Eye-specification genes in the bacterial light organ of the bobtail squid *Euprymna scolopes,* and their expression in response to symbiont cues

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Abstract

The squid *Euprymna scolopes* has evolved independent sets of tissues capable of light detection, including a complex eye and a photophore or 'light organ', which houses the luminous bacterial symbiont *Vibrio fischeri*. As the eye and light organ originate from different embryonic tissues, we examined whether the eye-specification genes, *pax6*, *eya*, *six*, and *dac*, are shared by these two organs, and if so, whether they are regulated in the light organ by symbiosis. We obtained sequences of the four genes with PCR, confirmed orthology with phylogenetic analysis, and determined that each was expressed in the eye and light organ. With *in situ* hybridization (ISH), we localized the gene transcripts in developing embryos, comparing the patterns of expression in the two organs. The four transcripts localized to similar tissues, including those associated with the visual system ~1/4 into embryogenesis (Naef stage 18) and the light organ ~3/4 into embryogenesis (Naef stage 26). We used ISH and quantitative real-time PCR to examine transcript expression and differential regulation in postembryonic light organs in response to the following colonization conditions: wild-type, luminescent *V. fischeri*; a mutant strain defective in light production; and as a control, no symbiont. In ISH experiments light organs showed down regulation of the *pax6, eya*, and *six* transcripts in response to wild-type *V. fischeri*. Mutant strains also induced down regulation of the *pax6* and *eya* transcripts, but not of the *six* transcript. Thus, luminescence was required for down regulation of the *six* transcript. We discuss these results in the context of symbiont-induced light-organ development. Our study indicates that the eye-specification genes are expressed in light-interacting tissues independent of their embryonic origin and are capable of responding to bacterial cues. These results offer evidence for evolutionary tinkering or the recruitment of eye development genes for use in a light-sensing photophore.

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1. Introduction

Research on visual systems has revealed remarkable conservation of eye-associated genes throughout much of the animal kingdom. Among such genes are *pax6* (paired box gene 6), *eya* (eyes absent), *six* (sine oculis), and *dac* (dachshund); transcription factors that interact in a regulatory network (Fig. 1A) and are critical for eye morphogenesis (Donner and Maas, 2004). Among other locations, these genes are expressed in both simple and complex eyes of diverse taxa, and are often referred to as "eye-specification genes" (e.g. Kumar and Moses, 2001). For example, the well-studied gene *pax6*, is found in animals with simple pigment-cup eyes (e.g., *Platynereis dumerilii*, Arendt et al., 2002), invertebrate compound eyes (e.g., *Drosophila* sp., Halder et al., 1995; Gehring and Ikeo, 1999), and vertebrate camera eyes (e.g., *Mus musculus*, Donner and Maas, 2004). Although eyes have been studied at the level of gene expression and in an evolutionary context (e.g., see Spady et al., 2005; Harzsch et al., 2006; Porter et al., 2012), few such studies have focused on photophores, light-emitting organs that have ocular attributes (but see Tong et al., 2009; Schnitzler et al., 2012).

The Hawaiian bobtail squid, *Euprymna scolopes* (Fig. 1B), is a model invertebrate species with complex eyes and a photophore or 'light organ' (Fig. 1C) that houses the luminous bacterial symbiont *Vibrio fischeri*. The light emitted by *V. fischeri* matches down-welling moonlight and starlight, and camouflages the squid while active at night in an anti-predatory phenomenon called counter-illumination (McFall-Ngai and Ruby, 1991; Jones and Nishiguchi, 2004; see also Johnsen et al., 2004). The bacterial symbionts, which are harvested anew each generation, enter through pores on either side of the light organ, and ultimately reside along the apical surfaces of polarized epithelia in the crypt spaces (Fig. 1D). Striking anatomical, biochemical, molecular, and physiological similarities exist between the eye and light organ. These similarities include a lens with crystallin proteins (Montgomery and McFall-Ngai, 1992), an analog of the tapetum with 'reflectin' proteins (Crookes et al., 2004), genes and proteins involved in phototransduction (Tong et al., 2009), and the physiological ability to respond to light (Tong et al., 2009). In addition, the ink sac, which surrounds a portion of the light organ, functions as both an iris and a choroid (McFall-Ngai and Montgomery, 1990). Such features in the light organ are thought to enable *E. scolopes* to detect and, in turn, control the light emitted by *V. fischeri*.

Although the eyes and light organ have notable similarities, they also have key differences. The eyes and light organ are not homologous, developing from ectoderm and mesendoderm, respectively (Montgomery and McFall-Ngai, 1993). The organs also develop at slightly different stages during embryogenesis (Table 1). In addition, light stimuli for the eyes and light organ come from different sources, environmental light and the luminous bacterial symbiont, respectively. Light stimulus is important in the development of both sets of tissues. Environmental light contributes to maturation of the vertebrate eye (e.g., see Grün, 1979; Tian 2004), and therefore might also influence squid eye development. Endogenous

light serves as a critical cue for morphogenesis of the *E. scolopes* light organ (Visick et al., 2000; Koropatnick et al., 2007).

In addition to luminescence, postembryonic development of the light organ depends on other morphogenic signals presented by *V. fischeri*, which are also molecules associated with animal immune responses. For example, the microbe-associated molecular patterns (MAMPs), specifically the cell-envelope molecules lipopolysaccharide (LPS) and tracheal cytotoxin (TCT), induce morphological changes throughout the first several days of development. These changes include apoptosis within and regression of the juvenile-specific light-organ appendages and ciliated ridges (Fig. 1D), which are surface tissues that facilitate symbiont colonization of the host (Foster et al., 2000; Koropatnick et al., 2004). In other host-microbe associations, immune-related responses contribute to development of host tissues with which the microbes interact (Stappenbeck et al., 2002; Bouskra et al., 2008). Thus, the *E. scolopes-V. fischeri* system offers an opportunity to examine the effects of microbes on the development of ocular-like tissues in the light organ.

We examined whether the eye-specification genes, *pax6*, *eya*, *six*, and *dac*, are expressed in the *E. scolopes* light organ during embryogenesis and whether they are regulated by symbiosis during early postembryonic development. After identifying the four genes in the eye and light organ, we quantified gene expression and differential regulation in the light organ in response to bacterial cues, including luminescence. Our study suggests that the eye-specification genes are present during development of light-interacting tissues, independent of their embryonic origin, and that these genes respond to bacterial cues, including light, that mediate morphogenesis of the light organ.

2. Experimental Procedures

2.1. Sample preparation and generation of full-length cDNA sequences

We used embryos and juveniles produced from mature, wild-caught *E. scolopes,* which were captured and maintained as previously described (Montgomery and McFall-Ngai, 1993). We stored excised tissues in RNAlater (Life Technologies) at −80°C until use in experiments. Unless otherwise noted, we used a TRIzol Reagent protocol (Life Technologies) for RNA extractions. We determined RNA concentration with a NanoDrop ND-1000 spectrophotometer and tested the quality of the RNA by agarose gel electrophoresis. We removed any contaminating DNA by DNase treatment with Ambion TURBO DNase Kit (Life Technologies).

Although annotation of a previously constructed EST database provided evidence that the *pax6*, *eya*, *six*, and *dac* transcripts are expressed in light-organ tissues (Chun et al., 2006), we sought to amplify full-length cDNA of all four genes from both eye and light-organ tissues. The full-length *pax6* cDNA sequence in *E. scolopes* had been determined previously from mRNA expressed in the eye, but not from light-organ mRNA (Hartmann et al., 2003). Therefore, we used standard PCR with primers designed to the eye sequence (Table S1) to determine if the same *pax6* gene was also present in the light organ. We used RACE-PCR (Rapid Amplification of cDNA Ends – PCR; Invitrogen GeneRacer Kit, Life Technologies) with gene-specific primers designed to the candidate light-organ sequences to generate the

eya, *six*, and *dac* cDNA sequences (Table S1). To determine whether the *eya*, *six*, and *dac* genes were present in the eye, we used standard PCR with primers designed to sequences known from light organs (Table S1). RNA extracted from ~50 juvenile light organs and 25 eyes was sufficient for the production of RACE-ready cDNA. For additional details see Supplementary Material section S1.1.

2.2. Sequence analysis and phylogenetic reconstructions

Analysis of the derived Pax6, Eya, Six, and Dac protein sequences allowed us to determine whether functional domains are conserved in *E. scolopes*. We aligned each *E. scolopes* protein sequence with those of other well-studied exemplar species from the Deuterostomia, Ecdysozoa, and Lophotrochozoa superphyla. The alignments were generated using CLC Sequence Viewer software and analyzed for domain structure using the Pfam website (http://pfam.sanger.ac.uk/). We next conducted phylogenetic analyses to test hypotheses of orthology between *E. scolopes* proteins and previously published eye-specification proteins. See Supplementary Material section S1.2 for detailed methods.

2.3. Whole-mount *in situ* hybridization

We examined expression patterns of the *pax6*, *eya*, *six*, and *dac* gene transcripts in *E. scolopes* during embryogenesis and in the light organ during early postembryonic development. For both sets of experiments, we used whole-mount colorimetric *in situ* hybridization (ISH). Details involving specific ISH steps are provided in the Supplementary Material section S1.3 and more briefly here as follows:

Embryonic development—We examined whether the four gene transcripts could be detected in both the eye and light organ of developing *E. scolopes* embryos. The embryonic stages of development spanned eye and light organ morphogenesis (Naef stages 18, 22, 26, 29, Table 1; Arnold et al., 1972; Lee et al., 2009). To minimize variation in expression patterns due to genetic effects, we tested each gene using samples from a single clutch in this and all subsequent ISH experiments. We performed two replicate experiments each using 6 to 10 embryos per condition per gene (*N*=14 to 19 total embryos per condition per gene). Reverse transcription-PCR (RT-PCR) confirmed the presence of each transcript in the various tissues at each of the four embryonic stages.

Postembryonic development—We also examined whether expression of the four gene transcripts in the postembryonic light organ were affected by *V. fischeri* cues. We distributed juvenile squid among four groups: newly hatched, and aposymbiotic (uncolonized) and symbiotic [colonized with either wild-type *V. fischeri* or with a *V. fischeri* mutant defective in light production, *lux* (Bose et al., 2008)] after 24 or 72 h of development (Fig. 1D). By convention, we use the term aposymbiotic and symbiotic to refer to animals exposed to environmental bacteria in the absence and presence of *V. fischeri*, respectively. To determine the effect of bacterial light on gene expression, we compared light organs colonized by wild-type *versus lux V. fischeri*. To determine the effect of bacterial cues other than light production on gene expression (e.g., MAMPs), we compared light organs colonized with wild-type and *lux V. fischeri versus* those left uncolonized. The bacterial colonization protocol was performed as described in a previous study (Ruby and Asato, 1993) using

5,000 colony-forming units of *V. fischeri* per mL of artificial seawater. We performed two replicate experiments (*N*=14 to 19 total juvenile light organs per condition per gene).

From preserved juvenile squid we used excised light organs for whole-mount ISH. Following probe-signal development, for each gene, we simultaneously terminated the reactions to determine if transcript expression differed statistically among the four experimental colonization conditions. Specifically, we examined transcript expression in the surface tissues of the light organ, including the appendages and ciliated ridges of the superficial ciliated epithelium, and the pores (see Fig. 1D). We scored labeling of each transcript in each light-organ tissue as presence or absence of signal. Because gene expression by ISH can be difficult to quantify, we did not attempt to score levels of signal other than presence or absence.

2.4. Sectioned light-organ *in situ* hybridization

Within the light organ, the crypt epithelia are the direct recipient tissues of *V. fischeri* MAMPs and luminescence. We were interested in whether the four gene transcripts localized to these tissues and whether they might be affected by such cues. By whole-mount ISH, the crypts are difficult to view. Therefore, we performed ISH with sectioned animals to test for transcript signal in the crypts. Preserved juvenile squid were embedded in paraffin wax, sectioned (5 μm), and mounted on slides at the University of Wisconsin, School of Veterinary Medicine Histology Laboratory. We performed two replicate experiments (*N*=12 to 18 total light organs per condition per gene). See Supplementary Material section S1.3 for remaining details.

2.5. Quantitative Real-Time PCR

We examined the *pax6*, *eya*, *six*, and *dac* transcripts by quantitative real-time PCR (qRT-PCR) to determine if differential regulation throughout the whole light organ was affected by symbiosis with *V. fischeri*. The qRT-PCR steps were performed following MIQE guidelines (Bustin et al., 2010). We collected juveniles from several clutches and divided them equally among four groups: newly hatched, 24-h aposymbiotic, and 24-h symbiotic colonized by either wild-type or *lux V. fischeri*. To obtain five independent biological replicates, we performed these collections on five independent days. Symbiotic animals were colonized as noted previously in section 2.3 with wild-type or *lux V. fischeri*. The qRT-PCR reactions of interest contained cDNA from the four light-organ colonization conditions: hatchlings, aposymbiotic, symbiotic with wild-type *V. fischeri*, and symbiotic with *lux V. fischeri*, each with five biological replicates and three technical replicates. We designed primers (see Table S1) such that amplicon sizes ranged from 81 to 188 bp and efficiencies ranged from 97 to 102%. The PCR reactions were run with a BioRAD CFX connect as follows: (Step 1) 95°C for 3 min; (Step 2) 95°C for 10 s, 60°C for 10 s, 68°C for 15 s ('Step 2' for 40 cycles); and (Step 3) 95°C for 10 s followed by a temperature gradient from 65°C to 95°C at 5°C intervals, 5 s per interval. In addition to the four eye-specification genes, we performed similar qRT-PCR reactions for two housekeeping genes, *ribosomal 40s* and *serine HMT* (Table S1). We normalized the expression of each eye-specification gene to the geometric mean of the expression of the two housekeeping genes. We used the

comparative method (C_q method) (Pfaffl, 2001) to analyze the data. For additional methods see Supplementary Material section S1.4.

2.6. Analysis of gene transcript expression and regulation data in the light organ

We used the statistical package R (Core Development Team, 2008) to test whether gene transcript expression (whole-mount ISH) and differential regulation (qRT-PCR) in the light organ differed among experimental colonization conditions. We used a Fisher Exact Test to test whether the number of light organs indicating presence *versus* absence of transcript signal at 24 h post hatching depended on colonization condition (see ISH data). This analysis provided a statistical means for quantifying the effect of bacterial cues on gene expression in light-organ surface tissues (i.e., anterior appendages, posterior appendages, ciliated ridges, pores). The model for gene expression in a given tissue type, E_t, as the dependent variable was:

$$E_t = \beta_0 + \beta_1 x_c,$$

where colonization condition (x_c) was the independent variable. We used an analysis of variance (ANOVA) to test whether differential regulation of each eye-specification gene depended on colonization condition and replicate (see qRT-PCR data). The model for normalized expression, R, as the dependent variable was:

$$R = \beta_0 + \beta_1 x_c + \beta_2 x_r,$$

where independent variables were colonization condition (x_c) and replicate (x_r). For each gene, x_r represented the five independent biological replicates, each consisting of the three pooled, technical replicates. For both tests, maximum likelihood parameter estimates were β_0, β_1, and β_2. All variables were treated as fixed effects. We performed Tukey pairwise comparisons for E_t and R between colonization conditions following the Fisher Exact Test and ANOVA, respectively.

3. Results

3.1. Conservation of eye-specification genes in the eye and light organ

Our experiments yielded evidence for full-length transcripts of the four eye-specification genes, *pax6*, *eya*, *six*, and *dac*, to be expressed in both the eye and light organ of *E. scolopes*. The predicted proteins contained conserved domains and functional amino acid residues characteristic of the eye-specification proteins (Fig. S1). In addition, phylogenetic analyses indicated orthology with known eye-specification genes (Figs. S2–S6). See also Supplementary Material sections S2.1 and S2.2.

3.2. Localization of gene transcripts throughout development

We observed transcript expression throughout embryonic and postembryonic development. During embryogenesis the eye develops at an earlier stage than the light organ (Table1). Thus, we predicted that the eye-specification gene transcripts would localize to the region of the head and eye early in development and to the light organ later in development.

Under the conditions of these experiments, the eye-specification genes generally showed patterns of expression similar to one another in developing embryos, although there was variation in the timing of labeling. By stage 18 (~1/4 of embryogenesis) the eye-specification gene transcripts localized to the eye primordia, and across the skin, including the tentacles and the shell gland, which gives rise to the mantle (Fig. 2, stage 18). We also observed transcript expression in the statocysts, funnel folds, and gill primordia (Fig. 2, stage 18). At stage 22 (~1/2 of embryogenesis) all four transcripts localized to the eye, optic lobe, nuchal organ, mantle, and except for *eya*, in the tentacles and fins (Fig. 2, stage 22). At stage 26 (~3/4 of embryogenesis) labeling was present in the optic nerve for the *pax6* and *six* transcripts, and in the olfactory organ for the *pax6*, *six*, and *dac* transcripts (Fig. 2, stage 26 dorsal and ventral views). In addition, all transcripts were detectable in the nuchal organ, mantle, distal tentacles, optic lobe, funnel, and fins (Fig. 2, stage 26 dorsal and ventral views). The sense controls showed very low or no detectable labeling (Fig. S7).

The four eye-specification gene transcripts also localized to the light organ (Fig. 3). Although development of the light organ begins at about stage 22 (Table 1), expression of the *pax6*, *six*, and *dac* transcripts was first discernable by ISH at stage 26 in the pores (Fig. 3, stage 26). At stage 26 labeling of the *eya* transcript was diffuse across the light organ and the different tissues were difficult to distinguish (Fig. 3, stage 26). By stage 29 all four transcripts localized to the anterior appendages (Fig. 3, stage 29). The *pax6*, *six*, and *dac* transcripts remained visible in the pores (Fig. 3, stage 29). In addition, the *pax6* and *dac* transcripts were expressed in the ciliated ridges (Fig. 3, stage 29). Upon hatching, the four transcripts were detectable in the anterior and posterior appendages, around the pores, and except for *eya*, in the ciliated ridges (Fig. 3, Hatchlings). The sense controls showed undetectable labeling of these tissues (Fig. S8).

3.3. Effect of symbiosis and symbiont light production on eye-specification genes

In response to symbiosis, the eye-specification genes showed loss of expression in surface tissues of the light organ, although the region of attenuation varied among the genes. In addition the genes differed in their response to *V. fischeri* cues. Scoring for the presence/absence of detectable labeling, we observed significant attenuation of labeling (i.e., no detection above background) of the *pax6* transcript in symbiotic light organs, both wild type and *lux V. fischeri*, relative to those aposymbiotic (Fisher Exact Test: *P*<0.00001 all tissue types) (Figs. 4, 5, comparisons 1, 2). Wild-type and *lux V. fischeri* colonized animals did not differ significantly from one another in *pax6* expression (Fig. 5, comparison 3). These data indicate an effect of the bacterium (e.g., MAMPs) on *pax6* expression. Similar to the *pax6* transcript, the *eya* transcript showed attenuation of labeling in symbiotic light organs with both wild-type and *lux V. fischeri* relative to those aposymbiotic, but with statistical significance only for the anterior (Fisher Exact Test: *P*<0.00001) and posterior appendages (Fisher Exact Test: *P*=0.00082) (Figs. 4, 5, comparisons 4, 5). Wild-type and *lux V. fischeri* colonized animals did not differ significantly from one another in *eya* expression (Fig. 5, comparison 6).

In contrast, we observed significant attenuation of labeling of the *six* transcript in light organs symbiotic with wild-type, but not *lux V. fischeri*, relative to those aposymbiotic

(Fisher Exact Test: Anterior appendages, *P*<0.00001; posterior appendages, *P*<0.00001; ciliated ridges, *P*=0.0003) (Figs. 4, 5, comparisons 7, 9). This trend occurred in all tissues except for the pores. Aposymbiotic light organs and those colonized with *lux V. fischeri* did not differ significantly from one another in *six* expression in the appendages and ciliated ridges (Fig. 5, comparison 8). Overall, these results indicate an effect of the light production by *V. fischeri* on *six* expression.

Finally, the *dac* transcript showed a trend toward attenuated labeling in aposymbiotic light organs and those symbiotic with wild-type, but not *lux V. fischeri*. However, this trend was only statistically significant at the pores (Fisher Exact Test: *P*=0.0056) (Figs. 4, 5, comparisons 11, 12). Aposymbiotic light organs and those symbiotic with wild-type *V. fischeri* did not differ significantly at the pores among colonization conditions (Fig. 5, comparison 10).

In addition to whole-mount ISH, we performed ISH on sectioned animals to determine if the eye-specification gene transcripts were expressed in the light-organ crypts that directly interact with *V. fischeri*. Using ISH on sectioned animals, all eye-specification gene transcripts localized to the apical surfaces of the crypt epithelia at 24 h post hatching (Fig. 6). However, the presence of transcript signal was not noticeably altered by symbiosis. The four gene transcripts continued to be expressed in the crypt epithelia at 72 h post hatching (Fig. 7), suggesting that these genes serve a role in the tissues where phototransduction develops for at least several days post hatching.

Transcriptomic studies of the juvenile light organ indicate that certain genes regulated by symbiosis can be detected when whole light organs are sampled (Chun et al., 2008). Thus, we sought to use qRT-PCR to determine whether changes in the eye-specification gene transcripts could be detected by this additional method. However, differential regulation of the *pax6*, *eya*, and *six* transcripts did not differ significantly among the different colonization conditions (ANOVA: *pax6*, *F*=0.20, d.f.=3, *P*=0.90; *eya*, *F*=2.6, d.f.=3, *P*=0.099; *six*, *F*=2.2, d.f.=3, *P*=0.14) (Fig. 8). In contrast, differential regulation of the *dac* transcript differed significantly among colonization conditions over the whole light organ (ANOVA: *F*=5.6, d.f.=3, *P*=0.012) (Fig. 8). The *dac* transcript was significantly up regulated in light organs colonized by *lux V. fischeri* compared to light organs of newly hatched juveniles (Tukey: *P*=0.0075). Thus, the *dac* gene might be involved in differential regulation of other host responses to bacterial light production (Chun et al., 2008).

4. Discussion

The results of this study indicated that four genes critical for eye development, *pax6*, *eya*, *six*, and *dac*, are expressed in both the eye and light organ of *E. scolopes*. The sharing of these genes between the two non-homologous sets of tissues offers evidence for evolutionary tinkering or co-option of a regulatory network at the developmental level (Jacob, 1977; Jacob, 2001; Monteiro, 2011). Within the light organ, gene expression patterns depended on different cues produced by the bacteria. Such differential responses suggest that the eye-specification genes are pivotal members of a greater network of genes regulated by *V. fischeri* cues, including its luminescence, that affect development of the light organ.

Although all four genes have been found in other mollusks, none have been isolated in a photophore. In addition, only *pax6* has been described in other cephalopods, including *Doryteuthis opalescens*, *Sepia officinalis*, and *E. scolopes* (Table 2). Thus, this study is the first to identify all four genes in a photophore and to describe the *eya*, *six*, and *dac* genes in a cephalopod.

4.1. Eye-specification genes through embryonic development

In general, the eye-specification gene transcripts were expressed in *E. scolopes* tissues and organs with shared homology to other organisms, especially other cephalopod mollusks (Fig. 2, Table 2). For example, in addition to being expressed in eye-associated tissues (e.g., eye, optic lobe; Fig. 2, Table 2), the four transcripts were expressed within other sensory-related tissues of *E. scolopes* similar to those of other organisms (e.g., olfactory organ *versus* nasal placode, statocysts *versus* inner ear; Fig. 2, Table 2). In the skin of *E. scolopes* (e.g., over the mantle), the four transcripts might be involved in development of the chromatophores, a group of light-interacting structures that are visible by late embryogenesis (~stages 26 to 30), or 'photosensitive vesicles', which occur in the mantle tissue of cephalopods (Young, 1991). Alternatively, there could be other features of the mantle that are regulated by these genes.

Our study also revealed that the eye-specification genes are present in the light-sensing photophore, or light organ, of *E. scolopes* (Fig. 3). Whereas many animal phyla have autogenic photophores, in which the animal produces the substrates for light production, only cephalopods, fishes, and a tunicate species have bacterial photophores (Herring, 1978). While both types of photophores also feature ocular structures similar to those in the eyes (e.g., anatomical, biochemical, molecular, physiological; Montgomery and McFall-Ngai, 1992; Dove et al., 1993; Nowell et al., 1998; Herring et al., 2002; Cavallaro et al., 2004; Crookes et al., 2004; Tong et al., 2009; Claes et al., 2011a; Claes et al., 2011b), to our knowledge, our study provides the first evidence that the eye-specification genes are present in a photophore. Shared expression between the eye and light organ in *E. scolopes* suggests that these genes are important for development of light-interacting tissues regardless of their embryonic origin, as the eye and light organ develop from ectodermal and mesendodermal tissues, respectively.

Because the eye-specification genes are associated with development of eyes of distantly related animals, and because the light organ is of much more recent origin (Lindgren et al., 2012), we can infer recent co-option of these genes for use in development of photophores. In addition to the eye-specification genes, phototransduction genes and proteins are identical in the eye and light organ even though the two structures serve different organismal functions (Tong et al., 2009). Whereas in the eyes these proteins respond to environmental light, in the light organ the same proteins likely respond to symbiont light production. The co-option of the eye-specification genes might drive the expression in the light organ of the phototransduction genes, which leads to physiological light responses (Tong et al, 2009).

Our ISH results on sectioned samples showed expression of the eye-specification gene transcripts within the crypt epithelia that are internal to the light organ and directly exposed to *V. fischeri* cues (Figs. 6, 7). Such expression might indicate that these genes are poised for

use in the development of phototransduction for perceiving the light emitted by *V. fischeri* (Tong et al., 2009). Later in development, the light organ lens forms, which is used for focusing light (Montgomery and McFall-Ngai, 1992; Weis et al., 1993). As the *pax6* and *six* genes are essential in the development of the lens in other organisms (Liu et al., 2006; Huang and Xie, 2010), these genes might also be employed for the same purpose in the *E. scolopes* light organ.

4.2. Expression of the eye-specification genes in response to bacterial cues

As the juvenile *E. scolopes* develops postembryonically, discrete surface regions of the light organ undergo distinct morphological changes for which the eye-specification genes could serve a role. Such morphogenesis is accelerated by the presence of *V. fischeri* (Montgomery and McFall-Ngai, 1994; Doino Lemus and McFall-Ngai, 2000). For example, cells of the anterior and posterior appendages experience apoptosis. This apoptosis leads to regression of the appendages and receding of the ciliated ridges, a process that in wild-type colonized juveniles is visible within 24 h and largely completed within several days post hatching (Fig. 1D). In addition, an initial set of three pores per side of the juvenile light organ eventually coalesces into one pore per side in the adult (McFall-Ngai and Montgomery, 1990). The adult pores and the entire musculature are used in a venting behavior to control the resident population of *V. fischeri* in the bi-lobed light organ. In the absence of wild-type *V. fischeri*, the normal morphological changes in these localized surface tissues either fail to occur, such as in aposymbiotic light organs, or proceed at a delayed rate, as in light organs colonized by *lux* *V. fischeri* (McFall-Ngai et al., 2012).

Our whole-mount ISH results suggested that expression of the *pax6* and *eya* transcripts in surface tissues is affected by *V. fischeri* cues other than light production (Figs. 4, 5; aposymbiotic *versus* symbiotic, and aposymbiotic *versus* *lux*). Such cues likely include microbe-associated molecular patterns (MAMPs), which induce light-organ morphogenesis (Foster et al., 2000; Koropatnick et al., 2004). In addition, MAMPs are potent inducers of immune responses that are conserved across the animal kingdom. Although studies involving the eye-specification genes in host-microbe associations are rare, our findings are in concordance with immune-related literature. For example, proliferation activity and expression of the Pax6 protein is reduced in the cortical progenitors of mouse embryos (*Mus musculus,* stage E17) following a maternal Poly I:C immunostimulant injection, impacting Pax6-related neurological development *in utero* (Soumiya et al., 2011). In the Eya protein, threonine-phosphatase activity at the N-terminus regulates immune responses to undigested DNA from apoptotic cells (Okabe et al., 2009; Sander and Blander, 2009). Determining whether analogous residues in the *E. scolopes* Eya protein are activated during programmed cell death, DNA fragmentation, and regression of the light-organ appendages would be intriguing, although beyond the scope of this study.

Although the *pax6* and *eya* transcripts could be down regulated in direct response to bacterial cues, such an effect could also be triggered by regulators of *pax6* and *eya*. The *pax6* gene is known to auto-regulate and to affect the expression of other genes (e.g., *eya*, *six*, *dac*; Fig. 1A). In addition, the *notch* gene is a known regulator of *pax6* expression (Kumar and Moses, 2001; Baker, 2001; Onuma et al., 2002; Mu and Klein 2004), and Notch

signaling can control apoptosis (e.g., *Drosophila*; Koto et al. 2011; Koto and Miura, 2011) and play a key role in inflammatory responses (Kim et al., 2008; Cao et al., 2011). Of particular interest, cultured cells exposed to the MAMP lipopolysaccharide (LPS) exhibit suppressed Notch signaling, and in turn, induce macrophage activity (Kim et al., 2008). Inhibition of Notch signaling also correlates with the production of inflammatory cytokines (e.g., microglial cells; Cao et al., 2011). Previous studies of *E. scolopes* show LPS to be an essential MAMP that initiates postembryonic development of the light organ in which the appendages undergo regression though cell apoptosis (Foster et al., 2000; Koropatnick et al., 2004). During this process, components of the inflammatory response, including peroxidase, nitric oxide, and NFκB occur in the light organ (Weis 1996; Davidson 2004; Goodson et al., 2005). For example, the presence of *V. fischeri* MAMPs are required for a symbiosis-induced attenuation of nitric oxide in light-organ tissues (Altura et al., 2011). In *E. scolopes*, Notch signaling might also be inhibited in response to *V. fischeri* LPS, enabling inflammatory reactions and apoptosis to occur in the appendages, accompanied by down regulation of the *pax6* and *eya* genes in these tissues. Although testing this prediction would require further investigation, we have identified a candidate *notch-delta* sequence in the annotated EST database of the *E. scolopes* light organs.

In contrast to the *pax6* and *eya* transcripts, we found down regulation of the *six* transcript in response to wild-type, but not *lux V. fischeri* (Figs. 4, 5; aposymbiotic *versus* wild-type *V. fischeri*, and *lux versus* wild-type *V. fischeri*), suggesting that the *six* transcript responded to bacterial luminescence. As mentioned previously in this section, in the absence of wild-type *V. fischeri* the light organ fails to develop at a normal rate. Although regression of the appendages occurs in light organs colonized with *lux V. fischeri*, the process proceeds more slowly. Failure of the *six* gene to be down regulated in the appendages of light organs colonized with *lux V. fischeri* (Figs. 4, 5) might contribute to such delayed regression. As the four eye-specification genes affect one another in a regulatory network (Fig. 1A), down regulation of *pax6* and *eya* expression in the appendages might, with time, induce down regulation of *six* expression, allowing for the eventual regression of the appendages of light organs colonized with *lux V. fischeri*. Interestingly, in the presence of *lux V. fischeri*, a symbiotic association is initiated with *E. scolopes*, but does not persist (McFall-Ngai et al., 2012; Heath-Heckmann et al., 2013), suggesting that luminescence, and possibly proper regulation of the eye-specification genes, are critical for the maintenance of the partnership.

Our ISH and qRT-PCR results were not in concordance. This discrepancy might have resulted from opposing developmental or gene-regulatory processes occurring on the surface *versus* internal crypt epithelia of the light organ. Whole-mount ISH detected the regulation of eye-specification genes in discrete regions of the surface epithelia of postembryonic light organs (Figs. 4, 5), which represent ~10% of the tissue of the whole organ. Discrete regions of the light organ are difficult to subsample for qRT-PCR, because of the sizes and relationships among the light organ tissues. As such, our qRT-PCR results represent amplified message from whole light organs (Fig. 8), including all tissue interacting with bacteria as well as the ink sac, ink gland, and hindgut. A recent transcriptomic study of the light organ, although analyzed at developmental times different from those used in this present study, demonstrated that the interaction of the bacteria with a few epithelial cells

causes global changes in transcription throughout the organ (Kremer et al., 2013). Thus, other portions of the organ may experience differential regulation of the eye-specification genes that would obscure the localized changes occurring in the surface epithelia.

4.3. Future studies

Our results suggest several avenues for future study of the convergence of organs that interact with light. For example, the system offers an ideal subject for comparisons of the coordination of the phototransduction and eye-specification genes in the eye and light organ. In addition, the bacterial MAMPs, such as LPS, induce responses in both the mammalian eye (see Pollreisz et al., 2011; Bordone et al., 2012) and squid light organ (for review see McFall-Ngai et al., 2010). Further analyses of the interface between the MAMPs and light-interacting tissues might provide insights into how the immune system tunes the eye in health and disease. From a technical viewpoint, laser capture microdissection of discrete regions of the light organ would likely resolve discrepancies between the ISH and qRT-PCR results.

4.4. Conclusions

Results of this study indicate that four genes essential for eye development, *pax6*, *eya*, *six*, and *dac*, are expressed in the photophore or light organ of the squid *Euprymna scolopes*. Such similarities between the eye and light organ provide evidence for evolutionary tinkering at the developmental level and the co-option of genes between sets of non-homologous tissues that differ in organismal function. Whether such conservation of the eye-specification genes between eyes and photophores is unique to *E. scolopes* or is prevalent throughout the animal kingdom is currently unknown, although the ctenophore *Mnemiopsis ledyi* also expresses phototransduction genes (Schniztler et al, 2012), which might also be regulated by eye-specification transcription factors. Study systems that involve microbe-driven photophores offer an unusual opportunity to examine the effects of bacteria on the development of ocular structures. We discovered that the eye-specification genes respond to bacterial cues that affect light-organ morphogenesis in *E. scolopes*, including programmed cell death. Although apoptosis is part of the normal developmental program of the light organ, our results raise questions as to whether microbes and the associated host immune responses affect the development and functioning of light-interacting structures, including highly vascularized tissues such as the eye, in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

The *pax6*, *eya*, *six*, and *dac* genes are expressed in the eye and photophore of a squid.

Eye and photophore similarities provide an example of evolutionary tinkering.

The eye-specification genes respond to bacterial cues, including luminescence.
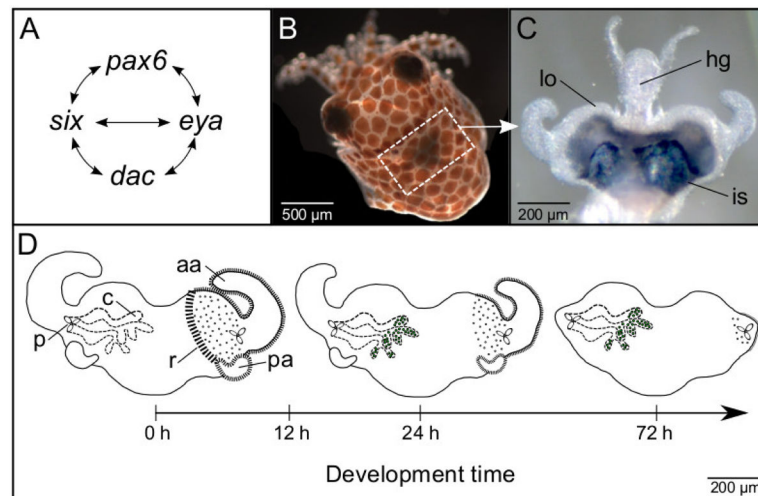
Figure 1.
Study system. (A) Regulatory network of the eye-specification genes (adapted from Donner and Maas, 2004). (B) Juvenile *E. scolopes*. The light organ is located within the white-dash boxed region and is associated with the ink sac, which is visible as a slightly darkened area. (C) Juvenile light organ (lo) surrounded by the ink sac (is) and attached to the hindgut (hg). (D) Early postembryonic development of the juvenile light organ. The juvenile light organ has three pores (p) that enable bacterial symbiont *V. fischeri* to enter the internal crypt spaces (c) (left side of light organ). *V. fischeri* are shown as green dots in the crypt spaces at 24 and 72 h of development. The surface tissues of the juvenile light organ include the anterior (aa) and posterior appendages (pa), and the ciliated ridges (r) (right side of light organ), all of which regress during the first several days of development. The developmental time of 12 h post hatching marks the point at which regression of the appendages is no longer reversible and can proceed in the absence of wild-type *V. fischeri*.
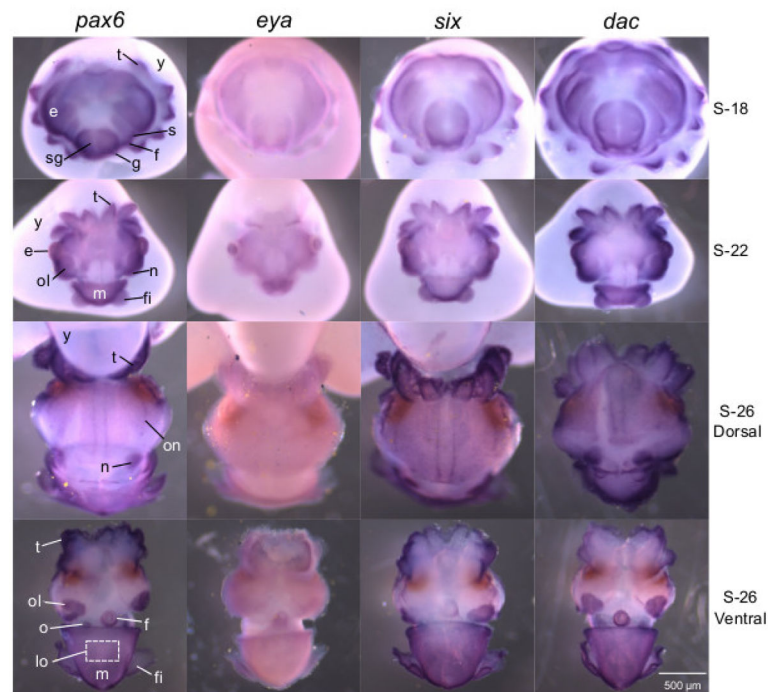
Figure 2.
Expression of the *pax6*, *eya*, *six*, and *dac* gene transcripts in developing embryos by whole-mount *in situ* hybridization. Embryos were sampled at progressive stages of development, S-18, S-22, and S-26. Tissues showing transcript signal include the eye (e), optic lobe (ol), tentacles (t), statocysts (s), shell gland (sg), mantle (m), funnel fold (f), gill (g), fin (f), nuchal organ (n), optic nerve (on), and olfactory organ (o). The developing light organ is located within the white-dash boxed region, but is covered by the mantle (m). Before hatching the embryo acquires its nutrition from the yolk (y), to which it is attached. See Supplementary Material for sense images (Fig. S7).
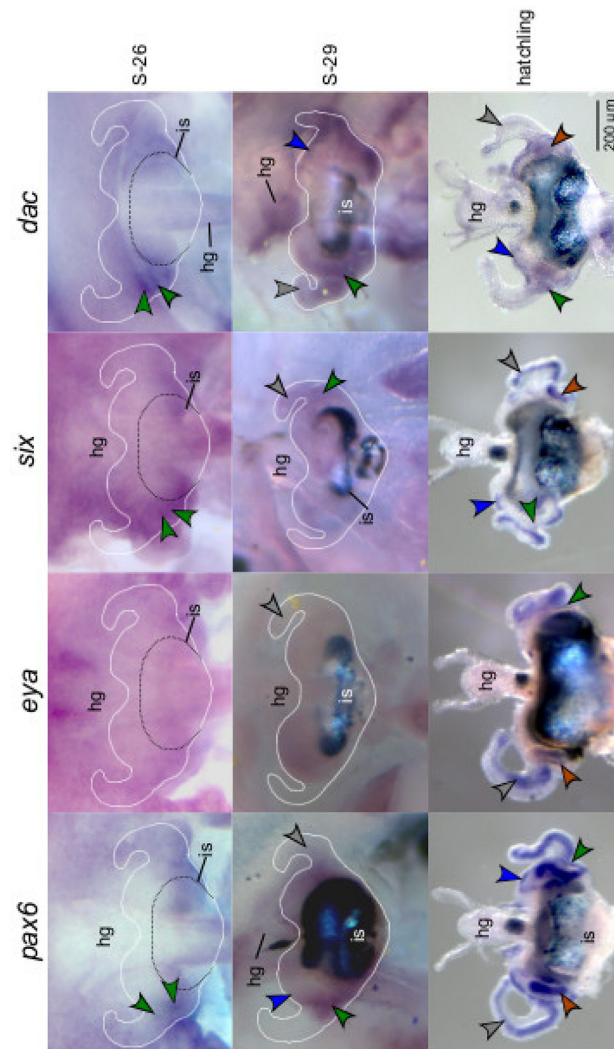
Figure 3.
Light organs showing *pax6*, *eya*, *six*, and *dac* gene transcript expression by whole mount *in situ* hybridization at different stages of development (S-26, S-29, hatching). The colored arrows indicate transcript signal in the anterior appendages (grey arrows), posterior appendages (brown arrows), ciliated ridges (blue arrows), and pores (green arrows). The hindgut (hg) develops alongside the light organ and the ink sac (is), visible by stage 29, surrounds a portion of the light organ. For clarity, an outline of the light organ, still attached to the embryos, is indicated at S-26 and S-29. See Supplementary Material for sense images (Fig. S8).
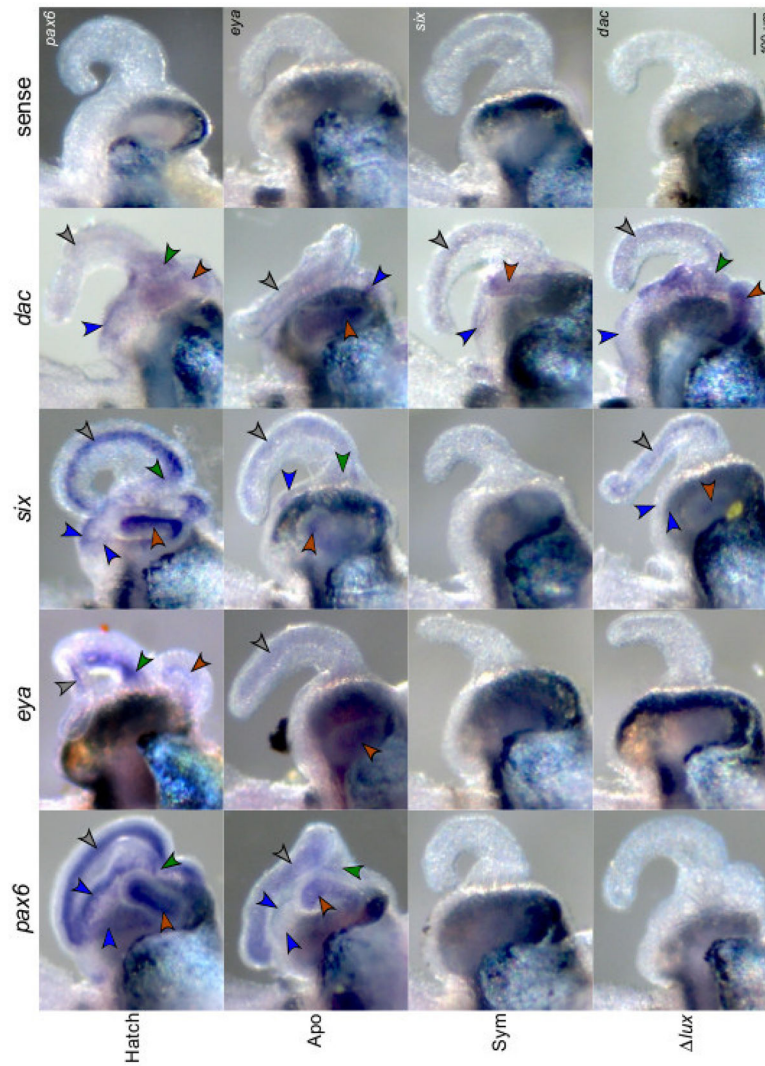
Figure 4.
Expression of the *pax6*, *eya*, *six*, and *dac* gene transcripts in surface tissues of light organs by whole-mount *in situ* hybridization. Light-organ tissues showing signal development include anterior appendages (grey arrows), posterior appendages (brown arrows), ciliated ridges (blue arrows), and pores (green arrows). Conditions consisted of newly hatched juveniles ('Hatch'), and 24 h juveniles aposymbiotic with no *V. fischeri* ('Apo'), symbiotic with wild-type *V. fischeri* ('Sym'), and symbiotic with *lux* *V. fischeri* (' *lux*'). Representative light organs exposed to the sense probes are included for each gene transcript.
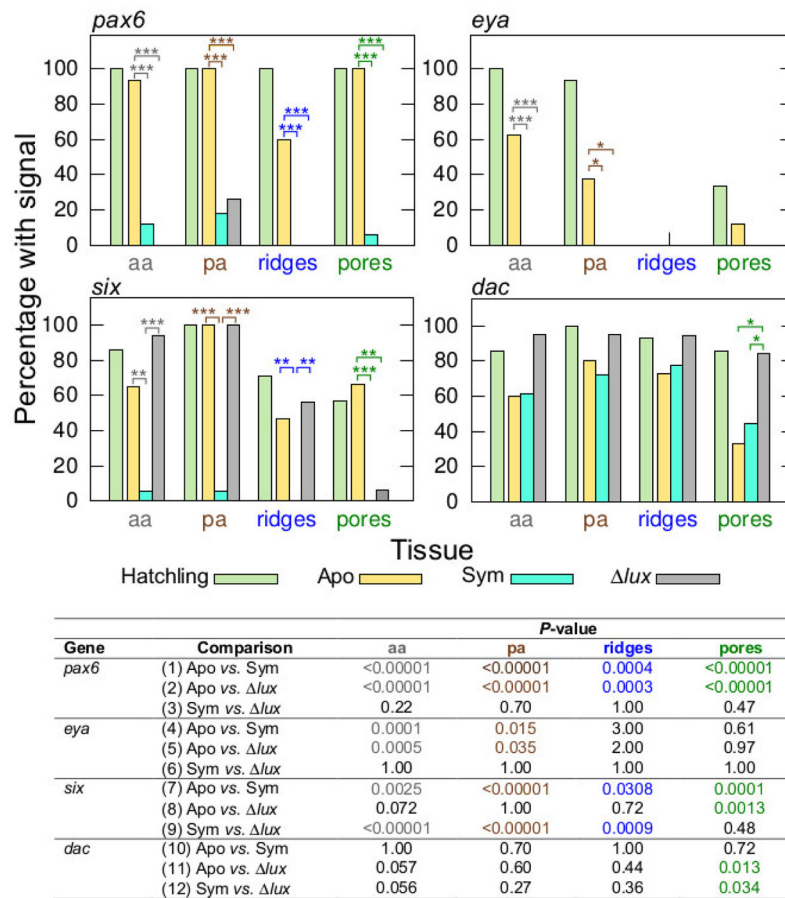
		P-value			
Gene	Comparison	aa	pa	ridges	pores
pax6	(1) Apo vs. Sym	<0.00001	<0.00001	0.0004	<0.00001
	(2) Apo vs. Δlux	<0.00001	<0.00001	0.0003	<0.00001
	(3) Sym vs. Δlux	0.22	0.70	1.00	0.47
eya	(4) Apo vs. Sym	0.0001	0.015	3.00	0.61
	(5) Apo vs. Δlux	0.0005	0.035	2.00	0.97
	(6) Sym vs. Δlux	1.00	1.00	1.00	1.00
six	(7) Apo vs. Sym	0.0025	<0.00001	0.0308	0.0001
	(8) Apo vs. Δlux	0.072	1.00	0.72	0.0013
	(9) Sym vs. Δlux	<0.00001	<0.00001	0.0009	0.48
dac	(10) Apo vs. Sym	1.00	0.70	1.00	0.72
	(11) Apo vs. Δlux	0.057	0.60	0.44	0.013
	(12) Sym vs. Δlux	0.056	0.27	0.36	0.034

Figure 5.

Percentage of light organs that indicated *pax6*, *eya*, *six*, and *dac* expression by whole-mount *in situ* hybridization in various surface tissues (see Fig. 4). Light-organ tissues of interest include anterior appendages ('aa', grey text), posterior appendages ('pa', brown text), ciliated ridges (blue text) and pores (green text). The colored text corresponds with the colored arrows in Fig. 4. Conditions consisted of newly hatched juveniles ('Hatchling'), and 24 h juveniles aposymbiotic with no *V. fischeri* ('Apo'), symbiotic with wild-type *V. fischeri* ('Sym'), and symbiotic with Δ*lux V. fischeri* ('Δlux'). Post hoc pairwise comparisons are for 24 h conditions only (***, $P<0.001$; **, $0.001<P<0.01$; *, $0.01<P<0.05$). Data consist of two replicates pooled. Sample sizes of light organs for the different conditions (Hatchling, Apo, Sym, Δ*lux*) consisted of the following, respectively, for each transcript: *pax6*, N=19, 15, 17, 19; *eya*, N=15, 16, 19, 15; *six*, N=14, 15, 17, 16; and *dac*, N=14, 15, 18, 19.
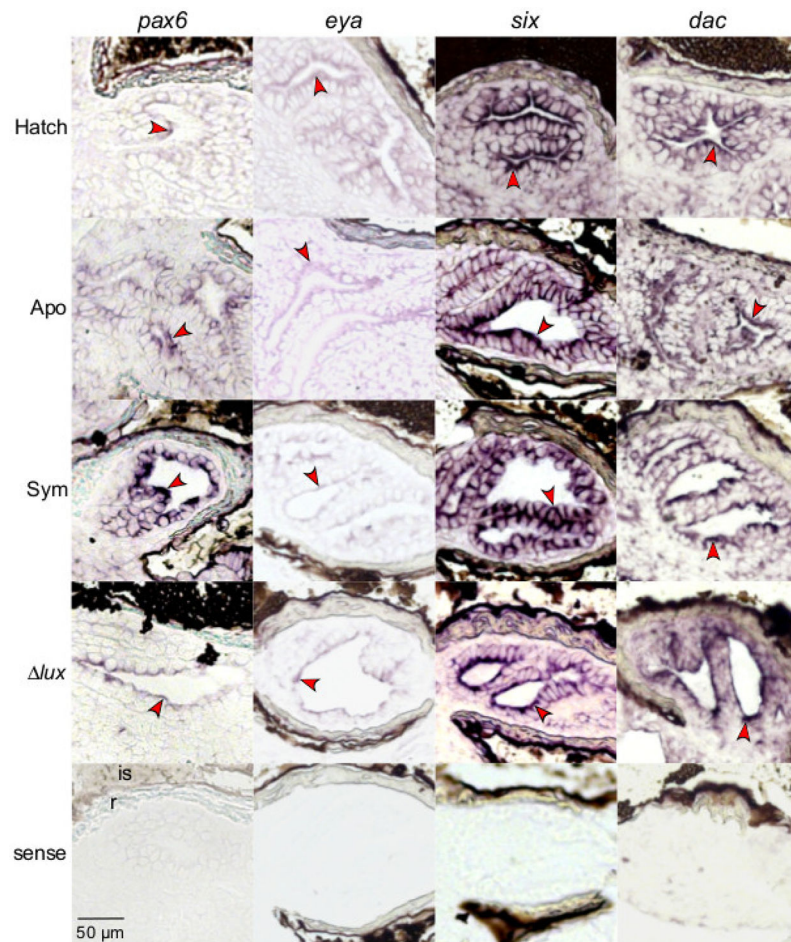
Figure 6.
In situ hybridization of sectioned light organs showing expression of the *pax6*, *eya*, *six*, and *dac* gene transcripts at 24 h post hatching. Red arrows indicate labeling of the crypt epithelia (antisense). Experimental conditions include light organs of juveniles aposymbiotic with no *V. fischeri* ('Apo'), symbiotic with wild-type *V. fischeri* ('Sym'), and symbiotic with *lux V. fischeri* ('*lux*') at 24 h post hatching. Newly hatched juveniles ('Hatch') are provided for comparison. The light organ reflector (r) and ink sac (is) are indicated in the representative *pax6* sense image.
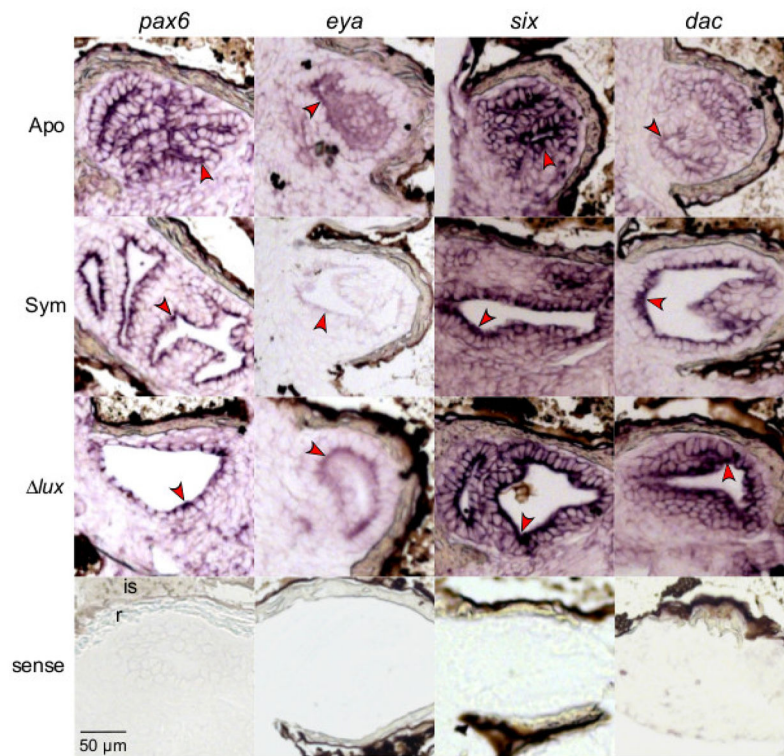
Figure 7.
In situ hybridization of sectioned light organs showing expression of the *pax6*, *eya*, *six*, and *dac* gene transcripts at 72 h post hatching. Red arrows indicate labeling of the crypt epithelia (antisense). Experimental conditions include light organs of juveniles aposymbiotic with no *V. fischeri* ('Apo'), symbiotic with wild-type *V. fischeri* ('Sym'), and symbiotic with Δ*lux V. fischeri* ('Δ*lux*') at 72 h post hatching. The light organ reflector (r) and ink sac (is) are indicated in the representative *pax6* sense image.
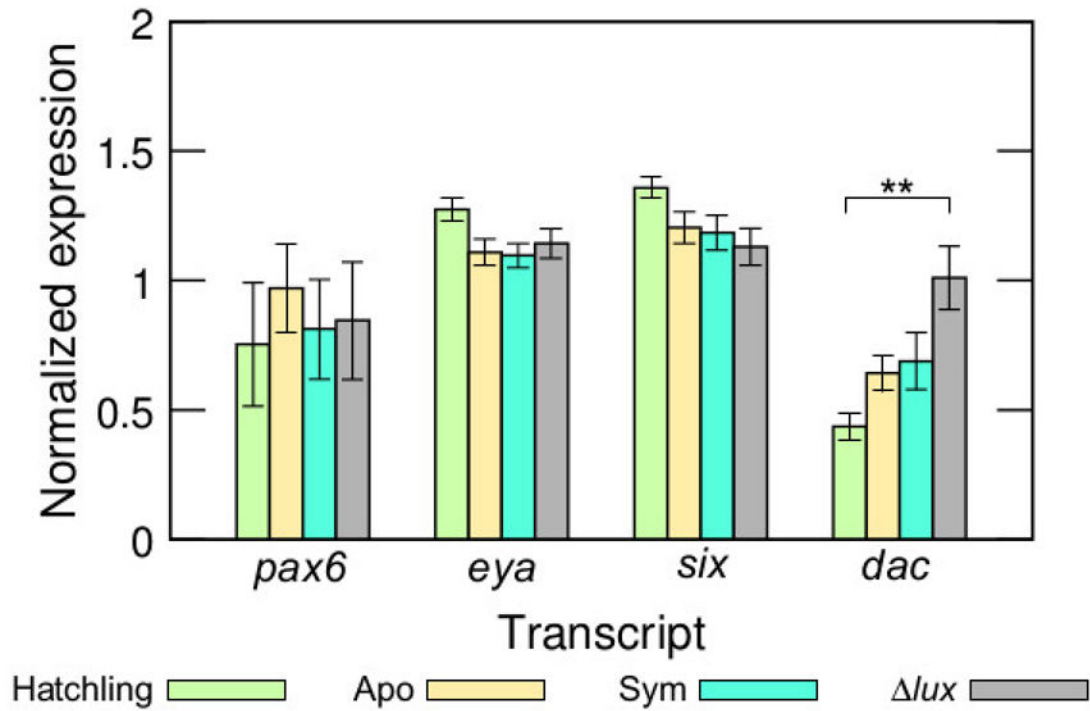
Figure 8.
Differential regulation of the *pax6*, *eya*, *six*, and *dac* transcripts among differently colonized light organs (**, 0.001<*P*<0.01). Colonization conditions were newly hatched juveniles ('Hatchling'), and 24 h juveniles aposymbiotic with no *V. fischeri* ('Apo'), symbiotic with wild-type *V. fischeri* ('Sym'), and symbiotic with *lux V. fischeri* (' *lux*'). Data consist of five biological replicates and three technical replicates combined per transcript.

Table 1

Stages of *E. scolopes* during embryogenesis and corresponding developmental events occurring in eye and light organ (Montgomery and McFall-Ngai, 1993; Arnold et al., 1972; Lee et al., 2009).

Stage	Event in Eye	Event in light organ
17	Eye primordia appear as two bilaterally positioned oval ectodermal thickenings	
19	Eye ectodermal placodes internalize as the annular folds thicken around periphery, forming the optic vesicle	Paired lateral mesoderm of hindgut-ink sac complex begins to proliferate
21	Retinas are pigmented; paired optic lobe primordia are evident	
22	Lens primordia are visible	Anterior epithelial appendage and first pair of crypts begins to form
23	Folds of iris have formed	
24	Pigmentation of retina continues to increase	Anterior and posterior appendages are evident and partially ciliated; epithelial cells of crypts form extensive brush borders; cells toward site of future ducts form cilia; reflector cells begin to differentiate
26		Second pair of crypts begins formation; epithelial cells forming crypts appear fully differentiated; many reflector cells differentiated; ink cells are functional
27	Eyes can move freely in orbits	
28	Primary lid covers the eye; patches of iridophores present on the eye	Third pair of crypts begins formation
30		Three crypts are present on each side; entire lateral surface of each side of light organ is ciliated

Table 2

Tissues in which the *pax6*, *eya*, *six*, and *dac* genes are expressed in other organisms.

Gene	Species	Tissues	References
pax6	*Euprymna scolopes*	Eye, Optic lobe, Statocysts, Olfactory organ, Tentacles, Mantle, Gills[*], Light organ[*]	Hartmann et al., 2003; Present study[*]
	Doryteuthis opalescens	Eye, Optic lobe, Olfactory organ, Tentacles, Mantle	Tomarev et al., 1997
	Sepia officinalis	Eye, Optic lobe, Ganglia, Tentacles, Gills, White body	Navet et al., 2009
	Platynereis dumerilii	Eye	Arendt et al., 2002
	Mus musculus	Eye, Brain, Olfactory epithelium	Duan et al., 2013; Davis and Reed, 1996; Chung et al., 2010
	Drosophila	Eye, Brain, Nerve cord	Quiring et al., 1994
	Danio rerio	Eye, Brain	Amirthalingam et al., 1995;
	Schmidtea polychroa	Eye	Martin-Duran et al., 2012
	Dugesia japonica	Eye	Dong et al., 2012
eya	*Euprymna scolopes*	Eye, Optic lobe, Statocysts, Olfactory organ, Tentacles, Mantle, Gills, Light organ	Present study
	Mus musculus	Eye, Ear, Nasal placode, Metanephric mesenchyme	Abdelhak et al., 1997; Xu et al., 1997, 2003;
	Drosophila sp.	Muscle	Heanue et al., 1999
	Schmidtea polychroa	Eye	Martin-Durán et al., 2012
	Dugesia japonica	Eye	Dong et al., 2012
six/so	*Euprymna scolopes*	Eye, Optic lobe, Statocysts, Olfactory organ, Tentacles, Mantle, Gills, Light organ	Present study
	Platynereis dumerilii	Eye	Arendt et al., 2002
	Mus musculus	Neural plate, Metanephric mesenchyme	Oliver et al., 1995; Xu et al., 2003
	Drosophila sp.	Muscle	Heanue et al., 1999
	Cladonema radiatum	Eye, Manubrium, Tentacles, Gonads, Umbrella	Stierwald et al., 2004; Graziussi et al., 2012
	Schmidtea polychroa	Eye	Martin-Durán et al., 2012
	Dugesia japonica	Eye	Dong et al., 2012
dac	*Euprymna scolopes*	Eye, Optic lobe, Statocysts, Olfactory organ, Tentacles, Mantle, Gills, Light organ	Present study
	Mus musculus	Eye, Optic cup, Neural crest, Brain, Limb, Otic vesicle, Genitalia	Hammond et al., 1998; Davis et al., 1999; Caubit et al., 1999
	Drosophila sp.	Eye, Limb, Muscle	Mardon et al., 1994; Heanue et al., 1999
	Oryzias latipes	Eye, Central nervous system, Pancreas, Finbuds	Loosli et al., 2002
	Schmidtea polychroa	Eye	Martin-Durán et al., 2012