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4

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39 ABSTRACT

40 Our understanding of maternal control of development in vertebrates remains incomplete. In  
41 this study, we investigated levels of maternal transcripts in good and poor quality eggs from  
42 artificially matured Japanese eel, using RNA-Seq and quantitative PCR (qPCR), to identify  
43 candidate maternal transcripts related to development. *De novo* assembly or mapping of  
44 reads to the eel draft genome yielded 619,029 contigs and 85,906 transcripts, respectively;  
45 normalized read counts to these assemblies were calculated using reads (RPKM) or  
46 fragments (FPKM) per kilobase of transcript per million mapped reads. *In silico* screening  
47 identified 1594 contigs and 150 transcripts with lower RPKM or FPKM in poor than in good  
48 quality eggs, 245 contigs and 85 transcripts of which could be annotated by BLASTx,  
49 respectively. From selected contigs or transcripts, 6 genes (*dnajb4*, *gnpat*, *card14*, *pdp1*,  
50 *fcgbp*, *ttn*) had significantly lower mRNA levels in poor than in good quality eggs by qPCR.  
51 Multiple regression analysis showed that 5 genes (*gnpat*, *b4galnt1*, *acsl6*, *rtkn*, *trim24*)  
52 significantly correlated with hatchability. Taken together, 10 genes were identified as  
53 candidate maternal transcripts, regulating development in Japanese eel. Our results  
54 contribute to understanding the molecular basis for maternal control of development in  
55 vertebrates.

## 1. INTRODUCTION

In vertebrates, maternally inherited transcripts (maternal transcripts) accumulate in the egg but are translationally suppressed during oogenesis. Upon fertilization, these transcripts are promptly translated to regulate early embryonic development in the transcriptionally quiescent zygote. Subsequently, a set of maternal transcripts is eliminated, coinciding with activation of *de novo* transcription from the genome of the zygote, referred to as zygotic genome activation (ZGA). This transfer of the control of development from products of maternal origin to those of an activated zygotic genome is known as the maternal-zygotic transition (MZT) (reviewed in Tadros & Lipshitz, 2009; Lee et al., 2014). A recent report has demonstrated that some maternal products are required to initiate the zygotic developmental program (Lee et al., 2013). Additionally, several maternal products continue to function into developmental stages beyond ZGA (see review in Marlow, 2010). Thus, maternal transcripts are essential not only for early, but also for late development.

Maternal-effect mutations make for a powerful tool to help dissect the maternal control of vertebrate embryogenesis (Dosch et al., 2004; Wagner et al., 2004; Pelegri et al., 2004). Accordingly, numerous maternal-effect mutants with morphological defects have been identified in zebrafish, a model vertebrate species that is highly suitable for study of developmental biology. Such studies have contributed to understanding of maternal control of development. Take, for example, maternal *lymphoid-restricted membrane protein* (*lrmp*), which plays an essential role in nucleus-centrosome attachment and pronuclear congression during fertilization. *Lrmp* has been identified from *futile cycle* zebrafish mutants which fail to undergo pronuclear congression and fusion (Lindeman & Pelegri, 2012). Another maternal transcript, *serine-threonine kinase mitogen activated protein kinase-activated protein kinase 2* (*mapkapk2*), which regulates progression of epiboly (gastrulation-specific movement of cells leading to an epithelial monolayer over the entire yolk mass) through modulation of the actin cytoskeleton, has been identified from *betty boop* mutants that display defects in epiboly (Holloway et al., 2009). Although several causative genes of maternal-effect mutants have accordingly been identified, in many cases their molecular identity remains unknown, so that understanding of the mechanisms for maternally controlled aspects of development in vertebrates remains incomplete.

Japanese eel, *Anguilla japonica*, is one of the most important aquaculture species, and

establishment of artificial propagation techniques is required to stably supply seedlings and conserve this natural resource in Japan (Tanaka, 2015). In captivity, fertilized eggs are obtained by exogenous hormone treatment, but the quality of eggs produced under these conditions is variable (Chai et al., 2010) – not unexpectedly, abnormal embryos appear at high frequencies from batches of poor quality eggs. It is empirically known that the phenotype of embryos produced from poor quality eel eggs resemble those of maternal-effect mutants in zebrafish (ex. Dosch et al., 2004; Wagner et al., 2004; Pelegri et al., 2004). In view of these observations, the study of maternal transcripts in poor quality eggs from Japanese eel could prove an effective novel approach to understanding the role of maternal transcripts during vertebrate development.

Previously, we investigated the maternal mRNA levels of 74 cell division-related genes in eggs from artificially matured Japanese eel (Izumi et al., 2016) and documented that the abundance of several of these mRNA species correlated with egg quality. Similarly, other studies on fish have identified differentially expressed maternal transcripts between good and poor quality eggs (reviewed in Sullivan et al., 2015; Zarski et al., 2017). Together, these reports have indicated that levels of accumulated maternal transcripts in the egg were associated with development competence. Therefore, it is plausible that maternal transcripts that are present in abnormal composition or abundance in poor quality eggs play an essential role in development.

In this study, we aim to identify candidate maternal transcripts that play a role in development in eggs from Japanese eel on the basis of their quantitative signature. We focused on transcripts that had lower abundance in poor than in good quality eggs. First, we subjected RNA from Japanese eel eggs of good and poor quality to next-generation sequencing (NGS) and then conducted a large-scale RNA-Seq analysis by comparing the number of mapped reads in egg samples to either *de novo* assembled contigs or to the eel draft genome sequence. We complemented these analyses by comprehensive quantitative PCR (qPCR) assays. Finally, we searched for maternal transcripts whose abundances were lower in poor quality eggs compared to eggs of good quality, as reflected in hatchability.

## 2. MATERIALS AND METHODS

### 2.1 Animals – methods, sampling and ethical conduct

Details on the source, maintenance and experimental manipulations of Japanese eels used in this study have been described in detail previously (Izumi et al., 2016); different subsamples from the same experiment were used in the present study. In brief, wild-caught elvers from Japanese eel were purchased and reared before being feminized by female sex hormone in experimental tanks. After a further two years of on-growing, feminized eels (500–1000 g) received weekly injections of exogenous pituitary homogenates to attain sexual maturity, as described in Chai et al. (2010). After 17,20 $\beta$ -dihydroxy-4-pregnen-3-one-induced ovulation (Kagawa et al., 1997), eggs from 13 artificially matured female Japanese eels were collected by strip-spawning and subsamples flash-frozen (n=12) in liquid nitrogen or preserved in RNALater (Thermo Fisher Scientific, Waltham, MA; n=1) until analysis. Parts of the egg batches (2 g) were dry-fertilized and incubated; fertilization and hatching rates were scored and these data used to consider batch quality as “good” or “poor” (see Table 1), exactly as described in Izumi et al. (2016). Eggs with fertilization rate >80% and hatching rate >80% were classified as good quality eggs (developmentally competent); eggs with fertilization rate >80% and hatching rate  $\leq$ 20% were classified as poor quality eggs (eggs producing viable morula embryos without hatching). All fish handling, husbandry and sampling methods were approved by the Institutional Animal Care and Use Committee of Hokkaido University.

### 2.2 RNA extraction and removal of ribosomal RNA for NGS

Total RNA was isolated from 6 egg samples (0.2–0.4 g per sample), 3 representing good eggs (Eel nos. 1, 2, 5; Table 1) and 3 representing eggs of poor quality (Eel nos. 10, 12, 13), after homogenization in ISOGEN reagent (Nippon Gene, Tokyo, Japan). Any remaining genomic DNA was digested on-column with DNase and RNA eluted using the Nucleospin RNA II Kit (Macherey-Nagel GmbH, Düren, Germany) in accordance with the manufacturer's instructions. RNA quantity and quality were subsequently evaluated using a Nano-Drop 2000 (Thermo Fisher Scientific) and an Agilent RNA Pico chip, run on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) – resulting RNA integrity numbers exceeded 8.0 for all samples.

Selective depletion of ribosomal RNA was achieved as described previously (Izumi et al., 2015; in Japanese): using Thermo Fisher Scientific's RiboMinus™ Eukaryote System v2, > 98% rRNA was removed after incubation of total RNA from eel eggs with biotinylated antisense probes to mammalian rRNA.

### **2.3 Library construction and sequencing**

Template RNA was quality-assessed on a 2100 Bioanalyzer to confirm the successful removal of rRNA (rRNA abundance < 2% of that in non-depleted samples, data not shown; also see Izumi et al., 2015). The Ovation® RNA-Seq System (NuGEN, Redwood City, CA) was then used to synthesize double-stranded cDNA. Paired-end sequencing of the cDNA libraries was performed using a HiSeq 2000 sequencer (Illumina, San Diego, CA), contracted to Hokkaido System Science Co Ltd, according to the manufacturer's instructions.

### **2.4 Assembly and mapping**

CASAVA version 1.8.2 (Illumina) was used for base calling, read filtering and cleaning of sequence data. Adapters and low quality bases were trimmed in CLC Genomics Workbench (Qiagen, Valencia, CA) with default-set parameters. To reduce bias, the trimmed reads were mapped to rRNA and mitochondrial genome sequences (GenBank Accession No. CM002536.1) using CLC Genomics Workbench as described in Izumi et al. (2015); unmapped-reads were used for subsequent analyses.

Two approaches, i.e., *de novo* assembly (Analysis 1) and reference-based assembly (Analysis 2), were performed in keeping with Martin & Wang (2011). Figure 1 shows a flow diagram of the experimental design. In Analysis 1, unmapped-reads shorter than 50 b were discarded and the longer reads were used for the *de novo* assembly to obtain contigs. *De novo* assembly was carried out using CLC Genomics Workbench at default settings. Thereafter, the reads from each of the 6 libraries were mapped to the *de novo* assembled contigs using CLC Genomics Workbench and converted into reads per kilobase of exon per million mapped reads (RPKM; Mortazavi et al., 2008). In Analysis 2, unmapped-reads from all samples were mapped to the draft genome sequence of the Japanese eel (Henkel et al., 2012) using TopHat (Trapnell et al., 2009), and the relative levels of each transcript were then calculated using the *cufflinks*, *cuffmerge* and *cuffdiff* commands in Cufflinks on the basis of

FPKM (fragments per kilobase of transcript per million mapped reads), as described in Trapnell et al. (2012).

## 2.5 *In silico* screening

To identify transcripts that had lower abundance in poor quality than in good quality eggs, we carried out *in silico* screening as our first approach, and then followed this up with qPCR. The screening design that was used in this study is described in Fig 1. In Analysis 1, contigs whose RPKM values were significantly down-regulated (at least two-fold;  $P < 0.05$ ) in poor eggs (nos. 10, 12, 13) compared to eggs of good quality (nos. 1, 2, 5) were identified. In addition, we identified contigs ( $> 200$  bp in length) of which no reads were found among poor egg quality samples (RPKM = 0), whereas RPKM  $> 0$  among the three batches of good eggs. These sequences were subjected to BLAST (blastx) against NCBI database (nr) using BLAST2GO software (Götz et al., 2008) and were abbreviated using the gene names used in accord with the Human Genome Organization Gene Nomenclature Committee (HGNC). To derive the potential function of a gene, individual contigs were manually annotated with the Gene Ontology (GO) term (biological process) in ZFIN (zebrafish database) and UniProtKB (human database). The GO term with the highest-ranked GO Evidence Code and involvement in embryogenesis was assigned to the respective contig.

In Analysis 2, we identified those transcripts for which FPKM from poor quality eggs = 0 and that from good eggs  $> 0$ . Additional stringent differential FPKM selection criteria were imposed, such that *i*) good egg FPKM  $> 1$  and *ii*) the ratio between FPKM of good eggs (lowest FPKM from among No. 1 and No. 2) and poor quality eggs (using the best sample amongst these, i.e., No. 10)  $< 0.65$ . These were annotated by BLASTx (Ref-Seq), and their potential function was presumed to be the same as described in the above methods.

## 2.6 qPCR analysis

To reduce the number of candidate genes to a manageable number for follow-up qPCR analyses, we performed small-scale qPCR analyses ( $n=3$ ) against the contigs identified by *in silico* screening in Analysis 1. For this purpose, cDNA was synthesized from rRNA-depleted RNA (i.e., that used for NGS) and subsequently amplified using the Ovation® Pico WTA System V2 (NuGEN).

For large-scale qPCR analysis (n=6 for each egg quality category), total RNA (nos.1–5, 7–13 in Analysis 1 and nos. 1–4, 6–13 in Analysis 2) was reverse-transcribed into single-stranded cDNA with random hexamer primers as described in Izumi et al. (2016). Genes that were identified by *in silico* screening and small-scale qPCR as being differentially expressed were analyzed by qPCR using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Osaka, Japan) or Thermo Fisher Scientific's Universal SYBR® Select Master Mix. Primers used for qPCR analyses and designed using the software package Primer Express v3.0.1 (Thermo Fisher Scientific) are listed in Supporting Information, Table S1, S2. All qPCR data were normalized over expression of  $\beta$ -actin (Izumi et al., 2016) using the  $\Delta C_t$  method (Pfaffl, 2001).

## 2.7 Statistical analysis

Hierarchical clustering using Ward's method and Euclidean distance was conducted using the RPKM or FPKM data from three good quality egg samples (nos. 1, 2, 5) and three poor quality egg samples (nos. 10, 12, 13) used for NGS. Expression data from small-scale experiments (n=3), both from qPCR and from *in silico* analysis (only Analysis 1), were analyzed with Student's *t*-test; differences were considered significant for  $P < 0.05$ . Mann-Whitney *U*-test was employed to analyze qPCR data from experiments with larger sample sizes (n=6), and differences were considered significant for  $P < 0.01$ . The Benjamini-Hochberg false discovery rate (FDR = 0.3) was adopted for multiple testing correction of qPCR data in Analysis 2. Cluster analysis and comparison of treatment means were done using R version 3.1.0. To determine whether maternal transcripts contributed to hatchability, stepwise multiple regression analysis was carried out using the Statistical Package for Social Sciences (IBM). The dependent variables were the transcript abundances for 36 and 72 genes quantified by qPCR analysis (n=6) in Analyses 1 and 2, respectively. The hatchability was used as independent variable. Predicted hatchability was subsequently estimated by multiple regression equations for Analyses 1 and 2, respectively. The value of qPCR analysis (n=6) was again used as dependent variable. Average of the calculated hatchability value was defined as the final predicted hatchability value. To test for relationships between the predicted hatchability and observed hatchability, Spearman's correlation coefficient was estimated using R. Significance was defined as  $P < 0.05$ .

### 3. RESULTS

#### 3.1 NGS statistics and cluster analysis

An average of around 60 million reads, not including those for rRNA and mtRNA, of nearly 100 bp in length was obtained for each library (35.53 Gb of sequence data; see Supporting Information, Table S3). These reads were *de novo* assembled into 619,029 contigs. The maximum contig length was 43,411 bp, the N50 equated to 296 bp, the average length to 260 bp and the approximate coverage depth was 81.6. When these reads were mapped to the draft eel genome - approximately 70% of all reads from all egg samples mapped to this genome – 85,906 transcripts were obtained.

In Analysis 1, the dendrogram derived from cluster analysis of relative levels of all transcripts showed that good and poor quality eggs clustered together (Fig. 2 a). In contrast, clustering on the basis of Analysis 2 produced two distinct clusters, one consisting of good quality eggs (nos. 1, 2, 5) and the other of poor quality eggs (nos. 10, 12, 13) (Fig. 2 b).

#### 3.2 Screening for qPCR analysis

Normalized read counts (RPKM) of 994 contigs were at least two-fold higher in good than in poor quality eggs from Japanese eel. When listing all contigs that yielded mapped reads in good eggs and no reads in batches of poor quality eggs, a total of 600 contigs was identified. Of these contigs, 148 and 97 contigs could be annotated by BLASTx, respectively (Supporting Information, Table S4, S5); however, 10 and 11 contigs, respectively, coded for unknown protein. Altogether, primers could be designed for 152 contigs for subsequent qPCR screening on reverse-transcribed total RNA from the same 6 samples as those used for NGS. Accordingly, the mRNA levels of 26 among these 152 candidate genes were significantly lower in poor quality relative to good quality eggs ( $P < 0.05$ ) (Table 2). A further 10 candidate genes tended to be differently expressed ( $0.059 < P < 0.168$ ). The resulting set of 36 candidate genes (Table 2) was analyzed for subsequent qPCR analysis on 6 batches of good (Nos. 1–5, 7) and poor quality eggs (Nos. 8–13).

When adopting the presence-absence approach of transcripts in good vs poor quality eggs against the draft Japanese eel genome, 150 transcripts, of which 85 could be annotated, were identified (Table 3). Three transcripts coded for unknown protein. In Analysis 2, 72 transcripts were selected as target for subsequent qPCR analysis (n=6).

### 3.3 Transcripts differentially expressed in the good and poor quality egg groups

Contig Nos. 107048, 71472, 64061, 112812, 156849, annotated as *card14*, *pdp1*, *gnpat*, *dnajb4* and *fcgbp*, respectively (see Table 2), were the only 5 among 36 contigs from Analysis 1 that displayed significantly lower mRNA levels in poor quality eggs than in good quality eggs ( $P < 0.01$ ) (Fig 3).

From the list of 72 transcripts from Analysis 2, only one transcript (transcript No. 47766, annotating *ttn*; see Table 3) was expressed at a significantly lower level in poor quality eggs as compared to good quality eggs ( $P = 0.0039$ , with false discovery rate  $< 0.3$ ) (Fig 4).

### 3.4 Transcripts contributed to hatchability

Stepwise multiple regression analysis of expression data from genes listed in Tables 2 and 3 was conducted to identify genes that could influence hatchability. In Analysis 1, contig No. 64061 annotated as *gnpat* and contig No. 47139 annotated as *b4galnt1* were positively associated with hatchability and the correlation coefficient ( $R^2$ ) was 0.811 ( $P < 0.001$ ; Table 4). *Gnpat* in particular had a strongly influence on hatchability, reflected in the standardized coefficient (B) of *gnpat* being higher than that of *b4galnt1*. In Analysis 2, hatchability was positively correlated with expression of 3 genes, i.e., transcript Nos. 8852, 40658 and 69150, annotated as *acsl6*, *rtkn* and *trim24*; the correlation coefficient ( $R^2$ ) was 0.921 ( $P < 0.001$ ; Table 4). The strongest predictor of hatching was *acsl6* expression.

The multiple linear regression models to quantify the relationship between hatchability and transcripts are listed as follows:

Analysis 1: Hatchability (%) =  $-74.17 + 87.43$  (*gnpat* mRNA level) +  $70.01$  (*b4galnt1* mRNA level)

Analysis 2: Hatchability (%) =  $-85.94 + 63.94$  (*acsl6* mRNA level) +  $46.57$  (*rtkn* mRNA level) +  $57.08$  (*trim24* mRNA level)

Observed and predicted hatchability values calculated from the above two models are

presented in Fig. 5 and proved to be strongly and positively correlated ( $R = 0.986$ ,  $P < 0.001$ ).

#### 4. DISCUSSION

In the present study, we investigated levels of maternal transcripts in good and poor quality eggs (those producing a viable morula embryo without hatching) from artificially matured Japanese eel using RNA-Seq and qPCR analysis. Accordingly, we aimed to identify maternal transcripts that may be related to development.

##### **Experimental approach**

In this study, two RNA-seq approaches were used in keeping with Martin & Wang (2011); reads were mapped to *de novo* assembled contigs or to the eel draft genome as reference sequence. Notably, the number of contigs obtained from *de novo* assembly was greater than the number of transcripts obtained from reference-based assembly. This suggests that the former generates a number of redundant contigs due to miss-assembly or to incomplete sequencing coverage. Although *de novo* assembly strategies tend to generate a number of redundant contigs due to sequence variations, which affect downstream experiments, *de novo* transcriptome analysis can recover transcripts that are transcribed from missing segments of the draft genome (Martin & Wang, 2011). Indeed, the published eel genome alignment (Henkel et al., 2012) was robust, but about 30% of reads were unmapped to the genome sequence. Thus, we carried out both approaches. The relative levels of each transcript were expressed as RPKM/FPKM in this study. It is later reported that RPKM can be inconsistent among samples (c.f., Wagner et al., 2012). It is further noted that statistics do not take batch effects (c.f., Leek et al., 2010) into consideration and therefore, such effects may be present in our RNA-Seq analyses. Regardless, our eventual qPCR analyses identified several transcripts that were significantly less abundant in poor than in good quality eggs. In order to gain a more detailed understanding, or further reinforce the molecular quantitative signature, of good *versus* poor quality eggs, increasing of the sample sizes and additional bioinformatic analysis of RNA-Seq are needed.

##### **Transcripts differentially expressed in good and poor quality egg groups**

Upon mapping reads to the *de novo* assembled transcriptome, good and poor quality egg

groups clustered together using hierarchical clustering analysis. It is very possible that contig redundancy affected the clustering analysis. Although several approaches for removal of redundant contigs have been proposed (Haznedaroglu et al., 2012; Ono et al., 2015), these were not employed in this study. Instead, we used a reference-based approach to analyse transcriptomes and observed that good and poor quality egg groups clearly separated into two clusters. Reference-based transcriptome analysis clearly proved more effective, that is, the result of clustering shows that the maternal transcript profile significantly differed between good and poor quality eggs. The drivers for differences in egg quality between female broodstock, which were all reared under the same artificial conditions, are unknown. Regardless, this quantitative signature of maternal transcripts in unfertilized eggs from artificially matured Japanese eel appear to be associated with development during the period from morula to hatching.

Our analyses identified a large number of genes that had lower abundance in poor than in good quality eggs by *in silico* screening. Many of these *in silico*-identified differentially expressed genes are likely to reflect developmental competence – indeed, a leading study by Chapman et al. (2014) on striped bass clearly highlights the complex nature of developmental competence and the notion that a single or a few genes governing this trait does not withstand scrutiny. This is also evident from our qPCR-derived gene expression dataset, in which we routinely noted 1–2 outliers among the 12 assayed samples – the outlying sample(s) were inconsistent, i.e., different egg batches served as outlier for different genes, which *i*) made it difficult to detect differences in expression between batches, and which *ii*) reinforced the notion that developmental competence is a complex trait. Nonetheless, the number of genes governing developmental competence may perhaps be reduced to a subset of genes with high predictive value (c.f. Chapman et al., 2014; Sullivan et al., 2015).

Genes that were differentially expressed *in silico* in the present study were initially assayed by qPCR at low replication (n=3), before subjecting a subset to higher replication (n=6). Accordingly, we detected lower levels of 6 maternal transcripts (*dnajb4*, *gnpat*, *card14*, *pdp1*, *fcgbp*, *ttn*) in poor quality eggs as compared to good quality eggs. This result suggests that the 6 maternal transcripts appear to be associated with development during the period from morula to hatching. Although several candidate maternal transcripts related to

developmental competence were identified in some aquaculture species by comparing mRNA levels between good and poor quality eggs/embryos (reviewed in Sullivan et al., 2015), the 6 transcripts represent novel candidate genes.

Dnajb4, dnaJ heat shock protein family (Hsp40) member B4, is involved in interaction with the actin cytoskeleton (Chen et al., 2010). Based on observations on zebrafish maternal mutants, regulation of the actin cytoskeleton is important for progression of epiboly (Holloway et al., 2009). Fcgbp, Fc fragment of IgG-binding protein, was implicated in the epithelial-mesenchymal transition, a cell conversion process crucial for cell movement during gastrulation (Xiong et al., 2014, review in Thiery et al., 2009). Although phenotypic analyses of eel embryos produced from poor quality egg was not conducted, previous reports render it possible that dnajb4 and fcgbp are involved in gastrulation by regulating cytoskeletal function in eel. Card14, caspase recruitment domain family member-14 can activate NF- $\kappa$ B (Bertin et al., 2001). In the zebrafish embryo, blocking NF- $\kappa$ B activity affects mesoderm development, resulting in embryos failing to undergo gastrulation or becoming dorsalized with partial or complete lack of tail formation (Correa et al., 2004). Thus, card14 is likely to be essential for normal development in eel. Pdp1, pyruvate dehydrogenase phosphatase catalytic subunit 1, is a component of the pyruvate dehydrogenase complex which plays a crucial role in energy metabolism (Wieland, 1983). Oxygen consumption increases as gastrulation progress in fish (Boulekbache, 1981), suggesting that insufficient *pdp1* mRNA accumulation may negatively impact gastrulation. In knockout mice, it was reported that Gnpat, glyceronephosphate O-acyltransferase, plays a role in development of eye and nervous system (Rodemer et al., 2003). Observations on zebrafish have suggested that some *ttn* exons are required for sarcomere assembly in the heart and somites (Seeley et al., 2007). How these transcripts mechanistically affect development during the period from morula to hatching remains a matter of speculation – regardless, our results suggest that sufficient amounts of these transcripts in unfertilized eggs are required to ensure hatching success in eel.

### **Transcripts contributing to hatchability**

Using multiple regression analysis, 5 transcripts (*gnpat*, *b4galnt1*, *acsl6*, *rtkn*, *trim24*) positively and significantly correlated with hatchability. This result suggests that two (*gnpat*

and *b4galnt1*) or three transcripts (*acsl6*, *rtkn*, *trim24*) co-operate to facilitate normal development and could contribute to hatchability.

Accordingly, *gnpat* was again highlighted for its association with hatchability. So too, was *B4galnt1*, which encodes  $\beta$ -1,4-N-acetyl-galactosaminyltransferase 1, an enzyme involved in the biosynthesis of glycosphingolipid essential for normal neural development and function in zebrafish (Boccuto et al., 2014). A role for *acsl6* in patterning is also possible, given the recent report that demonstrated that *acsl4a*, a protein closely related to *acsl6*, regulated dorsoventral patterning in the zebrafish embryo (Miyares et al., 2013). It is reported that several maternal products regulate dorsoventral patterning of zebrafish and loss of this function brings about abnormal phenotype, characterized by failed gastrulation, ventralization, and lacking anterior structures and notochord (Reim & Brand, 2006; Nojima et al., 2010; Ge et al., 2014). Lastly, Rhotekin (*Rtn*) interacts with active GTP-bound Rho proteins which regulate several cellular processes, such as cytokinesis, differentiation, cell polarity and cell movement. In zebrafish, Rho mediates cleavage furrow protein assembly during cytokinesis and cellular migration during gastrulation (Lai et al., 2005). Thus, it is likely that *rtkn* is an upstream regulator that plays an essential role in development of eel. Finally, *Trim24*, transcription intermediary factor (TIF) 1  $\alpha$ , modulates the first wave of transcription during early ZGA in the mouse (Torres-Padilla & Zernicka-Goetz, 2006). The ZGA is a prerequisite for gastrulation in zebrafish (Kane & Kimmel, 1993). A mouse study also has demonstrated that the *Trim24* transcript is notably abundant in the egg and embryo before ZGA. We pose that our findings - hatchability being positively correlated with mRNA levels of *trim24* - is reasonable. Indeed, it is evident that sufficiently high levels of maternal *trim24* mRNA in eggs are important for ZGA and subsequent development in fish.

Of note, our analyses showed that hatchability is adequately predicted by the levels of 5 maternal transcripts that accumulated in unfertilized eggs of artificially matured Japanese eel. These maternal transcripts, thus, are likely to be needed for development and can be used as markers of egg quality – the molecular mechanisms detailing *how* these transcripts contribute to hatchability remain to be elucidated in future studies.

Fish egg quality can be readily defined as the ability of an egg to be fertilized, to hatch and to then develop into a vital larva. However, when it comes to molecular signature (e.g. transcript abundance) or biochemical composition, egg quality is much more difficult to define

or describe. The loss of egg quality remains an ongoing problem in the artificial seedling production of aquaculture species, including Japanese eel. In aquaculture, many factors, such as environment, hormone, inappropriate timing of inducing ovulation etc, can negatively influence egg quality (Brook et al., 1997; Adachi, 2000; Bobe & Labbé, 2010). However, the complex interaction between multiple factors make it very difficult to identify the cause of compromised egg quality in many cases. Moreover, the molecular mechanisms leading to loss of egg quality remain unclear. Here, we have reported on a suite of transcripts implicated in hatchability that may prove useful as predictors of egg quality in eel. Correct prediction of egg quality in the unfertilized egg may help advance solutions to the issue of loss of egg quality in aquaculture species.

#### **Candidate maternal transcripts associated with development**

We identified 10 candidate genes whose transcripts are stored as maternal investment in the egg and whose quantitative signature may reflect developmental competence in artificially matured Japanese eel. Most of these candidate genes have not been previously studied in the context of maternal control of development. Our findings reinforce the importance of *trim24*, which was previously characterized as a key maternal transcript for development in the mouse (Torres-Padilla & Zernicka-Goetz, 2006); our results further reinforce that our experimental approach is effective in exposing maternal transcripts related to development. Table 5 shows the presumed function of these genes during embryogenesis, which were deemed to mainly concern ZGA, cell differentiation, patterning, gastrulation, and development of tissues. We propose that insufficient or reduced levels of 10 maternal transcripts in the egg may negatively affect these aspects of development, leading to hatching failure in artificially matured Japanese eel. In vertebrates, the timing of translation and the function of all identified transcripts, except *trim24*, during development have remained unclear. Further detail on the function of these transcripts will advance our understanding of maternal control of development in vertebrates and expose the molecular mechanisms responsible for reducing egg quality in species important for aquaculture.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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TABLE

**Table 1.** Quality, estimated by fertilization and hatching rates, and purpose of egg samples from artificially matured Japanese eel.

| No.            | Fertility (%) | Hatchability (%) | Egg quality | Purpose    |
|----------------|---------------|------------------|-------------|------------|
| 1              | 98.9          | 93.5             | good        | NGS & qPCR |
| 2              | 97.5          | 93.3             | good        | NGS & qPCR |
| 3              | 100           | 87.8             | good        | qPCR       |
| 4              | 91.9          | 82.1             | good        | qPCR       |
| 5 <sup>†</sup> | 85 <          | 85.2             | good        | NGS & qPCR |
| 6              | 91.4          | 81.5             | good        | qPCR       |
| 7              | 98.4          | 92.9             | good        | qPCR       |
| 8              | 95.2          | 20               | poor        | qPCR       |
| 9              | 86            | 18               | poor        | qPCR       |
| 10             | 90.5          | 15.5             | poor        | NGS & qPCR |
| 11             | 85.8          | 10.1             | poor        | qPCR       |
| 12             | 87.4          | 0                | poor        | NGS & qPCR |
| 13             | 86.7          | 0                | poor        | NGS & qPCR |

<sup>†</sup>Sample preserved RNA Later

681 **Table 2.** List of 36 transcripts selected by *in silico* screening (Analysis 1) and qPCR screening.

| Contig No. | Length | Description (BLASTx)                                      | E-value | Biological process                          | Fold change | P value |
|------------|--------|-----------------------------------------------------------|---------|---------------------------------------------|-------------|---------|
| 346537     | 265    | lamin-b receptor                                          | 5.7E-12 | cell migration involved in gastrulation     | 0.4         | 0       |
| 107048     | 1535   | caspase recruitment domain-containing protein 14          | 1.9E-93 | regulation of apoptotic process             | 0.25        | 0.002   |
| 47139      | 1843   | beta- n-acetylgalactosaminyltransferase 1-like            | 0       | lipid glycosylation                         | 0.19        | 0.002   |
| 81492      | 139    | protein fam57b-like                                       | 5.2E-15 | central nervous system development          | 0.42        | 0.002   |
| 186064     | 279    | solute carrier family 40 member 1                         | 3.2E-11 | iron ion transport                          | 0.13        | 0.002   |
| 180644     | 351    | novel protein human titin                                 | 6.6E-12 | skeletal muscle tissue development          | 0.39        | 0.004   |
| 113183     | 598    | testicular haploid expressed gene                         | 1.1E-41 | spermatogenesis                             | 0.15        | 0.009   |
| 35937      | 141    | pantothenate kinase 3                                     | 1.9E-15 | coenzyme A biosynthetic process             | 0.38        | 0.01    |
| 8543       | 174    | spermidine synthase-like                                  | 7.5E-28 | amine metabolic process                     | 0.39        | 0.01    |
| 123154     | 440    | von willebrand factor d and egf domain-containing protein | 2.5E-62 | -                                           | 0.23        | 0.01    |
| 44548      | 432    | calponin-1                                                | 7.7E-25 | actomyosin structure organization           | 0.22        | 0.013   |
| 36581      | 368    | gtpase imap family member 7-like                          | 1.8E-29 | -                                           | 0.11        | 0.014   |
| 25493      | 5433   | otoancorin- partial                                       | 0       | cell-matrix adhesion                        | 0.13        | 0.018   |
| 71472      | 1502   | pyruvate dehydrogenase                                    | 3.6E-06 | protein dephosphorylation                   | 0.18        | 0.024   |
| 187717     | 272    | roundabout homolog 3 isoform x3                           | 8.6E-43 | axon guidance                               | 0.44        | 0.024   |
| 64061      | 187    | dihydroxyacetone phosphate acyltransferase-like           | 1.4E-14 | cellular lipid metabolic process            | 0.45        | 0.027   |
| 185635     | 215    | astrotactin-1-like isoform x1                             | 2.4E-43 | neuron cell-cell adhesion, neuron migration | 0.52        | 0.027   |

|        |      |                                                                      |          |                                                                 |      |       |
|--------|------|----------------------------------------------------------------------|----------|-----------------------------------------------------------------|------|-------|
| 61146  | 306  | bromodomain and wd repeat-containing protein 3-like isoform x1       | 3.0E-07  | cytoskeleton organization, regulation of cell shape             | 0.06 | 0.03  |
| 90079  | 530  | matrix metalloproteinase-20                                          | 7.9E-08  | proteolysis                                                     | 0.19 | 0.031 |
| 98904  | 956  | sodium hydrogen exchanger 5-like                                     | 1.7E-155 | regulation of intracellular pH                                  | 0.43 | 0.032 |
| 32498  | 249  | mesothelin-like partial                                              | 4.1E-13  | cell-matrix adhesion                                            | 0.12 | 0.034 |
| 112812 | 106  | dnaj homolog subfamily b member 4-like                               | 5.6E-13  | protein folding                                                 | 0.33 | 0.035 |
| 7830   | 4035 | parathyroid hormone parathyroid hormone-related peptide receptor     | 0        | dorsal aorta development                                        | 0.37 | 0.041 |
| 131862 | 487  | scan domain-containing protein 3-like                                | 2.9E-06  | regulation of transcription, DNA-templated                      | 0.64 | 0.041 |
| 69803  | 339  | perilipin-2 isoform x3                                               | 4.3E-31  | long-chain fatty acid transport                                 | 0.55 | 0.044 |
| 105031 | 344  | cub and sushi domain-containing protein 1-like                       | 4.9E-50  | -                                                               | 0.24 | 0.049 |
| 54188  | 1235 | kelch-like protein 38-like                                           | 1.1E-151 | protein ubiquitination                                          | 0.18 | 0.059 |
| 228141 | 265  | low quality protein: titin                                           | 1.0E-45  | skeletal muscle tissue development                              | 0.32 | 0.095 |
| 184082 | 254  | ras rap gtpase-activating isoform x1                                 | 1.4E-17  | negative regulation of Ras protein signal transduction          | 0.31 | 0.113 |
| 216371 | 209  | slit homolog 3                                                       | 5.8E-33  | central nervous system projection neuron axonogenesis           | 0.21 | 0.115 |
| 51153  | 202  | tumor necrosis factor receptor superfamily member 14-like isoform x2 | 1.8E-14  | regulation of apoptotic process, response to lipopolysaccharide | 1.78 | 0.116 |
| 218733 | 270  | alpha-catulin                                                        | 1.4E-47  | cell adhesion                                                   | 0.3  | 0.116 |
| 40760  | 268  | matrix metalloproteinase-19-like                                     | 1.8E-30  | proteolysis                                                     | 0.36 | 0.133 |
| 223030 | 480  | titin-like                                                           | 1.7E-62  | skeletal muscle tissue development                              | 0.31 | 0.138 |
| 156849 | 231  | c-binding protein                                                    | 2.5E-24  | -                                                               | 0.12 | 0.156 |

|        |     |                                                            |         |               |      |       |
|--------|-----|------------------------------------------------------------|---------|---------------|------|-------|
| 177733 | 456 | junctional protein associated with coronary artery disease | 1.0E-37 | cell adhesion | 0.17 | 0.168 |
|--------|-----|------------------------------------------------------------|---------|---------------|------|-------|

682

683 **Table 3.** List of 85 transcripts selected by *in silico* screening (Analysis 2).

| Transcript No. | Length (b) | genome locus                              | Description (BLASTx)                                                               | E-value  | Biological process                                                             |
|----------------|------------|-------------------------------------------|------------------------------------------------------------------------------------|----------|--------------------------------------------------------------------------------|
| 47766          | 230        | gi 546466767 gb KI307198.1 :40381-42518   | low quality protein: titin                                                         | 3.3.E-34 | skeletal muscle tissue development                                             |
| 32885          | 1587       | gi 546463338 gb KI309797.1 :9705-14172    | solute carrier family 12 member 4-like isoform x1                                  | 0        | chemical synaptic transmission                                                 |
| 8852           | 287        | gi 546447610 gb KI322079.1 :6642-8285     | long-chain-fatty-acid-- ligase 6-like                                              | 5.8.E-55 | long-chain fatty acid metabolic process                                        |
| 51979          | 723        | gi 546467663 gb KI306639.1 :645-4603      | spermatogenesis-associated protein 13 isoform x2                                   | 4.8.E-78 | regulation of Rho protein signal transduction                                  |
| 74897          | 583        | gi 546470452 gb KI304769.1 :145862-147457 | von willebrand factor a domain-containing protein 3a                               | 1.0.E-39 | -                                                                              |
| 14109          | 198        | gi 546453374 gb KI317089.1 :12877-13507   | receptor-type tyrosine-protein phosphatase zeta isoform x3                         | 8.5.E-25 | dephosphorylation                                                              |
| 19392          | 1601       | gi 546457140 gb KI314184.1 :7157-19322    | ras gtpase-activating protein 1-like                                               | 0        | blood vessel development                                                       |
| 35139          | 191        | gi 546464182 gb KI309311.1 :321-5864      | calcium uniporter mitochondrial-like isoform x1                                    | 7.2.E-38 | regulation of heart contraction, convergent extension involved in gastrulation |
| 40658          | 245        | gi 546465458 gb KI308266.1 :114072-116308 | rhotekin-like isoform x2                                                           | 2.9.E-35 | signal transduction                                                            |
| 52511          | 297        | gi 546467781 gb KI306555.1 :226-29854     | titin isoform x5                                                                   | 1.0.E-41 | skeletal muscle tissue development                                             |
| 70699          | 379        | gi 546470120 gb KI304996.1 :39143-44808   | epidermal growth factor receptor kinase substrate 8-like protein 2-like isoform x2 | 9.7.E-61 | positive regulation of ruffle assembly                                         |
| 822            | 145        | gi 546401872 gb KI360409.1 :0-532         | tight junction protein zo-1-like isoform x2                                        | 1.9.E-22 | pigmentation                                                                   |
| 74065          | 338        | gi 546470376 gb KI304810.1 :184478-188419 | cyclin-dependent kinase-like 1                                                     | 1.4.E-75 | protein phosphorylation                                                        |
| 11598          | 1087       | gi 546451022 gb KI319089.1 :584-14381     | plexin-a1-like isoform x2                                                          | 0        | signal transduction                                                            |
| 19102          | 2436       | gi 546456976 gb KI314297.1 :22368-32455   | gem-associated protein 5-like                                                      | 0        | spliceosomal snRNP assembly                                                    |

|       |      |                                           |                                                                   |           |                                                                               |
|-------|------|-------------------------------------------|-------------------------------------------------------------------|-----------|-------------------------------------------------------------------------------|
| 32022 | 2485 | gi 546463143 gb KI309992.1 :7634-19903    | peptidyl-prolyl cis-trans isomerase fkbp10-like                   | 0         | chaperone-mediated protein folding                                            |
| 48754 | 941  | gi 546467007 gb KI307054.1 :94-9086       | protein hook homolog 2-like isoform x1                            | 3.9.E-157 | endosome to lysosome transport                                                |
| 19151 | 454  | gi 546456996 gb KI314284.1 :9679-18283    | hepatoma-derived growth factor                                    | 7.3.E-61  | negative regulation of transcription from RNA polymerase II promoter          |
| 31311 | 3671 | gi 546462934 gb KI310169.1 :44205-53406   | telomere length regulation protein tel2 homolog                   | 0         | protein stabilization, regulation of TOR signaling                            |
| 42044 | 1727 | gi 546465682 gb KI308042.1 :2024-42167    | focal adhesion kinase 1 isoform x1                                | 0         | lens fiber cell morphogenesis                                                 |
|       | 735  | gi 546399759 gb KI362139.1 :15-922        | phosphatidylinositol 4-kinase alpha-like isoform x1               | 7.5.E-39  | phosphatidylinositol-mediated signaling                                       |
| 21621 | 1605 | gi 546458616 gb KI313236.1 :0-12784       | zinc finger protein ozf-like                                      | 2.2.E-98  | regulation of transcription, DNA-templated                                    |
| 23638 | 312  | gi 546459803 gb KI312454.1 :8718-9436     | nesprin-1 isoform x4                                              | 9.4.E-32  | cytoskeletal anchoring at nuclear membrane                                    |
| 37240 | 189  | gi 546464742 gb KI308900.1 :25879-27085   | alkaline ceramidase 3                                             | 3.4.E-30  | ceramide metabolic process                                                    |
| 67483 | 1322 | gi 546469875 gb KI305212.1 :9404-47383    | leukocyte elastase inhibitor-like isoform x2                      | 2.8.E-158 | neutrophil degranulation                                                      |
|       |      |                                           |                                                                   |           | epiboly involved in gastrulation with mouth forming second, single            |
| 1067  | 163  | gi 546406455 gb KI356682.1 :306-469       | PREDICTED: cadherin-1                                             | 1.1.E-04  | organismal cell-cell adhesion,                                                |
|       |      |                                           |                                                                   |           | neuron migration                                                              |
| 3199  | 113  | gi 546429263 gb KI337706.1 :139-473       | ubiquitin carboxyl-terminal hydrolase 34- partial                 | 2.2.E-16  | positive regulation of canonical Wnt signaling pathway                        |
| 10477 | 861  | gi 546449739 gb KI320182.1 :7419-17054    | cytosolic carboxypeptidase 1-like                                 | 1.8.E-100 | chordate embryonic development, T cell differentiation in thymus              |
| 11307 | 281  | gi 546450697 gb KI319373.1 :37205-38100   | protein inscuteable homolog isoform x1                            | 8.5.E-15  | nervous system development                                                    |
| 21147 | 2058 | gi 546458320 gb KI313396.1 :2066-11024    | ap-1 complex subunit gamma-1-like                                 | 5.6.E-90  | pectoral fin development                                                      |
| 22694 | 604  | gi 546459307 gb KI312793.1 :30352-35119   | rcc1 and btb domain-containing protein 1                          | 6.1.E-131 | retina vasculature morphogenesis in camera-type eye, blood vessel development |
| 29117 | 290  | gi 546462094 gb KI310726.1 :24176-41307   | pre-mrna 3 end processing protein wdr33 isoform x8                | 1.5.E-61  | mRNA cleavage                                                                 |
| 30567 | 3871 | gi 546462656 gb KI310344.1 :44822-56454   | protein o-linked-mannose beta- -n-acetylglucosaminyltransferase 1 | 0         | protein glycosylation                                                         |
| 64640 | 552  | gi 546469589 gb KI305405.1 :106647-198499 | glycine receptor subunit alpha-4- partial                         | 7.1.E-42  | neuropeptide signaling pathway                                                |

|       |      |                                         |                                                                     |          |                                                               |
|-------|------|-----------------------------------------|---------------------------------------------------------------------|----------|---------------------------------------------------------------|
| 79333 | 1373 | gi 546470891 gb KI304576.1 :60912-77508 | bcl2 adenovirus e1b 19 kda protein-interacting protein 2 isoform x1 | 0        | apoptotic process                                             |
| 20857 | 190  | gi 546458113 gb KI313529.1 :11706-14316 | creb-binding partial                                                | 1.5.E-33 | histone acetylation                                           |
|       |      |                                         |                                                                     |          | cartilage development involved in endochondral bone           |
| 5428  | 292  | gi 546440436 gb KI328487.1 :3290-7399   | protein jagged-1b-like                                              | 2.3.E-53 | morphogenesis, liver development, pancreas development,       |
|       |      |                                         |                                                                     |          | thyroid gland development, face morphogenesis                 |
| 10737 | 164  | gi 546450072 gb KI319918.1 :5654-5986   | protein fam92b isoform x1                                           | 2.0.E-14 | -                                                             |
| 14719 | 812  | gi 546453904 gb KI316654.1 :4876-27344  | gtpase imap family member 7-like                                    | 1.1.E-75 | -                                                             |
| 22105 | 421  | gi 546458974 gb KI313006.1 :31151-36012 | calcium calmodulin-dependent protein kinase type 1d-like            | 2.5.E-76 | chordate embryonic development                                |
| 31852 | 444  | gi 546463110 gb KI310025.1 :68937-81387 | nuclear pore complex protein nup93- partial                         | 2.4.E-39 | nuclear pore complex assembly                                 |
| 54956 | 1839 | gi 546468211 gb KI306286.1 :28060-50504 | integrator complex subunit 8 isoform x1                             | 0        | snRNA processing                                              |
| 58632 | 357  | gi 546468847 gb KI305914.1 :11007-20940 | tight junction protein zo-3 isoform x1                              | 5.2.E-42 | multicellular organismal homeostasis, water homeostasis       |
| 69150 | 1440 | gi 546469987 gb KI305100.1 :64856-91293 | transcription intermediary factor 1-alpha-like isoform x1           | 0        | regulation of apoptotic process                               |
| 1479  | 244  | gi 546412573 gb KI351313.1 :401-962     | haus augmin-like complex subunit 8                                  | 5.9.E-10 | spindle assembly, centrosome organization                     |
| 14712 | 938  | gi 546453904 gb KI316654.1 :4876-27344  | gtpase imap family member 7-like                                    | 1.8.E-61 | -                                                             |
| 23549 | 194  | gi 546459771 gb KI312481.1 :4298-5306   | serine threonine-protein kinase mtor                                | 9.6.E-27 | regulation of myelination, skin development                   |
| 728   | 141  | gi 546399452 gb KI362415.1 :774-915     | zinc finger swim domain-containing protein 7-like                   | 8.6.E-04 | double-strand break repair via homologous recombination       |
| 29131 | 739  | gi 546462098 gb KI310722.1 :91-35794    | peptidyl-glycine alpha-amidating monooxygenase-like                 | 2.6.E-76 | oxidation-reduction process, peptide metabolic process        |
| 36664 | 356  | gi 546464594 gb KI309018.1 :61825-82426 | slit-robo rho gtpase-activating protein 1-like                      | 2.1.E-58 | negative regulation of cell migration                         |
| 39183 | 548  | gi 546465173 gb KI308551.1 :34818-37937 | cytochrome c oxidase subunit mitochondrial-like                     | 8.1.E-39 | response to cadmium ion                                       |
| 59652 | 543  | gi 546468959 gb KI305814.1 :13123-25297 | yeats domain-containing protein 2 isoform x1                        | 6.0.E-42 | regulation of transcription, DNA-templated                    |
| 60426 | 2211 | gi 546469035 gb KI305763.1 :74515-88014 | spartin-like isoform x1                                             | 0        | cell division, regulation of mitochondrial membrane potential |
| 2414  | 229  | gi 546423577 gb KI342752.1 :43-1073     | rho guanine nucleotide exchange factor 12-like isoform x3           | 9.9.E-33 | G-protein coupled receptor signaling pathway                  |

|       |      |                                         |                                                           |           |                                                                                                   |
|-------|------|-----------------------------------------|-----------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------------|
| 12712 | 383  | gi 546452144 gb KI318107.1 :1691-14650  | high mobility group protein hmgi-c                        | 1.1.E-13  | base-excision repair                                                                              |
| 43730 | 2138 | gi 546465958 gb KI307770.1 :4503-12983  | non-receptor tyrosine-protein kinase tyk2 isoform x2      | 0         | cell differentiation, regulation of cell proliferation, cell migration,<br>innate immune response |
| 4854  | 543  | gi 546438664 gb KI330226.1 :82-3897     | mitogen-activated protein kinase 8                        | 4.0.E-129 | phosphorylation                                                                                   |
| 16140 | 1182 | gi 546454873 gb KI315840.1 :2469-15234  | inositol -trisphosphate receptor type 2                   | 3.0.E-67  | release of sequestered calcium ion into cytosol                                                   |
| 17593 | 1341 | gi 546455882 gb KI315080.1 :2702-12533  | protein mon2 homolog isoform x4                           | 0         | Golgi to endosome transport                                                                       |
| 35572 | 317  | gi 546464323 gb KI309213.1 :6543-21515  | thioredoxin domain-containing protein 16-like             | 4.9.E-30  | cell redox homeostasis                                                                            |
| 41542 | 1374 | gi 546465596 gb KI308128.1 :300-8041    | tubby-related protein 4- partial                          | 0         | protein localization to cilium                                                                    |
| 9465  | 1258 | gi 546448481 gb KI321307.1 :1943-4142   | peflin                                                    | 1.1.E-16  | response to calcium ion                                                                           |
| 14289 | 860  | gi 546453504 gb KI316980.1 :4007-10361  | vacuolar protein sorting-associated protein 4a            | 0         | endosomal transport                                                                               |
| 20462 | 151  | gi 546457878 gb KI313690.1 :22752-25883 | unconventional myosin-xviii                               | 5.1.E-16  | cell migration, actomyosin structure organization, Golgi<br>organization                          |
| 21232 | 307  | gi 546458366 gb KI313372.1 :11188-19175 | sjogren syndrome nuclear autoantigen 1 homolog            | 6.7.E-04  | G2/M transition of mitotic cell cycle, ciliary basal body docking                                 |
| 55810 | 1030 | gi 546468390 gb KI306202.1 :38233-54470 | pc-esterase domain-containing protein 1a-like             | 3.1.E-106 | -<br>activation of cysteine-type endopeptidase activity involved in                               |
| 79079 | 1304 | gi 546470877 gb KI304586.1 :17093-36390 | mitochondrial ubiquitin ligase activator of nfkb 1-a-like | 5.7.E-81  | apoptotic process, mitochondrial fission, mitochondrion<br>localization                           |
| 769   | 104  | gi 546400551 gb KI361484.1 :536-940     | ornithine decarboxylase                                   | 6.0.E-13  | eye photoreceptor cell development                                                                |
| 18562 | 302  | gi 546456596 gb KI314587.1 :18319-18621 | type i inositol -bisphosphate 4-phosphatase- partial      | 1.5.E-06  | signal transduction                                                                               |
| 39088 | 1949 | gi 546465157 gb KI308567.1 :77779-95914 | serine--trna mitochondrial-like                           | 2.4.E-179 | seryl-tRNA aminoacylation                                                                         |
| 54702 | 1899 | gi 546468173 gb KI306309.1 :13997-23346 | alpha- and gamma-adaptin-binding protein p34-like         | 2.2.E-160 | small GTPase mediated signal transduction                                                         |
| 54769 | 655  | gi 546468183 gb KI306303.1 :76568-87792 | terf1-interacting nuclear factor 2 isoform x1             | 6.0.E-29  | negative regulation of epithelial cell proliferation                                              |
| 1170  | 215  | gi 546408290 gb KI355226.1 :131-658     | casein kinase ii subunit beta                             | 9.2.E-30  | signal transduction                                                                               |

|       |      |                                           |                                                                            |           |                                                                                                   |
|-------|------|-------------------------------------------|----------------------------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------------|
| 2419  | 265  | gi 546423662 gb KI342682.1 :1283-1998     | protein fam184a-like isoform x1                                            | 2.9.E-21  | -                                                                                                 |
| 5279  | 207  | gi 546439970 gb KI328920.1 :65-272        | dual specificity protein phosphatase cdc14a-like                           | 6.6.E-38  | cilium assembly                                                                                   |
| 7184  | 830  | gi 546444665 gb KI324638.1 :4579-9883     | uncharacterized kda                                                        | 2.0.E-05  | -                                                                                                 |
| 16685 | 2606 | gi 546455280 gb KI315514.1 :2361-16743    | rap guanine nucleotide exchange factor 2 isoform x7                        | 0         | signal transduction                                                                               |
| 35444 | 491  | gi 546464291 gb KI309242.1 :14932-15423   | PREDICTED: uncharacterized protein LOC102076953, partial                   | 1.4.E-05  | -                                                                                                 |
| 43733 | 2099 | gi 546465958 gb KI307770.1 :4503-12983    | non-receptor tyrosine-protein kinase tyk2 isoform x2                       | 0         | cell differentiation, regulation of cell proliferation, cell migration,<br>innate immune response |
| 56001 | 313  | gi 546468440 gb KI306181.1 :75455-75768   | dual specificity protein phosphatase 13 isoform a-like                     | 5.6.E-25  | protein dephosphorylation                                                                         |
| 71876 | 1128 | gi 546470215 gb KI304925.1 :766-49335     | arf-gap with ank repeat and ph domain-containing protein 3-like isoform x2 | 0         | small GTPase mediated signal transduction                                                         |
| 63840 | 203  | gi 546469508 gb KI305473.1 :136004-146252 | PREDICTED: uncharacterized protein LOC104934648                            | 4.4.E-12  | -                                                                                                 |
| 67268 | 990  | gi 546469861 gb KI305226.1 :15073-50557   | anoctamin-9-like                                                           | 1.3.E-150 | chloride transport                                                                                |
| 79332 | 3866 | gi 546470891 gb KI304576.1 :29882-59308   | autophagy-related protein 13 isoform x3                                    | 0         | autophagosome assembly                                                                            |
| 82757 | 1870 | gi 546471130 gb KI304459.1 :354247-368538 | immunoglobulin superfamily member 21-like isoform x2                       | 2.5.E-108 | -                                                                                                 |

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686 **Table 4.** Stepwise multiple regression analyses to identify factors influencing hatchability.

| Analysis | Independent variable |                                                           | Unstandardized coefficients | Standardized coefficients | Validity of the regression model |          |
|----------|----------------------|-----------------------------------------------------------|-----------------------------|---------------------------|----------------------------------|----------|
|          | No.                  | Description (BLASTx)                                      | B                           | $\beta$                   | $R^2$                            | F        |
| 1        | 64061                | dihydroxyacetone phosphate acyltransferase-like           | 87.43                       | 0.808***                  | 0.811                            | 24.65*** |
|          |                      | beta- n-acetyl                                            |                             |                           |                                  |          |
|          | 47139                | galactosaminyltransferase 1-like                          | 70.01                       | 0.412*                    |                                  |          |
| 2        | 8852                 | long-chain-fatty-acid-- ligase 6-like                     | 63.94                       | 0.627***                  | 0.921                            | 43.64*** |
|          | 40658                | rotenone-like isoform x2                                  | 46.57                       | 0.542***                  |                                  |          |
|          | 69150                | transcription intermediary factor 1-alpha-like isoform x1 | 57.08                       | 0.364**                   |                                  |          |

687 Data marked with asterisk were statistically different (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

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**Table 5.** List of candidate maternal transcripts related to development in Japanese eel and presumed gene function during embryogenesis.

| Characteristic                                               | Symbol <sup>†</sup> | Biological process                      | Presumed gene function during embryogenesis    |
|--------------------------------------------------------------|---------------------|-----------------------------------------|------------------------------------------------|
| Differentially expressed transcripts (PQ < GQ <sup>‡</sup> ) | <i>dnajb4</i>       | protein folding                         | Gastrulation                                   |
|                                                              | <i>gnpat</i>        | cellular lipid metabolic process        | Eye development, Development of nervous system |
|                                                              | <i>card14</i>       | regulation of apoptotic process         | Cell differentiation                           |
|                                                              | <i>pdp1</i>         | protein dephosphorylation               | Energy metabolism                              |
|                                                              | <i>fcgbp</i>        | -                                       | Gastrulation                                   |
|                                                              | <i>ttn</i>          | skeletal muscle tissue development      | Heart development, Somite development          |
| Transcripts contributed to hatchability                      | <i>gnpat</i>        | cellular lipid metabolic process        | Eye development, Development of nervous system |
|                                                              | <i>b4galnt1</i>     | lipid glycosylation                     | Development of nervous system                  |
|                                                              | <i>acs16</i>        | long-chain fatty acid metabolic process | Patterning                                     |
|                                                              | <i>rtkn</i>         | signal transduction                     | Cytokinesis, Gastrulation                      |
|                                                              | <i>trim24</i>       | regulation of apoptotic process         | ZGA                                            |

<sup>†</sup>For all genes, gene symbols identical to those in humans were given when possible.

<sup>‡</sup>GQ, good quality egg, PQ, poor quality egg.

FIGURE LEGEND

**Figure 1.** Work flow. Two approaches, i.e., *de novo* assembly (Analysis 1) and reference-based assembly (Analysis 2), were performed in keeping with Martin & Wang (2011). GQ, good quality egg, PQ, poor quality egg.

**Figure 2.** Dendrogram derived from hierarchical cluster analysis of RPKM (a) or FPKM values (b) of all transcripts in three batches of good quality eggs (No.1, 2, 5) and three of poor quality eggs (No.10, 12, 13). The vertical scale represents the distance between clusters.

**Figure 3.** Relative levels of transcripts selected by Analysis 1 screening in good quality and poor quality eggs. Open columns, good quality eggs (No. 1, 2, 3, 4, 5, 7); solid columns, poor quality eggs (No. 8, 9, 10, 11, 12, 13). Data marked with asterisk were statistically different ( $P < 0.01$ ).

**Figure 4.** Relative levels of the transcripts selected by Analysis 2 screening in good quality and poor quality eggs. Open columns, good quality eggs (No. 1, 2, 3, 4, 6, 7); solid columns, poor quality eggs (No. 8, 9, 10, 11, 12, 13). Data marked with asterisk were statistically different ( $P < 0.01$ ).

**Figure 5.** Relationship between predicted and observed hatchability. The predicted hatchability was estimated using Analyses 1 and 2 equations of multiple linear regression.

LEGENDS FOR SUPPLEMENTARY TABLES

**Table S1.** Primer sequence (Analysis 1)

**Table S2.** Primer sequence (Analysis 2)

**Table S3.** Summary of sequencing result

**Table S4.** List of 148 transcripts selected by *in silico* screening (Analysis 1) and results of qPCR analyses

**Table S5.** List of 97 transcripts selected by *in silico* screening (Analysis 1) and results of qPCR analyses †RPKM values among the three batches of good eggs.

Fig. 1

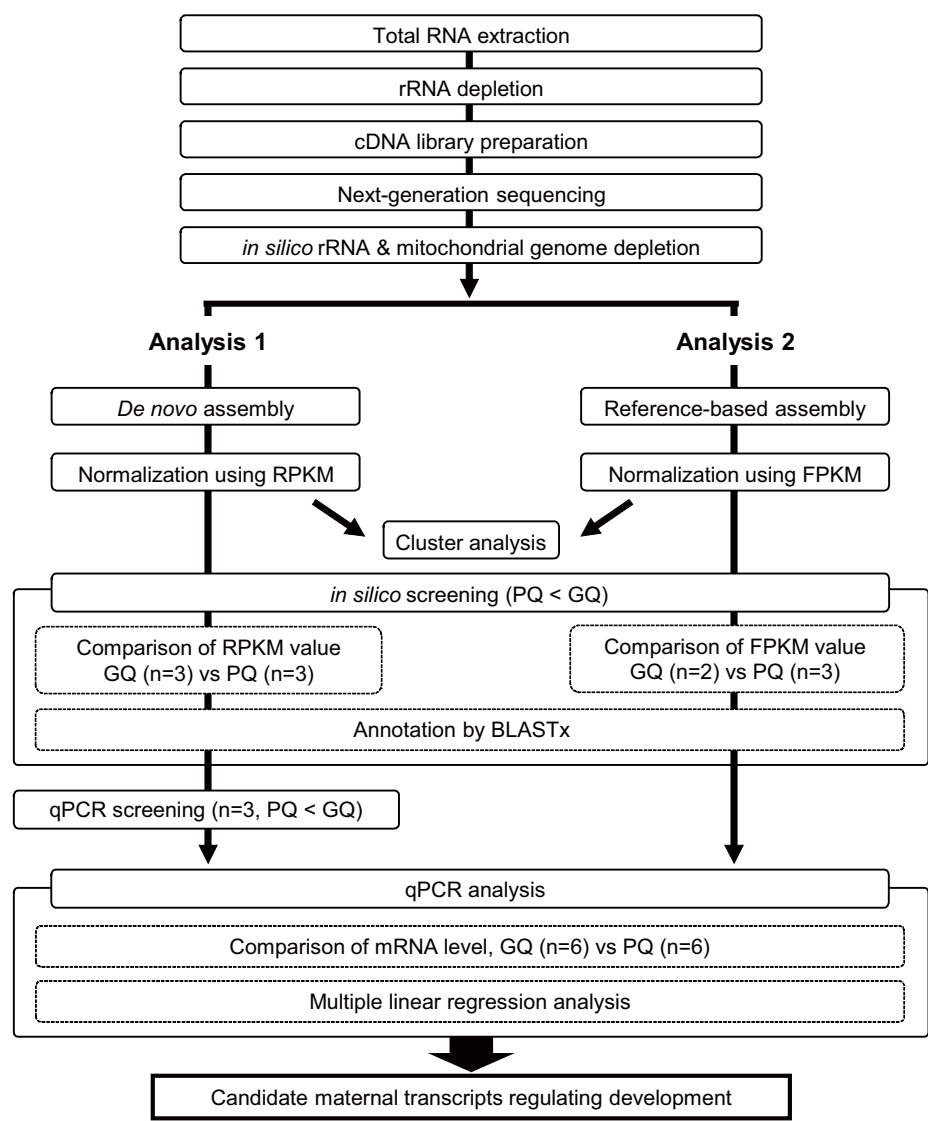


Fig. 2

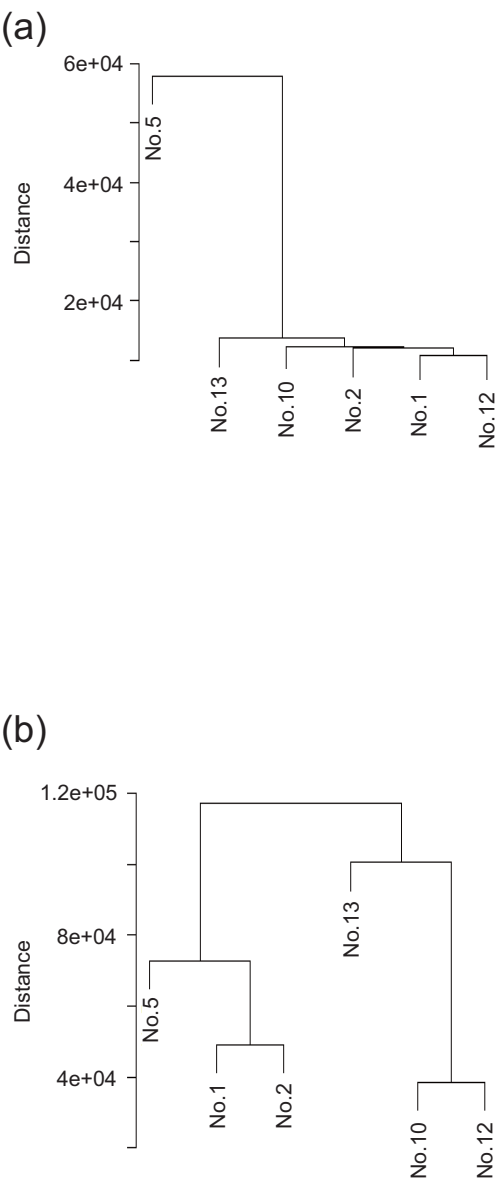
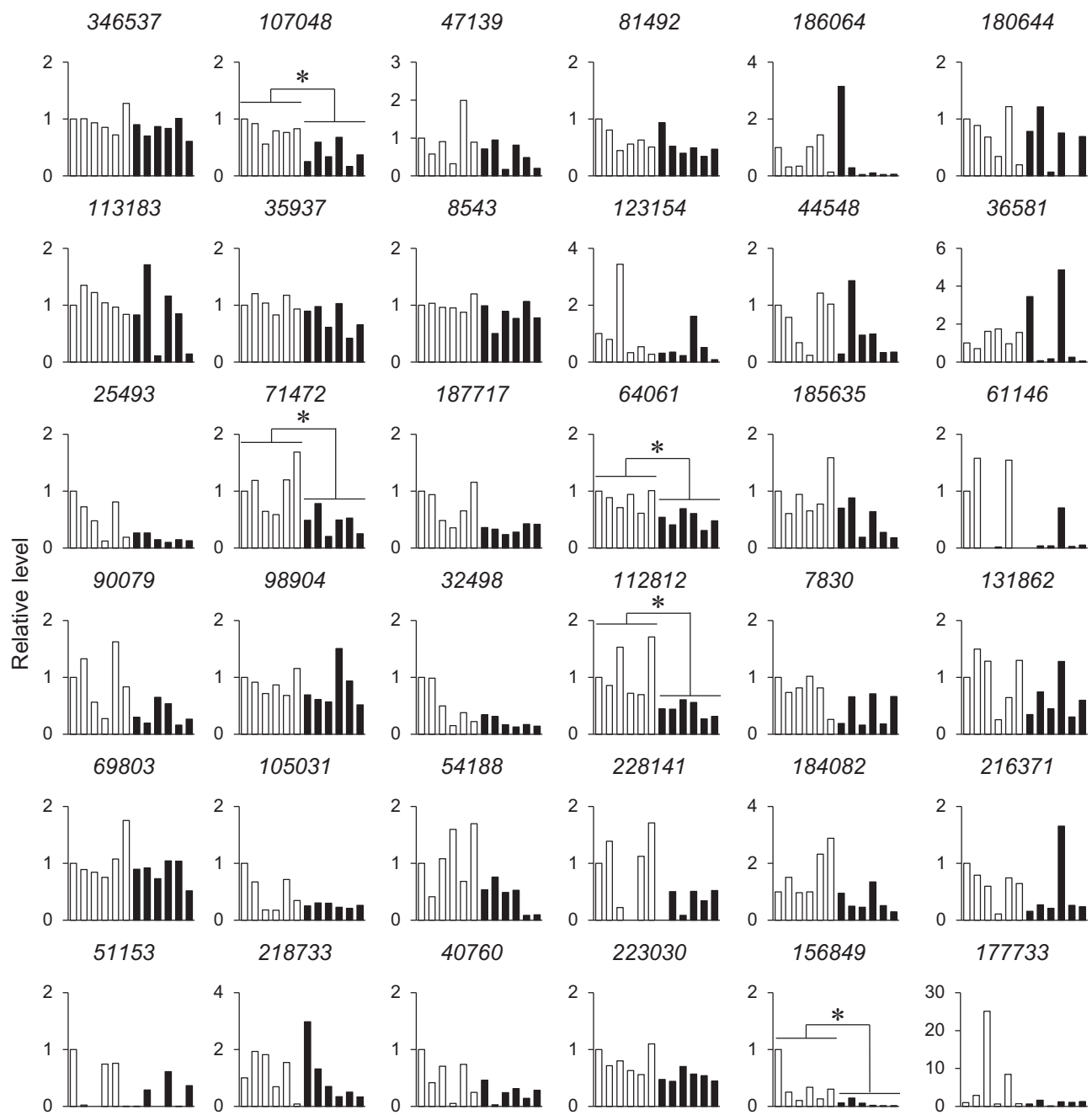
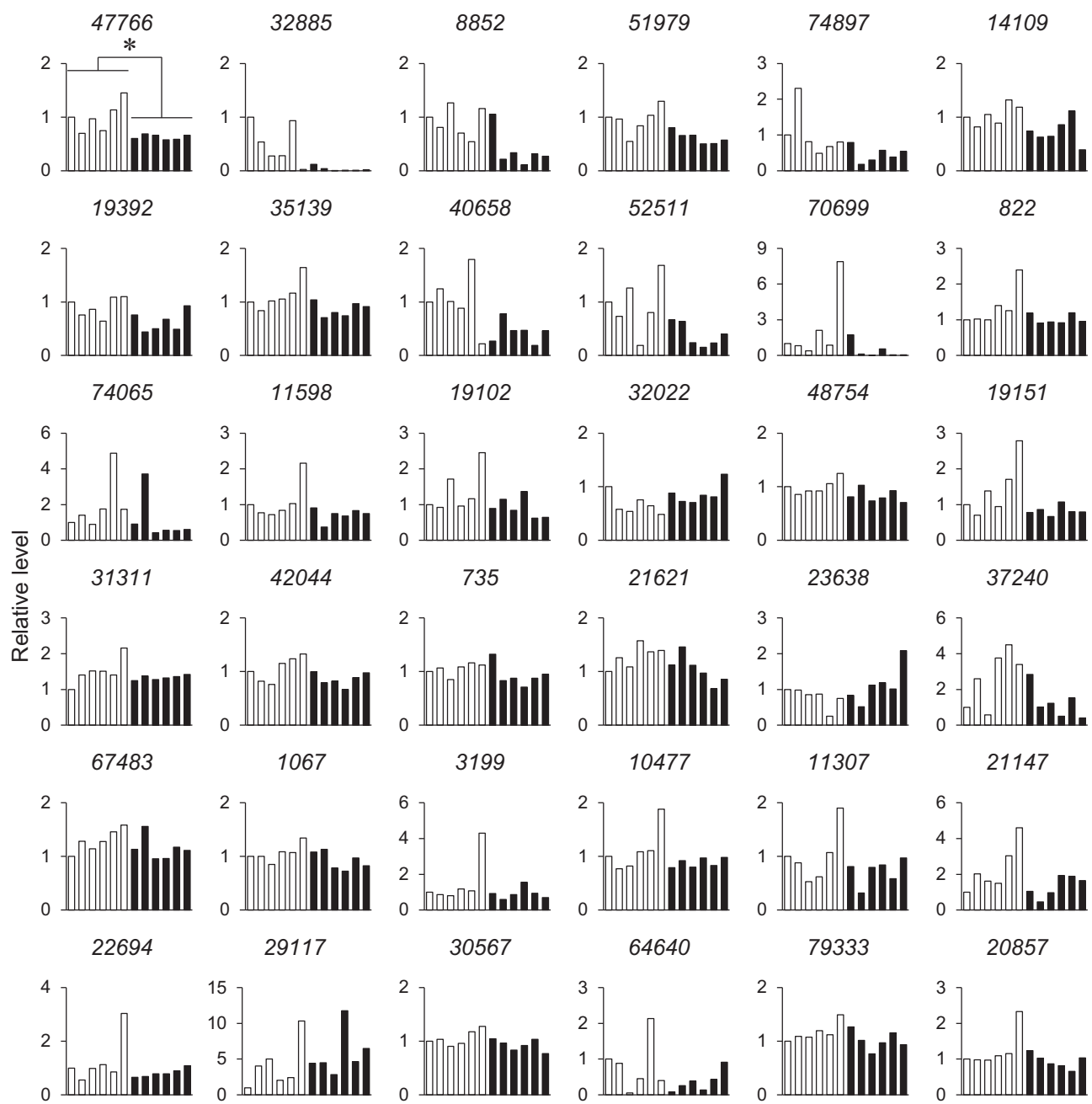


Fig. 3



**Fig. 4**



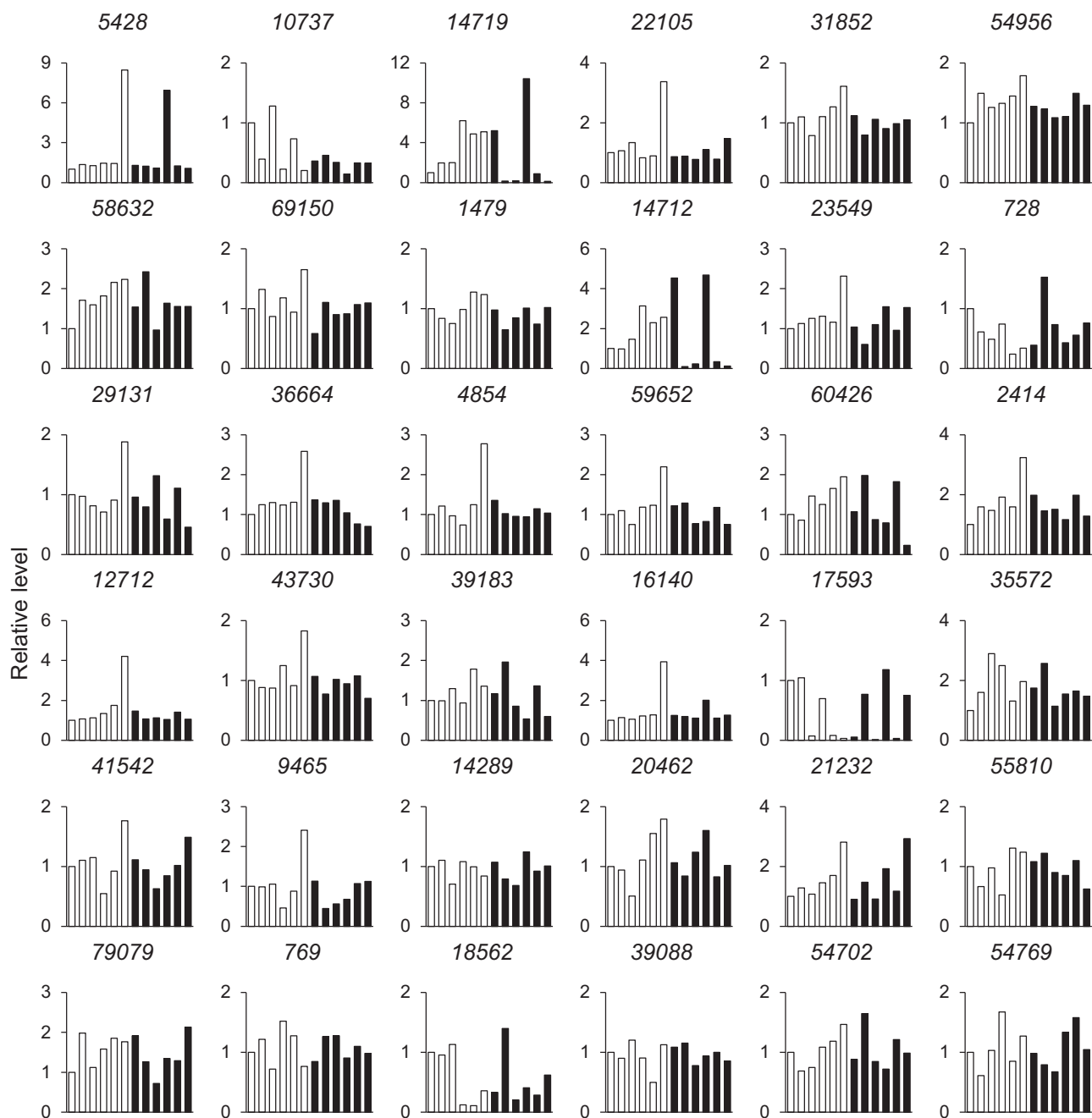


Fig. 5

