvantage. Leiden University recently patented hormone (LH/FSH) producing cells which can be implanted. Other approaches involve GnRH implants that stimulate the pituitary to produce LH and FSH or gene transfer.