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.