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Transcriptomic profiling of male European eel (*Anguilla anguilla*) livers at sexual maturity

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ABSTRACT

The European eel *Anguilla anguilla* has a complex life cycle that includes freshwater, seawater and morphologically distinct stages as well as two extreme long distance migrations. Eels do not feed as they migrate across the Atlantic to the Sargasso Sea but nevertheless reach sexual maturity before spawning. It is not yet clear how existing energy stores are used to reach the appropriate developmental state for reproduction. Since the liver is involved in energy metabolism, protein biosynthesis and endocrine regulation it is expected to play a key role in the regulation of reproductive development. We therefore used microarrays to identify genes that may be involved in this process. Using this approach, we identified 231 genes that were expressed at higher and 111 genes that were expressed at lower levels in sexually mature compared with immature males. The up-regulated set includes genes involved in lipid metabolism, fatty acid synthesis and transport, mitochondrial function, steroid transport and bile acid metabolism. Several genes with putative enzyme functions were also expressed at higher levels at sexual maturity while genes involved in immune system processes and protein biosynthesis tended to be down-regulated at this stage. By using a high-throughput approach, we have identified a subset of genes that may be linked with the mobilization of energy stores for sexual maturation and migration. These results contribute to an improved understanding of eel reproductive biology and provide insight into the role of the liver in other teleosts with a long distance spawning migrations.

1. Introduction

Teleost reproduction involves complex interactions between endocrine, physiological and developmental factors. The timing and coordination of these factors are key to reproductive success because they ensure that both males and females reach the appropriate developmental state at the same time and place. Much remains to be understood about the endocrine pathways controlling aspects of reproduction such as puberty and gametogenesis in teleosts. Gonad development and gametogenesis are influenced by external signals such as temperature and photoperiod (Zanuy et al., 1986; Taranger et al., 2010) and, in eels, can be influenced by swimming behavior and hydrostatic pressure (Sébert et al., 2007; Palstra et al., 2008). These external signals trigger the release of gonadotropin releasing hormone (GNRH) by the hypothalamus which controls the release of pituitary follicle stimulation hormone (FSH) and luteinizing hormone (LH). FSH and LH act directly on the gonads and stimulate steroidogenesis which controls reproductive behavior and gonadogenesis by acting on other tissues such as the liver (reviewed in Schulz et al., 2010; Zohar et al., 2010).

The European eel *Anguilla anguilla* has a complex life cycle that includes two trans-oceanic migrations and both freshwater and seawater stages (reviewed in van den Thillart et al., 2009; van Ginneken and Maes, 2005). After spawning in the Sargasso Sea, eel larvae (leptocephali) make use of oceanic currents to cross the Atlantic to European coastal waters. Larvae undergo metamorphosis into glass eels upon arrival at

Abbreviations: ACAT1, acetyl-coenzyme A acetyltransferase 1-like; ACP, acyl carrier protein; ABC, ATP-binding cassette; ABCA1, ATP-binding cassette sub-family A member 1; ABCD3, ATP-binding cassette sub-family D member 3; DAGT1, diacylglycerol O-acyltransferase 1-like; ELOVL1, elongation of very long chain fatty acids 1; FSH, follicle stimulation hormone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GNRH, gonadotropin releasing hormone; hCG, human chorionic gonadotropin; HPA, hypothalamic–pituitary–adrenal; HSD11B, 11β-hydroxysteroid dehydrogenase; HSDL2, hydroxysteroid dehydrogenase protein 2-like; LH, luteinizing hormone; MID1IP1L, mid1-interacting protein 1-like; PARGL, poly (ADP-ribose) glycohydrolase-like; PPAR, peroxisome proliferator-activated receptor; SPARC, secreted protein acidic and rich in cysteine; SPTLC2, serine palmitoyltransferase 2; SLC6A6, sodium and chloride taurine transporter-like; SULT2A1, sulfotransferase 2A1; SULT3, sulfotransferase 3; SULT6B1, sulfotransferase 6B1; TNF, tumor necrosis factor.

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the continental shelf and enter freshwater habitats where they become yellow eels. After an extensive feeding period of 5–30 years, eels undergo a second metamorphosis in which they become adults. This "silvering metamorphosis" involves morphological (color, eye size, body length and weight) and physiological modifications such as the regression of digestive tract, gonad development and metabolic changes (van Ginneken et al., 2007). These changes are in preparation for the second migration across the Atlantic where eels spawn and eventually die.

Silver eels do not feed during migration to the spawning site in the Sargasso Sea but nevertheless become sexually mature. This trans-Atlantic migration lasts several months; therefore, eels rely heavily on the use of and re-distribution of fat stores for swimming, gonad development and sexual maturation (Lewander et al., 1974; Larsson et al., 1990; van Ginneken et al., 2005). After three months without feeding, liver triglycerides serve as the primary energy source and liver glutamate oxaloacetate transaminase activity is increased (Larsson and Lewander, 1973). In the subsequent months, both liver and muscle triglycerides as well as liver glycogen are used as fuel. About 67% of fat stores are believed to be required for reproduction (Palstra and van den Thillart, 2010). Thus, in addition to its role in metabolism (e.g., protein synthesis, hormone production and lipid digestion), detoxification and endocrine regulation, the liver is expected to play a key role in energy metabolism during migration and reproductive development. The liver may also coordinate the redistribution of metabolic resources from, for example, the intestinal tract to the gonads; as the gonads develop, the digestive tract degenerates (Pankhurst and Sorensen, 1984).

In female eels, a significant proportion of fat stores are used for egg yolk formation during vitellogenesis and, although the process may slow down during swimming (Palstra et al., 2010), lipids are incorporated into oocytes as they develop (Palstra et al., 2007). As with females, gonad development in males is stimulated by swimming. In males, the liver may be involved in fatty acid metabolism that is important for spermatogenesis (Baeza et al., 2014b). Silvering is associated with increased levels of free fatty acids in the blood (van Ginneken et al., 2007) and sexual maturation is accompanied by changes in the fatty acid composition in the liver (Mazzeo et al., 2010; Baeza et al., 2014a). In some fish, androgen precursors are metabolized from cortisol in the liver (Yaron and Levavi-Sivan, 2011) and it has been suggested that an interrenal–liver–gonad axis for the synthesis of androgens exists in fish (Kime, 1978).

While much progress has been made in the field of eel biology, sexually mature eels have never been caught in the wild and many aspects of eel reproduction, particularly in males, are not yet well-understood. The process by which eels make use of existing energy stores to reach sexual maturity is not yet known. The goal of this study was to identify gene expression changes in the liver at sexual maturation in order to identify candidates that may be involved in spermatogenesis and gonad development. As maturation in eels is accompanied by a shift in bile acid metabolism that is gender specific (Huertas et al., 2010), we were also particularly interested in identifying genes that may be responsible for this switch. Since bile acids in lampreys act as sex pheromones (Li et al., 2002) and eels can smell bile acids (Huertas et al., 2010), the byproducts of liver metabolism are excellent candidates for future chemical communication experiments. If a gene in the liver has a role in sexual maturation, then it is expected to be expressed at higher levels in sexually mature males than sexually immature males. To test this, we used microarrays to compare the liver gene expression profiles of sexually mature males to the liver expression profiles of immature males.

2. Materials and methods

2.1. Animals

About 400 sexually immature eels with body weights of approximately 80–90 g were obtained from a local distributor in Seville, Spain (Pescafial). Eels were acclimatized in a 2000 L freshwater tank (0 PSU salinity) under natural photoperiod and temperature conditions. One hundred and twenty fish were transferred to 500 L recirculation tanks after two weeks at densities of approximately 30 fish per tank. Here, fish were allowed to acclimatize to seawater for two weeks. These tanks were maintained at 20 °C under a 12:12 h light/dark photoperiod. Eels were fed commercial pellets (DIBAQ No. 3, Proaqua, Spain) at 3% body-weight per day. Sexual maturation was induced in males in one of the seawater units by weekly intramuscular injection of 2000 IU hCG/kg (human chorionic gonadotropin, Sigma-Aldrich Chemical) in 0.9% saline over 140 days. Control males were kept separately and were injected weekly with 0.9% NaCl over the same period.

2.2. Sample preparation and microarray experiments

At the end of the injection period, eels were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222, Sigma Aldrich) then decapitated. The livers were dissected from 12 sexually mature and 12 immature fish and frozen immediately in liquid nitrogen. The tissue samples used in this study are from the fish used in our previous survey (Churcher et al., 2014) where the characterization of maturational state is described. Before shipping on dry ice, tissues were then placed in RNAlater® (Sigma R0901) and kept overnight at 4 °C then stored temporarily at −20 °C. All animal care and experimental procedures were done in accordance with the national legislation for the use of laboratory animals ("Group-1" license issued by the Ministry of Agriculture, Rural Development and Fisheries of Portugal).

RNeasy Mini Kit (Qiagen) was used to extract RNA from the livers. To reduce the cost associated with chip processing and reduce in effect of individual variation, samples from three different fish were pooled for each replicate. Therefore, each replicate (n = 4 sexually immature, n = 4 sexually mature) contains RNA from three fish. Total RNA concentrations were determined using the Nanodrop ND-100 spectrophotometer (NanoDrop Technologies) and sample integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with RNA integrity values (RIN) of 7.5 or greater were used.

An *A. anguilla* specific microarray containing 14,913 probes that were designed based on transcripts from a diversity of tissues (Pujolar et al., 2012) was used for microarray analysis. The probe sequences and details of the array and platform can be retrieved from the GEO database using the accession number GPL15124. All labeling and hybridization procedures were conducted using the methods described in (Milan et al., 2013). Briefly, the Agilent One-Color Microarray-Based Gene Expression Analysis protocol was used for sample labeling and hybridization. Hybridized slides were scanned at 5 μm resolution using an Agilent DNA microarray scanner and two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). Images generated from the two different sensitivity levels were analyzed together. Data extraction and background subtractions were done using the standard procedure in Agilent Feature Extraction (FE) software v. 9.5.1. Flag values were used to evaluate hybridization success and values that were not equal to one were excluded to ensure that only probes with signals above background were included. Quantile normalization was used for data normalization and SAM (Significance Analysis of Microarrays, version 4.0) (Tusher et al., 2001) was used to identify differentially expressed genes across samples. The two-class unpaired test was used to compare the expression levels of sexually mature males to immature males. A false discovery rate of 5% was used and only genes with a minimum fold change of 1.5 were considered. The array and methods used in this study have previously been verified using qPCR and a subset of eight genes in Churcher et al. (2014).

2.3. Functional annotation

For functional annotation with DAVID (Huang et al., 2008), we first used the eel transcriptome assembly contigs reported in (Pujolar et al.,