Chapter 1

General introduction

Into extremes: the intriguing life cycle of freshwater eels

Freshwater eels (*Anguilla* spp.) have intrigued scientists for centuries, even since the times of Aristotle, who believed eels emerged spontaneously from the mud. With the recently discovered species *A. luzonensis* the genus *Anguilla* currently comprises 19 species and subspecies (Ege, 1939; Minegishi et al., 2005; Watanabe et al., 2005, 2009). The genus *Anguilla* belongs to the superorder Elopomorpha, which are primitive fish species at the base of the teleost lineage (Greenwood et al., 1966; Inoue et al., 2004).

Eels are known for their fascinating catadromous life-cycle (Figure 1). Once eggs are laid in the depths of the oceanic waters, they hatch after about two days and change their body plan within a few weeks from small round larvae to the leaf-shaped leptocephalus larvae (Miller, 2009). This leptocephalus stage is a feature only seen in the teleost superorder Elopomorpha. Leptocephalus larvae were considered a separate species (*Leptocephalus brevirostris*), until Grassi and his colleague Calandruccio showed that leptocephali were the larval form of the European eel (*A. anguilla*, formerly known as *A. vulgaris*) (Grassi, 1896). The larvae drift along with the oceanic currents for ca. 8-12 months (Arai et al., 2000; Wang & Tzeng, 2000), and before entering the continental freshwaters they metamorphose into the rounded transparent glass eels (Tesch, 2003; van Ginneken & Maes, 2005; Aoyama, 2009). In the freshwaters they become pigmented and start their growth phase as elvers and yellow eels for 5-50 years (Tesch, 2003, van Ginneken & Maes, 2005).

At a certain time, eels start to adapt to life in the oceanic waters by changing morphological and physiological characteristics; a process that is called 'silvering' (Tesch, 2003). During silvering, the skin colour of the dorsal side changes from yellowish green to dark gray-black, while the ventral side becomes silvery white. The eye diameter enlarges (Pankhurst, 1982; Pankhurst & Lythgoe, 1983) and the visual sensitivity of the retinal pigment changes from green to blue (Archer et al., 1995), which corresponds to the light that penetrates deepest into the oceanic waters. Originally, the process of silvering was assumed to be a second metamorphosis. However, it was shown that silvering is primarily induced by the gonadotropic axis, indicating that silvering is actually the process of puberty instead of a 'true' metamorphosis (Aroua et al., 2006; Rousseau et al., 2009).

In autumn, when light intensity fades and ambient temperature start to drop, full grown eels cease feeding and start their spawning migration covering hundreds to thousands of kilometres (Tesch, 2003; Tsukamoto, 2009). During the journey to their spawning areas they also display extensive daily vertical migration, experiencing strong fluctuations in hydrostatic pressure and temperature (Jellyman & Tsukamoto, 2002; 2005; 2010; Tesch, 2003; Aarestrup et al., 2009; Manabe et al., 2011). The European eel (*A. anguilla*), for example, is assumed to spawn somewhere in the Sargasso Sea, approximately 6000 km from the European coasts (Schmidt, 1923, McCleave, 2003). Presumably, this distance is covered within 6 months, as the peak migration occurs in autumn and the smallest larvae were obtained in spring (Schmidt, 1923).

As silver eels cease feeding prior to their migration, they need to rely completely on their energy stores for migration as well as for gonad development (Tesch, 2003). Therefore, eels need to swim very efficiently and have a high endurance. Biomechanical studies suggest however a high energy cost for anguilliform swimming based on a low thickness-over-length ratio (Videler, 1993). However, during the last decade several studies showed that female eels swim remarkably efficiently as indicated by their low cost of transport (i.e. the oxygen consumed per distance swum), even up to 6 times more efficient than rainbow trout. It was calculated that female eels use approximately 40% of their initial fat reserves (20-30% of their total body weight) for migration, leaving 60% for the development of eggs (van Ginneken & van den Thillart, 2000; van den Thillart et al., 2004, van Ginneken et al., 2005a; Palstra et al., 2008a). In addition, calculations using the optimal swimming speed (i.e. the swimming speed at lowest cost of transport) indicate that females may be able to cover the distance to the spawning area even faster than previously assumed, namely in 3.5 instead of 6 months. when swimming at their optimal swimming speed of ca. 0.65 m s^{-1} (Palstra et al., 2008a). Although these studies showed the swimming ability only for a short term (max. of several weeks), van Ginneken and colleagues (2007a) showed that farmed female eels were able to swim continuously for 6 months at 0.5 body length per second (BL s^{-1}) covering a total of 5500 km.

These previous studies all focused on swimming capacity of female eels. The swimming capacity of males has been studied less extensively (Sébert et al.,

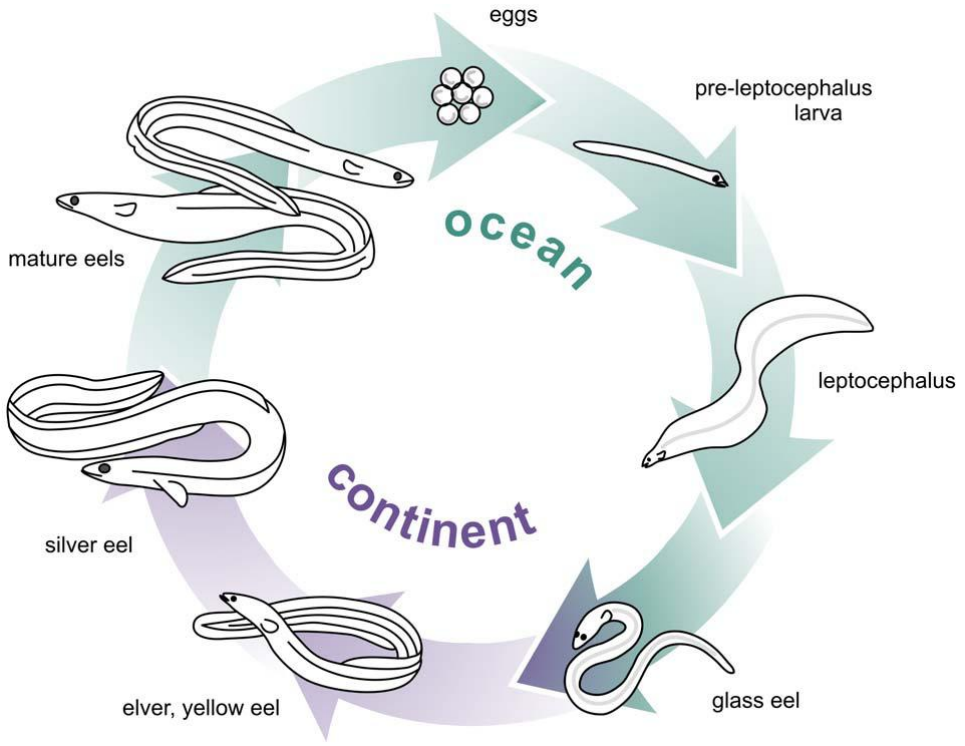

Figure 1. The life cycle of the European eel. After hatching, presumably in the Sargasso Sea, cylindrical larvae develop into leaf-shaped leptocephalus larvae, which after drifting on the Gulf Stream for approximately one year metamorphose into glass eels close to the European coast. The glass eels may stay at the coast or migrate upriver, where they stay as juveniles (elvers and yellow eel) for many years (depending on the region: males 4–6 years, females 8–12 years). Finally, they develop into migrating silver eels; the cause and timing of silvering is not well understood. They mature during or after migration to the spawning grounds (From Henkel et al., 2012).

2009; Quintella et al., 2010). It is important to note that males are much smaller in size than the females; in the case of the European eel, males have approximately half the body length (ca. 30-40 cm) and about a tenth of the body weight (ca. 100-150 g) of full grown females (Tesch, 2003). This smaller size is assumed to affect the swimming ability as the cost of transport increases with a decrease in body size (Schmidt-Nielsen, 1972; Beamish, 1978). Recently, it has been shown that the critical swimming speed (i.e. the highest aerobic swimming speed; Brett, 1964;

Beamish, 1978; Videler, 1993) was similar for male and female silver eels, indicating that both sexes may reach the spawning area in the same time (Quintella et al. 2010). Scaion et al. (2008) showed that the respiratory rate of male eels was higher than that of females. When male eels were subjected to swimming exercise up to a maximum of ca. 0.40 m s^{-1}, a ca. 1.23 times higher oxygen consumption rate, as compared to females of a different study (Palstra et al., 2008a), was observed (Sébert et al., 2009). In addition, it has been shown that high hydrostatic pressure resulted in a decrease of oxygen consumption (Sébert et al., 2009). However, the use of relatively small swimming chambers in those studies may have affected the results. It was recently shown that fish can obtain higher swimming speeds in larger swim tunnels (Tudorache et al., 2007). In conclusion, the swimming capacity of males needs to be further elucidated.

Decline of the population: call for eel management and aquaculture

Freshwater eels, especially the European and Japanese eel (*A. japonica*), are of high economic value and in many countries part of the traditional cuisine. Since the 1980s, a worldwide collapse of eel populations was observed; the yearly glass eel influx of several species (European, Japanese, American eel (*A. rostrata*)) showed a dramatic decrease, even up to 99% (Dekker et al., 2003; Stone, 2003). This decline may be due to a combination of anthropological, biotic and abiotic factors (van den Thillart & Dufour, 2009).

Anthropological factors include overexploitation, migration barriers (e.g. hydrodams and pumps), reduction of habitats and pollution of waters. There are indications that dioxin-like contaminants, such as PCBs, negatively affect embryonic development (Palstra et al., 2006). Also heavy metal contaminants, such as cadmium, could disrupt endocrine pathways and thereby the eel's reproductive capacity (Pierron et al., 2008). Biotic and abiotic factors that may contribute to the decline include changes in oceanic conditions (e.g. displacement of salinity and thermal fronts) within the spawning area and the area for early larval development (Kimura et al., 2001; Friedland et al., 2007), diseases e.g. caused by EVEX virus (van Ginneken et al., 2004; 2005b) and infection with swimbladder parasites, *Anguillicola crassus* (Palstra et al., 2007a; Székely et al., 2009). It was shown that severe infection with the swimbladder parasite negatively

affected the swimming capacity of female eels, indicating that those infected eels will fail to reach the spawning area (Palstra et al., 2007a; Székely et al., 2009). Also, it was suggested that due to the low energy reserves found in a large proportion of female silver eels, many will not succeed to reach their spawning area or may be too exhausted for reproduction (Svedäng & Wickström, 1997; Clevestam et al., 2011).

Recently, the European eel was added to the IUCN red list of threatened species (Freyhof & Kottelat, 2008). The strong decline of the eel populations over the last decades resulted in an urgent call for eel management and sustainable aquaculture in order to save the wild eel population. Protective measures include reduction of fishing pressure, allowing the escape of silver eels, and blocking the export of European glass eels. The Eel Management Plan of the Netherlands (2012) includes a closed season of fishing during the peak migration period (1 September – 1 December), reduction of migration barriers, and restocking of waters with glass eels and elvers from eel farms. Sustainable aquaculture of eels bred in captivity would reduce the fishing pressure on population recruitment. However, eel aquaculture is still far from sustainable. Major problems occur due to the fact that in captivity eels do not mature and reproduce. Artificial reproduction is possible to some extent (see below), however, most obtained larvae die within a few days due to lack of knowledge of rearing conditions and larval feeds.

Reproductive endocrinology of eels

The hypothalamus-pituitary-gonad axis

Sexual maturation in teleosts is a complex process and is still not completely understood. The major endocrine control of sexual maturation is via the hypothalamus-pituitary-gonad axis (HPG-axis, Figure 2). Sexual maturation is induced by internal or external factors (e.g. photothermal period, age, adiposity and social factors) triggering this axis (see reviews Migaud et al., 2010; Taranger et al., 2010; Zohar et al., 2010). When the HPG-axis is activated, gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the synthesis and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) by gonadotropic cells in the pituitary. In many adult

teleosts the release of LH is inhibited by dopamine (DA) and stimulated by GnRH (Peter et al., 1986; Dufour et al., 2005).

FSH is a key factor in the induction of vitellogenesis in females and of spermatogenesis in males (Nagahama, 1994; Nagahama & Yamashita, 2008; Planas & Swanson, 2008). It acts on the follicle cells of the gonads and stimulates the production of testosterone (T), which testosterone (T), which is either aromatized 17β-estradiol (E2) or converted into 11-ketotestosterone (11-KT) (Figure 3; Nagahama & Yamashita, 2008; Kazeto et al., 2011). In females, E2 controls the development of the ovaries among others by inducing the vitellogenin production by the liver (Nagahama & Yamashita, 2008, Kazeto et al., 2011). In males, E2 regulates the renewal of spermatogonial stem cells (Miura et al., 2003). 11-KT plays a critical role in the induction of spermatogenesis in males (Miura et al., 1991, 2003) and was shown to influence pre-vitellogenic and early vitellogenic oocyte development in females, which was for the first time observed in female *A. australis* and *A. dieffenbachii* (Lokman et al., 1998). In general, LH is involved in the stimulation of final maturation by production of 17α-20β-hydroxy-4-pregnen-3-one (DHP). DHP induces germinal vesicle breakdown in the oocyte and ovulation (Nagahama, 1994; Nagahama & Yamashita, 2008). It is suggested that DHP is also involved in the regulation of sperm maturation (Miura et al., 2003).

Puberty in eels

As mentioned above, during silvering, eels adapt to their new conditions of life in oceanic waters as morphological and physiological characteristics change (Todd, 1981; Lokman et al., 1998; Durif et al., 2005; van Ginneken et al., 2007b, 2007c). At the onset of migration there are large differences between eel species concerning their maturation status as based on gonadosomatic index (GSI). In several species, including the European eel, pre-vitellogenic oocyte growth has only just started at the onset of their migration (e.g. Dufour et al., 2003; Versonnen et al., 2004), while other species are in more advanced stages in vitellogenesis (Todd, 1981; Lokman et al., 1998; Hagihara et al., 2012). This is probably related to the migration distance to their spawning grounds, with the European eel migrating presumably the longest distance (Todd, 1981, Dufour et al., 2003). During puberty , from yellow to silver eel, significant increases in sex

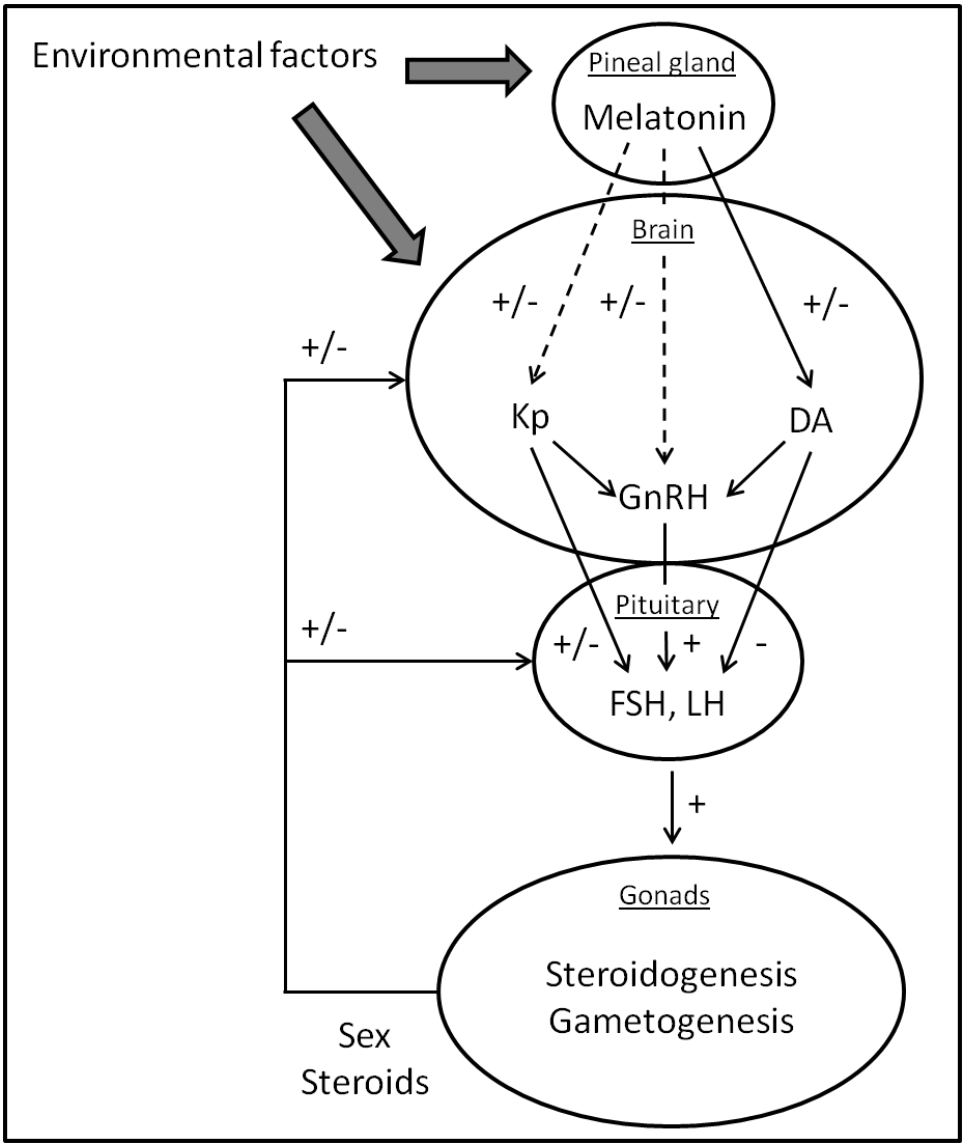

Figure 2. Schematic representation of endocrinological control of reproduction by the brain-pituitary-gonad axis in teleosts. In general, the reproductive axis is induced by internal or external factors (e.g. photothermal period, age, adiposity and social factors). Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus resulting in the synthesis and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH activate gonadal activity, i.e. steroidogenesis and gametogenesis. Kisspeptin (Kp), a gatekeeper of puberty, regulates GnRH and

Figure 2 continued. gonadotropins. There are indications that in eels LH expression is suppressed by Kp. In several teleosts, especially in eels, the synthesis and release of LH is under inhibitory control of dopamine (DA). Positive and negative feedbacks are exerted by sex steroids (e.g. 17ß-estradiol and testosterone) at different levels of the BPG axis. Melatonin mediates the effects of environmental factors on the central nervous system by modulating the activity of DA neurones. In addition, melatonin may interact with the Kp system as in mammals, but this still needs to be investigated. (Based on Dufour et al. 2010 and Migaud et al. 2010).

steroids profiles – E2, T and 11-KT – coinciding with ovarian development are observed in several *Anguilla* spp (Lokman et al., 1998; Han et al., 2003; Aroua et al., 2005; van Ginneken et al., 2007c; Sudo et al., 2011b, Setiawan et al., 2012). Additionally, during silvering, pituitary expression levels of FSHβ and LHβ are increased up to 15-fold (Aroua et al., 2005; Sudo et al., 2011b, Setiawan et al., 2012). In hormone treated eels, on the other hand, LHβ expression levels are even ca. 1000-fold increased (Vidal et al., 2004).

Dopaminergic inhibition in eels

At the onset of their migration silver eels are still in a prepubertal stage (e.g. Dufour et al., 2003; Versonnen et al., 2004). Therefore, vitellogenesis and final maturation must occur during their migration or at the spawning area (Dufour et al., 2003). When silver eels are captured before initiating their spawning migration, further maturation stops. Suppression of maturation is possibly due to insufficient stimulation of GnRH secretion and inhibition of synthesis and release of LH by dopamine at the pituitary level (Dufour et al., 1993; 2003; 2005, 2010; Vidal et al., 2004; Weltzien et al., 2006; 2009). In eels, this dopaminergic blockade is rather extreme as compared to other fish species, which may be related to their special life-cycle (Dufour et al., 2003, 2005; Vidal et al., 2004). Treatment with GnRH-analogue (GnRHa), T or a DA-receptor antagonist alone or in combination does not result in the release of the dopaminergic inhibition in female eels. Only a combined treatment using those three factors resulted in increased LH synthesis and release, increase of plasma vitellogenin levels, and ovarian vitellogenesis (Vidal et al., 2004).

Regulation of FSH and LH by sex steroid feedbacks

Current knowledge on the reproductive endocrinology of eels is mainly based on artificial maturation using injections with pituitary extracts, purified hormones and sex steroids. In Japanese and European eel, an opposite regulation of FSHβ and LHβ expression was found during induced ovarian maturation (Suetake et al., 2002; 2003; Dufour et al., 2003; Schmitz et al., 2005). During silver eel pre-vitellogenesis, the FSH expression level is relatively high but decreases during vitellogenesis and final oocyte maturation. LH expression level starts low and increases during stages of vitellogenesis and oocyte maturation (Suetake et al., 2002, 2003; Schmitz et al., 2005). The sex steroids E2 and T were found to exert a differential feedback on the expression of FSHβ and LHβ; i.e. a positive feedback on LH by E2, and a negative feedback on FSH by T (Schmitz et al., 2005). Treatments with sex steroids *in vitro* (primary cultures of eel pituitary cells) and *in vivo* (female eels) showed contrasting results (Aroua et al., 2007). *In vivo*, E2 treatment stimulated LHβ expression and resulted in a slight decrease of FSHβ mRNA expression levels. T treatment showed no effect on LHß and caused a slight decrease of FSHβ expression. *In vitro*, FSHβ expression was found increased after treatment with E2, whereas LHβ expression was not affected. T treatment resulted in an increased LHβ level, however, it did not affect the FSHβ expression level. The differences found were explained by the involvement of cerebral control (Aroua et al., 2007). The results from those previous studies clearly indicate that FSH and LH are under control of sex steroids by positive and negative feedback mechanisms.

In addition, in female eels, 11-KT has an important controlling function on pre-vitellogenic oocyte development and on silvering-related changes as shown in various studies (Lokman et al., 1998; 2007; Rohr et al., 2001; Matsubara et al., 2003; Sudo et al., 2011a, 2011b, Setiawan et al., 2012). It was shown that treatments with an 11-KT implant resulted in a reduction of FSHβ pituitary expression, and an increase of FSHβ-receptor expression in the gonads and E2 plasma levels. It was stated that 11-KT may be responsible for sensitizing the pre-vitellogenic follicle to FSH (Setiawan et al., 2012). Interestingly, pituitary FSH was decreased in eels treated with 11-KT, which is in contrast with natural conditions (Setiawan et al., 2012). However, the reduction of FSH in the pituitary may have resulted from secretion into the blood plasma, as gonadotropic cells produce and

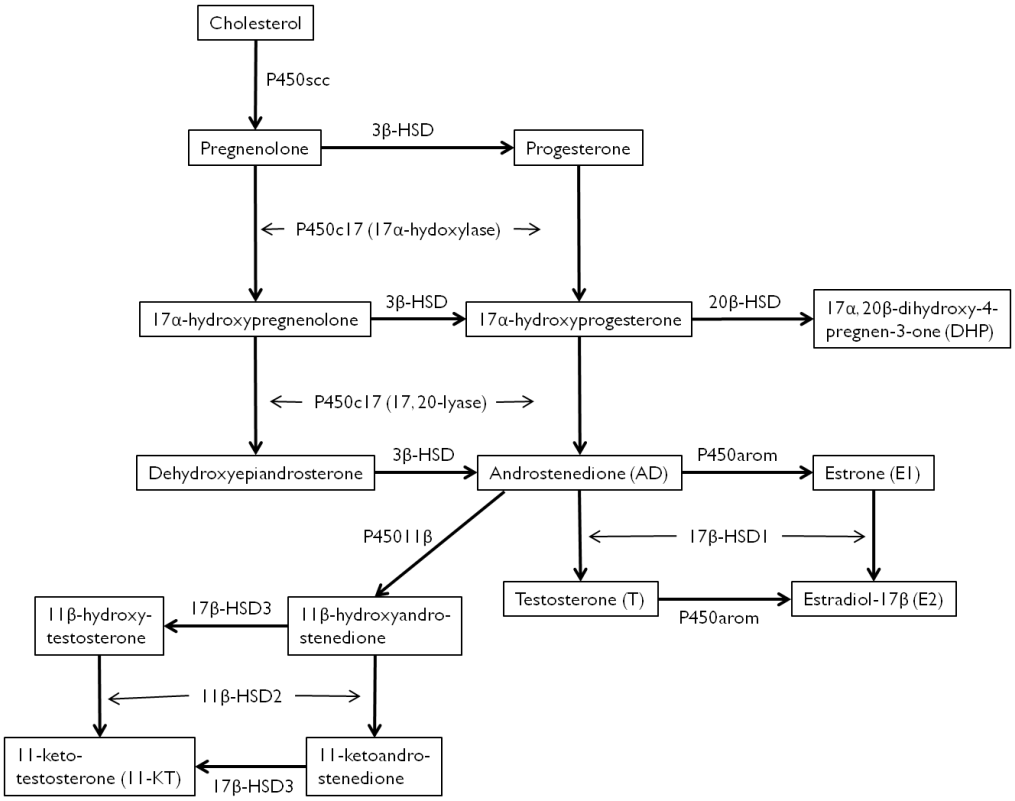

Figure 3. Steroidogenesis. Major steroidogenic pathway depicting the production of testosterone (T), 17β-estradiol (E2) and 11-ketotestosterone (11-KT). Adapted from Nagahama & Yamashita (2008) and Kazeto et al. (2011).

store gonadotropins that are secreted into the system according to requirements (e.g. Farnworth, 1995).

KiSS/GPR54 system

Recently, the involvement of the KiSS/GPR54 system on the reproductive cycle was shown, acting upstream of GnRH and subsequently on gonadotropins. Kisspeptin is considered a gatekeeper for puberty in mammals as well as in fish (e.g. Elizur, 2009; Tena-Sempere et al., 2012, Zohar et al., 2010). The effect of kisspeptin on expression of gonadotropins in the pituitary was studied *in vitro* on primary cultures of pituitary cells of female eels (Pasquier et al., 2011). It was shown that kisspeptin inhibited LHß expression; other studied pituitary hormone

subunits – FSHβ, GPHα, TSHβ – were not affected. However, *in vivo* kisspeptin may have a different effect due to cerebral control. Interestingly, during artificial maturation, expression of kisspeptin receptors (*Kissr-1 and Kissr-2*) in the pituitary decreases. As pituitary LHβ level during maturation increases it is suggested that the inhibitory control of kisspeptin on LHβ expression found *in vitro* could be removed by down-regulation of the receptors (Pasquier et al., 2012).

Melatonin – a hormone produced during the night by the pineal organ, – is believed to stimulate the expression of KiSS (Migaud et al., 2010). Interestingly, female eels treated with melatonin showed a clear decrease in pituitary FSHβ and LHβ expression levels, and blood plasma levels of 11-KT. These results suggest that melatonin inhibits maturation (Sébert et al., 2008). Currently, it is suggested that melatonin mediates the effects of environmental factors on the central nervous system (Dufour et al., 2010).

Methods for artificial reproduction

Weekly hormone injections

Artificial reproduction of freshwater eels has been studied for approximately 80 years, starting in the 1930s with the French scientist Fontaine who used injections with urine from pregnant women, containing human chorionic gonadotropin (hCG), to induce maturation and spermiation in male European eels (Fontaine, 1936). Twenty-eight years later in 1964, Fontaine and colleagues succeeded to induce maturation and ovulation of female European eels using injections of carp pituitary extract (CPE) (Fontaine et al., 1964). Eel larvae were first obtained for the Japanese eel in the 1970s (Yamamoto & Yamauchi, 1974). The reproduction protocols were optimized after the introduction of DHP that induces ovulation and increases the success of fertilization (Ohta et al, 1996). The main components to induce maturation are carp or salmon pituitary extracts (PE) for females and hCG for males. In the past decades, it has been shown that with the same protocol also embryos and larvae can be obtained from other *Anguilla* species; *A. anguilla* (Boëtius & Boëtius, 1980; Bezdenezhnykh et al., 1983; Pedersen, 2003; 2004; Palstra et al., 2005), New Zealand short-finned eel, *A. australis,* and New Zealand long-finned eel, *A. dieffenbachii* (Lokman & Young, 2000), and American

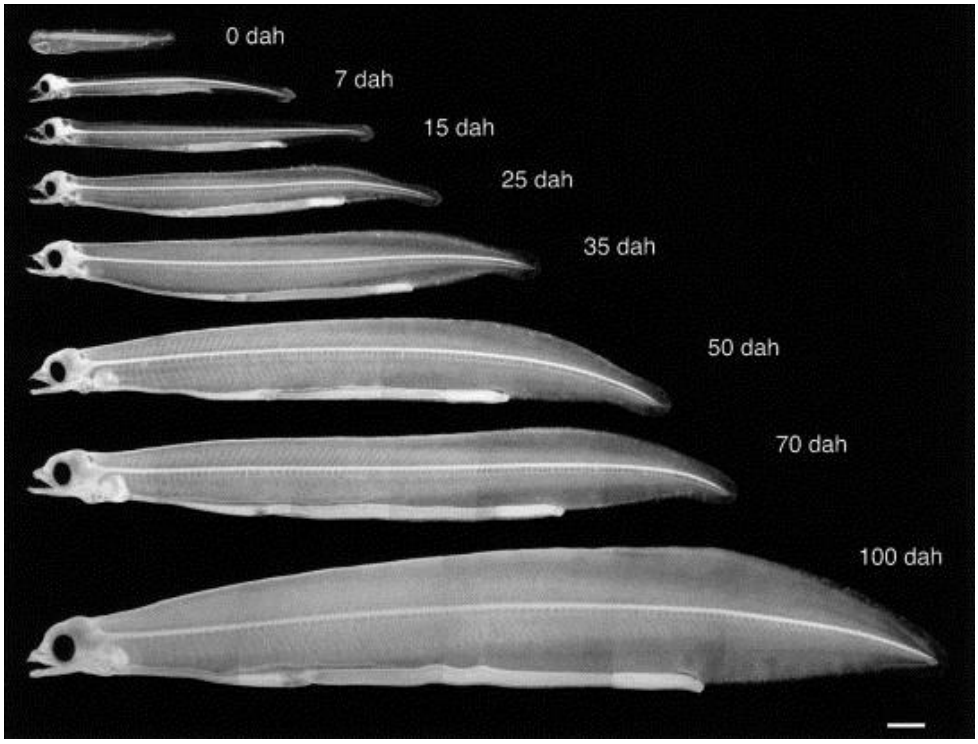

Figure 4. Transition from preleptocephalus to leptocephalus of captive-bred Japanese eel (*Anguilla japonica*). Age in days after hatching (dah). Scale bar 1 mm (from Tanaka et al., 2001).

eel, *A. rostrata* (Oliveira & Hable, 2010). However, major problems occur during larval rearing.

Tanaka and co-workers succeeded to feed Japanese eel larvae using an artificial mixture based on shark egg powder, krill extracts, minerals and vitamins (Tanaka et al., 2001). Over the last decade considerable progress has been made using this artificial feed, e.g. obtaining feeding leptocephalus larvae (Tanaka et al., 2001, Figure 4), glass eel (Tanaka et al., 2003: Kagawa et al., 2005) and recently a F2 generation (Ijiri et al., 2011). Still, success rates are low and far from a sustainable aquaculture. Presumably, major problems such as low egg quality and fertilization rates, are caused by the unnatural stimulation of maturation; the weekly injections result in high transient hormone levels (Sato et al., 2000; 2003)

and handling stress. It is shown that stress negatively affects success rates of reproduction in fish (e.g. Schreck, 2010).

Slow release systems

It is expected that under natural circumstances plasma hormone levels change gradually, which is in contrast to the high transient peaks resulting from weekly hormonal treatments. Recently, it was shown that maturation of male and female Japanese eels can be induced using osmotic pumps (Figure 5) that slowly release hCG or pituitary extract into the circulatory system (Kagawa et al., 2009; 2012, 2013). However, implantation of these pumps requires surgery, and the pumps last for a maximum of 6 weeks. In the case of female European eels this method will require multiple surgeries as the hormone treatments could last 4-6 months. European eels show a much slower and more variable response to the hormonal treatments (Pedersen, 2003; 2004; Palstra et al., 2005) as compared to other species, such as the Japanese and New Zealand short-finned eel (Ohta et al., 1996; 1997; Lokman & Young, 2000). In order to increase success rates, new methods based on slow release systems inducing maturation in female European eels need to be developed.

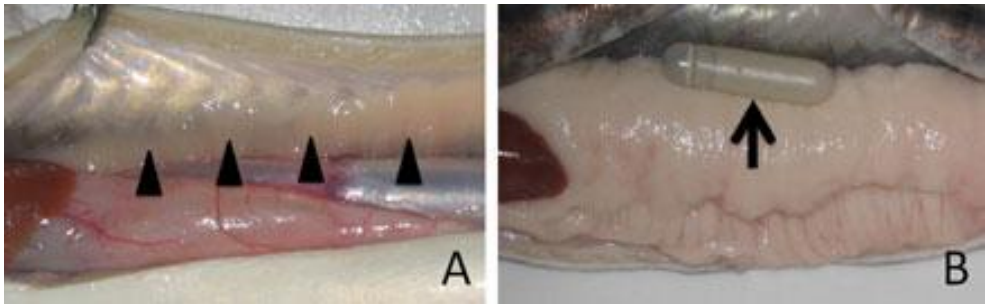

Figure 5. Representative photographs of the ovary of a female Japanese eel before (a) and after (b) implantation of a single osmotic pump loaded with salmon pituitary extract (3 mg day^{-1} $fish^{-1}$). The arrowheads indicate the immature ovary, and the arrow indicates the osmotic pump. Developed ovary possessing full- grown oocytes occupies the abdomen (b)(from Kagawa et al., 2013).

Natural triggers inducing maturation

Major barriers in several phases of the reproductive cycle such as the induction of early maturation (e.g. gonadal growth, vitellogenesis) could possibly be overcome using natural triggers (e.g. photoperiod, temperature, exercise). Currently, reproduction of many fish species can be controlled by manipulation of natural conditions, especially using changes in photoperiod and temperature (Taranger et al., 2010; Wang et al., 2010). Surprisingly, candidate natural triggers to induce maturation and ovulation in eels still are insufficiently studied and information is scarce, which is mainly due to the fact that the oceanic life phase is still largely unknown. However, from several studies using archival tags and pop-up tags, it is pointed out that during their spawning migration eels show clear diel vertical migrations (DVMs) between ca. 200-600 m (Aarestrup et al., 2009; Jellyman & Tsukamoto, 2002, 2005, 2010; Manabe et al., 2011; Tesch, 2003). During the day migrating silver eels descent into deeper waters, and during the night they migrate towards the water surface. Due to these DVMs eels will encounter daily fluctuations in temperature, pressure and, presumably, light intensity. It is hypothesized that DVM results from predator avoidance, maintenance of metabolism or delaying gonad development (Aarestrup et al., 2009).

Photoperiod and temperature

Annual cycles of reproduction are often linked to changes in photoperiod and temperature regimes. For many fish species (e.g. rainbow trout, salmon, pike perch, turbot) the effects of photothermal period on the induction of vitellogenesis and maturation have been extensively studied and used to manipulate the reproductive cycle (reviewed by Taranger et al., 2009; Wang et al., 2010). Based on a telemetry study European silver eels encounter water temperatures between 8 and 13°C, with a daily average of 10.1°C at least during the first part of their spawning migration (Aarestrup et al., 2009). It is hypothesized that these relatively low temperatures delay gonadal development. However, in several fish species, e.g. pike perch, a so-called cold period is necessary to induce vitellogenesis (Hermelink et al., 2011; Wang et al., 2010).

Sato et al. (2006) and Perez et al. (2011) found that changes in temperature affected the induction of vitellogenesis in Japanese and European eels. It needs to be noted however, that those studies were conducted in combination

with weekly pituitary extract injections, which stimulate the maturation process. Sato et al. (2006) concluded that: "… water temperature is an important factor for the artificial induction of ovarian maturation, and an effective temperature for the induction of ovarian development is 20°C". As compared to 20°C, ovarian development was slow at 10°C, and final maturation and ovulation could not be induced within 13 weeks. However, in the study of Perez et al. (2011) a temperature regime from 10 to 20°C accelerated development until week 8 as shown by higher levels of FSHβ, LHβ and estrogen receptor 1 (esr1) expression, and of plasma E2. These results are probably due to the effect of the weekly pituitary extract injections.

Recently, the effect of temperature decrease on maturation of cultured female Japanese eels was studied (Sudo et al., 2011a). It was shown that oocyte diameter increased and that oocytes showed an accumulation of oil droplets when the temperature was decreased over a 50 day period from 25 to 15°C. Although FSHβ and LHβ pituitary expression decreased, 11-KT blood levels increased. 11-KT is found to induce previtellogenic oocyte growth and is proposed as one of the important factors that stimulate gonadal development (Lokman et al., 1998, 2007; Rohr et al., 2001). Further maturation starting with full vitellogenesis was not observed, indicating the requirement of other environmental cues participating in the process (Sudo et al., 2011a). Interestingly, Sébert et al. (2008) showed that melatonin inhibits maturation by stimulation of the dopaminergic system in female European eel. In addition, low temperature could down-regulate secretion of melatonin in eel (Sébert et al., 2008). Moreover, it was shown that blue wavelengths decrease melatonin plasma levels in sea bass (Bayarri et al., 2002) and zebrafish (Ziv et al., 2007). During their spawning migration female eels show DVMs, thereby encountering changes in temperature and possibly light intensities or photoperiod. It is likely that temperatures and light intensity levels or photoperiod are controlled by DVMs, which could have an effect on melatonin secretion, and consequently maturation.

Hydrostatic pressure

With the DVMs over approximately 200-600 m eels encounter daily fluctuations in hydrostatic pressures (HP) during their migration. There are only two studies focused on the effect of HP on maturation (Fontaine et al., 1985; Sébert et al.,

2007). In the first study, encaged female European eels were sunken at a depth of 450 m in the Mediterranean Sea for 3 months, resulting in an increased GSI and pituitary LH content (Fontaine et al., 1985). In the second study, females and males were subjected to 101 ATA – an equivalent to 1000 m depth – for respectively 3 and 7 weeks. Females of the HP group showed significantly larger oocyte diameter, and higher E2, 11-KT and vitellogenin plasma levels as compared to the control group. In addition, the LHβ/FSHβ ratio of females of the HP group was significantly higher; FSHβ expression was lower than the control group but not significant. Males subjected to HP showed on average a higher plasma 11-KT level, and a higher LHβ and lower FSHβ expression levels; however those changes were not statistically significant. Based on these results, it was concluded that HP plays a positive role in the sexual maturation of eels, but other factors are needed for completing sexual maturation (Sébert et al., 2007).

Swimming exercise

Swimming exercise was proposed as an important natural trigger to induce maturation, and over the last decade the effect of swimming on maturation was studied for female as well as male eels (reviewed by Palstra et al., 2009; Palstra & van den Thillart, 2010). In short, a significantly higher GSI, eye index (EI), oocyte stage and number of fat droplets deposited in the oocyte were found for wild females subjected to swimming exercise in fresh water for 2-6 weeks (Palstra et al., 2007b). A significantly larger oocyte diameter was observed after a 5500 km swim trial, however no significant changes in GSI or other maturation parameters were observed in farmed females after long term swimming (van Ginneken et al., 2007a). Based on those studies it was concluded that swimming exercise might stimulate initial oocyte development. However in the study of Palstra et al. (2007b), the compared resting and swimming females were initially in different maturation stages as based on the silver index described by Durif et al. (2005). Several females within the swim group were already assigned to a higher silver index including pre-migrant and migrant stages, while resters were all yellow stage. As the GSI is positively correlated with silver index it can be expected that females in the swim group had a higher GSI and oocyte stage at the start of the trial. In addition, the increase in EI found within the females of the swim group

may have been an effect of time. Therefore, those results are inconclusive concerning the effect of swimming on maturation.

It was shown by Palstra et al. (2008b) that in female eels swimming in seawater for 3 months (corresponding to ca. 1400km) resulted in a regression of maturation as a decrease of LHβ expression in the pituitary, GSI and oocyte diameter were found. In addition, swimming in seawater was found to suppress vitellogenesis as the mRNA expression of *estrogen receptor 1*, *vitellogenin1* and *vitellogenin2* decreased over time (Palstra et al., 2010a). This inhibitory effect of swimming exercise on maturation in fish is supported by recent results of rainbow trout subjected to swimming exercise (Palstra et al., 2010b).

The stimulatory effect of swimming exercise on maturation found in wild male eels is intriguing (Palstra et al., 2008b). After 3 months of swimming covering ca. 900 km (corresponding to ca. 0.12 m s^{-1}), male eels had increased LHβ expression in the pituitary and an increased GSI. Additionally, injection with GnRHa resulted in a similar response as swimming exercise. Hence, the authors stated that endurance swimming may result in natural maturation and spermiation when eels are subjected to longer swim trials as during this trial only a sixth of the total distance to the spawning area was covered.

Thesis outline

Over the last decades, the population strength of several species of freshwater eels is under pressure as shown by dramatic decreases in glass eel recruitments of up to 99%. Still to date, eel farms rely on the recruitment of wild caught glass eels for on-growing. Recently, the European eel was added to the IUCN list of threatened species, resulting in an urgent call for eel management and reproduction. However, natural reproduction of these extraordinary species occurs in the deep pelagic layers of the oceanic waters, and can be still considered as a black box. Currently, artificial reproduction of several eel species is feasible, albeit with low success rates.

This thesis focuses on oceanic migration, maturation and reproduction of eels. New tools are developed that may improve artificial maturation and reproduction protocols for eel aquaculture.

Chapter 1 provides a general overview concerning the impressive migration, physiology of maturation and methods for the artificial reproduction of eels.

Many fish species migrate in groups, which provide various advantages, such as defense against predation, enhancing foraging success and reduction of the costs of transport. Until now, the effects of group-wise swimming were mainly studied on non-anguilliform fish. The migration of anguilliform swimming eels is of high interest. Studies conducted in the past mainly focused on female eels. It was shown that female eels (*A. anguilla*) swim much more efficient than non-anguilliform swimmers. As swimming in groups decreases the costs of transport even further, we hypothesized that swimming in groups might also be advantageous for male eels. In **Chapter 2**, the swimming efficiency of farmed male silver eels and the effect of swimming in groups were studied using respirometry. It was expected that oxygen consumption and costs of transport would be reduced when swimming group-wise. In addition, the group-wise swimming pattern of anguilliform swimmers was also studied.

Eels swim long distances and before leaving the continental waters, they are in a prepubertal state resulting from both a deficient release of GnRH and inhibition by dopamine (Dufour et al., 1983, Vidal et al., 2004, Aroua et al., 2005). As the onset of migration and maturation are closely intertwined in eels, it was hypothesized that swimming exercise may release this inhibition by dopamine. Female eels appear to be stimulated in their maturation by swimming (van Ginneken et al., 2007; Palstra et al., 2010). However, vitellogenesis was found to be suppressed by swimming. Males on the other hand, show an increase of the gonad mass and LH expression levels after swimming a relative short distance of ca. 900km. Hence, it was concluded that males are not under influence of the dopaminergic inhibition as the females are and it was hypothesized that males may be triggered to fully mature by swimming alone (Palstra et al., 2008). In **chapter 3** farmed males were subjected to endurance swimming for 6 months to test the hypothesis whether males become fully mature by exercise alone. In addition, it was tested whether males were able to swim continuously for 6 months at approximately 1 body length per second (BL s^{-1})

Success rates of artificial reproduction of European eels are still low, as approximately 50% of the female eels do not respond (i.e. produce viable eggs) to

the hormonal treatments. Compared to other freshwater eel species, female European eels show a much slower and more variable response, which is presumably due to their initial state of maturation prior to the treatment. Therefore, selection of broodstock (i.e. distinguish non-responders from responders) before or during the early stages of the treatment could improve success rates of artificial reproduction. Eel genomics will provide valuable information for all research areas, including reproductive physiology. However, current knowledge on eel genomics is sparse. In **chapter 4**, we provided the first draft genome sequence of the European eel. This genome was used as reference for various transcriptomic analyses. Recent RNAseq analysis of gonad tissue at different maturation stages showed that particularly enzymes involved in the steroidogenic pathway are differentially expressed. **Chapter 5** describes possible methods to select female broodstock based on molecular marker genes. Farmed female eels were subjected to a weekly hormonal treatment and sampled at consecutive time points. After 4 weekly injections responders and non-responders were identified on basis of their relative GSI. Subsequently, biomarkers were obtained using a custom-made micro-array, which was based on the European eel genome sequence. In addition, blood plasma steroid hormone levels were analyzed using ELISA. It was expected that genes involved in the steroidogenic pathway and plasma sex steroids would reflect the maturation status of eels providing selection possibilities for broodstock.

The European eel is, together with the Japanese eel (*A. japonica*), one of the economically most interesting species. Due to the decline of the population there is an urgent call for artificial reproduction, as the eel farms are still totally dependent on wild caught glass eels. Artificial reproduction should relieve the pressure of overexploitation of the wild population. Artificial reproduction of the European eel is rather complicated as vitellogenesis is not yet induced at the start of the oceanic migration, and less advanced in maturity stage as compared to other *Anguilla* species, such as the Japanese eel and New Zealand short-finned eel (*A. australis*). The European eel shows a long maturation trajectory of up to six months. Shortening the artificial trajectory may overcome vitellogenic abnormalities resulting in higher gamete quality, and higher success rates of fertilization, hatching and larval development. Therefore, hybridization of the European eel with a species that has a shorter maturation period, such as *A.*

australis, may be suitable for aquaculture purposes. In **chapter 6** we reproduced *A. australis* and studied its early ontogeny. Female *A. australis* was hybridized with male *A. anguilla* species.

At the moment, maturation and reproduction of eels can be stimulated by weekly injections with pituitary extracts or human chorionic gonadotropin. These continuous injections influence adult eels and also the quality of the eggs due to transient hormone peak levels in the eel. At the moment, this method is the only one applicable for the eels, and therefore new methods need to be developed. In **chapter 7** a new method is developed for the stimulation of maturation of eels, using a single injection of hormone producing cell implants. The implants work as a slow release mechanism providing a more natural flow of hormone levels in the circulatory of the eel.

Chapter 8 provides a summary of the presented work in this thesis.