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°C and 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.

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

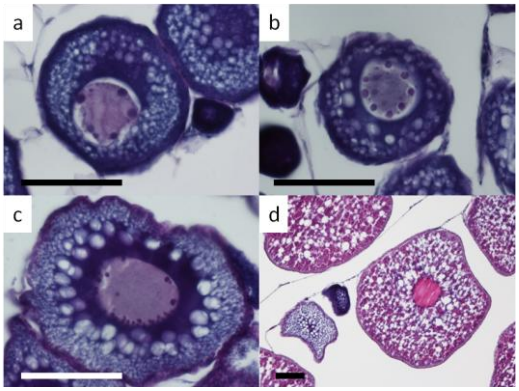

Figure 1. Histological sections of HE-stained oocytes. a. Initial control showing furthest oocyte stage: cortical alveoli stage 3. b. Non-responder after 4 weekly injections showing similar oocytes as initial control. c. Responder after 4 weekly injections showing enlarged oocytes with increased incorporation 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 ($p>0.05$).

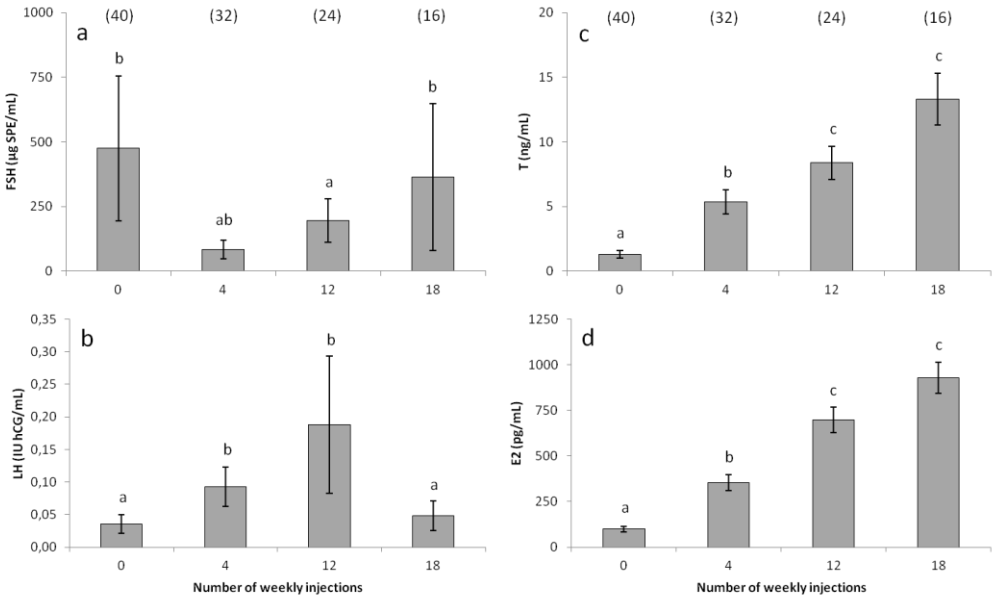

Figure 2. Changes in plasma hormone levels during artificially induced maturation in cultured female eels. a. Follicle-stimulating hormone (FSH). b. Luteinizing hormone (LH). c. Testosterone (T). d. 17β-Estradiol (E2). FSH and LH show a clear opposite regulation. FSH decreases over the first 12 weeks, 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

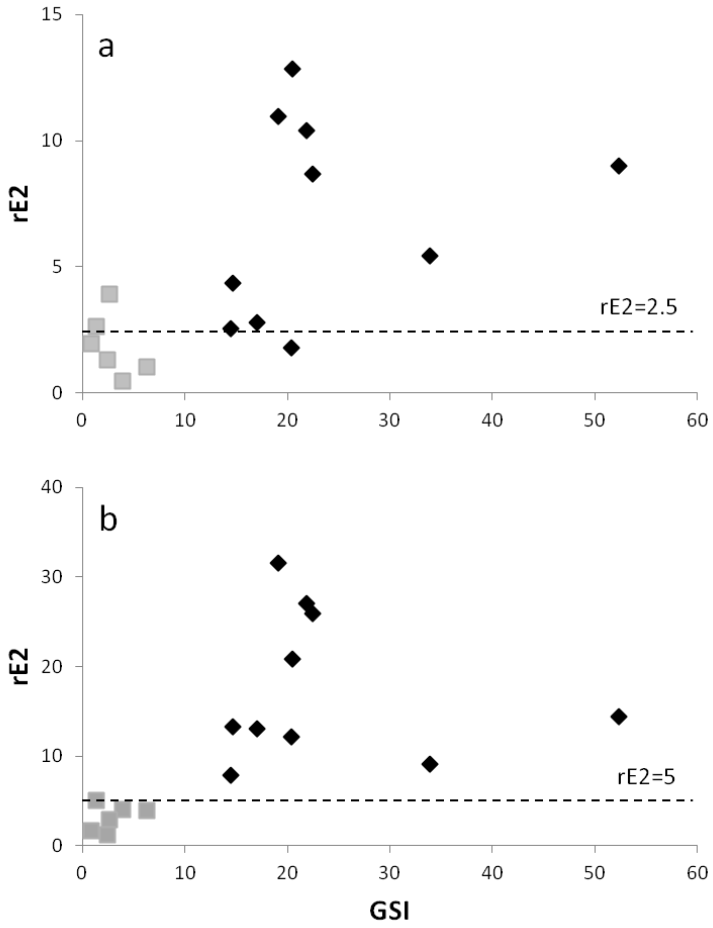

Figure 3. Relative fold increase of 17β-estradiol blood plasma level (rE2) at 4 weeks (a) and 12 weeks (b) as compared to initial measurements (t=0) of females sampled after ovulation or after 18 weekly injections with salmon pituitary extract. Responders (diamonds) and non-responders (squares) were 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.

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