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Citation

Dirks, R. P., Burgerhout, E., Brittijn, S. A., De Wijze, D. L., Ozupek, H., Tuinhof-Koelma, N., … Van den Thillart, G. E. (2014). Identification of molecular markers in pectoral fin to predict artificial maturation of female European eels (Anguilla anguilla). *General And Comparative Endocrinology*, *204*, 267-276. doi:10.1016/j.ygcen.2014.06.023

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Downloaded from:	https://hdl.handle.net/1887/3674201

Note: To cite this publication please use the final published version (if applicable).

General and Comparative Endocrinology 204 (2014) 267–276

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

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

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ARTICLE INFO

Article history:
Available online 30 June 2014

Keywords:
European eel
Artificial maturation
Pectoral fin
Predictive markers
Minimally invasive

ABSTRACT

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

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1. Introduction

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

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

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http://dx.doi.org/10.1016/j.ygcen.2014.06.023
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migration barriers and/or climate change, and the species is now critically endangered (IUCN Red List). European eel is an important consumption fish and most of the marketed eel is produced in aquaculture farms; however, farming of European eel is still fully dependent on wild-caught juveniles (glass eels), since artificial reproduction of European eel in captivity has not yet been achieved. Successful closure of the eel's life cycle in captivity will probably result in sustainable aquaculture, which is expected to relieve pressure on wild stocks and contribute to restoration of the natural population.

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

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

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

2. Materials and methods

2.1. Animals, morphometrics, hormone treatment, and sampling

All experiments conducted during this study complied with the Dutch law on animal experiments and were approved by the animal experimental committee of Leiden University (DEC# 11093).

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

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

Morphometric data was found normally distributed (Kolmogorov–Smirnov, $p > 0.05$) and was tested for significance at consecutive sampling points using two-tailed ANOVA with post hoc Bonferroni correction. Statistical difference was considered significant at $p < 0.05$. In all cases values are expressed as average ± standard error.

2.2. RNA isolation and Illumina RNAseq analysis

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

analyzer 2100 total RNA Nano series II chip (Agilent). Illumina RNAseq libraries were prepared from 2 µg total RNA using the Illumina TruSeq™ RNA Sample Prep Kit v2 according to the manufacturer's instructions (Illumina Inc.). All RNAseq libraries were sequenced on an Illumina HiSeq2000 sequencer as 2 × 50 nucleotides paired-end reads according to the manufacturer's protocol. Image analysis and base calling were done by the Illumina pipeline.

2.3. Illumina data processing

Reads were aligned to the draft genome sequence of 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 (version 0.5.3p9) (Anders et al., 2014). In order to make comparisons across samples possible, these fragment counts need to be corrected for the total amount of sequencing performed for each sample. As a correction scaling factor, we employed library size estimates determined using the R/Bioconductor (release 2.11) package DESeq (Anders and Huber, 2010). Read counts were normalized by dividing the raw counts obtained from HTSeq by its scale factor. Detailed read coverage for individual genes was extracted from the TopHat alignments using SAMtools. For manual re-annotation of candidate marker genes, CLC bio's *de novo* assembler was used to generate cDNA contigs from all pectoral fin RNAseq reads (fin contigs) or from our in-house collection of multiple organ RNAseq reads (multi-organ contigs). Improved versions of the automatically predicted genes were obtained via BLASTN searches of the predicted cDNAs against the *de novo* assembled pectoral fin and multi-organ cDNA contigs and via BLASTX searches of the *de novo* assembled cDNA contigs and genomic scaffolds against the non-redundant NCBI database. New Illumina read alignments were generated for re-annotated genes, which were then quantified and normalized as before. A FASTA file with all manually re-annotated fin clip marker contigs can be downloaded from www.zfgenomics.com/sub/eel (see also Supplementary file 1).

3. Results

3.1. Artificial maturation trial

A schematic representation of the artificial maturation trial is shown in Fig. 1. Based on EI, all eels that were used in this experiment were defined as silver eels before starting the hormonal treatment (EI > 6.5; Pankhurst, 1982). The morphometric data of the trial are shown in Table 1. The initial control group ($n = 8$) had a GSI of 1.01 (from 0.68 to 1.37). The remaining group of 14 females received weekly SPE injections as described in materials and methods. A total of 7 eels showed a 10% increase in body weight, namely after 14 ($n = 2$), 16 ($n = 2$) and 17 ($n = 3$) weekly hormone injections. These females received an SPE boost and a DHP treatment, which resulted in ovulation of 6 animals. One female did not ovulate after DHP injection and was sampled the day after expected spawning. The 7 remaining eels showed no or insufficient increase in body weight and were sampled at one week after the 18th SPE injection. The GSI of the group of 14 SPE-treated females was 15.56 and showed a large individual variation from 0.88 to 52.40, which is consistent with a mixture of non-responders and responders. The ΔEI (EI at time of sampling minus initial EI) was 4.14 (14–18 weeks), the SI increased from 3.38 (0 weeks) to 4.69 (14–18 weeks) and the HSI increased from 0.85 (0 weeks) to 1.31 (14–18 weeks). The ΔPFLI (PFLI at time of sampling minus PFLI at start of the experiment) was 0.28, whereas the DTSI decreased from 1.57 (0 weeks) to 0.47 (14–18 weeks).

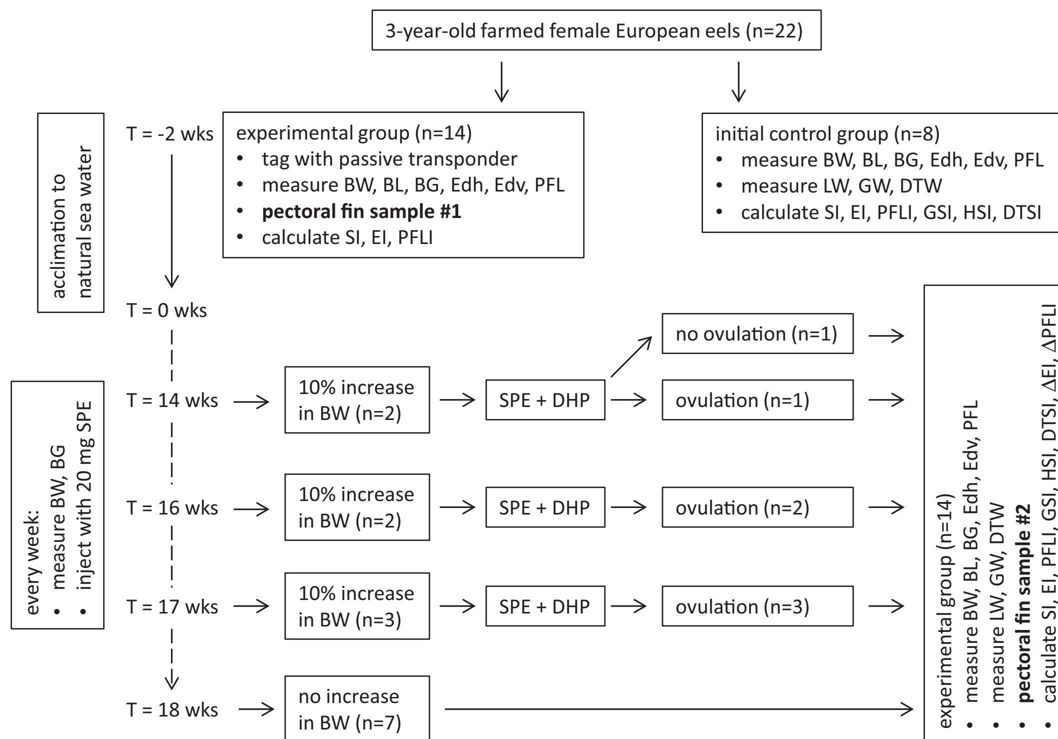


Fig. 1. Schematic overview of the artificial reproduction trial. Three year old female European eels ($n = 22$) were obtained from a commercial farm. An initial control group ($n = 8$) was sampled directly after transport to the lab facility, including morphometrics and measurement of multiple internal parameters. The remaining eels ($n = 14$) were tagged, morphometrics was performed and fin clip sample #1 was obtained from each individual. Subsequently, the experimental group was acclimated to natural seawater for two weeks and then subjected to weekly injections with SPE. Morphometrics of each individual was performed prior to the weekly hormone injections. Eels were sampled either at one day after ovulation or at one 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 fin clip sample #2 from each individual.

Table 1
Artificial maturation of female European eels. Farmed silver eels were either sampled at the start of the trial (initial control group; $n = 8$) or subjected to weekly SPE injections for 14–18 weeks (SPE-treated animals; $n = 14$). Animals are sorted per group from high to low GSI. Asterisks indicate eels that were selected for pectoral fin transcriptome analysis. *Abbreviations:* Ave ± SE: average ± standard error; SPE: salmon pituitary extract; GSI: gonadosomatic index; Sp: spawning; EI_{init}: initial eye index; ΔEI = eye index at time of sampling minus EI_{init}; ΔPFLI: pectoral fin length index at time of sampling minus initial pectoral fin length index; SI: silver index; HSI: hepatosomatic index; DTSI: digestive tract somatic index; n/a: not applicable; ND: not determined.

Initial control group

Nr.	GSI	EI_{init}	SI	HSI	DTSI
4	1.37	8.0	3	1.09	2.67
6	1.21	11.0	4	0.92	1.27
3	1.16	10.4	4	0.91	1.81
1	1.10	9.5	4	0.80	1.25
2	0.97	7.0	3	0.93	1.53
5	0.85	9.2	3	0.57	0.92
8	0.72	8.1	3	0.95	2.18
7	0.68	8.6	3	0.61	0.96
Ave ± SE	1.01 ± 0.09	9.0 ± 0.5	3.38 ± 0.18	0.85 ± 0.06	1.57 ± 0.22

SPE-treated animals

Nr.	SPE (wks)	GSI	Sp	EI_{init}	ΔEI	ΔPFLI	SI	HSI	DTSI
4A1D*	14	52.40	No	9.2	4.5	0.0	4	0.79	0.21
A46C*	16	22.48	Yes	8.9	5.6	0.1	5	1.60	0.32
4CAB*	18	21.87	No	9.1	3.9	0.2	4	1.45	0.32
OFCC*	14	20.44	Yes	12.9	3.5	0.0	5	2.01	0.29
2842	17	20.36	Yes	9.1	4.1	0.2	5	2.01	0.44
E94A	17	19.09	Yes	10.1	4.5	0.3	5	1.84	0.45
1A1D	18	16.99	No	9.3	5.3	0.5	5	1.32	0.29
EE09	16	14.65	Yes	8.1	3.8	0.3	5	1.70	0.32
5CE2	17	14.50	Yes	11.0	6.1	0.1	5	2.00	0.45
F091	18	6.25	No	9.9	4.6	0.6	5	0.96	0.38
F758	18	3.94	No	10.6	6.3	0.7	5	0.77	0.46
11AA*	18	2.61	No	11.5	3.2	0.3	5	0.71	0.53
C63F*	18	1.40	No	7.5	0.6	0.4	3	0.52	0.85
004C*	18	0.88	No	9.3	2.0	ND	ND	0.62	1.25
Ave ± SE		15.56 ± 3.34		9.75 ± 0.35	4.14 ± 0.39	0.28 ± 0.05	4.69 ± 0.16	1.31 ± 0.14	0.47 ± 0.07

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 × 50-nt reads. *Abbreviations:* GSI: gonadosomatic index after 14–18 weeks of treatment with SPE; SPE: salmon pituitary extract.

Tag	GSI	Category	SPE (wks)	Sequence clusters	Mapped RNAseq reads (%)[1]
4A1D	52.40	Responder	0	5,427,892	3,229,156 (59.5%)
			14	11,734,446	6,924,100 (59.0%)
A46C	22.48	Responder	0	10,910,993	6,098,514 (55.9%)
			16	11,678,479	7,424,285 (63.6%)
4CAB	21.87	Responder	0	15,404,243	9,035,231 (58.7%)
			18	9,990,432	6,121,807 (61.3%)
OFCC	20.44	Responder	0	10,663,299	6,487,170 (60.8%)
			14	14,729,262	8,930,477 (60.6%)
11AA	2.61	Non-responder	0	11,273,235	6,867,514 (60.9%)
			18	10,861,634	6,619,356 (60.9%)
C63F	1.40	Non-responder	0	8,660,826	5,278,272 (60.9%)
			18	10,840,380	6,417,050 (59.2%)
004C	0.88	Non-responder	0	10,316,152	6,095,875 (59.1%)
			18	12,862,757	7,900,153 (61.4%)

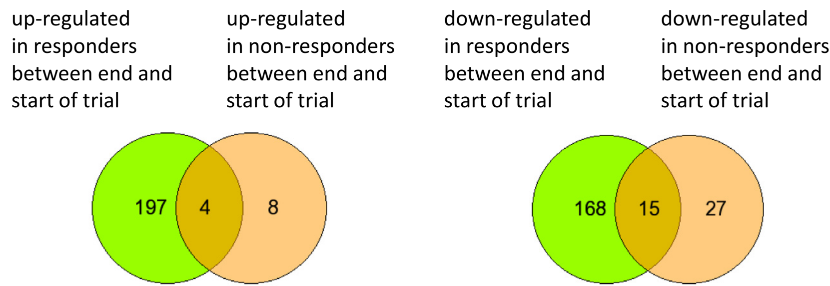
[1] Alignment of at least one read of each paired read.

3.2. Pectoral fin transcriptomes

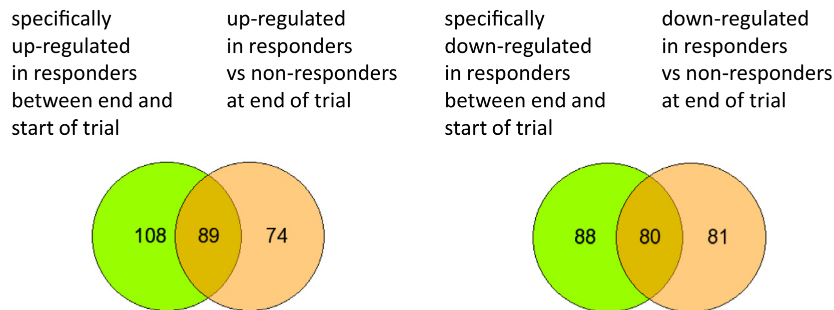
Based on the GSI and oocyte stages at the end of the trial, the 14 females of the hormone-treated group (14–18 SPE injections) were divided into three categories: non-responders (GSI < 3.0) have oocytes that lack yolk granules and are in a pre- and early-vitellogenic stage; slow-responders (GSI 3.0–10.0) have oocytes at pre-, early- and mid-vitellogenic stage; responders (GSI > 10.0) have oocytes at early-, mid-, and late vitellogenic stage and include spawning eels (Burgerhout, data not shown). The four responders with the highest GSI and the three non-responders with the lowest GSI (indicated by asterisks in Table 1) were selected for maturation marker discovery. Early ($t = 0$ weeks) and late ($t = 14–18$ weeks) pectoral fin samples derived from the seven selected eels were subjected to Illumina RNAseq analysis, resulting in 14 datasets of between 5.4 and 15.4 million clusters of paired-end 50-nucleotide reads. As summarized in Table 2, an average of 60% of all sequence clusters could be mapped to the list of 45,975 predicted European eel cDNA sequences (Henkel et al., 2012) using Tophat (Trapnell et al., 2009).

DESeq analysis was used to identify genes that were differentially expressed ($p < 0.00001$) in pectoral fin after 14–18 weekly SPE injections (end of trial) compared with fin samples obtained at the start of the trial. The responders showed ~17 times more up-regulated genes and ~4 times more down-regulated genes than the non-responders (Fig. 2A): in the responders, 384 genes were

A Differentially expressed between end and start of trial



B Differentially expressed at end of trial



C Differentially expressed at start of trial

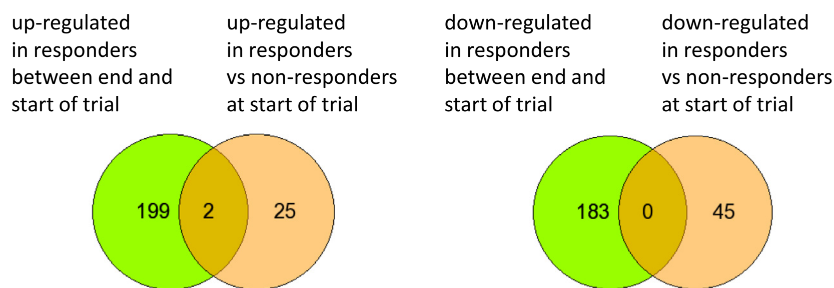


Fig. 2. Illumina RNAseq analysis of pectoral fin samples. Female European eels received weekly injections with SPE for up to 18 weeks and pectoral fin samples obtained from responders ($n = 4$) and non-responders ($n = 3$) at the start and the end of the trial were subjected to Illumina RNAseq analysis. The sequence reads were aligned to the European eel genome (Henkel et al., 2012) and differentially expressed genes were identified using DESeq. The significance cut-off was set at $p < 0.00001$. (A) Venn diagrams showing the differentially expressed genes between the end and the start of the trial in responders (green) and non-responders (orange). The number of genes with up-regulated expression is shown in the left panel and with down-regulated expression in the right panel. The intersection of responders and non-responders is shown in the overlaps. (B) Venn diagrams showing the differentially expressed genes between responders and non-responders at the end of the trial (orange) and the genes that are differentially expressed between the end and the start of the trial, exclusively in the responders (green). The number of genes with up-regulated expression is shown in the left panel and with down-regulated expression in the right panel. The intersection of genes that are differentially expressed specifically in the responders between the end and the start of the trial and between the responders and non-responders at the end of the trial is shown in the overlaps. (C) Venn diagrams showing the differentially expressed genes between future responders and non-responders at the start of the trial (orange) and the genes that are differentially expressed in responders between the end and the start of the trial (green). The number of genes with up-regulated expression is shown in the left panel and with down-regulated expression in the right panel. The intersection of genes that are differentially expressed in future responders at the start of the trial and the genes that are differentially expressed in responders between the end and the start of the trial is shown in the overlaps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentially expressed (201 up-regulated and 183 down-regulated), whereas in the non-responders only 54 genes were differentially expressed (12 up-regulated and 42 down-regulated). Thus, this first analysis resulted in a preliminary list of 365 responder-specific candidate maturation markers genes (384 minus the intersections in Fig. 2A). To further increase the stringency of candidate marker selection, DESeq was used to also identify genes that were differentially expressed between responders and non-responders at the end of the trial (after 14–18 SPE injections). In total 324 genes were differentially expressed ($p < 0.00001$): 163 genes were up-regulated and 161 down-regulated (Fig. 2B). The intersection of the 197 genes that were specifically up-regulated in responders between the end and start of the trial (Fig. 2A) and the 163 genes that were up-regulated between responders and non-responders at the end of the trial resulted in a list of 89 up-regulated candidate marker genes (overlap in Fig. 2B, left panel). Similarly, the

intersection of the 168 genes that were specifically down-regulated in responders between the end and start of the trial (Fig. 2A) and the 161 genes that were down-regulated between responders and non-responders at the end of the trial yielded a list of 80 down-regulated candidate marker genes (overlap in Fig. 2B, right panel). Candidate genes that ended up outside the intersections of Fig. 2B did not comply to the stringent p value ($p < 0.00001$), mostly as a result of big differences in read counts between individual fin samples of the responder or non-responder group. Finally, DESeq was used to identify genes that were already differentially expressed between future responders and non-responders at the beginning of the trial (Fig. 2C). In total 72 genes were differentially expressed: 27 genes were up-regulated and 45 down-regulated. The intersection of the 201 genes that were up-regulated in responders between end and start of the trial (Fig. 2A) and the 27 genes that were up-regulated between responders and non-responders at the start of the trial resulted in 2 genes (overlap in Fig. 2C, left panel), whereas the intersection of the 183 genes that were down-regulated in responders between end and start of the trial and the 45 genes that were down-regulated between responders and non-responders at the start of the trial yielded 0 genes (overlap in Fig. 2C).

3.3. Selection and correction of maturation marker genes

DESeq analysis resulted in 89 up-regulated and 80 down-regulated genes (overlaps in Fig. 2B). The expression level of an up-regulated marker should be sufficiently high at the end of the trial and that of a down-regulated marker sufficiently high at the start of the trial to allow reliable quantification of changes in expression level via a simple assay, such as quantitative PCR. Therefore, another selection criterion was applied based on an arbitrarily chosen minimum DESeq-BaseMean value of 100 at the end of trial (up-regulated genes) or start of trial (down-regulated genes), resulting in 53 up-regulated and 57 down-regulated candidate markers. A suitable maturation marker should also show a big change in expression level between the end and start of the maturation protocol in the responders and very little or no change in the non-responders. To meet this criterion, only those candidate markers were selected for which the ratio of fold-change expression in responders versus non-responders was at least 10. This resulted in a final selection of 28 up-regulated (Supporting Table S1) and 29 down-regulated (Supporting Table S2) maturation marker genes.

The list of 45,975 cDNA sequences that was used for initial mapping of the pectoral fin RNAseq reads was automatically predicted from the draft European eel genome sequence using AUGUSTUS software (Henkel et al., 2012; www.zfgenomics.com/sub/eel). Part of the predicted genes consist of incorrectly fused exons, which causes errors in the read counts of the RNAseq analysis. Therefore, all 57 marker genes were manually re-annotated via BLAST analysis against three different references: (1) a list of *de novo* assembled contigs derived from all pectoral fin RNAseq reads; (2) a list of *de novo* assembled contigs derived from our in-house collection of multiple organ RNAseq reads; and (3) BLASTX analysis of the corresponding genomic scaffolds of the European eel against the NCBI database. In this manner, 22 out of 28 up-regulated and 18 out of 29 down-regulated genes could be replaced with manually re-annotated genes (Supporting Tables S3 and S4). Indeed, four predicted cDNAs (g15077, g809, g5257 and g5806) turned out to be chimeras derived from two neighbouring genes and could be replaced with the eight individual cDNA sequences. Eventually, this resulted in a list of 56 re-annotated genes and their best BLASTX hits in the non-redundant NCBI database (Supporting Tables S3 and S4). All pectoral fin RNAseq reads were then aligned again to the re-annotated sequences, yielding a list of corrected read counts for all seven individual eels at the start and end of the maturation trial (data not shown). Based on DESeq analysis of the corrected read counts 12 out of 56 re-annotated genes could be removed from the list of marker genes, because they no longer met the stringent selection criteria that were used in the first DESeq analysis round: (1) minimum DESeq-BaseMean value of 100 at the end (up-regulated genes) or start of the trial (down-regulated genes); and (2) at least 10 times higher fold-change expression in responders versus non-responders. To be able to sort the markers based on combined highest expression value and highest fold-change ratio in responders versus non-responders, we assigned them a quality score, the Marker Index (MI), according to the following formulas:

$$\text{For up-regulated markers}: MI_{up} = BM_{EOTresp} \times \frac{FC_{resp}}{FC_{nonresp}}$$

$$\text{For down-regulated markers}: MI_{down} = BM_{SOTresp} \times \frac{FC_{resp}}{FC_{nonresp}}$$

where $BM_{EOTresp}$ is the DESeq BaseMean value in responders at the end of the trial, $BM_{SOTresp}$ is the DESeq BaseMean value in responders at the start of the trial, FC_{resp} is the fold-change expression in responders and $FC_{nonresp}$ is the fold-change expression in non-reponders. The 23 up-regulated and 21 down-regulated, re-annotated, maturation marker genes, sorted by decreasing marker index, are shown in Tables 3 and 4, respectively.

4. Discussion

We used Illumina RNAseq analysis followed by a highly stringent selection procedure to identify 23 up-regulated and 21 down-regulated pectoral fin marker genes that can specifically discriminate female European eels responding to pituitary extract-induced sexual maturation from those that do not respond to this treatment. A quality score, the Marker Index, was assigned to each marker based on a combination of the highest expression level and the highest ratio of fold change expression in responders versus non-responders. The up-regulated marker genes can be placed into five functionally related groups, whereas the down-regulated markers can be placed into two groups.

4.1. Up-regulated pectoral fin marker genes

The first main category of up-regulated marker genes codes for a set of proteases. According to the Marker Index, the Golgi-associated plant pathogenesis-related protein 1 (GAPR1) gene is by far the best marker gene for responders (MI = 2.02E+07). It has the highest BaseMean value at the end of the trial (57529.72), the highest fold change expression between the start and the end of the trial (1434-fold up-regulated) and the second best ratio of fold-change expression in responders versus non-responders (350.5). Relatively little is known about the activity and biological function of the GAPR1 protein: it has serine protease activity (Milne et al., 2003) and anti-fungal properties (Niderman et al., 1995) and mammalian GAPR1 is highly expressed in immune-related tissues and cells and may play a role in the innate immune system (Eberle et al., 2002). The second best marker for responders is also a protease, namely the meprin A subunit beta-like gene (MEP1B; MI = 3.15E+06). The MEP1B protein is the beta subunit of the metalloproteinase meprin and is associated with inflammation and angiogenesis and with diseases such as cancer, fibrosis and neurodegeneration. Meprins are also overexpressed in fibrotic skin tumors, characterized by massive accumulation of fibrillar collagens and procollagen III is processed to its mature form by meprins, which is an essential step in the assembly of collagen fibrils

Table 3
Up-regulated maturation markers (manually re-annotated sequences). *Abbreviations:* BM-SOT, DESeq BaseMean at start of trial; BM-EOT, DESeq BaseMean at end of trial; FC, fold change; FC-resp, fold change in responders; FC-nonresp, fold change in non-responders; MI_{up}, Marker Index of up-regulated maturation markers ($BM_{EOT} \times \frac{FC_{resp}}{FC_{nonresp}}$).

Predicted cDNA	Original name	Re-annotated sequence		Non-responders (*n* = 3)			Responders (*n* = 4)			FC_{resp}/ $FC_{nonresp}$	Marker Index (MI_{up})
		Contig/Scaffold Nr.	Gene description	BM_{SOT}	BM_{EOT}	FC	BM_{SOT}	BM_{EOT}	FC		
g17944	gapr1	19943 (mo)	Golgi-associated plant pathogenesis-related protein 1	14.58	59.66	4.1	40.12	57529.72	1433.9	350.5	2.02E+07
g15118	mep1b	6694 (fin)	Meprin A subunit beta-like	59.42	32.06	0.5	17.39	5440.05	312.8	579.8	3.15E+06
g26339	ajl2	13216 (sc)	Lactose-binding lectin l-2	537.88	613.70	1.1	531.95	31853.97	59.9	52.5	1.67E+06
g34657	ajl2	1516358 (sc)	Lactose-binding lectin l-2	426.07	483.72	1.1	316.37	15040.52	47.5	41.9	6.30E+05
g809 (chimera)	can14	730 (mo)	Calpain-1 catalytic subunit-like isoform X2	207.11	315.32	1.5	316.70	10783.22	34.0	22.4	2.42E+05
g26704	flo11-like	66774 (mo)	Mucin-22-like	3.52	19.75	5.6	4.01	2193.17	547.5	97.7	2.14E+05
g809 (chimera)	can14	1515 (mo)	DDB1- and CUL4-associated factor 6	176.17	253.60	1.4	286.75	7764.64	27.1	18.8	1.46E+05
g5257 (chimera)	ccr9	27129 (fin)	Keratin	4.28	19.68	4.6	6.25	1863.36	298.2	64.9	1.21E+05
g9254 g9682	tyrp2	18384 (mo)	L-dopachrome tautomerase-like	20.86	19.89	1.0	15.30	1104.39	72.2	75.7	8.36E+04
g7065	gch1	58973 (mo)	GTP cyclohydrolase 1	4.94	20.66	4.2	7.35	1544.30	210.1	50.3	7.77E+04
g29311	n/a	55922 (fin)	Unknown	1.05	4.07	3.9	1.83	712.85	389.7	100.4	7.16E+04
g15077 (chimera)	prm1a	28671 (mo)	Fibroblast growth factor-binding protein 1 precursor	74.58	105.67	1.4	38.00	1350.29	35.5	25.1	3.39E+04
g8076	es31 l	47320 (mo)	Fatty acyl-CoA hydrolase precursor, medium chain-like isoform X1	10.35	15.30	1.5	10.67	709.59	66.5	45.0	3.19E+04
g17158	b4gn2	3469 (sc)	Beta-1,4 N-acetylgalactos-aminyltransferase 2-like	159.86	50.37	0.3	79.23	745.37	9.4	29.9	2.23E+04
g25582	b4gn2	21084 (mo)	Beta-1,4 N-acetylgalactos-aminyltransferase 2-like	290.20	223.45	0.8	170.11	1544.75	9.1	11.8	1.82E+04
g9054	pgh1	9641 (mo)	Prostaglandin G/H synthase 1-like	9.30	24.49	2.6	19.96	834.96	41.8	15.9	1.33E+04
g41537	ion3	1169032 (sc)	Keratin 8	70.44	79.05	1.1	49.19	799.34	16.2	14.5	1.16E+04
g16435	vmat2	52367 (fin)	Vesicular monoamine transporter 2	7.90	9.30	1.2	6.03	239.85	39.8	33.8	8.11E+03
g18188	fhl3	4212 (scaf)	Four and a half LIM domains protein 3	25.85	27.63	1.1	23.70	388.04	16.4	15.3	5.94E+03
g13055	tmpsd	46867 (fin) 42988 (fin) 1977 (scaf)	Transmembrane protease serine 13-like	10.04	5.81	0.6	7.21	122.66	17.0	29.4	3.61E+03
g209	grb1l	33703 (mo) 61711 (fin)	Growth regulation by estrogen in breast cancer-like	2.21	4.34	2.0	3.89	110.12	28.3	14.4	1.59E+03
g20173	mucin-22-like	12324 (mo)	Mucin-22-like	0.00	2.33	infinite	0.22	227.61	1033.1	n/a	n/a
g8444	k1c13	29902 (mo)	Keratin	0.00	0.87	infinite	1.81	1294.68	716.7	n/a	n/a

Table 4
Down-regulated maturation markers (manually re-annotated sequences). *Abbreviations:* BM_{SOT}, DESeq BaseMean at start of trial; BM_{EOT}, DESeq BaseMean at end of trial; FC, fold change; FC_{resp}, fold change in responders; $FC_{nonresp}$, fold change in non-responders; MI_{down}, Marker Index of down-regulated maturation markers ($BM_{SOT} \times \frac{FC_{resp}}{FC_{nonresp}}$).

Predicted cDNA	Original name	Re-annotated sequence		Non-responders (n = 3)			Responders (n = 4)			FC_{resp}/ $FC_{nonresp}$	Marker Index (MI_{down})
		Contig/Scaffold Nr.	Gene description	BM_{SOT}	BM_{EOT}	FC	BM_{SOT}	BM_{EOT}	FC		
g27587	fa55c	2053 (fin)	NXPE family member 3-like	707.71	877.81	0.8	879.97	1.14	769.1	954.0	8.39E+05
g5806 (chimera)	exos7	1454 (mo)	Tetranectin-like	526.59	1405.80	0.4	2099.61	20.65	101.7	271.4	5.70E+05
g36553	ajl2	640298 (sc)294190 (sc)135815 (sc)	Lactose-binding lectin l-2	10671.87	811.33	13.2	17734.28	51.94	341.5	26.0	4.61E+05
g34395	ajl2	178 (fin)	Lactose-binding lectin l-2	10145.29	716.33	14.2	14014.35	38.69	362.2	25.6	3.59E+05
g45747	ajl2	624896 (sc)	C-type lectin 1	19272.27	1140.77	16.9	19630.26	65.27	300.8	17.8	3.49E+05
g45606	ajl2	585697 (sc)	C-type lectin 1	11140.55	276.21	40.3	18376.10	28.38	647.4	16.1	2.96E+05
g28202	hbaa	2256 (fin)	Hemoglobin anodic subunit alpha	4381.18	3786.52	1.2	4941.75	238.94	20.7	17.9	8.85E+04
g30174	hbba	1550 (fin)	Hemoglobin anodic subunit beta	5831.37	4811.72	1.2	6079.90	371.85	16.4	13.5	8.21E+04
g45886	n/a	1544 (fin)	Unnamed protein product	1525.29	562.77	2.7	3038.36	47.64	63.8	23.5	7.14E+04
g45138 g37175	hbac	7276 (fin)	Hemoglobin cathodic subunit alpha	3062.14	4357.83	0.7	3735.23	336.93	11.1	15.8	5.90E+04
g44952 g41104	hbbc	693 (mo)10220 (mo)	Hemoglobin cathodic subunit beta	3086.52	4164.03	0.7	3785.24	334.15	11.3	15.3	5.79E+04
g5800	ret4b	367 (mo)	Retinol-binding protein 2 precursor	177.45	683.82	0.3	1305.56	132.76	9.8	37.9	4.95E+04
g1061	sbp1	5957 (mo)	Selenium-binding protein 1 isoform X1	652.88	467.09	1.4	1076.68	20.10	53.6	38.3	4.12E+04
g28115	samh1	18174 (sc)	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	2094.62	1470.14	1.4	2753.86	130.45	21.1	14.8	4.08E+04
g34564	n/a	793194 (sc)	unknown	37.86	77.05	0.5	157.27	3.29	47.8	97.2	1.53E+04
g28927	samh1	21638 (sc)	SAM domain and HD domain-containing protein 1-like	441.48	292.90	1.5	860.76	44.43	19.4	12.9	1.11E+04
g30582	samh1	32278 (sc)	SAM domain and HD domain-containing protein 1-like	109.21	47.82	2.3	282.16	4.23	66.8	29.2	8.24E+03
g28082	samh1	18060 (sc)	SAM domain and HD domain-containing protein 1-like	313.38	151.70	2.1	443.59	18.15	24.4	11.8	5.23E+03
g32786	ajl1	62384 (sc)	Ancestral Congerin Con-anc	331.21	221.45	1.5	394.13	22.53	17.5	11.7	4.61E+03
g33776	samh1	114763 (sc)	SAM domain and HD domain-containing protein 1-like	405.83	329.80	1.2	468.92	34.62	13.5	11.0	5.16E+03
g15713	st3a1	15485 (fin)	Sulfotransferase family 3, cytosolic sulfotransferase 3	81.71	154.72	0.5	219.40	41.05	5.3	10.1	2.22E+03

(reviewed in Broder and Becker-Pauly, 2013). The list of up-regulated markers contains two more proteases, namely a calpain-related gene (MI = 2.42E+05) and transmembrane protease serine 13-like (MI = 3.61E+03).

The second main category of up-regulated marker genes codes for structural components of the skin and the mucus layer, including two lactose-binding lectin genes, two mucin genes and three keratin genes. This suggests that females that respond to the hormone treatment undergo a significant reorganization of their skin structure. Since keratins, mucins and lectins are all associated with defense against pathogens (e.g. Hobbs et al., 2012; Nielsen and Esteve-Gassent, 2006; Suzuki et al., 2003) this could mean that hormone-induced sexual maturation of female eels results in major changes in the eel's immune system.

A third category of up-regulated markers is associated with steroid hormone signaling: "DDB1- and CUL4-associated factor 6" (MI = 1.46E+05) is a ligand-dependent coactivator of nuclear receptors, whereas "growth regulation by estrogen in breast cancer-like" (GREB1L; MI = 1.59E+03) was recently identified as an estrogen-specific estrogen receptor (ER) cofactor (Mohammed et al., 2013). These genes may play an essential role in the response of peripheral tissues to the sex hormone 17β-estradiol that is produced by the ovary upon successful response to the gonadotropin treatment.

Three up-regulated markers fit into a fourth category linked to tyrosine/dopamine metabolism: (1) GTP cyclohydrolase 1 (GCH1; MI = 7.77E+04) is the first enzyme in the three-step enzymatic pathway that converts GTP into tetrahydrobiopterin, which plays an important role in tyrosine/dopamine biosynthesis; (2) L-dopachrome tautomerase (MI = 8.36E+04) is a tyrosinase-related protein involved in regulating eumelanin and phaeomelanin levels; (3) vesicular monoamine transporter 2 (VMAT2; MI = 8.11E+03) is an intracellular transporter of monoamines, including dopamine. Up-regulation of these three genes is likely involved in the increased pigmentation of the pectoral fins that is commonly observed in females that respond to the hormone treatment (Braasch et al., 2007).

A fifth category of up-regulated markers is linked to lipid metabolism: prostaglandin G/H synthase 1-like (pgh1/PTGS1-like; MI = 1.33E+04) is related to PTGS1, a key enzyme in prostaglandin biosynthesis that may play a role in steroid synthesis and/or the production of the mucus layer; fatty acyl-CoA hydrolase medium chain (MI = 3.19E+04) is a thioesterase that has been shown to play a role in estradiol-induced production of 3-hydroxy fatty acid diester female pheromones (Bohnet et al., 1991); beta-1,4 N-acetylgalactos-aminyltransferase 2 (B4GALNT2; two genes, MI = 2.23E+04 and MI = 1.82E+04) is a blood-group-related glycosyltransferase (Dall'olio et al., 2013).

Only two up-regulated markers could not be fit into one of these five categories: fibroblast growth factor-binding protein 1 (FGFBP1; MI = 3.39E+04) is a secreted protein that can reversibly bind to FGF1 and FGF2 and plays a role in angiogenesis; Four and a half LIM domains protein 3 (FHL3; MI = 5.94E+03) is a member of the four and a half LIM domains (FHL) family of proteins and may function as a transcriptional coactivator and regulator of the cytoskeleton.

4.2. Down-regulated pectoral fin marker genes

The down-regulated marker genes can be placed into two main categories, namely blood markers and lectin-related genes. According to the Marker Index, the best down-regulated marker is neurexophilin and PC-esterase domain (NXPE) family member 3-like (NXPE3; MI = 8.39E+05), the expression of which decreases by 769-fold in the responders and even shows a slight increase in the non-responders (ratio of fold change expression in responders versus non-responders is 954). NXPE3 fits into the category of blood markers, together with the four eel hemoglobin subunits anodic subunit alpha (MI = 8.85E+04), anodic subunit beta (MI = 8.21E+04), cathodic subunit alpha (MI = 5.90E+04) and cathodic subunit beta (MI = 5.79E+04), the retinol-binding protein 2 (MI = 4.95E+04), the selenium-binding protein 1 isoform (MI = 4.12E+04) and five genes related to the deoxynucleoside triphosphate triphosphohydrolase SAMHD1 (MI = 4.08E+04, 1.11E+04, 8.24E+03, 5.23E+03, 5.16E+03, respectively). The sequence reads corresponding to these typical blood markers must be derived from blood cells that were present in the pectoral fin samples. One possible explanation for the strongly reduced expression of these blood markers in the pectoral fins of responding female eels is that transcription of these blood marker genes is actively down-regulated during sexual maturation. This can be tested via transcriptome analysis of whole blood samples obtained from the circulatory system. Another explanation is that the percentage of blood cells relative to the rest of the fin cells decreases during sexual maturation. The size of the pectoral fin may increase without a corresponding increase in vasculature; however, in the current artificial maturation trial the pectoral fin length index did not significantly increase in the responders (Table 1), although we cannot rule out that the thickness of the pectoral fin increased during sexual maturation. Alternatively, the number of blood cells per total blood volume (hematocrit) may decrease in time. We have already observed that long-term treatment of female European eels with pituitary extracts is associated with decreasing hematocrit values (unpublished data), but additional research is required to determine whether a reduced hematocrit can account for such a strong reduction in the expression of blood marker genes. The reduction in expression of the twelve blood marker genes ranges from 9.8-fold (retinol-binding protein 2) to 769-fold (NXPE3) with an average of 86-fold reduced expression. E.g., in the responders, the down-regulation of the hemoglobin anodic subunit alpha (20.7-fold) and beta (16.4-fold) genes is much stronger than that of the cathodic subunit alpha (11.1-fold) and beta (11.3-fold) genes, whereas the down-regulation is even much stronger for the selenium-binding protein 1 isoform gene (53.6-fold). This suggests that active regulation of gene expression, either at the level of transcription or at the level of mRNA stability, plays at least some role in the down-regulation of these genes.

The second category of down-regulated markers consists of lectin-related genes, namely tetranectin-like (MI = 5.70E+05) and two other C-type lectins (MI = 3.49E+05 and 2.96E+05), two lactose-binding lectins (MI = 4.61E+05 and 3.59E+05) and ancestral Congerin Con-anc (MI = 4.61E+03). Since lactose-binding lectins are also among the best up-regulated markers, we can conclude that hormone induced maturation of female eels is associated with a significant reorganization of the spectrum of lectins that are expressed in the pectoral fin. Lectins are thought to play an important role in the defense against pathogens (Nielsen and Esteve-Gassent, 2006), supporting the theory mentioned above that sexually maturing female eels undergo major changes in their immune system.

In conclusion, the list of pectoral fin markers identified in this study may not only help in discriminating between female European eels that do or do not respond to hormone treatment, but also provides new fundamental insight into several processes associated with sexual maturation of female European eels, such as the response to steroid hormones, restructuring of the skin and mucus layer, pigmentation of the skin, and production of pheromones. Fin clip transcriptomes of the eel appear to be an unexpectedly rich source of information about their physiological status and we expect that similar studies on other fish species will result in additional minimally invasive methods for monitoring the physiological status of fish in general. E.g., a recent transcriptome analysis of caudal fin biopsies of rainbow trout resulted in new molecular

markers that can be used to monitor the exposure of salmonids to environmental pollutants such as endocrine disruptors and heavy metals (Veldhoen et al., 2013).

Acknowledgments

Mieke and John van Dooren (Passie voor Vis, Sevenum, The Netherlands) are greatly acknowledged for providing the farmed silver eels. We thank Dr. Hans Jansen for helpful discussions. This study was supported by Grants from the Dutch Ministry of Economic Affairs/SenterNovem (Grant No. 359 SII081030) and from the EU commission (PROEEL, Grant Agreement No. 245257).

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.2014.06.023.

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