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Light-dependent transcriptional events during resting egg hatching of the rotifer *Brachionus manjavacas*

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Abstract

Rotifer resting eggs often have to endure harsh environmental conditions during the diapause phase. They are stimulated by light to hatch. In order to study the hatching mechanism, we observed resting eggs and measured their transcriptional expression under different light exposure periods (total darkness, and after 30 min, and 4h light). By using differential-display reverse transcription PCR (DDRT-PCR), we isolated 80 genes that displayed different expression patterns in response to the three light treatments: 20 genes were expressed in total darkness, 40 different genes were differentially expressed under 30 min light, and 20 further genes were expressed after 4h of light. The resting eggs showed no phenotypic differences in embryonic development during the 4h illumination period. In general, the expression patterns of the analyzed genes in resting eggs were differentially modulated by light exposure time. In total darkness, resting eggs mainly express genes encoding cell defense and homeostasis functions. In the 30 min illumination group, we found enriched expression of genes encoding fatty acid metabolism-related components, including Acyl-CoA dehydrogenase (*ACAD*). Genes encoding cellular and embryonic developmental functions were highly observed in the 30 min-illuminated group but were not observed in the 4h-illuminated group. Real-time RT-PCR revealed that several transcripts such as encoding V-type H(+)-translocating pyrophosphatase (*V-PPase*) and *Meckelin* had prolonged expression levels when exposed to light for 4 hours. In the 4h illuminated group, the RecQ protein-like 5 *(RECQL5)* gene was enriched. This *RECQL5* gene may be expressed to protect the developing embryo from continuous light exposure. The data presented in this study indicate that DDRT-PCR-aided gene screening can be helpful to isolate candidate genes involved in the hatching process. Therefore, this paper provides new insights regarding the light-mediated molecular response of rotifer resting egg hatching.

Keywords: Rotifera, Resting egg, Hatching, Light, Transcriptional expression.

1. Introduction

Under stressful conditions such as extremes in temperature, or inadequate nutritional resources, some animals migrate away, while others modify their behaviors and/or habitats to reduce environmental stress. Dormancy is another adaptive mechanism to minimize the impact of stressors and to protect organisms from harsh environmental conditions (Lubzens et al., 2010). Dormancy includes diapause and quiescence that are distinguished from a que of endogenous or exogenous control, respectively (Ricci, 2001). During dormancy, organisms' physiological and metabolic activities (including growth, development and reproduction) temporarily cease in order to conserve energy for the following active season. In the hydrosphere, planktonic metazoa such as monogonont rotifer, *Artemia*, copepods and cladocera have the ability to become dormant by entering the resting egg (cyst) stage (Dahms, 1995). Among those microscopic aquatic invertebrates, rotifers frequently used as model laboratory organism because of their small size, simple organization, quick growth (<24h) and ease of mass cultivation (Dahms et al., 2011). In addition, the dormancy mechanism has been more clearly described in rotifers than in other species (Gilbert, 2003). During rotifer dormancy, two phenomena may occur, called diapause and quiescence (Ricci, 2001).

Rotifers have a heterogenic life cycle that includes both sexual (mictic) and asexual (amictic) reproduction (Snell, 1987; Hagiwara et al., 1989; Gilbert, 2003). The asexual reproduction dominates and amictic females parthenogenetically produce exclusively female offspring. Sexual reproduction in rotifers is initiated by environmental cues known as mixis inducers. Mixis inducers include both external factors (e.g. population density, temperature, salinity, food, juvenile hormone, serotonin) and internal factors (e.g. genetic variation, culture history, cumulative generation, aging) (Snell, 1987; Gallardo et al., 2000; Gilbert, 2003). Once sexual reproduction is triggered, mictic females undergo meiosis, which yields haploid gametes. If a male fertilizes these gametes, diploid resting eggs result. The resulting resting eggs are remarkably tolerant to unfavorable conditions and can retain viability for decades (reviewed in Gilbert, 2004 and Hagiwara et al., 2007). A prior study

suggested that several genes are associated with dormancy and desiccation tolerance in the monogonont rotifer *Brachionus plicatilis* (Denekamp et al., 2009). Despite these data, the underlying molecular mechanism and gene profile of dormancy and resting egg hatching in Rotifera is still incompletely understood.

Many researchers have extensively studied the environmental conditions, including light, temperature and salinity, that may affect resting egg hatching (Hagiwara et al., 1989, 1995; Hagiwara and Hirayama, 1993; Hagiwara, 1994). It is currently well known that in favorable environments, light stimulation triggers resting eggs to hatch as amictic females (Gilbert, 1974, 2007; Pourriot and Snell, 1983; Hagiwara and Hino, 1989; Wallace et al., 2006). Rotifer resting eggs, in particular, cannot initiate embryonic development without light. The eggs will not hatch in the absence of light, even if they are incubated under optimal conditions for hatching. Previously, it was thought that light triggered hatching via light-induced peroxide production in seawater and fatty acid oxidation to prostaglandins (E_1, E_2 or $F_{2\alpha}$) inside of the embryo. These hypotheses were supported by the fact that both hydrogen peroxide and prostaglandins can trigger resting egg hatching, even in darkness (Hagiwara et al., 1995). However, empirical studies have yet to substantiate these hypotheses.

Our objective was to better define the mechanism between diapause and hatching. In order to do so, we measured the transcription expression of resting monogonont rotifer *Brachionus manjavacas* eggs under different periods of light irradiation (total darkness, 30 min, and 4 h) using differential-display reverse transcription PCR (DDRT-PCR). This method has been used previously to isolate differentially enriched genes in response to various stressors in a short time period (Callejas and Gutiérrez, 2003; Jeon et al., 2010; Medini et al., 2009; Qin et al, 2011; Siquieroli et al., 2009). The results were validated using real-time RT-PCR. Finally, we determined the putative function of each transcript expressed during embryonic development using bioinformatics-aided characterization of the enriched genes. This paper provides new insight into the light-mediated molecular hatching process in the rotifer resting egg.

2. Materials and methods

2.1. Resting egg preparation

This study employed the Australian stain of *B. manjavacas*, which demonstrates active induction of sexual reproduction (Araujo and Hagiwara, 2005; Kim and Hagiwara, 2011). In order to produce a sufficient number of resting eggs, the rotifers were cultured at 25^{o}C in 40L of 11 parts per thousand (ppt) artificial seawater (Hagiwara et al., 1988; Hagiwara and Lee, 1991). These conditions are optimal for producing resting eggs. The rotifers were fed daily with a mixture of *Tetraselmis tetrathele* ($0.12x10^6$ cells/ml) and *Nannochloropsis oculata* ($3.5x10^6$ cells/ml). Two rotifer diets were independently cultured at 25^{o}C in 100L of 11 ppt artificial seawater with continuous fluorescent light (2000 lx) and were mixed immediately before feeding. The rotifers were cultured for 12 days and the resting eggs were harvested on the last day. The harvested resting eggs were preserved in a 4^{o}C refrigerator (in total darkness) for four months. Prior to sample preparation, the hatchability of the preserved resting eggs was confirmed. The threshold light condition to induce hatching was 4400 lx for 30 min. In addition, illumination for 4h had no effect on embryonic development with regard to morphological traits (unpublished data). Fifty resting eggs were randomly selected from the preserved stock. These eggs were transferred into a 6-well polystyrene microplate, incubated for 24h under continuous fluorescent light (3000 lx), and maintained under the same conditions as used to form the resting eggs (25°C, 11 ppt). Three 20W white fluorescent bulbs (FL20SW, Toshiba, Japan) in the incubator were used to irradiate these 50 eggs at 3000 lx. A mean of six replicates was used to calculate the hatching rate.

2.2. Morphological observation

The preserved resting eggs (n~18,000 eggs) were transferred into 500 mL glass beakers (from 4 to 25°C) containing 200 mL of 11 ppt diluted natural seawater. The three prepared beakers were incubated at 25^{o}C under the following illuminance conditions; in total darkness (0 min, control) and 3000 lx fluorescent light for 30 min and 4h. After light illumination, the morphological characteristics including diameter (width and length) and cell division of the resting eggs were compared between the control and the 4h treatment groups using microscopic observation.

2.3. RNA extraction and cDNA synthesis

The light-treated (0, 30 min, and 4h) resting eggs were rinsed several times with milli-Q water (millipore 0.24 μm) to remove saline water and feces. Total RNA was extracted with ISOGEN (Nippon gene, Tokyo, Japan) based on the supplied protocol and was suspended in an appropriate volume of autoclaved milli-Q water. RNAqueous® (Small Scale Phenol-Free Total RNA Isolation Kit, Ambion®, Tokyo, Japan) was used to recover the total RNA with enzyme reactions according to the instruction manual. The total amount of isolated RNA was quantified using UV spectrophotometry (Gene Spec III, Naka instruments Co., Ltd, Ibaraki, Japan). Total RNA isolated from some tissues often contains relatively high levels of genomic DNA. Therefore, 5 μg of total RNA in each sample were treated with TURBO DNA-free™ (Ambion®, Carlsbad, CA, USA) in order to remove the genomic DNA and to subsequently remove DNase I and divalent cations from the samples. cDNA was synthesized with 0.1 μg of isolated RNA using Advantage® RT-for PCR kit (Clontech, Tokyo, Japan) with the following ingredient mixture in a 500μL reaction tube: RNA, oligo(dT)$_{18}$ primer, milli-Q water and mineral oil to prevent evaporation. These mixtures were incubated at 70^{o}C for 3 min. Next, a 5x reaction buffer, dNTP mix (10 mM each) and MMCV-RT reverse transcriptase were added to the tubes. Reverse transcription was performed at 42^{o}C for an hour. When this process was finished, the reaction mixture was heated to 75^{o}C for 10 min to inactivate the reverse transcriptase. The synthesized cDNA was kept at -20^{o}C until use.

2.4. Differential display reverse transcription-PCR (DDRT-PCR)

DDRT-PCR analysis was performed using the Delata™ Differential Display Kit (Clontech, Tokyo, Japan). The total volume of the PCR reaction (20 μL) contained the following ingredients: 1 μL cDNA template, 0.4 μL 50x dNTP mix, 2 μL 10x KlenTaq PCR reaction buffer, 1 μL 20 pmol/μL of T-primer, 1 μL 20 pmol/μL of P-primer, 0.4 μL 50x Advantage KlenTaq Polymerase and 14.2 μL Milli-Q water. The PCR cycle included one cycle at 94ºC for 5 min, 40ºC for 5 min, 68ºC for 5 min; two cycles at 94ºC for 30 sec, 40ºC for 30 sec, and 68ºC for 5 min; 30 cycles at 94ºC for 20 sec, 60ºC for 30 sec, 68ºC for 2 min; and one cycle at 68ºC for 7 min. The 30 cycles of synthesis were carried out with an annealing temperature of 60ºC. The amplified cDNA in the PCR products was separated using agarose gel electrophoresis (2.0% agarose gel, Metaphor® Agarose, Cambrex, Rockland, USA). The gels were stained with SYBR Green (Molecular Probes Inc., Invitrogen, Carlsbad, CA, USA) for 30 min and photos were taken to compare the gene transcription among different treatments. The differentially expressed gene fragments were excised from the gel and preserved at -20ºC.

2.5. Reamplification and purification

The excised gene fragments containing cDNA fragments were melted and crushed with a micro-pipette; 1 μL of paste was extracted. Reamplification was performed in the same way as was the prior amplification. Eighty reamplified cDNA fragments were electrophoresed on 1.8% of agarose gel (Agarose-LE, Classic type, nacalai tesque, Kyoto, Japan). The gels were stained with 0.5 μg/mL of ethidium bromide for 30 min and photos were taken under UV light. Gel bands containing the target cDNA fragments were excised and purified using Wizard® SV Gel and the PCR Clean-Up System (Promega, Tokyo, Japan) according to the technical manual. The extracts of purified cDNA

fragments were kept at -20ºC.

2.6. TA-Cloning and sequencing

Purified cDNA fragments were cloned using the pGEM®-T and pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). Based on blue/white selection, the cDNA library recombination was performed efficiently. Four white colonies were selected per cDNA fragment. Colony direct PCR was performed using the General Reaction Mixture for PCR (Takara, Shiga, Japan) and the M13 forward primer and M13 reverse primer (Promega). The PCR cycle included one cycle at 95ºC for 3 min, and 95ºC for 20 sec, 55ºC for 20 sec, and 72ºC for 1 min of 35 cycles. The reamplified cDNA fragments were sequenced with ABI Big Dye 3.1 chemistry using M13 forward and reverse primers. The fragments were then eluted on the ABI PRISM 310 Genetic Analyzer (Life Technologies, Tokyo, Japan).

2.7. Gene annotation and GO analysis

Sequenced data were compared with DNA and protein sequences from NCBI's non-redundant (NR) protein sequence database (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blastblas) (**Supplementary Table 1**). The results with the highest score (>50) and E-value (<1.0xe-13) were used to infer the putative function of the identified gene associated with light exposure time.

The gene ontology (GO) were assigned by Blast2GO automated sequence annotation tool (http://www.blast2go.org) (Conesa et al., 2005). Three main categories for the biological process, cellular component, and molecular function were obtained after comparing similarities with default parameters.

2.8. Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

The transcriptional levels of certain genes from each sample were validated using real-time RT-PCR. Sample preparation was performed as previously described. The primers for each gene were designed after comparing the exon/intron boundary to the genomic DNA using GENRUNNER software (Hastings Software, Inc., Hastings, NY, USA). They were then confirmed using the Primer 3 program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). In order to determine the amplicon identity, all of the PCR products were cloned into the pCR2.1 TA vector (Invitrogen) and were sequenced with an ABI 3700 DNA analyzer (Bionics Co., Seoul, South Korea). Optimized conditions were applied according to the following CFX96™ real-time PCR protocol (Bio-Rad, Hercules, CA, USA). A no template control (NTC) reaction was included in every run for each primer pair in order to exclude DNA contamination in buffers/solutions and to assess primer dimers. Controls that did not have the reverse transcriptase enzyme from the cDNA synthesis reaction (no RT controls) were also used to test for genomic DNA contamination. All real-time RT-PCR experiments were carried out in unskirted low 96-well clear plates (Bio-Rad). A total of 2 μg of total RNA was used to synthesize cDNA for real-time RT-PCR. In each reaction, 0.2 μM of both forward and reverse primers for each gene was employed (**Supplementary Table 2**). The reaction conditions to detect specific PCR products were as follows: 94°C/4 min; 35 cycles of 94°C/30 sec, 55°C/30 sec, 72°C/30 sec; and 72°C/10 min. SYBR Green (Molecular Probes Inc., Invitrogen) was used to detect specific amplified products. In order to confirm the amplification of specific products, cycles were continued under the following conditions: 95°C/1 min, 55°C/1 min, and 80 cycles of 55°C/10 s with 0.5°C increase per cycle. All PCR products were sequenced at Bionics Co. Amplification and detection of SYBR Green-labeled products were performed using the CFX96 real-time PCR system (Bio-Rad). Data from triplicate experiments were expressed relative to the expression of the internal control *18S rRNA* gene used to normalize for any difference in reverse transcriptase efficiency. Each transcriptional level was determined by the $2^{-\Delta\Delta C}$t method

235 (Livak and Schmittgen, 2001).

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237 **2.9. Statistical analysis**

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239 The SPSS ver. 17.0 (SPSS Inc., IL, USA) software package was used for statistical analysis.
240 Data are expressed as means ± S.D. Significant differences between the total darkness and the light-
241 exposed groups were analyzed with one-way ANOVA followed by Tukey's test. P-values < 0.05
242 were considered as statistically significant.

243

244 **3. Results**

245

246 **3. 1. Morphological characteristics**

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248 The resting eggs had 91% hatchability under 24h of illumination. These eggs showed no
249 microscopic phenotypic differences in diameter (width 124.30 ± 6.25 μm and length 162.09 ± 3.18
250 μm for total darkness; width 125.73 ± 14.39 μm and length 170.34 ± 12.93 μm for 4h illumination)
251 or cell division compared to those of eggs under total darkness (0 min) or 4h irradiation (**Fig. 1**).

252

253 **3. 2. Transcriptional expression analysis using DDRT-PCR**

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255 There was variable expression of 80 genes under different lighting periods (0, 30 min, and 4 h)
256 in the resting eggs. After colony PCR was performed, a total of 145 cDNA fragments were selected
257 to compare gene expression. The BLASTX results (**Supplementary Table 1**) include 136 putative
258 genes that were classified into seven putative functional classes based upon their different light-
259 dependent patterns (**Fig. 2**). There were 80 differentially modulated transcripts depending upon
260 the light exposure. Twenty fragments were enriched in total darkness, 40 with 30 min of irradiation,

and 20 with 4h of light. There was also variable expression of genes that encoded certain enzymes in different light settings (**Table 1**). For example, the gene that encoded oxidase was actively expressed under 30 min of light (43% among expressed enzymes). However, there was no enrichment of this gene from eggs sampled in total darkness. Instead, in total darkness, the gene encoding hydrolase (80%) was actively expressed in the resting eggs. In addition, 13% of the enriched genes isolated from the total darkness samples had a putative function such as in cell defense or homeostasis. This fraction was decreased with an increase of light exposure, resulting in only 10% of the enriched genes after 30 min of illumination and 4% in the samples exposed to light for 4h, respectively. Genes that significantly contribute to embryonic development were actively expressed with any illumination period between 0 and 30 min (**Fig. 2, Table 2**). The transcripts that code for the regulatory proteins *LuxR*, *S*-formylglutathione hydrolase (*FGH*), and glutathione *S*-transferase (*GST*) were observed in total darkness. These genes had highest scores and E-values. With 30 min of illumination, V-type H(+)-translocating pyrophosphatase (*V-PPase*), acyl-CoA dehydrogenase (*ACAD*), and Meckelin were expressed. With 4h of light, the transcripts of RecQ protein-like 5 (*RECQL5*) and proton/sodium-glutamate symporter protein (*gltT*) were observed.

3. 3. Transcript profile validation using real-time RT-PCR

In order to test as to whether the modulation patterns inferred from the DDRT-PCR study can be validated by an independent method, we selected several genes and tested their transcription changes using real-time RT-PCR (**Fig. 3**). The overall expression dynamics obtained with RT-PCR were similar to the trends that were inferred from the DDRT-PCR results. RT-PCR revealed that several transcripts have prolonged expression patterns. The transcriptional levels of *LuxR*, *FGH*, and *GST* were highly detected in total darkness. The *V-PPase* and *ACAD* transcripts were highly expressed with 30 min of illumination. The mRNA levels of *Meckelin* and *RECQL5* were higher in both the 30 min and 4h illuminated samples than they were in the total darkness sample. The *gltT*

transcription levels were highest with 4h of illumination. Overall, most genes showed similar expression patterns with RT-PCR to their respective patterns obtained using DDRT-PCR.

4. Discussion

We used DDRT-PCR to isolate genes that were enriched under different light stimulations (0, 30 min, and 4 h) after transfer from 4°C to 25°C. There was no embryonic development observed microscopically within four hours of irradiation (**Fig. 1**). Interestingly, even at total darkness, the transcriptional expression was modulated by temperature change. From eggs sampled in total darkness, we isolated 22 enriched genes that were differentially expressed in response to temperatures ranging between 4°C and 25°C. Compared to the eggs exposed to the other two light treatments, cell defense and homeostasis genes were dominantly expressed in the total darkness group with increasing temperature (**Fig. 2**). The temperature increase in the absence of light stimulation probably stresses the resting eggs, and leads to expression of genes that defend against thermal protein damage. This suggests that these organisms use a molecular signaling system to minimize damage caused by sudden temperature changes.

In aquatic invertebrates, oxidative stress is generally mediated by reactive oxygen species (ROS), which are formed after exposure to environmental factors like heat, salinity, UV radiation, and nutritional imbalance (Abele et al., 2007). Increasing temperature, or thermal stress, significantly influences oxidative stress biomarkers in marine ectotherms (Abele et al., 1998, 2002; Keller et al., 2004; Vinagre et al., 2014). Glutathione (GSH) is an important molecule that plays a role in hydrogen peroxide removal. Several researches have suggested that GSH may accumulate during temperature-triggered oxidative stress in animals (Almeselmani et al., 2006; An and Choi, 2010). Therefore, in the rotifer *B. manjavacas*, elevated temperature might also induce genes such as *S*-formylglutathione hydrolase (*FGH*) and glutathione *S*-transferase (*GST*) (**Table 2**), both of which are involved in the GSH-mediated metabolism. It is possible that rotifers have sensitive

molecular systems that trigger the expression of cellular defense and homeostasis genes (e.g. cell defense system, antioxidant system, heat shock protein family) in response to environmental stressors (e.g. temperature, UV radiation, salinity, toxicants) (Kim et al., 2011, 2013; Kim et al., 2014; Rhee et al., 2011; Wheelock et al., 1999). The exact mechanism of such stress-triggered molecular changes is not yet fully understood in the rotifer. Regardless, they act to protect and conserve resting eggs at threatening, environmental changes.

In total darkness, the expression of luminescence-regulatory protein (*LuxR*) (**Table 2**) is modulated by halogenated furanones associated with a bioluminescence signal in the *Vibrio* species (Kolibachuk and Greenberg, 1993; Egland and Greenberg, 1999; Manefield et al., 2002). At elevated temperatures, the rotifer *B. manjavacas LuxR* gene detects chemical signals from luminescence, and can initiate embryo development under light stimulation. These findings indicate that resting eggs recognize changes in their ambient environment. After oviposition, changes in egg nuclei and shell layers may determine their preparation for subsequent diapause or immediate hatching (Hagiwara et al., 1995). The opsin-relevant genes in rotifers were conserved across species to sense luminescence (Kim et al., 2014). This suggests that a light-triggered molecular cascade would be available with *LuxR* involvement. In addition, among the analyzed genes (**Supplementary Table 1**), *Pcryo_1876* is one that functions as a component of heat-shock proteins (Hsp90, Anderson et al., 2010; García-Descalzo et al., 2011), protecting cells from elevated temperature (Cooper, 2000). In contrast, the *Sacsin* gene is a Hsp70 co-chaperone that may also be a biomarker in the autosomal recessive human disease spastic ataxia (Parfitt et al., 2009). Although sacsin has not yet been functionally characterized in invertebrates, it likely has chaperoning activity in response to temperature-triggered detrimental effects in *B. manjavacas*.

The largest number of isolated enriched genes came from eggs after 30 min of illumination. Most of these genes encode putative functional proteins of embryonic development (**Fig. 2**). One example is V-type H(+)-translocating pyrophosphatase (*V-PPase*), which is involved in plant cell growth and development (Sarafian et al., 1992). More specifically, the V-PPase is a unique

electrogenic proton pump that couples pyrophosphate (PPi) hydrolysis. The molecular function of the *V-PPase* gene is still unclear in aquatic invertebrates. However, it is known to play a critical role in insect embryogenesis and the hatching process. Acid hydrolases are able to degrade yolk granules with the proton pumping ability of V-PPase (Motta et al., 2009). In mice, the *meckelin* gene plays an important role in the intraciliary transport of phototransduction molecules during photoreceptor morphogenesis and maintenance (Collin et al., 2012) (**Table 2**). Acyl-CoA dehydrogenase (*ACAD*) catalyzes the initial step of fatty acid β-oxidation, the process of breaking long fatty acid chains into acetyl CoA molecules, in the mitochondria. Fatty acid oxidation was previously suggested to be involved in resting egg hatching (Hagiwara et al., 1995). Therefore, the transcriptional induction of the *ACAD* gene may be significant.

None of the genes associated with both light stimulation and embryonic development were expressed during 4h of illumination. In the 4h illuminated group, interestingly, the RecQ protein-like 5 (*RECQL5*) gene was significantly expressed. In *Escherichia coli*, the *RECQL5* gene functions in DNA repair and recombination after UV-light exposure (Courcelle and Hanawalt, 1999). Light is required for resting egg hatching. However, the light used in this study was more intense than that which would reach eggs resting on the bottom of estuaries. Therefore, it is likely that the experimental eggs expressed RECQL5 in order to protect themselves from the strong light. Similarly, the rotifer has a conserved, inducible defense system that involves replication of the protein A complex, and is used during UV irradiation (Kim et al., 2011).

Half of the genes that were enriched after 4h of illumination encode putative transporters and enzymes (**Fig. 2**). Many proton and chloride pumps are known to be light-driven transporters in relation to the rhodopsin metabolism (e.g., proton/sodium-glutamate symporter protein; gltT) (**Table 2**). Numerous studies have also suggested that light induces the expression of diverse enzyme-relevant genes. Given the importance of light for the expression of several genes, we hypothesized that it would similarly important for embryonic development and the subsequent hatching process in resting eggs.

Expression of oxidase related to mitochondrial activity initially appeared with light stimulation (**Table 1**). Mitochondria produce cellular energy (ATP) by oxidation, which also contributes to cellular metabolism, signaling, and the control of cell cycle and growth (Henze and Martin, 2003; McBride et al., 2006). Therefore, high levels of oxidase expression may indicate active embryonic development. Hagiwara et al. (1995) first suggested that a photochemical process may be involved in resting egg hatching. Hagiwara proposed that UV photolysis oxidizes polyunsaturated fatty acids (PUFA) in a resting embryo, which leads to the generation of prostaglandins and subsequent resting egg hatching. Acyl-CoA dehydrogenase is important for fatty acid metabolism, and oxidation (Ghisla and Thorpe, 2004; Leaver et al., 2011) (**Table 2**). Its expression was only observed with 30 min of light stimulation.

In this study, we investigated differential gene expression in resting rotifer eggs in response to three different illumination periods (but independent of time). The expression patterns of enzymes involved in fatty acid metabolism suggest that hatching is closely related to the photochemical process. An overall enriched gene set suggests that eggs are sensitive to light stimulation even in diapause which may trigger subsequently a molecular signaling cascade. However, further detailed studies are required to substantiate this hypothesis.

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Table 1

The transcriptional expression of putative enzymes. Numbers indicate the percentage of expressed enzyme transcripts among the total expressed transcripts (%) with regard to light exposure time.

Enzyme	0 min	30 min	4 h
Oxidase	0	43	17
Hydrolase	80	14	50

Table 2

Putative significant genes related to the resting egg hatching process. The score and E-value are results from BLASTX based on the NCBI database.

Lighting period	Accession number	Clone size (bp)	Gene product name or probable function	Score	E-value
0 min	ZP_06070805.1	570	*S*-formylglutathione hydrolase (*FGH*)	214	3E-54
	ZP_06067585.1	660	Glutathione *S*-transferase (*GST*)	137	7E-31
	YP_004126814.1	580	Regulatory protein *LuxR*	134	6E-30
30 min	ZP_04762976.1	680	V-type H(+)-translocating pyrophosphatase (*V-PPase*)	369	7E-94
	EAW83836.1	640	hCG2014340	213	1E-24
	ZP_05359921.1	500	Acyl-CoA dehydrogenase (*ACAD*)	165	3E-39
	NP_808529.2	730	Meckelin	68	7E-10
4 h	NP_001135548.1	680	RecQ protein-like 5 (*RECQL5*)	60	7E-16
	ZP_06065255.1	560	Proton/sodium-glutamate symporter protein (*gltT*)	164	5E-39

Figure legends

Fig. 1. Morphological observation related to the light exposure period (0 and 4 h). There were no morphological differences using microscopic observation between (A) a resting egg kept in total darkness (0 min) and (B) an egg exposed to light for 4h.

Fig. 2. GO classification for differentially enriched genes against three light exposure periods (0, 30 min, and 4 h). One hundred thirty six genes obtained by DDRT-PCR and sequencing methods were separated into several categories that are expected to have putative functions. The numbers located above the pie charts describe the lighting periods. Each area of the pie chart represents expression abundance of the gene indicated.

Fig. 3. A) Transcriptional levels of genes involved in resting egg dormancy and hatching in total darkness, 30 min, and 4h of illumination. B) Enlarged figure for *FGH* transcript level in total darkness, 30 min, and 4h illumination. The values are the means of three replicates. The *18S rRNA* gene was used as a reference gene to normalize the transcript level. Significant differences (*) of the 30 min and 4h illuminated groups against the total darkness sample were ascertained when $P < 0.05$.

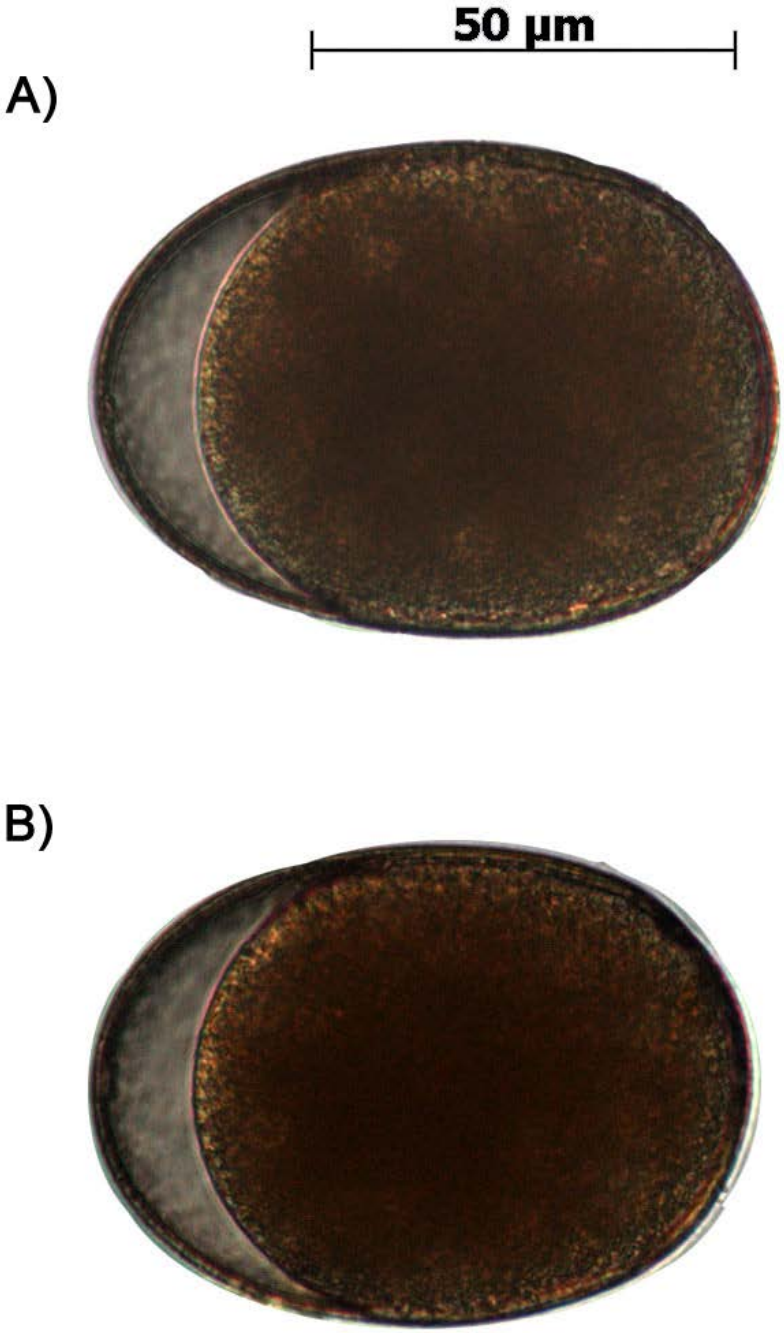

Fig. 1

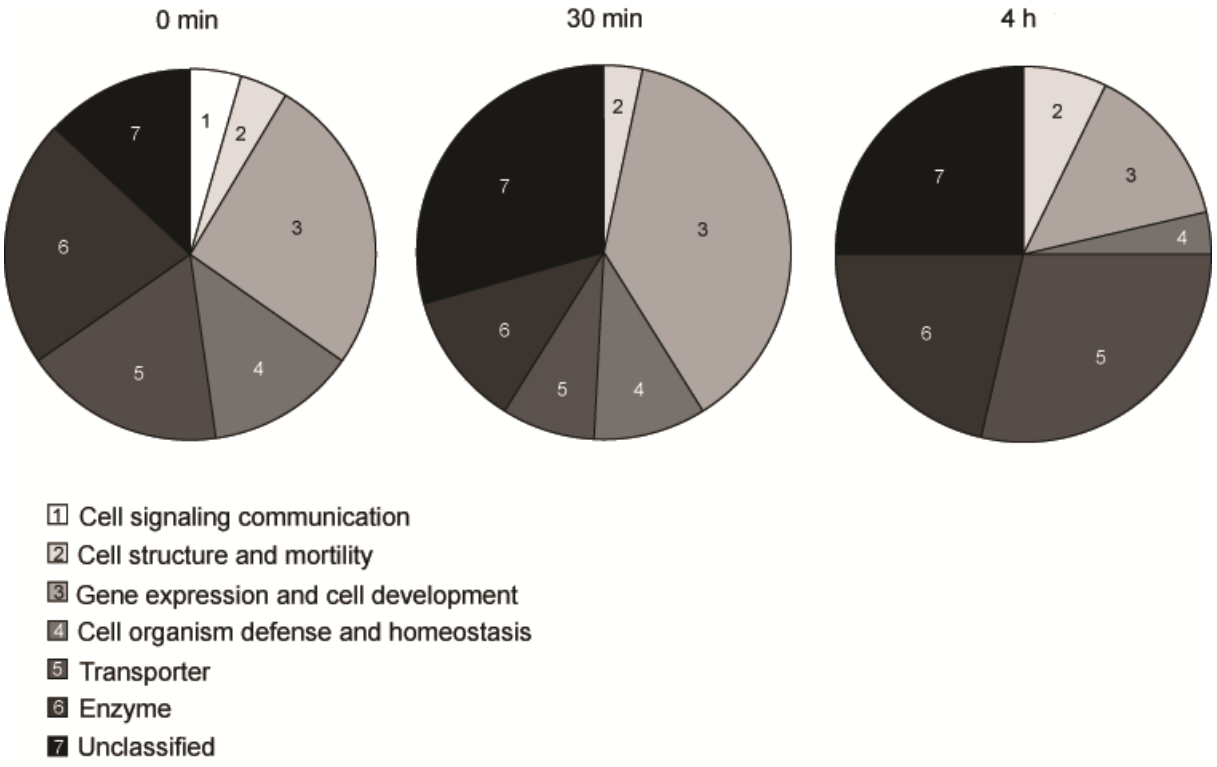
0 min
30 min
4 h
1
2
3
4
5
6
7
1 Cell signaling communication
2 Cell structure and mortility
3 Gene expression and cell development
4 Cell organism defense and homeostasis
5 Transporter
6 Enzyme
7 Unclassified

Fig. 2

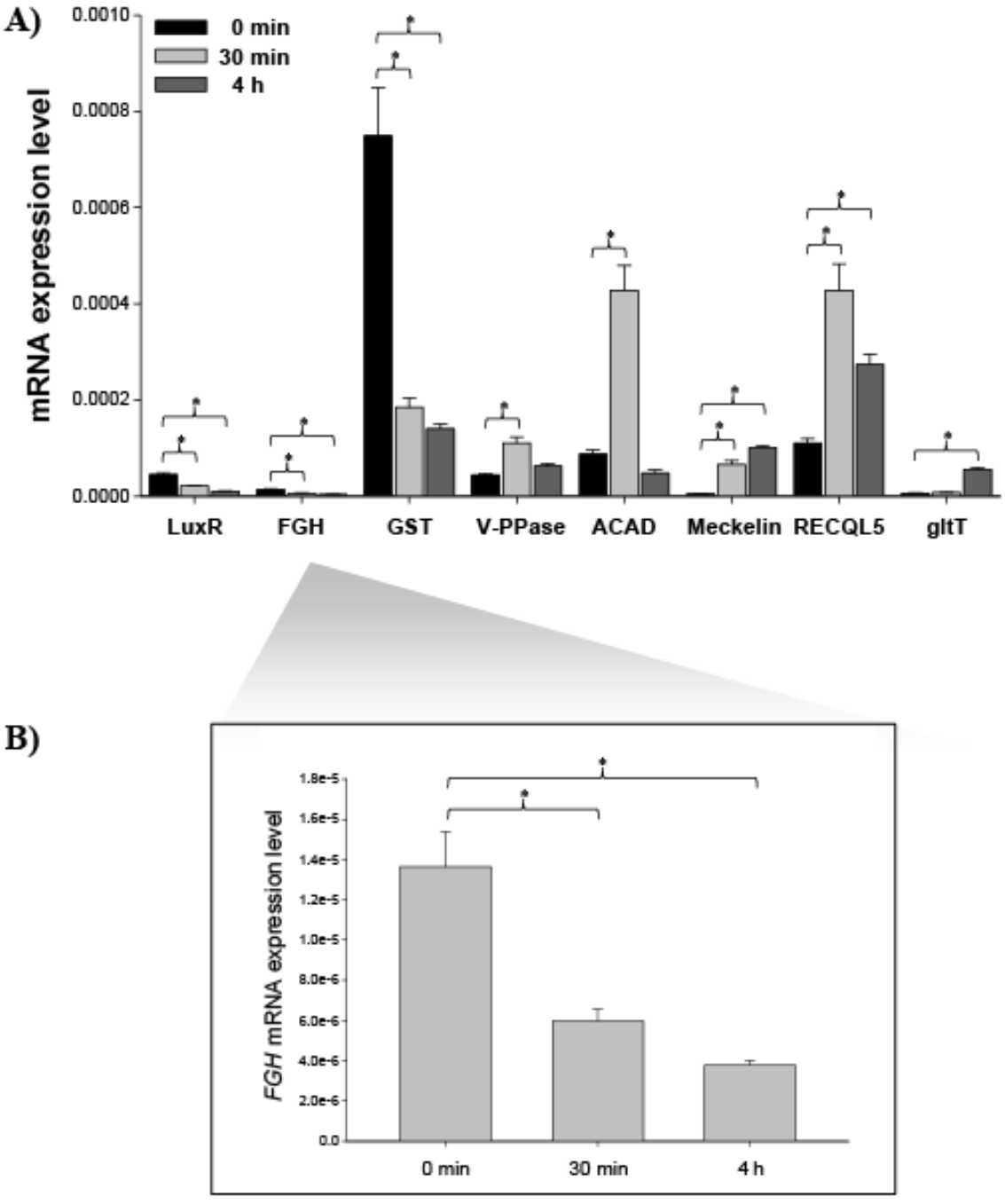
A)
0 min
30 min
4 h
mRNA expression level
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
LuxR
FGH
GST
V-PPase
ACAD
Meckelin
RECQL5
gltT
B)
FGH mRNA expression level
1.8e-5
1.6e-5
1.4e-5
1.2e-5
1.0e-5
8.0e-6
6.0e-6
4.0e-6
2.0e-6
0.0
0 min
30 min
4 h

Fig. 3

Supplementary Table 1. Annotation of transcripts identified using DDRT-PCR analysis.

Light exposure time	Accession No.	Putative function	Score	E value
0 min	ADZ11617.1	hypothetical protein RIA_0448	90.5	1.00E-16
	BAB33421.1	putative senescence-associated protein	189	8.00E-40
	ZP_06727215.1	TonB-dependent outer membrane ferric citrate receptor	221	1.00E-49
	XP_002425070.1	conserved hypothetical protein	77.8	6.00E-13
	YP_984277.1	extracellular solute-binding protein	83.2	1.00E-14
	ZP_03246583.1	FeS assembly protein SufB	114	1.00E-23
	ZP_05824154.1	multidomain protein	365	2.00E-99
	ZP_05823907.1	Permease	77.4	6.00E-13
	ZP_06063953.1	hypothetical membrane protein	103	1.00E-20
	CAR56929.1	hemagglutinin esterase	54.3	6.00E-06
	XP_002920712.1	PREDICTED: sacsin-like	130	6.00E-29
	XP_002739357.1	PREDICTED: hypothetical protein	138	3.00E-31
	YP_003126693.1	hypothetical protein Cpin_7091	134	3.00E-30
	YP_047633.1	putative GTPase	174	4.00E-42
	ZP_06062585.1	conserved hypothetical protein	97.8	6.00E-19
	ACA04850.1	senescence-associated protein	115	1.00E-35
	ZP_06063025.1	ABC-type nitrate/sulfonate/bicarbonate transport system protein	218	3.00E-55
	XP_002072862.1	GK13463	225	3.00E-57
	ZP_03698704.1	thioesterase superfamily protein	95.9	3.00E-42
	ACI33310.1	Transmembrane protein 127	71.6	6.00E-11

	ABO26659.1	peptidyl-prolyl cis-trans isomerase	125	3.00E-27
	YP_001231214.1	transposase, IS204/IS1001/IS1096/IS1165 family protein	89.7	1.00E-16
	BAC86222.1	unnamed protein product	140	8.00E-32
	ZP_06070805.1	S-formylglutathione hydrolase	214	3.00E-54
	CAR56929.1	hemagglutinin esterase	54.3	6.00E-06
	ZP_06067585.1	glutathione S-transferase	137	7.00E-31
	YP_004126814.1	regulatory protein LuxR	134	6.00E-30
	YP_581137.1	hypothetical protein Pcryo_1876	86.7	1.00E-15
	ZP_06071045.1	excinuclease ABC, A subunit	38.9	0.01
	NP_883401.1	hypothetical protein BPP1082	162	2.00E-38
	ACL54967.1	MADS FLC-like protein 3	57.8	5.00E-07
	ACL54966.1	MADS FLC-like protein 2	53.1	1.00E-05
	ZP_07045260.1	sulphate transporter	79.7	2.00E-13
	CAR56929.1	hemagglutinin esterase	55.8	4.00E-06
30 min	ADV40159.1	putative 60S ribosomal protein L5	152	2.00E-35
	CBZ53235.1	hypothetical protein NCLIV_030220	483	0.61
	ZP_04946149.1	hypothetical protein BDAG_02075	159	2.00E-37
	YP_003276378.1	ATP synthase F1, beta subunit	321	4.00E-86
	CAR56929.1	hemagglutinin esterase	54.3	1.00E-05
	YP_003122430.1	peptidase S41	205	2.00E-26
	NP_001127521.1	SNW domain-containing protein 1	171	8.00E-41
	YP_004154142.1	membrane protein	59.3	3.00E-07
	YP_001565978.1	alpha/beta hydrolase fold protein	76.3	3.00E-12
	ZP_06064153.1	branched chain amino acid ABC transporter permease	193	2.00E-47

ADV40159.1	putative 60S ribosomal protein L5	208	5.00E-52
ZP_06487984.1	hypothetical protein XcampvN_25860	76.3	3.00E-12
EFA06094.1	hypothetical protein TcasGA2_TC008936	122	9.00E-16
ADZ11617.1	hypothetical protein RIA_0448	90.9	1.00E-16
YP_004045765.1	hypothetical protein Riean_1098	92.8	3.00E-17
ABM53547.1	conserved hypothetical protein	113	3.00E-07
EFZ09311.1	hypothetical protein SINV_14484	53.5	5.6
EAW83836.1	hCG2014340	213	1.00E-24
EAW90874.1	hCG2024782	125	9.00E-21
CAR56929.1	hemagglutinin esterase	54.3	7.00E-06
XP_002608990.1	hypothetical protein BRAFLDRAFT_84796	65.9	2.00E-09
YP_970479.1	major facilitator superfamily transporter	155	2.00E-36
YP_004234128.1	hypothetical protein Acav_1643	150	8.00E-35
YP_001565451.1	porin	126	1.00E-37
XP_003085827.1	PREDICTED: probable ATP-dependent RNA helicase DDX5-like	122	2.00E-26
CAR56929.1	hemagglutinin esterase	52.8	2.00E-05
YP_305346.1	hypothetical protein Mbar_A1825	116	1.00E-24
ZP_03246583.1	FeS assembly protein SufB	114	9.00E-24
BAB33421.1	putative senescence-associated protein	163	1.00E-38
ZP_06067625.1	copper-translocating P-type ATPase	144	5.00E-33
XP_002723895.1	PREDICTED: hypothetical protein	107	6.00E-22
CAL64823.1	putative helicase	202	2.00E-50
XP_001887546.1	predicted protein	52	4.00E-05
ZP_06429925.1	branched-chain-amino-acid transaminase	221	3.00E-57

ZP_04762976.1	V-type H(+)-translocating pyrophosphatase	369	7.00E-94
YP_981935.1	membrane-bound proton-translocating pyrophosphatase	355	2.00E-89
YP_002326369.1	Rhs element Vgr family protein	196	1.00E-51
NP_808529.2	Meckelin	68.2	7.00E-10
ZP_03967762.1	conserved hypothetical protein	119	3.00E-25
AAW29072.1	DEAD box helicase PL10	287	5.00E-63
XP_001627306.1	predicted protein	287	5.00E-63
ZP_05133098.1	conserved hypothetical protein	117	1.00E-24
ZP_06061844.1	starvation-induced protein	212	4.00E-64
ZP_07745133.1	efflux transporter, RND family, MFP subunit	87	2.00E-23
AAZ39216.1	putative bacterial transcriptional regulator	224	5.00E-57
YP_003375424.1	GTP binding protein	221	1.00E-57
ZP_06066286.1	RumB protein	224	4.00E-57
ZP_06895378.1	ABC superfamily ATP binding cassette transporter, ABC protein	34.3	6.2
CAR56929.1	hemagglutinin esterase	54.3	6.00E-06
YP_003126495.1	DNA repair protein RecN	154	6.00E-36
AAO61995.1	nef attachable protein	91.7	5.00E-17
ZP_06064635.1	predicted protein	197	2.00E-50
YP_315413.1	transmembrane protein	130	8.00E-29
ZP_06844629.1	diguanylate cyclase	77.8	2.00E-24
ZP_06972937.1	hypothetical protein Krac_1658	186	5.00E-21
ZP_04765427.1	alpha/beta hydrolase fold protein	139	3.00E-31
YP_986353.1	cyclohexanone monooxygenase	333	4.00E-90
YP_003048632.1	general secretion pathway protein H	85.1	4.00E-15

	AAZ22389.1	phosphate transporter	127	9.00E-28
	ZP_06062492.1	Zn-dependent protease with chaperone function	111	2.00E-26
	ACH70991.1	ribosomal protein L8-2	142	4.00E-32
	YP_003749752.1	polygalacturonase	200	7.00E-50
	YP_996398.1	tRNA 2-selenouridine synthase	166	1.00E-43
	ZP_05359907.1	NADPH-dependent 7-cyano-7-deazaguanine reductase	68.6	3.00E-10
	ZP_06070795.1	predicted protein	74.3	5.00E-12
	ZP_07720640.1	protein-L-isoaspartate O-methyltransferase	112	3.00E-30
	CAL47051.1	transposase IS630	45.1	0.006
	ZP_05359921.1	acyl-CoA dehydrogenase	165	3.00E-39
4 h	NP_001187474.1	probable O-sialoglycoprotein endopeptidase	306	2.00E-68
	YP_002553509.1	ATP-dependent protease la	152	2.00E-35
	ZP_06063257.1	homoserine acetyltransferase	345	2.00E-86
	NP_001025761.1	ubiquitin carboxyl-terminal hydrolase BAP1	72.4	4.00E-11
	XP_002808753.1	ubiquitin transferase	72.8	1.4
	CAR56929.1	hemagglutinin esterase	54.3	1.00E-05
	YP_004241946.1	periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transporter	89.7	2.00E-16
	CAQ43070.2	putative puroindoline b protein	68.9	2.00E-10
	ADX94387.1	Transposase	111	4.00E-23
	YP_002945780.1	basic membrane lipoprotein	160	1.00E-37
	ZP_06064616.1	conserved hypothetical protein	264	4.00E-69
	ACO15045.1	3-oxoacyl-acyl-carrier-protein reductase	158	3.00E-37
	CAR56929.1	hemagglutinin esterase	54.3	7.00E-06
	CAY12635.1	SRY-related HMG box C protein	132	2.00E-29

YP_003126495.1	DNA repair protein RecN	150	9.00E-35
YP_001847243.1	ABC-type enterochelin transport system, permease component	205	1.00E-62
NP_001135548.1	RecQ protein-like 5	60.1	7.00E-16
EFN69699.1	Dynamin	210	5.00E-69
YP_003087805.1	efflux transporter, RND family, MFP subunit	139	1.00E-31
ZP_03544526.1	5-methyltetrahydropteroyltriglutamate/homocysteine S-methyltransferase	292	1.00E-77
YP_003977184.1	hypothetical protein AXYL_01125	327	4.00E-88
ZP_06337182.1	conserved hypothetical protein	100	1.00E-19
XP_002723895.1	PREDICTED: hypothetical protein	107	5.00E-22
CAR56929.1	hemagglutinin esterase	54.3	9.00E-06
XP_002647021.1	Hypothetical protein CBG24044	72	2.00E-24
ZP_06067821.1	predicted protein	109	1.00E-22
YP_003123007.1	transporter, hydrophobe/amphiphile efflux-1 (HAE1) family	273	8.00E-72
XP_968933.1	PREDICTED: similar to GA12251-PA	60.8	1.00E-07
ZP_04762460.1	major facilitator superfamily MFS_1	120	7.00E-26
ZP_08178243.1	hypothetical protein XVE_2176	92.8	3.00E-17
ZP_06057078.1	alpha/beta hydrolase fold family protein	207	1.00E-51
BAH37027.1	AT rich interactive domain 1D protein	64.7	6.00E-09
ZP_06063317.1	conserved hypothetical protein	121	4.00E-26
ZP_06065255.1	proton/sodium-glutamate symporter protein	164	5.00E-39

Supplementary Table 2. Information regarding primer sequence and amplification efficiency for each gene used in real-time RT-PCR.

Primer name	Primer direction	Sequence (5'→3')	Efficiency (%)
LuxR	Forward	CAG CAG CAC ATC AAA CTC GT	99.6
	Reverse	GTG CTG CTG ATT GAT GAC GA	
FGH	Forward	CTT CAA GAA AAT CAT CAG CAG TT	97.61
	Reverse	GTG AAA CCG AAA CTT TAT GGA	
GST	Forward	GTT ACG ATT GGC AAG GCA TA	103.25
	Reverse	ACT AAA TGC TGT ATG GTG TGC TC	
V-PPase	Forward	GCT TCA TTA CGC ATA TCT TCC	103.27
	Reverse	CGG TCA CTT CAC TTT GAT GTT	
ACAD	Forward	GTC ACT GGT ACC TTC ATA GAT TTG	98.69
	Reverse	TTT CCT CAG AGA TGG CAG AG	
Meckelin	Forward	TTT GCT AAA TAC GAC TAC GGT G	101.25
	Reverse	TTG GCA TTA CAT TCT ACG CA	
RECQL5	Forward	GAT TGC GTG CTT ATT GTA GAC T	102.54
	Reverse	CGT CCA TTT GAT TAT CCC TC	
gltT	Forward	AAG ATG GAG ATG AGC GAC GA	99.61
	Reverse	ACT GGT TTC TTC TGG TTT CTC G	