Changes in ovarian gene expression profiles and plasma hormone levels in maturing European eel (Anguilla anguilla) biomarkers for broodstock selection

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Changes in ovarian gene expression profiles and plasma hormone levels in maturing European eel (*Anguilla anguilla*); Biomarkers for broodstock selection

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ABSTRACT

Complete sexual maturation of European eels (*Anguilla anguilla*) in captivity can only be achieved via injections with gonadotropins. For female eels this procedure takes 4–6 months and the response ranges from "unresponsive" to final maturation and ovulation. Reproductive success could be significantly increased via early selection of responders based on predictive markers and minimally invasive sampling methods. To get a better understanding of the genetic background of ovarian maturation of the European eel we performed a pilot deep-sequencing transcriptome analysis of ovarian tissue derived from a yellow eel, a prepubertal silver eel and a post-spawning matured eel. Two key players in steroidogenesis were strongly correlated with advanced sexual maturation, namely P450c17 and liver receptor homolog-1, suggesting that blood plasma steroids might qualify as minimally invasive markers for early detection of responders. Since the predictive value of plasma sex steroid levels for final maturation of the European eel had not yet been carefully examined, we performed an extensive artificial maturation trial. Farmed silver eels were treated with pituitary extracts and sampled at multiple time intervals. Expression of steroidogenesis-related genes in ovarian tissue of responding and non-responding eels after four weekly injections with pituitary extract was compared using a custom-built microarray and RNAseq. Increased expression of 17β-hsd1 was strongly linked to sexual maturation. Blood plasma levels of sex steroids were measured using ELISAs. We show that a 2.5-fold increase in blood-plasma estradiol level after 4 weekly pituitary extract injections is a strong predictor of final sexual maturation of female European eel.

1. Introduction

The catadromous European eel (*Anguilla anguilla*) shows a complicated and intriguing life-cycle. In freshwater, juvenile (yellow) eels start their growth phase, which lasts for 5–50 years (Tesch, 2003). Migration occurs from the European and North African freshwaters to the spawning area in the Sargasso Sea (Schmidt, 1923; Tesch, 2003). This spawning migration is preceded by a variety of morphological and physiological transformations, collectively known as the silvering process. Morphologically, the silvering process includes changes in body coloration, enlargement of eyes and pectoral fins, and degeneration of the alimentary tract (Acou et al., 2005; Durif et al., 2005; Pankhurst, 1982; Pankhurst and Lythgoe, 1982; also reviewed by Aoyama and Miller, 2003; Lokman et al., 2003). Physiologically, this phase involves a slight but significant increase of the gonadosomatic index (GSI) and of plasma steroid hormone (estradiol, testosterone, 11-ketotestosterone) and vitellogenin levels, elevated expression of pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) genes, and increased number of the fat droplets in and the diameter of the oocytes (Aroua et al., 2005; Durif et al., 2005; Palstra et al., 2011; Sbaihi et al., 2001; Van Ginneken et al., 2007). Silver eels are still at a prepubertal stage, far from sexual maturation, and thus supposed to mature during and/or after their oceanic migration to the spawning areas in nature (Dufour et al., 2003). However, the exact process and

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mechanism of sexual maturation and reproduction of the European eel are shrouded in mystery, because migrating and spawning specimens have never been caught close to or at the spawning area.

Over the last decades, the populations of several eel species have shown a drastic decline (Dekker et al., 2003; Stone, 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). The European eel and the Japanese eel are listed as, respectively, "critically endangered" (Freyhof and Kottelat, 2010) and "endangered" (Jacoby and Gollock, 2014) species on the IUCN red list. Therefore, management of the wild eel populations and development of successful artificial reproduction protocols resulting in sustainable aquaculture are very urgent matters. However, eels do not mature naturally in captivity and artificial maturation is currently only possible via treatment with gonadotropins (Butts et al., 2014; Kagawa et al., 2005; Lokman and Young, 2000; Ohta et al., 1996, 1997; Oliveira and Hable, 2010; Palstra et al., 2005; Pedersen, 2003, 2004; Tanaka et al., 2001, 2003). Complete artificial reproduction of the European eel has been attempted for several decades, but so far without success. Recently, the life cycle of the Japanese eel (*Anguilla japonica*) in captivity was closed by producing a second generation of glass eel, but the success rates are still low (Ijiri et al., 2011).

Artificial maturation of female European eels is induced by 11–29 weekly hormone injections with pituitary extracts (Palstra et al., 2005; Pedersen, 2003, 2004). There is a wide variety in responsiveness to the hormonal treatment and often broodstock females do not reach full maturation even after 30 weekly injections (Palstra and Van den Thillart, 2009). Response to treatment is suggested to be dependent on the initial maturation status of the female individuals. For the Japanese eel it was shown that yellow eels do not respond to hormone treatment, while silver stage 1 and stage 2 eels respond with approximately 80% and 100% efficiency, respectively (Okamura et al., 2007, 2008). However, the maturation status of silver stage 2 Japanese eels is more advanced than that of silver European eels, which is reflected by differences in GSI at the onset of their migration, namely 2–4% for Japanese eel and <2% for European eel (Durif et al., 2005; Palstra et al., 2005). This indicates that the silvering stages are not a good indicator of the true status of gonadal maturation and that there is a need for biomarkers that reflect the actual status of ovarian maturation and can be used for a reliable pre-selection of broodstock.

To increase the reproductive success rate and to reduce the costs (labor, hormones, space and time) associated with non-responding animals, one would like to be able to preselect female European eels with the highest chance of responding to the long-term hormone treatment. To this end, we first need to gain a better understanding of the entire genetic background of maturation of the European eel. The whole genetic basis behind the sexual maturation has never been comprehensively investigated, although the maturation process of this species has been well studied by histological observations, analysis of plasma hormone levels and expression patterns of some specific genes related to maturation. Moreover, it is essential that predictive markers for broodstock selection can be measured using minimally invasive sampling of readily accessible tissues, such as blood, fin, muscle and skin (mucus). Recently, we identified molecular markers in pectoral fin that can be used to predict artificial maturation of female European eels (Dirks et al., 2014). Here, we describe our parallel search for predictive markers that can be detected in blood samples. To map the gene expression profiles during maturation and to find leads for such predictive maturation markers based on these expression profiles, we first performed a pilot deep-sequencing transcriptome analysis of ovarian tissue derived from three stages of female European eel, namely yellow eel, prepubertal silver eel and post-spawning, fully matured eel. Two key genes in the steroidogenesis-related pathway were among the best leads resulting from this screen. Subsequently, we performed an extensive artificial maturation trial and used custom-designed microarrays and RNA sequencing to study changes in gene expression during the early stages of artificial maturation, since the response to hormone treatment is dependent on the initial maturation status of an individual eel as stated above. Finally, we show that the fold-increase in blood-plasma estradiol level is a strong predictor of successful final sexual maturation of the female European eel.

2. Materials and methods

2.1. Illumina RNAseq analysis

Ovaries from three maturation stages were used for Illumina RNAseq analysis: a wild immature yellow eel, a wild silver eel, and an artificially matured eel (immediately after spawning). The yellow and silver eel were captured in Lake Veere and Lake Grevelingen, respectively, in the Netherlands. The fully matured eel was from Lake Veere, The Netherlands, and subsequently kept in a 2300 L recirculation system with seawater (30 ppt salinity) at 21 °C and 16:8 L:D photoperiod (white light). This eel received weekly injections with salmon pituitary extract (SPE, 20 mg) for 17 weeks to induce sexual maturation and then received 17α, 20β-dihydroxy-4-pregnen-3-one (DHP, 2 mg kg-1) for ovulation induction following the protocol described in Burgerhout et al. (2011). Detailed information about the animals is shown in Table 1.

Tissues were taken from anterior, posterior and middle parts of gonad from each individual, and stored in RNAlater (Ambion) at −80 °C till RNA extraction. The different parts of gonad tissue of each individual were pooled. Total RNA was isolated from the pooled samples using the Qiagen miRNeasy kit according to the manufacturer's instructions (Qiagen), and analyzed with an Agilent Bioanalyzer 2100 total RNA Nano series II chip (Agilent). A transcriptome library for each individual was prepared from 10 μg total RNA, using the Illumina mRNAseq Sample Preparation Kit following the manufacturer's instructions (Illumina Inc.). All RNAseq libraries were sequenced on an Illumina GAIIx instrument as 2 × 76 nucleotides paired-end reads according to the manufacturer's protocol (Illumina, Inc.). Image analysis and base calling were done by the Illumina pipeline.

Reads were aligned to the draft genome sequence of the European eel (Henkel et al., 2012) using TopHat (version 2.0.5) (Trapnell et al., 2009). The resulting files were filtered using SAMtools (version 0.1.18) (Li et al., 2009) to exclude secondary alignment of reads. Aligned fragments per predicted gene were counted from SAM alignment files using the Python package HTSeq (Anders et al., 2014). Differentially expressed genes were identified using DESeq (Anders and Huber, 2010) and data were further processed using Microsoft Excel with the cut-off set at $p < 0.01$. Genes were categorized into GO terms based on provisional annotation of the European eel genome by Henkel et al. (2012) using CateGOrizer (Hu et al., 2008).

To confirm the microarray data (Section 2.5), the ovarian RNA samples from four responders and two non-responders were also analyzed via Illumina RNAseq. RNA sequencing and data analysis were essentially performed as described above, except that RNAseq libraries were prepared from 1 μg total RNA and sequenced as 1 × 50-nt single-reads on an Illumina HiSeq2500 sequencer.

The raw RNAseq data of the ovaries from the three wild specimens and the six farmed specimens have been deposited in the NCBI GEO repository with accession numbers GSE70381 and GSE70382, respectively.

Table 1
Sampling information and morphometric parameters of wild eel specimens.

Parameters	Yellow eel	Silver eel	Spawned eel
Sampling date	April 21, 2010	December 7, 2008	May 26, 2010
Sampling location	Lake Veerse The Netherlands	Lake Grevelingen The Netherlands	Lake Veerse The Netherlands
Total length (cm)	84.0	88.4	77.9
Body weight (g)	891.3	1371.3	573.7
Eye diameter (horizontal) (mm)	8.8	10.8	11.8
Eye diameter (vertical) (mm)	8.0	11.0	10.5
Pectoral fin length (mm)	38.9	42.4	35.6
Condition factor	0.15	0.20	0.12
Eye index	6.6	10.6	12.5
Silver index	3	4	5
GSI (%)	0.9	2.0	15.48

2.2. Artificial maturation trial and sampling procedure

A schematic representation of the artificial maturation trial is shown in Fig. 1. All experiments that were 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 silver 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). Upon transport from the farm to the research facility, all females were anesthetized in clove oil (dissolved 1:10 in 96% ethanol, dosage 1 mL/L) and measured for morphometry including: body weight (BW), body length (BL), body girth (BG), 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 min at 4 °C at 13,200 RPM, and afterwards stored in a −80 °C freezer until further analysis. Eight eels (initial control group) were randomly selected and immediately sampled. These eels were euthanized using clove oil (dissolved 1:10 in 96% ethanol, dosage 5 mL/L) followed by decapitation. The weight of the gonads (GW), liver (LW) and digestive tract (DTW) were measured. Gonadosomatic index (GSI), hepatosomatic index (HSI) and digestive tract somatic index (DTSI) were calculated with the following formula: Tissue index = (tissue weight/body weight) × 100. Gonad samples for histological analysis were preserved overnight in paraformaldehyde (PFA) and subsequently stored in 70% ethanol. All remaining eels ($n = 32$) were PIT-tagged (Trovan, DorsetID, Aalten, the Netherlands), transferred to a 1500 L tank connected to a recirculation system and acclimated to natural seawater (32 ± 1 ppt, 21 ± 0.5 °C) for 2 weeks. During acclimation and subsequent hormone treatment eels were kept at 21 °C and 16:8 L:D photoperiod (white light). Eels were not fed during acclimation and during the trial.

Females ($n = 32$) were measured for morphometry (BW, BG) and injected with 20 mg salmon pituitary extract (SPE) once per week following the protocol described by Burgerhout et al. (2011). Eight females were sampled after 4 weekly hormone injections according to the sampling scheme described above for the initial control group. Gonad samples for microarray analysis were stored in RNA-later (Ambion). These samples were kept overnight at 4 °C and then stored in a −80 °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. From the other eels, a blood sample was obtained for the measurement of plasma hormone levels and external parameters were measured prior to the weekly SPE injection (7 days after the previous SPE injection). Blood samples were treated as described above. Another group of eight females was sampled after 12 weekly hormone injections according to the same sampling protocol. Final sampling of the 16 remaining eels was performed either after 18 weekly SPE injections or at 1 day after ovulation, whichever occurred first. Upon observing a 10% increase in BW, ovulation was induced using 2 mg kg-1 of DHP according to the protocol described by Burgerhout et al. (2011). Palstra et al. (2005) showed that the majority of hormone-responsive European eel females ovulated after 12–18 weekly hormone injections and therefore 18 injections was set to the maximum in this maturation trial.

2.3. Histology

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). After rehydration, the nuclei and cytoplasm were stained using Mayer's haematoxylin and eosin (H–E) staining method. Stages of gametogenesis were determined according to the most advanced ooctye stage as described (Palstra et al., 2007; Wallace and Selman, 1981).

2.4. Plasma hormone measurements

To observe the changes in blood plasma hormone levels throughout the artificial maturation process, the hormone levels were measured using the samples taken from all sampling points, including the ones obtained before the start of the artificial maturation trial. Blood plasma testosterone (T) and 17β-estradiol (E2) concentrations were measured using T and E2-specific ELISAs (HUMAN GmBH Worldwide Diagnostics).

2.5. Microarray analysis

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 gene predictions (Henkel et al., 2012). 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 67,063 and 17,869 probes, respectively.

The second approach was to use Tophat v1.1.4 (Trapnell et al., 2009) to map the embryo (unpublished data) and gonad RNAseq reads on the European eel genome scaffolds and unscaffolded contigs and use Cufflinks v0.9.3 (Trapnell et al., 2010) 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 89,912 probes. The final custom Agilent array design contained 4 times 174,844

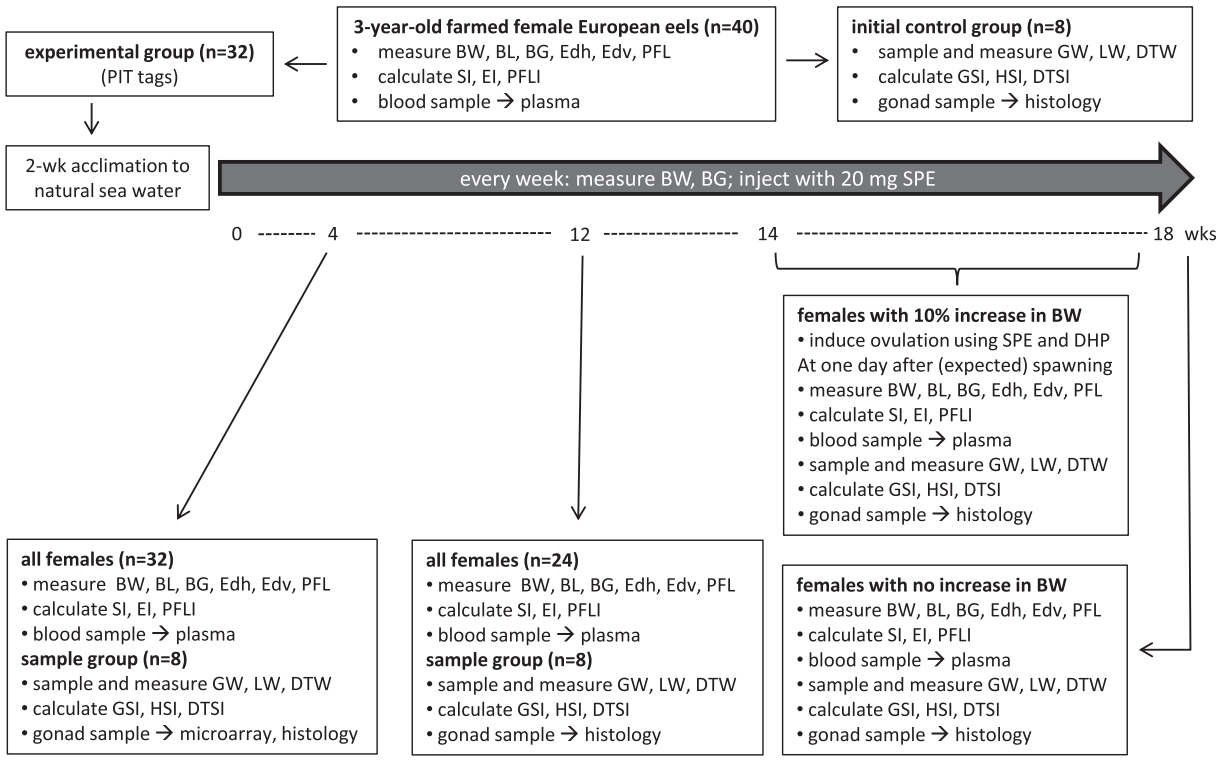

Fig. 1. Schematic overview of the artificial reproduction trial. Three year old female European eels ($n = 40$) were obtained from a commercial farm. An initial control group ($n = 8$) was sampled directly after transport to the lab facility, including morphometrics and measurement of multiple internal parameters. The remaining eels ($n = 32$) were tagged, morphometrics was performed and a blood sample was obtained from each individual. Subsequently, the experimental group was acclimated to natural seawater for 2 weeks and then subjected to weekly injections with SPE. Morphometrics of each individual was performed prior to the weekly hormone injections. Eels were sampled after 4 weeks ($n = 8$), after 12 weeks ($n = 8$) and either at 1 day after ovulation or at 1 week after the 18th SPE injection, whichever occurred first. Sampling included morphometrics, measurement of multiple internal parameters, obtaining an egg sample for oocyte staging and obtaining a blood sample from each individual. *Abbreviations:* BG: body girth; BL: body length; BW: body weight; DTSI: digestive tract somatic index; DHP: 17α, 20β-dihydroxy-4-pregnen-3-one; DTW: digestive tract weight; Edh: eye diameter horizontal; Edv: eye diameter vertical; EI: eye index; GSI: gonadosomatic index; GW: gonad weight; HSI: hepatosomatic index; LW: liver weight; PFL: pectoral fin length; PFLI: pectoral fin length index; SI: silver index; PIT: Passive Integrated Transponder; SPE: salmon pituitary extract.

probes excluding internal quality controls designed by Agilent (Table S1).

The ovarian samples obtained after four weekly SPE injections were used for 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. These samples were labeled 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: 120,660 probes aligned to 43,435 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 and Speed, 2003). If multiple probes assayed a single annotated transcript, spot values were averaged.

2.6. 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 Bonferroni correction. As the data of blood plasma hormones 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 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).

3. Results

3.1. Deep sequencing of ovarian transcriptomes from yellow, silver and artificially matured eel

To map the entire genetic background of maturation and to search for leads for maturation-stage-specific biomarkers we used Illumina RNAseq technology for deep sequencing analysis of ovarian samples from three wild eels, namely a yellow eel, an untreated silver eel and an artificially matured silver eel. The sampling information and morphometric data of the three animals are shown in Table 1. The Illumina run generated between 25.1 and 33.2 million paired-end 76-nucleotides reads per library (Table 2), about 60% of which could be aligned to the 45,975 transcripts predicted from the European eel genome (Henkel et al., 2012). A complete overview of all mapped reads is presented in Table S2. In total 320

Table 2
Alignment of RNAseq reads. RNAseq reads were mapped to the 45,975 cDNA sequences predicted from the *A. anguilla* genome using TopHat and mapped reads were quantified using HTseq. Percentages of mapped reads are relative to the total sequenced clusters of paired-end 2 × 76-nt reads.

Ovarian sample	76-nt sequence reads	data yield	Mapped RNAseq reads (%)[a]
Wild yellow eel	2 × 25,106,160	3.82 Gb	27,811,764 (55.4%)
Wild silver eel	2 × 33,190,800	5.05 Gb	41,426,808 (62.4%)
Wild artificially matured eel	2 × 25,365,240	3.86 Gb	30,137,882 (59.4%)

[a] Alignment of at least one read of each paired read.

out of 45,975 predicted genes (0.7%) were differentially expressed between yellow and silver eel (Fig. 2; $p < 0.01$; 193 with higher and 127 with lower expression level in silver eel), 621 genes (1.4%) were differentially expressed between yellow and matured eel (Fig. 2; $p < 0.01$; 371 with higher and 250 with lower expression level in matured eel), and 563 genes (1.2%) were differentially expressed between silver and matured eel (Fig. 2; $p < 0.01$; 269 with higher and 294 with lower expression level in matured eel). From these lists we could identify 49 genes with lower expression in matured eel compared with both yellow and silver eel, and none of these had lower expression in silver than in yellow eel (Fig. 2, lower Venn diagram). In total 144 genes had higher expression in matured eel compared with both yellow and silver eel, only one of which also had higher expression in silver than in yellow eel (Fig. 2, upper Venn diagram). This gene (cp17a) codes for the enzyme steroid 17-alpha-hydroxylase/17,20 lyase (P450c17, Cyp17a1), which is one of the key enzymes in androgen and estrogen steroidogenesis. We also identified 14 genes that were exclusively expressed in the ovary of matured eel with an arbitrarily chosen minimum RPKM value of 2 (Table 3). Two out of these 14 genes correspond with the liver receptor homolog-1 gene (lrh-1, also known as nr5a2), which codes for transcription factor Lrh-1 (Nr5a2) that plays a critical role in steroidogenesis. In conclusion, three of the most stringently selected leads for maturation-specific markers are associated with steroidogenesis.

Since we were especially interested in predictive markers that could be detected via minimally invasive blood sampling, and sex steroids can be easily detected via ELISA assays in blood samples, we studied the ovarian expression profiles of the genes related to steroidogenesis in more detail in the three wild eel specimens. Based on the provisional gene and functional annotation of

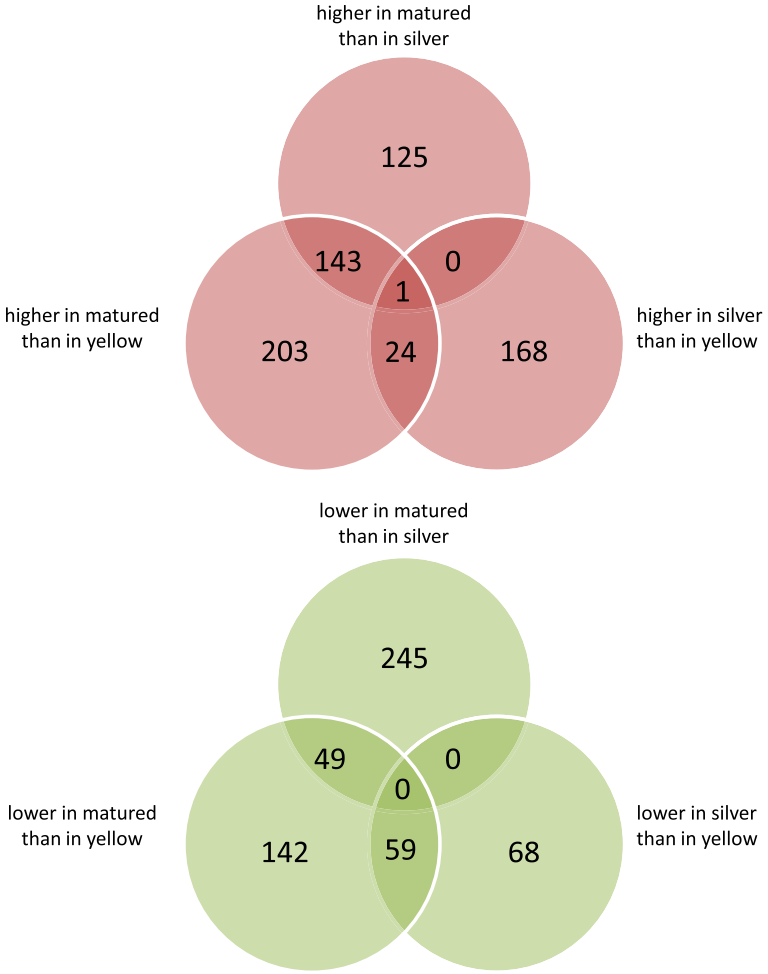

Fig. 2. Illumina RNAseq analysis of ovarian samples from wild female European eels. The two Venn diagrams show the number of differentially expressed genes between the three maturation stages. (A) A total of 193 genes showed higher expression in silver than in yellow eel, 371 genes showed higher expression in matured than in yellow eel and 269 genes had higher expression in matured than in silver eel. We could identify 1 gene (overlap of all three categories) that showed higher expression in matured eel compared with yellow and silver eel and that also had higher expression in silver eel compared with yellow eel. (B) A total of 127 genes had lower expression in silver eel than in yellow eel, 250 genes had lower expression in matured eel than in yellow eel and 294 genes had lower expression in matured than in silver eel. None of these genes (overlap of all three categories) showed both lower expression in matured eel compared with yellow and silver eel and lower expression in silver compared with yellow eel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Genes that are exclusively expressed in ovary of matured eel and have a minimum RPKM value of 2. The two features (g28942, g8202) corresponding with liver receptor homolog-1 (lrh-1/nr5a2) are shown in bold.

Gene	Name	Description	Total length	# reads in matured eel	RPKM value
g42816	leg4	Galectin-4-like	145	38	8.70
g40717	leg7	Galectin-7	475	111	7.75
g13804		Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2 isoform X4	853	144	5.60
g38780	cmah	Cytidine monophosphate-n-acetylneuraminic acid hydroxylase	138	20	4.81
g28942	**nr5a2**	**Nuclear receptor subfamily 5 group A member 2**	**148**	**19**	**4.26**
g13683	ada	Adenosine deaminase	524	56	3.55
g25954		Probable e3 ubiquitin-protein ligase rnf144a-a	213	21	3.27
g37222	rbm47	Rna-binding protein 47	335	32	3.17
g8202	**nr5a2**	**Nuclear receptor subfamily 5 group A member 2**	**623**	**58**	**3.09**
g32381	aplp2	Amyloid-like protein 2 short = aplp-2	128	11	2.85
g45799	gli3	Transcriptional activator GLI3	105	8	2.53
g33252	z385a	Zinc finger protein 385a	111	8	2.39
g35424	pvrl1	Poliovirus receptor-related protein 1	255	17	2.21
g43900	cola1	Collagen alpha-1 chain flags: precursor	178	11	2.05

the European eel genome (Henkel et al., 2012), a subset of 140 genes related to steroidogenesis was identified (Table S3). Ovarian steroidogenesis has been well documented in the Japanese eel (e.g. Kazeto et al., 2011; Nagahama and Yamashita, 2008). Expression profiles of the genes that are supposedly involved in the major steroid synthesis pathway suggested by previous studies were investigated (Fig. 3). Several genes showed consistently increasing expression levels correlating with the transition from yellow to silver eel and from silver to fully matured (spawned) eel (Fig. 3), namely cytochrome P450 cholesterol side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase/17,20 lyase (P450c17), 11beta-hydroxylase (P45011β), 17-beta hydroxysteroid dehydrogenase I (17β-hsd1), one of the two 17-beta hydroxysteroid dehydrogenase III genes (17β-hsd3) and 11-beta hydroxysteroid dehydrogenase II (11β-hsd2).

3.2. Artificial maturation of farmed eel; morphometry

To examine whether sex steroids have predictive value with respect to the successful response of farmed European eel to treatment with gonadotropins, we performed an extensive artificial maturation trial (schematically depicted in Fig. 1). Based on EI, all 40 female eels that were used in the artificial maturation trial were defined as silver eels (EI > 6.5, Pankhurst, 1982). At the start of the experiment, one of the females was assigned to the yellow stage 2, 28 were assigned to the premigrant silver stage 3, and 11 were assigned to the 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 4). The PFLI showed a significant increase after 18 weeks as compared to the PRE measurements (Table 4). As compared to the initial control the gonad weight (GW) and GSI were significantly increased, whereas the digestive tract weight (DTW) and DTSI were significantly decreased after 4, 12 and 18 weekly injections (Table 4). Based on the increase in GSI at the time point 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 4). Morphometric data of all individual animals are shown in Table S4.

3.3. Histological analysis of oocytes from artificially maturing eels

The furthest developed oocytes of the initial control ($t = 0$) represented the cortical alveoli stage 3 (Fig. 4a). Incorporated lipid droplets were dispersed around the nucleus. The nucleus was found centered with nucleoli in the periphery. Yolk granules were not present, indicating that vitellogenesis was not initiated.

After 4 weekly injections, oocytes were in pre- or early vitellogenic stage. Two females with a relatively low GSI (0.62 and 0.82) showed small oocytes with incorporated lipid droplets similar to those found in the initial control (Fig. 4b). 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 twofold enlarged. Yolk granules were not present or became present in the periphery (Fig. 4c).

Based on the presence of yolk granules, early and mid-vitellogenic stage oocytes were observed after 12 weekly injections in gonad tissue of females with GSI of 8.5 and above (Fig. 4d). 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 weeks group), a range of different responses to the treatment could be distinguished (Table S4). Six out of 16 eels of the 18 weeks group 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 pre- and early vitellogenic stage. Slowly responding females (GSI 3.9–6.3) showed oocytes at early vitellogenic stages.

3.4. Microarray and RNAseq profiling of genes related to steroidogenesis

The RNAseq analysis of wild female European eel (Section 3.1; Figs. 2 and 3, Tables 3, S2 and S3) showed that the main differentially expressed genes during natural maturation (yellow and silver stages) and artificial maturation (spawned stage) within ovarian tissue are involved in the steroidogenic pathway. To examine if changes in expression of steroidogenesis-related genes already occur at the beginning of the artificial maturation process of hormone-responsive farmed eels, ovarian RNA of eels that had received four weekly SPE injections was analyzed using microarrays Comparison of the mRNA expression levels of 140

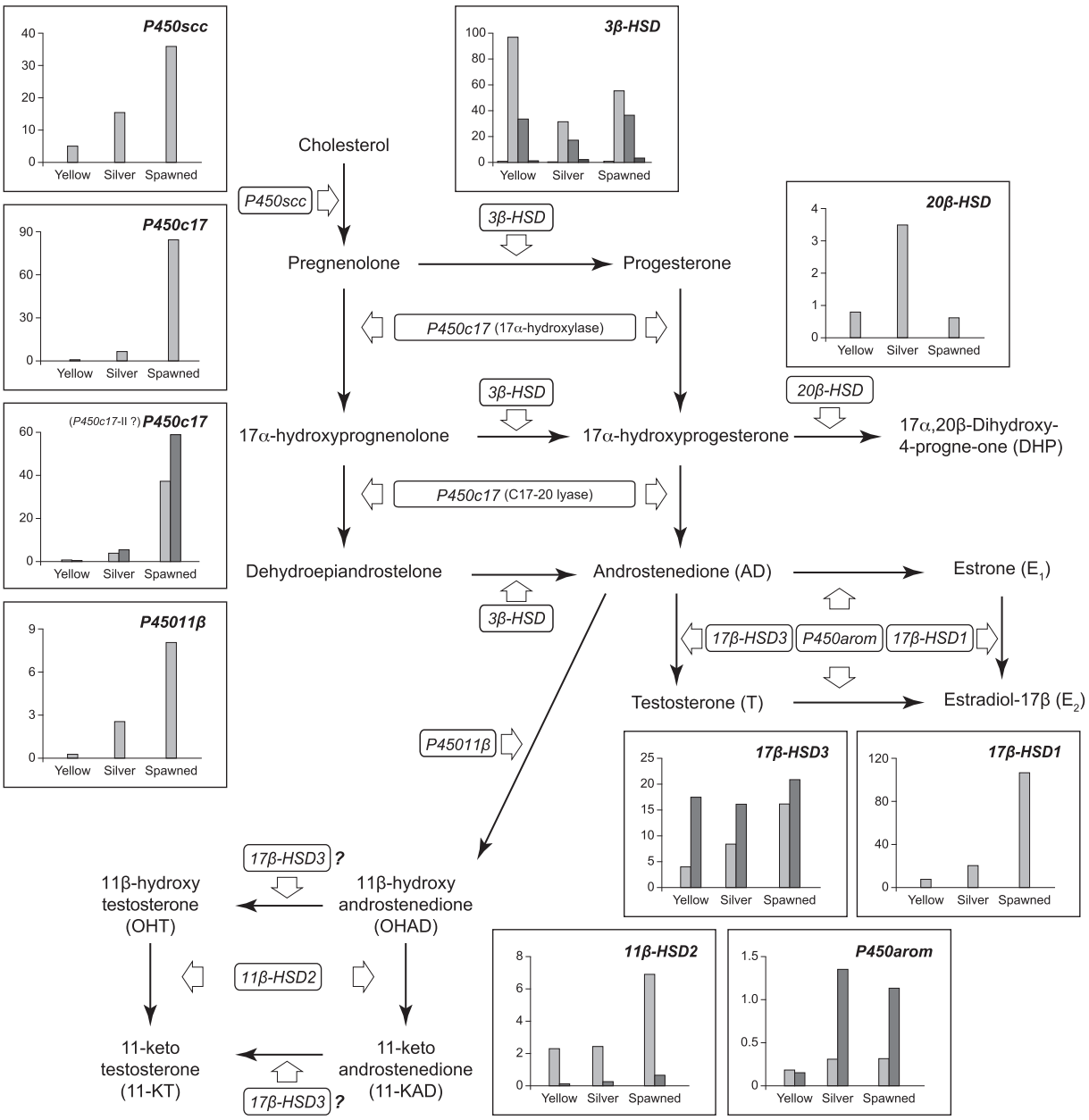

Fig. 3. Major pathway of steroidogenesis and the expression levels of the related genes after Kazeto et al. (2011) and Nagahama and Yamashita (2008). Different colours in expression levels (light and dark gray) show provisionally differentially annotated loci. The *y*-axis indicates the RPKM values. Note that the scale of the *y*-axis is different in each panel. See also Table S3 for more details.

steroidogenesis-related genes (Table S3) between four responders (GSI > 1.5) and two non-responders (GSI < 1.0) revealed that only 3 genes were significantly differentially expressed (Table 5; $p < 0.01$):farnesyltransferase beta (fntb) and 17-beta hydroxysteroid dehydrogenase I (17β-hsd1) were up-regulated and cytochrome P450-family 27-subfamily B (cp27b) was down-regulated. To further check the microarray data we analyzed the same ovarian RNA samples (four responders and two non-responders) via Illumina RNAseq. A complete overview of all mapped reads is presented in Table S5. Up-regulation of fntb and 17β-hsd1 was confirmed ($p < 0.01$), whereas down-regulation of cp27b was not statistically significant (Table 5). RNAseq analysis also revealed a significant up-regulation ($p < 0.01$) of arylsulfatase (arsj), which was less pronounced ($p < 0.05$) in the microarray analysis. Up-regulation of liver receptor homolog-1 (lrh-1/nr5a2) was also detected via RNAseq analysis ($p < 0.05$), but not via microarray analysis. Expression of LH receptor and FSH receptor mRNA was barely detectable via RNAseq and did not allow to calculate differential expression between responders and non-responders (Table 5).

3.5. Blood plasma steroid hormone levels and correlation with GSI

We examined whether steroid hormone levels in blood plasma correlated with the maturation status of the gonads. ELISA analysis of plasma levels of sex steroids T and E2 (Fig. 5a and b) 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 ($p > 0.05$). Significant positive correlations with GSI were found for E2, its absolute (ΔE2) and relative (rE2) changes after 4 weekly injections, and for E2 and ΔE2

Table 4
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 (*). *Abbreviations:* BL: body length; BW: body weight; DTSI: digestive tract somatic index; DTW: digestive tract weight; EI: eye index; GSI: gonadosomatic index; GW: gonad weight; HSI: hepatosomatic index; LW: liver weight; PFLI: pectoral fin length index; SI: silver index; RESP: responders; SE: standard error.

	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 (g)	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 (cm)	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 (g)	6.8	0.7			6.0	0.6			6.3	0.7			5.7	1.1
GW (g)	8.2	1.0			13.3*	2.4			36.1*	11.8			50.5*	19.9
DTW (g)	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
# RESP.					6				3				10	

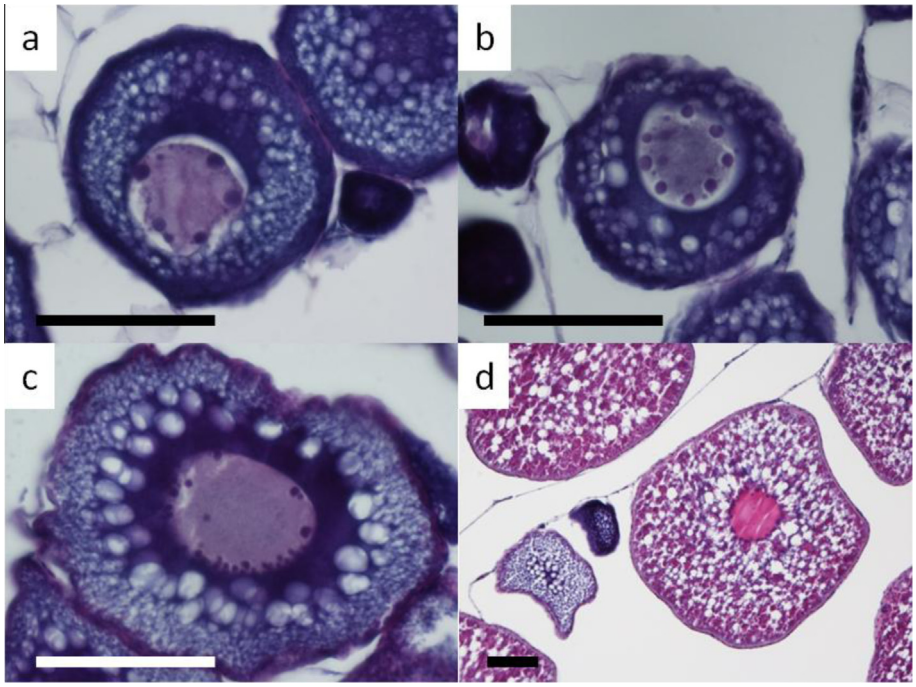

Fig. 4. Histological sections of HE-stained oocytes. (a) Initial control showing furthest oocyte stage: cortical alveoli stage 3. (b) Non-responder after 4 weekly injections showing similar oocytes as initial control. (c) Responder after 4 weekly injections showing enlarged oocytes with increased incorporation 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.

after 12 and 18 weekly injections. T and ΔT were found significantly positively correlated with GSI after 12 and 18 weekly injections (Table 6). It was found that rE2 could be used to distinguish responders from non-responders after 4 (Fig. 6a) and 12 (Fig. 6b) weekly injections with a reliability of ~80% and ~99%, respectively. Absolute E2 and ΔE2 levels showed a wide variation due to initial plasma levels and were therefore not suitable to distinguish between future responders and non-responders (data not shown).

4. Discussion

The large variation in the response to weekly hormonal treatments of female European eels might be one of the reasons for the low reproductive success rates. The maturation protocol of female European eels takes approximately 4–6 months, and the average success rate is around 50% with respect to egg production and even below 10% with respect to hatching. This causes unnecessary use of animals, waste of time, space and expensive hormones. Early selection of responders based on predictive markers and minimally invasive sampling methods could contribute to better reproductive success rates. During the present study we first performed RNAseq analysis of ovaries from wild eels. Two steroidogenesis-related genes, lrh-1 (Nr5a2) and cp17a (P450C17, cyp17a1), turned out to be the best leads for maturation-specific biomarkers. Then, female eels were subjected to a hormonal treatment for a maximum of 18 weeks. A custom-built microarray, Illumina RNAseq and blood plasma analyses were used to further explore the predictive value of gene expression profiles and plasma sex steroid levels for hormone-induced maturation.

Table 5
Significant changes in expression levels of steroidogenesis-related genes and gonadotropin receptors (FSH-r and LH-r) between responders ($n = 4$) and non-responders ($n = 2$) after 4 weekly SPE injections. Responders and non-responders were distinguished based on GSI (Table S4). β-Actin 1 was added as reference gene. Gene ID refers to codes of predicted genes (see www.zfgenomics.com). FC is the relative fold change in expression value between responders and non-responders. Expr. is the average expression value between responders and non-responders in the microarray analysis and baseMean is the average expression value between responders and non-responders in the RNAseq analysis. Significant *p*-values are shown in bold ($p < 0.01$).

Microarrays analysis					RNAseq		
Gene ID	Name	FC	Expr.	*p*-Value	FC	BaseMean	*p*-Value
g1697	fntb	2.49	397.3	**0.002**	7.47	21.2	**0.000**
g10882	cp27b	0.38	278.1	**0.003**	0.54	2.5	0.555
g40434	hsd17b1	1.97	1955.3	**0.004**	2.96	109.4	**0.000**
g7619	arsj	1.57	267.2	0.026	57.02	18.1	**0.001**
g8768	nr5a2	0.95	69.8	0.781	1.91	67.4	0.043
g10501	FSH-r	0.91	89.9	0.501	1.70	5.1	0.577
g12514	LH-r	1.66	72.0	0.080	n/a	0.2	1.000
g40338	LH-r	0.89	975.5	0.412	n/a	0.3	1.000
g144	β-Actin 1	1.15	55878.3	0.420	0.90	14254.6	0.590
g22021	β-Actin 1	1.12	36107.2	0.428	0.91	981.0	0.672

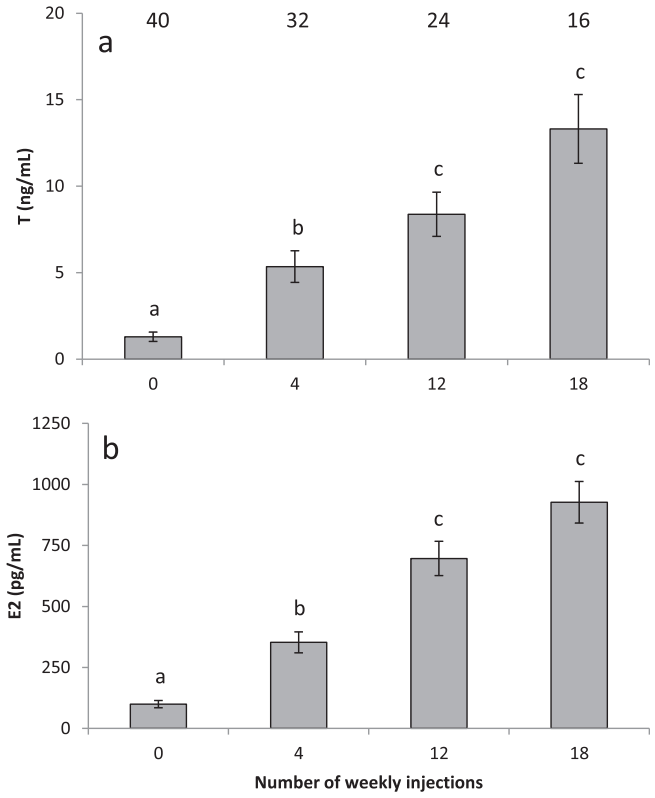

Fig. 5. Changes in plasma hormone levels during artificially induced maturation in cultured female eels. (a) Testosterone (T). (b) 17β-Estradiol (E2). Significant differences in hormone plasma levels between consecutive sample points are indicated by letters (Mann–Whitney *U* test; $p < 0.05$). Number of females measured is indicated by values (n) at the top of the figure above each bar.

An increase of plasma steroid levels during artificial maturation has already been described (e.g. Chiba et al., 2007; Lokman et al., 1998, 2001; Matsubara et al., 2005). In those studies the increased expression of cp17a correlates closely with the increase in plasma steroid levels during artificial maturation (e.g. Chiba et al., 2007; Lokman et al., 1998, 2001; Matsubara et al., 2005).

Table 6
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 week	4 week	12 week	18 week
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

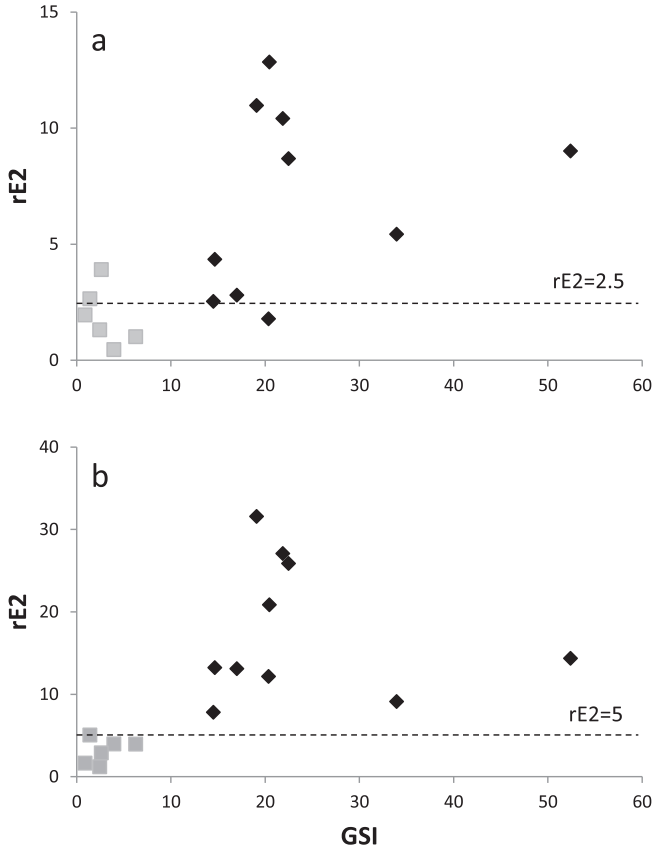

Fig. 6. Relative fold increase of 17β-estradiol blood plasma level (rE2) at 4 weeks (a) and 12 weeks (b) as compared to initial measurements ($t = 0$) of females sampled after ovulation or after 18 weekly injections with salmon pituitary extract. Responders ($n = 10$; diamonds) and non-responders ($n = 6$; squares) were distinguished based on GSI > 10. Dotted line indicates a suggested threshold to select responders from non-responders. At 4 weeks a 2.5-fold increase of E2 levels excludes 2/3 of the non-responders, while at 12 weeks a 5-fold increased level discriminates all responders from non-responders.

4.1. Ovarian gene expression profiles

The global gene expression profiles obtained in this study revealed that relatively few (<1.5%) of the protein-coding genes of the European eel genome seem to be directly involved in ovarian maturation and that a small subset thereof consists of steroidogenesis-related genes (see below and Table S2). Slight changes in physiological conditions can already affect gene expression profiles in tissues and organs of individual eels. Subtle differences in expression levels of key regulatory genes may play a significant role in controlling eel maturation and in the responsiveness of individual eels to treatment with gonadotropins. The initial response of female eels to gonadotropins is mediated by

gonadotropin receptors in the ovary. Ovarian LH receptor and FSH receptor mRNA levels were extremely low in responders and non-responders and did not allow to calculate differential expression (Table 5). Differential responsiveness to gonadotropins could also be caused by individual differences in protein stability, post-translational modifications or differences in the downstream signal transduction machinery of the gonadotropin receptors. One of the genes that was significantly and reproducibly up-regulated in the responders after four weekly injections with gonadotropins codes for the beta subunit of protein farnetyltransferase (Table 5; fntb). Farnesyltransferases add an isoprenoid farnesyl group to target proteins, often to stimulate membrane binding and function of signaling proteins (reviewed by Zverina et al., 2012). The exact function of gonadotropin-induced up-regulation of farnesyltransferase in the ovary remains to be determined.

Overexpression of Liver receptor homolog-1 (LRH-1)/NR5A2 in human mesenchymal stem cells induces differentiation into steroid hormone-producing cells. It also induces expression of CYP17A1 via three LRH-1 binding sites in the CYP17A1 promoter. This suggests that LRH-1 is a key regulator of the steroidogenic lineage in mesenchymal stem cells and plays a vital role in steroid hormone production (Yazawa et al., 2009). Our RNAseq results indicate that Lrh-1 may play a similar role in ovary of the European eel. Recently, it was shown that Lrh-1 down-regulates the expression of CYP19A1 (P450arom) in the mature ovary of the orange-spotted grouper (Lu et al., 2014). In the present analysis of wild eel specimens, lrh-1 was exclusively expressed in the maturing eel ovary (Table 3) and the expression level of P450arom was fairly low throughout the gonadal maturation (Fig. 3), which may indicate the presence of the regulation mechanism of P450arom expression by Lrh-1 as found in the orange-spotted grouper. On the other hand, Ijiri et al. (2003) reported that plasma E2 concentration is dependent on CYP19 expression levels in the Japanese eel, which did not correspond with our observation in this study that plasma E2 level was gradually increased throughout the artificial maturation trial (Fig. 5). According to our RNAseq analysis, Lrh-1/nr5a2 mRNA was also specifically up-regulated in responders after four weekly injections with gonadotropins (Table 5; $p < 0.05$), although this was not detected via the microarray analysis. Discrepancies between the microarray and RNAseq results may be caused by non-specific binding of labeled cDNA to microarray probes, which could especially affect signals of genes with very low expression levels (Guo et al., 2013).

Among those genes playing important roles in steroidogenesis, *in silico* annotation identified three loci that showed the highest BLAST hit with P450c17 (cp17a) of teleosts (Tables S2 and S3; features g10692, g22995, g38561); Kazeto et al. (2000) isolated the Japanese eel P450c17 and found that it possesses both activities of 17α-hydroxylase and 17,20 lyase similar to P450c17-I of other fish species; however, P450c17-II of medaka and tilapia possesses only 17α-hydroxylase activity (Zhou et al., 2007). Furthermore, based on their temporally different gene expression patterns during the spawning cycle it was suggested that P450c17-I is mainly involved in oocyte growth, while P450c17-II is required for oocyte maturation (Nagahama and Yamashita, 2008; Zhou et al., 2007). Although different types of P450c17 may have different functions, the RNAseq results in the present study showed that the expression profiles of all three European eel P450c17 loci were similar (Fig. 3). Since the provisional annotation of the European eel genome indicates that the differently identified loci are truly different genes, future studies will need to clarify the presence of functionally different isoforms of P450c17 in eels.

Microarray and RNAseq analysis of responders and non-responders showed that 2 out of 140 steroidogenesis-related genes were reproducibly and significantly up-regulated in the responders, namely 17β-HSD1 and fntb (Table 5; $p < 0.01$). In the developing ovary 17β-HSD1 is responsible for the conversion of estrone (E1) into E2 (Kazeto et al., 2011; Nagahama and Yamashita, 2008). Increasing expression levels of 17β-HSD1 were also observed via RNAseq analysis of ovarian transcriptomes derived from advancing maturation stages of wild eel specimens (Section 3.1; Figs. 2 and 3) and is in line with the increase in blood plasma E2 levels found in the present study. Apart from the fairly low level of P450arom expression discussed above, the increased expression of 17β-HSD1 and the elevated plasma level of E2 possibly indicate the presence of the E2 synthetic pathway from androstenedione (AD) via E1 in the European eel, as was also suggested for the Japanese eel (Kazeto et al., 2011). Since it has been reported that the expression of P450arom is affected by temperature regime (Mazzeo et al., 2014; Pérez et al., 2011), the E2 production from AD via E1 may be dominant over the other pathway through P450arom from AD via T depending on temperatures. One of the most pronounced up-regulated genes (Table 5; FC = ~57-fold) in the RNAseq analysis of the gonadotropin-treated responders was arylsulfatase J (arsj). Arylsulfatases catalyze the removal of sulfate from a wide variety of substrates, including steroids (reviewed by Purohit et al., 2011). Very little is known about arylsulfatase J and its exact substrate and function in the ovary of the maturing eel remain to be determined.

We have recently identified multiple maturation marker genes in the pectoral fin of hormone-responsive female eels, including two genes that are also associated with steroid hormone signaling (Dirks et al., 2014). The gene "DDB1- and CUL4-associated factor 6" codes for a ligand-dependent coactivator of nuclear receptors and the gene "growth regulation by estrogen in breast cancer-like" (GREB1L) encodes an estrogen-specific estrogen receptor (ER) cofactor. These genes may play an essential role in the response of peripheral tissues to 17β-estradiol derived from the ovary, upon successful response to the gonadotropin treatment. This implies the coordinated regulation of production of steroid hormones in the gonads and the responsiveness of peripheral tissues to these hormones.

4.2. Sex steroids as biomarkers

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. Chiba et al., 2007; Lokman et al., 1998, 2001; Matsubara et al., 2005). A significant correlation was found between GSI and E2, ΔE2 and rE2, already after 4 weekly injections (Table 6). The rE2 levels of responders and non-responders sampled after ovulation or after 18 weekly injections indicate that ~80% and ~99% of the females responding to the treatment may be selected after 4 and 12 weeks, respectively (Fig. 6). At 4 weeks, a 2.5-fold increase of E2 levels excludes more than 60% of the non-responders, whereas at 12 weeks, a 5-fold increase of E2 levels discriminates all responders from non-responders. 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.

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. Furthermore, responsive female eels may already be selected after 4 weekly injections based on a 2.5-fold increase in E2 blood plasma levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.08.006.

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