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Identification of molecular markers in pectoral fin to predict artificial maturation of female European eels (*Anguilla anguilla*)

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ABSTRACT

The European eel is a critically endangered species that cannot be reproduced in captivity yet. Artificial maturation of female European eels can be achieved via a laborious and expensive procedure, including weekly injections with pituitary extracts for up to 6 months. The success rate is highly variable and a minimally invasive method for early selection of responsive eels would prevent the unnecessary and lengthy treatment of non-responding individuals. Since sexual maturation of European eels is accompanied by morphological changes of the pectoral fin, we examined whether fin could be used to monitor the response to the hormone treatment. Farmed eels were subjected to weekly injections with pituitary extracts and representative groups were sampled at 0 and 14–18 weeks of hormone treatment. Responders and non-responders were identified based on the gonado-somatic index. Transcriptomes of pectoral fin samples obtained at the start and end of the trial were mapped using Illumina RNAseq. Responders showed 384 and non-responders only 54 differentially expressed genes. Highly stringent selection based on minimum expression levels and fold-changes and a manual re-annotation round yielded 23 up-regulated and 21 down-regulated maturation marker genes. The up-regulated markers belong to five categories: proteases, skin/mucus structural proteins, steroid hormone signaling, tyrosine/dopamine metabolism and lipid metabolism. The down-regulated markers are either blood markers or lectin-related genes. In conclusion, pectoral fin transcriptomes are a rich source of indicator markers for monitoring hormone induced sexual maturation of female European eels. In addition, these markers provide important new insight into several fundamental processes in eel biology.

1. Introduction

The European eel (*Anguilla anguilla*) is a catadromous fish species. Hitherto unknown factors trigger the transition of immature yellow eels to pre-pubertal silver eels, a process called silvering, and subsequent migration from fresh water habitats in Europe and North-Africa to the presumed spawning area in the Sargasso Sea (Tesch, 2003). The silvering process is characterized by multiple external and internal changes, such as skin coloration, enlargement of the eyes, increased pectoral fin length, darkening of the pectoral fins, degeneration of the gastrointestinal system, increased plasma sex steroid and vitellogenin levels and increased oocyte diameter (Acou et al., 2005; Durif et al., 2005; Palstra et al., 2011; Pankhurst, 1982; Pérez et al., 2011; Sbaihi et al., 2001; van Ginneken et al., 2007; also reviewed by Aoyama and Miller, 2003; Lokman et al., 2001). Nonetheless, at the onset of their migration silver eels are still far from sexually mature. Full maturation is blocked by strong dopaminergic inhibition in the brain and must take place during the oceanic migration or upon arrival at the spawning area, although this has never been observed in nature (reviewed by Dufour et al., 2003).

The natural population of European eels is threatened by multiple known and unknown factors, such as overfishing, parasites,

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migration barriers and/or climate change, and the species is now critically endangered (IUCN Red List). European eel is an important consumption fish and most of the marketed eel is produced in aquaculture farms; however, farming of European eel is still fully dependent on wild-caught juveniles (glass eels), since artificial reproduction of European eel in captivity has not yet been achieved. Successful closure of the eel's life cycle in captivity will probably result in sustainable aquaculture, which is expected to relieve pressure on wild stocks and contribute to restoration of the natural population.

Natural triggers, such as temperature, water pressure, social interactions and swimming can be used to induce some of the early phases of sexual maturation in European eel (Huertas et al., 2006; Palstra et al., 2008; Pérez et al., 2011; Sébert et al., 2007); however, full artificial maturation can only be reached via treatment with gonadotropins. Induction of full maturation of males is very efficient and spermiation can be easily achieved using human chorionic gonadotropin, often even via a single injection (Boëtius and Boëtius, 1967; Khan et al., 1987). In the past couple of years, methods for artificial maturation of male eels have been further refined, resulting in standardized methods for evaluating sperm density and motility (Gallego et al., 2013; Sørensen et al., 2013), and definition of optimal sperm to egg ratio and best fertilization time point for maximum fertilization success rates (Butts et al., 2014). In contrast with the relatively easy induction of full maturation of male eels, inducing complete artificial maturation of females is a laborious and expensive procedure starting with weekly injections with carp pituitary extract (CPE) or salmon pituitary extract (SPE) over a period of 3 to 6 months (Boëtius and Boëtius, 1980; Burgerhout et al., 2011; Fontaine et al., 1964; Palstra et al., 2005; Pedersen, 2003, 2004). The success rate is highly variable, ranging from less than 10% to more than 90% responders, and probably depends on the initial maturation status, age and quality of the broodstock.

Unsuccessful hormone treatment of female eels results in a considerable waste of time, money, effort and fish housing facilities. Thus, there is a strong demand for a minimally invasive method to discriminate between future non-responders and responders and avoid the unnecessary and lengthy treatment of non-responding eels. Ideally, predictive selection markers should allow the removal of future non-responders at an early stage in the artificial maturation protocol.

Artificial maturation of European eels is accompanied by external changes, similar to those observed during the natural silvering process, such as enlargement of the eyes (Pankhurst, 1982). Lengthening of the pectoral fin during artificial maturation was observed by some researchers (Palstra et al., 2010), but not by others (Durif et al., 2006) and gradual darkening of the pectoral fin at later stages in the maturation process has often been observed (e.g. our unpublished results). Wild female Japanese eels (*A. Anguilla japonica*) also show a clear correlation between coloration of the pectoral fin and their maturation stage (Okamura et al., 2007). Since pectoral fin samples can be easily obtained via a simple clipping procedure, we examined whether molecular markers in the pectoral fins would be suitable for monitoring the response of female eels to the hormone treatment. The draft genome sequence of the European eel was recently published and ~46,000 genes were provisionally annotated (Henkel et al., 2012). This has created a whole new toolbox for molecular research on artificial maturation and reproduction of the eel, including the possibility for deep sequencing analysis of tissue transcriptomes. Here, we used Illumina RNAseq analysis to identify a set of marker genes in the pectoral fins that are specific for the responders to hormone-induced maturation.

2. Materials and methods

2.1. Animals, morphometrics, hormone treatment, and sampling

All 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).

Three year old farmed female European eels ($n = 22$, 714.9 ± 28.2 g; 67.9 ± 0.7 cm (mean ± standard error)) were obtained from a commercial eel farm (Passie voor Vis, Sevenum, The Netherlands). An initial control group of 8 animals was sampled directly after transport to the lab facility: eels were euthanized using an overdose of clove oil (dissolved 1:10 in 96% ethanol, dosage 5 mL/L) followed by decapitation, and the body weight (BW), body length (BL), body girth (BG), eye diameter horizontal and vertical (Edh and Edv, respectively), pectoral fin length (PFL), liver weight (LW), gonad weight (GW) and digestive tract weight (DTW) were measured. The morphometric data were 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). The gonadosomatic index (GSI), hepatosomatic index (HSI) and digestive tract somatic index (DTSI) were calculated by the following formula: Tissue index = (tissue weight/*body weight*) × 100. Prior to the maturation trial, all remaining eels (n = 14) were anesthetized in clove oil (dissolved 1:10 in 96% ethanol, dosage 1 mL/L), tagged with passive transponders with unique identification numbers (Trovan, EID Aalten BV, Aalten, The Netherlands) and measured for external morphometrics including: (BW, BL, BG, Edh, Edv and PFL. The morphometric data were used to calculate the SI (Durif et al., 2005), EI (Pankhurst, 1982) and PFLI (Durif et al., 2005). Using surgical scissors, a first fin clip sample of 0.25 cm^2 was obtained from the distal side of the right pectoral fin. The fin clips were transferred to RNAlater (Ambion), kept overnight at 4 °C and subsequently stored at −80 °C.

Subsequently, the 14 eels were housed in a 1500 L tank connected to a recirculation system, and acclimated to natural seawater (32 ± 1ppt, 21 ± 0.5 °C) for 2 weeks. Eels were not fed during acclimation and during the trial. After the acclimation period, eels were subjected to weekly injections with 20 mg salmon pituitary extract (SPE; Argent Labs, Redmond, WA, USA) according to the protocol described by Burgerhout et al. (2011). External morphometrics (BW, BG) of all animals was performed prior to the weekly hormone injections. The eels were euthanized and sampled as described above for the initial control group, including a second fin clip sample from the distal side of the right pectoral fin and an egg sample for oocyte staging. Eels were sampled either at one day after ovulation or at one week after the 18th SPE injection, whichever occurred first. Ovulation was induced using 2 mg kg^{-1} of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP; Sigma–Aldrich BV, Zwijndrecht, The Netherlands).

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 Bonferroni correction. Statistical difference was considered significant at $p < 0.05$. In all cases values are expressed as average ± standard error.

2.2. RNA isolation and Illumina RNAseq analysis

Total RNA was isolated from the pectoral fin clip samples using the Qiagen miRNeasy kit according to the manufacturer's instructions (Qiagen). Integrity of the RNA was checked on an Agilent Bio-