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Tools and Triggers for Eel Reproduction

Erik Burgerhout

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Dissertation Leiden University

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Tools and Triggers for Eel Reproduction

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Weinig vissen zijn er, wier leven zo sterk tot de verbeelding van de mens spreekt, als dat van de aal of paling. Lang lag over zijn bestaan een duistere, verhullende sluier en pas in de loop der eeuwen is daar telkens een tipje van opgelicht. Er zijn tijden geweest, dat men de paling met zijn slangachtig voorkomen niet voor een vis wenste te verslijten en dat is geenszins onbegrijpelijk: bij rechtschapen vrouwelijke vissen toch moet te eniger tijd kuit kunnen worden vastgesteld en geen visser was er, wien dit bij de paling ooit was gelukt.

Trouwens: van voortplantingsorganen was ook geen spoor te ontdekken. De palingen, zo ging men concluderen, moeten dan wel uit het niet, zó maar geboren worden. Het denkbeeld van de generatio spontanea vond dan ook bij menigeen ingang. Maar de geleerden konden met deze al te gemakkelijke oplossing geen genoegen nemen; her en der in de wereld zonnen zij op middelen om achter het geheim te komen en hun energiek voortgezette pogingen hebben er inderdaad toe geleid, dat men het palingprobleem grotendeels onder de knie heeft gekregen.

From: Het wonderlijke leven van de aal, in Kijk uit je ogen! IV Vissen by Dr. W.M. Kruseman and Rinke Tolman, Born's Uitgeverij NV, Assen, 1946

Ever since those early days when mankind first began to sit up and take notice, the eel has been an object of great curiosity. No one had ever found eggs, let alone an egg-bearing adult, and the idea was fostered that eels developed spontaneously from either the mud (according to Aristotle) or from the hair of horse tails (according to Pliny). That is how the matter stood for centuries, and people accepted those theories as facts. In the last century, when people became more "natural history" minded, further theories were propounded and disagreed with, in fact many were the acrimonious battles waged through the columns of the popular science journals of those days.

Gradually, however, the barrier of secrecy surrounding the eel was broken down and bit by bit, from facts gathered here and there, the full story has been revealed. To no one person or country does the honour belong of solving the problem, the scientists of the world pooled their knowledge to the common end; indeed, a list of the people who had a hand in the game reads like an all-Europe football team. To a German, Johan Schmidt, belongs the honour of assembling all the known facts and, with other facts gathered himself, he was able to outline the career of the Common Eel "from the cradle to the grave".

From: The Common Eel, in The Observer's Book of Freshwater Fishes by A. Laurence Wells, Frederick Warne & Co. LTD, London, 1958

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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.,

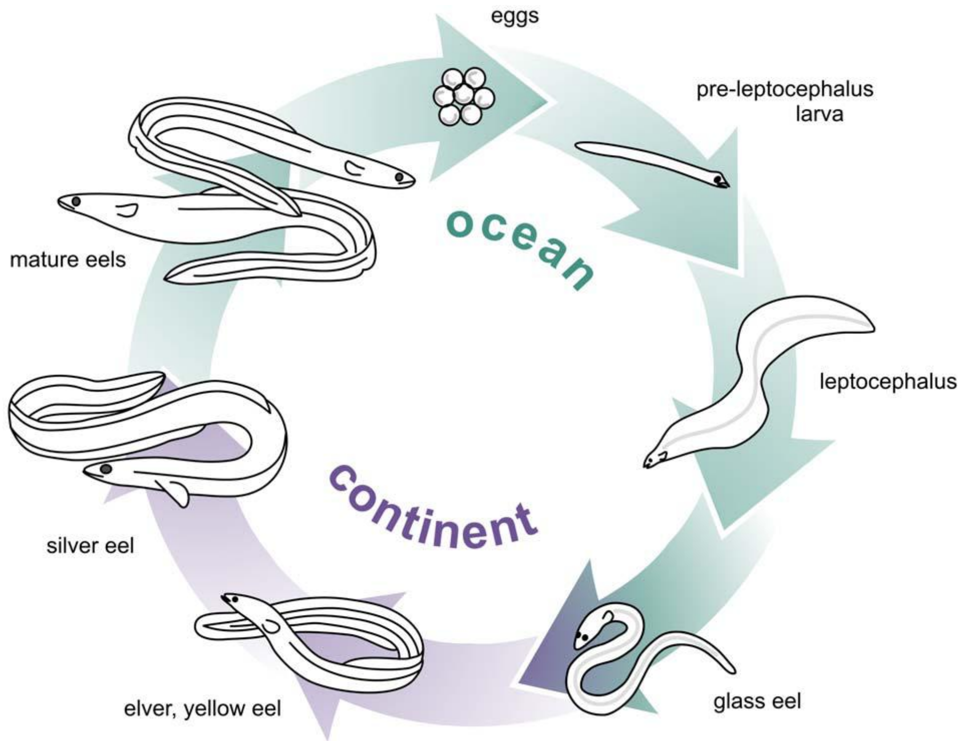

Figure 1. The life cycle of the European eel. After hatching, presumably in the Sargasso Sea, cylindrical larvae develop into leaf-shaped leptocephalus larvae, which after drifting on the Gulf Stream for approximately one year metamorphose into glass eels close to the European coast. The glass eels may 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

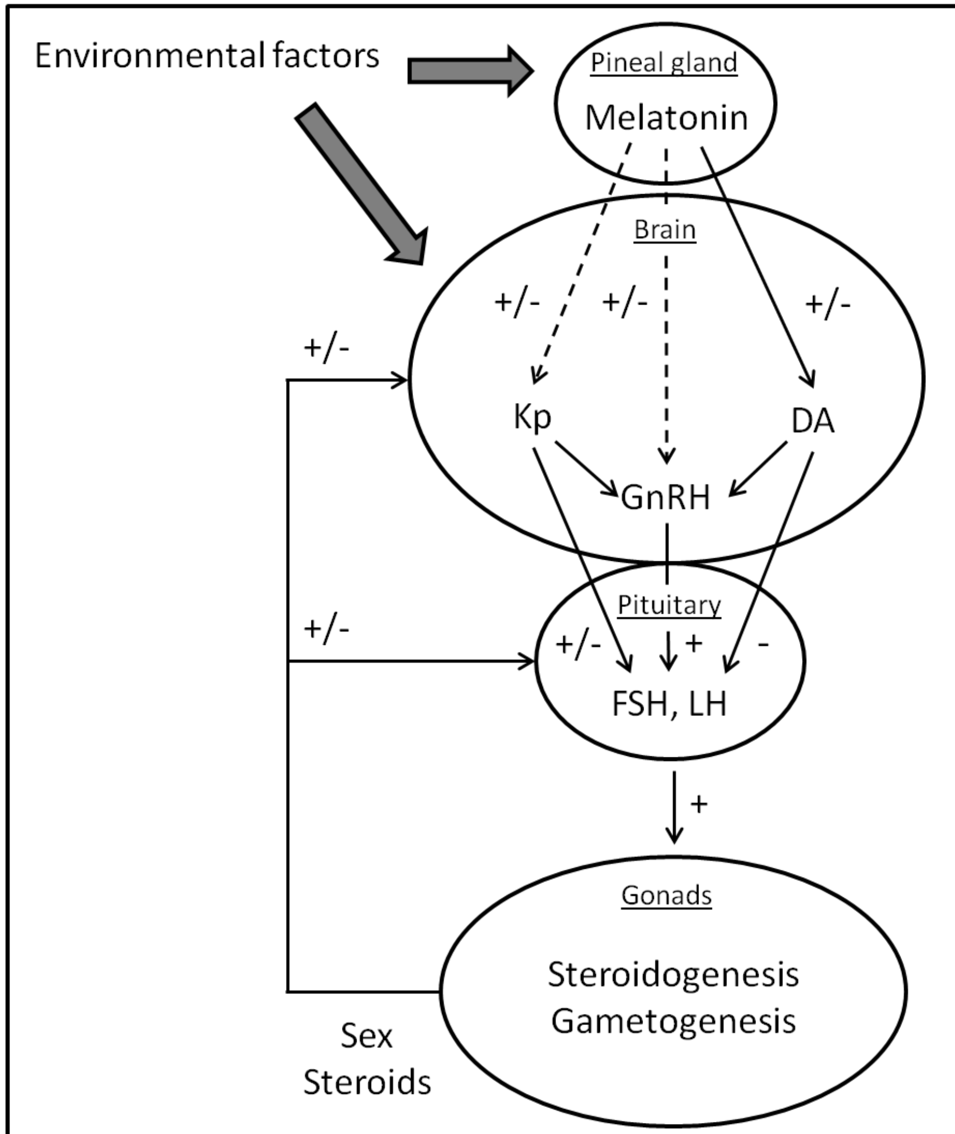

Figure 2. Schematic representation of endocrinological control of reproduction by the brain-pituitary-gonad axis in teleosts. In general, the reproductive axis is induced by internal or external factors (e.g. photothermal period, age, adiposity and social factors). Gonadotropin-releasing hormone (GnRH) 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

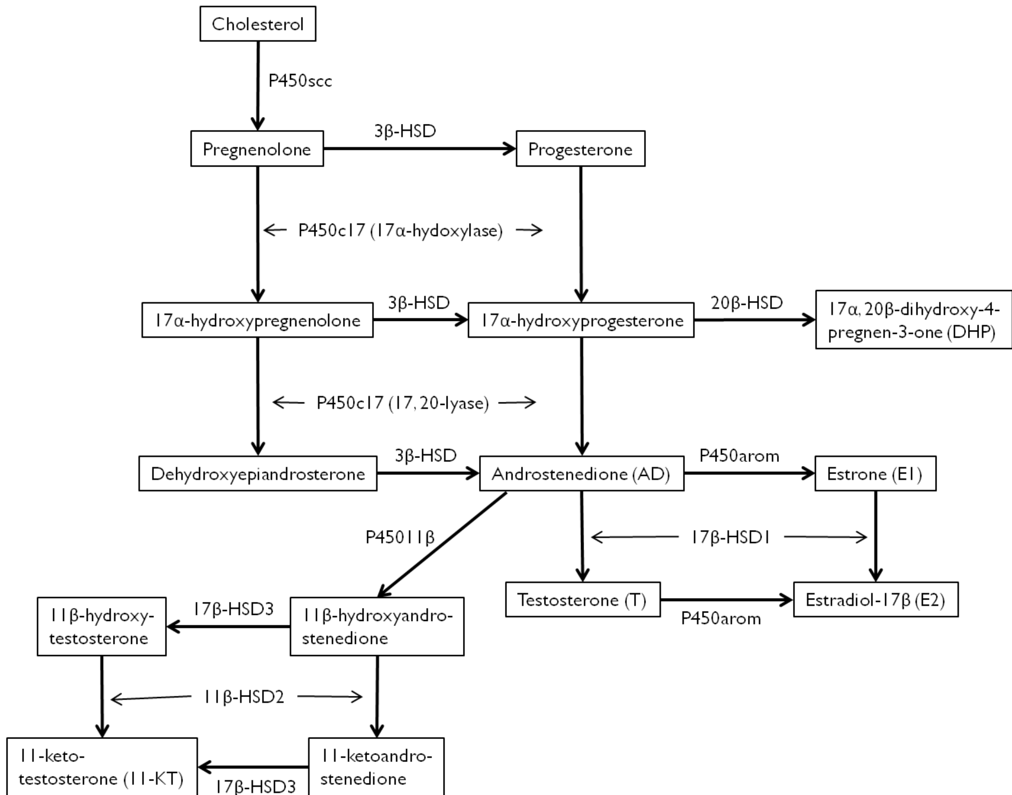

Figure 3. Steroidogenesis. Major steroidogenic pathway depicting the production of testosterone (T), 17β-estradiol (E2) and 11-ketotestosterone (11-KT). Adapted from Nagahama & Yamashita (2008) and Kazeto et al. (2011).

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

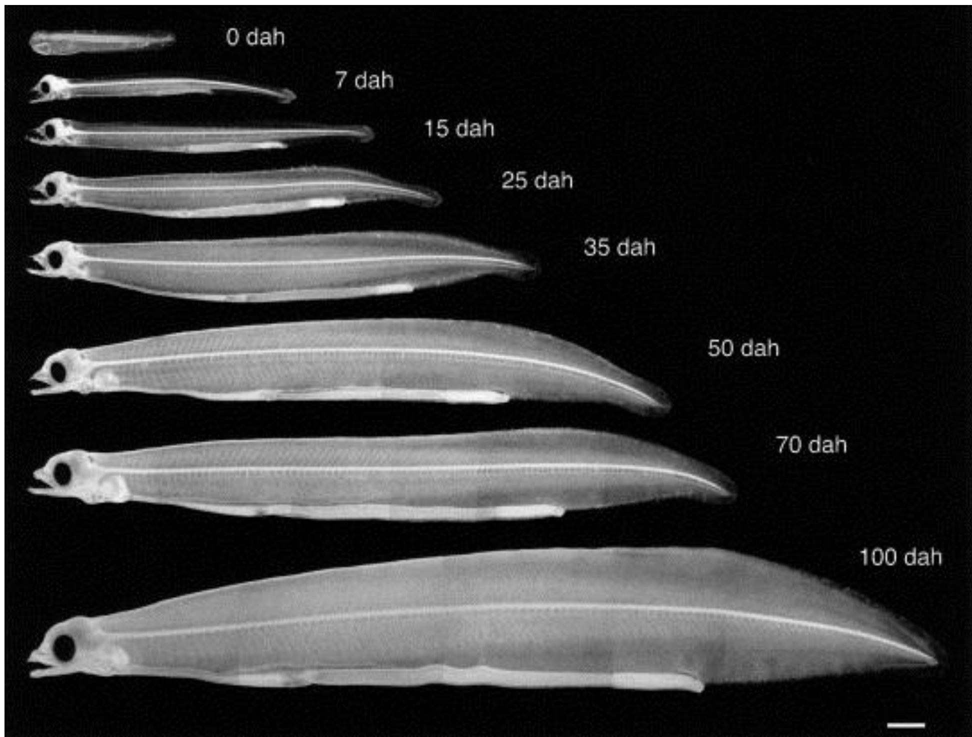

Figure 4. Transition from preleptocephalus to leptocephalus of captive-bred Japanese eel (*Anguilla japonica*). Age in days after hatching (dah). Scale bar 1 mm (from Tanaka et al., 2001).

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.

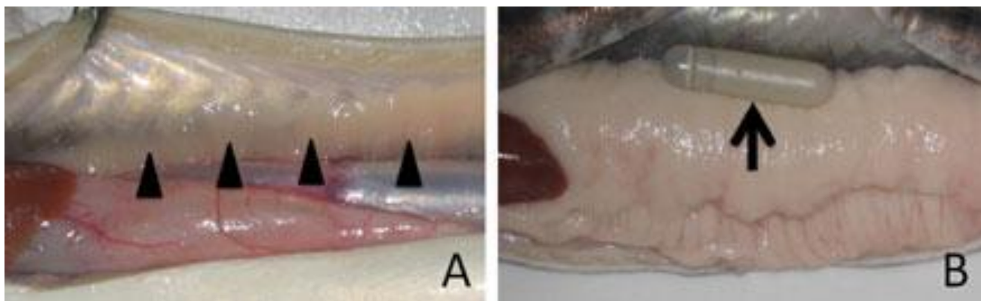

Figure 5. Representative photographs of the ovary of a female Japanese eel before (a) and after (b) implantation of a single osmotic pump loaded with salmon pituitary extract (3 mg day^{-1} $fish^{-1}$). The arrowheads indicate the immature ovary, and the arrow indicates the osmotic pump. Developed ovary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

Chapter 2

Schooling reduces energy consumption in swimming male European eels, *Anguilla anguilla* L.

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Abstract

Swimming in schools provides fish with a number of behavioural and ecological advantages, including increased food supply and reduced predation risk. Previous work shows that carangiform and tunniform group-wise swimming result in energetic advantages for individuals using a diamond swimming formation. However, little is known about the potential energetic advantage associated with schooling for anguilliform fish, such as European eel. European eels migrate a long distance to their spawning area in the Sargasso Sea and may experience energetic advantages when swimming in schools. In this study the effect of group-wise swimming on the cost of transport was tested. In addition, the swimming pattern of eels swimming in groups was studied.

Male silver eels were individually subjected to an increased velocity test (0.4-0.9 m s^{-1}) with increments of 0.1 m s^{-1} every 2 hours. Groups comprising of seven males were swum following the same protocol. Video recordings were obtained to analyze tail beat frequency at the various water speeds.

At all swimming speeds the oxygen consumption was significantly lower in group-wise vs. individually swimming males. The cost of transport at the optimal swimming speed of group-wise swimming males was significantly lower than that of the individually swimming males (21.3 ± 3.2 vs. 32.0 ± 0.6 mg O_2 kg^{-1} h^{-1}, respectively). The optimal swimming speeds however, were not significantly different (0.57 ± 0.02 and 0.52 ± 0.04 m s^{-1} respectively). At speeds of 0.50 m s^{-1} and above, tail beat frequency was lower in males swimming in groups than in males swimming individually (2.6 ± 0.1 and 3.8 ± 0.1, respectively). As compared to the 'diamond' shape pattern of many group-wise swimming fish, eels tend to swim in a synchronized fashion parallel to each other. It is concluded that male eels in groups swim energetically more efficient than males swimming individually by the synchronized parallel swimming mode.

Introduction

When migrating, many fish species swim in groups, named schools, defined as a social aggregation of fish, swimming in the same direction and maintaining near-constant spacing relative to neighbouring conspecifics (Pitcher & Parrish, 1993). Schooling is a common migration behaviour in fishes, with school sizes often varying between species, locations and seasons from a few individuals to several million swimming together (e.g. Coetzee 2000). The advantages of migrating in groups are thought to be predation avoidance (Breder, 1967; Seghers, 1981; Godin & Morgan, 1985; Magurran & Higham, 1988), increase of foraging success (Pitcher et al., 1982; Wolf, 1987; Ranta & Lindstrom, 1990), or reduction of migration costs (Parker, 1973; Weihs, 1973; Webb, 1975; Ross & Backman, 1992; Herskin & Steffensen, 1998; Fish, 1999; Svendsen et al., 2003; Liao, 2007; Johansen et al, 2010; Killen et al., 2011). Also, schooling behaviour was reported to be a response to many ecological and physiological factors such as temperature (Dommasnes et al., 1994) and oxygen availability (Domenici et al., 2002).

On basis of the degree of body use for undulation during swimming, Breder (1926) divided swimming fish in four different classes: tunniform, carangiform, ostaciiform and anguilliform. In the past, schooling studies concerned only carangiform, ostraciiform or tunniform swimming fish, such as saithe, herring, tuna, salmon or mackerel, (Partridge et al., 1980; 1983, Hoar, 1953; Castonguay & Gilbert, 1995; Hansen & Jonsson, 1985). When swimming in schools during migration these fish are known to be distributed in a rhomboid or 'diamond' pattern (Weihs, 1973) induced by the hydrodynamic advantages by swimming in the wake vortices preceding school members (Breder, 1965; Weihs, 1973). By the undulatory movements of a swimming fish thrust-type vortices are generated and shed into the wake (Rosen, 1959; Müller et al., 1997). These vortices are thought to affect the swimming efficiency of a following fish depending on its position within the school (Breder, 1965). Previous studies have suggested hydrodynamic interactions of schooling fish as a possible energy conserving mechanism (Breder, 1965; Belyayev & Zuyev, 1969; Zuyev & Belyayev, 1970; Weihs, 1973; Partridge et al., 1983; Abrahams & Colgan, 1985, 1987; Pitcher & Parrish, 1993), and it has repeatedly been shown that schooling fish swimming in a rhomboid distribution have energetic advantages over fish swimming alone (Parker, 1973; Weihs, 1973; Webb, 1975; Ross & Backman, 1992; Herskin & Steffensen, 1998; Fish, 1999;

Svendsen et al., 2003; Liao, 2007; Johansen et al, 2010; Killen et al., 2011). In contrast to other swim types, vortices produced by anguilliform swimmers, as eels, are directed sideways, suggesting a different form of thrust (Tytell & Lauder, 2004). Energetic advantages of schooling in anguilliform swimmers were thus far not studied.

The swimming capacity of migrating European eels (*Anguilla anguilla* L., 1758) is subject of increasing scientific interest (van Ginneken & van den Thillart, 2000; van den Thillart et al., 2004; van Ginneken et al., 2005; Palstra et al., 2008; Sébert et al., 2009; Burgerhout et al., 2011; Methling et al., 2011). The spawning area of the European eel is assumed to be situated in the Sargasso Sea (Schmidt, 1923), which implies an average migration distance of about 6000 km from the European coast. During their spawning migration, eels do not feed and rely therefore entirely on their body reserves for energy supply (Tesch, 2003). A low cost of transport has therefore a high advantage for ecological fitness of European eels. Indeed, a very low cost of transport was observed for swimming female eels, i.e. more than 5 times lower than that of rainbow trout (van Ginneken et al., 2005). Eels swim in an anguilliform mode, i.e. they use the whole body for propulsion, with at least one wave-length present in the body (Breder, 1926, Webb, 1971). Currently, there is no information or model that predicts whether sideway vortices generated by swimming eels can be used by others to reduce their cost of transport.

Although, current knowledge on group-wise swimming of eels during the oceanic phase is lacking, there are reports that during their exodus eels tend to aggregate in large groups (Tesch, 2003). Considering the tremendous effort associated with such a long distance migration, it would be advantageous for eels to migrate in groups when this reduces swimming cost of transport. The present study examined whether European eels swimming in groups show a reduction of energy consumption compared to eels swimming individually. In addition, the swimming behaviour within a group of anguilliform swimming eels was investigated.

Methods

Animals and housing

Farmed male silver eels (n = 89; 106.6 ± 2.1 g; 38.4 ± 0.2 cm) were obtained from a commercial eel farm (Nijvis-Holding B.V., Nijmegen, The Netherlands), where they were kept in fresh water at 24°C. Prior to the swimming trials, the eels were acclimated for four weeks and housed in a ca. 8000L recirculation system, supplied with natural seawater (30 ± 1 ppt) at 18 ±1°C. The fish were kept in the dark before and during the trials, except during filming. As the eels cease feeding when silvering, they were not fed during the whole period of time.

Swimming capacity

Seven 127 L Blazka-type swimming tunnels as described by van den Thillart et al. (2004) were used. The tunnels were connected to a recirculation system with running natural seawater (28 ± 1 ppt; 18 ± 1°C) with a total volume of ca. 4000 L.

Five days prior to the trials the eels were introduced into the swimming tunnels to acclimate. The eels were anesthetized with clove oil (1:10 dissolved in 96% ethanol, 1 mL in 1 L water); body weight (BW, g) and body length (BL, cm) were measured before placing them into the tunnels. To determine the range of swimming velocities a preliminary test was conducted at water speeds from 0.1-0.9 m s^{-1}. The eels were still able to swim steadily at 0.9 m s^{-1}, therefore 0.9 m s^{-1} (~ 2.2 BL s^{-1}) was taken as the maximum velocity in the trials.

For the swimming trials with individuals, seven male eels (104.9 ± 6.8 g; 37.5 ± 0.6 cm) were subjected to a series of swimming speeds ranging from 0.4-0.9 m s^{-1} (0.4-0.6 m s^{-1} on the first day and 0.7-0.9 m s^{-1} on the second) with increments of 0.1 m s^{-1} at 120 minutes intervals in seven separate swimming tunnels. During the two-day trial the eels were kept in their separate tunnels. Oxygen consumption was measured during the first 90 minutes after each increase in flow speed, which was sufficient to reach a significant slope in the $[O_2]$ decline ($p<0.05$) with an $r^2 \geq 0.80$. Thereafter the tunnels were flushed for 30 minutes with well oxygenated water at 5-7 L min^{-1}. During flushing the speed was lowered to 0.05 m s^{-1}. Because of irregular swimming behaviour – i.e. changes in tail beat frequency and position within the swimming tunnel – at low swimming speeds (< 0.4 m s^{-1}), the standard metabolic rate (SMR) was determined by

extrapolating the oxygen consumption curve to zero swimming speed using the formula $\dot{M}O_2$ = SMR+aU^b; where $\dot{M}O_2$ is the weight specific oxygen consumption, U swimming speed, and a and b dimensionless constants.

For group-wise swimming trials, seven separate groups consisting of seven eels each (104.2 ± 8.0 g; 38.4 ± 0.9 cm), were placed in the seven swimming tunnels and acclimated for 5 days. The protocol was identical as the one for the swimming trials with individuals. The group size of seven individuals was estimated the largest number that could fit in the tunnels without disturbed swimming behaviour.

Swimming kinematics

A HD video camera (Panasonic, HDC-SD90, Panasonic Inc., Japan) was mounted 0.6 m above the swimming section. To compensate the spherical aberration caused by the cylindrical shape of the swimming tunnel, a Perspex adapter box with a flat surface and filled with water was placed on top of the tunnel. The eels were filmed for 10 minutes at each speed (range 0.4-0.9 m s^{-1}). Per speed, 3 movie file sequences of 20 s, randomly chosen from the beginning the middle and end of the 10 minute video recordings were used for further analysis (for details see Tudorache et al., 2009). In short: The period of ten minutes was divided in three period of 200 s. Each of these 200 s was then divided in ten periods of 20 s. One period of 20 s was chosen using mathematical randomisation (Microsoft Excel:Mac 2011, version 41.2.2, Microsoft inc., Seattle, USA). The selection criteria for a suitable video sequence were at least two individuals swimming steadily in front and one individual in the back of the tunnel during the entire period of 20 s. If the randomly chosen period did not fulfil these criteria, another period was chosen according to the same method. From each of the three sections of 20 s, tail beat frequency (*f*), and amplitude (*a*) were measured using the tracking program Vernier Logger Pro (v3.6, Vernier Software and Technology, USA), resulting in a total of 9 measurements per swimming speed. From the videos of the group-wise swimming eels, *f* and *a* values of two randomly chosen eels in the front third and of one eel in the back third of the swimming tunnel were analysed.

Statistics

First, all data were checked for normal distribution by Kolmogorov-Smirnoff tests. As the data were not normally distributed (Kolmogorov-Smirnoff; $p>0.05$) a Mann-Whitney U non-parametric test was used to analyze the results. Differences in oxygen consumption ($\dot{M}O_2$), standard metabolic rate, minimum cost of transport, optimal swimming speeds, tail beat frequency and tail beat amplitude between individual swimmers and group-wise swimmers were tested. Costs of transport per velocity were tested within the individual and group-wise swimming groups as well as between the two groups. Statistical difference was considered significant at $p<0.05$. In all cases values are expressed as average ± standard error.

Ethics Statement

The experiments complied with the Dutch law on animal experiments and were approved by the animal ethical committee of Leiden University (DEC# 09020).

Results

Behavioural observations

At low water speeds below 0.4 m s^{-1}eels, individuals as well as groups, swam irregularly and remained often motionless at the rear end of the swimming section curled up against the grid. When increasing the water speed the eels started to show regular swimming activity at water speeds of 0.30 m s^{-1}, while at 0.40 m s^{-1} all eels were swimming regularly. When swimming group-wise, a few eels swam parallel to each other in the front swimming in synchronized phase and with the same tail beat frequency, however with regular changes in and out of synchronization (Fig. 1). In contrast, eels swimming at the back of the tunnel did not show this type of synchronized swimming behaviour. The eels in the front regularly changed places with eels swimming in the rear. Thus all eels swam under similar conditions. There were no drop-outs; all fish were able to finish the trials.

Respirometry

Oxygen consumption during swimming was significantly higher ($p<0.05$) for males swimming individually as compared to males swimming group-wise at all swimming speeds (Figure 2a). The graph of oxygen consumption ($\dot{M}O_2$) versus swimming

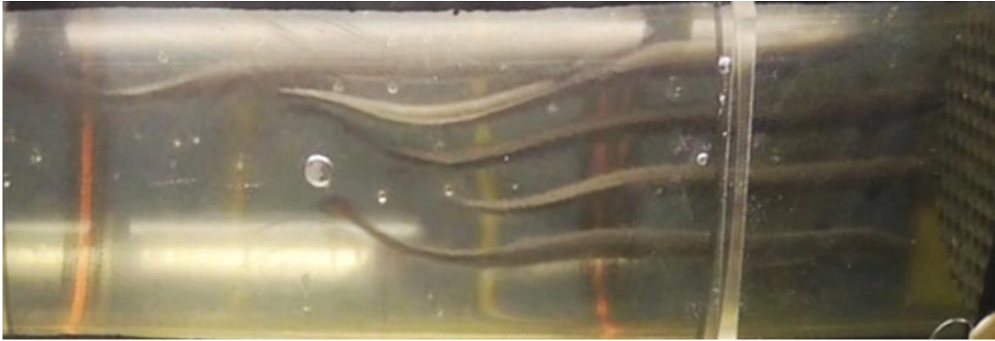


Figure 1. Anguilliform swimming motion. This photographic image shows the anguilliform swimming motion performed by group-wise swimming males in a parallel swimming formation at 0.40 m s^{-1}. For further explanation see text.

speed (U) fitted the formula $\dot{M}O_2 = SMR + aU^b$ with an r^2 of 0.99 for individuals as well as for groups. The extrapolated standard metabolic rate (SMR) showed no difference between males swimming individually and those swimming group-wise, i.e. 29.7 ± 6.2 vs. 30.6 ± 4.1 mg O_2 kg^{-1} h^{-1} (Table 1). At all speeds, the cost of transport (COT) of males swimming individually was significantly higher than the COT of males swimming group-wise (Figure 2b). However, when comparing the COT-values within each series, no significant differences were observed between speeds 0.4 – 0.8 m s^{-1}, indicating a rather flat curve.

There was no significant difference in the optimal swimming speed (U_{opt}) between individuals and groups (respectively 0.52 ± 0.04 and 0.57 ± 0.02 m s^{-1}, $p>0.05$). The minimum cost of transport, i.e. the cost of transport at U_{opt} (COT_{min}), was significantly higher in males swimming individually compared to males swimming group-wise; 31.99 ± 0.56 and 21.25 ± 3.24 mgO_2 kg^{-1} km^{-1} ($p<0.05$) respectively (Table 1).

Kinematics

Tail beat frequency (*f*) plotted against swimming speed (U) revealed a linear relationship; $f = a+bU$, with a being the intercept and b being the slope of the curve. At swimming speeds above 0.5 m s^{-1}, *f* was significantly higher in males swimming individually compared to males swimming group-wise at the same swimming speed (Figure 2c). There was no difference between *f* of eels swimming

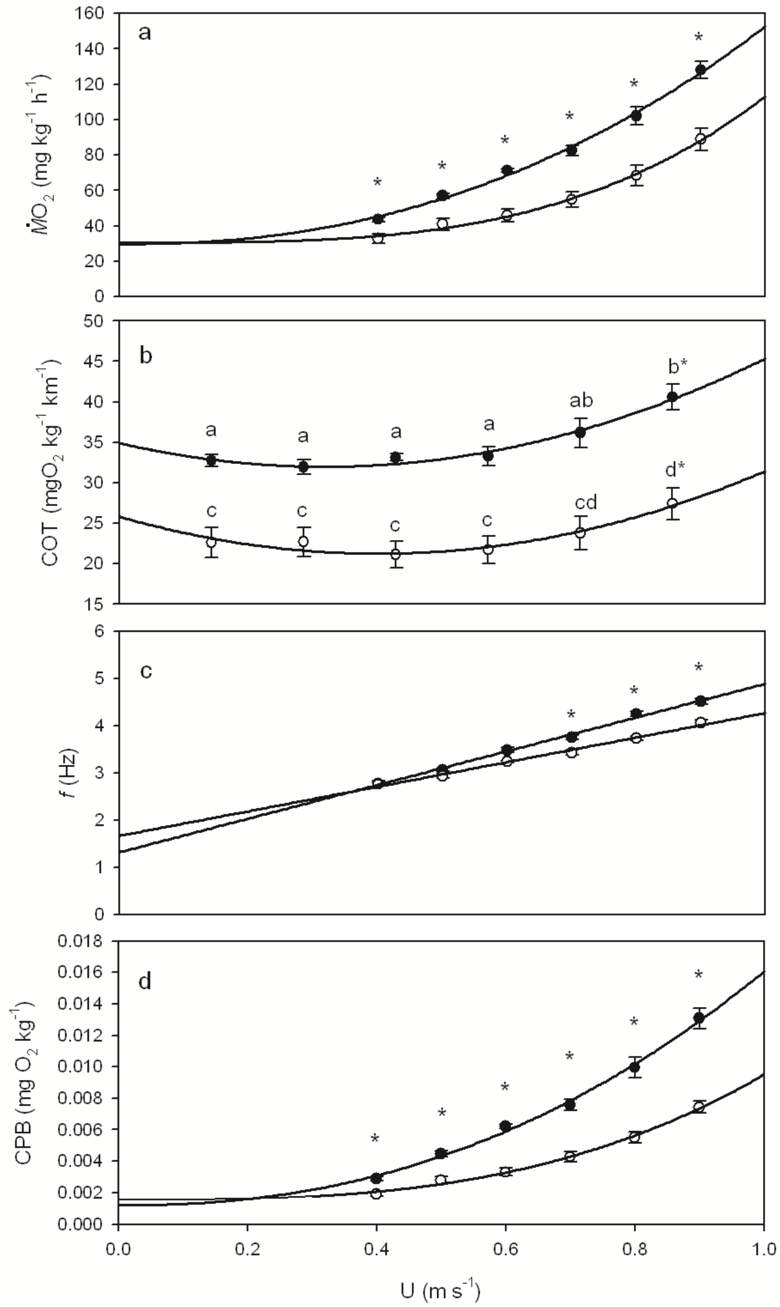
at the front versus those swimming in the rear of the swimming section. The tail beat amplitude (*a*) was not affected by swimming in groups or individually and remained constant across all swimming speeds at 5.1 ± 0.1 cm (pooled data of 3 movie file sequences of 20 s, Table 1). The width of the swimming tunnel was 4 times the tail amplitude (i.e. 20 cm) and thus the eels had their full range of motion without any obstructions at all applied speeds (Steffensen, 1989).

In order to determine the cost per fin beat (CPB in mg O2 kg^{-1}), standard metabolic rate (SMR) values were subtracted from oxygen uptake ($\dot{M}O_2$) values and the resulting numbers were divided by fin beat frequency (*f*) values per speed. Data were plotted using the power function CPB=a+bUc with a b and c being constant. The values for a are 0.0012±0.009 and 0.0016±0.003 mg O2 kg^{-1}, for b 0.0148±0.0007 and 0.0079±0.0003, and for c 2.2580±0.3783 and 2.9816±0.4324, for males swimming individually and for males swimming group wise, respectively (Figure 2d). Only values for b were significantly different from each other. The ratio of CPB values per swimming speed of group wise and individually swimming males did not differ with speed and resulted in the pooled value of 0.59±0.02.

Discussion

In order to establish the advantages of group-wise anguilliform swimming, male silver eels were swum over a range of 0.4 to 0.9 m s^{-1} individually and in groups of seven in swim tunnels. This study shows for the first time that group-wise anguilliform swimming, measured in terms of cost of transport (COT), is energetically more advantageous than swimming individually by ca. 30% (31.99 ± 0.56 vs. 21.25 ± 3.24 mg O_2 kg^{-1} km^{-1}, respectively). These results for anguilliform swimmers agree with observations on other fish species representing carangiform, ostaciiform and tunniform swimming modes (Parker, 1973; Webb, 1975; Ross & Backman, 1992; Herskin & Steffensen, 1998; Fish, 1999; Svendsen et al., 2003; Liao, 2007; Johansen et al, 2010; Killen et al., 2011).

Video analysis of swimming eels revealed that when swimming group-wise eels have a lower tail beat frequency (*f*) than when swimming individually. As *f* is almost linearly related to swimming speed (Figure 1c), this suggests that eels in a group take advantage of the wake of their neighbours. In addition, the amplitude of the tail beat (*a*) was not significantly different between individual and group-wise swimming. So, the amount of thrust force is only dependent on *f*.


a
b
c
d
$\dot{M}O_2$ (mg kg^{-1} h^{-1})
COT (mgO$_2$ kg^{-1} km^{-1})
f (Hz)
CPB (mg O$_2$ kg^{-1})
U (m s^{-1})
a
a
a
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ab
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Figure 2 (opposite page). Energetics and kinematics of male silver eels swimming individually and group-wise. Swimming energetics and kinematics of male silver eels swimming individually (closed circles, n=7) and male silver eels swimming group-wise (n=7, 7 males per group, open circles). a. Oxygen consumption ($\dot{M}O_2$, mg kg $^{-1}$ h^{-1}) as a function of swimming speed (U; m s^{-1}) with the formula $\dot{M}O_2$ = SMR+aU^b. Significant differences are indicated by an asterisk (*); Mann-Whitney U test, n=7, p<0.05). b. Cost of transport (COT, mgO_2 kg $^{-1}$ km $^{-1}$) as a function of U (m s^{-1}) with the formula COT = $\dot{M}O_2$ U^{-1}. Letters indicate significant differences between data points; an asterisk indicates significant difference between X and COT_{min} (see Table 1; Mann-Whitney U test, n=7, p<0.05). c. Tail beat frequency (*f*, Hz) as a function of U (m s^{-1}) with the formula f= a+ bU. Significant differences are indicated by an asterisk (*); Mann-Whitney U test, n=7, p<0.05). All lines (a, b and c) are regression lines (refer to Table 1 for regression values). Abbreviations: SMR: standard metabolic rate. d. Costs per fin beat (CPB, mg kg^{-1}) as a function of swimming speed (U; m s-1) with the formula CPB = a+bUc. Significant differences are indicated by an asterisk (*); Mann-Whitney U test, n=7, p<0.05).

Additionally, group-wise swimming suggests a lower mean cost per fin beat (CPB) at the same speed (Figure 1d) and thus should reduce the COT (Table 1). Indeed, a lower CPB was observed in group-wise swimming eels.

Also, the present study shows that male silver eels were able to swim steadily at 0.9 m s^{-1} (corresponding to ca. 2.3 BL s^{-1}). A preliminarily conducted velocity test showed that they also swim steadily at speeds even above 0.9 m s^{-1} (E. Burgerhout et al., unpublished). Quintella et al. (2010) observed a critical swimming speed (U_{crit}) of 0.66 m s^{-1} for wild male silver eels. Our results indicate that male eels can have sustained swimming speeds above 0.9 m s^{-1}, with a calculated optimal swimming speed (U_{opt}) of above 0.50 m s^{-1} (Table 1). The differences in results between the two studies might be due a difference in e.g. origin (wild versus farmed), conditioning or handling protocol. Stress may be an important factor as farmed eels are less sensitive to handling stress than wild eels. Furthermore, the present study used swimming tunnels with a longer swimming section; it has been shown that fish can obtain higher speeds in longer swimming tunnels (Tudorache et al., 2007) As shown in this study male silver eels can reach endurance speeds, even above 0.9 m s^{-1} (~2 BL s^{-1}).

Table 1. Energetics and kinematics of male silver eels swimming individually and group-wise. Oxygen consumption (mgO_2 kg^{-1} h^{-1}) was expressed as a function of swimming speed (U, m s^{-1}) with the formula $\dot{M}O_2$ = SMR+aU^b. SMR, standard metabolic rate; U_{opt}, optimal swimming speed (speed at the lowest cost of transport); COT_{min}, minimum cost of transport. Tail beat frequency (*f*, Hz) was expressed as a function of swimming speed (U, m s^{-1}) with the formula *f* = a + bU. Values are mean ± s.e.. P-values <0.05 are considered significantly different, and are expressed in bold.

Energetics	**Individual**	**Group-wise**	**Mann-Whitney U**
SMR (mgO_2 kg^{-1} h^{-1})	29.72±6.16	30.62±4.05	p=0.91
constant a	122.88±5.54	82.39±3.61	**p<0.01**
constant b	2.25±0.37	3.38±0.43	**p<0.01**
U_{opt} (m s^{-1})	0.52±0.04	0.57±0.02	p=0.28
COT_{min} (mgO_2 kg^{-1} km^{-1})	31.99±0.56	21.25±3.24	**p<0.01**
Kinematics			
frequency slope (b)	3.8±0.1	2.6±0.1	**p<0.05**
frequency intercept (a)	1.31±0.02	1.66±0.31	p=0.27
amplitude (cm)	5.1±0.1	5.6±0.6	p=0.43

In addition, we observed the behaviour of eels swimming group-wise, i.e. the swimming pattern in group-wise swimming eels, as many carangiform, ostraciiform or tunniform swimming species tend to swim in a rhomboid or 'diamond' shape pattern when swimming group-wise (Breder, 1965; Weihs, 1973; Partridge et al., 1980; 1983, Hoar, 1953; Castonguay & Gilbert, 1995; Hansen & Jonsson, 1985). Remarkably, instead of this rhomboid or 'diamond' schooling pattern, eels swam rather close to each other in a temporarily synchronized anguilliform swimming motion (Fig. 1). In studies on the hydrodynamics of eel

swimming, it was shown that the jet forces are directed laterally to the swimming direction (Tytell & Lauder, 2004; Kern & Koumoutsakos, 2006; Lauder & Tytell, 2006). Therefore, it is assumed that the lower energy consumption of the group-wise swimmers was at least in part due to this type of synchronized swimming motion where the individuals in the group use the lateral forces of the neighbours. This mechanism can be compared to the so called Karman gait, where thrust can be generated entirely passively by a foil when placed in an oscillating flow (reviewed in Liao, 2007). The actual mechanism of how eels use the water flow in group-wise swimming still needs to be studied in further detail using kinematic techniques.

In conclusion, the present study shows for the first time that group-wise swimming in eels is energetically more advantageous than swimming individually by the significant reduction of cost of transport. The synchronized swimming mode observed in group-wise swimming eels is expected to contribute to the lower energy consumption per capita.

Acknowledgements

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Chapter 3

Male European eels are highly efficient long distance swimmers: Effects of endurance swimming on maturation

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Abstract

Eels do not mature naturally in captivity only by using hormonal treatments. Low gamete quality and survival of offspring may reflect the unnatural treatments. The use of natural triggers to induce maturation may improve gamete quality and therefore be of high interest for eel aquaculture. European eels (*A. anguilla*) migrate ~6000 km from the European and North-African coasts towards their spawning area in the Sargasso Sea. Eels are still premature at the onset of this migration. It was hypothesized that simulation of the migration by swimming exercise would be the natural trigger inducing maturation. A previous study showed that maturation in wild male silver eels was stimulated when subjected to swimming exercise for 3 months covering ~900 km. It was therefore hypothesized that male eels will become fully mature when covering a longer distance or swimming for a longer period of time.

In the present study two groups of farmed male silver eels were subjected to either endurance swimming or resting for a maximum of 6 months. It was found that male eels were able to swim continuously for a total distance of 6670 km within 6 months, corresponding to swimming at ca. 1.0 BL s^{-1}. This is the first study that shows that male eels are able to cover the distance to the Sargasso Sea within expected time. In contrast to our expectation, swimming exercise did not induce maturation in farmed male silver eels, suggesting that swimming is not sufficient as a trigger for sexual maturation.

Introduction

Reproduction of freshwater eels (*Anguilla* spp.) intrigued many scientists and is studied for almost 80 years. Over the last decade progress has been made especially for the Japanese eel (*A. japonica*), e.g. by obtaining feeding larvae (Tanaka et al., 2001), glass eels (Tanaka et al., 2003: Kagawa et al., 2005) and recently an F2 generation (Ijiri et al., 2011). However, the success rates are far from suitable creating a sustainable eel aquaculture. Presumably, major problems such as low egg quality and poor fertilization rates, are caused by the unnatural stimulation of maturation by weekly injections of gonadotropins and pituitary extracts (Fontaine, 1936; Fontaine et al., 1964; Ohta et al., 1997), causing e.g. high transient hormonal peak levels (Sato et al., 2000, 2003), and possibly asynchronous oocyte development (Palstra et al., 2005).

Hormonal treatment is necessary as eels do not mature naturally in captivity. At the onset of their reproductive migration they are still in a prepubertal state (Dufour et al., 2003); i.e. oocytes are still in a pre-vitellogenic stage (Versonnen et al., 2004). Maturation in female eels is suppressed by a deficit gonadotropin-releasing hormone (GnRH) secretion and inhibition by dopamine (dopaminergic inhibition). Dopamine inhibits synthesis and secretion of luteinizing hormone (LH) (Dufour et al., 1988; Vidal et al., 2004, Weltzien et al., 2006, 2009). The neurohormone GnRH stimulates the pituitary to release the gonadotropins follicle-stimulating hormone (FSH) and LH. However, injections with GnRH is not effective in silver eels, indicating that blockage of sexual maturation is more complex in eels as compared to other teleosts (Vidal et al., 2004; Dufour et al., 2005). Internal and external stimuli are of importance in triggering the brain to relieve this dopaminergic inhibition. Relieving this inhibition takes place during or after migration to the spawning area, leading to complete maturation.

Major barriers in several phases of the reproductive cycle such as induction of early maturation (e.g. previtellogenic growth, vitellogenesis) and release of the dopaminergic inhibition might be overcome by using natural triggers (e.g. photoperiod, temperature, pressure, swimming exercise. Maturation and reproduction of many fish species can be controlled by manipulation of natural conditions, especially by changing photoperiod and temperature (Taranger et al., 2010; Wang et al., 2010). Natural triggers to induce maturation in eels are still insufficiently studied (e.g. Palstra et al., 2007; Sébert et al., 2007; van Ginneken et

al., 2007, Perez et al., 2011; Sudo et al., 2011). In addition, information from the field is scarce, which is mainly due to the fact that the natural conditions encountered during their oceanic phase are still for the larger part unknown (e.g. Jellyman & Tsukamoto, 2002; Tesch, 2003; Aarestrup et al., 2009, Manabe et al., 2011).

The catadromous eels migrate long distances to their spawning grounds; in the case of the European eel (*A. anguilla*) even over 6000 km, which is assumed to be covered within 6 months. The latter is based on the time difference between the onset of the migration in autumn and the occurrence of larvae in spring (Schmidt, 1923; Tesch, 2003). It was hypothesized that simulation of the migration by swimming exercise would release the dopaminergic inhibition and thereby induce maturation. Several studies showed that swimming exercise induced early maturation in female eels, such as incorporation of fat into the oocytes (van Ginneken et al., 2007; Palstra et al., 2007; reviewed by Palstra & van den Thillart, 2010). However, vitellogenesis and final maturation remained suppressed during longterm swimming exercise (Palstra et al., 2008; Palstra et al., 2010a). Similar effects of swimming exercise on maturation were recently found for rainbow trout (Palstra et al., 2010b).

In contrast to female silver eels, wild male silver eels showed a different response when subjected to long term swimming exercise. Palstra et al. (2008) found a significant increase of the GSI in male silver eels after three months of swimming, covering ca. 900 km (average velocity of 0.12 m s^{-1}). In addition, the expression of luteinizing hormone β subunit (LHβ) in the pituitary was two to three-fold higher as compared to resting males. Spermatogenesis was also stimulated; demonstrated by >80% spermatogonia of late type b, and one male even showing spermatocytes. These results suggest that swimming stimulates maturation in male silver eels, and that full maturation may occur when swimming for a longer period or distance (Palstra et al., 2008). In addition, it was stated that the dopaminergic inhibition as found in female eels, may not be effective in male eels based on the maturation response to injections with GnRH analogue (GnRHa).

It is currently unknown whether male eels are able to swim continuously covering a distance of ~6000 km within 6 months, which corresponds to a mean speed of 0.40 m s^{-1} (ca. 1 BL s^{-1}). Recently, it was found that farmed male eels are

efficient swimmers; i.e. relative low costs of transport and an optimal swimming speed of ca. 0.6 m s^{-1} (ca. 1.5 BL s^{-1}) (Burgerhout et al., unpublished data). Van Ginneken et al. (2007) showed that female eels were able to swim continuously for 6 months covering ca. 5500 km. The optimal swimming speed of males was found to be similar to that of females while the cost of transport was even lower (Tudorache, Burgerhout & van den Thillart, unpublished data). Therefore, it was expected that also male eels would be able to cover ca. 6000 km within 6 months.

During the present study a long term swimming trial was performed with farmed male silver eels for a maximum of 6 months to test two hypotheses: 1) farmed males will be stimulated to full maturation when subjected to continuous long term swimming, and 2) male eels are able to cover 6000km within 6 months. When spermiation is induced by a natural trigger such as swimming it may result in improved sperm quality, and therefore will be of much interest for eel aquaculture.

Methods

Animals and housing

Farmed male silver eels (*Anguilla anguilla*; n = 70; 119.9 ± 2.2 g; 38.2 ± 0.3 cm; average ± SE) were obtained from a commercial eel farm (Nijvis-Holding B.V., Nijmegen, The Netherlands), where they were kept in fresh water at 24°C. Prior to the trial, the eels were acclimated for two weeks and housed in a ca. 2500L recirculation system, supplied with natural seawater (32 ± 1 ppt) at 18 ± 0.5°C. The fish were kept under red light conditions (670nm, bandwidth 20nm), 12:12 L:D. This wavelength is likely invisible for silver eels as during silvering eye pigment changes to a blue spectral band (Pankhurst & Lythgoe, 1983). Light intensity above the experimental set-up was 0.06 lx. As silver eels do not feed, they were not fed. Black conservation sacks (120 x 80cm, Spro Strategy Conservation Sack) were added as shelter. The experiments complied with the Dutch law on animal experiments and were approved by the animal ethical committee of Leiden University (DEC# 09020).

Swimming exercise trial

At the start of the trial, morphometric data - including body length (BL), body weight (BW), eye diameter horizontal and vertical (Edh and Edv, respectively) and the pectoral fin length (PFL) - of all eels were obtained. Data were used to calculate the eye index following Pankhurst (1982) and the silver index following Durif et al. (2005). The males were not PIT-tagged to identify them individually, as the tag might have an influence on the swimming performance due to possible muscle damage.

Eels were randomly divided into seven groups (n=10 per group). One group was sampled (see next section) as an initial control (t=0), the other six groups were each introduced into six 127 L Blazka-type swimming tunnels (described by van den Thillart et al., 2004). The swimming tunnels were covered with plastic sheets which reduced the light intensity to 0.02 lx.

Three groups were subjected to a water velocity of 0.57 m s^{-1} (swim group), which is the optimal swimming speed found for males in a study with individuals as well as with groups (Burgerhout et al., unpublished data). The other three groups were kept resting (rest group) at a water velocity of 0.05 m s^{-1}. The latter was necessary to keep the water within the tunnel well mixed, while low enough for the eels to remain rested.

When individuals within a swim group started to fall back, the velocity was decreased to 0.40 m s^{-1} for the rest of the experiment. This velocity is the estimated minimum speed to cover 6000 km within 6 months. Eels that could not sustain this speed were removed from the tunnel within ca. 15 minutes. After 1.5, 3, and 6 months, eels from the respective swim and rest groups were taken out of the tunnel, anesthetized, dissected and sampled (see next section).

Sampling procedure

The eels were sacrificed using an overdose of clove oil (1:10 dissolved in 96% ethanol, dose 5 mL L^{-1}), followed by decapitation. Blood was obtained from the tail (dorsal aorta or caudal vein), using a heparin (10.000 IU in 0.9% saline) rinsed needle and syringe. Blood was centrifuged for 5 minutes at 13.000 RPM at 4°C) to obtain the blood plasma, which was stored at -80°C until further analysis. A testis sample (left side) for histological analysis was fixed overnight in 4% paraformaldehyde (PFA), and afterwards stored in 70% ethanol at 4°C.

Blood analysis

The levels of the gonadotropins were measured in the blood plasma using the recently developed bioassays based on the eel-specific FSH and LH receptors (Minegishi et al., 2012). Briefly, the bioassays consist of human embryonic kidney cells (HEK293 cells), which stably express the LH receptor of the European eel or the FSH receptor of the Japanese eel and contain a stably integrated luciferase reporter gene driven by a cAMP responsive-element. After incubation at 37°C for five hours for cell stimulation, a luciferase assay was performed using Steadylite plus Reporter Gene Assay System (Perkin Elmer, Waltham, MA, USA). The luminescence signal was measured on a multilabel plate reader (Victor, PerkinElmer). A serial dilution of pure hCG (Sigma-Aldrich) in DPBS (Invitrogen, Paisly, UK) or salmon pituitary extract in eel blood plasma was used as a reference for LH and FSH, respectively.

Blood plasma levels of testosterone were measured using a Testosterone ELISA kit (HUMAN Diagnostics Worldwide GmBH) following manufacturer's instructions.

Histological analysis

The testis samples were first dehydrated in a series of ethanol (70%-80%-90%-100%), followed by incubation in 100% Histo-Clear (National Diagnostics) and a 1:2 mixture of Histo-Clear (National Diagnostics, Biozym TC B.V., The Netherlands) with paraffin (Paraclean, Klinipath B.V., The Netherlands), respectively. Afterwards, the tissue samples were then embedded in paraffin. Sections (7 µm thick) were obtained using a Leica microtome (Leica RM2165) and, after rehydration, nuclei and cytoplasm were stained using Mayer's haematoxylin and eosin (H-E) staining. The stages of the testis were determined following Peñaranda et al. (2010).

Statistics

All data were checked for normal distribution by Kolmogorov-Smirnoff tests. As the data were not normally distributed (Kolmogorov-Smirnoff; $p<0.05$) a Mann-Whitney U non-parametric test was used to analyze the results. Differences in biometry (EI, HSI, GSI, DTWI) and blood plasma levels of FSH, LH, T were tested between and within the groups (swimmers and resters) over the consecutive time

points (0, 1.5, 3 and 6 months). At $p<0.05$ the statistical difference was considered significant. In all cases values are expressed as average ± standard error (SE).

Results

Endurance swimming

During the first 26 days of the trial, three groups of males (n=10 per group) swam against a water flow of 0.57 m s^{-1}. Four swimmers (two of the 1.5 months group, one of the 3 months group and one of the 6 months group) that dropped out after 4 weeks were removed from the tunnels. The water velocity in all tunnels of the swim groups was thereafter decreased to 0.40 m s^{-1} for the rest of the trial. After 1.5 month 8 out of 10 males had swum 1970 km. In the period between 1.5 and 3 months, three swimmers of the 6 months group, and one rester of the 3 months group had dropped out, and were taken out of the tunnel. After 3 months of swimming 9 out of 10 eels had covered a total of 3525 km. Six remaining males of the swim group continued swimming for 6 months, covering 6670 km. Drop-outs in the swim groups were probably occurred due to fatigue. The one resting eel that dropped out showed several wounds along its body probably due to frequent contact with the rear part of the tunnel.

Swimming males tended to aggregate in the front section of the tunnel close to the water inlet. As observed in a previous study, they often swam parallel to each other in a synchronized phase. The eels in the front frequently changed position with the eels in the rear of the tunnel as observed earlier (Burgerhout et al., unpublished data). Resting males showed three different kinds of behaviour: they moved around in the tunnel, kept oriented against the stream, or stayed clumped together at the rear of the tunnel. While resting in a clump at the rear, they still kept moving along each other.

Biometry

The results of the biometry data can be found in Table 1. All males used in this study were assigned silver eels following the silver index of Durif et al. (2005).

At all consecutive sample points, average body weight (BW, Fig. 1) and condition factor (CF) decreased in both swim and rest groups as compared to the pre-measurements. After 3 and 6 months of swimming exercise average BW was

significantly decreased in the swim group as compared to the pre-swim measurements and the initial control ($p<0.05$). After 1.5 and 6 months of resting the average body weight (BW) decreased significantly in the rest group as compared to the pre-rest measurements ($p<0.05$), and at 6 months also to the initial control ($p<0.05$).

In all groups the CF decreased significantly as compared to the pre-measurements and initial control ($p<0.05$). In addition, the average monthly BW decrease was calculated. At 1.5 months the decrease was 10.1 and 7.8 g per month for the swim and rest group, respectively. The rest group showed on average a reduction in monthly BW decrease at 6 months as compared to 1.5 months, while that of the swim group was similar (3.3 g and 9.9 g per month, respectively). At 6 months, swim and rest group showed a monthly BW decrease of 3.7 g and 4.6 g per month, respectively.

An increase of the eye index (EI) is associated with an increased production of sex steroids and gonadal development (Pankhurst, 1982; Sbaihi et al., 2001; Peñaranda et al., 2010), and therefore an external marker for maturation. In both swim and rest groups average EI increased at all consecutive sample points. Although on average the EI increase was higher in the rest group as compared to the swim group, EI was not significantly different between the swim and rest groups ($p>0.05$).The average EI of the swim group was significantly increased at 6 months as compared to the initial measurements ($p<0.05$); the average EI of the rest group was significantly increased as compared to the initial measurements and initial control at 1.5, 3 and 6 months ($p<0.05$).

Maturation in eels is accompanied by increases in liver weight (LW) and gonad weight (GW), and a decrease of the digestive tract weight (DTW) (e.g. Pankhurst & Sorensen, 1984; Rohr et al., 2001, Durif et al., 2005). Between the swim and rest groups no significant differences were found in liver weight (LW), gonad weight (GW) and digestive tract weight (DTW) at all consecutive sample points. The LW of the swim and rest groups was found significantly lower than the initial control at 6 months, and at 3 and 6 months, respectively ($p<0.05$). As compared to the DTW of the initial control, the DTW of the swim and rest groups were found significantly lower at 1.5, 3 and 6 months, and 3 and 6 months, respectively ($p<0.05$). The GW of the swim and the rest groups was significantly lower as compared to the initial control group at 3 and 6 months ($p<0.05$).

	Initial control	Swim 1.5 months		Rest 1.5 months		Swim 3 months		Rest 3 months		Swim 6 months		Rest 6 months	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	n=10	n=10	n=8	n=10	n=10	n=10	n=9	n=10	n=9	n=10	n=6	n=10	n=10
BW	115.4 ± 5.3	115.5 ± 5.7	103.9 ± 6.1	131.5 ± 4.0*	**116.4 ± 5.6**	126.0 ± 4.4*	**96.3 ± 2.8***	109.2 ± 6.4	99.3 ± 6.9	120.0 ± 6.1	**98.1 ± 3.8***	121.4 ± 7.4	**93.8 ± 8.2*[b]**
BL	37.3 ± 0.6	37.8 ± 0.7	38.2 ± 0.7	39.4 ± 0.4	39.3 ± 0.4	39.1 ± 0.5	38.3 ± 0.5	37.5 ± 0.8	37.6 ± 0.9	38.0 ± 0.8	38.9 ± 0.7	38.2 ± 0.8	37.8 ± 0.9
CF	0.22 ± 0.01	0.21 ± 0.00	**0.18 ± 0.00***	0.22 ± 0.00	**0.19 ± 0.01***	0.21 ± 0.01	**0.17 ± 0.01***	0.21 ± 0.01	**0.19 ± 0.01***	0.22 ± 0.01	**0.17 ± 0.01***	0.22 ± 0.00	**0.17 ± 0.01*[b]**
EI	9.6 ± 0.5	10.4 ± 0.6	11.1 ± 0.7	9.7 ± 0.2	**11.2 ± 0.3***	9.4 ± 0.4	10.6 ± 0.7	9.7 ± 0.4	**11.9 ± 0.3***	9.1 ± 0.4	**11.1 ± 0.8**	9.3 ± 0.4	**11.6 ± 0.3***
PFLI	4.9 ± 0.1	4.8 ± 0.2	4.9 ± 0.1	4.7 ± 0.1	4.9 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.2	4.8 ± 0.1	5.1 ± 0.1[c]
LW	1.29 ± 0.05		1.29 ± 0.12		1.49 ± 0.10		1.37 ± 0.24		0.96 ± 0.04*[a]		1.02 ± 0.07*		0.90 ± 0.11*[b]
GW	0.10 ± 0.00		0.09 ± 0.01		0.08 ± 0.02		0.06 ± 0.01*[a]		0.07 ± 0.01*		0.06 ± 0.01*		0.07 ± 0.01*
DTW	2.21 ± 0.11		1.59 ± 0.15*		1.79 ± 0.17		1.51 ± 0.16*		1.59 ± 0.26*		1.32 ± 0.16*		1.58 ± 0.16*
HSI	1.13 ± 0.04		1.25 ± 0.10		1.33 ± 0.15		1.47 ± 0.32		1.00 ± 0.06		1.05 ± 0.08		0.96 ± 0.07*[b]
GSI	0.09 ± 0.00		0.09 ± 0.01		0.06 ± 0.01*		0.06 ± 0.01*[a]		0.06 ± 0.01*		0.06 ± 0.01*		0.07 ± 0.01*
DTSI	1.93 ± 0.08		1.55 ± 0.13*		1.54 ± 0.11*		1.60 ± 0.22		1.57 ± 0.19		1.33 ± 0.12**		1.70 ± 0.10

Table 1 (opposite page): Biometry of males subjected to endurance swimming (Swim) or resting (Rest) for 1.5, 3 and 6 months. Prior the experiment an initial control group was sampled (t=0), and external morphometry data of all eels were obtained. Significant differences ($p<0.05$) are indicated in bold (Pre vs Post conditioning), or by * (swim or rest vs initial control) or by ** (swim vs rest and initial control). Changes in biometric data over time of male eels subjected to endurance swimming (Swim) or resting (Rest) for 1.5, 3 and 6 months. Significant differences ($p<0.05$) are indicated by letters a (1.5 vs 3), b (1.5 vs 6) or c (3 vs 6). Abbreviations: BW: body weight; BL: body length; CF: condition factor; EI: eye index (Pankhurst, 1982); PFLI: pectoral fin index (Durif et al., 2005); LW: liver weight; GW: gonad weight; DTW: digestive tract weight; HSI: hepatosomatic index; GSI: gonadosomatic index; DTSI: digestive tract somatic index.

When body weight was taken into account to calculate the somatic indices of liver (HSI), gonads (GSI) and digestive tract (DTSI), only the DTSI was found significantly lower in the swim group as compared to the rest group at 6 months. No significant differences in HSI and GSI were found between the swim and rest groups at all consecutive sample points. The HSI of the rest group was significantly lower than the initial control at 6 months ($p<0.05$). The DTSI of the swim group was significantly lower than the initial control at 1.5 and 6 months, and significantly lower than the rest group at 6 months ($p>0.05$). The rest group showed as compared to the initial control a significantly lower DTSI at 1.5 months ($p<0.05$).

Plasma hormone levels

Testosterone is an important marker for sexual maturation in fish (Nagahama, 1994) and was measured in the blood plasma using an ELISA. No significant differences were observed between the swim and rest groups at all consecutive sample points ($p>0.05$, Fig. 2a). A significantly lower in T plasma level was observed after 6 months in resting group as compared to the initial control group, and after 1.5 months of swimming and resting ($p<0.05$, Fig 2a).

The processes of spermatogenesis can be induced by human chorionic gonadotropin (hCG, Miura et al., 1991a), a hormone analogue to luteinizing hormone (LH). Stimulation of spermatogenesis is suggested one of the major functions of follicle stimulating hormone (FSH) (reviewed by Planas & Swanson,

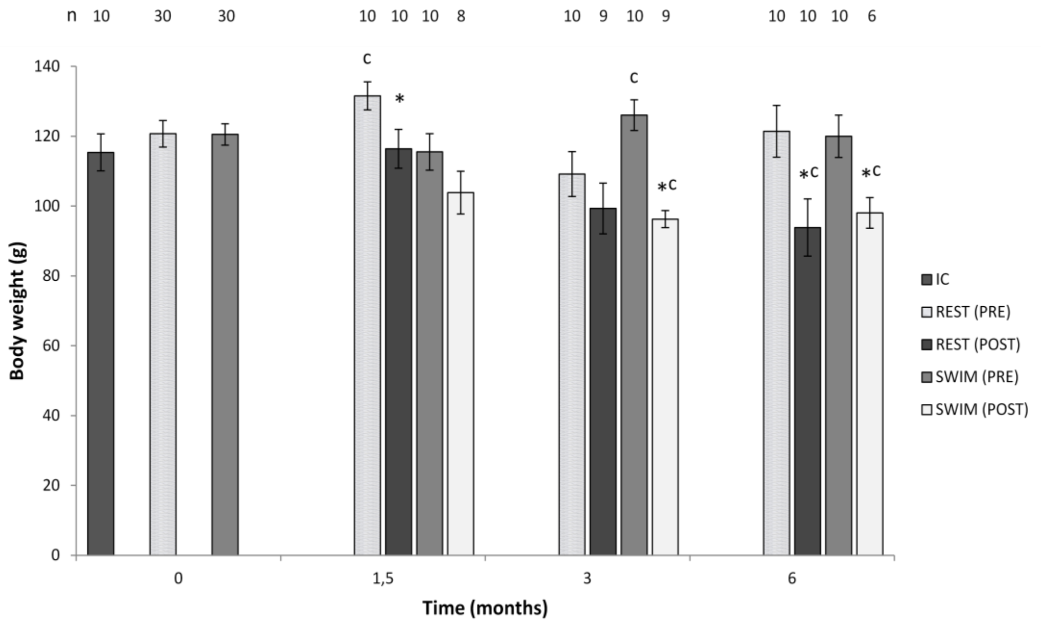

Figure 1. Body weight overview. Initial body weight (g; average ± SE) measurements (t=0 months, PRE) and final measurements (POST) at consecutive sample points (t=1.5, 3 6 months) after swimming and resting treatment (Swim and Rest, respectively). Statistical differences (p<0.05) between PRE and POST are indicated asterisk (*). Significant differences (p<0.05) as compared to initial control (IC) are indicated by letter c. Number of males measured is indicated by values (n) at the top of the figure above each bar.

2008). Both hormones were therefore measured in blood plasma. No significant differences were found in the blood plasma levels of FSH for all groups at all consecutive sample points based on fluorescence counts per second (CPS) (p> 0.05, Fig 2b). After 1.5 months of swimming and resting, the blood plasma levels of LH were significantly higher than the LH levels of the initial control based on CPS (p<0.05, Fig. 2c). However, after 3 and 6 months of swimming the LH levels were significantly lower than the values of the swim and rest group at 1.5 months, and significantly lower than the swim group at 3 months, respectively (p<0.05). The LH levels of the rest group at 6 months was significantly lower as compared to the levels of the swim and rest group at 1.5 months (p<0.05). No significant

differences in LH levels were found between the initial control and the swim and rest group at 3 and 6 months ($p>0.05$).

Histological analysis

Testis showed spermatogonia type a and b as the most advanced developmental stages in all groups, corresponding to testis development stages 1-2 as described by Penaranda et al. (2010). No further development such as formation of spermatocytes was observed. No differences in testis development were observed between swimmers and resters at all consecutive sample points (data not shown).

Discussion

The application of natural triggers, e.g. photothermal stimulation, pressure or exercise, may ultimately lead to breakthroughs in eel reproduction, but they are still hardly studied. Maturation is suppressed due to a deficit release of GnRH and inhibition by dopamine acting on the synthesis and release of LH (Dufour et al., 1988; Vidal et al., 2004; Weltzien et al., 2006, 2009). Simulation of migration by swimming exercise was hypothesized to be a natural trigger by releasing eel from this deficiency and inhibition (van Ginneken et al., 2007; Palstra et al., 2007). Palstra et al. (2008) showed that swimming exercise stimulated maturation in wild male silver eels. Based on the latter study, it was hypothesized that, when wild male eels would be subjected to endurance swimming covering a larger distance or longer time period, full maturation may be expected. In the present study, farmed male silver European eels were subjected to long-term swimming exercise for up to 6 months to test the effects of long-term swimming on maturation.

Endurance swimming and efficiency

It is still uncertain whether eels swim continuously during their ca. 6000 km spawning migration, or whether they use the oceanic currents, as suggested by Fricke & Kaese (1995). Female eels were shown to be able swimming 5500 km in 6 months (van Ginneken et al., 2007). The present study showed for the first time that farmed male silver eels are able to swim continuously for 6 months covering a total distance of 6670 km (corresponding to 0.42 cm s^{-1}, ca. 1.0 BL s^{-1}). Although males were subjected to their optimal swimming speed of 0.57 m s^{-1} (Burgerhout

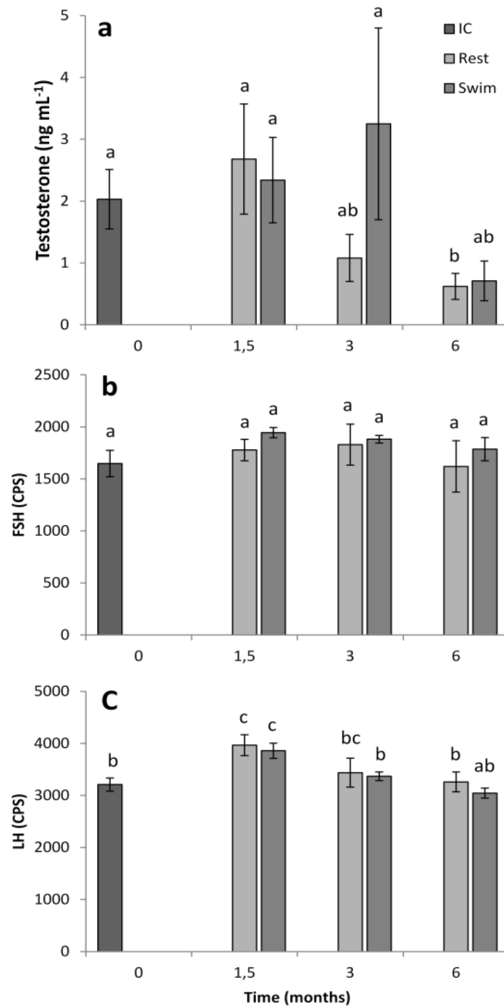

Figure 2. Changes in blood plasma hormone levels. Plasma samples were obtained from initial control (IC) and after swimming or resting (Swim and Rest, respectively). Testosterone (a) was measured by ELISA. FSH (b) and LH (c) were measured using the bioassay as described in Minegishi et al. (2012) and expressed as fluorescence counts per second (CPS). There were no significant differences ($p>0.05$) in plasma hormone levels between the swim and rest groups at all consecutive sample points. Significant differences ($p<0.05$) over time between groups (Swim and Rest) and as compared to the initial control (IC) are indicated by letters.

et al., unpublished data), during the trial several males dropped out possibly due to fatigue. As the applied swimming speed was based on short-term swimming trials (Burgerhout et al., unpublished data), the drop-outs after 4 weeks indicate that the optimal swimming speed (U_{opt}) is probably lower for long-term swimming. However, U_{opt} might be different between individuals, and during their natural spawning migration it may be expected that not all males will survive, due to e.g. insufficient energy stores (Svedäng & Wickström, 1997; Clevestam et al., 2011).

At 1.5 and 6 months there were no significant differences in the average monthly BW decrease between the swim and rest groups, indicating that the swimming costs at the applied speeds tested were close to the routine metabolic rate. The drop-outs in the swim groups were not included in this comparison. It is quite possible that they had a higher metabolic rate, thus using up their energy stores faster than the others. Recently we found that the oxygen consumption per individual of group-wise swimming males at a velocity of 0.40 m s^{-1} was not significantly higher than the standard metabolic rate (Burgerhout et al., unpublished data). The present results indicate that the energy costs during resting (e.g. spontaneous activity, social interaction) are similar as those for swimming at 0.40 m s^{-1}.

The rest group at 1.5 months and the swim group at 3 months had initially a significantly higher BW as compared to the swim and rest group at 1.5 and 3 months, respectively. All other measured parameters did not differ between groups. Interestingly, in both groups the level of BW decrease was relatively higher as compared to the other groups. This phenomenon may be explained by a higher standard metabolic rate (SMR) for males with a relatively higher BW. However, it is currently unknown whether eels with a relative higher BW have a higher SMR. In conclusion, from almost similar costs of swimming and resting it is shown that farmed male silver eels are extremely efficient swimmers.

Induction of maturation

No significant differences in the maturation parameters – EI, GSI, plasma levels of T, FSH and LH – were found between the swimming and resting eels, clearly in contrast to our expectations. The increase in eye index in both swimmers and

resters at all consecutive sample points, suggests a progressed maturation, which might be due to the transition from freshwater to seawater prior to the trial.

The presented results indicate that endurance swimming is not sufficient to induce sexual maturation in farmed male silver eels. However, there were differences between the present study and the study of Palstra et al. (2008), which may explain the differences in final results.

First, the major difference between our study and the study performed by Palstra et al. (2008) is the use of farmed instead of wild eels. Those wild migratory males were obtained from Greece and caught in February (Palstra et al., unpublished data). Possibly, the stimulatory response shown by the wild males to swimming exercise could be due to the fact that maturation had farther progressed, which is reflected by a relatively high GSI found for several males of the initial control (GSI of 0.3; Palstra et al., unpublished data). In addition, after 3 months the resting males in the study of Palstra et al also showed a progression in maturation of the testis, although less apparent than in the swimming males (Palstra et al., 2008). Based on those data we suggest that before the trial those wild males have been stimulated in their maturation by other environmental factors (e.g. changes in photothermal period) which caused this progressed state of maturation. In many fish species, changes in photothermal period affects maturation (see reviews Taranger et al., 2010; Wang et al., 2010). It was also recently found for female eels that temperature decrease induces pre-vitellogenic growth. However, further sexual maturation was not observed indicating that other environmental factors, such as salinity or photoperiod, are involved (Sudo et al., 2011).

Second, the males in the study of Palstra et al. (2008) swam at an average velocity of 0.12 m s^{-1} (i.e. 912 km in 90 days). During the present study males were subjected to a much higher velocity – initially 0.57 m s^{-1} and 0.40 m s^{-1} after 26 days for the rest of the trial. The difference in swimming velocity may have contributed to the contrasting results concerning maturation found in the present study and the study of Palstra et al. (2008). This suggests that swimming at a low velocity (ca. 0.12 m s^{-1}), which was slightly above the resting velocity in our study (0.05 m s^{-1}), appears to stimulate maturation.

In addition, it was found that wild males responded to a GnRH-agonist (GnRHa) with increased expression of LHβ in the pituitary and with an increase of

the GSI (Palstra et al., 2008). Therefore, it was stated that the dopaminergic blockage as described for female eels (Dufour et al., 1988; Vidal et al., 2004; Weltzien et al., 2006, 2009) is not effective in male eels (Palstra et al., 2008). However, GnRHa did not affect the maturation (i.e. increase of GSI) in farmed male Japanese eels (Kagawa et al., 2009), suggesting that in farmed male eels maturation is blocked by dopamine. Therefore it appears that the major difference is the use of wild versus farmed male eels; i.e. the initial maturation status of the wild males used in the study of Palstra et al. (2008) being more advanced and therefore responsive to GnRHa treatments and swimming.

Although the dopaminergic system in male eels is still insufficiently studied, based on our present results, and the results of Palstra et al. (2008) and Kagawa et al. (2009), we hypothesize that inhibition of dopamine may be effective in males and released by environmental triggers than swimming exercise. Future studies should elucidate whether maturation in males is also suppressed by dopamine as found in females.

With the present study we have shown that one of the proposed natural triggers, i.e. swimming exercise, was found not sufficient to induce maturation in farmed male eels. We recommend that future research studies should focus more on other possible triggers (e.g. photoperiod, temperature). It may be expected that, as compared to the current artificial reproduction procedures by hormonal treatments, natural induction of maturation increases gamete quality and efficiency rates, which is a major priority for a sustainable aquaculture.

Conclusions

This is the first study that showed that farmed male silver eels are able to swim continuously for 6 months covering a total distance of 6670 km. The extreme swimming efficiency of male eels is indicated by similar decreases in body weight as found for resting eels. Furthermore our results suggest that swimming exercise does not trigger sexual maturation in farmed male silver eels in contrast to earlier observed effects on wild male silver eels.

Acknowledgements

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Chapter 4

Primitive Duplicate Hox Clusters in the European Eel's Genome

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Abstract

The enigmatic life cycle and elongated body of the European eel (*Anguilla anguilla* L., 1758) have long motivated scientific enquiry. Recently, eel research has gained in urgency, as the population has dwindled to the point of critical endangerment. We have assembled a draft genome in order to facilitate advances in all provinces of eel biology. Here, we use the genome to investigate the eel's complement of the Hox developmental transcription factors. We show that unlike any other teleost fish, the eel retains fully populated, duplicate Hox clusters, which originated at the teleost-specific genome duplication. Using mRNA-sequencing and *in situ* hybridizations, we demonstrate that all copies are expressed in early embryos. Theories of vertebrate evolution predict that the retention of functional, duplicate Hox genes can give rise to additional developmental complexity, which is not immediately apparent in the adult. However, the key morphological innovation elsewhere in the eel's life history coincides with the evolutionary origin of its Hox repertoire.

Introduction

The life history of the European eel (*Anguilla anguilla* L., 1758) involves two distinct ocean-dwelling larval stages, a protracted juvenile phase in European continental freshwater, and finally sexual maturation coincident with migration to spawning grounds in the Atlantic Ocean, presumably the Sargasso Sea (see Chapter 1, Figure 1) (Tesch, 2003). The complexity and geographical range of this life cycle have long inspired evolutionary and physiological studies, especially on the structure of the eel's single, randomly mating (panmictic) population (Als et al., 2011), interspecific hybridization with the American eel (*A. rostrata*, which shares the same oceanic spawning grounds (Avise et al., 1990)), its hidden migrations (Schmidt, 1923; Aarestrup et al., 2009; van den Thillart et al., 2009), and the development of fertility (van den Thillart et al., 2009).

Its catadromous migratory behaviour, long life, serious habitat reduction, pollution, and overfishing may be amongst the causes of the catastrophic collapse of the European eel population observed over the past decades (Freyhof & Kottelat, 2008). So far, *Anguilla* species have resisted efforts directed at efficient and sustainable artificial breeding (Tanaka et al., 2003). As knowledge on the eel's genetic makeup is sparse, physiological studies aimed at understanding maturation, reproduction and the sustenance of successive larval stages have not been able to take full advantage of gene expression profiling. In order to alleviate this shortcoming, we have sequenced and assembled its genome.

While the draft genome will be an important tool in reproduction research, it also offers new perspectives for fundamental studies in eel biology, as well as a resource for the comparative interpretation of model fish genomes (e.g. zebrafish and medaka). Here, we investigate its repertoire of Hox genes in a comparative genomics context.

The Hox genes encode transcription factors, which throughout the animal kingdom are involved in the developmental patterning of the body plan. In vertebrates, Hox genes are tightly organized into clusters which exhibit colinearity between gene position and temporal and spatial expression along the primary body axis: genes at the 3' ends of clusters are expressed earlier in development, and more anterior, than genes at the 5' ends of clusters (Duboule, 2007). The

organization of Hox clusters has been extensively documented for many groups of vertebrates (Kuraku & Meyer, 2009).

A. anguilla is a member of the superorder Elopomorpha (Greenwood et al., 1966; Inoue et al., 2004), a major teleost group of 856 species (Nelson, 2006). As such, elopomorphs presumably share the inferred whole-genome duplication at the base of the teleost lineage (Jaillon et al., 2004; Meyer & van de Peer, 2005). This teleost-specific genome duplication (TSGD) event is most apparent when considering the Hox genes in extant species (Kuraku & Meyer, 2009; Crow et al., 2006; Amores et al., 1999). In tetrapods and coelacanths, approximately forty genes are organized in four ancestral vertebrate clusters. In theory, teleosts could have retained eight duplicate clusters. However, whereas tetrapod Hox loci are relatively stable, teleost genomes show dramatic gene loss, such that all species examined in detail retain at most seven of these clusters, each with on average about half their original gene content (Duboule, 2007; Kuraku & Meyer, 2009). A PCR-based survey of the Hox clusters of the Japanese eel *A. japonica* found evidence for the conservation of eight clusters and 34 genes (Guo et al., 2010).

As the Elopomorpha represent an early branch on the teleost tree (Inoue et al., 2004), the eel Hox gene complement may expose constraints on the evolution of morphological complexity in teleost fish, and in vertebrates in general. Furthermore, analysis of the eel's Hox clusters may shed light on the developmental mechanisms and evolutionary history of its life cycle and body plan. In particular, they may provide evidence regarding the evolutionary novelty of the eel's indirect development.

Methods

Eel embryos

Wild female and male silver short-finned silver eels (*A. australis*) from Lake Ellesmere, New Zealand, were held together in a 2,300 L recirculation system with seawater (30 ppt salinity) at 21 °C. Sexual maturation was induced as described (Burgerhout et al., 2011). Briefly, males received nine weekly injections with 250 IU human chorionic gonadotropin and females were injected once a week with 20 mg salmon pituitary extract. Eggs and milt were stripped and the

eggs were dry fertilized. Embryos were reared in glass beakers with UV-sterilized seawater (35 ppt) at 21 °C. At 26, 48 and 96 hpf embryos were fixed in 4% paraformaldehyde and stored in 100% methanol.

Total RNA was isolated from 27 hpf embryos using the Qiagen miRNeasy kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany), and analyzed with an Agilent Bioanalyzer 2100 total RNA Nano series II chip (Agilent, Santa Clara). A transcriptome library was prepared from 10 µg total RNA, using the Illumina mRNA-Seq Sample Preparation Kit according to the manufacturer's instructions (Illumina Inc., San Diego, USA).

Genome size determination

Blood samples taken from two eels (*A. anguilla* and *A. australis*) were washed with physiological salt and fixed in cold ethanol. Prior to analysis the cells were collected, resuspended in physiological salt and stained with propidium iodide. After 30 minutes of incubation the cells were analyzed by FACS, using human blood cells as a size reference (3.05 Gbp haploid). The eel genome size was calculated by (human size)/(mean fluorescence human)×(mean fluorescence eel). Both *Anguilla* genomes were determined to be 1.1 Gbp in size (haploid).

Genomic DNA libraries

Genomic DNA was isolated from blood of a female European yellow (*A. anguilla*, caught in Lake Veere, The Netherlands) using the Qiagen Blood and Tissue DNeasy kit according to the manufacturer's description. Paired-end libraries were prepared from 5 µg of isolated gDNA using the Paired-End Sequencing Sample Prep kit according to the manufacturer's description. Either a 200 bp band or a 600 bp band was cut from the gel (libraries PE200 and PE600, Table S1). After amplification for 10 cycles the resulting libraries were analyzed with a Bioanalyzer 2100 DNA 1000 series II chip.

Mate pair libraries were prepared from 10 µg of isolated gDNA using the Mate Pair Library Prep Kit v2 (Illumina Inc.). Either a 3,000 bp band or a 10,000 bp band was cut from gel (libraries MP3K and MP10K, Table S1). After the first gel purification the fragment length was analyzed using a Agilent Bioanalyzer 2100 DNA 12000 chip. After circularization, shearing, isolation of biotinylated

fragments, and amplification, the 400 to 600 bp fraction of the resulting fragments was isolated from gel. Finally, the libraries were examined with an Agilent Bioanalyzer 2100 DNA 1000 series II chip.

Illumina sequencing

All libraries were sequenced using an Illumina GAIIx instrument according to the manufacturer's description. Genomic paired-end libraries were sequenced with a read length of 2×76 nucleotides (to ~20-fold genome coverage), genomic mate-pair libraries with a read length of 2×51 nucleotides (to ~33-fold genome span), and the mRNA-Seq library with a read length of 2×76 nucleotides (Table S1). Image analysis and base calling was done by the Illumina pipeline.

Genome assembly

Sequencing reads from both paired-end libraries were used in building the initial contigs (Figure S1). Both sets were preprocessed to eliminate low quality and adapter contamination. Whenever possible, PE200 pairs were merged into longer single reads. For initial contig assembly, we employed the De Bruijn graph-based *de novo* assembler implemented in the CLC bio Genomics Workbench version 3.6.5 (CLC bio, Aarhus, Denmark). A run with a k-mer length of 25 nt resulted in an assembly a total length of 969 Mbp and a contig N50 of 1672 bp.

Initial contigs were oriented in larger supercontigs (scaffolds) using SSPACE (Boetzer et al., 2011). In scaffolding the contigs, we decided to exclude low-quality and highly repetitive contigs as much as possible. SSPACE was used in a hierarchical fashion, employing first links obtained from the PE600 library to generate intermediate supercontigs, which were used as input for subsequent runs with links from the MP3K and MP10K libraries, respectively. At each stage, a minimum of three non-redundant links was required to join two contigs. This procedure resulted in a final scaffold set with a total length of 923 Mbp and an N50 of 77.8 Kbp (Table S1). AUGUSTUS (version 2.4) was used to predict genes (Stanke et al., 2008), which were provisionally annotated using Blast2GO (version 2.4.8) (Götz et al., 2008). The draft assembly is available at *www.eelgenome.org*.

In order to obtain more information on flanking genes for the analysis of conserved synteny (Figure 5), scaffolds were subjected to a further round of

linking by SSPACE using reduced stringency (two instead of three non-redundant links required to join scaffolds). This resulted in extended scaffolds with an N50 of 169 Kbp.

Hox genes

Hox contigs in the short-finned eel embryonic transcriptome (generated using CLC bio's *de novo* assembler) were identified via Blast (Altschul et al., 1990) searches at the NCBI website (*www.ncbi.nlm.nih.gov*). European eel genomic scaffolds were annotated using CLC bio's DNA Workbench. Remaining Hox genes and genes flanking the Hox clusters were identified using Blast, based on AUGUSTUS/Blast2GO predictions. Annotated Hox scaffolds have been submitted to GenBank (accession numbers JF891391–JF891400).

MicroRNAs were identified by Blast using *H. sapiens* and *D. rerio* miR-10 and miR-196 sequences (precursors and mature) retrieved from miRBase release 18 (*www.mirbase.org*, (Kozomara & Griffiths-Jones, 2011)).

Phylogenetic methods

Species and Hox gene accession numbers used are listed in Table S4. Amino acid sequences of Hox genes were aligned using Clustal X (Larkin et al., 2007) and checked manually. After excluding ambiguous alignments, ProtTest 2.4 (Abascal et al., 2005) was used to choose an optimum substitution model, based on the Akaike information criterion. The aligned sequences were subjected to maximum likelihood analysis using RAxML version 7.2.6 (Stamatakis et al., 2008) with 1000 rapid bootstrap replicates (-f a option).

For the analysis of Hox9 genes (Figure 3), 70 aligned residues were used and analyzed using a JTT + I + Γ model (Jones et al., 1992). All other alignments were fitted using a JTT + Γ model. The multi-gene analyses of HoxA, HoxB, HoxC and HoxD (Figure 4) were based on alignments of 427, 493, 935 and 308 amino acid residues, respectively. The phylogenetic trees of sarcopterygian and actinopterygian Hox9 paralogues (Figure S2) were based on 151 (HoxA9), 210 (HoxB9), 248 (HoxC9), and 136 (HoxD9) residues.

Synteny was analyzed using *D. rerio* and *O. latipes* genomic contexts extracted from Ensembl release 65 (*www.ensembl.org*), based on Zv9 and

MEDAKA1 genome assemblies, respectively (Table S5). Pairwise alignments were generated by NCBI tblastx and analyzed using genoPlotR (Guy et al., 2010).

Whole mount in situ hybridization

Chromosomal DNA was isolated from *A. australis* blood using a DNeasy Blood & Tissue Kit (Qiagen). Riboprobe template fragments, including a T7 RNA polymerase promoter, were PCR amplified from chromosomal DNA using the following primer sets: *HoxB9a* forward (5'-TGAAACCGAAGACCCGAC-3'), *HoxB9a* reverse (5'-GAAATTAATACGA CTCACTATAGGGCTGAGGAAGACT CCAA), *HoxD12b* forward (5'-TAATCTTCTCAGTCCTGGCTATG-3'), *HoxD12b* reverse (5'-GAAATTAATACGACTCACTATA GATCCAAGTTTGAAAATTCA TATTTGC-3'), *HoxC13a* forward (5'-CACCTTGA TGTACGTGTATGAAAA-3'), *HoxC13a* reverse (5'-GAAATTAATACGACTCACTATAGGCTCCGTGTATTTC TCTGACG-3'). Digoxygenin-labelled riboprobes were made according to standard protocols using T7 RNA polymerase. Whole mount *in situ* hybridization with labelled riboprobes was performed at 70 °C, according to a slightly modified version of a standard protocol (Hiroi et al., 2004). Hybridizing riboprobes were made visible using anti-Digoxigenin AP and BM Purple AP substrate (Roche). Stained embryos were bleached using hydrogen peroxide (Sigma-Aldrich) and photographed using a Leica M205 FA stereo microscope.

Results

Genome assembly of the European eel

We have sequenced and assembled the genome of a female juvenile *A. anguilla* specimen caught in the brackish Lake Veere, the Netherlands in December 2009. Its haploid genome size was determined to be 1.1 Gbp. Because of the impossibility of breeding *A. anguilla*, no genetic linkage information is available. We therefore employed Illumina Genome Analyzer sequencing technology only in the assembly of a draft genome. Based on a *de novo* genome assembly, we constructed 923 Mbp of scaffolds with a length-weighed median fragment length (N50) of 78 Kbp (Figure S1 and Table S1). An additional 179 Mbp of initial contigs, which are

either very small or highly repetitive, were excluded from scaffolding, but included in all further analyses.

Identification of Hox transcripts and genes

To identify *A. anguilla* Hox genes, we used a *de novo* assembled transcriptome of a 27 hours post-fertilization (hpf) embryo of the short-finned eel, *A. australis*. This species is closely related to *A. anguilla* (Minegishi et al., 2005), yet produces viable embryos more easily (Burgerhout et al., 2011). We compared Hox-like sequences from the transcriptome to the genome assembly using Blast (Altschul et al., 1990), which yielded ten genomic scaffolds (Table S2) that were further examined for the presence of additional genes. This resulted in the identification of 73 Hox genes (twice as many as found in *A. japonica* in a previous study using PCR fragments (Guo et al., 2010)), including three pseudogenes, organized in eight clusters (Figure 2 and Table S3). The flanking regions of these eight clusters contain an additional 24 predicted genes (Figure 2). No further protein-coding genes were found within the Hox clusters.

Conserved microRNAs were discovered using Blast searches with human and zebrafish homologues (Figure 2). miR-10 is present posterior of Hox4 in six clusters (all except Aa and Ab). miR-196 is found between Hox9 and Hox10 in five clusters (all except Bb, Da and Db). This arrangement is consistent with that found in other vertebrates (Yekta et al., 2008; He et al., 2011).

Hox cluster identity

We based preliminary identification of clusters on homology between *A. anguilla* and *Danio rerio* protein sequences and comparisons with all sequences in the NCBI non-redundant protein database (Table S3). Whereas the two *A. anguilla* HoxA clusters can easily be matched to their corresponding HoxAa and HoxAb orthologues in *D. rerio*, each of the two HoxB and HoxC clusters of *A. anguilla* most closely resembles *D. rerio* HoxBa and HoxCa, respectively. Both *A. anguilla* HoxD clusters predictably match *D. rerio* HoxDa only, since the zebrafish HoxDb cluster has lost all protein-coding genes (Woltering & Durston, 2006).

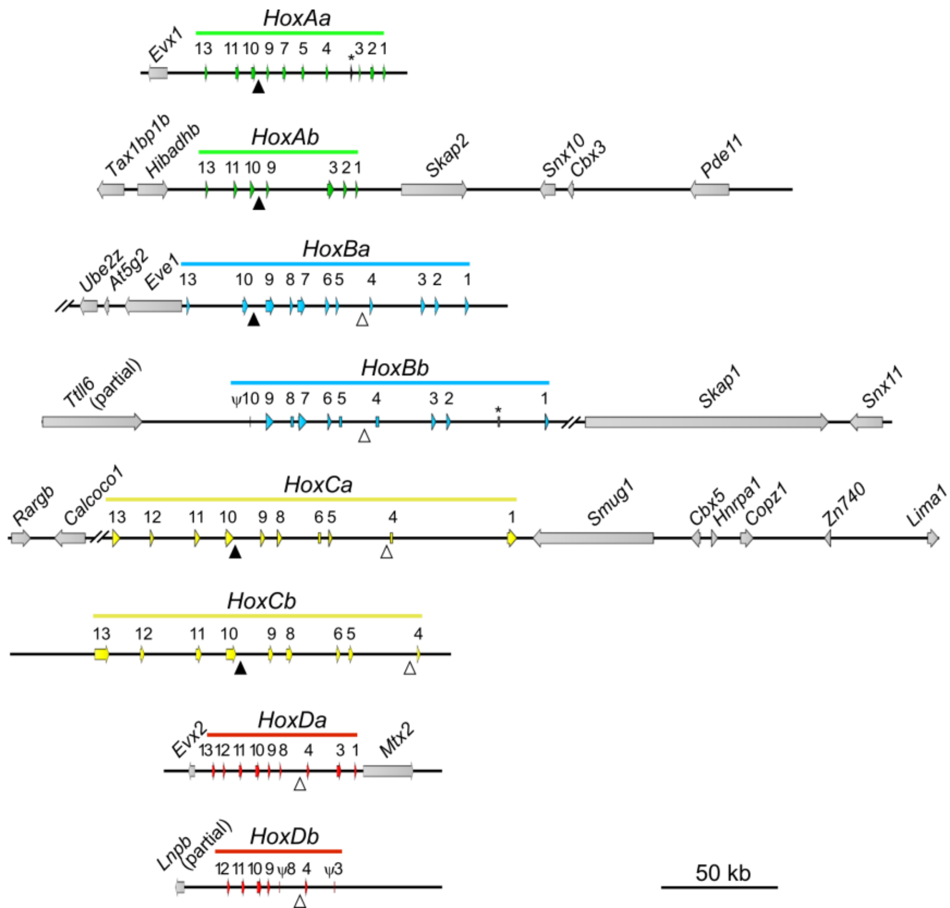


Figure 2. Genomic organization of the Hox gene clusters of the European eel. Scaffolds are indicated by black lines and asterisks represent two gaps between scaffolds. Hox genes are indicated by colored arrows that are numbered according to their paralogous groups. Three pseudogenes are indicated by the symbol ψ. Neighboring genes are indicated by grey arrows. Conserved microRNA genes are indicated by triangles: miR-196 (closed triangles) and miR-10 (open triangles).

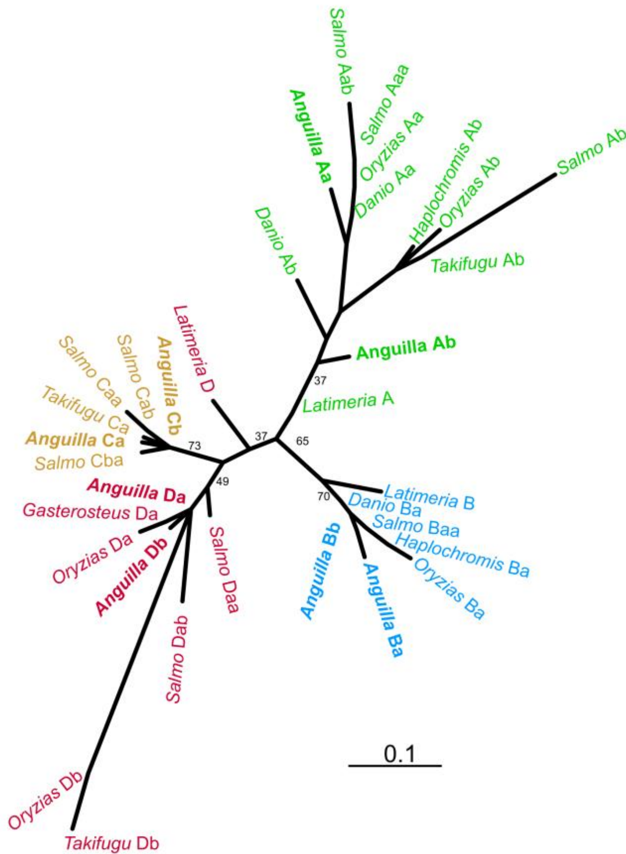


Figure 3. Classification of the European eel Hox clusters. An unrooted phylogenetic tree showing the relationships between *A. anguilla* and fish Hox9 paralogues. Numbers indicate bootstrap support.

To more precisely assign the Hox genes to proper cluster orthologues, we generated unrooted maximum likelihood phylogenetic trees for paralogous group 9 (Figures 3 and S2), of which *A. anguilla* possesses all eight copies. These confirmed the preliminary classification in A, B, C and D paralogous groups, but failed to validate the identity of teleost a and b cluster duplicates (with the exception of HoxAa and HoxAb). Likewise, phylogenetic trees based on multi-gene alignments do not conclusively indicate either a or b cluster membership for HoxB, HoxC and HoxD (Figure 4). In general, there appears to be a lack of

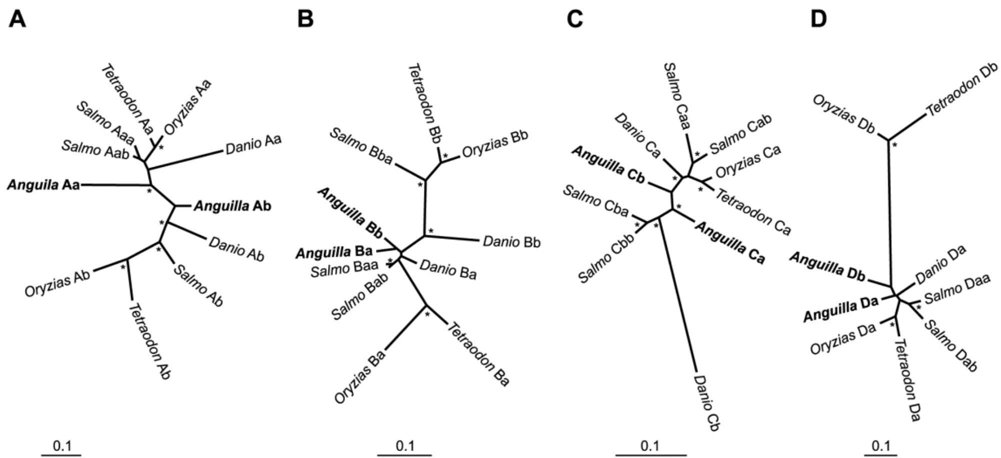


Figure 4. Phylogeny of Hox clusters of the European eel. Unrooted phylogenetic trees based on alignments combining multiple Hox genes per cluster. A) Cluster A relationships, based on HoxA9, HoxA11 and HoxA13 genes. B) Cluster B relationships, based on HoxB1, HoxB5 and HoxB6 genes. C) Cluster C relationships, based on HoxC6, HoxC11, HoxC12 and HoxC13 genes. D) Cluster D relationships, based on HoxD4 and HoxD9 genes. Species included: *A. anguilla*, *Salmo salar* (Atlantic salmon), *Danio rerio* (zebrafish), *Oryzias latipes* (medaka), and *Tetraodon nigroviridis* (green spotted puffer). Asterisks indicate bootstrap support > 90%.

sequence divergence between eel Hox gene duplicates, which makes classification based on coding sequence alone inaccurate.

Final orthologous relationships could only be established on the basis of conserved local synteny between Hox clusters and flanking genes (Figure 5). In addition to both HoxA clusters, eel HoxBa and HoxBb appear orthologous with their respective teleost equivalents. This identification is further supported by the absence of miR-196 from both *D. rerio* and *A. anguilla* HoxBb clusters. The affinities of HoxC and HoxD duplicates remain difficult to resolve because of conserved synteny around a and b cluster duplicates, and extensive cluster reduction and deletion in other teleosts (Figure 5c, d).

Hox gene expression

In order to confirm the transcriptional activity of the Hox genes, we determined relative expression levels by aligning transcriptomic reads of the 27 hpf embryo against the Hox protein-coding regions (Figure 6a). Transcriptome reads mapped unambiguously to 71 out of 73 Hox genes, including one pseudogene (ψ *HoxD3b*), suggesting that all *A. anguilla* Hox protein-coding genes are functional. The relative expression levels vary over five orders of magnitude with the lowest expression observed for the posterior paralogous groups 12 and 13, and the highest expression for paralogous groups 7–9, but with particularly high expression levels for *HoxB1a*, *HoxB1b*, *HoxB4b* and *HoxD1a*.

Full mRNA-seq read alignment to the entire Hox clusters indicated that transcriptional activity is not restricted to protein coding regions (Figure S3). In fact, intergenic expression sometimes exceeds intragenic levels, supporting the observation that complete Hox clusters function as meta-genes (Duboule, 2007; Mainguy et al., 2007).

At 27 hpf, expression of posterior Hox genes is very low (Figure 6a). We therefore confirmed transcriptional activity of posterior Hox paralogues by whole mount *in situ* hybridizations (Figure 4b). *HoxB9a* is expressed at 26 and 48 hpf, with an anterior expression boundary coinciding with somite number 5/6. Expression of *HoxD12b* and *HoxC13a* is not yet detectable at 48 hpf, but clearly visible at 96 hpf with anterior expression boundaries located at somite numbers 65/70 and 90/95 for *HoxD12b* and *HoxC13a*, respectively. For these Hox genes, expression in the eel embryo appears to conform to the expected spatio-temporal pattern (colinearity between cluster organization and developmental timing and localization), with expression of Hox12 and Hox13 paralogues appearing later in development, and more posterior than Hox9.

The evolution of Hox cluster organization

The early branching of the Elopomorpha from the main teleost trunk allows a new reconstruction of ancestral Hox cluster architectures (Figure 7), which are strongly constrained by the limited organizational divergence between eel HoxB, C and D duplicates.

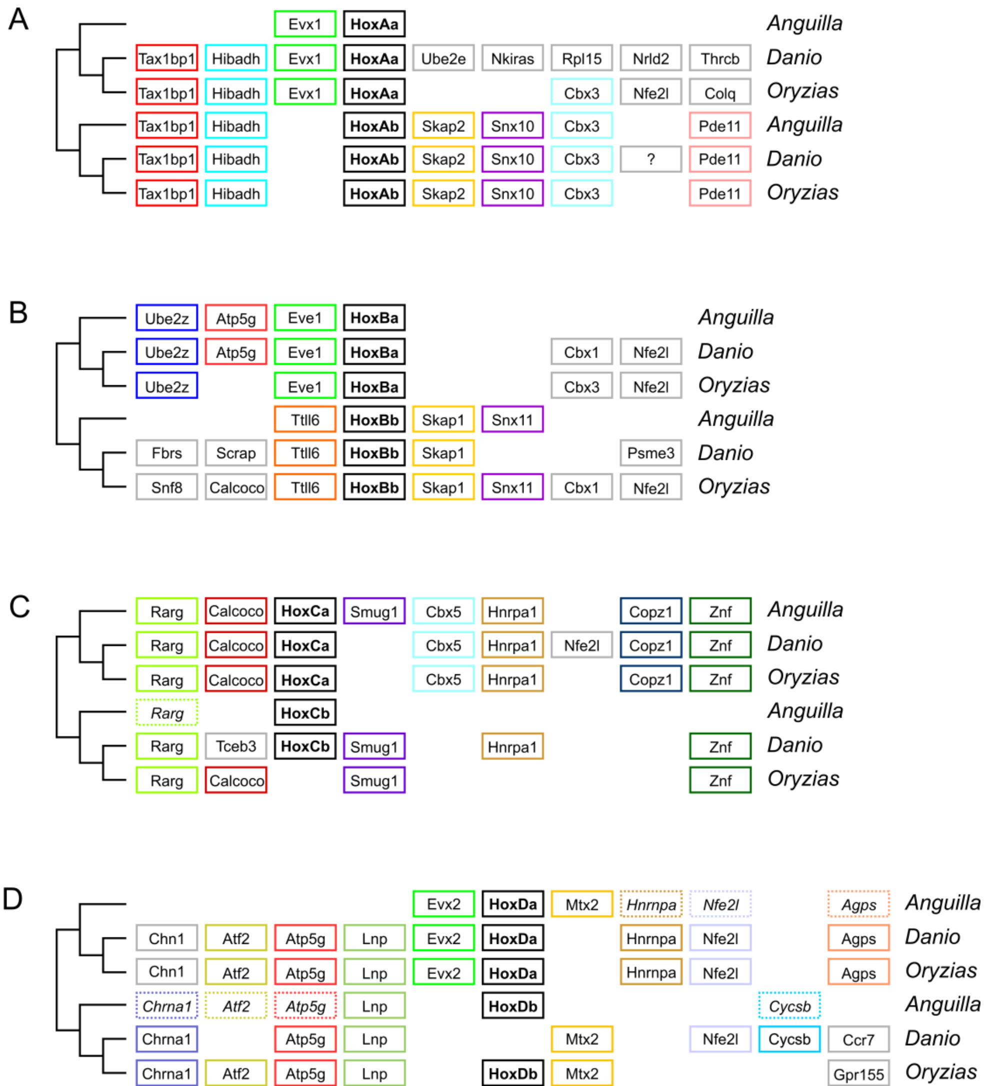

A
Evx1 HoxAa Anguilla
Tax1bp1 Hibadh Evx1 HoxAa Ube2e Nkiras Rpl15 Nrld2 Thrcb Danio
Tax1bp1 Hibadh Evx1 HoxAa Cbx3 Nfe2l Colq Oryzias
Tax1bp1 Hibadh HoxAb Skap2 Snx10 Cbx3 Pde11 Anguilla
Tax1bp1 Hibadh HoxAb Skap2 Snx10 Cbx3 ? Pde11 Danio
Tax1bp1 Hibadh HoxAb Skap2 Snx10 Cbx3 Pde11 Oryzias
B
Ube2z Atp5g Eve1 HoxBa Anguilla
Ube2z Atp5g Eve1 HoxBa Cbx1 Nfe2l Danio
Ube2z Eve1 HoxBa Cbx3 Nfe2l Oryzias
Ttll6 HoxBb Skap1 Snx11 Anguilla
Fbrs Scrap Ttll6 HoxBb Skap1 Psme3 Danio
Snf8 Calcoco Ttll6 HoxBb Skap1 Snx11 Cbx1 Nfe2l Oryzias
C
Rarg Calcoco HoxCa Smug1 Cbx5 Hnrpa1 Copz1 Znf Anguilla
Rarg Calcoco HoxCa Cbx5 Hnrpa1 Nfe2l Copz1 Znf Danio
Rarg Calcoco HoxCa Cbx5 Hnrpa1 Copz1 Znf Oryzias
Rarg HoxCb Anguilla
Rarg Tceb3 HoxCb Smug1 Hnrpa1 Znf Danio
Rarg Calcoco Smug1 Znf Oryzias
D
Evx2 HoxDa Mtx2 Hnrnpa Nfe2l Agps Anguilla
Chn1 Atf2 Atp5g Lnp Evx2 HoxDa Hnrnpa Nfe2l Agps Danio
Chn1 Atf2 Atp5g Lnp Evx2 HoxDa Hnrnpa Nfe2l Agps Oryzias
Chrna1 Atf2 Atp5g Lnp HoxDb Cycsb Anguilla
Chrna1 Atp5g Lnp Mtx2 Nfe2l Cycsb Ccr7 Danio
Chrna1 Atf2 Atp5g Lnp HoxDb Mtx2 Gpr155 Oryzias

Figure 5 (opposite page). Synteny around Hox clusters. Conservation of flanking genes supports the classification of *A. anguilla* clusters into different orthologous subgroups. The eel clusters and up to seven flanking genes are compared with the genomic organization in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Coloured box outlines indicate preserved synteny between eel and the two other species, dotted outlines denote flanking genes found on extended eel scaffolds (see Methods). Interpretation should take into account residual synteny between a and b paralogous clusters. Limited data is available on HoxCb (lost in *O. latipes*, possibly misassembled in *D. rerio*) and HoxDb (lost in *D. rerio*) clusters.

Since teleost fish are believed to have experienced the TSGD event early in their evolutionary history (Jaillon et al., 2004; Meyer & van de Peer, 2005), their genomes should in theory possess up to eight cluster duplicates. However, all teleosts examined in detail retain at most seven clusters of protein-coding genes (Duboule, 2007): a HoxC duplicate was lost in the lineage leading to medaka and pufferfish, a HoxD duplicate in the lineage represented by zebrafish. The high number of clusters in salmon is the result of relatively recent further duplications (Mungpakdee et al., 2008).

The main teleost lineages diverged briefly after the TSGD (Crow et al., 2006). The reconstruction in Figure 7 demonstrates that nearly all post-duplication gene loss events in the eel's ancestry occurred within this interval, followed by millions of years of stasis. Only the HoxAb cluster appears to have accumulated major changes in pre-branching, post-genome duplication teleosts. Alternative hypotheses, in which a whole-genome duplication is not shared between elopomorphs and advanced teleosts, or in which the genome duplication is followed by successive deletion and duplication of specific clusters in the eel, are less parsimonious and not consistent with local conservation of synteny (Figure 5).

Discussion

Two rounds of Hox cluster duplications in chordates are believed to be responsible for important vertebrate novelties (e.g. brains, heads, jaws) and increases in complexity (Holland et al., 1994). A plausible mechanism invokes a

temporary relaxation of meta-genic cluster constraints after duplication, paving the way for innovation (Wagner et al., 2003; Lynch & Wagner, 2008). In contrast, the TSGD-associated third duplication of vertebrate Hox clusters theoretically endowed teleost fish not with additional complexity within individuals, but with increased prospects for morphological diversification between individuals and species (Duboule, 2007; Kuraku & Meyer, 2009). In support of this hypothesis, advanced teleosts have extensively pruned their Hox surplus, leading to significant diversity in cluster structure (Figure 7). In all examined representatives (with the exception of salmon (Mungpakdee et al., 2008)), the residual number of Hox genes is not much higher than the non-duplicated count in tetrapods. The resulting teleost Hox cluster architectures have been interpreted as an evolutionary choice for developmental flexibility in a trade-off with robustness(Duboule, 2007). By proving that it is possible for a vertebrate to stably preserve eight densely populated (Figure 2) and functional (Figure 6) Hox clusters, the eel genome presents an exception to these models, and a third alternative in the evolution of vertebrate complexity.

For hundreds of millions of years, *A. anguilla* and its ancestors have maintained the highest ontogenic potential of any vertebrate, indicative of continuous selective pressure. However, as adults, they do not display markedly more complex bodies than other fish or tetrapods. The eel's distinctive life cycle and body plan suggest three (not mutually exclusive) explanations for this cryptic complexity.

Hox genes are involved in the primary patterning of the body axis, which implies a functional role for *A. anguilla*'s Hox surplus in axial elongation. Alterations in Hox genes have been associated with elongated body plans (Woltering et al., 2009; Di-Poï et al., 2010), however the changes observed are of a regulatory nature, and do not involve extra genes. For example, elongation of the body axis in snakes has been linked to a spatial relaxation in the posterior end of Hox clusters facilitated by the insertion of transposable elements between genes (Di-Poï et al., 2010). In addition, even the elongate members of the Elopomorpha (which also includes non-elongated tarpons, bonefish and others) display considerable diversity in the developmental mechanisms resulting in axial lengthening (Mehta et al., 2010). Hence, the eel's adult body plan cannot explain

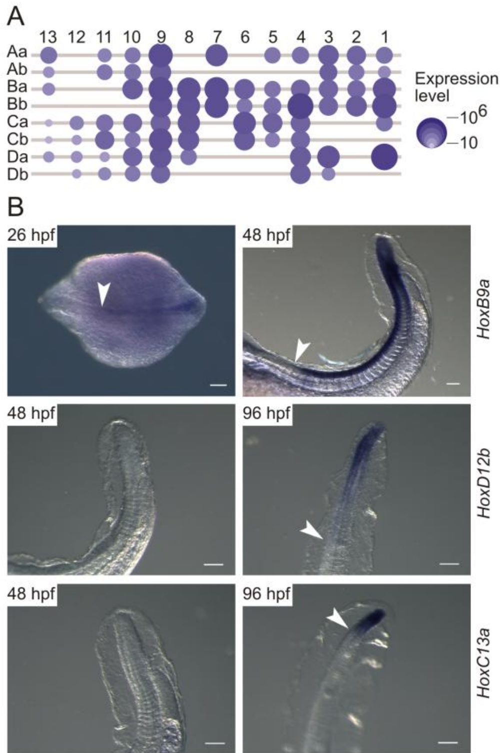
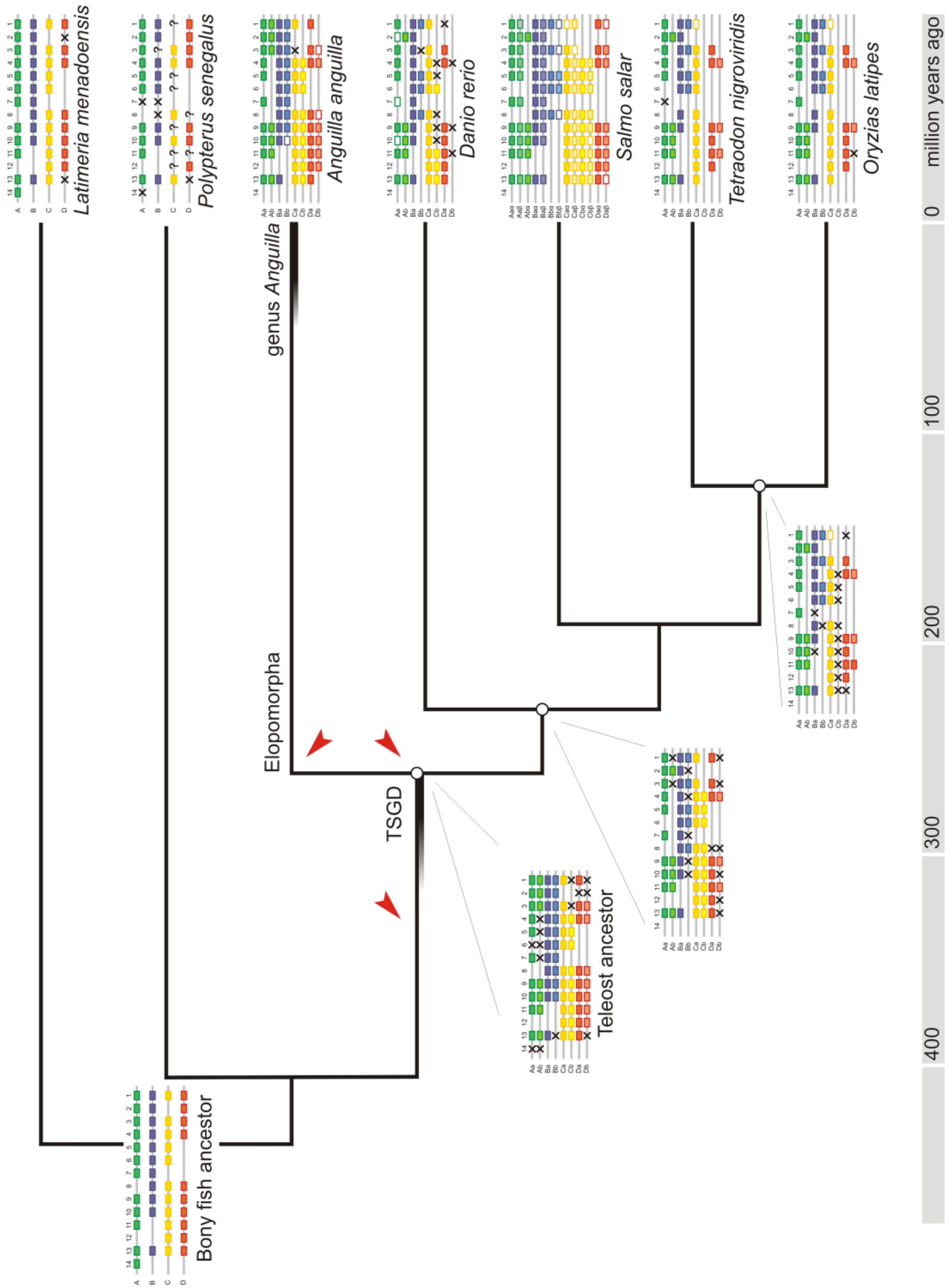


Figure 6. Hox gene expression in *A. australis* embryos. A) mRNA-seq-based gene expression in a 27 hpf embryo. B) Whole mount *in situ* hybridizations showing the expression of *HoxB9a*, *HoxD12b* and *HoxC13a*. *HoxB9a* expression can be detected in 26 hpf (dorsal view) and 48 hpf (lateral tail region view) embryos. *HoxD12b* and *HoxC13a* display expression in the tail region (lateral views) at 96 hpf, but not at 48 hpf. White arrowheads indicate anterior expression boundaries. Scale bars correspond to 100 μm.

the preservation of primitive Hox clusters between the TSGD (226–316 million years ago (Hurley et al., 2007)) and the origin of the genus *Anguilla*, estimated at 20–50 million years ago (Minegishi et al., 2005). Similarly, if the European eel may at present experience singular evolutionary forces because of its panmictic population (Als et al., 2011), any explanation these offer does not extend beyond the genus *Anguilla* of freshwater eels (Inoue et al., 2010).

Even if for most of their lives eels are eel-shaped, as ocean-dwelling larvae (Miller, 2009) their body plan is radically different (See Chapter 1, Figure 1). In fact, until the late nineteenth century, these large, long-lived, laterally compressed leptocephali were considered to be autonomous pelagic species (Cunningham, 1897). Fully transparent and slowly metabolizing, a leptocephalus provides considerable survival benefits (Pfeiler, 1999; Bishop & Torres, 1999). After approximately one year, they undergo a dramatic metamorphosis (Otake, 2003),



Latimeria menadoensis
Polypterus senegalus
Anguilla anguilla
Danio rerio
Salmo salar
Tetraodon nigroviridis
Oryzias latipes
genus Anguilla
Elopomorpha
TSGD
Teleost ancestor
Bony fish ancestor
million years ago
0
100
200
300
400

Figure 7 (opposite page). Model for the evolution of teleost Hox gene organization. Schematic Hox clusters (Kuraku & Meyer, 2009; Mungpakdee et al., 2008; Raincrow et al., 2011) are superimposed on a species phylogeny with estimates of divergence times (Inoue et al., 2005; Matschiner et al., 2011) – which vary considerably between studies (Hurley et al., 2007). Ancestral architectures are inferred on the basis of maximum parsimony, i.e. the number of cluster duplications and gene loss events is minimized. *Salmo salar* (Atlantic salmon) has presumably lost several duplicate clusters (Mungpakdee et al., 2008) (not shown). Deduced gene loss in a lineage is illustrated by a cross, question marks denote uncertainty about cluster gene content in the pre-TSGD actinopterygian *Polypterus senegalus* (bichir). Arrows indicate the possible origins of the leptocephalus body plan.

including extensive tissue remodelling and shortening of the body, resulting in cylindrical juveniles. In the early embryos investigated here (Figure 6), nearly all Hox genes are expressed and presumably functionally involved in determining cell fate. Logically, a high gene and cluster count can be explained by the assumption that the eel's two body plans are simultaneously outlined at this stage.

Leptocephali are the fundamental developmental innovation shared by all Elopomorpha (Greenwood et al., 1966; Inoue et al., 2004; Nelson, 2006), and therefore arose either before or soon after the TSGD, or at the base of the lineage (arrows in Figure 7). The last alternative is the most parsimonious (no loss of developmental complexity in advanced teleosts), especially since no member of the Elopomorpha is known to have ever discarded the leptocephalous larval stage (Greenwood et al., 1966; Nelson, 2006). Regardless, either of the post-TSGD origins is compatible with an intercalation model of indirect development (Raff, 2008), in which a temporary excess of developmental potential was permanently recruited for the conception of an additional body plan. Although speculative, an explanation invoking the morphological challenges associated with a complex life history is consistent with the stable high Hox gene and cluster count found in the anadromous Atlantic salmon (Mungpakdee et al., 2008).

Further functional studies on eel development will become possible once *A. anguilla*'s life cycle can be completed in captivity. In particular, there exists considerable variation in development (timing, number of somites) between leptocephali of related and interbreeding *Anguilla* species (Tesch, 2003; Miller,

2009), which can only be studied when these larvae can be raised under controlled conditions (Tanaka et al., 2003; Oliveira & Hable, 2010).

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Chapter 5

Biomarkers for broodstock selection of farmed female European eels (*Anguilla anguilla*)

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Abstract

In captivity, European eels (*Anguilla anguilla*) can only be reproduced artificially by applying hormonal treatments with pituitary extracts or purified gonadotropins, which takes 4-6 months. However, female eels show a high individual variation in responsiveness to the treatment, ranging from no response at all to the production and release of eggs. The number of non-responders is often higher than 50%. Response to treatment is probably related to the initial maturation state of the female. In order to increase reproductive success, broodstock needs to be selected for responsive females, either prior to or in the early phase of the treatment. The goal of the present study was to identify markers for broodstock selection.

Farmed silver eels were treated with pituitary extract and sampled at different time intervals. Expression of steroidogenic genes in ovarian tissue of responding and non-responding eels was examined using a custom-built microarray based on the European eel genome sequence. In addition, blood plasma levels of sex steroid hormones were measured using ELISA. By using those techniques, we were able to identify candidate biomarkers to distinguish responders from non-responders.

It is concluded that the expression level of steroidogenic enzymes within ovarian tissue may be used as broodstock selection marker. Additionally, based on relative fold increase of E2 blood plasma levels after 4 weekly injections responsive female eels may be selected.

Introduction

The catadromous European eel (*Anguilla anguilla*) shows an intriguing life-cycle. Migration occurs from the European and North-African freshwaters to the spawning area in the Sargasso Sea (Schmidt, 1923, Tesch, 2003). In freshwater, juvenile (yellow-stage) eels start their growth phase, which lasts for 5-50 years (Tesch, 2003). Prior to their return to the spawning area, the yellow eels change morphologically and physiologically, thereby adapting to the oceanic phase; a process called silvering (silver-stage) (Tesch, 2003; Durif et al., 2005).

Over the last decades, the populations of several eel species have shown a drastic decline (Stone, 2003; Dekker, 2003), which is probably due to a combination of several anthropogenic factors (e.g. overexploitation, pollution, migration barriers) and biotic factors (e.g. swim bladder parasite, viruses) (reviews in van den Thillart et al., 2009). As the European eel (*Anguilla anguilla*) was recently added to the IUCN red list of endangered species (Freyhof & Kottelat, 2008), management of the eel population and sustainable aquaculture have a high priority.

Eels do not mature naturally in captivity and artificial reproduction is currently only possible by applying hormonal treatments with gonadotropins (GtH) and pituitary extracts (PE) (Ohta et al., 1996, 1997; Tanaka et al., 2001, 2003; Pedersen, 2003, 2004; Palstra et al, 2005, Kagawa et al., 2005; Oliveira & Hable, 2010). Although recently the life cycle of Japanese eel (*A. japonica*) in captivity was closed by producing a second generation of glass eel, success rates are still low (Ijiri et al., 2011).

Artificial maturation of female European eels is induced by 11-29 weekly hormone injections (Pedersen, 2003, 2004; Palstra et al., 2005). There is a wide variety in responsiveness to the hormonal treatment (Pedersen, 2003, 2004; Palstra et al., 2005) and often >50% of broodstock females do not reach full maturation (referred to as non-responding eels; Palstra & van den Thillart, 2009; Burgerhout et al., unpublished data). Non-responding eels cause a waste of time, labour, space and expensive hormones and especially unnecessary use of animals. For the Japanese eel it was shown that yellow eels do not respond, while silver stage 1 and stage 2 eels respond with approximately 80% and 100% efficiency, respectively (Okamura et al., 2008). However, silver stage 2 Japanese eels are more advanced in their maturation state as compared to silver European eels, which is reflected by the differences in gonadosomatic index at the onset of their

migration, namely 2-4% for Japanese eel and <2% for European eel (Durif et al., 2005; Okamura et al., 2007). Response to treatment is therefore suggested to be dependent on the initial state of maturation of the females (see Durif et al., 2006).

In order to increase reproductive success rates of European eels, broodstock needs to be selected for females responding to the hormonal treatment, either prior to or early during the treatment. The recently published draft genome sequence of the European eel (Henkel et al., 2012) provides a perfect tool for gene expression profiling. Recently, RNA-seq transcriptomic analysis of gonad tissue derived from different maturation stages of female European eel (yellow, silver and after spawning), revealed that 2% of the expressed genes were specific for a maturation stage (Minegishi et al., unpublished data). Interestingly, the majority of those differentially expressed genes were involved in the steroidogenic pathway (Minegishi et al., unpublished data). In addition, it was shown that during artificial maturation, levels of sex steroids – 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) – increase significantly (e.g. Lokman et al., 1998, 2001; Matsubara et al., 2005, Chiba et al., 2007). Therefore, it was hypothesized that steroidogenic gene expression levels and sex steroid levels may provide biomarkers for broodstock selection.

In the present study, expression of genes involved in steroidogenesis was examined by microarray analysis of ovarian tissue derived from responding and non-responding eels subjected to hormonal treatment. In addition, it was examined whether blood plasma levels of sex steroids could be linked to those steroidogenic genes studied and whether endogenous GtH levels would be suitable as selection markers.

Methods

Animals

Three year old cultured female European eels (n=40, 700.5 ± 21.9 g; 67.6 ± 0.6 cm (mean ± standard error)) were obtained from a commercial eel farm (Passie voor Vis, Sevenum, The Netherlands). As an initial control, 8 females were sampled directly after transport (see Sampling procedure). Other eels were housed in a 1500L tank connected to a recirculation system, and acclimated to natural seawater (32 ± 1ppt, 21 ± 0.5°C) for two weeks. Eels were not fed during

acclimation and during the trial. The experiments conducted during this study complied with the Dutch law on animal experiments and were approved by the animal experimental committee of Leiden University (DEC# 11093).

Morphometry

Prior the trial, all eels were anesthetized in clove oil (dissolved 1:10 in 96% ethanol, dosage 1mL/L) and measured for morphometry including: body weight (BW), body length (BL), eye diameter horizontal and vertical (Edh and Edv, respectively) and pectoral fin length (PFL). The morphometric data was used to calculate the silver index (SI; Durif et al., 2005), eye index (EI; Pankhurst, 1982) and pectoral fin length index (PFLI, Durif et al., 2005). A blood sample was obtained using a heparin flushed needle and syringe (Sigma-Aldrich, Zwijndrecht, The Netherlands; 10.000 IU/mL, dissolved in 0.9% saline). Blood plasma was obtained by centrifuging the blood for 5 minutes at 4°C at 13200 RPM, and afterwards stored in a -80°C freezer until further analysis.

Maturation trial

Females (n=32) were weekly injected with 20mg salmon pituitary extract (SPE) following the protocol as described by Burgerhout et al. (2011). Ovulation was induced using 2mg kg^{-1} of 17α, 20β-dihydorxy-4-pregnen-3-one (DHP). Palstra et al. (2005) showed that 80-90% of wild European eel females ovulated between 12-18 weekly injections, therefore 18 injections was the maximum.

Sampling procedure

Eels were euthanized using clove oil (dissolved 1:10 in 96% ethanol, dosage 5 mL/L) followed by decapitation. Weight of the liver, gonads and digestive tract were measured. Gonad samples were stored in RNA-later (Ambion). These samples were kept overnight at 4^{0}C and stored in a -80^{0}C freezer. In addition, gonad samples from the same individuals were preserved overnight in paraformaldehyde (PFA) for histological analysis, and subsequently stored in 70% ethanol.

Gonadosomatic index (GSI), hepatosomatic index (HSI) and digestive tract somatic index (DTSI) were calculated with the following formula: Tissue index = (tissue weight / body weight) x 100.

After 4 and after 12 weekly injections, 8 females were sampled. From the other eels, a blood sample was obtained and external parameters were measured (see Morphometry) prior to the weekly SPE injection (7 days after the previous SPE injection). Finally, the 16 remaining eels were totally sampled either after 18 weekly injections or one day after ovulation.

Microarray probe design

Probes were designed using the eArray software from Agilent Technologies (earray.chem.agilent.com/earray) using the following settings: base composition methodology, best probe methodology and design with 3_ bias. Design was based on transcripts predicted from genome and transcriptome data for which two approaches were chosen. The first was to run AUGUSTUS v2.3.1 on the European eel genome scaffolds and on the unscaffolded contigs using RNAseq data from embryos and from gonads to validate gen predictions. This resulted in two fasta sequence files with predicted transcripts. One with the transcripts derived from the scaffolds and one with transcripts predicted in the unscaffolded contigs yielding 67063 and 17869 probes respectively.

The second approach was to use Tophat v1.1.4 to map the embryo and gonad RNAseq reads on the European eel genome scaffolds and unscaffolded contigs and use Cufflinks v0.9.3 to derive gene models from the mapped reads. The resulting annotations were used to extract the transcript sequences from the scaffolds and unscaffolded contigs. This resulted in one fasta sequence file with the Cufflinks predicted transcripts resulting in 89912 probes. The final custom Agilent array design contained 4 times 174844 probes excluding internal quality controls designed by Agilent.

Microarray analysis

After four weekly SPE injections, the ovaries from four females with a relatively high GSI (1.71-2.93) and two females with a relatively low GSI (0.62-0.82) were used for RNA isolation. One ovary sample of a female with relatively low GSI was used in duplo. These samples were labelled and hybridized with the custom designed microarrays according to Agilent's standard procedures (Microarray Department, University of Amsterdam).

Prior to analysis, probe names were reannotated by sequence alignment to predicted European eel transcripts. 120660 probes aligned to 43435 unique gene annotations. One array of the duplo samples was discarded after visual inspection of array images. The array data were analyzed in R/Bioconductor version 2.10, using the limma package version 3.12.3 (Smyth, 2005). Arrays were background corrected, normalized within arrays using the loess method, and quantile normalized using A-values (average spot intensities) between arrays (Smyth & Speed, 2003). If multiple probes assayed a single annotated transcript, spot values were averaged.

Plasma hormone measurements

FSH and LH levels were measured in blood plasma using a recently developed eel specific bioassay, based on the eel FSH- and LH-receptor (Minegishi et al., 2012). Human chorionic gonadotropin (hCG, Sigma-Aldrich, The Netherlands) is homologous to LH and was used as a positive control for the amount of plasma LH. SPE was used as a positive control for the amount of FSH in blood plasma. Blood plasma testosterone (T) and 17β-estradiol (E2) were measured using a T ELISA or E2 ELISA (HUMAN GmBH Worldwide Diagnostics) following manufacturer´s instructions.

Histological analysis

Gonad samples were dehydrated in a series of ethanol (70%-80%-90%-100%), followed by incubation in 100% Histo-Clear (National Diagnostics) and a 1:2 mixture of Histo-Clear and paraffin (Paraclear), respectively. The tissue samples were then embedded in paraffin. Sections (7 μm thick) were obtained using a Leica microtome (Leica RM2165) and, after rehydration, nuclei and cytoplasm were stained using Mayer's haematoxylin and eosin (H-E) staining. Stages of gametogenesis were determined according to the most advanced ooctye stage as described by Wallace & Selman (1981, see also Palstra et al., 2007).

Statistical analysis

First, all data was examined for normality using a Kolmogorov-Smirnov test. Morphometric data was found normally distributed (Kolmogorov-Smirnov, $p>0.05$) and was tested for significance at consecutive sampling points using two-

tailed ANOVA with post-hoc Bonferoni correction. As data of blood plasma hormones FSH, LH, E2 and T were not normally distributed (Kolmogorov-Smirnov, $p<0.05$), a Mann-Whitney U non-parametric test was used to analyze those results. Correlation analysis of blood plasma LH, FSH, E2 and T, and their absolute and relative changes with GSI was performed using two-tailed Pearson correlation tests. Statistical difference was considered significant at $p<0.05$. In all cases values are expressed as average ± standard error.

For microarray analysis, differential expression between responders (n=4) and non-responders (n=2) was calculated by fitting a linear model and calculating empirical Bayes statistics (Smyth, 2004).

Results

Morphometry

Based on EI, all 40 female eels that were used in this experiment were defined as silver eels (EI>6.5, Pankhurst, 1982). At the start of the experiment, one of the females was assigned yellow stage 2, 29 were assigned premigrant silver stage 3, and 11 were assigned migrant silver stage 4-5 following the silver index of Durif et al. (2005). At all consecutive sample points, the EI increased significantly as compared to the initial measurements (PRE) of each group that were taken prior to the weekly SPE injections (Table 1). The PFLI showed a significant increase after 18 weeks as compared to the PRE measurements (Table 1). As compared to the initial control gonad weight (GW), GSI, digestive tract weight (DTW) and DTSI were found significantly increased and decreased, respectively after 4, 12 and 18 weekly injections (Table 1). Based on increase in GSI at time of sampling after 4, 12 and 18 weekly SPE injections, approximately 60% of the females (19 out of 32) showed a response to the hormonal treatment (Table 1).

Histological analysis

The furthest developed oocytes of the initial control (t=0) represented the cortical alveoli stage 3 (Figure 1a). Incorporated lipid droplets were dispersed around the nucleus. The nucleus was found centred with nucleoli in the periphery. Yolk granules were not present, indicating that vitellogenesis was not initiated.

After 4 weekly injections, oocytes were still in previtellogenic stage. Two

Table 1. Overview of morphometric parameters of farmed female eels treated with pituitary extracts, i.e. 0, 4, 12 and 18 weeks. As initial control eight females were sampled. PRE: data at t=0 from corresponding individuals sampled at t=4, 12 and 18 weeks. POST: sampled after 4, 12, 18 weeks. The 18 weeks group includes females 1 day after ovulation. Response indicate responding females showing a relative increase in GSI. Bold characters indicate statistical differences between PRE and POST measurements; statistical differences between POST and initial control are indicated by asterisk (*).

	Initial control (n=8)		4 weeks (n=8)				12 weeks (n=8)				18 weeks (n=16)			
			PRE		POST		PRE		POST		PRE		POST	
	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
BW	794,0	41,7	702,5	45,1	686,5	44,7	684,9	60,2	627,0*	51,8	612,5	38,6	531,3*	38,4
BL	69,2	1,3	68,4	1,6	68,8	1,6	67,0	1,8	67,6	1,7	65,6	1,2	65,7	1,4
EI	9,0	0,5	8,5	0,3	**10,1**	0,5	8,8	0,6	**11,4***	0,6	9,8	0,5	**13,8***	1,1
PFLI	36,6	1,5	35,1	1,7	38,6	0,7	36,0	1,2	37,7	2,7	37,8	0,6	**42,3***	0,8
SI	3,4	0,2	3,1	0,1	3,4	0,3	3,3	0,3	4,0	0,3	3,3	0,3	4,6*	0,3
LW	6,8	0,7			6,0	0,6			6,3	0,7			5,7	1,1
GW	8,2	1,0			13,3*	2,4			36,1*	11,8			50,5*	19,9
DTW	12,4	1,7			4,8*	0,5			4,0*	0,7			3,0*	0,7
HSI	0,8	0,1			0,9	0,1			1,0	0,1			1,0	0,2
GSI	1,0	0,1			1,9*	0,3			5,9*	2,0			8,6*	2,9
DTSI	1,6	0,2			0,7*	0,1			0,6*	0,1			0,6*	0,1
RESPONSE					6				3				10	

females with a relatively low GSI (0.6 and 0.8) showed small oocytes with incorporated lipid droplets similar to those found in the initial control (Figure 1b). Oocytes of females with highest GSI (1.7-2.9) showed an increase of incorporated lipid droplets, which were more dispersed in the cytoplasm. The diameter of those oocytes was up to two-fold enlarged. Yolk granules were not present (Figure 1c).

Based on presence of yolk granules, early and mid-vitellogenic stage oocytes were observed after 12 weekly injections in gonad tissue of females with a GSI of 8.5 and above (Figure 1d). Oocytes of females with a GSI<1.6 were found in pre-vitellogenic stage as no yolk granules were incorporated. Few yolk granules were observed in the periphery of the ooplasm in oocytes of females with GSI>3.3, indicating early vitellogenic stage.

At the end of the trial, i.e. after ovulation or after a maximum of 18 weekly injections (18 week group), females showing a full response to the treatment could be distinguished. Six out of 16 eels of the 18 week group

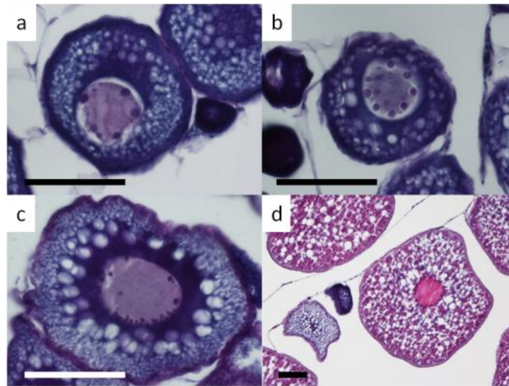

Figure 1. Histological sections of HE-stained oocytes. a. Initial control showing furthest oocyte stage: cortical alveoli stage 3. b. Non-responder after 4 weekly injections showing similar oocytes as initial control. c. Responder after 4 weekly injections showing enlarged oocytes with increased incorporation of lipid droplets as compared to initial control. d. Responder after 12 weekly injections showing mid- or late vitellogenic stage oocytes with incorporated yolk granules. Scale bar = 100 µm.

ovulated after a final DHP injection. One female did not ovulate after DHP injection and was sampled the day after expected spawning. Three of the 16 females showed a partial response to the hormonal treatment, namely an increase in gonad weight and a GSI of 17.0 and higher (17.0-34.0), and oocytes up to late vitellogenic stage were observed. However, these females did not ovulate after 18 weekly injections. Six females did not respond or slowly responded to the treatment after 18 weekly injections as the relative GSI did not increase above 6.3 (range 0.9-6.3). Non-responding females (GSI 0.9-2.6) showed oocytes still in previtellogenic stage. Oocytes of slowly responding females (GSI 3.9-6.3) showed pre- and early vitellogenic stages.

Gene expression profile: steroidogenesis

Recent RNA-seq transcriptomic analysis of wild female European eel showed that the major differentially expressed genes during natural maturation (yellow and silver stages) and artificial maturation (spawned stage) within ovarian tissue are involved in the steroidogenic pathway (Minegishi et al., unpublished data). In this study, ovarian RNA of eels that were injected with four weekly SPE injections was

analyzed using microarrays. Minegishi et al. (unpublished data) identified 207 genes as putatively involved in the steroidogenic pathway, 140 of which were assayed using the microarray. We focused on differences in expression level of steroidogenic genes between responders and non-responders (Table 2). Based on fold change (FC) and average expression levels of the following genes was found up-regulated in responders as compared to non-responders: cholesterol side-chain cleavage cytochrome P450 (P450scc), cytochrome P450c17 (17α-hydroxylase and 17, 20-lyase), 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1), cytochrome P45011β, 17β-HSD3, 11β-HSD2. The expression level of one of the 4 genes encoding P450c17 was found up-regulated, although not significantly. The expression level of one of four genes encoding 3β-HSD was found down-regulated as based on FC values, however the difference between responders and non-responders was not significant. Other examined genes within the steroidogenic pathway showed no significant difference, and low FC values (^{2}logFC<0.20).

In addition, the expression level of one gene encoding for the LH-receptor was found up-regulated in responders as compared to non-responders. No significant difference was found for the expression of the FSH-receptor between responders and non-responders ($p>0.05$). The expression of estradiol-receptor 1 (esr1) was found significantly down-regulated in responders as compared to non-responders.

Table 2 (next page). Changes in expression levels of genes encoding steroidogenic enzymes, gonadotropin receptors (FSH-r and LH-r) and estradiol receptors (esr1) between responders (n=4) and non-responders (n=2) after 4 weekly injections. Responders and non-responders were distinguished based on GSI. β-Actin1 was added as reference gene. Gene ID refers to codes of predicted genes (see www.zfgenomics.com). Note that several gene IDs refer to the same gene. These are either different genes encoding similar proteins or the same gene fragmented on different contigs. The two gene IDs referring to P450arom are most likely the same gene. FC is the relative fold change in expression value between responders and non-responders expressed as ^{2}Log (FC>0 indicates a higher expression in responders, FC<0 indicates a lower expression in responders). Expr. is the average expression value between responders and non-responders expressed as ^{2}Log.

Gene ID	Name	FC	Expr.	p-value
g4682	P450scc	0,7333	7,09	0,104
g3996	3β-HSD	-0,7188	8,68	0,214
g15248	3β-HSD	0,1152	6,66	0,559
g33843	3β-HSD	-0,1548	12,69	0,605
g34859	3β-HSD	-0,1733	12,6	0,45
g22995	P450c17	0,3159	8,23	0,254
g10692	P450c17	1,1658	7,21	0,024
g38561	P450c17	1,1095	8,22	0,028
g16247	P45011β	0,6133	5,76	0,091
g33336	17β-HSD3	0,7038	10,19	0,017
g4873	17β-HSD3	-0,0132	9,08	0,945
g40434	17β-HSD1	0,9771	10,93	0,004
g9213	20β-HSD	-0,0961	10,27	0,59
g9348	P450arom	0,0307	8,2	0,869
g9349	P450arom	-0,0172	8,49	0,954
g12369	11β-HSD2	0,7004	9,22	0,036
g16135	11β-HSD2	-0,0719	8,13	0,733
g10501	FSH-r	-0,1403	6,49	0,501
g12514	LH-r	0,7287	6,17	0,08
g40338	LH-r	-0,1669	9,93	0,412
g19754	esr1	-1,4093	6,17	0,019
g23190	esr1	-0,1575	6,71	0,539
g144	β-actin 1	0,2043	15,77	0,42
g22021	**β-actin 1**	**0,1583**	**15,14**	**0,428**

Hormone blood plasma levels

We examined whether peptide and steroid hormone levels in blood plasma correlated with the maturation status of the gonads. The gonadotropins FSH and LH were measured using an eel-receptor specific bioassay (Minegishi et al., 2012). Mean FSH blood plasma levels (Figure 2a) significantly decreased over 12 weekly injections ($p<0.05$), which was followed by a significant increase in females sampled after ovulation or after 18 weekly SPE injections ($p<0.05$). Average LH

plasma levels (Figure 2b) showed a significant increase over 12 weekly injections ($p<0.05$), which was followed by a significant decrease afterwards ($p<0.05$).

ELISA analysis of plasma levels of sex steroids T and E2 (Figure 2c-d) revealed that both hormones increased significantly over 12 weekly injections ($p<0.05$). Although, average steroid plasma levels slightly increased afterwards, this was not significant as compared to the plasma levels at 12 weekly injections ($p>0.05$).

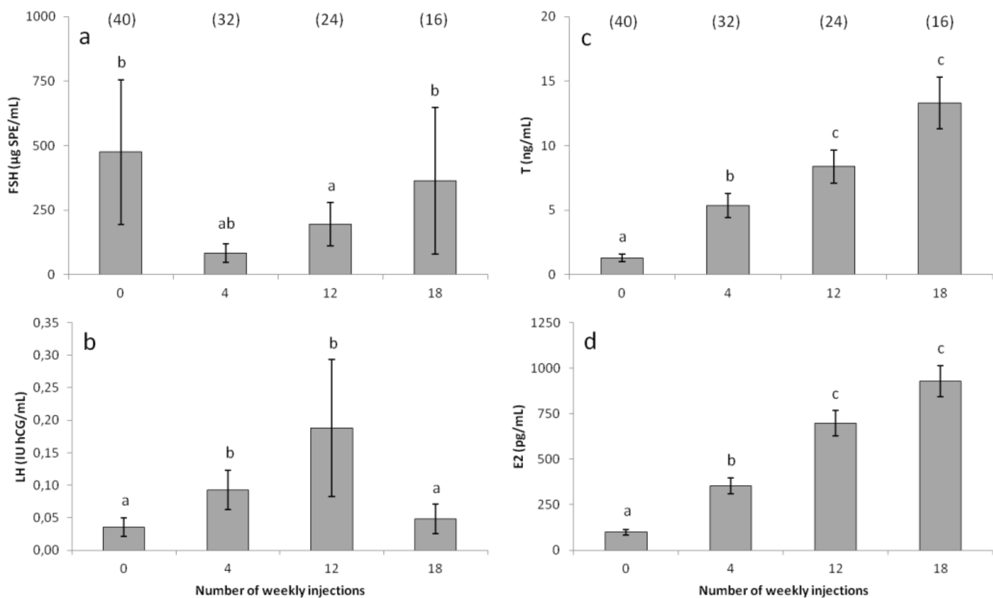

Figure 2. Changes in plasma hormone levels during artificially induced maturation in cultured female eels. a. Follicle-stimulating hormone (FSH). b. Luteinizing hormone (LH). c. Testosterone (T). d. 17β-Estradiol (E2). FSH and LH show a clear opposite regulation. FSH decreases over the first 12 weeks, while LH increases. Subsequently, FSH increases and LH decreases. T and E2 show both a significant increase in blood plasma levels over the first 12 weeks, followed by a slight non-significant increase after 18 weeks. Significant differences in hormone plasma levels between consecutive sample points are indicated by letters (Mann-Whitney U test; $p<0.05$). Number of males measured is indicated by values (n) at the top of the figure above each bar.

No significant correlations were found for LH, FSH and their absolute and relative changes at any sample point (data not shown). Significant positive correlations with GSI were found for E2, ΔE2 and rE2 after 4 weekly injections, and for E2 and ΔE2 after 12 and 18 weekly injections. T and ΔT were found significantly positively correlated with GSI after 12 and 18 weekly injections (Table 3). It was found that rE2 could be used to distinguish responders from non-responders after 4 (Figure 3a) and 12 (Figure 3b) weekly injections with a reliability of 80% and ca. 99%, respectively. Absolute E2 and ΔE2 levels showed a wide variation due to initial plasma levels (data not shown).

Discussion

The large variation in the response to weekly hormonal treatments of female European eels possibly results in low reproductive success rates. The maturation protocol of female European eels takes approximately 4-6 months, and the success rate is often less than 50%. This causes for example unnecessary use of animals, waste of time, space and expensive hormones. Therefore, it is of much interest for eel aquaculture to be able to select females prior to the hormone treatment or within the first few weeks of treatment. During the present study, female eels were subjected to a hormonal treatment for a maximum of 18 weeks. A custom-built microarray and blood plasma analyses were used to identify possible selection biomarkers.

Genetic biomarkers

As a limited number of samples were run on the microarray (responders, n=4; non-responders, n=2), the present results need to be considered as an indication for the found differences in responding and non-responding females. Microarray analysis of responders and non-responders showed that 5 out of 17 genes encoding steroidogenic enzymes were significantly up-regulated (Table 2). It needs to be noted that several predicted gene IDs refer to the same gene. These are either different genes encoding similar proteins or the same gene fragmented on different contigs. The two gene IDs referring to P450arom are most likely the same gene.

Based on average expression level and FC, 17β-HSD1 was found highly expressed in responders as compared to non-responders. In the developing ovary

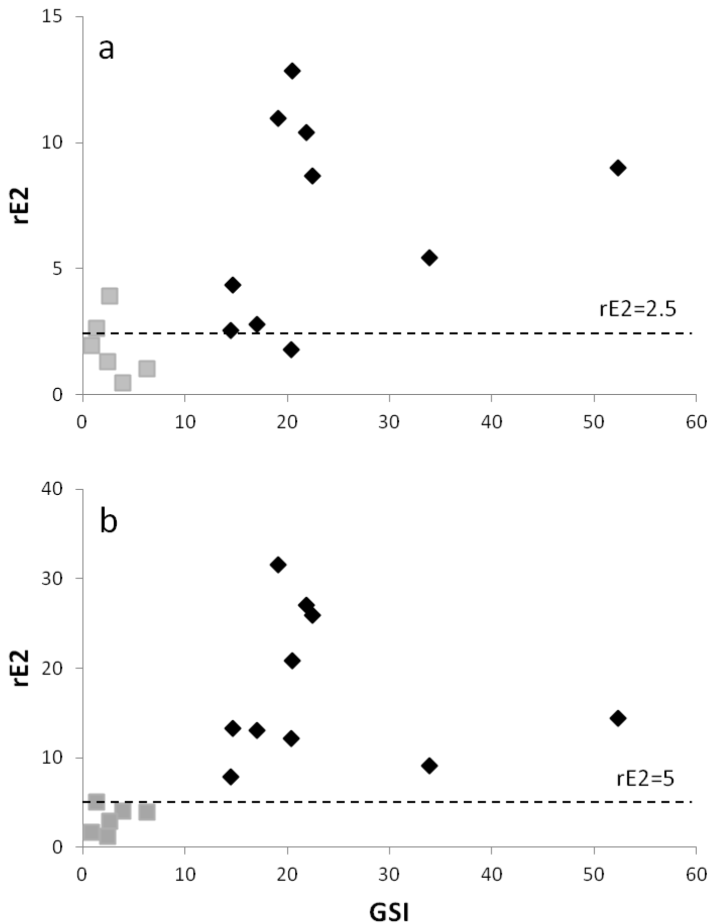

Figure 3. Relative fold increase of 17β-estradiol blood plasma level (rE2) at 4 weeks (a) and 12 weeks (b) as compared to initial measurements (t=0) of females sampled after ovulation or after 18 weekly injections with salmon pituitary extract. Responders (diamonds) and non-responders (squares) were distinguished based on GSI>10. Dotted line indicates a suggested threshold to select responders from non-responders.

this enzyme is responsible for the conversion of estrone (E1) into E2, and androstenedione (AD) into T (Nagahama & Yamashita, 2008; Kazeto et al., 2011). A high expression level of 17β-HSD1 was also previously observed in advancing maturation stages by RNA-seq transcriptome analysis (Minegishi et al.,

unpublished data) and is in line with the increase in E2 and T blood plasma levels found in the present study.

Although a higher expression of P45011β was found in responders as compared to non-responders, its average expression was low. P45011β is responsible for the conversion of AD into 11β-hydroxy-androstenedione (OHAD), which can be converted into either 11β-hydroxy-testosterone (OHT) or 11-keto-androstenedione (11-KAD) by 17β-HSD3 and 11β-HSD2, respectively (Kazeto et al., 2011). Further, OHT and 11-KAD can be converted into the non-aromatizable androgen 11-ketotestosterone (11-KT) by 11β-HSD2 and 17β-HSD3, respectively (Kazeto et al., 2011). 11-KT is found an important factor in previtellogenic and early vitellogenic oocyte growth (Lokman et al., 1998; Rohr et al., 2001; Sbaihi et al., 2001; Kazeto et al., 2011). Microarray analysis showed that steroidogenic enzymes involved in the production of 11-KT (11β-HSD2, 17β-HSD3) were highly expressed in responders as compared to non-responders. This increased expression indicates that in responding females pre- or early vitellogenic growth was initiated, which was confirmed by histological analysis (Figure 1).

Microarray analysis also revealed that the expression level of esr1 was significantly reduced in responding females as compared to non-responding females. Plasma E2 levels, on the other hand, significantly increased, suggesting a negative intra-gonadal feedback mechanism. The actual function of E2 on gonad tissue yet remains unclear, and was not further studied during this research.

The present data are similar to previously observed results of RNA-seq transcriptome analysis of advancing maturation stages from yellow to silver and spawned females (Minegishi et al., unpublished data). Further investigation of steroidogenic enzymes in response to hormonal treatment may confirm our present results.

Sex steroids as biomarkers

During artificial maturation, the gonadotropins FSH and LH showed to be oppositely regulated (Figures 2a,b). FSH blood plasma level decreased over 12 weeks and was followed by an increase, while LH blood plasma level showed an increase over 12 weeks followed by a decrease. Other studies also showed an opposite regulation of pituitary FSHβ and LHβ expression in Japanese and European eel (Suetake et al., 2002, 2003; Schmitz et al., 2005).

Table 3. Correlations between GSI and hormone plasma levels of 17β-estradiol (E2) and testosterone (T), its absolute (Δ) and relative (r) change prior (0) and after 4, 12 and 18 weekly injections. Significant differences are indicated by * (p<0.05) and ** (p<0.01).

	0	4	12	18
E2	0.035	0.793*	0.861**	0.681**
ΔE2		0.901**	0.761*	0.698**
rE2		0.730*	0.285	0.439
T	0.043	0.341	0.920**	0.565*
ΔT		0.391	0.884**	0.582*
rT		0.295	0.265	0.407

Pituitary extracts (PEs) contain both FSH and LH (see e.g Minegishi et al., 2012). In general, the half-life of those gonadotropic hormones is relatively short; at seven days after injection, plasma levels of exogenous LH are back to base-line (Sato et al., 2000; 2003). During the present study blood plasma samples were obtained prior the weekly PE injection. Therefore, our results suggest that the measured plasma levels of FSH and LH may be of endogenous origin. It was shown recently that sex steroids E2 and T exert a differential feedback on the expression of FSHβ and LHβ; i.e. a positive feedback on LHβ expression by E2, and a negative feedback on FSHβ expression by T (Schmitz et al., 2005, Aroua et al., 2007). The results of the present study suggest a similar process as previously found. Maturation is initiated PE, which results in the production of sex steroids. Subsequently, steroids inhibit endogenous FSH production and stimulate the endogenous LH production.

Moreover, it was found that between 12 and 18 weekly PE injections FSH plasma levels had increased significantly. During artificial reproduction, female eels can be stimulated to produce multiple batches of eggs (e.g. Burgerhout et al., 2011), a feature probably reflecting natural conditions (Tsukamoto et al., 2011).

FSH is an important inducer of vitellogenesis (Nagahama & Yamashita, 2008). An increase of FSH levels during the final phases of oocyte development may indicate a preparation for a future batch of eggs or that FSH has a different, yet unknown, function during final oocyte maturation.

Sex steroid plasma levels of E2 and T increased significantly during artificial maturation, as also shown in the present study and various other studies (e.g. Lokman et al., 1998, 2001; Matsubara et al., 2005; Chiba et al., 2007). A significant correlation was found between GSI and E2, ΔE2 and rE2 already after 4 weekly injections (Table 2). The rE2 levels of responders and non-responders sampled after ovulation or after 18 weekly injections indicate that ca. 80% and 99% of the females responding to the treatment may be selected after 4 and 12 weeks, respectively (Figure 2). The present results show that the relative change in E2 blood plasma levels between 4 and 12 weekly injections may be a reliable candidate for broodstock selection. However, future studies are necessary to validate our current findings.

Conclusions

Broodstock selection of female European eels to increase success rates is of great interest for eel aquaculture. Our findings suggest that increased expression levels of several steroidogenic enzymes may be used as broodstock selection marker. On the other hand, responsive female eels may be selected after 4 weekly injections based on relative fold increase of E2 blood plasma levels.

Acknowledgements

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Chapter 6

First artificial hybrid of the eel species *Anguilla australis* and *Anguilla anguilla*

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Abstract

Studies on artificial hybridization of different *Anguilla* species were conducted recently, i.e. female *A. australis* with male *A. dieffenbachii*, and female *A. japonica* with male *A. anguilla*. The existence of these artificial hybrids was however not demonstrated by independent genetic methods. Two species - *A. anguilla* and *A. australis* - that are phylogenetically close but have different sexual maturation times (12-25 weeks and 6-8 weeks, respectively), were expected to produce favourable hybrids for reproduction studies.

A modification of the protocol for the reproduction of *Anguilla japonica* was used to produce eight-day *Anguilla australis* larvae, with a success rate of 71.4%. Thus ten out of 14 females produced eggs that could be fertilized, and three batches resulted in mass hatching. Hybrid larvae from female *A. australis* x male *A. Anguilla* survived for up to seven days post fertilization (dpf). The early development of the hybrid showed typical characteristics of *A. anguilla* tail pigmentation at 50 hours post fertilization (hpf), indicating expression of genes derived from the father.

In this paper we describe the first production of hybrid larvae from male *A. anguilla* and female *A. australis* and their survival for up to 7 dpf. A species-specific nucleotide difference in the 18 S rDNA gene confirmed that genes from both *A. australis* and *A. anguilla* were present in the hybrids. The developmental stages of the hybrid eel embryos and larvae are described using high resolution images. Video footage also indicated a heart beat in 5-dpf larva.

Introduction

A number of research groups have attempted artificial reproduction in various species of eel: *A. japonica* (Yamamoto & Yamauchi, 1974; Yamamoto et al., 1976; Tanaka et al., 1995; Ohta et al., 1996, 1997), *A. anguilla* (Bezdenezhnykh et al., 1983; Pedersen, 2003, 2004; Palstra et al., 2005, Tomkiewicz, unpublished data), *A. dieffenbachii* (Lokman & Young, 2000), *A. australis* (Lokman & Young, 2000; Kurwie, unpublished data), and *A. rostrata* (Oliveira & Hable, 2010). Some Japanese scientists have also overcome major problems associated with developing artificial feeds for larvae and have successfully produced leptocephalus larvae (Tanaka et al., 2001) and glass eels (Tanaka et al., 2003; Kagawa et al., 2005). Tanaka and his co-workers used a mix of shark egg powder, soya peptide, minerals, vitamins and krill paste (Tanaka et al., 2001) to develop a successful feed for *A. japonica*. Further research is, however, needed to develop suitable diets and rearing techniques for the production of larvae of other *Anguilla* species and their hybrids.

European eel (*A. anguilla*) females have a much slower, and widely-variable, response to hormonal stimulation (Palstra et al., 2005) when compared to females of other freshwater eel species (e.g. *A. japonica* and *A. australis*). At the onset of the natural spawning migration, the gonadosomatic index (GSI) of *A. anguilla* females is close to 2% (A Palstra, unpublished data) and they are still in a previtellogenic state when they migrate to sea. However, females of *A. australis* have a higher GSI, of up to 4% (Lokman et al., 1998), indicating that they are sexually more advanced than *A. anguilla* at the same stage The same holds true for *A. japonica*, which has a GSI of up to 4% at the commencement of its spawning migration (Okamura et al., 2007a). Induction of vitellogenesis and final maturation in *A. australis* requires approximately six to eight weekly hormonal injections (Lokman & Young, 2000; Kurwie, unpublished data) while 9-12 injections (Ohta et al., 1996), or 6-15 weekly injections (Tanaka et al., 2001), are required for *A. japonica* and up to 12-25 weekly injections for *A. anguilla* (Pedersen, 2003, 2004; Palstra et al., 2005).

There are several reasons for testing hybridization between European and New Zealand short finned eels. There are large differences in silver eel maturation states between these species. In contrast to the stage reached by *A. australis*, silver eels of *A. anguilla* have not yet commenced vitellogenesis. Shortening the artificial trajectory may overcome vitellogenic abnormalities,

resulting in higher gamete quality and higher success rates of fertilization, hatching and larval development.

Anguilla anguilla is listed by the IUCN as critically endangered (Freyhof & Kottelat, 2008), which raises some problems in association with the culture of this species. Farming is reliant on the influx of wild glass eel, thereby pressurizing wild stocks. Breeding for aquaculture is, nevertheless, supposed to take pressure off wild stocks. Therefore, the hybridization of *A. anguilla* with a species such as *A. australis*, that has a short artificial trajectory, may be a suitable option for aquaculture. Since maturation levels at silvering are very different in the parent species, it is quite possible that the maturation level of the hybrid at the silver stage would be far more advanced than that of the European silver eel. Furthermore, since *A. australis* lives in the southern hemisphere, its migration is in January-June (Todd, 1981), in contrast to *A. anguilla*, which migrates in October-November. To gain insights into the combination of the properties of *A. australis* and *A. anguilla* present in hybrids, it is useful to study eel reproduction and to compare the early ontogeny of these species including their hybrids.

A number of studies on hybridization of various eel species have been previously conducted: for example, female *A. australis* with male *A. dieffenbachii* (Lokman & Young, 2000), and female *A. japonica* with male *A. anguilla* (Okamura et al., 2004). The existence of the artificial hybrids has not, however, been demonstrated by independent genetic methods. In contrast, genetic evidence for natural hybrids between the Atlantic species *A. rostrata* and *A. anguilla* has been demonstrated (Albert et al., 2006). Since *A. anguilla* and *A. australis* are phylogenetically more closely related than some other hybrids (for example, *A. anguilla* and *A. japonica* (Minegishi et al., 2005), we hypothesized that hybridization between the former two species would be possible. In this paper we describe experiments on the hybridization of *A. anguilla* and *A. australis* and post-fertilization survival levels. Investigations into the18 S rDNA gene – for the purpose of genetic validation – are also described.

Methods

Eel collection

Silver females (n = 14; 80.1 ± 0.4 cm; 978.1 ± 19.5 g) and males (n = 8; 45.6 ± 1.4 cm; 172.5 ± 14.9 g) of New Zealand short-finned eels (*A. australis*) were caught in Lake Ellesmere in Christchurch, New Zealand, and transported to The Netherlands in aired plastic bags with a small amount of water, fitted into polystyrene boxes. Silver male European eels (*A. anguilla*) were purchased from the eel farm Royaal BV (Helmond, The Netherlands).

Anguilla australis females and males were kept together in a 1000 L tank filled with natural seawater, collected from Lake Grevelingen (30 ppt), and coupled to a 1500 L recirculation system (salinity 30 ppt, 21°C). The daily cycle was set with blue light (Philips special TLD Blue 36W/18) at 16:8 L:D. To compensate for the 11 h time difference between The Netherlands and New Zealand, the daily cycle was changed stepwise (1 h per week) to Central European Time (CET). *Anguilla anguilla* males (n = 15; 40.4 ± 0.6 cm; 118.8 ± 4.9 g) were kept in a 1500 L tank connected to a 2400 L recirculation system, in natural seawater (30 ppt, 21°C), under a complete dark regime. PVC pipes were introduced into both systems to provide refuges for animals. All animals were starved throughout the experiment, and treated on a weekly basis with Melafix (API aquarium pharmaceuticals, MARS Fishcare North America Inc., Chalfont, PA, USA) against infections. Prior to treatment, eels were anesthetized with 1-2 mL/L 10% clove oil (oil mixed 1:10 with absolute ethanol). At the start of this study all eels were tagged with passive transponders with unique identification numbers (Trovan, EID Aalten BV, Aalten, The Netherlands). This experiment was approved by the animal ethical commission of the Leiden University (DEC# 08112).

Hormone treatments

Female eels were distributed into four groups, with the starting point of hormonal treatment for each group being shifted one week forward, on a weekly basis. Treatment followed a modified version of Ohta's protocol (Ohta et al., 1996, 1997). On the first day of the week, females were weighed and injected intramuscularly (IM), at a point approximately 1 cm below the rostral attachment of the dorsal fin, with 20 mg salmon pituitary extract (SPE; Argent Labs, Redmond,

WA, USA) per kg dissolved in 0.9% saline. When a 5% increase in body weight (BW) -- with respect to initial BW -- was reached, females were transferred to a separate 400 L tank, connected to the same system. The BW was measured the day after the transfer and/or 2 days later. When a 10% increase in BW was reached, an oocyte sample was collected by means of inserting a cannula (polyethylene tube, inner diameter 1.4 mm) through the oviduct. Oocytes were checked under a microscope to ascertain developmental stages. When migration of the germinal vesicle – still with many oil droplets in the oocytes (stage 3/4 in European eel according to (Palstra et al., 2005) – was observed, the female was primed with a single injection of SPE (20 mg/kg dissolved in 1 mL 0.9% saline). After 24 h, ovulation was induced by means of intraperitoneal injections of 17, 20β-dihydroxy-4-pregnen-3-one (DHP 2 mg/kg, Sigma-Aldrich BV, Zwijndrecht, The Netherlands) dissolved in DMSO, administered at six to eight locations.

Male New-Zealand short-finned eels were treated according to a modified version of Ohta's protocol (Ohta et al., 1996, 1997). They were subjected to a weekly IM injection procedure (at a site approximately 0.5-1 cm below the rostral attachment of the dorsal fin) with 250 IU human chorionic gonadotropin (hCG, Sigma-Aldrich BV, Zwijndrecht, The Netherlands) dissolved in 0.1 mL 0.9% saline. Males were injected every week for up to 9 weeks and checked for spermiation by hand stripping. After 9 weeks, all males produced milt and injections were stopped until a female was ready to spawn. (This reduced handling stress; it was also noted that the sperm quality did not decline much during the "holding" period). On the day before the eggs were to be stripped, two to three males that demonstrated high sperm motility were selected and stimulated with a single booster dose of 500 IU dissolved in 0.1 mL saline. Sperm motility was determined visually under a microscope, after mixing a drop of sperm with a drop of seawater. Only sperm with at least 50% motility (continuous activity of > 50% of spermatozoa) was used for fertilization.

For the production of a hybrid between *A. australis* and *A. anguilla*, 15 farmed male European eels received weekly intraperitoneal (IP) hCG injections, according to the protocol for European eel (Palstra et al., 2005), at a dose of 200 IU/male, followed by a booster dose of 1,000 IU hCG (in 0.2 ml 0.9% saline) 24 h before a fertilization trial.

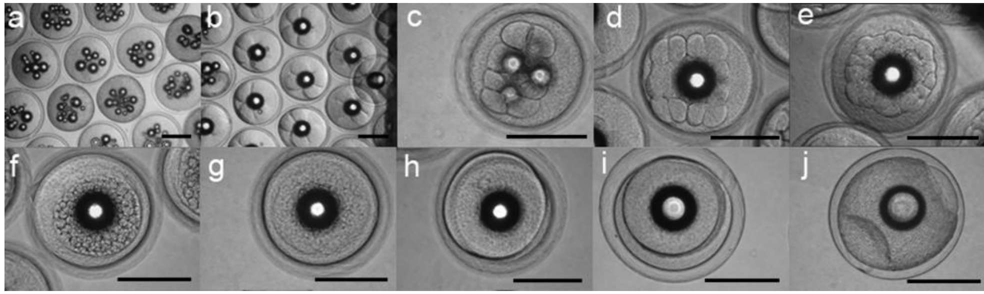

Figure 1. Embryogenesis of *A. australis*. Early ontogeny until the late gastrula stage: a) Fertilized eggs, ~0.5 hours post fertilization (hpf); b) 4-cell stage, ~1.5 hpf; c) 8-cell stage, ~2 hpf; d) 16-cell stage, ~2.5 hpf; e) 32-cell stage, ~4 hpf; f) morula stage, ~6hpf; g) blastula stage, ~7 hpf; h) and i) early gastrula stage (shield stage), 9-13 hpf; j) late gastrula stage, 20 hpf. (Scale bar = 1 mm).

Artificial fertilization and larval rearing

Two to three males per species were hand stripped 24 h after the hCG booster injection. Milt was collected in a syringe (10 mL) and kept on ice or in the refrigerator for a maximum of 48 h. Sperm motility was checked prior to fertilization by means of microscopic examination. Females were expected to ovulate between 11 and 15 h after the final injection with DHP. The artificial fertilization programme was terminated in cases when female spawning only commenced after more than 18 h after the final injection, which is indicative of low fertility and hatchability (Ohta et al., 1997). After the final injection, females were checked hourly for egg release, by gently pressing on the abdomen near the vent. When a female showed an ovarian plug, the plug was gently removed. Eggs were collected in plastic, pre-weighed, sterilized bowls. The first flow of eggs (~50 g) was not used for fertilization. The combined egg weight was determined after all eggs had been stripped.

The collected sperm was added to dry eggs in bowls and mixed. Fresh seawater (35 ppt, 20°C) was added, and after approximately 3-4 min the eggs were transferred into buckets with fresh (sterile) seawater (~20 L). A net (of mesh size 600 μm) was used to separate floating eggs from sinking eggs. The former were transferred, after 30-45 min, to another bucket containing fresh seawater. Finally, the eggs were transferred to 1 L glass beakers and/or 200 mL Petri dishes, for observation. At this stage they were kept in complete darkness at

21°C and a salinity of 35 ppt. Approximately 24 hours after incubation, the water was refreshed by transferring the still-floating eggs into new glass beakers or Petri dishes. During the trial, all white or sunken eggs were removed. A portion of eggs from one batch were also reared at 25°C.

DNA analysis

As described by Frankowski & Bastrop (2009), parental species and their hybrids can be identified by means of polymorphism in nuclear 18 S rDNA. For our study we used a slightly different protocol, as described below.

Total DNA was isolated and purified from ten whole hybrid larvae and fin-clips of parental specimens, using a DNeasy Blood & Tissue Kit (Qiagen). The polymerase chain reaction (PCR) was performed using the FastStart High Fidelity PCR System protocol (Roche) and an amplification profile consisting of denaturation for 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, followed by 5 min at 72°C for final extension. Amplification was carried out according to the manufacturer's instructions in 50 μL 1 × FastStart High Fidelity Reaction Buffer containing 1.8 mM $MgCl_2$, 2.5 U FastStart High Fidelity Enzyme Blend, 1 μg chromosomal DNA, 0.4 μM of the 18 S rDNA forward and reverse primers, and 0.2 mM dNTPs.

The sense (5'-AGC ATA TGC TTG TCT CAA AGA TTA AG-3') and antisense (5'-CTG CTG CCT TCC TTG GAT GTG G-3") primers were based on NCBI accession numbers FM946133 (*A. australis*) and FM946070 (*A. anguilla*) (Okamura et al., 2007b). The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and 0.5 μg of the purified fragments were digested with two units of the restricting enzyme *BssH*II (New England Biolabs Inc) according to the manufacturer's instructions. Restriction enzyme digestion was conducted in 10 μL reaction buffer for 1 h at 37°C. DNA fragments were made visible using a 2% agarose gel.

Results

Reproduction

After 4 weeks of injections with hCG, the first *A. australis* male started to spermiate and all males had spermiated within 6 weeks. After 9 weeks the

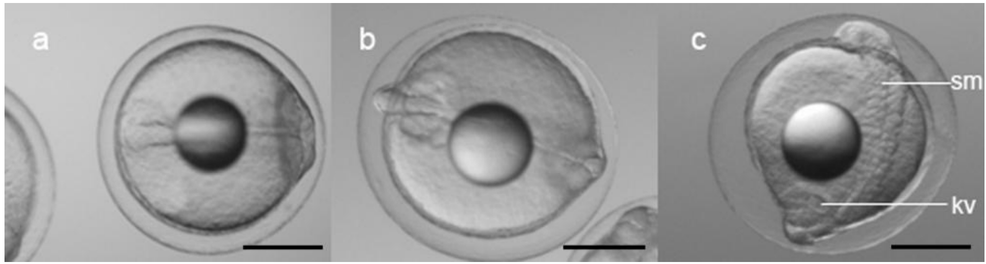

Figure 2. Somitogenesis in *A. australis* embryos. Stages of somitogenesis: a) 6-7 somites, ~24 hpf; b) 11 somites, ~26 hpf; c) 17 somites, ~30 hpf. (Scale bar = 0.5 mm; kv = Kuppfer's vesicle; sm = somites). Note: due to limited depth of field of the microscope in relation to the size of the embryos, not all structures can be shown simultaneously.

injections were stopped. Three of the 15 *A. anguilla* males produced sperm after 5 weekly injections and all males of *A. anguilla* had produced sperm after 6 weekly injections. Before use, the males received a booster hCG injection to reactivate spermiation.

In most females, hormone treatment resulted in a rapid increase in body weight after 9-13 injections with salmon pituitary extract (SPE). Ten of the 14 females (71.4%) ovulated once and seven females ovulated twice during this study. The second ovulation was induced 2 weeks after the first ovulation by a single injection of 20 mg SPE/kg dissolved in 1 mL 0.9% saline, one priming injection of SPE, and an injection with 17, 20β-dihydroxy-4-pregnen-3-one (DHP) one day later. Three females did not respond to the SPE treatment and one female died after the DHP injection, just before ovulation. In total, three out of the ten batches of eggs produced larvae (33.3%). The larvae of two of the three batches from *A. australis* x *A. australis* stayed alive for 5 dpf, and the larvae from one batch of the hybrid *A. australis* x *A. anguilla* stayed alive for 7 dpf. Some of the eggs from one batch of *A. australis* were reared at 25°C, which resulted in larvae that survived until 8 dpf.

Embryogenesis and early larval development of A. australis

After fertilization (Figure 1a), developing eggs floated to a level just below the surface of the water, resulting in a clear separation from undeveloped eggs, which sank. Cell divisions occurred every 30-60 minutes. The 4-cell and 8-cell states

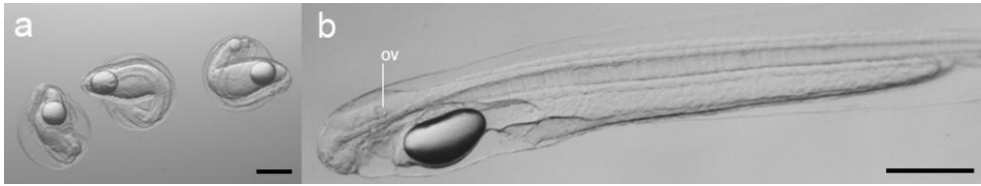

Figure 3. Hatching of *A. australis*. Structures in larvae: a) hatching larvae, ~45 hpf; b) larva at 2.5 dpf. (Scale = 0.5 mm; ov = otic vesicle with otoliths).

were observed at the 1.5 hpf stage (Figure 1b) and the 2 hpf stage (Figure 1c), respectively. The 16-cell stage and 32-cell stage were observed at 2.5 hpf (Figure 1d) and 4 hpf (Figure 1e), respectively. The morula stage (Figure 1f) and the blastula stage (Figure 1g) were observed at 6 hpf and 7 hpf, respectively. An embryonic shield started to form between 9 and 13 hpf (Figure 1h and 1i), and the late gastrula stage was observed at about 20 hpf (Figure 1j). Somitogenesis started between 20 and 24 hpf (Figure 2). The Kuppfer's vesicle (Figure 2c, for description see (Essner et al., 2005) was observed at about 26 hpf, and the first heartbeat occurred at about 43 hpf.

Neutral buoyancy of the eggs was observed just before hatching. At approximately 43 hpf the embryos started to hatch and at that time had approximately 40 somites (Figure 3a; 45 hpf). After hatching (Figure 3b; 2.5 dpf) the larvae (at first C-shaped) were positioned upright in the water column, probably due to the position of the oil droplet. It appeared that the larvae were neutrally buoyant at 35 ppt. The larvae were immobile, except when disturbed by light or vibrations, which caused very fast and short horizontal movements. During sampling procedures (by pipette) the larvae avoided suction, and swam in the opposite direction at speeds of up to several body lengths per second. Sampling caused mechanical damage to larvae, followed by death within a short period. Discoloration of the brain and neural tube was observed within a few seconds, followed by cellular breakdown of the larvae.

Larvae elongated during development and lateral neuromast cells on the flank were observed at 68 hpf. Head development showed remarkable changes over time, especially a decrease in volume of the 4th ventricle between 5 and 6.5 dpf (Figure 4a, b), the protrusion of the mouth and development of teeth between 5 and 8 dpf, and pigmentation of the eyes at 8 dpf (Figure 4c). The angle of the

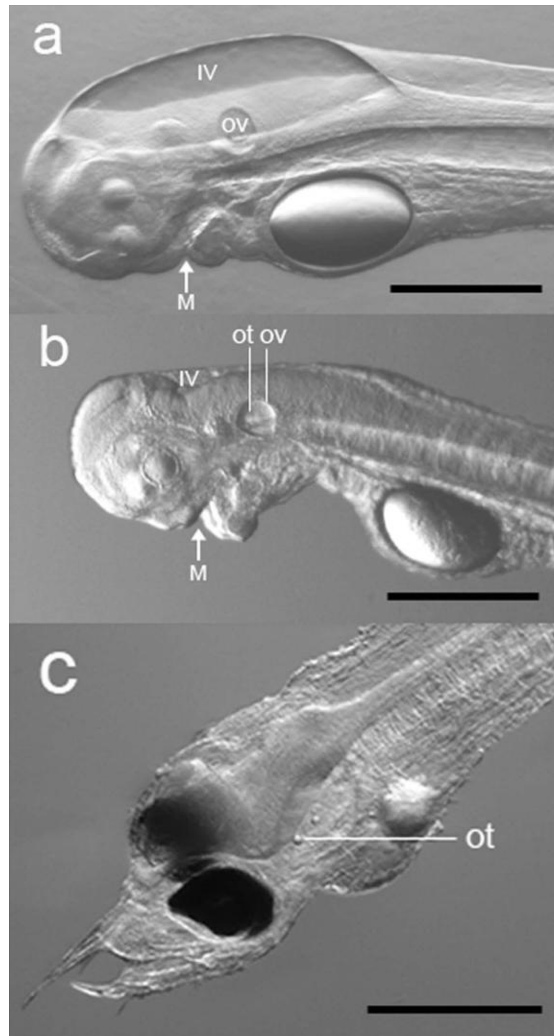

Figure 4. Head development of *A. australis*. Head structures of *A. australis*: a) 5 dpf larva; b) 6.5 dpf larva; c) 8 dpf larva reared at 25°C (representing a stacking of two illustrations of the same larva). Note the remarkable differences between 5 and 6.5 dpf larvae in the development of the 4th ventricle (IV)(with a decrease in volume) and the protrusion of the mouth (M). Between 6.5 dpf and 8 dpf, the formation of teeth commences, the angle of the head increases, and the eyes become pigmented. (Scale bar = 0.5 mm; ov = otic vesicle with otoliths; ot = otoliths).

head also increased in such a way that the mouth protruded anteriorly. At 8 dpf the larvae showed well developed teeth and a straightened head, indicating that they had reached the feeding stage. No visual differences in development between larvae from *A. australis* and the hybrid species were observed, except for the development of tail pigment cells, which were already present at about 2 dpf in the hybrid. In contrast, the pigmented cells appeared much later (5-6 dpf) in *A. australis* (Figure 5a, b)

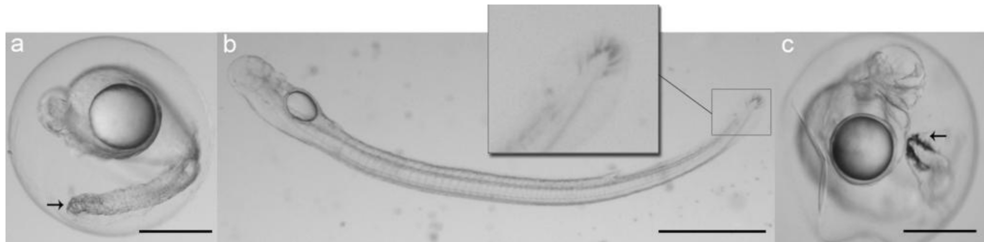

Figure 5. Timing of tail pigmentation. Differences in timing of tail pigmentation in *A. australis* and the *A. australis* x *A. anguilla* hybrid: a) hybrid species at ~50hpf; b) *A. australis* at 6 dpf; c) *A. anguilla* at 60 hpf. (Scale bar in a,c = 0.5 mm, in b = 1 mm; arrows indicate pigmentation of the tail).

Video recordings were made of several larvae, to observe the heart beat. The heart is bent in an S-shape, contracting regularly at a frequency of about 54 beats per minute in a 5 dpf larva (Additional file 1). There were no significant differences between the heart beat rates of 2 dpf and 5 dpf larvae, suggesting that the rate is based on the innate rhythm of heart muscle tissue.

DNA analysis

A species-specific nucleotide difference in the 18 S rDNA genes of *A. australis* and *A. anguilla* (Figure 6) allowed us to provide genetic evidence that we had produced hybrid offspring from the two species. The PCR product amplified from the 18 S rDNA gene is 428 bp long and has a single mismatch between *A. anguilla* and *A. australis* at position 222, resulting in a *BssH*II restriction site, specific for the *A. australis* product (Figure 6). *BssH*II digestion of the PCR product from *A. australis* therefore results in 207 bp and 221 bp fragments, whereas the 428 bp PCR product from *A. anguilla* is not digested by *BssH*II. As the hybrid species must

contain both the *A. anguilla* and the *A. australis* 18 S rDNA genes, three fragments were expected. Figure 7 clearly shows that the parental species *A. anguilla* and *A. australis*, as well as their hybrid, can be identified using the 18 S rDNA gene.

Discussion

In this study, we succeeded in breeding *Anguilla australis* under artificial conditions and produced free swimming larvae of this species. In addition, hybrids of *A. australis* x *A. anguilla* were produced for the first time and genetic methods were used to confirm the existence of this hybrid. The hybrid larvae were kept alive for a maximum of 7 dpf and the larvae of *A. australis* for a maximum of 8 dpf.

```
                      *         *         *         *        50
 A_anguilla   AGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTACACACGG
A_australis   AGCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTACACACGG
                      *         *         *         *       100
 A_anguilla   CCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTTT
A_australis   CCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTTT
                      *         *         *         *       150
 A_anguilla   GATCGCTCCAACGTTACTTGGATAACTGTGGCAATTCTAGAGCTAATACA
A_australis   GATCGCTCCAACGTTACTTGGATAACTGTGGCAATTCTAGAGCTAATACA
                      *         *         *         *       200
 A_anguilla   TGCCGACGAGCGCTGACCCTCCCAGGGGATGCGTGCATTTATCAGACCCA
A_australis   TGCCGACGAGCGCTGACCCTCCCAGGGGATGCGTGCATTTATCAGACCCA
                      *         *         *         *       250
 A_anguilla   AAACCCATCCGGGGTGCCTCGTGCGCCCCGGCCGCTTTGGTGACTCTAGA
A_australis   AAACCCATCCGGGGTGCCTCGCGCGCCCCGGCCGCTTTGGTGACTCTAGA
                      *         *         *         *       300
 A_anguilla   TAACCTCGGGCCGATCGCACGCCCTCCGTGGCGGTGACGTCTCATTCGAA
A_australis   TAACCTCGGGCCGATCGCACGCCCTCCGTGGCGGTGACGTCTCATTCGAA
                      *         *         *         *       350
 A_anguilla   TGTCTGCCCTATCAACTTTCGATGGTACTTTCTGCGCCTACCATGGTGAC
A_australis   TGTCTGCCCTATCAACTTTCGATGGTACTTTCTGCGCCTACCATGGTGAC
                      *         *         *         *       400
 A_anguilla   CACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAA
A_australis   CACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAA
                      *         *    428
 A_anguilla   CGGCTACCACATCCAAGGAAGGCAGCAG
A_australis   CGGCTACCACATCCAAGGAAGGCAGCAG
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Figure 6. PCR fragments from the 18 S rDNA genes. Alignment of the PCR fragments amplified from the 18 S rDNA genes of *A. anguilla* and *A. australis* (location of forward and reverse PCR primers is single underlined; the restriction site for *BssH*II (position 220-225) is thick underlined; species-specific nucleotide difference at position 222 is highlighted grey).

Larvae of *A. japonica* reach the feeding stage at approximately 7 days after hatching (Tanaka et al., 1995). This development is strongly temperature dependent and for *A. japonica* the optimal temperature was shown to be about 25°C (Chang et al., 2004; Okamura et al., 2007b; Kurokawa et al., 2008). It was noted that hatching also occurred at about 25°C (Tsukamoto et al., 2009) at the spawning site, suggesting that this may be the optimal temperature for early development. It was, however, noted that within a few days the larvae were distributed over an extremely large area at much lower temperatures (Tsukamoto et al., 2009), suggesting that early larvae are able to develop at a wide range of temperatures.

In our study, the larvae raised at 25°C (Figure 4c) were sufficiently developed to start feeding. In contrast, the 7 dpf larvae that were raised at 21°C had not yet reached the feeding stage and the head was still in a tilted position. The ten dpf larvae of *A. australis* – collected by T. Kurwie at (illustrated in Dufour & van den Thillart, 2009) a prevailing temperature of 21°C – were slightly more developed, although the 4th ventricle was still large and the eyes were not as fully pigmented, as in the 8 dpf larvae reared at 25°C (Figure 4d). This clearly indicated that the main differences occur in the development of the head. Compared to 8 dpf larvae reared at 25°C, the mouths of 10 dpf larvae reared at 21°C were not fully developed, the teeth were just starting to form, and the mouth angle was not fully protruded anteriorly. This shows that development is highly temperature dependent and that larval development cannot be indicated by age alone.

Compared to other species, such as zebrafish (Kimmel et al., 1995) and medaka (Iwamatsu, 2004), development of the *Anguilla* head seems relatively slow. On the other hand, the appearance of the lateral neuromast cells, which are part of the mechanosensory system, was already observed on the flanks at approximately 1 dpf. After hatching, the larvae were very sensitive to vibrations, indicating that mechanoreception is well developed at this stage. Similar results were also reported for *A. australis* (Lokman & Young, 2000) and *A. japonica* (Okamura et al., 2002).

There were no visual differences in development and appearance between the two species other than the difference in timing of tail pigmentation, which occurred a few days earlier in the hybrid species than in *A. australis*. This seems to be a trait of the European eel, as a similar appearance of tail pigmentation was

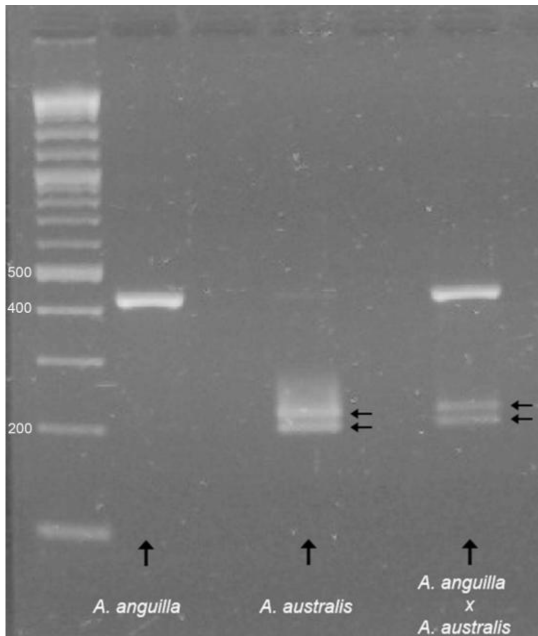

Figure 7. PCR runs showing species-specific differences. Identification of both parental species, *A. anguilla* and *A. australis*, and their hybrid, *A. anguilla* × *A. australis*, based on the species-specific nucleotide difference in nuclear 18 S rDNA. Arrows indicate the two fragments after restriction with the enzyme BssHII; the first lane indicates the position of the DNA size marker.

observed in *A. anguilla* (A Palstra, unpublished data) (Figure 5c). The reason for tail pigmentation occurring at this early stage remains unknown.

In studies on the natural hybrid of *A. anguilla* with *A. rostrata* (Albert et al., 2006; Frankowski & Bastrop, 2009), hybridization was validated by an independent method. This was not the case for recent artificial *Anguilla* hybridizations performed by Okamura et al. (2004) and Lokman & Young (2000). Our results (Figure 7), based on the species-specific nucleotide difference in nuclear 18 S rDNA, show that both *A. anguilla* and *A. australis* and their hybrid *A. anguilla* × *A. australis* can been identified according to the method of Frankowski & Bastrop (2009), in which the following fragments were produced: a single 18 S rDNA fragment for *A. anguilla*, two fragments for *A. australis*, and three fragments for the hybrid species, indicating that two alleles from both parent species were present in the hybrid.

There are still problems with artificial reproduction and larval rearing of Anguilla species, partly due to high individual variability in response to hormone treatments, and partly due to selecting the correct feed for larvae. Tanaka et al. (2001) developed a reasonably successful feed for Japanese eel larvae although almost all larvae died before, or shortly after, the first feeding stage, which may

have been due to the unnatural feeding methods that were employed. Possibly due to negative phototaxis, the larvae swim downwards towards the shark egg paste, where they encounter the food (Tanaka et al., 2001; Yoshimatsu, personal communication).

It is assumed that the natural food sources for leptocephalus larvae of *Anguilla spp*. are the oikopleura larvacean shelters (or 'marine snow'), which have been found in larval digestive tracts of several *Anguilloid* species (Mochioka & Iwamizu, 1996). Recent analysis of A. *anguilla* larval gut contents indicated that the diet of the smallest larvae consisted of a variety of plankton organisms, with Hydrozoa and Polycystinea species occurring most frequently (Riemann et al., 2010). So it seems that eel larvae may take a variety of available food from their immediate environment.

Conclusions

The applied reproduction method resulted in healthy embryos and larvae of New Zealand short finned eels (*A. australis*) as well as hybrids from male European eels (*A. anguilla*) crossed with female New Zealand short finned eels. The developmental stages of eel embryos and larvae are described by means of high resolution images. In this paper we described the first production of hybrid larvae from male *A. anguilla* and female *A. australis* and their survival for up to 7 days post fertilization (dpf). Evidence for hybridization is based on a single nucleotide difference in the 18 S rDNA gene of both species. This is the first time that an artificial *Anguilla* hybrid has been validated by means of a genetic tool. Future work with this hybrid may provide further understanding of the reproductive mechanisms that affect breeding of the European eel, which is now on the CITES list of critically endangered species.

Acknowledgements

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Chapter 7

A cellular implant system stimulates the early phase of sexual maturation in European eel

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Abstract

The European eel (*Anguilla anguilla*) is a critically endangered species, therefore there is an urgent need for reliable artificial reproduction protocols. Male eel spermiation can be efficiently induced via 6-8 weekly injections of purified human chorionic gonadotropin (hCG). Sexual maturation of female European eels can be induced via weekly injections of salmon or carp pituitary extract for up to 6 months. The weekly hormone injections result in handling stress and high transient hormone peaks in the blood plasma that negatively affect sexual maturation. In addition, fish pituitary extract is poorly defined and a potential source of pathogens. Most of these problems can be solved with a defined hormone preparation that slowly releases the required gonadotropins at the right dose into the circulation. However, the currently available slow release pumps require surgery of the fish and large amounts of purified hormone. We have now developed a slow release system consisting of live fish cells that constitutively secrete a specified gonadotropic hormone. In a proof-of-principle experiment, male European eels were administered a single injection with hCG-producing cellular implants. Using a recently developed bioassay, we could demonstrate that hCG was detectable in the eel blood plasma for up to 14 days after intraperitoneal injection of the cellular implant. The implant resulted in significantly increased blood testosterone levels and an increased eye index, both of which are strong indicators of sexual maturation. This proof-of-principle experiment shows that the cellular implant system functions as a slow release system and induces the early phase of sexual maturation in male eels.

Introduction

According to current knowledge, natural maturation of European eels (*Anguilla anguilla*) only occurs during and/or after the migration to the spawning area in the Sargasso Sea (Schmidt, 1923; Tesch, 2003). The influx of glass eels has dramatically declined in the past decades (Dekker, 2003) and the European eel is now even listed as critically endangered on the IUCN "Red List of Threatened Species" (Freyhof & Kottelat, 2008). Artificial reproduction may contribute to the restoration of the natural populations by reducing the pressure on the wild stock and allow sustainable eel aquaculture in the future. Artificial reproduction of eels, including European eels, has already been accomplished (Lokman & Young, 2000; Ohta et al., 1996, 1997; Oliveira & Hable, 2010; Palstra et al., 2005; Pedersen, 2003; Yamamoto & Yamauchi, 1972); however, until now full closure of the life cycle has only been achieved for Japanese eel (*Anguilla japonica*) (Ijiri et al., 2011). The main bottleneck toward closing the life cycle is lack of in depth knowledge on the maturation process and on the proper food for the larvae. Recently, the first insight into the feeding biology of eel larvae in their natural habitat was obtained, suggesting that specific plankton composition is essential for feeding and growth of these larvae (Riemann et al., 2010). To increase the chances of finding the correct food composition, frequent production of large numbers of high quality larvae are required, which are until now not available. As for artificial maturation of eels, the current standard protocols are based on treating males with human chorionic gonadotropin (hCG) (Fontaine, 1936; Miura et al., 1991; Ohta et al., 1996, 1997a), and females with pituitary extracts (PEs) from salmon or carp (SPE or CPE, respectively) (Fontaine et al., 1964; Kagawa, 2003; Kagawa et al., 2005; Ohta et al., 1997a).

Induction of maturation in male eels can be accomplished via 6-8 injections of hCG with success rates close to 100% (Miura et al., 1991; Ohta et al., 1997a; Pedersen, 2003). Induction of full maturation of female eels requires multiple weekly injections with PEs: Japanese eels (Yamamoto & Yamauchi, 1972; Ohta et al., 1996; 1997a) and New Zealand shortfinned eels (*Anguilla australis*) require 6-15 injections (Lokman & Young, 2000; Burgerhout et al., 2011), while European eels need 11-29 injections (Palstra et al., 2005; Pedersen, 2003, 2004). However, usually more than 50% of injected European female eels do not respond to the treatment (Burgerhout et al., unpublished data). In addition, egg quality and

fertilization rates are very low. In teleosts, there is a clear negative correlation between exposure to stressors and the success rate of reproduction (Barton & Iwama, 1991; Foo & Lam, 1993; Pottinger et al., 1991; Schreck, 2010). The multiple PE injections cause stress as a result of handling and high transient hormone peaks in the blood plasma (Sato et al., 2000). Another disadvantage is that the composition of PEs (luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL) and other compounds) is variable and poorly defined. Furthermore, the crude extracts may contain pathogens (Oidtmann et al., 2003; Rivera-Milla et al., 2003).

The ultimate goal of artificial maturation is that the protocols result in similar physiological conditions as those occurring in nature. Under natural circumstances, plasma hormone levels show gradual changes (Suetake et al., 2003), rather than the high transient peaks that occur after each injection with hormones (Sato et al., 2000). Therefore, slow release systems with defined hormone preparations would better mimic the natural situation. Currently available slow release pumps that last up to six weeks require surgery on the fish (Kagawa et al., 2009, 2013). Other slow release methods, like the lipophilized gelatin emulsion (Sato et al., 1995, 1997) only dampen the peak and still have to be injected weekly.

Here, we present a newly developed slow release system consisting of cellular implants from fish cells that secrete constitutive levels of a gonadotropic hormone. These implants have a defined hormone production, are pathogen free, and can be directly injected into the peritoneal cavity.

Methods

Recombinant DNA constructs

The building blocks for the hCG expressing plasmid were obtained from the following sources. The full length cDNA clone IRAUp969C05103D, which contains the code for the hCGβ chain, was purchased from RZPD. The plasmid pBC-hCGsynα that contains a synthetic DNA fragment encoding the human glycoprotein hormone alpha chain, codon-optimized for expression in teleost cells (hCGsynα) and flanked by a 5' *Bst*XI site and a 3' *Sna*BI site, was purchased from

BaseClear BV (Leiden, The Netherlands). The Gateway-based Tol2kit plasmids p5E-bactin2, p3E-IRES-nlsEGFPpA, pME-MCS and pDestTol2pA2 were described elsewhere (Kwan et al., 2007).

The hCG expressing plasmid was constructed from the building blocks, according to the following cloning scheme. The *Bst*XI-*Sna*BI fragment of pBC-hCGsynα was cloned into the *Bst*XI and *Sna*BI sites of p3E-IRES-nlsEGFPpA, thereby replacing the nlsEGFP code with the hCGsynα code and resulting in p3E-IRES-hCGsynα. The hCGβ code was cloned into the pME-MCS vector, yielding pME-hCGb. The plasmid pBactin-hCGβ-IRES-hCGsynα-polyA, hereafter referred to as pZFS010 (Fig. 1), was made via a recombination event between p5E-bactin2, pME-hCGβ, p3E-IRES-hCGsynα and pDestTol2pA2 using the Gateway LR Clonase kit according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands).

The neomycin selection plasmid was constructed according to the following cloning scheme. The lacZ gene was PCR amplified from pMP2838 (a kind gift from Dr. Jeroen Bakker) using forward primer (5'-AGC TAA GCT TGA ATT CAC CAT GGA AGA TCC CGT CGT TTT ACA ACG TCG-3') and reverse primer (5'-AGC TGG ATC CCC TGA CAC CAG ACC AAC TGG TAA TG-3') and digested with *Hin*dIII and *Bam*HI and subsequently cloned into the *Hin*dIII and *Bam*HI sites of pcDNA5/FRT (Invitrogen), yielding pcDNA5/FRT-lacZ. The neomycin resistance gene was also PCR amplified from pMP2838 using forward primer (5'- AGC TGG ATC CCC CGG GCT GCA GCC AAT ATG GGA TCG GCC ATT GAA CAA GAT GGA TTG CAC GCA G-3') and reverse primer (5'-AGC TCT CGA GTC AGA AGA ACT CGT CAA GAA GGC G-3'), digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI and *Xho*I sites of pcDNA5/FRT-lacZ, yielding pZFS013 (Figure 1). The neomycin resistance gene in pZFS013 also confers resistance against geneticin and was used to select for stably transfected clones.

Large-scale plasmid purification was performed using the EndoFree Plasmid Maxi Kit (Qiagen, Venlo, The Netherlands). All plasmids were sequence verified (BaseClear, Leiden, The Netherlands). The pZFS10 and pZFS013 plasmids were linearized with *Afe*I and *Psi*I, respectively, and the linearized plasmids were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Diegem, Belgium).

Generation of hormone-producing cellular implant

Fat head minnow cells (FHM, *Pimephales promelas*), a kind gift from the Hubrecht Laboratory (Utrecht, The Netherlands), were cultured at 25°C in 67% L15 medium supplemented with 10% fetal bovine calf serum 100 IU/mL penicillin and 100 µg/mL streptomycin. The FHM cells were transfected with a 5:1 volume ratio of the linearized pZFS010 (hCG code) and pZFS013 (neomycin code) plasmids, respectively, using FuGENE6 Transfection Reagent in serum-free medium according to the manufacturer's protocol (Roche, Basel, Switzerland). At 8 hours after transfection, the serum-free medium was replaced with serum-containing medium. At two days after transfection, cells were transferred to 12-well microplates and stably transfected cells were selected for by culturing in the presence of 500 µg/mL geneticin (G418; Duchefa, Haarlem, The Netherlands). After approximately one month of selection, four individual clones of G418-resistant cells were obtained, named FHM-hCG clones 2.1 to 2.4.

The production of biologically active hCG by the FHM-hCG clones was measured using a recently developed bioassay (Minegishi et al., 2012). The FHM-hCG clones were cultured for 16 hours in serum-free medium, since the serum could interfere with the bioassay. The conditioned medium was added to HEK293 cells that stably express the European eel's LH receptor and contain a stably integrated luciferase reporter gene driven by a cAMP responsive-element. After incubation at 37°C for five hours for cell stimulation, a luciferase assay was performed using Steadylite plus Reporter Gene Assay System (Perkin Elmer, Waltham, MA, USA) The luminescence signal was measured on a multilabel plate reader (Victor, PerkinElmer). A serial dilution of pure hCG (Sigma-Aldrich) in DPBS (Invitrogen, Paisly, UK) was used as a reference.

Stable FHM-hCG clone 2.1, hereafter referred to as "2.1", and untransfected wild type FHM cells, hereafter referred to as "WT", were used for the trial. Three and a half weeks before the trial, 4 flasks of 75cm^2 were seeded to upscale the number of cells. One and a half week later, two Hyperflasks (Corning, Tewksbury, USA) of WT and 2.1 cells were seeded according to manufacturer's protocol to obtain sufficient cells for the *in vivo* trial.

Animals

Male European eels (n=28; 110.1 ± 3.6 g body weight (BW) ± standard error (SE); 38.8 ± 0.4 cm body length (BL) ± SE) were obtained from a commercial eel farm (Nijvis-Holding B.V., Nijmegen, The Netherlands). All eels used during this trial were silver eels according and the silver index that was calculated as indicated by Durif et al. (2005). After transport, the eels were immediately transferred from fresh water to natural sea water (32 ± 1ppt) in a ca. 6000L tank connected to a recirculation system. Animals were kept at 21 ± 1°C in the dark and acclimated to these new conditions for 1 month prior to the trial. As silver eels cease feeding during maturation they were not fed during the experiment. All animal experiments complied with the current laws of the Netherlands and were approved by the animal experimentation committee of Leiden University (DEC #11004).

Implant injection experiment

In this study male eels were chosen, because (1) they respond relatively fast to a single hCG injection, (2) there are only few non-responders compared to the female eels, and (3) testosterone levels are a reliable indication of maturation in male eels (Ohta et al., 1997b; Peñaranda et al., 2010). An outline of the experimental design is shown in Figure 1. At the start of the trial, all eels were tagged with a small passive transponder with a unique identification code (Trovan, EID Aalten B.V., Aalten, The Netherlands). In addition, a blood sample was taken and the following morphometric parameters were measured: total body length (BL), body weight (BW), eye diameter horizontal and vertical (EDh and EDv), and pectoral fin length (PFL). The blood was centrifuged at 13,000 x g for 5 minutes at 4°C to obtain blood plasma that was stored at -80°C until measurement. The eye index (EI) was calculated using the formula: $EI = 100^* (((EDh + EDv)/4)^2 \pi/(10^*BL))$ (Pankhurst, 1982).

For each of WT and 2.1 cellular implants, a total of ~ 10^9cells were harvested from Hyperflasks using trypsin, after washed with PBS and resuspended in culture medium to ~ 70 * 10^6 cells/mL. The 28 male eels were randomly divided into 4 groups of 7 individuals and intraperitoneally injected according to the following scheme: the first negative control group (saline group) where each individual was injected with 2 mL 0.9% saline and the positive control group (hCG group) where

each individual was injected with 1000 IU hCG dissolved in 2 mL 0.9% saline (Sigma-Aldrich, Zwijndrecht, The Netherlands). Eels of the second negative control group (WT group) were injected with 2 mL resuspended WT cells and eels of the experimental group (2.1 group) were injected with 2 mL of the 2.1 cells.

Sampling of blood (200 μL) and morphometric measurements occurred at 1, 3, 5, 7, 14, 21, 35 and 42 days after injection. In addition, at each time point, the eels were checked for production of milt. At every sampling point, the animals were anesthetized with clove oil (1 mL of a 10% solution in 96% ethanol added to 1 L of sea water). At the end of the trial (42 days after injection) the animals were sacrificed using an overdose of clove oil (5 mL of a 10% solution added to 1 L sea water), followed by decapitation. After sacrificing the eels, the gonad and liver were weighed to calculate the gonadosomatic index (GSI; 100 * (weight gonads / BW) and the hepatosomatic index (HSI; 100 * (weight liver / BW). The gonads were preserved overnight in 4% paraformaldehyde (PFA, buffered in PBS), and transferred to 70% ethanol for histological analysis.

Plasma hormone measurements

The hCG concentration in the blood plasma samples was measured using the bioassay as described above (Section Generation of hormone-producing cellular implant. Pure hCG (Sigma-Aldrich) that was serially diluted in blood plasma obtained from a negative control eel was used as a reference. To inactivate the eel toxins that affect the HEK293 cells of the bioassay the plasma samples were heated overnight at 37°C prior to measurements as described elsewhere (Minegishi et al., 2012). In the first week after injection, the hCG levels in undiluted plasma samples obtained from the hCG group were too high for the linear range of the standard curve. Therefore, we also measured 10-fold and 50-fold dilutions of these samples in the bioassay. The levels of testosterone in the plasma samples were measured using a Testosterone ELISA kit (HUMAN Diagnostics Worldwide GmbH). The absorbance of the ELISA plates and luminescence of the bioassay plates were measured on a multilabel plate reader (Victor, PerkinElmer).

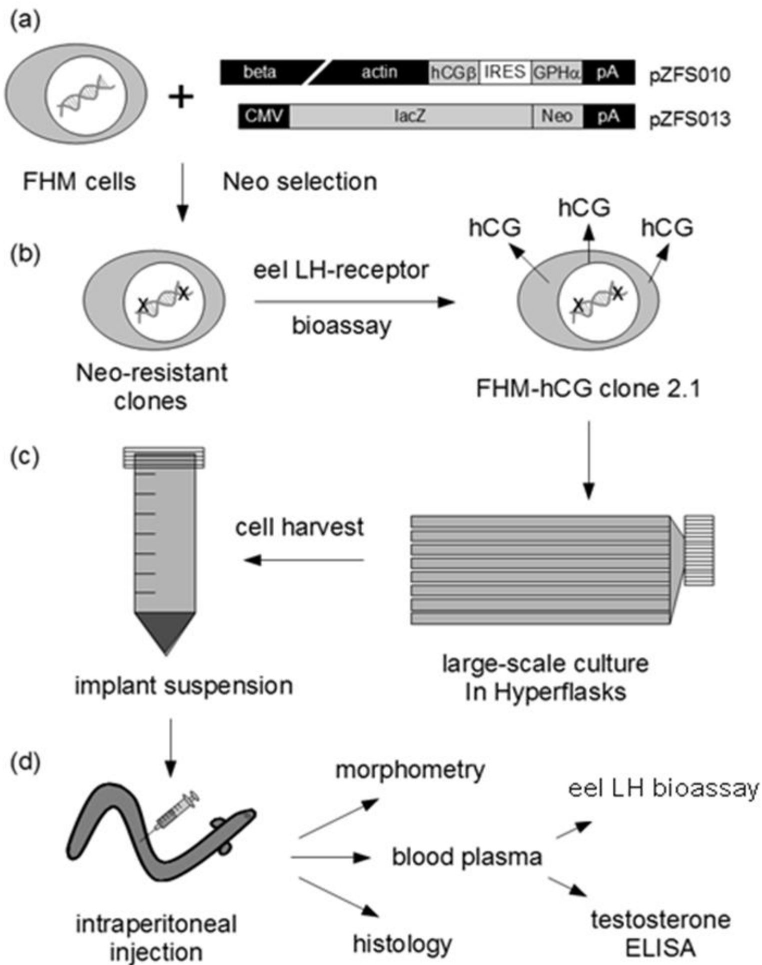

Figure 1. Schematic overview of the workflow. (a) FHM cells were stably transfected with a mixture of a bicistronic expression construct encoding both peptide chains of hCG (pZFS010) and a neomycin resistance selection construct (pZFS013). (b) The amount of hCG in conditioned medium of neomycin-resistant clones was analysed using a bioassay based on the eel LH receptor. (c) The clone that produced the highest amount of hCG (clone 2.1) was expanded in Hyperflasks and concentrated into a small volume. (d) Male eels were intraperitoneally injected with 2.1 cells or with a control substance (wild type cells, pure hCG, or saline). Immediately before injection and at eight time points after injection, the eye index was measured, blood samples were obtained, and eels were checked for production of milt. The hCG and testosterone levels in the blood plasma

Figure 1 (continued). were determined using the eel LH bioassay and an ELISA, respectively. After 42 days, all eels were sacrificed, their hepatosomatic and gonadosomatic indices were determined, and their testes were microscopically analysed.

Histological analysis

The gonad samples were dehydrated in a series of ethanol (70%-80%-90%-100%), followed by incubation in 100% Histo-Clear (National Diagnostics) and a 1:2 mixture of Histo-Clear and paraffin, respectively. The tissue samples were then embedded in paraffin. Sections (7 μm thick) were obtained using a Leica microtome (Leica RM2165) and, after rehydration, nuclei and cytoplasm were stained using Mayer's haematoxylin and eosin (H-E) staining. Testis stages were determined following Peñaranda et al. (2010).

Statistics

A Kolmogorov-Smirnov test was used to test for normality of all data. As all data were not normally distributed (Kolmogorov-Smirnoff; $p>0.05$) a Mann-Whitney U non-parametric test was used to analyze the results. Differences in eye index, plasma levels of testosterone and hCG were tested between the groups and within the groups over time. Statistical difference was considered significant at $p<0.05$. In all cases values are expressed as average ± standard error.

Results and Discussion

Hormone-producing implants

Current maturation protocols with European eels are performed at temperatures between 18°C and 20°C (Palstra et al., 2005; Pedersen, 2003, 2004). Since Fathead Minnow cells (FHM) have optimal growth characteristics at this temperature (unpublished data), these cells were chosen as starting material for the construction of hormone producing cellular implants. The standard protocol for the induction of sexual maturation of male eels is based on injections with purified hCG (Ohta et al., 1996, 1997a) which is a heterodimer of an alpha and a beta chain. In order to get high constitutive expression of both polypeptide chains in FHM cells, we designed the plasmid pZFS010 that is based on the zebrafish

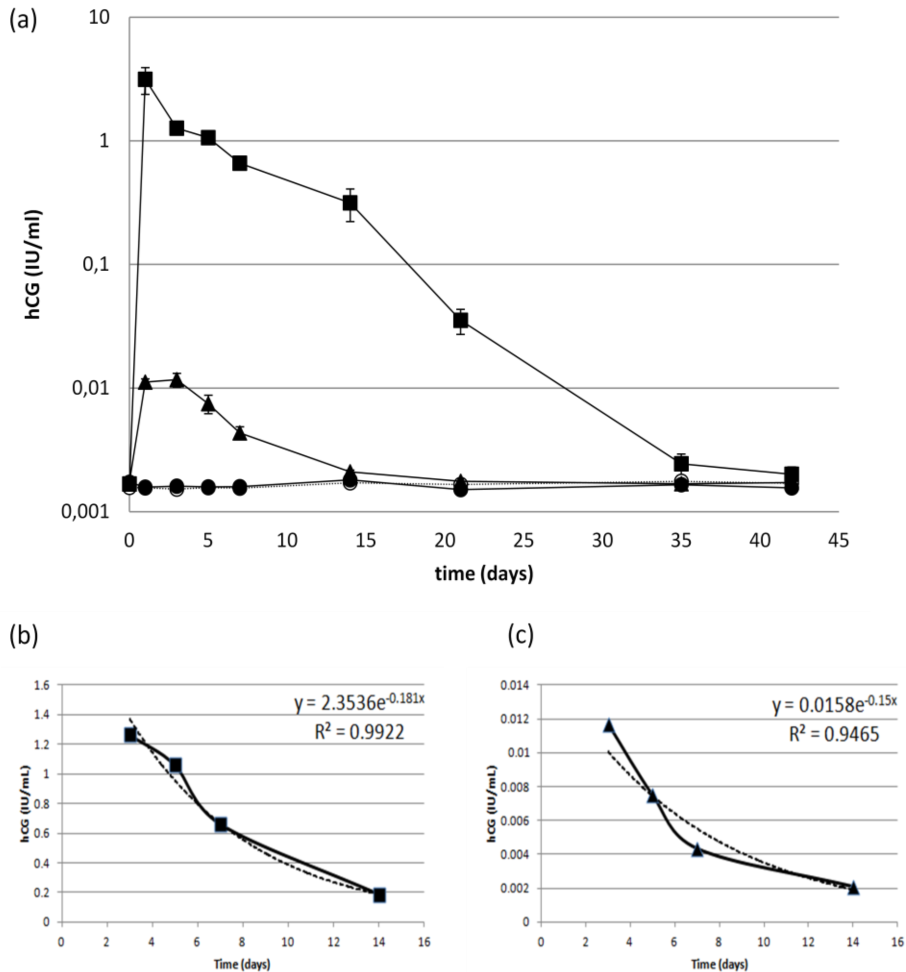

Figure 2: (a) Concentration of blood plasma hCG after a single intraperitoneal injection. Male eels (n=7 per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲). Blood samples were taken immediately before and at 1, 3, 5, 7, 14, 21, 35, and 42 days after injection. The blood plasma hCG concentration was determined using a bioassay based on the eel LH receptor. (b) Trend line (dotted line) of hCG plasma levels between 3 and 14 days after injection of 1000 IU pure hCG (■). (c) Trend line (dotted line) of hCG plasma levels between 3 and 14 days after injection of hCG-producing FHM 2.1 cells (▲).

beta-actin promoter and an expression cassette in which an IRES element is flanked by the codes for the alpha and beta chains of hCG (Figure1). Antibiotic selection of FHM cells transfected with pZFS010 and the selection plasmid pZFS013 resulted in four stably transfected clones. To determine the production level of biologically active hCG by the FHM clones, we used a recently developed bioassay based on the eel LH receptor. This bioassay shows a linear response to a more than 100,000-fold concentration range of hCG diluted in saline (Minegishi et al., 2012). The two clones with highest activity, clones 2.1 and 2.4, produced similar amounts of hCG (91 and 94 mIU hCG/day/10^6 cells, respectively). Since clone 2.1 showed better growth rates, this clone was selected for the trial in male European eels.

The hormone production of implants in male European eels

To determine the *in vivo* effects of hCG-producing cellular implants on maturation of male eels we performed the following trial (see also Figure 1 and experimental design in Methods). Four groups of male silver eels received a single injection of 1000 IU hCG (hCG group), 0.9% saline (saline group), untransfected wild type FHM cells (WT group) or FHM 2.1 cells (2.1 group). The injected amount of 2.1 cells was equivalent to an *in vitro* production of ~13 IU hCG per day, which corresponds with a theoretical daily *in vivo* maximum dose of ~120 mIU/g BW (~13 IU/110 g/day). Before and after administration of the injections, morphometric measurements and blood samples were obtained at nine consecutive time points covering a period of 42 days. The blood plasma hCG levels were measured using the bioassay as described above. At 1 and 3 days after injection of the cellular implant, the hCG concentration in the eel plasma was approximately 10 mIU/mL (Figure 2a, 2.1 group). This was the highest plasma hCG concentration measured in this group, although the actual peak level may have been higher between 1 and 3 days after injection. After 3 days, the plasma hCG concentration gradually decreased, but remained significantly higher ($p < 0.05$) than the background signal of the saline and WT groups for up to 14 days after injection (Fig. 2a). A single dose of 1000 IU pure hCG (~9 IU/g BW) resulted in a plasma hCG concentration of approximately 5 IU/mL at 1 day post injection (Figure 2a, hCG group), although the actual peak hCG plasma level could again have been reached before or after this time point. After 3 days, the plasma hCG

level gradually decreased and the hormone level was still significantly above background ($p < 0.05$) at 42 days after injection.

To determine the *in vivo* half-life of hCG in eel, trend lines were generated based on the plasma hCG levels at 3, 5, 7, and 14 days post injection (Figure 2b,c). According to the trend line formula, pure hCG had an *in vivo* plasma half-life of 3.83 days (Figure 2b). Injection of the FHM 2.1 cells resulted in a plasma hCG half-life equivalent to 4.62 days, which is longer than both values found for pure hCG and which suggests that hCG was synthesized *de novo* by the injected cells. At 1 day after injection of FHM 2.1 cells, a plasma hCG concentration of 10 mIU/mL was observed. This is less than one-tenth of the calculated theoretical daily maximum dose of ~120 mIU/g BW. Since the hCG spreads throughout the eel's entire body, it would not be reliable to calculate the total amount of implant-derived hCG that is present in the eel from the concentration of hCG that was measured in the blood plasma. Instead, we estimated the amount of pure hCG that would result in similar plasma levels as the amount of hCG produced by the implant. The single injection of pure hCG (~9 IU/g BW) resulted in a plasma hCG concentration of 5 IU/mL after 1 day. From these results we could estimate that the actual daily *in vivo* production level of the FHM cells was equivalent to a single injection of ~18 mIU hCG/g BW (((10 mIU/mL) / (5 IU/mL)) * (9 IU/g BW)). This is almost seven times lower than the theoretical daily maximum *in vivo* dose of ~120 mIU hCG/g BW based on a daily *in vitro* production of 13 IU hCG. The lower *in vivo* production might be the result of a combination of factors, such as (1) damage of the cells during the injection, (2) limited *in vivo* access to nutrients and growth factors, (3) inefficient release of hormone to the circular system, and (4) attack of the cellular implant by the immune system of the eel.

Effects of the cellular implant on sexual maturation of the European male eels

An increasing eye index (EI) is an external marker of sexual maturation in eels and associated with increased production of male sex steroids (e.g. Pankhurst, 1982; Durif et al., 2005; Peñaranda et al., 2010). The EI of the 2.1 group continuously increased after injection of the cellular implant and reached a maximum level at 21 days post injection ($\Delta EI = 2.22 \pm 0.27$), whereas the EI in the WT and saline group did not significantly change (Figure 3). After 21 days, the EI of the 2.1 group showed some regression; however, it remained at a significantly higher level (p <

0.05) compared with the WT and saline groups until the end of the trial. The hCG group (positive control) showed a stronger increase in EI, which reached a plateau level (ΔEI = 3.94 ± 0.40) at 28 days after injection and stayed at that high level until the end of the trial.

Testosterone (T) and its oxidized form 11-keto testosterone (11-KT) are important steroids for sexual maturation in eel (Nagahama, 1994; Peñaranda et al., 2010). Therefore, the plasma T values at the different time points were measured using ELISA. At one day after injection of the cellular implant, the plasma T concentration was already significantly increased as compared to the saline and WT negative control groups ($p < 0.05$, Figure 4). The plasma T level reached a maximum value of ~13 ng/mL at 7 days after injection, thereafter it gradually decreased. The plasma T concentrations in the positive control group (hCG)

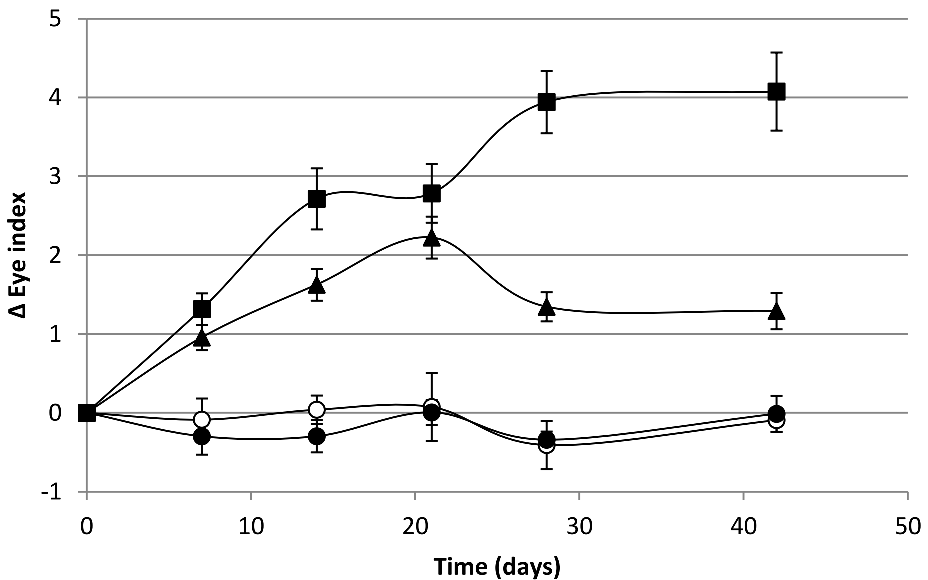

Figure 3: Change of the eye index (ΔEI). Male eels (n=7 per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲). The EI was determined at 7, 14, 21, 28, and 42 days after injection. The ΔEI was calculated by subtracting the average EI measured immediately before injection from the average EI measured at the indicated time points.

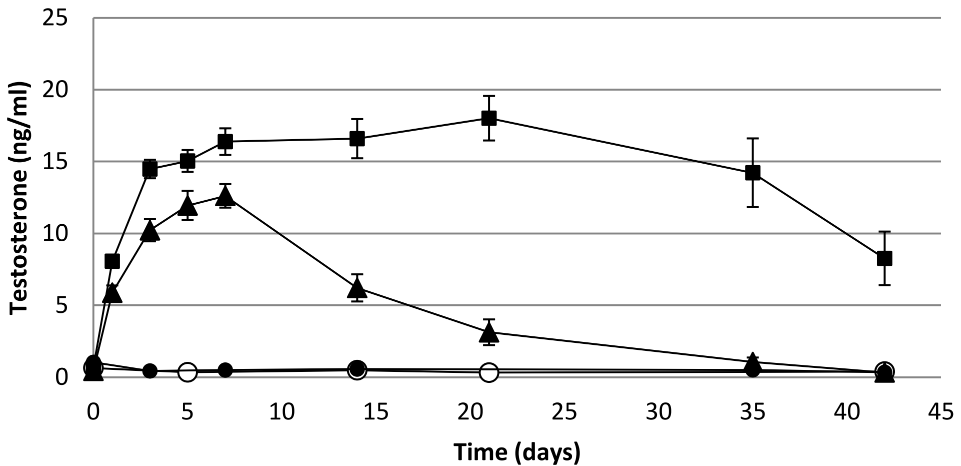

Figure 4: Temporal change of plasma testosterone (T) levels. Male eels (n=7 per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲).Blood samples were taken immediately before and at 1, 3, 5, 7, 14, 21, 35, and 42 days after injection. The blood plasma T levels were measured using ELISA.

were always significantly higher than those of the cellular implant group ($p < 0.05$). The highest concentration (~ 18 ng/mL) was measured at 21 days after hCG injection and even at 42 days post injection the plasma T concentration was still ~8 ng/mL. Although 11-KT, an oxidized form of T, also plays a major role in fish reproduction, the plasma 11-KT was not measured due to limited plasma sample amounts. Repeated sampling from 100g animals didn't allow sufficient sample size for additional assays.

Throughout the trial, all eels were checked for the production of milt. At 28 days after hCG injection, the first eels of the positive control group started the production of milt and at 42 days after injection, five out of six (83%) of the positive control eels produced milt (data not shown). In contrast, none of the eels from the other groups produced milt at any time point. At the end of the trial (t=42 days), gonads and livers from all males were weighed in order to calculate the GSI and HSI (data not shown). In addition, the histology of the gonads was studied in more detail using microscopy. The HSI did not significantly differ

between the positive control (hCG), negative control (WT and saline groups) and the cellular implant (2.1) groups. The GSI of the positive control group (hCG) was more than fifty times higher as compared to that of the negative control groups, which is not unexpected based on the highly efficient milt production. Advanced maturation of the positive control group was further confirmed by histological analysis, which showed mature spermatozoa of stage 4-6 in the lumen of the testis (Peñaranda et al., 2010). In contrast, the GSI of the cellular implant group was not increased as compared to the negative control groups and the testes were immature (stage 1-2) in all these three groups (data not shown).

In the current experiment FHM cells were used, whereas species-specific cells would probably be more suited. Eel cells are more likely to survive longer inside the eel and might be better adapted to the extracellular environment in the coelomic lumen of the eel. Although it is not known how the European eel's immune system responds to the cellular implant, our results show significantly increased plasma hCG levels in the implant group for up to 14 days after injection. It is possible that the FHM cells were broken down by the host's immune response or died as a result of lack of nutrition.

Conclusions and future prospects

In this study we demonstrated a new approach to induce maturation in eels by hormone producing cellular implants. The male eels showed a clear biological response to the implants. However, the hormone production of the cells within the host was too low and needs to be optimized.

There are several ways to significantly increase the production of hCG by the cells. Firstly, by using more powerful transcriptional promoters. In the cell line described in the current study the expression of hCG is driven by the zebrafish beta-actin promoter. Recently we sequenced the transcriptomes of multiple eel tissues (unpublished data), which revealed many genes that are expressed at a much higher abundance than the actin gene. We expect that using the promoters of those genes will result in much higher hormone production levels in the near future. Secondly, optimization of the intracellular ratio of the hCG alpha versus the beta polypeptide chains may increase the hormone production by the cell (Dr. Yves Combarnous, pers. comm.).

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Chapter 8

Summary and future perspectives

Summary

Freshwater eels (*Anguilla spp.*) have fascinated many scientists for centuries. Their incredible life cycle involves e.g. two distinct larval stages and a long distance migration, which may cover thousands of kilometers (even over 6000 km for the European eel, *A. anguilla*). The farming of eels is still dependent on the influx of wild glass eels, because eels do not mature naturally in captivity. Since the 1980s, the glass eel influx of several eel species drastically declined (Dekker et al., 2003), due to e.g. overexploitation, migration barriers, parasites and pollution (reviewed in van den Thillart & Dufour, 2009). The European eel was recently listed by the IUCN as a critically endangered species (Freyhoff & Kottelat, 2008), therefore there is an urgent call for artificial reproduction. Artificial reproduction may contribute to restoration of the eel population by releasing the current fishing pressures on the wild stocks, and allowing a sustainable eel aquaculture in the future.

Eels are still in a premature state at the onset of their spawning migration. Silvering that occurs before the start of migration is hypothesized a pubertal event (Aroua et al., 2005; Rousseau et al., 2009). Sexual maturation is inhibited due to insufficient release of gonadotropin-release hormone (GnRH) and blockage by dopamine acting on the synthesis and release of luteinizing hormone (LH) (Dufour et al., 1993, 2003, 2005; Vidal et al., 2004). Therefore, the sexual maturation of silver eels must take place during or after the spawning migration. Currently, reproduction of eels can only be achieved using a treatment with pituitary hormones, thereby circumventing the endogenous inhibition.

It was proposed that swimming exercise induces maturation in eels, by releasing the dopaminergic inhibition. Early stages of oocyte maturation appeared to be stimulated in females by swimming (Palstra et al., 2007; van Ginneken et al., 2007); however, further maturation such as vitellogenesis were found to remain inhibited in females (Palstra et al., 2008b, Palstra et al., 2010). On the other hand, spermatogenesis of wild male silver eels was found to be stimulated after swimming ca. 900 km (Palstra et al., 2008b). It was hypothesized that swimming for a longer distance equal to the full migration distance will result in full maturation in male eels. In order to test the above stated hypothesis, we first established the swimming capacity (i.e. optimal swimming speed, cost of transport)

of farmed male silver European eels (**Chapter 2**). In addition, we studied the group-wise swimming of eels. Earlier studies on the swimming capacity of eels was mainly focused on females (van Ginneken & van den Thillart, 2000; van Ginneken et al., 2005, Palstra et al., 2008a), which showed that females are very efficient swimmers, 4-6 times more efficient than rainbow trout. Based on our results of the costs of transport of male silver eels, we can conclude that males can swim even more efficient than females (Tudorache, Burgerhout & van den Thillart, unpublished data). This is in contrast with earlier proposed hypothesis that the much smaller males would consume more energy than females. In addition, when males were swimming group-wise we found a ca. 30% reduction of the cost of transport at all speeds tested. We also observed that during group-wise swimming males prefer a phase synchronized swimming mode which may represent a way to reduce the energy cost of swimming.

In **Chapter 3**, the hypothesis that spermiation of male silver eels will occur after longterm swimming was tested. Farmed male silver European eels were subjected to swimming exercise for a maximum of 6 months, covering a total distance of 6670 km. We have shown that there was no effect of swimming exercise on maturation in farmed male silver eels, which is a major difference with the previous study (Palstra et al., 2008b). The latter showed a marked stimulatory effect of swimming on maturation of wild silver males, respectively caught in brackish water during their migration. Data of the initial control of those wild males showed progression in spermatogenesis, indicating that maturation was already induced and that the dopaminergic inhibition was already released. This was quite different from the farmed eels used in our study. We concluded that swimming exercise is not the natural trigger for inducing maturation in farmed male silver eels.

Since the last decade the use of genomics increased rapidly. Physiological studies on maturation and reproduction of eels may benefit from gene expression profiling. Therefore, we sequenced and assembled a draft of the eel genome (**Chapter 4**). Here, we focused on the Hox genes, genes encoding transcription factors which are involved in the developmental patterning of the body plan. We showed that unlike any other teleost fish the eel retains fully populated, duplicate Hox clusters, those duplicate Hox clusters originated from the teleost-specific

genome duplication. All copies of Hox genes were found expressed in embryos (27 hour post fertilization) by RNA-seq transcriptomic analysis and *in situ* hybridzations. This draft of the eel genome will be a perfect reference for future transcriptomic analyses within all fields of biology.

During the maturation trajectory of female European eels, often >50% do not respond to the hormonal treatment (non-responders) that comprises ca. 3-6 months (Pedersen, 2003, 2004; Palstra et al., 2005). Selection of female broodstock prior or early during the maturation trajectory will increase efficiency of artificial reproduction. As response is related to the initial state of the female it is necessary to obtain non-invasive biomarkers. Based on an earlier study (Minegishi et al., unpublished data), it was expected that genes within the steroidogenic pathway and sex steroids were possible candidate markers. In **Chapter 5**, we conducted a reproduction trial to obtain specific biomarkers indicating the response of female eels. We correlated invasive markers with 'non'-invasive markers, using gonad tissue and blood plasma, respectively. For the first time, an eel-specific microarray analysis based on the European eel genome (Henkel et al., 2012) was used to analyze differences in transcriptomics of gonad tissue between responders and non-responders after 4 weekly injections. Blood analysis showed that the change in blood plasma levels of 17β-estradiol (E2) after 4 weekly injections significantly correlated with the gonadosomatic index (GSI). We concluded that the relative change in E2 plasma levels after 4 weeks as compared to initial measurements may be used as a biomarker to distinguish responders from non-responders for ca. 80%.

As the maturation trajectory of females of the New Zealand short-finned eels (*A. australis*) is much shorter than that of the European eel (2-4 months (Lokman & Young, 2000) vs 3-6 months (Pedersen, 2003, 2004; Palstra et al., 2005), respectively), hybridization of *A. anguilla* with a species such as *A. australis* may be a suitable option for eel aquaculture. In **Chapter 6** we produced viable larvae of *A. australis* up to 8 days post fertilization (dpf), and of a hybrid species between female *A. australis* and male *A. anguilla* up to 7dpf. We described the early ontogeny of short-finned eel and the hybrid species, and validated the production of a hybrid species using a specific difference in the 18S rDNA between the two species. Studying the reproduction of closely related species and early ontogeny of

the hybrid species will provide further understanding of the mechanisms of reproduction, and thereby possibly helpful for the breeding of the European eel.

Currently, the standard protocols for maturing and reproducing eels involve the use of weekly injections of pituitary extracts or purified gonadotropins. These weekly treatments causes handling stress and transient hormone peaks in blood plasma (Sato et al., 2000), which have negative effects on gametogenesis, affecting quality of eggs and larvae. Slow release systems are expected to solve the above problems by reducing the handling stress and physiological stress. However, the current slow release systems available require surgery and large amounts of purified hormones (Kagawa et al., 2009). We have developed a slow release system based on hormone producing fish cells. **Chapter 7** shows a proof-of-principle experiment where male eels were administered with a single injection with hCG-producing implants. hCG plasma levels were detectable up to 14 days after injection. The implant resulted in a significant increase of blood plasma testosterone levels and an increase of the eye index. These results show that the cellular implant induces sexual maturation in male eels. However, the hormone production of the implants needs to be optimized as they did not result in full spermiation.

Future perspectives

Success rates of artificially reproduced eels are still far from sufficient to create a sustainable eel aquaculture. Major problems, such as low egg quality and fertilization rates, are probably caused by the unnatural stimulation of maturation. Clearly, the protocols for eel reproduction are not yet suited for eel aquaculture.

Current reproduction protocols use pituitary extracts which contain a cocktail of FSH and LH from fish species other than eel, such as salmon or carp. In general, FSH is mainly involved in vitellogenesis and LH in final oocyte maturation (Nagahama & Yamashita, 2008). The frequently observed disruptions in egg development may be caused by continuously injecting both hormones. The use of slow release systems (Chapter 7, Kagawa et al., 2009) may contribute to a more natural stimulation of maturation.

In order to improve current protocols of artificial maturation and reproduction for freshwater eels, the unnatural hormonal treatment needs to be

replaced by the use of natural triggers. However, natural triggers inducing maturation in eels are still insufficiently studied, which is probably due to limited knowledge on the natural conditions encountered during the oceanic spawning migration.

The major crux of inducing or advancing maturation in eels appears to be the reproductive inhibition by dopamine, which is rather extreme in comparison to other fish species (Dufour et al., 2005, 2010; Vidal et al., 2004). Knowledge on the (neuro)-endocrinological pathways during natural maturation is of crucial importance to understand the mechanism of the dopaminergic inhibition and consequently the release of this inhibition. Information on the induction of vitellogenesis and final maturation may be obtained by studying closely related eel species that are far more advanced at the onset of oceanic migration, such as New Zealand longfinned eels (*A. dieffenbachii*; Lokman et al., 1998) or *A. celebesensis* (Hagihara et al., 2012).

Interestingly, the administration of melatonin to female eels resulted in a stimulation of the dopaminergic system, thereby inhibiting maturation (Sébert et al., 2008). It is therefore suggested that a decrease of melatonin levels may result in the release of the dopaminergic inhibition and thereby stimulate maturation. Melatonin levels may be decreased by changes in environmental factors such as salinity (López-Olmeda et al., 2009), temperature (Sébert et al., 2008; Porter et al., 2001), photoperiod (Porter et al., 1999; Hansen et al., 2001; Taranger et al., 1998), blue wave lengths (Bayarri et al., 2002; Ziv et al., 2007) or a combination of those. Additionally, it is believed that melatonin stimulates the KiSS/GPR54 system (Migaud et al., 2010), which was shown to be involved in the reproductive cycle acting upstream of GnRH and subsequently on gonadotropins. Kisspeptin is considered as an important gatekeeper for puberty in mammals as well as in fish (e.g. Elizur, 2009; Tena-Sempere et al., 2012; Zohar et al., 2010). Kisspeptin inhibited LHβ expression in primary cultures of pituitary cells, suggesting an inhibiting effect of kisspeptin on maturation (Pasquier et al., 2011). However, *in vivo* kisspeptin may have a different effect due to cerebral control, which still needs to be elucidated.

Based on the various studies currently available, induction and further stimulation of maturation is possibly mediated through the melatonin system,

which consequently may affect the KiSS/GPR54 system and thereby the puberty event. As melatonin is mainly regulated by photoperiod and temperature it is highly recommended for future studies to investigate the effects of those two environmental cues on maturation. The draft genome sequence (Henkel et al., 2012) may be helpful as reference for future transciptomic analyses yielding insight into the maturation pathways at a genetic level.

References

Aarestrup, K., Økland, F., Hansen, M.M., Righton, D., Gargan, P., Castonguay, M., Bernatchez, L., Howey, P., Sparholt, H., Pedersen, M.I., McKinley, R.S. 2009. Oceanic spawning migration of the European eel (*Anguilla anguilla*). *Science* **325**: 1660.

Abascal, F., Zardoya, R., Posada, D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**: 2104-2105.

Abrahams, M.V., Colgan, P.W. 1985. Risk of predation, hydrodynamic efficiency and their influence on school structure. *Env Biol Fish* **13**, 195-202.

Abrahams, M.V., Colgan, P.W. 1987. Fish schools and their hydrodynamic function: a reanalysis. *Env Biol Fish* **20**, 79-80.

Albert, V., Jonsson, B., Bernatchez, L. 2006. Natural hybrids in Atlantic eels (*Anguilla anguilla*, *A. rostrata*): evidence for successful reproduction and fluctuating abundance in space and time. *Mol Ecol* **15**:1903-1916.

Als, T.D., Hansen, M.M., Maes, G.E., Castonguay, M., Riemann, L., Aarestrup, K., Munk, P., Sparholt, H., Hanel, R., Bernatchez, L. 2011. All roads lead to home: panmixia of the European eel in the Sargasso Sea. *Mol Ecol* **20**: 1333-1346.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Amores, A., Force, A., Yan, Y-L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y-L., Westerfield, M., Ekker, M., Postlethwait, J.H. 1999. Zebrafish *hox* clusters and vertebrate genome evolution. *Science* **282**: 1711-1714.

Aoyama, J. 2009. Life history and evolution of migration in catadromous eels (genus *Anguilla*) Aqua-*BioSci. Monogr. (ABSM)* **2**: 1-42.

Arai, T., Otake, T., Tsukamoto, K. 2000. Timing of metamorphosis and larval segregation of the atlantic eels Anguilla rostrata and A. anguilla, as revealed by otolith microstructure and microchemistry. *Mar Biol* **137**: 39-45.

Archer, S., Hope, T., Partridge, J.C. 1995. The molecular basis for the green-blue sensitivity shift in rod visual pigments of the European eel. *Proc R Soc Lond B* **262**: 289-295

Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S. 2005. Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. *Neurendocrinology* **82**: 221-232.

Aroua, S., Weltzien, F-A., Le Belle, N., Dufour, S. 2007. Development of real-time RT-PCR assays for eel gonadotropins and their application to the comparison of in vivo and in vitro effects of sex steroids. *Gen Comp Endocr* **153**: 333-343.

Avise, J.C., Nelson, W.S., Arnold, J., Koehn, R.K., Williams, G.C., Thorsteinsson, V. 1990. The evolutionary genetic status of Icelandic eels. *Evolution* **44**: 1254-1262.

Barton, B.A., Iwama, G.K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann Rev Fish Dis* **1:** 3-26.

Bayarri, M.J., Madrid, J.A., Sanchez-Vazquez, F.J. 2002. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. *J Pin Res* **32**: 34-40.

Beamish, F.W.H. 1978. Swimming capacity. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, Vol. VII, Academic Press, New York pp 101-187.

Belyayev, V.V., Zuyev, G.V. 1969. Hydrodynamic hypothesis of school formation in fishes. *Probl Ichthyol* **9:** 578-584.

Bezdenezhnykh, V.A., Prokhorcik, G.A., Petrikov, A.M., Petukov, V.B., Plyuta, M.V. 1983. Obtaining the larvae of European eel *Anguilla anguilla* L. (Pisces, Anguillidae), under experimental conditions. *Dokl Akad Nauk SSSR* **268:** 1264-1266 (In Russian).

Bishop, R.E., Torres, J.J. 1999. Leptocephalus energetics: metabolism and excretion. *J Exp Biol* **202**: 2485-2493.

Boëtius, J., Boëtius, L. 1980. Experimental maturation of female silver eels, *Anguilla anguilla*. Estimates of fecundity and energy reserves for migration and spawning. *Dana* **1**: 1-28.

Boetzer, M., Henkel, C.V., Jansen, H.J., Butler, D., Pirovano, W. 2011. Scaffolding preassembled contigs using SSPACE. *Bioinformatics* **27**: 578-579.

Breder, C.M. 1926. The locomotion of fishes. *Zoologica (N.Y.)* **4**: 159-297.

Breder, C.M. 1967. On the survival value of fish schools. *Zoologica* **52**: 25-40.

Brett, J.R. 1964. The respiratory metabolism and swimming performance of Young sockeye salmon. *J Fish Res Board Canada* **21**: 1183-1226.

Burgerhout, E., Manabe, R., Brittijn, S.A., Aoyama, J., Tsukamoto, K., van den Thillart, G.E.E.J.M. 2011a. Dramatic effect of pop-up satellite tags on eel swimming. *Naturwissenschaften* **98**: 631-634.

Burgerhout, E., Brittijn, S.A., Kurwie, T., Decker, P., Dirks, R.P., Palstra, A.P., Spaink, H.P., van den Thillart, G.E.E.J.M. 2011b. First artificial hybrid of the eel species *Anguilla australis* and *Anguilla Anguilla*. *BMC Dev Biol* **11**: 16.

Castonguay, M., Gilbert, D. 1995. Effects of tidal streams on migrating Atlantic mackerel, *Scomber scombrus* L.. *ICES J Mar Sci* **52**: 941-954.

Chang, S.L., Kou, G.H., Liao, I.C. 2004. Temperature adaptation of the Japanese eel (*Anguilla japonica*) in its early stages. *Zool Stud* **43**: 571-579.

Chiba, H., Iwatsuki, K., Hayami, K., Hara, A., Yamauchi, K. 2007. Changes in serum steroid hormones and vitellogenin levels in cultured female European eels *Anguilla anguilla* during artificially induced ovarian development. *J World Aquacult Soc* **25**: 553-560.

Clevestam, P.D., Ogonowski, M., Sjöberg, N.B., Wickström, H. 2010. Too short to spawn? Implications of small body size and swimming distance on successful migration and maturation of the European eel *Anguilla anguilla. J Fish Biol* **78***:* 1073-1089.

Coetzee, J. 2000. Use of a shoal analysis and patch estimation system (SHAPES) to characterise sardine schools. *Aquat Living Resour* **13**: 1-10.

Crow, K.D., Stadler, P.F., Lynch, V.J., Amemiya, C., Wagner, G.P. 2006. The "fish-specific" Hox cluster duplication is coincident with the origin of teleosts. *Mol Biol Evol* **23**: 121-136.

Cunningham, J.T. 1897. The discovery of the larva of the common eel. *Nature* **55**: 467-468.

Dekker, W. 2003. Did lack of spawners cause the collapse of the European eel, *Anguilla anguilla*? *Fisheries Manag Ecol* **10**: 365-376.

Dekker, W., Casselman, J.M., Cairns, D.K., Tsukamoto, K., Jellyman, D., Lickers, H. 2003. Worldwide decline of eel resourses necessitates immediate action. *Fisheries* **28**: 28-30.

Di-Poï, N., Montoya-Burgos, J.I., Miller, H., Pourquié, O., Milinkovitch, M.C., Duboule, D. 2010. Changes in *Hox* genes' structure and function during the evolution of the squamate body plan. *Nature* **464**: 99–103.

Domenici, P., Ferrari, R.S., Steffensen, J.F., Batty, R.S. 2002. The effect of progressive hypoxia on school structure and dynamics in Atlantic herring *Clupea harengus*. *Proc R Soc Lond B.* **269**: 2103-2111.

Dommasnes, A., Rey, F., Røttingen, I. 1994. Reduced oxygen concentrations in herring wintering areas. *ICES J Mar Sci* **51**: 63-69.

Duboule, D. 2007. The rise and fall of Hox gene clusters. *Development* **134**: 2549-2560.

Dufour, S., van den Thillart, G. 2009. Reproduction capacity of European eels. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel, reproduction index, a useful tool for conservation management., Springer, Dordrecht pp. 465-475.

Dufour, S., Lopez, E., Le Menn, F., Le Belle, N., Baloche, S., Fontaine, Y.A., 1988. Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. *Gen Comp Endocr* **70**: 20-30.

Dufour, S., Montero, M., Le Belle, N., Bassompiere, M., King, J.A., Millar, R.P. 1993. Differential distribution and response to experimental maturation of two forms of brain gonadotropin-releasing hormone (GnRH) in the European eel, *Anguilla anguilla*. *Fish Physiol Biochem* **11**: 99-106.

Dufour, S., Burzawa-Gerard, E., Le Belle, N., Shaihi, M., Vidal, B. 2003. Reproductive endocrinology of the European eel, *Anguilla anguilla.* In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer-Verlag, Tokyo pp. 373-383.

Dufour, S., Weltzien, F-A., Sébert, M-E., Le Belle, N., Vidal, B., Vernier, P., Pasqualini, C. 2005. Dopaminergic inhibition of reproduction in teleost fishes – Ecophyiological and evolutionary implications. *Ann NY Acad Sci.* **1040**: 9-21.

Dufour, S., Sebert, M-E., Weltzien, F-A., Rousseau, K., Pasqualini, C. 2010. Neuroendocrine control by dopamine of teleost reproduction. *J Fish Biol* **76**: 129-160.

Durif, C., Dufour, S., Elie, P. 2005. The silvering process of the eel: a new classification from the yellow resident stage to the silver migrating stage. *J Fish Biol* **66**: 1-19.

Durif, C., Dufour, S., Elie, P. 2006. Impact of silvering stage, age, body size and condition on reproductive potential of the European eel. *Mar Ecol Prog Ser* **327**: 171-181

Eel Management Plan of the Netherlands. 2012. Available: http://www.rijksoverheid.nl/documenten-en-publicaties/rapporten/2012/06/01/implementation-of-eel-management-plan-emp-in-the-netherlands.html

Ege, V. 1939. A revision of the genus *Anguilla* Shaw, a systematic, phylogenetic and geographical study. *Dana Rep* **16:** 1-256.

Elizur, A. 2009. The KiSS1/GPR54 system in fish. *Peptides* **30**: 164-170.

Essner, J.J., Amack, J.D., Nyholm, M.K., Harris, E.B., Yost, J. 2005. Kuppfer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Development* **132**:1247-1260.

Fish, F.E. 1999. Energetics of swimming and flying in formation. *Comm Theor Biol* **5**: 283-304.

Fontaine, M. 1936. Sur la maturation complete des organs génitaux de l'anguille male et l'émission spontanée. *C r hebd Séanc Acad Sci Paris* **202**:132-134.

Fontaine, M., Betrand, E., Lopez, E., Callamand, O. 1964. Sur la maturation des organs génitaux de l'anguille femelle (*Anguilla anguilla* L.) et l'émission spontanée des oeufs en aquarium. *Comp Rend Acad Sci* **259**: 2907-2910.

Fontaine, Y.A., Dufour, S., Alinat, J., Fontaine, M., 1985. L'immersion prolongée en profondeur stimule la fonction hypophysaire de l'Anguille européenne (*Anguilla anguilla* L.) femelle. *Comp Rend Acad Sci Paris série III* **2:** 83-87.

Foo, J.T.W., Lam, T.J. 1993. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone levels in the tilapia *Oreochromis mossambicus. Aquaculture* **115:** 145-158.

Frankowski, J., Bastrop, R. 2009. Identification of *Anguilla anguilla* (L.) and *Anguilla rostrata* (Le Sueur) and their hybrids based on a diagnostic single nucleotide polymorphism in nuclear 18 S rDNA. *Mol Ecol Resour* **10**:173-176.

Freyhof, J., Kottelat, M. 2008. *Anguilla anguilla*. IUCN Red List of Threatened Species. Version 2010.4. Available: http://www.iucnredlist.org

Fricke, H., Kaese, R. 1995. Tracking of artificially matured eels (*Anguilla anguilla*) in the Sargasso Sea and the problem of the Eel's Spawning Site. *Naturwissenschaften* **82**: 32-36.

Friedland, K.D., Miller, M.J., Knights, B. 2007. Oceanic changes in the Sargasso Sea and declines in recruitment of the European eel. *ICES J Mar Sci* **64**: 519-530.

Godin, J-G.J., Morgan, M.J. 1985. Predator avoidance and school size in a cyprinodontid fish, the banded killifish (*Fundulus diaphanus* Lesueur). *Behav Ecol Sociobiol* **16**: 105-110.

Götz, S., García-Gómez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talón, M., Dopazo, J., Conesa, A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* **36**: 3420-3435.

Grassi, G.B. 1896. The reproduction and metamorphosis of the common eel (*Anguilla vulgaris*). *Proc R Soc London* **60**: 262-271.

Greenwood, P.H., Rosen, D.E., Weitsman, S.H., Myers, G.S. 1966. Phyletic studies of teleostean fishes, with a provisional classification of living forms. *Bull Am Mus Nat Hist* **131:** 339-456.

Guo, B., Gan, X., He, S. 2010. Hox genes of the Japanese eel *Anguilla japonica* and Hox cluster evolution in teleosts. *J Exp Zool (Mol Dev Evol)* **314B**: 135-147.

Guy, L., Kultima, J.R., Andersson, S.G. 2010. genoPlotR: comparative gene and genome visualization in R. *Bioinformatics* **26**: 2334-2335.

Hagihara, S., Aoyama, J., Limbong, D., Tsukamoto, K. 2012. Morphological and physiological changes of female tropical eels, *Anguilla celebesensis* and *Anguilla marmorata*, in relation to downstream migration. *J Fish Biol* **81**: 408-426.

Han, Y-S., Liao, I-C., Tzeng, W-N., Huang, Y-S., Yu, J.Y-L. 2003. Serum estradiol-17*β* and testosterone levels during silvering in wild Japanese eel *Anguilla japonica*. *Comp Biochem Physiol B* **136:** 913-920.

Hansen, L.P., Jonsson, B. 1985. Downstream migration of hatchery-reared smolts of Atlantic salmon (*Salmo salar* L.) in the river Imsa, Norway. *Aquaculture* **45**: 237-248.

Hansen, T., Karlsen, Ø., Taranger, G.L., Hemre, G-I., Holm, J.S., Kjesbu, O.S. 2001. Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. *Aquaculture* **203**: 51-67.

He, X., Yan, Y-L., Eberhart, J.K., Herpin, A., Wagner, T.U., Schartl, M., Postlethwait, J.H. 2011. miR-196 regulates axial patterning and pectoral appendage initiation. *Dev Biol* **357**: 463-477.

Henkel, C.V., Burgerhout, E., de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J., Spaink, H.P., Dufour, S., Weltzien, F-A., Tsukamoto K., van den Thillart, G.E.E.J.M. 2012. Primitive duplicate Hox clusters in the European eel's genome. *PLoS ONE* **7**: e32231.

Hermelink, B., Wuertz, S., Trubiroha, A., Rennert, B., Kloas, W., Schulz, C. 2011. Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. *Gen Comp Endocr* **172**: 282-292.

Herskin, J., Steffensen, J.F. 1998. Energy savings in sea bass swimming in a school: measurements of tail beat frequency and oxygen consumption at different swimming speeds. *J Fish Biol* **53**: 366–376.

Hiroi, J., Maruyama, K., Kawazu, K., Kaneko, T., Ohtani-Kaneko, R., Yasumasu, S. 2004. Structure and developmental expression of hatching enzyme genes of the Japanese eel *Anguilla japonica*: an aspect of the evolution of fish hatching enzyme gene. *Dev Genes Evol* **214**: 176-184.

Hoar, W.S. 1953. Control and timing of fish migration. *Biol Rev* **28**: 437-452.

Holland, P.W., Garcia-Fernàndez, J., Williams, S.A., Sidow, A. 1994 Gene duplications and the origins of vertebrate development. Development Supplement: 123-133.

Hurley, I.A., Lockridge, Mueller, R,, Dunn, K.A., Schmidt, E.J., Friedman, M., Ho, R.K., Prince, V.E., Yang, Z., Thomas, M.G., Coates, M.I. 2007. A new time-scale for ray-finned fish evolution. *Proc R Soc B* **274**: 489-498.

Ijiri, S., Tsukamoto, K., Chow, S., Kurogi, H., Adachi, S., Tanaka, H. 2011. Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. *Aquaculture Europe* **32**: 13-17.

Inoue, J.G., Miya, M., Tsukamoto, K., Nishida, M. 2004. Mitogenomic evidence for the monophyly of elopomorph fishes (Teleostei) and the evolutionary origin of the leptocephalus larva. *Mol Phylogenet Evol* **32**: 274-286.

Inoue, J.G., Miya, M., Venkatesh, B., Nishida, M. 2005. The mitochondrial genome of Indonesian coelacanth *Latimera menadoensis* (Sarcopterygii: Coelacanthiformes) and divergence time estimation between the two coelacanths. *Gene* **349**: 227-235.

Inoue, J.G., Miya, M., Miller, M.J., Sado, T., Hanel, R., Hatooka, K., Aoyama, J., Minegishi, Y., Nishida, M., Tsukamoto, K. 2010. Deep-ocean origin of the freshwater eels. *Biol Lett* **6**: 363-366.

Iwamatsu, T. 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev* **121**: 605-618.

Jaillon, O., Aury, J-M., Brunet, F., Petit, J-L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C.,, Ozouf-Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, et al. 2004. Genome duplicaton in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* **431**: 946–957.

Jellyman, D., Tsukamoto, K. 2002. First use of archival transmitters to track migrating freshwater eels *Anguilla dieffenbachii* at sea. *Mar Ecol Prog Ser* **233**: 207–215.

Jellyman, D., Tsukamoto, K. 2005. Swimming depths of offshore migrating longfin eels *Anguilla dieffenbachii*. *Mar Ecol Prog Ser* **286**: 261-267.

Jellyman, D., Tsukamoto, K. 2010. Vertical migrations may control maturation in migrating female *Anguilla dieffenbachii. Mar Ecol Prog Ser* **404:** 241-247.

Johansen, J.L., Vaknin, R., Steffensen, J.F., Domenici, P. 2010. Kinematics and energetic benefits of schooling in the labriform fish, striped surfperch *Embiotoca lateralis*. *Mar Ecol Prog Ser* **420**: 221-229.

Jones, D.T., Taylor, W.R., Thornton, J.M. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* **8**: 275-282.

Kagawa, H. 2003. Artificial induction of oocyte maturation and ovulation. In Aida, K., Tsukamoto, K., Yamauchi, Y. (Eds.), Eel Biology, Springer, Tokyo. pp. 401-414.

Kagawa, H., Tanaka, H., Ohta, H., Unuma, T., Nomura, K. 2005. The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. *Fish Physiol Biochem* **31**: 193-199.

Kagawa, H., Kasuga, Y., Adachi, J., Nishi, A., Hashimoto, H., Imaizumi, H., Kaji, S. 2009. Effects of continuous administration of human chorionic gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in male Japanese eel, *Anguilla japonica*. *Aquaculture* **296**: 117-122.

Kagawa, H., Sakurai, Y., Horiuchi, R., Kazeto, Y., Gen, K., Imaizumi, H., Masuda, Y. 2013. Mechanism of oocyte maturation and ovulation and its application to seed production in the Japanese eel. *Fish Physiol Biochem* **39**: 13-17.

Kazeto, Y., Tosaka, R., Matsubara, H., Ijiri, S., Adachi, S. 2011. Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation in the Japanese eel. *J Steroid Biochem Mol Biol* **127**: 149-154.

Kern, S., Koumoutsakos, P. 2006. Simulations of optimized anguilliform swimming. *J Exp Biol* **209**: 4841-4857.

Killen, S.S., Marras, S., Steffensen, J.F., McKenzie, D.J. 2011. Aerobic capacity influences the spatial position of individuals within fish schools. *Proc R Soc B* doi: 10.1098/rspb.2011.1006.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. *Dev Dynamics* **203**: 253-310.

Kimura, S., Inoue, T., Sugimoto, T. 2001. Fluctuation in the distribution of low-salinity water in the North Equatorial Current and its effect on the larval transport of the Japanese eel. *Fish Oceanogr* **10**: 51–60.

Kozomara, A., Griffiths-Jones, S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* **39**: D152-D157.

Kuraku, S., Meyer, A. 2009. The evolution and maintenance of Hox gene clusters in vertebrates and the teleost-specific genome duplication. *Int J Dev Biol* **53**: 765–773.

Kurokawa, T., Okamoto, T., Gen, K., Uji, S., Murashita, K., Unuma, T., Nomura, K., Matsubara, H., Kim, S., Ohta, H., Tanaka, H. 2008. Influence of water temperature on morphological deformities in cultured larvae of Japanese eel, *Anguilla japonica*, at completion of yolk resorption. *J World Aquacult Soc* **39**: 726-735.

Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., Parant, J.M., Yost, H.J., Kanki, J.P., Chien, C.B. 2007 The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* **236**: 3088-3099.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Lauder, G.V., Tytell, E.D. 2006. Hydrodynamics of undulatory propulsion. In: Shadwick, R.E., Lauder, G.V., (Eds.), Fish Biomechanics, Elsevier Academic Press, Amsterdam pp. 425-468.

Liao, J.C. 2007. A review of fish swimming mechanics and behaviour in altered flows. *Phil Trans R Soc B* **362**: 1973-1993.

Lokman, P.M., Young, G. 2000. Induced spawning and early ontogeny of New Zealand freshwater eels (*Anguilla dieffenbachii* and *A. australis*). *N Z. J Mar Freshw* **34**: 135-145.

Lokman, P.M., Vermeulen, G.J., Lambert, J.G.D., Young, G. 1998. Gonad histology and plasma steroid profiles in wild New Zealand freshwater eels (Anguilla dieffenbachii and A. australis) before and at the onset of the natural spawning migration. I. Females. *Fish Physiol Biochem* **19**: 325–338.

Lokman, P.M., Wass, R.T., Syter, H.C., Scott, S.G., Judge, K.F., Young, G. 2001. Changes in steroid hormone profiles and ovarian histology during salmon pituitary-induced vitellogenesis and ovulation in female New Zealand longfinned eels, *Anguilla dieffenbachii* Gray. *J Exp Zool* **289**: 119-129.

Lokman, P.M., George, K.A., Divers, S.L., Algie, M., Young, G. 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. *Reproduction* **133**: 955-967.

López-Olmeda, J.F., Oliveira, C., Kalamarz, H., Kulczykowska, E., Delgado, M.J., Sánchez-Vázquez, F.J. 2009. Effects of water salinity on melatonin levels in plasma and peripheral tissues and on melatonin binding sites in European sea bass (*Dicentrarchus labrax*). *Comp Biochem Physiol A* **152**: 486-490.

Lynch, V.J., Wagner, G.P. 2008. Resurrecting the role of transcription factor change in developmental evolution. *Evolution* **62**: 2131–2154.

Magurran, A.E., Higham, A. 1988. Information transfer across fish shoals under predator threat. *Ethology* **78**: 153–158.

Mainguy, G., Koster, J., Woltering, J., Jansen, H., Durston, A. 2007. Extensive polycistronism and antisense transcription in the mammalian Hox clusters. *PLoS ONE* **2**: e356.

Manabe, R., Aoyama, J., Watanabe, K., Kawai, M., Miller, M.J., Tsukamoto, K. 2011. First observations of the oceanic migration of Japanese eel, from pop-up archival transmitting tags. *Mar Ecol Prog Ser* **437**: 229-240.

Matschiner, M., Hanel, R., Salzburger, W. 2011. On the origin and trigger of the notothenioid adaptive radiation. *PLoS ONE* **6**:e18911.

Matsubara, H., Lokman, P.M., Senaha, S., Kazeto, Y., Ijiri, S., Kambegawa, A., Hirai, T., Young, G., Todo, T., Adachi, S., Yamauchi, K. 2003. Synthesis and possible function of 11-ketotestosterone during oogenesis in eel (*Anguilla* spp.). *Fish Physiol Biochem* **28:** 353–354.

Matsubara, H., Lokman, P.M., Kazeto, Y., Adachi, S., Yamauchi, K. 2005. Serum steroid profiles in artificially maturing female Japanese eel, *Anguilla Japonica*. *Aquaculture* **243**: 393-402.

McCleave, J.D. 2003. Spawning areas of the Atlantic eels. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer-Verlag, Tokyo pp. 141-156.

Mehta, R.S., Ward, A.B., Alfaro, M.E., Wainwright, P.C. 2010. Elongation of the body in eels. *Integr Comp Biol* **50**: 1091-1105.

Methling, C., Tudorache, C., Skov, P.V., Steffensen, J.F. 2011. Pop-up satellite tags impair swimming performance and energetics of the European eel (*Anguilla anguilla*). *PLoS ONE* **6**: e20797.

Meyer, A., van de Peer, Y. 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *BioEssays* **27**: 937-945.

Migaud, H., Davie, A., Taylor, J.F. 2010. Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. *J Fish Biol* **76**: 27-68.

Miller, M.J. Ecology of anguilliform leptocephali: remarkable transparent fish larvae of the ocean surface layer. *Aqua-BioSci Monogr (ABSM)* **2:** 1-94.

Minegishi, Y., Aoyama, J., Inoue, J.G., Miya, M., Nishida, M., Tsukamoto, K. 2005. Molecular phylogeny and evolution of the freshwater eels genus Anguilla based on the whole mitochondrial genome sequences. *Mol Phylogen Evol* **34**: 134-146.

Minegishi, Y., Dirks, R.P., de Wijze, D.L., Brittijn, S.A., Burgerhout, E., Spaink, H.P., van den Thillart, G.E.E.J.M. 2012. Quantitative bioassays for measuring biologically functional gonadotropins based on eel gonadotropic receptors. *Gen Comp Endocr* **178**: 145-152.

Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y. 1991a. Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *Proc Natl Acad Sc* **88**: 5774-5778.

Miura, T., Yamauchi, C., Nagahama, Y., Takahashi, H. 1991b. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. *Zool Sci* **8**: 63-73.

Miura, T., Miura, C., Yamauchi, K. 2003. Spermatogenesis in the Japanese eel. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer-Verlag, Tokyo pp. 319-330.

Mochioka, M., Iwamizu, M. 1996. Diet of anguilloid larvae: leptocephali feed selectively on larvacean houses and fecal pellets. *Mar Biol* **125**: 447-452.

Müller, U.K., van den Heuvel, B.L.E., Stamhuis, E.J., Videler, J.J. 1997. Fish foot prints: morphology and energetics of the wake behind a continuously swimming mullet (*Chelon Labrosus* Risso). *J Exp Biol* **200**: 2893-2906.

Mungpakdee, S., Seo, H-S., Angotzi, A.R., Dong, X., Akalin, A., Chourrout, D. 2008. Differential evolution of the 13 Atlantic salmon Hox clusters. *Mol Biol Evol* **25**: 1333-1343.

Nagahama, Y. 1994. Endocrine regulation of gametogenesis in fish. *Int J Dev Biol* **38**: 217-229.

Nagahama, Y., Yamashita, M. 2008. Regulation of oocyte maturation in fish. *Dev Growth Differ* **50**: S195-S219.

Nelson, J.S. 2006. Fishes of the world, 4th edition. Wiley, Hoboken.

Ohta, H., Tanaka, H. 1997. Relationship between serum levels of human chorionic gonadotropin (hCG) and11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel. *Aquaculture* **153**: 123-134.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K. 1996. Changes in fertilization and hatching rates with time after ovulation induced by 17,20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica***.** *Aquaculture* **139:** 291-301.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., Hirose, K. 1997. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiol Biochem* **17**: 163-169.

Oidtmann, B., Simon, D., Holtkamp, N., Hoffmann, R., Baier, M. 2003. Identification of cDNAs from Japanese pufferfish (Fugu rubripes) and Atlantic salmon (Salmo salar) coding for homologues to tetrapod prion proteins. *FEBS Letters* **538**: 96-100.

Okamura, A., Oka, H.P., Yamada, Y., Utoh, T., Mikawa, N., Horie, N., Tanaka, S. 2002. Development of lateral line organs in leptocephali of the freshwater eel *Anguilla japonica* (Teleostei, Anguilliformes). *J Morph* **254**: 81-91.

Okamura, A., Zhang, H., Utoh, T., Akazawa, A., Yamada, Y., Horie, N., Mikawa, N., Tanaka, S., Oka, H.P. 2004. Artificial hybrid between *Anguilla anguilla* and *A. japonica*. *J Fish Biol* **64**: 1450-1454.

Okamura, A., Yamada, Y., Yokouchi, K., Horie, N., Mikawa, N., Utoh, T., Tanaka, S., Tsukamoto, K. 2007a. A silvering index for the Japanese eel *Anguilla japonica*. *Env Biol Fish* **80**: 77-89.

Okamura, A., Yamada, Y., Horie, N., Utoh, T., Mikawa, N., Tanaka, S., Tsukamoto, K. 2007b. Effects of water temperature on early development of Japanese eel *Anguilla japonica*. *Fish Sci* **73**: 1241-1248.

Okamura, A., Yamada, Y., Horie, N., Utoh, T., Mikawa, N., Tanaka, S., Tsukamoto, K. 2008. Effects of silvering state on induced maturation and spawning in wild female Japanese eel *Anguilla japonica. Fish Sci* **74**: 642-648.

Oliveira, K., Hable, W.E. 2010. Artificial maturation, fertilization, and early development of the American eel (Anguilla rostrata) *Can J Zool* **88**: 1121-1128.

Otake, T. 2003. Metamorphosis. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds), Eel biology, Springer-Verlag, Tokyo pp. 61-74.

Palstra, A., van den Thillart, G. 2009. Artificial maturation and reproduction of the European eel. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel, reproduction index, a useful tool for conservation management., Springer, Dordrecht pp. 309-331

Palstra, A.P., van den Thillart, G.E.E.J.M. 2010. Swimming physiology of European silver eels (*Anguilla anguilla*, L.): energetic costs and effects on sexual maturation and reproduction. *Fish Physiol Biochem* **36**: 297-322.

Palstra, A.P., Cohen, E.G.H., Niemantsverdriet, P.R.W., van Ginneken, V.J.T., van den Thillart, G.E.E.J.M. 2005. Artificial maturation and reproduction of European silver eel: Development of oocytes during final maturation. *Aquaculture* **249:** 533-547.

Palstra, A.P., van Ginneken, V.J.T., Murk., A.J., van den Thillart, G.E.E.J.M. 2006. Are dioxin-like contaminants responsible for the eel (*Anguilla anguilla*) drama? *Naturwissenschaften* **93**: 145-148

Palstra, A.P., Heppener, D.F.M., van Ginneken, V.J.T., Székely, C., van den Thillart, G.E.E.J.M. 2007a. Swimming performance of silver eels is severely impaired by the swim-bladder parasite *Anguillicola crassus*. *J Exp Mar Biol Ecol* **352**: 244-256.

Palstra, A., Curiel, D., Fekkes, M., de Bakker, M., Székely, C., van Ginneken, V., van den Thillart, G. 2007b. Swimming stimulates oocyte development in European eel. *Aquaculture* **270**: 321-332.

Palstra A., van Ginneken, V., van den Thillart, G. 2008a Cost of transport and optimal swimming speed in farmed and wild European silver eels (*Anguilla anguilla*). *Comp Biochem Physiol A* **151**: 37-44.

Palstra, A.P., Schnabel, D., Nieveen, M.C., Spaink, H.P., van den Thillart, G.E.E.J.M. 2008b. Male silver eels mature by swimming. *BMC Physiol* **8**: 14.

Palstra, A.P., Schnabel, D., Nieveen, M.C., Spaink, H.P., van den Thillart, G. 2010a. Swimming suppresses hepatic vitellogenesis in European female silver eel as shown by expression of the estrogen receptor 1, vitellogenin1 and vitellogenin2 in the liver. *Reprod Biol Endocrin* **8**: 27.

Palstra, A.P., Crespo, D., van den Thillart, G.E.E.J.M., Planas, J.V. 2010b. Saving energy to fuel exercise: swimming suppresses oocyte development and downregulates ovarian transcriptomic response of rainbow trout *Oncorhynchus mykiss*. *Am J Physiol Regul Integr Comp Physiol* **299**: R486-R499.

Pankhurst, N.W. 1982. Relation of visual changes to the onset of sexual maturation in the European eel *Anguilla anguilla* L. *J Fish Biol* **21**: 127-140.

Pankhurst, N.W., Lythgoe, J.N. 1983. Changes in vision and olfaction during sexual maturation in the European eel *Anguill anguilla* (L.). *J Fish Biol* **23**: 229-240.

Pankhurst, N.W., Sorensen, P.W. 1984. Degeneration of the alimentary tract in sexually maturing European *Anguilla anguilla* (L.) and American eels *Anguilla rostrata* (Lesueur). *Can J Zool* **62**: 1143-1149.

Parker, F.R.J. 1973. Reduced metabolic rates in fishes as a result of induced schooling. *Trans Am Fish Soc* **102**: 125-131.

Partridge, B.L., Pitcher, T., Cullen, J.M., Wilson, J. 1980. The three-dimensional structure of fish schools. *Behav Ecol Sociobiol* **6**: 277-288.

Partridge, B.L., Johansson, J., Kalish, J. 1983. The structure of schools of giant bluefin tuna in Cape Cod Bay. *Environ Biol Fish* **9:** 253-262.

Pasquier, J., Lafont, A-G., Leprince, J., Vaudry, H., Rousseau, K., Dufour, S. 2011. First evidence for a direct inhibitory effect of kisspeptins on LH expression in the eel, *Anguilla anguilla*. *Gen Comp Endocr* **173**: 216–225.

Pasquier, J., Lafont, A-G., Jeng, S.R., Morini, M., Dirks, R., van den Thillart, G., Tomkiewicz, J., Tostivint, H., Chang, C-F., Rousseau, K., Dufour, S. 2012. Multiple kisspeptin receptors in early osteichthyans provide new insights into the evolution of this receptor family. *PLoS ONE* **7**: e48931.

Pedersen, B.H. 2003. Induced sexual maturation of the European eel *Anguilla anguilla* and fertilization of the eggs. *Aquaculture* **224:** 323-338.

Pedersen, B.H. 2004. Fertilization of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture* **237:** 461-473.

Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F. 2010. Molecular and physiological study of the artificial maturation process in European eel males: from brain to testis. *G Comp Endocr* **166**: 160-171.

Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., van den Thillart, G.E.E.J.M., Asturiano, J.F. 2011. Influence of temperature regime on endocrine parameters and

vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. *Gen Comp Endocr* **174**: 51-59.

Peter, R.E., Calvayrac, R., Nahorniak, C.S., Omeljianuk, R.J., Sokolowska, M., Shih, S.H., Billard, R.E. 1986. Interactions of catecholamines and GnRH in regulation of gonadotropic secretion in teleost fish. *Rec Prog Horm Res* **42**: 513-548.

Pfeiler, E. 1999. Developmental physiology of elopomorph leptocephali. *Comp Biochem Physiol A* **123**: 113–128.

Pierron P., Baudrimont M., Dufour S., Elie P., Bossy A., Baloche S., Mesmer N., Gonzalez P., Bourdineaud J.-P., Massabuau J.-C. 2008. How Cadmium could compromise the completion of the European eel's reproductive migration. *Environ Sci Technol* **42**: 4607-4612.

Pitcher, T.J., Magurran, A.E., Winfield, I.J. 1982. Fish in larger shoals find food faster. *Behav Ecol Sociobiol* **10**: 149-151.

Pitcher, T. J., Parrish, J. K. 1993. Functions of shoaling behaviour in teleosts. In: Pithcher, T.J. (Ed). Behaviour of teleost fishes, Chapman & Hall, London, pp. 363-439.

Planas, J.V., Swanson, P. 2008. Physiological function of gonadotropins in fish. In: Rocha, M.J., Arukwe, A., Kapoor, B.G. (Eds), Fish Reproduction, Science Publishers, Enfield (NH), pp. 37-66.

Porter, M.J.R., Duncan, N., Mitchell, D., Bromagea, N.R. 1998. The use of cage lighting to reduce plasma melatonin in Atlantic salmon (*Salmo salar*) and its effects on the inhibition of grilsing. *Aquaculture* **176**: 237-244.

Porter, M.J.R., Duncan, N., Handeland, S.O., Stafansson, S.O., Bromage, N.R. 2001. Temperature, light intensity and plasma melatonin levels in juvenile Atlantic salmon. *J Fish Biol* **58**: 431-438.

Pottinger, T.G., Campbell, P.M., Sumpter, J.P. 1991. Stress induced disruption of the salmonid liver–gonad axis. In Scott, A.P., Sumpter, J.P., Kime D.E., Rolfe, M.S. (Eds.), Reproductive Physiology of Fish, FishSymp 91, Sheffield pp. 114–116.

Quintella, B.R., Mateus, C.S., Costa, J.L., Domingos, I., Almeida, P.R. 2010. Critical swimming speed of yellow- and silver-phase European eel (*Anguilla anguilla*, L.). *J Appl Ichthyol* **26**: 432–435.

Raff, R.A. 2008. Origins of the other metazoan body plans: the evolution of larval forms. *Phil Trans R Soc B* **363**: 1473–1479.

Raincrow, J.D., Dewar, K., Stocsits, C., Prokasha, S.J., Amemiya, C.T., Stadler, P.F., Chiu, C-H. 2011. Hox clusters of the bichir (Actinopterygii, Polypterus senegalus) highlight unique patterns in sequence evolution in gnathostome phylogeny. *J Exp Zool (Mol Dev Evol)* **316B**: 451–464.

Ranta, E., Lindström, K. 1990. Assortative schooling in three-spined sticklebacks? *Ann Zool Fennici* **27**: 67-75.

Riemann, L., Alfredsson, H., Hansen, M.M., Als, T.D., Nielsen, T.G., Munk, P., Aarestrup, K., Maes, G.E., Sparholt, H., Petersen, M.I., Bachler, M., Castonguay, M. 2010. Qualitative assessment of the diet of European eel larvae in the Sargasso Sea resolved by DNA barcoding. *Biol Letters* **6**: 1-4.

Rivera-Milla, E., Stuermer, C.A., Malaga-Trillo, E. 2003. An evolutionary basis for scrapie disease: identification of a fish prion mRNA., *Trends Genet* **19** : 72–75.

Rohr, D.H., Lokman, P.M., Davie, P.S., Young, G. 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis*. *Comp Biochem Physiol A* **130**: 701–714.

Rosen, M.W. 1959. Water flow about a swimming fish. *Naval Ordnance Test Station, China Lake, California.*

Ross, R.M., Backman, T.W.H. 1992. Group–size mediated metabolic rate reduction in American shad. *Trans Am Fish Soc* **121**: 385–390.

Rousseau, K., Aroua, S., Schmitz, M., Elie, P., Dufour, S. 2009. Silvering: metamorphosis or puberty? In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel: reproduction index, a useful tool for conservation management, Springer, Dordrecht, pp.39-63.

Sato, N., Kawazoe, I., Shiina, Y., Furukawa, K.,. Suzuki, Y., Aida, K. 1995. A novel method of hormone administration of inducing gonadal maturation in fish. *Aquaculture* **135**: 51-58.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K. 1997. Development of an emulsion prepared with lipophilized gelatin and its application for hormone administration in the Japanese eel *Anguilla japonica*. *Fish Physiol Biochem* **17**: 171-178.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K. 2000. Time-course response of plasma testosterone and estradiol-17β to salmon gonadotropin treatment in the Japanese eel during ovarian development. *Fish Sci* **66**: 792–794.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K. 2003. Induction of vitellogenesis. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer-Verlag, Tokyo pp. 387-399.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K. 2006. Effects of temperature on vitellogenesis in Japanese eel *Anguilla japonica*. *Fish Sci* **72**: 961-966.

Sbaihi, M., Fouchereau-Peron, M., Meunier, F., Elie, P., Mayers, I., Burzawa-Gerard, E., Vidal, B., Dufour, S. 2001. Reproductive biology of the conger eel from the south coast of Brittany, France and comparison with the European eel. *J Fish Biol* **59**: 302-318.

Scaion, D., Belhomme, M., Sébert, P. 2008. Pressure and temperature interactions on aerobic metabolism of migration European silver eel. *Resp Physiol Neurobiol***164:** 319-322.

Schmidt, J. 1923. Breeding places of the eel. *Nature* **111**: 51-54.

Schmidt-Nielsen, K. 1972. Locomotion: Energy cost of swimming, flying, and running. *Science* 177: 222-228.

Schmitz, M., Aroua, S., Vidal, B., Le Belle, N., Elie, P., Dufour, S. 2005. Differential regulation of luteinizing hormone and follicle-stimulating hormone expression during ovarian development and under sexual steroid feedback in the European eel. *Neuroendocrinology* **81**: 107–119.

Schreck, C.B. 2010. Stress and fish reproduction: the roles of allostasis and hormesis. *Gen Comp Endocrinol* **165**: 549-556.

Sébert, M-E., Amérand, A., Vettier, A., Weltzien, F-A., Pasqualini, C., Sébert, P., Dufour, S. 2007. Effects of hydrostatic pressure on the pituitary-gonad axis in the European eel, *Anguilla anguilla* (L.). *Gen Comp Endocr* **153**: 289-298.

Sébert, M-E., Legros, C., Weltzien, F-A., Malpaux, B., Cheminaeu, P., Dufour, S. 2008. Melatonin activates brain dopaminergic systems in the eel with an inhibitory impact on reproductive function. *J Neuroendocrin* **20:** 917-929.

Sébert, P., Scaion, D., Belhomme, M. 2009. High hydrostatic pressure improves the swimming efficiency of European migrating silver eel. *Resp Physiol Neurobiol* **165**: 112-114.

Seghers, B.H. 1981. Facultative schooling behavior in the spottail shiner (*Notropis hudsonius*): possible costs and benefits. *Env Biol Fish* **6**: 21-24.

Setiawan, A.N., Ozaki, Y., Shoae, A., Kazeto, Y., Lokman, P.M. 2012. Androgen-specific regulation of FSH signalling in the previtellogenic ovary and pituitary of the New Zealand shortfinned eel, *Anguilla australis. Gen Comp Endocr* **176**: 132-143.

Smyth, G.K. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**: 3.

Smyth, G.K. 2005. Limma: linear models for microarray data. In: Gentleman, R., Carey, V., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and Computational Biology Solutions using R and Bioconductor, Springer, New York, pp. 397-420.

Smyth, G.K., Speed, T. 2003. Normalization of cDNA microarray data. *Methods* **31**: 265-273.

Stamatakis, A., Hoover, P., Rougemont, J. 2008. A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol* **57**: 758–771.

Stanke, M., Diekhans, M., Baertsch, R., Haussler, D. 2008. Using native and syntenically mapped cDNA alignments to improve *de novo* gene finding. *Bioinformatics* **24**: 637-644.

Steffensen, J.F. 1989. Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish Physiol Biochem* **6**: 49-59.

Stone, R. 2003. Freshwater eels are slip sliding away. *Science* **302**: 221-222.

Sudo, R., Tosaka, R., Ijiri, S., Adachi, S., Suetake, H., Suzuki, Y., Horie, N., Tanaka, S., Aoyama, J., Tsukamoto, K. 2011a. Effect of temperature decrease on oocyte development, sex steroids, and gonadotropin b-subunit mRNA expression levels in female Japanese eel *Anguilla japonica*. *Fish Sci.* **77**: 575-582.

Sudo, R., Suetake, H., Suzuki, Y., Utoh, T., Tanaka, S., Aoyama, J., Tsukamoto, K. 2011b. Dynamics of reproductive hormones during downstream migration in females of the Japanese eel, *Anguilla japonica*. *Zool Sci* **28**: 180-188.

Suetake, H., Okubo, K., Sato, N., Yoshiura, Y., Suzuki, Y., Aida, K. 2002. Differential expression of two gonadotropin (GTH) b subunit genes during ovarian maturation induced by repeated injection of salmon GTH in the Japanese eel *Anguilla japonica*. *Fish Sci* **68**: 290-298.

Suetake, H., Okubo, K., Yoshiura, Y., Aida, K. 2003. GTH and GnRH molecules and their expression in the Japanese eel. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer-Verlag, Tokyo pp. 351-372.

Svedäng, H., Wickström, H. 1997. Low fat contents in female silver eels: indications of insufficient energetic stores for migration and gonadal development. *J Fish Biol* **50**: 475-486.

Svendsen, J.C., Skov, J., Bildsoe, M., Steffensen, J.F. 2003. Intra-school positional preference and reduced tail beat frequency in trailing positions in schooling roach under experimental conditions. *J Fish Biol* **62**: 834-846.

Székely, C., Palstra, A., Molnár, K., van den Thillart, G. 2009. Impact of the swim-bladder parasite on the health and performance of European eels. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel: reproduction index, a useful tool for conservation management, Springer, Dordrecht pp: 201-226.

Tanaka, H., Kawaga, H., Ohta, H., Okuzawa, K., Hirose, K. 1995. The first report of eel larvae ingesting rotifers. *Fish Sci* **61**: 171-172.

Tanaka, H., Kagawa, H., Ohta, H. 2001. Production of leptocephali of Japanese eel (*Anguilla japonica*) in captivity. *Aquaculture* **201**: 51-60

Tanaka, H., Kagawa, H., Ohta, H., Unuma, T., Nomura, K. 2003. The first production of glasseel in captivity: fish reproductive physiology facilitates great progress in aquaculture. *Fish Physiol Biochem* **28**: 493-497.

Taranger, G.L., Haux, C., Stefensson, S.O., Björnsson, B.T., Walther, B. Th., Hansen, T. 1998. Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and oestradiol-17β profiles in Atlantic salmon, *Salmo salar*. *Aquaculture* **162**: 85-98.

Taranger, G.L., Carrillo, M., Schultz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F-A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E., Hansen, T. 2010. Control of puberty in farmed fish. *Gen Comp Endocrinol* **165**: 483-515.

Tena-Sempere, M., Felip, A., Gómez, A., Zanuy, S., Carrillo, M. 2012. Comparative insights of the kisspeptin/kisspeptin receptor system: Lessons from non-mammalian vertebrates. *Gen Comp Endocrinol* **175**: 234–243.

Tesch, F-W. 2003. The Eel. Blackwell Science Lt, Oxford.

Todd, P.R. 1981a. Morphometric changes, gonad histology, and fecundity estimates in migrating New Zealand freshwater eels (*Anguilla* spp). *N Z J Mar Freshw Res* **15:** 155-170.

Todd, P.R. 1981b. Timing and periodicity of migrating New Zealand freshwater eels (*Anguilla* spp,). *N Z J Mar Freshw Res* **15**: 225-235.

Tsukamoto, K. 2009. Oceanic migration and spawning of anguillid eels. *J Fish Biol* **74**: 1833-1852.

Tsukamoto, K., Yamada, Y., Okamura, A., Kaneko, T., Tanaka, H., Miller, M.J., Horie, N., Mikawa, N., Utoh, T., Tanaka, S. 2009. Positive buoyancy in eel leptocephali: an adaptation for life in the ocean surface layer. *Mar Biol* **156**: 835-846.

Tsukamoto, K., Chow, S., Otake, T., Kurogi, H., Mochioka, N., Miller, M.J., Aoyama, J., Kimura, S., Watanabe, S., Yoshinaga, T., Shinoda, A., Kuroki, M., Oya, M., Watanabe, T., Hata, K., Ijiri, S., Kazeto, Y., Nomura, K., Tanaka, H. 2011. Oceanic spawning ecology of freshwater eels in the western North Pacific. *Nature Comm* **2**: 179.

Tudorache, C., Viaenen, P., Blust, R., de Boeck, G. 2007. Longer flumes increase critical swimming speeds by increasing burst and glide swimming duration in Carp (*Cyprinus carpio* L.). *J Fish Biol* **71**: 1630-1638.

Tudorache, C., Jordan, A.D., Svendsen, J.C., Domenici, P., DeBoeck, G., Steffensen, J.F. 2009. Pectoral fin beat frequency predicts oxygen consumption during spontaneous activity in a labriform swimming fish (*Embiotoca lateralis*). *Environ Biol Fish* **84**: 121-127.

Tytell, E.D., Lauder, G.V. 2004. The hydrodynamics of eel swimming. I. Wake structure. *J Exp Biol* **207**: 1825-1841.

van Ginneken, V.J.T., van den Thillart, G.E.E.J.M. 2000. Eel fat stores are enough to reach the Sargasso. *Nature* **403**: 156-157.

van Ginneken, V.J.T., Maes, G.E. 2005. The European eel (*Anguilla anguilla*, Linneaus), its lifecycle, evolution and reproduction: a literature review. *Rev Fish Biol Fisheries* **15**: 367-398.

van Ginneken, V., Haenen, O., Coldenhoff, K., Willemze, R., Antonissen, E., van Tulden, P., Dijkstra, S., Wagenaar, F., van den Thillart, G. 2004. Presence of virus infections in eel populations from various geographic areas. *Bull Eur Ass Fish Pathol* **24**: 268.

van Ginneken, V., Antonissen, E., Müller, U.K., Booms, R., Eding, E., Verreth, J., van den Thillart, G. 2005a. Eel migration to the Sargasso: remarkably high swimming efficiency and low energy costs. *J Exp Biol* **208**: 1329-1335.

van Ginneken, V., Ballieux, B., Willemze, R., Coldenhoff, K., Lentjes, E., Antonissen, E., Haenen, O., van den Thillart, G. 2005b. Hematology patterns of migrating European eels and the role of EVEX virus. *Comp Biochem Physiol C* **140**: 97-102.

van Ginneken, V., Dufour, S., Sbaihi, M., Balm, P., Noorlander, K., de Bakker, M., Doornbos, J., Palstra, A., Antonissen, E., Mayer, I., van den Thillart, G. 2007a. Does a 5500-km swim trial stimulate early sexual maturation in the European eel (*Anguilla anguilla*, L.)? *Comp Biochem Physiolt A* **147**: 1095-1103.

van Ginneken, V., Durif, C., Balm, S.P., Boot, R., Verstegen, M.W.A., Antonissen, E., van den Thillart, G. 2007b. Silvering of European eel (*Anguilla anguilla* L.): seasonal changes of morphological and metabolic parameters. *Anim Biol* **57:** 63-77.

van Ginneken, V., Durif, C., Dufour, S., Sbaihi,M., Boot, R., Noorlander, K., Doornbos, J., Murk, A.J., van den Thillart, G. 2007c. Endocrine profiles during silvering of the European eel (*Anguilla anguilla* L.) living in salt water. *Anim Biol* **57**: 453-465.

van den Thillart, G., Dufour, S. 2009. How to estimate the reproductive success of European silver eels. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel: reproduction index, a useful tool for conservation management, Springer, Dordrecht pp: 3-9.

van den Thillart, G., van Ginneken, V., Körner, F., Heijmans, R., van der Linden, R., Gluvers, A. 2004. Endurance swimming of European eel. *J Fish Biol* **65**: 1-7.

van den Thillart, G., Dufour, S., Rankin, J.C (Eds). 2009. Spawning migration of the European eel: reproduction index, a useful tool for conservation management, Springer, Dordrecht.

Versonnen, B.J., Goemans, G., Belpaire, C., Janssen, C.R. 2004. Vitellogenin content in European eel (*Anguilla anguilla*) in Flanders, Belgium. *Environ Pollut* **128**: 363-371.

Vidal, B., Pasqualini, C., Le Belle, N., Claire, M., Holland, H., Sbaihi, M., Vernier, P., Zohar, Y., Dufour, S., 2004. Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: a neuroendocrine lock for the onset of puberty. *Biol Reprod* **71**: 1491-1500.

Videler, J.J. 1993. Fish Swimming. Chapman & Hall, London, 260 pp.

Wagner, G.P., Amemiya, C., Ruddle, F. 2003. Hox cluster duplications and the opportunity for evolutionary novelties. *Proc Natl Acad Sci USA* **100**: 144603-14606.

Wallace, R.A., Selman, K. 1981. Cellular dynamic aspects of oocyte growth in teleosts. *Am Zool* **21**: 325-343.

Wang, C.H., Tzeng, W.N. 2000. The timing of metamorphosis and growth rates of American and European leptocephali: a mechanism of larval segregative migration. *Fish Res* **46**: 191-205.

Wang, N., Teletchea, F., Kestemont, P., Milla, S., Fontaine, P. 2010. Photothermal control of the reproductive cycle in temperate fishes. *Rev Aquacult* **2**: 209-222.

Watanabe, S., Aoyama, J., Nishida, M., Tsukamoto, K. 2005. A molecular genetic evaluation of the taxonomy of eels of the genus *Anguilla* (Pisces: Anguilliformes). *Bull Mar Sci* **76**: 675-690.

Watanabe, S., Aoyama, J., Tsukamoto, K. 2009. A new species of freshwater eel *Anguilla luzonensis* (Teleostei: Anguillidae) from Luzon Island of the Philippines. *Fish Sci* **75**: 387-392.

Webb, P.W. 1971. The swimming energetic of trout. I. Thrust and power output at cruising speeds. *J Exp Biol* **55**: 489-520.

Webb, P.W. 1975. Hydrodynamics and energetics of fish propulsion. *Bull Fish Res Board Can* **190**: 1-159.

Weihs, D. 1973. Hydromechanics of fish schooling. *Nature* **241**: 290-291.

Weltzien, F-A., Pasqualini, C., Sébert, M-E., Vidal, B., Le Belle, N., Kah, O., Vernier, P., Dufour, S. 2006. Androgen-dependent stimulation of brain dopaminergic systems in the female European eel (*Anguilla anguilla*). *Endocrinology* **147**: 2964-2973.

Weltzien, F-A., Sébert, M-E., Vidal, B., Pasqualini, C., Dufour, S. 2009. Dopamine inhibition of eel reproduction. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning migration of the European eel: reproduction index, a useful tool for conservation management, Springer, Dordrecht pp: 279-307.

Wolf, N.G. 1987. Schooling tendency and foraging benefit in the ocean surgeonfish. *Behav Ecol Sociobiol* **21**: 59-63.

Woltering, J.M., Durston, A.J. 2006. The zebrafish hoxDb cluster has been reduced to a single microRNA. *Nat Genet* **38**: 601-602.

Woltering, J.M., Vonk, F.J., Müller, H., Bardine, N., Tuduce, I.L., de Bakker, M.A.G., Knöchel, W., Sirbu, O., Durston, A.J., Richardson, M.K. 2009. Axial patterning in snakes and caecilians: evidence for an alternative interpretation of the *Hox* code. *Dev Biol* **332**: 82-89.

Yamamoto, K., Yamauchi, K.. 1974. Sexual-maturation of Japanese eel and production of eel larvae in aquarium. *Nature* **251**: 220-221.

Yamamoto, K., Morioka, T., Hiroi, O., Omori, M. 1976. Artificial maturation of female Japanese eels by injection of salmonid pituitary. *Bull Jap Soc Sci Fish* **40**: 1-7.

Yekta, S., Tabin, C.J., Bartel, D.P. 2008. MicroRNAs in the Hox network: an apparent link to posterior prevalence. *Nat Rev Genet* **9**: 789-796.

Ziv, L., Tovin, A., Strasser, D., Gothilf, Y. 2007. Spectral sensitivity of melatonin suppression in the zebrafish pineal gland. *Exp Eye Res* **84**: 92-99.

Zohar, Y., Munoz-Cueto, J.A., Elizur, A., Kah, O. 2010. Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrin* **165**: 438-455.

Zuyev, G., Belyayev, V.V. 1970. An experimental study of the swimming of fish in groups as exemplified by the horsemackerel (*Trachurus mediterraneus ponticus* Aleev). *J Ichthyol* **10:** 545-549.

Samenvatting

Al eeuwen hebben 'zoetwater' palingen of alen (*Anguilla* spp.) vele wetenschappers gefascineerd. Hun intrigerende levenscyclus bestaat onder andere uit een tweetal verschillende larvale stadia en een lange afstandsmigratie welke soms wel duizenden van kilometers beslaat (zelfs meer dan 6000 km voor de Europese paling, *A. anguilla*). De kweek van palingen is nog steeds afhankelijk van de invoer van glasaal, omdat palingen in gevangenschap niet op natuurlijke wijze matureren (afrijpen/volwassen worden). Sinds de jaren '80 van de vorige eeuw is de instroom van glasaal van een aantal palingsoorten sterk achteruit gegaan (Dekker et al., 2003) door onder andere overexploitatie, migratie barrières, parasieten en vervuiling (zie overzicht in van den Thillart & Dufour, 2009). Recentelijk is de Europese paling als bedreigde diersoort op de IUCN rode lijst geplaatst (Freyhoff & Kottelat, 2008). Daarom is er een urgente noodzaak tot kunstmatige reproductie. Kunstmatige reproductie zou namelijk een bijdrage kunnen leveren aan het herstel van de palingpopulatie door de visserijdruk op de wilde populatie te verminderen en daarmee een toekomstige duurzame paling aquacultuur mogelijk maken.

Aan het begin van de reproductieve migratie zijn palingen nog in een prematuur stadium. Voor de start van de migratie worden palingen 'schier' (schieraal); een proces dat waarschijnlijk is te vergelijken met puberteit (Aroua et al., 2005; Rousseau et al., 2009). Seksuele maturatie is geïnhibeerd doordat gonadotropin-releasing hormone (GnRH) onvoldoende wordt afgegeven en doordat dopamine het synthetiseren en afgeven van luteïniserend hormoon (LH) blokkeert (Dufour et al., 1993, 2003, 2005; Vidal et al., 2004). Seksuele maturatie moet daarom plaatsvinden tijdens of na de reproductieve migratie. Op dit moment is de kunstmatige voortplanting van palingen alleen mogelijk door middel van wekelijkse hormoonbehandelingen waarbij de endogene inhibitie wordt omzeild.

In eerdere studies was gesuggereerd dat langeafstandzwemmen de dopaminerge inhibitie zou opheffen en daarbij maturatie zou induceren. Het lijkt erop dat langdurig zwemmen de eerste stadia van ei-ontwikkeling in vrouwtjes stimuleert (Palstra et al., 2007; van Ginneken et al., 2007), maar dat verdere ontwikkeling zoals vitellogenese blijft geblokkeerd (Palstra et al., 2008b; Palstra et al., 2010). In wilde schiere mannetjes palingen bleek de spermatogenese

gestimuleerd na het zwemmen van ca. 900 km (Palstra et al., 2008b). Op basis hiervan werd verondersteld dat het zwemmen van de complete afstand tot de Sargassozee (circa 6000 km) zou resulteren in volledige maturatie in mannetjes palingen. Om deze veronderstelling te testen hebben we eerst de zwemcapaciteit (i.e. optimale zwemsnelheid, kosten van transport) van gemeste schiere mannetjespalingen bepaald (**Hoofdstuk 2**). Tevens hebben we het groepsgewijs zwemmen van palingen bestudeerd. Voorgaande studies betreffende de zwemcapaciteit van palingen waren voornamelijk gericht op vrouwtjes (van Ginneken & van den Thillart, 2000; van Ginneken et al., 2005; Palstra et al., 2008a). Deze studies toonden aan dat vrouwtjes zeer efficiënte zwemmers zijn, zelfs 4-6 keer efficiënter dan regenboogforel. Gebaseerd op onze resultaten met betrekking tot de kosten van transport van schiere mannetjespalingen kunnen we concluderen dat mannetjes zelfs nog efficiënter kunnen zwemmen dan vrouwtjes (Tudorache, Burgerhout & van den Thillart, ongepubliceerde data). Dit is in tegenstelling tot de eerdere verwachting dat de veel kleinere mannetjes meer energie zouden verbruiken dan vrouwtjes. Daarnaast hebben we gevonden dat bij mannetjes die groepsgewijs zwommen de kosten van transport met nog eens 30% werden gereduceerd bij ieder geteste snelheid. We hebben tevens geobserveerd dat mannetjes tijdens groepsgewijs zwemmen een in-fase gesynchroniseerde zwemmodus prefereren, welke mogelijk een manier is om de energiekosten van het zwemmen te reduceren.

In **Hoofdstuk 3** werd de hypothese getest dat spermiatie zou optreden bij schiere mannetjespalingen na langdurig zwemmen. Gemeste schiere Europese mannetjespalingen werden blootgesteld aan zwemmen voor een maximum van 6 maanden, waarbij een totale afstand van 6670 km werd afgelegd. We hebben aangetoond dat er geen effect was van langeafstandzwemmen op maturatie in mannetjes, wat in contrast is met een voorgaande studie (Palstra et al., 2008b). De laatste toonde een stimulerend effect van zwemmen aan in wilde schiere mannetjes, welke waren gevangen in brakwater tijdens de migratie. Data van de initiële controle van deze wilde mannetjes lieten een progressie in spermatogenese zien, wat aangeeft dat de maturatie al was geïnduceerd en dat de dopaminerge inhibitie al was opgeheven. Dit is nogal verschillend ten opzichte van de gemeste palingen die in onze studie zijn gebruikt. We hebben daarom

geconcludeerd dat zwemmen alleen niet voldoende is om maturatie te induceren in gemeste schiere mannetjespalingen.

Het gebruik van genomics is het afgelopen decennium sterk toegenomen. Fysiologische studies betreffende maturatie en reproductie van palingen zouden profijt kunnen hebben van genexpressie ‘profiling’. Daarom hebben we een eerste versie van de DNA-volgorde van het palinggenoom samengesteld (**Hoofdstuk 4**). In dit hoofdstuk hebben we ons gericht op de Hox genen; genen welke coderen voor transcriptiefactoren die van belang zijn in het ontwikkelingspatroon van het bouwplan. We hebben laten zien dat, verschillend van andere teleost vissen, de paling volledig gedupliceerde Hox clusters heeft behouden. Deze gedupliceerde Hox clusters zijn voortgekomen uit de teleost-specifieke genoom duplicatie. We vonden door middel van RNA-seq transcriptoom analyse en *in situ* hybridisaties dat alle kopieën van de Hox genen tot expressie komen in embryo’s (27 uur na fertilisatie). Deze schets van het genoom zal een perfecte referentie zijn voor toekomstige transcriptoom analyses binnen alle velden van de biologie.

Tijdens het maturatietraject van Europese vrouwtjespalingen reageert vaak meer dan 50% van de dieren niet op de hormonale behandeling (non-responders) welke ca. 3-6 maanden duurt (Pedersen, 2003, 2004; Palstra et al., 2005). Selectie van vrouwelijke broodstock voorafgaand aan of vroeg tijdens het maturatietraject zal de efficiëntie van kunstmatige reproductie verhogen. Omdat de respons gerelateerd is aan de initiële staat van het vrouwtje is het van belang om non-invasieve biomerkers te verkrijgen. Op basis van een eerdere studie (Minegishi et al., ongepubliceerde data) was de verwachting dat genen in de steroidogenese cascade en de seks-steroïden zelf mogelijke kandidaat merkers zijn. In **Hoofdstuk 5** hebben we een reproductie experiment uitgevoerd om specifieke biomerkers te identificeren, welke een indicatie van de respons van vrouwtjes geven. We hebben invasieve met minder-invasieve merkers gecorreleerd door gebruik van, respectievelijk, ovariumweefsel en bloedplasma. Voor het eerst is er gebruik gemaakt van een palingspecifieke microarray analyse, gebaseerd op het Europese palinggenoom (Henkel et al., 2012), om verschillen te analyseren in transcriptomen van ovariumweefsel tussen responders en non-responders na 4 wekelijkse injecties. Analyse van bloed toonde aan dat een verandering in bloedplasma niveaus van 17β-oestradiol (E2) na 4 wekelijkse

injecties significant gecorreleerd was met de gonadosomatic index (GSI). We concludeerden dat de relatieve verandering in E2 plasma niveaus na 4 weken ten opzichte van de initiële waarden gebruikt kan worden als biomerker om met circa 80% zekerheid onderscheid te maken tussen responders en non-responders.

Aangezien het maturatietraject van vrouwtjes van de ‘New Zealand short-finned eel’ (*A. australis*) veel korter is dan dat van de Europese paling (respectievelijk 2-4 maanden (Lokman & Young, 2000) vs 3-6 maanden (Pedersen, 2003, 2004; Palstra et al., 2005)), zou hybridisatie tussen *A. anguilla* en een soort als *A. australis* een mogelijke optie zijn voor paling aquacultuur. In **Hoofdstuk 6** hebben we levensvatbare larven van *A. australis* geproduceerd tot 8 dagen post fertilisatie (dpf) en larven van een hybride soort tussen vrouwelijke *A. australis* en mannelijke *A. anguilla* tot 7 dpf. We hebben de eerste ontogenie beschreven van de ‘short-finned eel’ en de hybride soort. De productie van de hybride soort werd gevalideerd door middel van een specifiek verschil in de 18S rDNA genen tussen de twee soorten. Het bestuderen van de reproductie van nauw verwante soorten en de eerste ontogenie van de hybride soort zal verder inzicht geven in het mechanisme van de reproductie en daarbij mogelijk een hulpmiddel zijn voor het kweken van de Europese paling.

Vooralsnog zijn de standaardprotocollen voor maturatie en reproductie van palingen gebaseerd op wekelijkse injecties met hypofyse extracten of gezuiverde gonadotropines. Deze wekelijkse behandelingen resulteren in hanteerstress en tijdelijke hormoonpieken in het bloedplasma (Sato et al., 2000), welke negatieve effecten hebben op gametogenese en daarbij de kwaliteit van eieren en larven. Naar verwachting zullen zogenaamde ‘slow-release’ systemen bovengenoemde problemen oplossen door het reduceren van de hanteerstress en fysiologische stress. Echter de huidig verkrijgbare ‘slow-release’ systemen vereisen chirurgische ingrepen en grote hoeveelheden gezuiverde hormonen (Kagawa et al., 2009). We hebben een ‘slow-release’ systeem ontwikkeld op basis van hormoon producerende viscellen. **Hoofdstuk 7** laat een ‘proof-of-principle’ experiment zien waarin mannetjespalingen werden behandeld via een enkele injectie met humaan choriongonadotropine (hCG) producerende implantaten. hCG plasma niveaus waren detecteerbaar tot 14 dagen na de injectie. Het implantaat resulteerde in een significante toename van bloedplasma testosteron

niveaus en een verhoging van de oogindex. Deze resultaten toonden aan dat het cellulaire implantaat seksuele maturatie induceert in mannetjes palingen. Echter, de hormoonproductie van de implantaten moet worden geoptimaliseerd aangezien de behandeling niet resulteerde in volledige spermiatie.

Tot slot, **Hoofdstuk 8** biedt, naast de samenvatting van dit proefschrift, handvatten voor toekomstig onderzoek ter verbetering van de huidige protocollen voor kunstmatige reproductie van palingen. Essentieel is het verminderen dan wel volledig vervangen van de onnatuurlijke hormoonbehandelingen. Dit zou bewerkstelligd kunnen worden door het gebruik van ‘slow release’ systemen, zoals de celimplantaten, of door middel van het gebruik van natuurlijke prikkels. Kennis met betrekking tot de (neuro)-endocrinologische cascades tijdens de natuurlijke maturatie is van vitaal belang om inzicht te verkrijgen in het mechanisme betreffende de dopaminerge inhibitie, evenals het opheffen hiervan.

Curriculum vitae

Erik Burgerhout was born on 12 February 1981 in Zwijndrecht, The Netherlands. After completing his secondary school education at Walburg College in Zwijndrecht, he started his doctoral study Biology at Leiden University in 2000. During his study, two research projects were performed at Leiden University. The first concerned mate-choice preferences of two closely related Lake Victoria cichlids at the department of Animal Ecology. The second was focused on the effects of hypoxia on embryonic development in Lake Victoria cichlids at the department of Integrative Zoology, thereby combining his main fields of interest: physiology, morphology and embryology. After his graduation in 2006 he started to work as a secondary school teacher in Biology at Gymnasium Haganum in The Hague for a year and also worked voluntarily as a research assistant at the Integrative Zoology department of the Institute Biology Leiden, Leiden University.

In the period of 2007-2008 he worked as a research assistant and zootechnician/animal caretaker at the same department, which was followed by a position as Junior Researcher. During this time, he started working on the artificial reproduction of freshwater eels (*Anguilla* spp.). Also, he co-organized the "Special Workshop on Eel Reproduction" that was held at the European Aquaculture Society 2007 in Istanbul, Turkey, and assisted with editing the book "Spawning Migration of European eel. *Reproduction index, a useful tool for conservation management*" (Eds. G. van den Thillart, S. Dufour and C. Rankin), Fish & Fisheries Series 30, Springer, 2009.

In 2009, he began his PhD research on artificial maturation and reproduction of eels at the company NewCatch BV (a spin-off company of Leiden University) and Leiden University, supervised by Dr. G. van den Thillart and Prof. Dr. H. Spaink. During the last year of his PhD research he worked for the European project PRO-EEL. After the completion of his PhD he is planning to continue his research within the field of reproductive physiology.

Publications

Vonk, F.J., Admiraal, J.F., Jackson, K., Reshef, R., de Bakker, M.A.G., Vanderschoot, K., van den Berge, I., van Atten, M., **Burgerhout, E.**, Beck, A., Mirtschin, P.J., Kochva, E., Witte, F., Fry, B.G., Woods, A. & Richardson, M.K. 2008. Evolutionary origin and development of snake fangs. *Nature* 454: 630-633.

Palstra, A.P., Tudorache, C., Rovira M, Brittijn, B., **Burgerhout, E.**, van den Thillart, G.E.E.J.M., Spaink, H.P. & Planas, J.V. 2010. Establishing zebrafish (*Danio rerio*) as a novel exercise model: swimming economy, swimming-enhanced growth and regulation of muscle growth marker gene expression. *PLoS ONE* 5: e14483

Burgerhout, E., Brittijn, S.A., Kurwie, T., Decker, P., Dirks, R.P., Palstra, A.P., Spaink, H.P. & van den Thillart, G.E.E.J.M. 2011. First hybrid of the eel species *Anguilla australis* and *Anguilla anguilla*. *BMC Developmental Biology,* 11: 16

Burgerhout, E., Manabe, R., Brittijn, S.A., Aoyama, J., Tsukamoto, K. & van den Thillart, G.E.E.J.M. 2011. Drastic effect of satellite tags on eel swimming. *Naturwissenschaften* 98: 631–634

Henkel, C., **Burgerhout, E.**, de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J., Spaink, H.P., Dufour, S., Weltzien, F-A., Tsukamoto, K. & van den Thillart, G.E.E.J.M. 2012. Primitve duplicate hox clusters in the European eel's genome. *PLoS ONE* 7: e32231

Minegishi, Y., Dirks, R.P., de Wijze, D.L., Brittijn, S.A., **Burgerhout, E.**, Spaink, H.P. & van den Thillart, G.E.E.J.M. 2012. Quantitative bioassays for measuring biologically functional gonadotropins based on eel gonadotropic receptors. *General and Comparative Endocrinology* 178: 145-152

Palstra, A.P., Beltran, S., **Burgerhout, E.**, Brittijn, S.A., Magnoni, L.J., Henkel, C.V., Jansen, H.J., van den Thillart, G.E.E.J.M., Spaink, H.P. & Planas, J.V. 2013. Deep RNA sequencing of the skeletal muscle transcriptome in swimming fish. *PLoS ONE* 8: e53171

Burgerhout, E., Tudorache, C., Brittijn, S.A., Palstra, A.P., Dirks, R.P. & van den Thillart, G.E.E.J.M. Schooling reduces energy consumption in swimming male European eels. *Accepted for publication in Journal of Experimental Marine Biology and Ecology.*

Ager-Wick, E., Dirks, R.P., **Burgerhout, E.**, Nourizadeh-Lillabadi, R., de Wijze, D.L., Spaink, H.P., van den Thillart, G.E.E.J.M., Tsukamoto, K., Dufour, S., Weltzien, F-A. & Henkel, C.V. The pituitary gland of European eel reveals massive expression of genes involved in the melanocortin system. *Submitted to PLoS ONE*

Tudorache, C., **Burgerhout, E.**, Brittijn, S.A. & van den Thillart, G.E.E.J.M. Eels with balls: the effect of additional drag on anguilliform swimming physiology, kinematics and behaviour. *In preparation.*

Burgerhout, E., Tudorache, C., Brittijn, S.A., de Wijze, D.L., Dirks, R.P. & van den Thillart, G.E.E.J.M. Male European eels are highly efficient long distance swimmers: Effect of endurance swimming on maturation. *In preparation.*

Burgerhout, E., Dirks, R.P. & van den Thillart, G.E.E.J.M. Natural triggers inducing maturation in freshwater eels (*Anguilla* spp.): a review. *In preparation*.