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Quantitative comparisons of maternal transcripts related to cell division between good and poor quality eggs from artificially matured Japanese eel *Anguilla japonica*

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Abstract: The current method of artificial egg production in Japanese eel does not always yield good quality eggs, but the molecular basis for the decrease in egg quality remains unclear. In this study, we compared the levels of 74 maternal transcripts related to cell division among good quality eggs, developing poor quality eggs (i.e., those producing a viable morula embryo without hatching), and non-developing poor quality eggs (i.e., developmentally incompetent eggs) to determine the molecular characteristics of poor quality eggs produced by artificially matured Japanese eel. Only one transcript (*lsm7*) had a lower level in developing poor quality than in good quality eggs, with the others being present at similar levels between the two types of egg. The levels of 11 transcripts (e.g., *cdc26*, *psma7*) were lower in non-developing poor quality than in good quality eggs. These results suggest that insufficient or reduced levels of maternal mRNA related to cell division are associated with the loss of egg quality in artificially matured Japanese eel, and that maternal characteristics other than quantity may affect the developmental competence of poor quality eggs.

Key words: Japanese eel; Egg quality; Maternal mRNA; Cell division

The Japanese eel *Anguilla japonica* is a major aquaculture species in Japan. The seedling stock for aquaculture is derived from glass eels caught in the wild. Recently, natural glass eel stocks have drastically diminished (Tanaka et al. 2006) and in 2014, the species was listed as endangered on the International Union for Conservation of Nature Red List (Jacoby and Gollock 2014). Therefore, artificial production of glass eels is necessary in order to ensure a seedling supply and preserve this natural resource. Fertilizable eggs and larvae were first produced by artificial hormone treatment in the 1970s (Yamamoto and Yamauchi 1974), but glass eels were not generated in captivity until much later (Tanaka et al. 2003), with second-generation larvae finally obtained by artificial production in 2010 (Tanaka 2011). However, there remain many challenges in the current method of artificial eel maturation. One problem is that many artificially matured eels produce poor quality eggs, which decreases larvae production efficiency, although this has recently seen some improvement owing to methodological advances (Kagawa et al. 2005; Unuma et al. 2011; Okamura et al. 2014; Tanaka et al. 2015).

Egg quality is assessed by the percentage of eggs that are successfully fertilized and hatch, as well as the rate of deformity during early

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development. Good quality eggs are those that exhibit normal developmental competence and have sufficient amounts of substrate required for embryogenesis including yolk proteins, cytoskeleton and membrane proteins, lipids, RNAs, hormones, and vitamins (Brook et al. 1997). In contrast, poor quality eggs are those that show reduced developmental competence and might have abnormal composition or abundance of these substances. In aquaculture, multiple factors can influence egg quality (see review Brook et al. 1997; Bobe and Labbé 2010). Environmental factors such as photoperiod, temperature, salinity, pH of the water, and diet affect oogenesis by altering the physiological condition of broodstock, ultimately dictating egg quality. Stress and reproductive age can also play a role. In some aquaculture species including Japanese eel in which ovulation must be artificially induced even if oogenesis is induced normally, eggs ovulated at the inappropriate time show low developmental competence (Adachi 2000; Unuma et al. 2011; Ishihara et al. 2014). This is the main problem in artificially matured fish. Even in eggs ovulated at the optimum timing, postovulatory aging, and post-stripping handling can directly impact eggs and undermine egg quality (Ohta et al. 1996a; Bobe and Labbé 2010; Nomura et al. 2013). Additionally, genetic factors such as breeding between genetically close individuals are known to have a major impact on egg quality (Brook et al. 1997). It is very likely that the above-described factors negatively influence egg components required for normal embryogenesis, resulting in poor quality eggs. However, the molecular basis for the loss of egg quality as a result of the above-described factors has not been widely investigated in aquaculture species, including Japanese eel.

Comparing the molecular characteristics of poor vs. good quality eggs can provide insight into the molecular basis for the loss of egg quality. One type of substance required for embryogenesis is maternally inherited transcript (maternal mRNA), which accumulates in eggs during oogenesis and is translated during final oocyte maturation and egg activation. After fertilization, maternal gene products drive early embryonic development in a transcriptionally silent embryo (Vasudevan et al. 2006). In fish, the onset of zygotic transcription—referred to as zygotic genome activation (ZGA)—occurs at the mid-blastula transition (MBT), which is characterized by the lengthening of the cell cycle and an asynchronous appearance after the first 10 synchronous cleavage divisions (Kane and Kimmel 1993; Lee et al. 2014). Maternal transcripts begin to degrade shortly before ZGA, and developmental control is transferred from maternal to zygotic gene products in a process known as the maternal-zygotic transition (MZT), which is a critical step for the progression of embryonic development (Tadros and Lipshitz 2009; Lee et al. 2014). Maternal factors are required for early stages of embryonic development as well as for MBT and ZGA (Shimuta et al. 2002; Dosch et al. 2004; Lindeman and Pelegri 2010; Lee et al. 2013). Additionally, maternal factors also influence subsequent development after MZT (Wagner et al. 2004). Therefore, abnormal composition or abundance of maternal mRNA in eggs is likely to result in reduced egg quality.

Several previous studies have investigated the relationship between egg quality and maternal mRNA abundance in aquaculture species (see review Sullivan et al. 2015). One transcriptome analysis of fully grown ovarian follicles from the striped bass *Morone saxatilis* identified 95 maternal transcripts related to cell division among the 133 informative genes that were correlated with egg quality (Chapman et al. 2014), leading the authors to propose that dysfunction of the cell cycle contributes to embryo mortality prior to MBT. This suggests that abnormal levels of maternal mRNA related to cell division are a molecular characteristic of poor quality eggs. However, quantitative PCR (qPCR) analysis of mRNA levels was not carried out in this report. In artificial maturation of aquaculture species, it is often observed that poor quality eggs develop into viable embryos at the synchronous cleavage stage but fail to hatch; however, maternal mRNA abundance in these eggs has not been investigated. Additionally,

there have been no studies to date examining the relationship between maternal mRNA in Japanese eel eggs and egg quality.

The present study investigated the molecular characteristics of poor quality eggs resulting from multiple factors, including inappropriate timing of ovulation induction in artificially matured Japanese eel. Egg quality was estimated by fertilization and hatching rates; eggs with high hatching rates were classified as good quality eggs; those with high fertilization and low hatching rates were classified as developing poor quality eggs; and those with low fertilization rate were classified as non-developing poor quality eggs. We compared the maternal mRNA levels of cell division-related genes identified in the striped bass study among good quality, developing poor quality, and non-developing poor quality eggs by qPCR.

Materials and Methods

Artificial maturation of eels

Feminized cultured eels treated with estradiol-17β (Tachiki and Nakagawa 1993) and male eels were acclimated to seawater and maintained without feeding in flow-through tanks holding 400 l of seawater at a constant water temperature of 20°C during artificial maturation. Feminized eels (500−1000 g) received weekly injections of salmon pituitary extract (20 mg pituitary powder/fish) to induce vitellogenesis, followed by 17α, 20β-dihydroxy-4-pregnen-3-one (2 mg/kg body weight) to induce final oocyte maturation and ovulation (Kagawa et al. 1997). Eggs were stripped from females immediately after ovulation and some were flash frozen in liquid nitrogen and stored at −80°C until RNA extraction. On the same time schedule, spermatogenesis and spermiation were induced in male eels (300−350 g) via weekly injections of human chorionic gonadotropin (250 IU/fish) as previously described (Ohta et al. 1996b). Sperm was diluted 100 fold in artificial seminal plasma (Ohta and Izawa 1996) and motility was assessed by microscopy prior to use.

Artificial fertilization

Fertilization and hatching rates were determined using an individual rearing method (Unuma et al. 2004) in which 2 g of ovulated eggs were inseminated with 1 ml of pre-diluted milt (sperm mortality >50%) and transferred to 100 ml filtered seawater. A 3-ml volume of seawater containing about 120 eggs was transferred to a glass dish, and the eggs were transferred to 48-well culture plates (one egg per well). Each well was filled with 1 ml filtered seawater containing 100,000 IU/l penicillin G potassium, 0.1 g/l streptomycin sulfate, and 1 μg/ml polyethylene glycol, and the eggs were incubated at 23°C. Normal morula embryos and hatched larvae were counted 4 h after insemination and 3 days after fertilization, respectively. Fertilization and hatching rates were calculated using the following formulae:

Fertilization rate (%) = 100 × number of normal morula embryos/number of incubated eggs

Hatching rate (%) = 100 × number of hatched larvae/number of incubated eggs

Egg quality was determined based on fertilization and hatching rates (Table 1). Eggs with fertilization rate >80% and hatching rate >80% were classified as developmentally competent

Table 1. Egg quality in 17 females estimated by fertilization and hatching rates

No.	Fertility (%)	Hatchability (%)	Egg quality
1	97.5	93.3	good
2	98.9	93.5	good
3	98.4	92.9	good
4	100.0	87.8	good
5	91.9	82.1	good
6	86.7	0	developing poor
7	87.4	0	developing poor
8	90.5	15.5	developing poor
9	85.8	10.1	developing poor
10	86.0	18.0	developing poor
11	95.2	20.0	developing poor
12	1.7	0	non-developing poor
13	1.5	0.8	non-developing poor
14	8.9	0	non-developing poor
15	0	0	non-developing poor
16	4.9	0	non-developing poor
17	9.3	0	non-developing poor

Table 2. List of 36 transcripts related to cell cycle regulation (direct regulator, UPS: ubiquitin-proteasome system, and CSN: COP9 signalosome), DNA replication, mitosis, and cytokinesis; and results of BLASTx analyses

Symbol	Description (HUGO gene)	Contig ID*	Length (b)	E-value	Species	Accession	Functional category
btg1	B-cell translocation gene 1, anti-proliferative	AJC013812_1	906	7E-78	*Epinephelus coioides*	ACM41870.1	direct regulator
ccnb3	cyclin B3	AJC001104_2	1170	1E-179	*Anguilla japonica*	BAD52074.1	direct regulator
ccne2	cyclin E2	AJC007038_1	700	6E-94	*Salmo salar*	NP_001133906.1	direct regulator
gdf9	growth differentiation factor 9	AJC015031_1	556	5E-47	*Danio rerio*	NP_001012383.1	direct regulator
mphosph10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	AJC004018_1	1118	1E-107	*Danio rerio*	XP_709654.1	direct regulator
anapc7	anaphase promoting complex subunit 7	AJC000827_1	1898	0	*Salmo salar*	NP_001139989.2	UPS
cdc26	cell division cycle 26	AJC024504_1	360	6E-24	*Danio rerio*	NP_001004005.1	UPS
cnpy2	canopy FGF signaling regulator 2	AJC000802_3	432	5E-34	*Danio rerio*	NP_001034915.2	UPS
cul3	cullin 3	AJC000415_1	1876	0	*Ornithorhynchus anatinus*	XP_001512511.1	UPS
g2e3	G2/M-phase specific E3 ubiquitin protein ligase	AJC011262_1	413	2E-54	*Danio rerio*	NP_001003822.1	UPS
ltn1	listerin E3 ubiquitin protein ligase 1	AJC024104_1	352	2E-62	*Gallus gallus*	XP_416690.2	UPS
pomp	proteasome maturation protein	AJC010955_1	608	1E-67	*Ictalurus punctatus*	ADO29311.1	UPS
psma7	proteasome (prosome, macropain) subunit, alpha type, 7	AJC001435_1	1055	1E-124	*Danio rerio*	NP_998331.1	UPS
psmd14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AJC001158_1	1378	1E-175	*Salmo salar*	NP_001135360.1	UPS
rchy1	ring finger and CHY zinc finger domain containing 1, E3 ubiquitin protein ligase	AJC010233_1	520	2E-89	*Danio rerio*	CAM13181.1	UPS
tollip	toll interacting protein	AJC007082_1	1183	1E-127	*Danio rerio*	XP_002663063.1	UPS
ube2l3	ubiquitin-conjugating enzyme E2L 3	AJC011390_1	535	2E-71	*Danio rerio*	CAM14365.1	UPS
ubl5	ubiquitin-like 5	AJC019269_1	417	6E-36	*Salmo salar*	NP_001134376.1	UPS
usp11	ubiquitin specific peptidase 11	AJC029994_1	466	6E-61	*Danio rerio*	XP_002663119.1	UPS
usp14	ubiquitin specific peptidase 14	AJC000663_1	1968	0	*Danio rerio*	NP_956267.1	UPS
cops5	COP9 signalosome subunit 5	AJC001703_5	903	1E-120	*Osmerus mordax*	ACO09632.1	CSN
cops6	COP9 signalosome subunit 6	AJC006328_2	884	1E-100	*Anoplopoma fimbria*	ACQ58159.1	CSN
cops8	COP9 signalosome subunit 8	AJC004981_1	730	9E-82	*Salmo salar*	NP_001139878.1	CSN
cdt1	chromatin licensing and DNA replication factor 1	AJC000193_2	2570	0	*Danio rerio*	XP_695164.3	DNA replication
msh6	mutS homolog 6	AJC000250_3	1851	0	*Danio rerio*	AAL04170.1	DNA replication
rad23a	RAD23 homolog A, nucleotide excision repair protein	AJC001516_1	1814	2E-68	*Salmo salar*	NP_001135284.1	DNA replication
rps3	ribosomal protein S3	AJC000252_2	827	1E-131	*Ictalurus punctatus*	ADO28834.1	DNA replication
cenpk	centromere protein K	AJC001303_1	1480	5E-76	*Danio rerio*	XP_693534.4	mitosis
kifc1	kinesin family member C1	AJC000135_3	2272	0	*Danio rerio*	CAD60638.1	mitosis
lmnb2	lamin B2	AJC013561_1	543	6E-76	*Carassius auratus*	BAB32978.1	mitosis
mapre1	microtubule-associated protein, RP/EB family, member 1	AJC000622_1	1518	1E-123	*Danio rerio*	NP_998805.1	mitosis
ncapg2	non-SMC condensin II complex, subunit G2	AJC028595_1	440	3E-61	*Ornithorhynchus anatinus*	XP_001511868.1	mitosis
setd8	SET domain containing (lysine methyltransferase) 8	AJC003441_2	776	2E-79	*Danio rerio*	NP_001038814.2	mitosis
ssx2ip	synovial sarcoma, X breakpoint 2 interacting protein	AJC000710_1	2144	1E-129	*Danio rerio*	CAK03613.1	mitosis
anln	anillin actin binding protein	AJC031317_1	1639	1E-110	*Danio rerio*	AAI34810.1	cytokinesis
arpc5	actin related protein 2/3 complex, subunit 5, 16kDa	AJC007311_2	754	4E-73	*Danio rerio*	NP_958917.1	cytokinesis

*Contigs were identified in the EST database (Ijiri et al. *unpublished*) using BLASTn algorithms.

(good quality); eggs with fertilization rate >80% and hatching rate ≤20% were classified as those producing viable morula embryos without hatching (developing poor quality); and eggs with fertilization rate <10% and hatching rate <1% were classified as developmentally incompetent (non-developing poor quality).

RNA extraction and cDNA synthesis

Total RNA was extracted from eggs (good quality, $n = 5$; developing poor quality, $n = 6$; non-developing poor quality, $n = 6$) using ISOGEN reagent (Nippon Gene Co., Ltd) according to the manufacturer's instructions, then treated with DNase and purified using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co., KG). First strand cDNA was reverse transcribed from 1 μg total RNA using the ReverTra Ace reverse transcriptase (Toyobo Co., Ltd) and random hexamer primers (Thermo Fisher Scientific Inc.) in 20-μl reactions.

In silico cloning of genes

Similarity searches were carried out in order to identify Japanese eel orthologs of the 95 genes related to cell division that were identified in the striped bass study (Chapman et al. 2014); mRNA sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI). Sequences were compared to an expressed sequence tag (EST) database derived from Japanese eel ovary (Ijiri et al. *unpublished*) using BLASTn algorithms. Sequences identified as similar were verified using Genbank BLASTx (NCBI) to ensure that the similarity was not greater than for any other gene. Gene information obtained from NCBI Gene and GeneCards (Rebhan et al. 1997) was used to determine potential gene functions according to the following 10 categories (Tables 2, 3): direct regulators of cell cycle; ubiquitin-proteasome system (UPS) and constitutive photomorphogenesis (COP)9 signalosome (CSN) that modulate the

Table 3. List of 44 transcripts related to transcription, RNA processing, translation, and signal transduction; and results of BLASTx analyses

Symbol	Description (HUGO gene)	Contig ID*	Length (b)	E-value	Species	Accession	Functional category
cebpd	CCAAT/enhancer binding protein (C/EBP), delta	AJC004301_2	600	9E-35	*Danio rerio*	AAH45282.2	transcription
cir1	corepressor interacting with RBPJ, 1	AJC022320_1	318	3E-40	*Danio rerio*	XP_689017.4	transcription
esr2	estrogen receptor 2 (ER beta)	AJC029675_1	469	1E-59	*Anguilla anguilla*	ABF50552.1	transcription
gtf2f1	general transcription factor IIF, polypeptide 1, 74kDa	AJC003870_1	899	2E-49	*Danio rerio*	CAI11479.1	transcription
hdac1	histone deacetylase 1	AJC000189_4	1692	0	*Takifugu rubripes*	AAL89665.1	transcription
ncl	nucleolin	AJC000352_1	2187	1E-165	*Tetraodon nigroviridis*	CAG03484.1	transcription
znf143	zinc finger protein 143	AJC000044_5	2170	0	*Danio rerio*	CAK11243.1	transcription
lsm1	LSM1 homolog, mRNA degradation associated	AJC001733_1	1107	3E-51	*Canis familiaris*	XP_532806.2	RNA-processing
lsm7	LSM7 homolog, U6 small nuclear RNA and mRNA degradation associated	AJC002803_1	674	1E-35	*Xenopus (Silurana) tropicalis*	NP_001165133.1	RNA-processing
nop10	NOP10 ribonucleoprotein	AJC027817_1	454	7E-29	*Danio rerio*	NP_001003868.1	RNA-processing
prpf39	pre-mRNA processing factor 39	AJC003888_1	1449	1E-142	*Danio rerio*	AAI16541.1	RNA-processing
rbm22	RNA binding motif protein 22	AJC000739_2	1623	0	*Ailuropoda melanoleuca*	XP_002918204.1	RNA-processing
smn1	survival of motor neuron 1, telomeric	AJC020033_1	392	3E-27	*Salmo salar*	ACM09638.1	RNA-processing
snrnp27	small nuclear ribonucleoprotein 27kDa (U4/U6.U5)	AJC003037_1	914	5E-31	*Esox lucius*	ACO13283.1	RNA-processing
srpk1	SRSF protein kinase 1	AJC000073_6	1025	4E-95	*Danio rerio*	NP_001074138.1	RNA-processing
tsen54	TSEN54 tRNA splicing endonuclease subunit	AJC026848_1	448	4E-35	*Tetraodon nigroviridis*	CAG02240.1	RNA-processing
utp18	UTP18 small subunit (SSU) processome component homolog (yeast)	AJC000796_1	561	1E-59	*Salmo salar*	NP_001133382.1	RNA-processing
wdr3	WD repeat domain 3	AJC012146_1	433	3E-56	*Danio rerio*	CAQ14989.1	RNA-processing
eif1ad	eukaryotic translation initiation factor 1A domain containing	AJC004522_2	580	2E-30	*Danio rerio*	NP_956762.1	translation
eif3e	eukaryotic translation initiation factor 3, subunit E	AJC001959_1	1468	0	*Osmerus mordax*	ACO08955.1	translation
eif3k	eukaryotic translation initiation factor 3, subunit K	AJC004567_1	1076	1E-120	*Salmo salar*	ACI66240.1	translation
metap2	methionyl aminopeptidase 2	AJC003143_2	880	1E-131	*Xenopus laevis*	NP_001080472.1	translation
mrpl32	mitochondrial ribosomal protein L32	AJC004774_1	723	3E-54	*Salmo salar*	ACI66019.1	translation
mrps30	mitochondrial ribosomal protein S30	AJC001058_1	1579	1E-164	*Oncorhynchus mykiss*	ACO08034.1	translation
rps6	ribosomal protein S6	AJC000145_5	1067	1E-118	*Osmerus mordax*	ACO08923.1	translation
srp54	signal recognition particle 54kDa	AJC000161_3	1952	0	*Danio rerio*	NP_957282.1	translation
arfgap2	ADP-ribosylation factor GTPase activating protein 2	AJC002483_1	1118	1E-106	*Danio rerio*	NP_001032507.1	signal transduction
cdc37	cell division cycle 37	AJC000904_1	2392	1E-156	*Tetraodon fluviatilis*	AF091237_1	signal transduction
cdc42bpb	CDC42 binding protein kinase beta (DMPK-like)	AJC002447_3	1519	0	*Danio rerio*	NP_001038590.1	signal transduction
ctnnb1	catenin (cadherin-associated protein), beta 1, 88kDa	AJC000055_1	3167	0	*Salmo salar*	NP_001167409.1	signal transduction
cyld	cylindromatosis (turban tumor syndrome)	AJC014216_1	584	5E-73	*Danio rerio*	XP_692599.3	signal transduction
fibp	fibroblast growth factor (acidic) intracellular binding protein	AJC000048_3	2039	0	*Danio rerio*	CAM12898.1	signal transduction
gna13	guanine nucleotide binding protein (G protein), alpha 13	AJC001605_2	1796	1E-175	*Danio rerio*	AAR25617.1	signal transduction
gnpda1	glucosamine-6-phosphate deaminase 1	AJC002515_1	1147	1E-138	*Salmo salar*	NP_001134003.1	signal transduction
hsp90ab1	heat shock protein 90kDa alpha (cytosolic), class B member 1	AJC030922_3	2703	0	*Oncorhynchus mykiss*	NP_001118063.1	signal transduction
jup	junction plakoglobin	AJC000055_3	3208	0	*Carassius auratus*	ACI02123.1	signal transduction
map2k2	mitogen-activated protein kinase kinase 2	AJC002015_2	1378	0	*Danio rerio*	NP_001032468.2	signal transduction
mbip	MAP3K12 binding inhibitory protein 1	AJC004372_1	834	6E-67	*Danio rerio*	AAH57252.1	signal transduction
nucb1	nucleobindin 1	AJC005923_1	1020	1E-135	*Salmo salar*	NP_001133883.1	signal transduction
pdcl	phosducin-like	AJC002235_1	1123	1E-103	*Anoplopoma fimbria*	ACQ58103.1	signal transduction
pfdn4	prefoldin subunit 4	AJC007709_1	833	1E-47	*Ictalurus punctatus*	ADO29007.1	signal transduction
ptges3	prostaglandin E synthase 3 (cytosolic)	AJC006707_1	754	2E-64	*Osmerus mordax*	ACO09759.1	signal transduction
rap1b	RAP1B, member of RAS oncogene family	AJC000179_2	700	1E-88	*Canis familiaris*	XP_851250.1	signal transduction
tcirg1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3	AJC008127_1	804	1E-101	*Danio rerio*	NP_998234.1	signal transduction

*Contigs were identified in the EST database (Ijiri et al. *unpublished*) using BLASTn algorithms.

functions of cell cycle regulators; DNA replication; mitosis; cytokinesis; transcription; RNA processing; translation; and signal transduction.

qPCR analysis

Maternal mRNA levels were measured by qPCR using the THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific Inc.). First-strand cDNA was diluted 6 fold and 2 μl were used in a 10-μl qPCR reaction. The cycling conditions were as follows: 95°C for 1 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; and a dissociation curve step. Transcript levels were normalized to that of β-*actin* mRNA. The relative quantity of each transcript was determined by the comparative Ct method (Livak and Schmittgen 2001). Gene-specific primers were designed from sequences identified in silico using Primer Express Software v.3.0 (Thermo Fisher Scientific Inc.) (Table 4).

Statistical analysis

Statistical analysis was carried out using Excel statistical Analysis 2012 (Social Survey Research Information Co., Ltd). Maternal mRNA levels were compared between groups with the Kruskal-Wallis test followed by the Steel-Dwass test. Data are expressed as mean ± SD, and differences were considered significant at $P < 0.05$.

Results

In silico cloning

A total of 80 Japanese eel orthologs of genes

Table 4. qPCR primer sequences

Symbol	Contig ID	Forward primer (5'-3')	Reverse primer (5'-3')
anapc7	AJC000827_1	CCTGCCGTGTGAGGACTGT	GACAAAAGCAGCAAAATAAGCGTAT
anln	AJC031317_1	TGAGGACGTTAGCGGCTTTG	GGTAGGTCCAGTAGGAGATGCAGTA
arfgap2	AJC002483_1	GGACATCGCTCAGTTCAAGCA	CGATCCTGAATTGTGTTCATCAC
arpc5	AJC007311_2	CCGACTTGAGGACCATGACTTT	GTACTCCTCTAGGTGTGGCAAGAAC
btg1	AJC013812_1	AGCCCCTCCAAAGCCTACAG	CCCGTCGGTATCGCTCTCT
ccnb3	AJC001104_2	GTTGGGCTCTCTCACCTCAGA	GGGATGCCTTTGGTTCTTGTAC
ccne2	AJC007038_1	ATGCTGACGCAGGAGGATGT	GAGGCAGGTGATGCCGATAA
cdc26	AJC024504_1	CCGCGCCTGTATGTTGCT	ATGTCATCCAGCTTCAGTTCCA
cdc37	AJC000904_1	ATGCGTCTGTACGCCAAAGC	GTCCAGCCACCTGTGTGTTG
cdc42bpb	AJC002447_3	GCCTTCGAGAGGAGGATCAA	CTGCCTGGGTGGGATTCTT
cdt1	AJC000193_2	TCGGGAGGCCAACTCTAACA	CAAGCTTCTTTCTGGCCGATT
cebpd	AJC004301_2	CGAAATATGGAAATGCAGCAAA	AAGCTCGCGGGTTAAATGGT
cenpk	AJC001303_1	TGAGTGACCTCCTGGAAGAACA	TGGGAGCAAATCCTTCCTTTT
cir1	AJC022320_1	GTGTGAAGAATGGCCTCAACTTT	TGATTCCCCCTCCTCCTTTG
cnpy2	AJC000802_3	GTGGATGAAATGGAGTGGGAAA	TCCGGATTGATCCTGAAGGA
cops5	AJC001703_5	AGCAGCAGCCTACGAGTACATG	ACCAGCCAATGGCGTTCTC
cops6	AJC006328_2	GTCACAGGCTTCCCGTTCTG	GCCATGAGCCCCACATCA
cops8	AJC004981_1	TGGAGGAGGCAGTGAAAGGA	TGGCTTCTGTGGCATAACCA
ctnnb1	AJC000055_1	GCTCGGACGACATCAACGT	ACCTGGCACACCATCATCTTG
cul3	AJC000415_1	CAGTCGGTGCGGGTTCAC	CAAACCGGTTCATCTTGGAAGA
cyld	AJC014216_1	ACAACCGGGCAGAATCAAAC	CGCAGAGGGTGGGAGTGTAC
eif1ad	AJC004522_2	GCAAATGGTGAACGCTTCCT	TGGGATCAACAATGACAAAATCA
eif3e	AJC001959_1	TTCTTCAACCACCCCAAAGG	GGGCACATGGTCTGAATGG
eif3k	AJC004567_1	CGTGAAGCCAAAGCGAATAGA	CGGTCGGAGGAAGAAAACG
esr2	AJC029675_1	TCATCTTTTCCCCAGACCTCAA	TTGCCGCCAGCACCAT
fibp	AJC000048_3	GGTGGATTCCCTGGCAAAA	AGTTACGGAGGTCGGCAGAA
g2e3	AJC011262_1	ATGTCCCTGCTGCCACAGTAG	TGAAGAAGAACATGGCAGCACTA
gdf9	AJC015031_1	TGACTGTGGCTTGTATGACTTCAG	TTGTGCGGAGCTATGATCCA
gna13	AJC001605_2	TGGCAGACGTGCAGAAGTTC	TGGTGGTAGAGTGGCTTCTGACT
gnpda1	AJC002515_1	TGCCCGAGAGGTGATGATTT	GCCGAGACGGTCCACATG
gtf2f1	AJC003870_1	AGCCCATGACCACCAAGGA	TGGGCCAGCACGTTGAC
hdac1	AJC000189_4	CGCAGTGAACTACCCTCTCAGA	TCACCTTCGCCATGATAGGTT
hsp90ab1	AJC030922_3	GGAAGTGCGGTCAATGAATGT	GGGCAACACAGGTCCACTAAC
jup	AJC000055_3	GGAAATCTTGGAGCGGATACAT	GGCAAGGCTAGGGTTTGAGA
kifc1	AJC000135_3	TGGAGCAAGAGCATTCAGATGT	GGCTCACAACAAGCACTTGACT
lmnb2	AJC013561_1	CCTGGAGAACCGCTGTCAGA	GCGGGTCTCGCGTACCT
lsm1	AJC001733_1	TGACAACTTGTGGGAGATGTTTG	AGTTTCGGTACGCCGTTCTCT
lsm7	AJC002803_1	CATGGAGGCCATTCCGAAT	TTTTTGGAGGGTCGAGGGATA
ltn1	AJC024104_1	TCACCAGAACGGAAGCATCAT	AGTCCTCCACGCCTTCGAA
map2k2	AJC002015_2	GTCGGATTTGACCGCTAACAA	CCGGTAGGCGTGATGTTCA
mapre1	AJC000622_1	ACAACTTGAGTCGCCATGACAT	GTAGGCCGCACCTGAGCATA
mbip	AJC004372_1	CTCTCCAGGCAACGGACGTA	ACCGGCTTCATCAGCAACTT
metap2	AJC003143_2	GCGAAGCGATTCAGGAAGTG	CGATGGAGTGGCCGTTTAAA
mphosph10	AJC004018_1	GAACCGGGCTGGAGACATC	GACGCTTCGCCAGCTTCTT
mrpl32	AJC004774_1	GACAGTTGGCCCCAGCATTA	CTGTGTTCCTGATGCTGTTGGT
mrps30	AJC001058_1	TATGCCCCCGACCTGCTT	CATGGGTCAGCAAGTTTGGA
msh6	AJC000250_3	GTGCTCACTCGCCGAAGAC	CGCGGGTTTCCTTTACAGTTC
ncapg2	AJC028595_1	GGACCTGCTGCTCAAGATCAA	GCCGGGCCAGGAGATG
ncl	AJC000352_1	GCATAGCGCCCCTCAGATC	AAAACACGGATAATGGAATGGAA
nop10	AJC027817_1	GCTGGCTAACGGAGATCGAA	CGATCCGGCTCCGTAGGTAT
nucb1	AJC005923_1	AGTGGCTGCTGCCCTTGTC	TTCCTGCGGCTTGGTATTGT
pdcl	AJC002235_1	AAGGTTGCTGTGTGTGTTTTTATCTC	CATCCAGAGTTGTCATGGTCACTT
pfdn4	AJC007709_1	AATTTGGAGGACGCCAGTGA	AAGACATCCCCGATTTGGTAAG
pomp	AJC010955_1	CATCATGGTGGAGCACAAGCT	TGGTTATGCGGTCACACACAA
prpf39	AJC003888_1	AGTCTCGCCTCGCCTTCTC	GGCAGACACCAGCGTGTTG
psma7	AJC001435_1	CGACGTAGGATGGCTGCAA	CCTGGGCATATTCAACTTGGA
psmd14	AJC001158_1	CTGAGGCTTGGAAGTGGAATG	GCGCCAGTGAAGAGATGTACAC
ptges3	AJC006707_1	GGAGTGATGGAGACCAGGAGTT	CGGTGTGGAACAGTGGATGA
rad23a	AJC001516_1	CTGAGAGCGAGAGCCATGCT	AAGAGGACCCTGTGCTTGCA
rap1b	AJC000179_2	TTGCAGGATGCGTGAATACAA	GGACAAACTGCACGGTCAGA
rbm22	AJC000739_2	CCATACATCCGCATGACCAA	CACCAGCGAAACACTGTGAAG
rchy1	AJC010233_1	CTGTGCCACGATGCTGAAGA	TGCCGTTTGGACCGTGTTA
rps3	AJC000252_2	GGGCATGTTATGGCGTTCTC	CCTGAGCTTCCCAGAAACCA
rps6	AJC000145_5	GAGCCGCGTCAGTCTCACA	CGTCCACTTCGATCAGCTTCT
setd8	AJC003441_2	AGACCCTTCTACGGGCTGCTA	TCTCTAGTGGCGTCCACACAGT
smn	AJC020033_1	GGAAAAAGTGACGACCCTGACA	TTCTTGAATGATGCGACAGCTT
snrnp27	AJC003037_1	GAAAAGGCCGTGAAGGAACA	TGCTGAAGCCCATGAGCTT
srp54	AJC000161_3	TGGACGTGGCGTCTGTGAT	TAGGACTCTTGGTGGCAGCAA
srpk1	AJC000073_6	TCCACATGACAGTGACTGAATCAG	TGGAGGGAGTTTCTCTGGGATAC
ssx2ip	AJC000710_1	CAGCAGTGGAGGCGACTGA	CCGACCCGCACATCCAT
tcirg1	AJC008127_1	GGCTGGTCATACTCGCTGTGT	GTTTCGGTGCCTGATAAGAAGGT
tollip	AJC007082_1	CAAAAACCCTCGCTGGAACA	CGTCGAAGATCTCCAGGTAGAAG
tsen54	AJC026848_1	AAGAGGCCCTTTACCTCATGGA	TAACCGTCCTGGATCGACAGA
ube2l3	AJC011390_1	TGACTTGGCAAGGGCTTATTG	GGCAGGGAAGATGATCTCGAT
ubl5	AJC019269_1	GGGACCAGGTGGGACAAGAT	TTCATCCCGTCGTGAATTTCA
usp11	AJC029994_1	GGAGCTTGAGACGGCTAGTGA	GCAATGGTGAGGGTTTGATGA
usp14	AJC000663_1	AAGCCAGAGCTGGTCGATGT	TCCTCCCGAACTTCATTCAAA
utp18	AJC000796_1	CCAGGTTGATGGAAAGACCAA	TGCTGAACTGGGCCTTGTG
wdr3	AJC012146_1	CAGCCCCAGCGGAGACTAC	GATGGGCTCCCTCGTTCTCT
znf143	AJC000044_5	ATGGTGGTCGGACGAAGGA	TTTGCGAGTCGCGGTTAATC
β -actin	-	GCCTTCCTTCCTGGGTATGG	AGTTGAAGGTGGTCTCGTGGATT

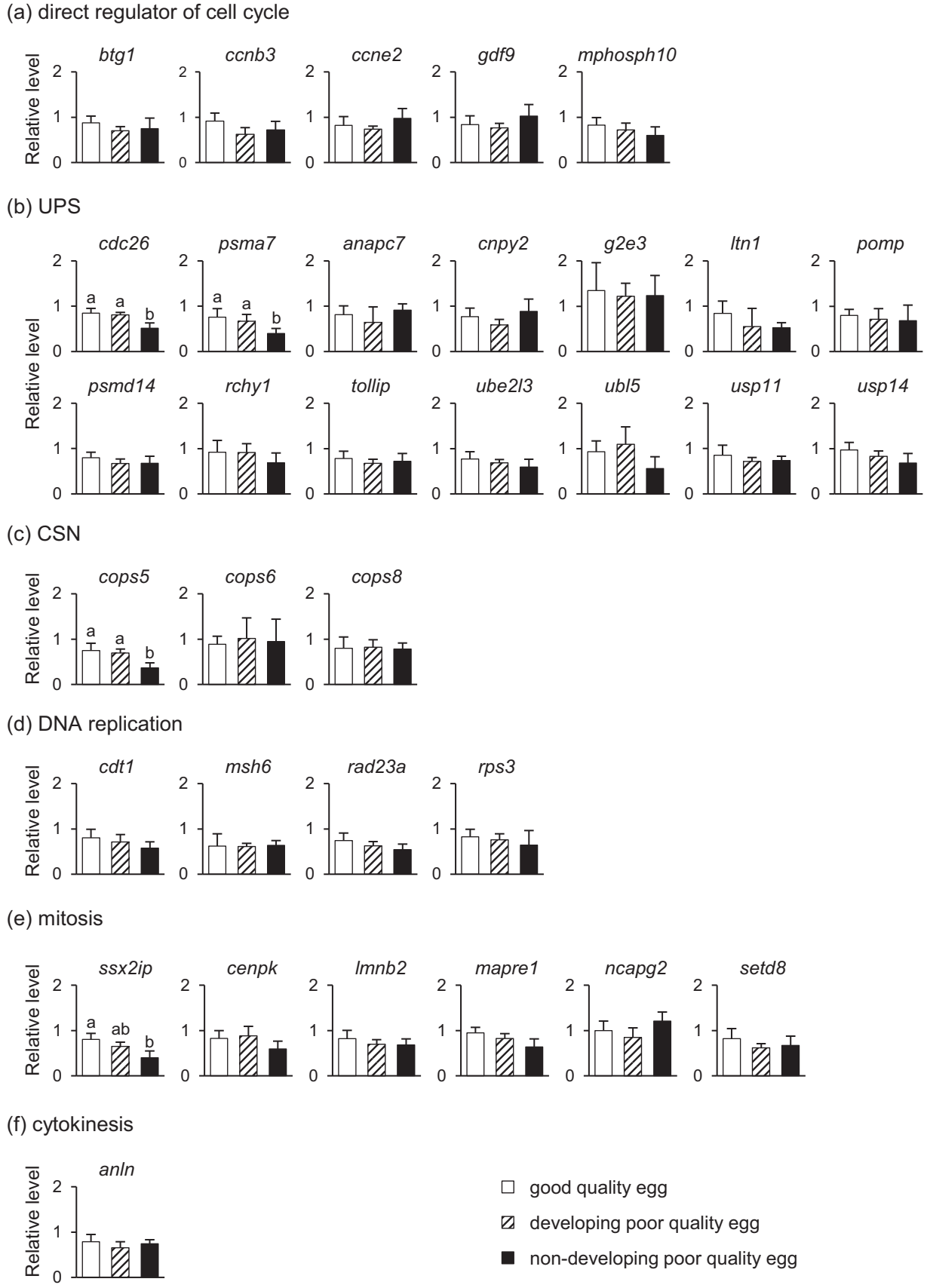

Fig. 1. Relative levels of transcripts related to (a) direct regulator of the cell cycle, (b) UPS (ubiquitin-proteasome system), (c) CSN (COP9 signalosome), (d) DNA replication, (e) mitosis, and (f) cytokinesis in good quality, developing poor quality, and non-developing poor quality eggs. Bars indicate standard deviation. Different letters indicate significant differences relative to good quality, developing poor quality, and non-developing poor quality eggs ($P < 0.05$).

related to cell division were identified in the assembled EST database (Ijiri et al. *unpublished*) by BLASTx analysis (e value range: 0 to 6E − 24) (Tables 2 and 3), while 15 orthologous genes (*apex1*, *cops4*, *ddb2*, *eif4e2*, *ergic2*, *fbxo9*, *mcl1*, *nudt21*, *prss27*, *snrpa*, *tbc1d25*, *tmbim6*, *tuba3e*, *ube2f*, and *ubox5*) were not in the database.

qPCR analysis

Of the 80 genes identified in silico, six (*arpc5*, *cul3*, *cyld*, *jup*, *kifc1*, and *srpk1*) were excluded from the qPCR analysis for methodological reasons (e.g., double amplification, low PCR efficiency, or no amplification). Significant differences in maternal mRNA level were observed among groups for 12 of the remaining 74 genes (Figs. 1–3).

There were no differences among groups with respect to genes that direct regulator of the cell cycle, DNA replication, or cytokinesis (Fig. 1a, d, f). Among UPS and CSN genes, maternal mRNA levels of *cdc26, psma7*, and *cops5* were lower in non-developing poor quality as compared to good and developing poor quality eggs (Fig. 1b, c). Among genes associated with mitosis, the level of *ssx2ip* was lower in non-developing poor quality as compared to good quality eggs (Fig. 1e).

Among genes involved in transcription, the

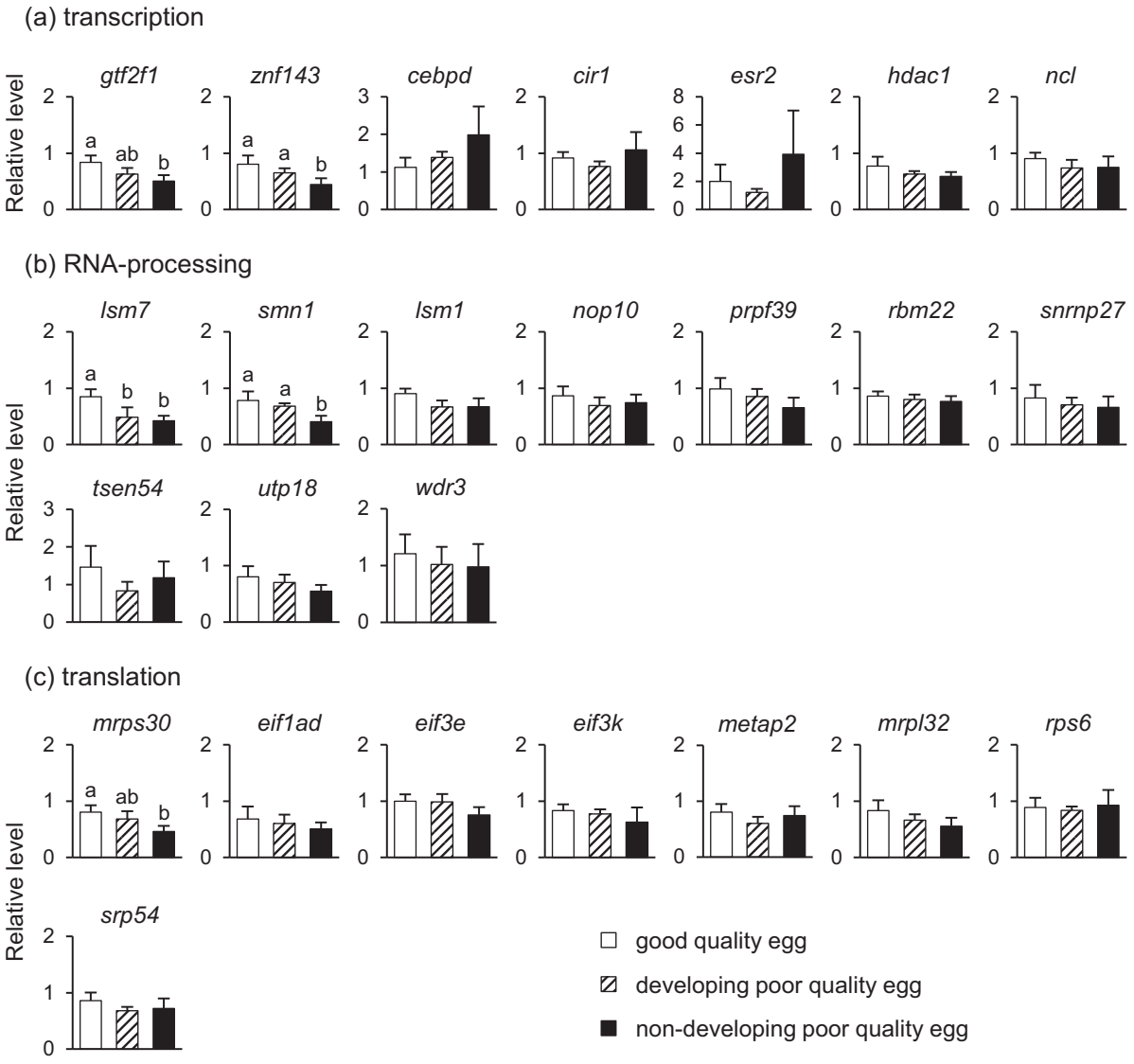

Fig. 2. Relative levels of transcripts related to (a) transcription, (b) RNA processing, and (c) translation in good quality, developing poor quality, and non-developing poor quality eggs. Bars indicate standard deviation. Different letters indicate significant differences relative to good quality, developing poor quality, and non-developing poor quality eggs ($P < 0.05$).

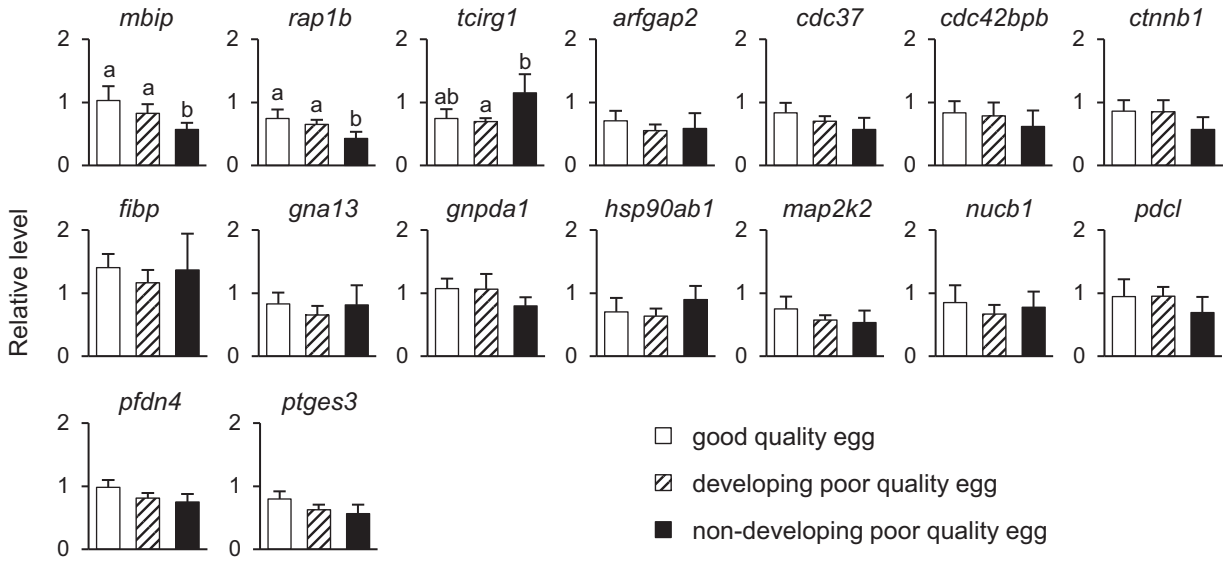

Fig. 3. Relative levels of transcripts related to signal transduction in good quality, developing poor quality, and non-developing poor quality eggs. Bars indicate standard deviation. Different letters indicate significant differences relative to good quality, developing poor quality, and non-developing poor quality eggs ($P < 0.05$).

level of *gtf2f1* was lower in non-developing poor quality relative to good quality eggs, whereas that of *znf143* was lower in non-developing poor quality eggs as compared to the two other groups (Fig. 2a). Among RNA processing genes, *lsm7* transcript level was higher in good as compared to poor quality eggs, and *smn1* level was lower in non-developing poor quality eggs relative to the other two groups (Fig. 2b). Among translation-related genes, *mrps30* mRNA level was lower in non-developing poor quality as compared to good quality eggs (Fig. 2c).

Among genes involved in signal transduction, mRNA levels of *mbip* and *rap1b* were lower in non-developing poor quality eggs relative to the other two groups (Fig. 3). In contrast, *tcirg1* transcript was higher in non-developing as compared to developing poor quality eggs, with no difference observed between good and poor quality eggs (Fig. 3).

Discussion

In the present study, environmental factors were unlikely to influence egg quality since eel broodstocks were reared under the same artificial conditions, although response to various external environmental stressors may have differed among individuals. Additionally, post-ovulatory aging (postovulatory overripe) and post-stripping handling were also unlikely to influence egg quality in this study, since eggs were stripped immediately after ovulation was observed and were fertilized shortly thereafter. Another remaining factor that affects the egg quality may be the induction of ovulation at the inappropriate time. This is first report investigating the molecular characteristics of poor quality eggs produced by the above-mentioned factors in artificially matured Japanese eel by comparing maternal mRNA content between good and poor quality eggs.

We investigated genes identified in a previous report of striped bass (Chapman et al. 2014) because in both species, artificial treatment is necessary to induce final oocyte maturation and ovulation and the quality of eggs produced under the same artificial conditions is variable. To precisely measure maternal mRNA levels in eggs, we modified the protocol of the striped bass study by using ovarian follicles as samples and oligo(dT) primers specific to polyadenylated mRNA to synthesize cDNA. It is reasonable to use eggs (ovulated oocytes) as samples given that contamination of somatic

follicular cells and loss of egg quality may result from inducing ovulation at the incorrect time. Moreover, maternal mRNAs stored in eggs have short poly(A) tails that are lengthened after fertilization for translation (Richter 1999; Mendez and Richter 2001). A recent high-throughput sequencing study reported that median poly(A) tail lengths are approximately 23 nucleotides in *Danio rerio* (zebrafish) embryos 2 h post-fertilization, with some mRNAs having very short poly(A) tail (<10 nucleotides) (Subtelny et al. 2014); it is likely that many maternal mRNAs in eggs have very short poly(A) tails prior to fertilization. It is therefore reasonable to synthesize cDNA from whole maternal mRNAs, including those with very short poly(A) tails. For these reasons, we used eggs as samples and synthesized cDNA using random primers for qPCR analysis in this study.

We determined maternal mRNA levels of 74 genes related to cell division in good quality, developing poor quality, and non-developing poor quality eggs. We detected lower levels of maternal mRNA of genes involved in modulating the functions of direct cell cycle regulators (*cdc26*, *psma7*, and *cops5*), transcription (*znf143*), RNA processing (*smn1*), and signal transduction (*mbip* and *rap1b*) in non-developing poor quality as compared to developing poor quality and good quality eggs, indicating that these maternal transcripts are closely related to developmental competence during early embryonic stages. Psma7 is a subunit of the 26S proteasome, which controls proteolysis in the UPS and modulates the functions of cell cycle regulators, including cyclins, cyclin-dependent kinases, and cell division cycle (Cdc) proteins (Elenich et al. 1999; Nakayama and Nakayama 2006; Kish-Trier and Hill 2013). Cdc26 plays a role in the assembly of the anaphase promoting complex/cyclosome (APC/C), a ubiquitin E3-ligase complex that is required for mitotic progression and exit (Castro et al. 2005; Wang et al. 2009). Cops5 is a subunit of the CSN, which regulates the APC/C (Wei and Deng 2003; Kob et al. 2009). Four of the identified genes—*znf143*, *smn1*, *mbip*, and *rap1b*—are upstream regulators of the cell cycle. Znf143 modulates the transcription of genes associated with DNA replication and the cell cycle (Izumi et al. 2010). Smn1 is involved in the biogenesis of small nuclear ribonucleoproteins that comprise the catalytic center of the pre-mRNA splicing reaction (Madhani and Guthrie 1994; Fischer et al. 1997); recent studies have reported alternative splicing of genes involved in cell cycle control (Moore et al. 2010; Prinos et al. 2011). Mbip and rap1b are components of a signaling pathway that controls cell proliferation (Fukuyama et al. 2000; Chrzanowska-Wodnicka et al. 2008; Wagner and Nebreda 2009). Therefore, insufficient or reduced maternal mRNA levels of these genes in eggs are likely to negatively influence cell cycle regulators after fertilization, thereby contributing to abnormal cell division during early embryogenesis.

In contrast, *tcirg1* maternal mRNA was present at higher levels in non-developing poor quality as compared to good quality eggs, although this difference was not statistically significant and levels varied across individuals. Tcirg1 is a subunit of the vacuolar proton ATPase that is essential for pH homeostasis in organelles (Forgac 2007) and is involved in cell proliferation (Cruciat et al. 2010; Niehrs and Acebron 2012) and oocyte hydration in marine teleosts (Selman et al. 2001; Matsubara et al. 2003). The latter is a process that occurs during oocyte maturation and controls egg buoyancy, which maintains embryos at the oxygen- and nutrient-rich water surface and thereby ensures their survival (Kagawa et al. 2009; Matsubara 2010). Excess *tcirg1* maternal mRNA in eggs may indicate incomplete oocyte hydration and therefore, egg immaturity. The timing of ovulation induction can influence egg quality: if it is too early, it can lead to the production of an immature egg, whereas late induction may result in preovulatory (intrafollicular) overripe eggs (Adachi 2000). Previous reports have indicated that artificially matured Japanese eel eggs ovulated at the wrong time had low fertilization rates (Adachi

2000; Unuma et al. 2011). In the present study, samples were unlikely to be contaminated by postovulatory overripe eggs since eggs were stripped immediately after ovulation. For this reason, poor quality eggs may include immature and intrafollicular overripe eggs in artificially matured Japanese eel. Assuming that Tcirg1 is a molecular marker of immature eggs, our observation that the maternal transcript levels of the gene varied across individual non-developing poor quality eggs implies that immature and intrafollicular overripe eggs coexisted in these samples. However, the relationship between maternal mRNAs and immature or intrafollicular overripe eggs requires clarification.

The maternal mRNA of *lsm7* was present at lower levels in developing as well as non-developing poor quality as compared to good quality eggs. We assessed egg quality in the morula preceding the blastula stage and hatching rate; therefore, developing low quality eggs would be expected to produce abnormal embryos between MZT and hatching stages. Lsm7 is involved in alternative splicing and mRNA degradation (Tharun et al. 2000; Wilusz and Wilusz 2005). In all animals, MZT is a critical step in embryonic development that consists of two events—namely, the degradation of maternal transcripts and ZGA (Tadros and Lipshitz 2009). Therefore, our results suggest that insufficient or reduced *lsm7* maternal mRNA in eggs would affect MZT and cause abnormal development at later embryonic stages. In addition, the abundance of transcripts for genes involved in mitosis (*ssx2ip*), transcription (*gtf2f1*), and translation (*mrps30*) decreased as a function of developmental competence, which would likely have a negative impact on early embryogenesis and subsequent development.

Although we found that 11 of 74 maternally inherited transcripts were lower in non-developing poor quality as compared to good quality eggs, some of these—i.e., ssx2ip, znf143, and rap1b—showed opposite trends to those identified in the striped bass study (Chapman et al. 2014). This may be due to methodological or species difference. Nevertheless, cdc26, psma7, cops5, gtf2f1, lsm7, smn1, mrps30, and mbip showed concordance with the striped bass study, suggesting that insufficient or reduced levels of these maternal transcripts in ovulated eggs may be a molecular signature of non-developing poor quality eggs in Japanese eel and other aquaculture fishes. Another recent study that focused on egg quality under the same captive conditions by transcriptome analysis and qPCR suggested that broodstock stress by cortisol treatment affected the abundance of maternal mRNAs, especially those related to cytogenesis in eggs and embryos (Kleppe et al. 2013). Given that stress may affect egg quality in artificially matured Japanese eel, further studies on these mRNAs are needed.

In summary, our findings reveal some of the molecular characteristics of poor quality eggs in artificially matured Japanese eel. Only one of 74 transcripts related to cell division was present at lower levels in developing poor quality relative to good quality eggs. We also found that the abundance of 11 maternal transcripts related to cell division was lower in non-developing poor quality eggs. These results suggest that insufficient or reduced levels of maternal mRNAs related to cell division are associated with the loss of egg quality in artificially matured Japanese eel. However, most maternal transcripts related to cell division showed normal levels in both types of poor quality egg, suggesting that the molecular basis for loss of egg quality cannot be solely explained by abnormal quantities of maternal mRNA and that transcripts of genes other than those associated with cell division may contribute to this phenomenon. Further research on the relationship between egg quality and additional maternal characteristics including mRNA localization, translation, and function is needed in order to clarify the molecular basis for the loss of egg quality in artificially matured Japanese eels, which could provide a basis for improving current aquaculture practices and thereby increasing fish production.

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人為催熟ニホンウナギの良質卵および不良卵における細胞分裂関連遺伝子群の母性 mRNA 量の比較

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ニホンウナギ人為催熟では常に良質卵が得られるとは限らず，また，卵質悪化の分子機構もわかっていない。そこで本研究では，人為催熟ニホンウナギの不良卵の分子生物学的特徴を明らかにするため，細胞分裂関連遺伝子の母性 mRNA に着目し，良質卵，発生不良卵（桑実胚まで発生するが孵化しない），非発生不良卵（発生しない）に含まれる母性 mRNA 量を比較した。調べた74個の細胞分裂関連遺伝子のうち，発生不良卵では１個（*lsm7*），非発生不良卵では11個（*cdc26*, *psma7* など）の母性 mRNA 量が良質卵と比較して有意に低値を示したが，それ以外の遺伝子で有意な差は認められなかった。この結果は，卵内の細胞分裂関連遺伝子の母性 mRNA 量の不足または減少が本種の卵質悪化に関与していることを示唆するものの，胚発生能には母性 mRNA の量以外の要因も影響している可能性を示している。