MOLECULAR ECOLOGY

Molecular Ecology (2013) doi: 10.1111/mec.12474

SPECIAL ISSUE: NATURE'S MICROBIOME

Features governing symbiont persistence in the squid–vibrio association

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Abstract

Experimental studies of the interaction between host and symbiont in a maturing symbiotic organ have presented a challenge for most animal–bacterial associations. Advances in the rearing of the host squid *Euprymna scolopes* have enabled us to explore the relationship between a defect in symbiont light production and late-stage development (*e.g.* symbiont persistence and tissue morphogenesis) by experimental colonization with specific strains of the symbiont *Vibrio fischeri*. During the first 4 weeks postinoculation of juvenile squid, the population of wild-type *V. fischeri* increased 100-fold; in contrast, a strain defective in light production (Δ*lux*) colonized normally the first day, but exhibited an exponential decline to undetectable levels over subsequent weeks. Co-colonization of organs by both strains affected neither the trajectory of colonization by wild type nor the decline of Δ*lux* levels. Uninfected animals retained the ability to be colonized for at least 2 weeks posthatch. However, once colonized by the wild-type strain for 5 days, a subsequent experimentally induced loss of the symbionts could not be followed by a successful recolonization, indicating the host's entry into a refractory state. However, animals colonized by the Δ*lux* before the loss of their symbionts were receptive to recolonization. Analyses of animals colonized with either a wild-type or a Δ*lux* strain revealed slight, if any, differences in the developmental regression of the ciliated light-organ tissues that facilitate the colonization process. Thus, some other feature(s) of the Δ*lux* strain's defect also may be responsible for its inability to persist, and its failure to induce a refractory state in the host.

Keywords: *Euprymna scolopes*, luminescence, maintenance, persistence, *Vibrio fischeri*

Received 8 May 2013; revision received 15 July 2013; accepted 18 July 2013

Introduction

Symbiosis is now recognized as a principal feature of biological systems (Gilbert *et al.* 2012; McFall-Ngai *et al.* 2013). To understand the 'rules' governing the establishment and persistence of symbiotic associations in animals, biologists have developed a series of models systems that lend themselves to experimental manipulation under laboratory conditions (Ruby 2008; Kostic *et al.* 2013). The integration of information gained through the study of these systems is enabling the community of biologists in this field to construct a conceptual framework that addresses the questions: (i) What biochemical, molecular and cellular features of symbiosis are conserved across the animal kingdom and (ii) How has evolutionary selection driven diversity in symbiotic relationships? In this contribution, we present the first experimental studies of maturation of the symbiotic state in the squid–vibrio model.

The binary symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and the marine luminous bacterium, *Vibrio fischeri*, has been exploited for much of the last 25 years as an experimental system for the study of the onset and early development of an animal–bacterial partnership (for review, see McFall-Ngai 2008). Similar to most symbiotic associations, the squid–vibrio

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system is horizontally transmitted, that is, acquired each generation, as the newly hatched animal harvests free-living *V. fischeri* cells from the bacterioplankton. The embryo develops a nascent light organ with juvenile-specific ciliated surfaces where the symbiont aggregates in the hours following hatching of the juvenile into seawater. At 3–4 h after hatching, the aggregated cells enter pores on the surface of the organ and travel through ducts to one of six internal crypt spaces, where the symbionts grow. Beginning with the first dawn and thereafter throughout life, the host animal responds to the environmental light cue with an expulsion of the majority of symbionts into the surrounding seawater (Lee & Ruby 1994). Concomitant with the first expulsion of symbionts is an irreversible morphogenetic signal, specifically, the presentation of high concentrations of cell envelope molecules (Koropatnick *et al.* 2004) in synergy with symbiont light production (Koropatnick *et al.* 2007), which triggers the loss of the superficial ciliated fields that potentiate the initial symbiont colonization. The symbionts also induce significant anatomical changes in the crypt epithelia with which they associate, including a swelling of these host cells and an increase in the density of the microvilli that interface directly with the symbionts.

Genetic tools have been developed in the bacterial partner of the squid–vibrio system (Ruby 2008). Microbiologists studying this system have identified three broad mutant classes whose defects affect early stages of the association: (i) initiation mutants, such as those defective in motility (Graf *et al.* 1994), which show no colonization of the organ; (ii) accommodation mutants, such as amino acid auxotrophs (Graf & Ruby 1998), which do not achieve normal colonization levels in the early symbiosis and (iii) persistence mutants, such as those defective in light production, which grow to normal levels initially, but whose populations in the organ subsequently decline (Ruby 2008). Perhaps the most widely studied of the *V. fischeri* persistence mutants are those defective in one or more of the 6 *lux* structural genes responsible for luminescence (*i.e. luxCDABEG*), including Δ*luxA*::*erm*, which is deleted for a subunit of the luciferase protein (Visick *et al.* 2000). The Δ*luxA*::*erm* and Δ*luxCDABEG* (Δ*lux*) mutations result in a complete loss of luminescence, and have been constructed in a number of *V. fischeri* strains (Bose *et al.* 2008). These mutations in the symbiont have specific effects on several aspects of early host development (for review see McFall-Ngai *et al.* 2012) including delays in regression of the light organ's superficial ciliated epithelia that facilitate colonization, and in haemocyte trafficking into these epithelial fields (Koropatnick *et al.* 2007), as well as defects in crypt-cell development, most notably a failure to induce host crypt-cell swelling (Visick *et al.* 2000).

Although research of the squid–vibrio system has principally focused on early development, that is, over the first few days after hatching, studies of the symbiosis have suggested that long-term maintenance of the association requires a maturation process that extends for weeks beyond the initiation of the partnership (Montgomery & McFall-Ngai 1998). The gross morphology of the light-organ progresses over this period from heart-shaped structure of the juvenile to the bilobed organization of the adult (Fig. 1). In addition, the tissues that modify bacterial light, including the reflector, lens and diverticula of the ink sac, while rudimentary in the juvenile, mature to prominent features of the adult organ. This maturation of the host organ dramatically changes the relationship of the symbionts to the host tissue and to the external environment. Specifically, whereas the symbionts in the juvenile's crypts are a few cell layers away from environment, the complex,

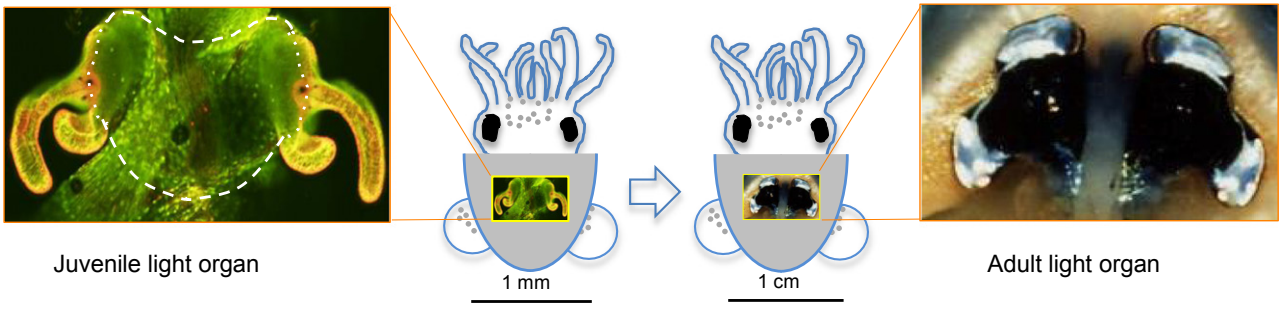

Fig. 1 The light organ of *E. scolopes*, which is located in the centre of the mantle cavity, undergoes morphogenesis from the hatchling to the mature condition. *Left*: The centre of the newly hatched juvenile light organ is heart-shaped (subtended by the white dashed line in the confocal micrograph image). This retained portion of the organ will undergo maturation over the first weeks following inoculation with the specific symbiont, *V. fischeri*. The unique features of the juvenile organ include transparent, superficial fields of ciliated epithelia (golden, with fluorescent label; CellTracker Orange, Molecular Probes, Inc.) that potentiate colonization by the symbiont. These fields begin to regress within days of the initiation of the symbiosis. *Right*: The bilobed, mature light organ of the adult animal.

protracted morphogenesis during organ maturation results in the symbionts in the crypts being remote from the organ surface. These structural changes are likely to influence host–symbiont interactions dramatically. Further, a recent project designed to characterize the host and symbiont transcriptome in the adult animal suggested pronounced metabolic shifts in the symbiont over the day–night cycle, which are associated with marked changes in crypt-cell microanatomy (Wier *et al.* 2010). Preliminary studies of the underlying mechanisms of this process suggest that they do not begin to occur until several weeks into the symbiotic association.

The suggestion of important maturation events in the squid–vibrio symbiosis has driven our interest in the development of protocols for the routine captive rearing of newly hatched host animals to enable studies of persistence in an animal–bacterial partnership. With modifications of *E. scolopes* culturing methods previously developed (Arnold *et al.* 1972; Hanlon *et al.* 1997; Claes & Dunlap 2000), we have successfully overcome the technical hurdles of raising the juveniles beyond the first week after hatching and established a reliable system for long-term culture of the host animal. Here, we present the studies of maturation events of this symbiosis, with particular emphasis on the role of symbiont persistence in driving the form and function of the fully developed organ.

Materials and methods

Bacterial strains and growth media

Vibrio fischeri strains TIM302 and TIM313 are derivatives of the wild-type symbiont strain ES114 (Boettcher & Ruby 1990), marked with chromosomal insertions for erythromycin resistance (erm^r) (Table 1). Strain TIM302 also contains a chromosomal insertion of a gene encoding green fluorescent protein (GFP) (Miyashiro *et al.* 2010). Strains TIM386 and TIM387 are derivatives of EVS102 (Bose *et al.* 2008), which has a deletion of the *luxCDABEG* genes; they also contain chromosomal insertions for erm^r. TIM387 encodes a chromosomal insertion of GFP. The erm^r and GFP encoding sequences in plasmids pEV107 and pTM242 were integrated into the Tn7 site within EVS102 using helper plasmids pEVS104 and pUX-BF13 as described elsewhere (McCann *et al.* 2003) to generate TIM386 and TIM387, respectively.

To prepare *V. fischeri* for colonization experiments, cells were grown in LBS medium (Stabb *et al.* 2001) containing (per litre) 950 mL deionized water, 50 mL 1M Tris-HCl buffer (pH 7.5), 10 g Difco bacto-tryptone, 5 g yeast extract and 20 g NaCl. LBS agar medium was solidified with 15 g of agar per litre. *V. fischeri* strains for culture competitions were grown in SWTO medium containing (per litre) 700 mL of a marine salts solution (Instant Ocean® aquarium sea salt mixture, IO), 300 mL deionized water, 10 g NaCl, 5 g bacto-tryptone and 3 g yeast extract (Bose *et al.* 2008). To select for antibiotic resistance strains, erythromycin (erm) was added to LBS agar at a concentration of 10 μg/mL.

Rearing E. scolopes juveniles to maturity

Fully mature *Euprymna scolopes* were collected in Maunalua Bay, Hawaii and transported to the University of Wisconsin-Madison. These animals were maintained in artificial seawater (IO) as previously described (McFall-Ngai & Montgomery 1990; Montgomery & McFall-Ngai 1993). Egg clutches produced by the breeding colony were maintained individually in a 2.5-gal aquarium at 23°C under heavy aeration and daily 50% water changes. Within 12 h of hatching, after approximately 23 days of embryogenesis, juvenile animals were placed in 100 mL of filter-sterilized IO containing (per ml) 3000–5000 CFU of the desired *V. fischeri* strain(s). Based on previous studies (Choe & Oshima 1963; Arnold *et al.* 1972; Hanlon *et al.* 1997), a reliable *E. scolopes* rearing

Table 1 Bacterial strains and plasmids used in this study

Strain	Relevant genotype	Source
ES114	Wild-type *V. fischeri* symbiont	Ruby *et al.* (2005); Mandel *et al.* (2008)
EVS102	ES114 *ΔluxCDABEG*	Bose *et al.* (2008)
TIM302	ES114 miniTn*7*:: *erm* P_{tetA}-*gfp*+	Miyashiro *et al.* (2010)
TIM313	ES114 miniTn*7*::*erm*	Miyashiro *et al.* (2010)
TIM386	EVS102 miniTn*7*::*erm*	This work
TIM387	EVS102 miniTn*7*:: *erm* P_{tetA}-*gfp*+	This work
Plasmid		
pEVS104	R6Kori RP4 *oriT trb tra kan*	Stabb & Ruby (2002)
pEVS107	R6Kori *oriT mini*-Tn*7 mob erm kan*	McCann *et al.* (2003)
pTM242	pEVS107 P_{tetA}-*gfp*+	Miyashiro *et al.* (2010)
pUX-BF13	R6Kori *tns bla*	Bao *et al.* (1991)

method was developed (see Supporting Information for details). The animals were exposed to symbiont cells for 14–16 h, and then transferred to 4-L rearing vessels at 25 to 60 squid/vessel (Table S1, Supporting information). Nonsymbiotic animals were placed in 100 mL of filtered IO without addition of *V. fischeri* and then transferred into rearing vessels at the same time and density as the symbiont-colonized animals. All animals were maintained on 12 h/12 h light/dark cycle throughout the experiment. Although we were able to raise squid through their entire life cycle, the majority of experiments in this study ended at 4 weeks. This timing was chosen as the experimental end point because, by 4 weeks, the animals have transitioned into adult diel behaviour, which is characterized by a nocturnal, active period and a diurnal, quiescent period (Hanlon *et al.* 1997; unpubl. obs.).

Colonization, co-colonization and persistence of wild-type and ΔluxCDABEG V. fischeri

For colonization experiments using single strains, the squid were inoculated simultaneously but separately with 3000–5000 CFU of either wild type or Δ*luxCDABEG* (Δ*lux*) per ml. The inoculum was plated on LBS agar before introduction of the squid. The number of *V. fischeri* symbionts in the light organs was determined at 1, 7, 14, 21 and 28 days after inoculation by plating squid homogenates (n = 10–30 squid per time point) on LBS agar (Stabb *et al.* 2001). *E. scolopes* were not fed the day of sampling and were rinsed for 1 h in filter-sterilized IO to reduce contamination. After rinsing, each squid was checked for luminescence using a TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA). The squid were then homogenized within 2 h prior to the scheduled lights-off period, and serial dilutions were plated onto LBS agar. The plates were incubated overnight at 28°C, and colonies were counted within 24–48 h. As in the initial publication describing this operon deletion (Bose *et al.* 2008), we were unable to construct a suitable genetic-complementation vector containing the entire 6-gene locus. However, their work showed that the same type and level of defect was observed with independently constructed Δ*lux* and *luxA*::*erm* mutants, in two different strain backgrounds. Thus, it seemed likely that the persistence phenotype of the Δ*lux* mutant resulted from the shared defect in light emission and not because of any second-site mutation that happened to appear in all strains.

For co-colonization experiments, the squid were inoculated at a 1:1 ratio of labelled wild type (TIM31 3) to Δ*lux* (TIM387); the combined inoculum was 3000–5000 CFU/mL. Previous work has shown that the single-copy labels of erm^r and GFP have no detectable effect on growth or colonization (Miyashiro *et al.* 2010). The inoculum was plated on LBS agar both just before introduction of the squid and after transferring the squid into the rearing tubs (12 h later) to assure that a 1:1 ratio of the two strains was maintained throughout the inoculation. The number of CFU per squid was determined to be 1, 3, 7, 11 and 15 days after inoculation. At each sampling, the animals were homogenized, and the diluted homogenate was plated on LBS agar (n = 20 squid per time point). After 48 h, the colonies were examined with a fluorescence microscope to identify Δ*lux* colonies by their green fluorescence. Plates were made with a thin (approximately 2 mm) layer of LBS agar to reduce the autofluorescence associated with nonfluorescent colonies. Both nonfluorescent and fluorescent colonies were counted to determine the CFU per light organ of each strain, and the ratio of the two strains (Δ*lux*: wild type) was calculated as the competitive index (CI).

To increase the ability to detect co-colonizing Δ*lux* cells as their percentage of the symbiotic population declines, an additional experiment was performed. The inoculation procedure was the same as above, only erm-sensitive ES114 was used in place of erm^r TIM313. In this way, a small percentage of the Δ*lux* TIM387 could be identified against a large background of wild-type ES114 in a symbiont population by plating the homogenate on LBS agar either with or without erm. Sampling on day 1 postinoculation showed that the ratio of strains was 1:1 as expected; at day 15, undiluted homogenate was plated on erm-containing plates to identify the small percentage of erm-resistant TIM387 CFU present.

Secondary colonization of cured light organs

To determine whether an initial colonization would render the light organ resistant to a subsequent colonization, newly hatched animals were placed into one of three conditions: (i) not colonized (nonsymbiotic), (ii) colonized by wild type (ES114) or (iii) colonized by Δ*lux* (EVS102). The initial symbiont was then removed from the light organ by treatment with the antibiotics gentamicin (20 μg/mL) and chloramphenicol (20 μg/mL) for 48 h (Nyholm *et al.* 2009). After curing, the animals were rinsed for 1 h in filter-sterilized IO, and wild-type colonized animals checked for the absence of luminescence as an indication of the loss (or great reduction) of symbionts. Because the Δ*lux*-colonized squid were similarly treated, we assumed those symbionts had also been eliminated by the treatment.

Sets of wild-type colonized and nonsymbiotic squid were then (re)inoculated at either 1, 5 or 10 days post-hatching, and Δ*lux*-colonized squid re-inoculated at 5 or

10 days posthatching (n = 5–30 squid per time point) (Table 2) with 3000–5000 CFU of GFP-labelled wild-type *V. fischeri* (TIM302) per mL. Colonies of this GFP-labelled wild-type strain were easily distinguished from those of the initial nonfluorescent colonizers (ES114 and EVS102). After an overnight inoculation, the squid were returned to the rearing containers and 24 and 48 h later, each squid was checked for luminescence. All the animals were then homogenized, and the diluted homogenate plated on LBS agar. The number of CFUs per squid for both the initial colonization and the secondary colonization was quantified. The effectiveness of the curing procedure was validated by the observation that the light organs of secondarily colonized animals only carried GFP-labelled symbionts. All colonization and curing procedures were performed at the same times, in parallel, with the same bacterial culture or antibiotic dilutions. This experiment was repeated twice using the time points described above, and a third time at 28 days posthatching, with the same outcome.

Development of the light-organ ciliated field

The morphology of the light-organ ciliated field was monitored over 4 weeks for animals under 3 conditions: (i) nonsymbiotic, (ii) colonized by wild type, or (iii) colonized by Δ*lux*. Animals were sampled at 1, 7, 14, 21 and 28 days. Samples were rinsed for 1 h in filtered IO and then prepared for scanning electron microscopy (SEM) by fixing in 4% paraformaldehyde in 1X marine phosphate-buffered saline (mPBS; 50 mM sodium phosphate, 0.45 M sodium chloride, pH 7.4). After 5 min in fix, the mantle was opened to allow proper fix penetration of the light organ. The squid were fixed for 14–16 h at room temperature and then washed twice for 10 min in mPBS and dehydrated through an ethanol series (Montgomery & McFall-Ngai 1993). The samples were dried for SEM, using a Tousimis Samdri 780 critical point drier and sputter coated with a SeeVac Auto conductavac IV. Samples were mounted on stubs and examined with a Hitachi S-570 LaB6 scanning electron microscope. In conjunction with the sampling of squid for SEM, three animals from each of the wild-type and Δ*lux* treatment groups were sacrificed to determine the number of *V. fischeri* CFUs present in the light organ and to ensure that the animals were colonized with the appropriate strains.

Results

While raising *E. scolopes* juveniles, we observed that the general behaviour of these squid gradually shifts during the first 4 weeks following hatching, as had been noted previously (Hanlon *et al.* 1997). Although no consistent day–night difference in behaviour occurred in the first days to weeks, by 28 days posthatch all animals had transitioned to the distinct pattern of diurnal quiescence and nocturnal activity that is characteristic of mature symbiotic *E. scolopes*. Thus, we chose to examine the relationship between the developing squid and its symbiotic bacteria during this important period of maturation. Specifically, we compared the colonization dynamics of wild-type luminescent symbionts with those of a nonluminescent mutant strain (Δ*lux*).

We first examined the growth trajectory of a symbiotic population of wild-type *V. fischeri* during the first 28 days following inoculation, relative to that of a Δ*lux* population (Fig. 2). The wild-type strain initially

Table 2 Effect of initial colonization on the ability of *E. scolopes* to become secondarily colonized

Initial treatment	Time of curing*	Time of secondary reinoculation†	Luminescence after secondary colonization‡		CFU/squid (48 h after secondary colonization)
			24 h	48 h	
Nonsymbiotic	Day 1	Day 3	5/5	5/5	10^5
	Day 5	Day 7	5/5	5/5	10^6
	Day 10	Day 12	10/10	10/10	10^6
Wild type	Day 1	Day 3	0/5	5/5	10^5
	Day 5	Day 7	0/10	0/10	<30
	Day 10	Day 12	0/29	0/29	<30
Δ*luxCDABEG*	Day 5	Day 7	2/5	4/5	10^5
	Day 10	Day 12	11/20	19/20	10^6

*Animals in each treatment group, including the nonsymbiotic, were cured with antibiotics at the indicated times after an initial colonization.

†Cured animals in each treatment group were secondarily exposed to wild-type *V. fischeri*.

‡Based on the production of luminescence, the number of animals (positives/total) recolonized at 24 or 48 h after reinoculation with wild type.

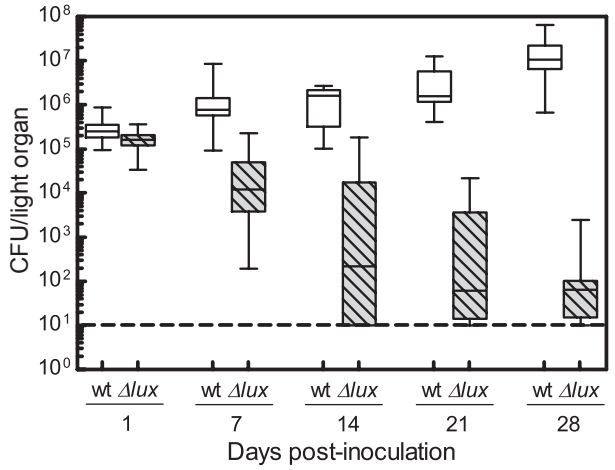

Fig. 2 Colonization dynamics of the light organ of *E. scolopes* by a wild-type (wt; open) or Δ*luxCDABEG* (Δ*lux;* hatched) strain of *V. fischeri* over the first 28 days (n = 10–30 squid per time point). The dashed line indicates the limit of detection (approximately 10 CFU/light organ). The median and 25th to 75th percentile limits are indicated by the horizontal line and box, respectively, and the bars indicate the range of all values.

colonized at approximately 10^5 CFU/squid. As the host grew, the light organs subsequently harboured approximately 10^6 CFU/squid by 7 days and approximately 10^7 CFU/squid by 28 days. This growth of the symbiont population correlated with the increase in volume of the crypt space in which *V. fischeri* is maintained (Montgomery & McFall-Ngai 1998). Previous studies of the symbiosis had demonstrated that while *V. fischeri* mutants defective in bioluminescence colonize normally over the first day following inoculation, they decline to about 25% of initial levels by 48–72 h (Visick *et al.* 2000; Bose *et al.* 2008). To determine whether a Δ*luxCDABEG* (Δ*lux*) strain is maintained at this reduced level or continues to decline in numbers as the organ develops, we monitored its population levels in the light organ through 28 days. In fact, the light-organ populations of the Δ*lux* strain continued to decline exponentially; by 7 days, they were at 10% of original colonization levels, and by 28 days, the populations were <0.1%, near the limit of detection (10 CFU/squid). The growth rates of wild-type and Δ*lux* strains were indistinguishable (Fig. S2, Supporting information).

We then asked what effect co-colonization by the Δ*lux* and wild-type strains would have on each other. Three general outcomes were possible: (i) the wild type could rapidly outcompete the Δ*lux* strain, such that its loss would be accelerated; (ii) the light-emitting wild-type strain could functionally complement the Δ*lux* such that this nonluminescent strain could now persist as a co-colonizer; or (iii) the two strains would not affect one another's growth patterns in the crypts. At 3 days following the co-inoculation, the Δ*lux* population was reduced to approximately 25% that of wild type, with an average competitive index (CI) of 0.65 (Fig. 3), similar to previous reports for this point in the symbiosis (Visick *et al.* 2000; Bose *et al.* 2008). As the host matured, the Δ*lux* populations continued to decline while wild type steadily increased (Fig. 3a). By 11 days postinoculation, the Δ*lux* strain, whose presence becomes increasingly difficult to discern during co-colonization, approached the limit of detection for these conditions (<500 CFU/squid); at 15 days, very few Δ*lux* CFU could be observed within the background of an average of 1.5×10^6 wild-type *V. fischeri* CFU/squid. More sensitive analyses using a selective medium (Materials and Methods) indicated that fewer than 10 Δ*lux* CFU/squid. Taken together, these data show that there is apparently no significant effect of co-colonization on the symbiotic trajectory of either of the two strains.

We then studied the effects of initial colonization on the ability of previously colonized animals to be recol-

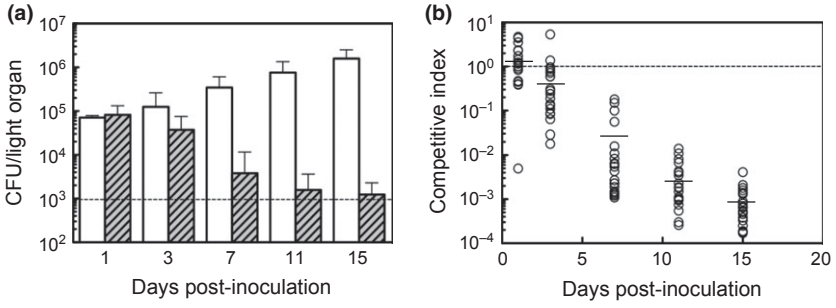

Fig. 3 Co-colonization of *E. scolopes* by a 1:1 mixed inoculum of wild type and Δ*luxCDABEG*. (a) Mean CFU per light organ of wild-type (open) and Δ*luxCDABEG* (hatched) portions of the symbiotic population (n = 20 squid per time point). The dashed line is the limit of detection (approximately 10^3 CFU per organ) and the standard error of the mean is displayed. (b) Competitive index (CI) of the Δ*luxCDABEG* strain relative to the wild type in the light organ populations (each circle represents the CI for one animal). The CI was calculated as the ratio of Δ*luxCDABEG*:wild type; the mean value at each time is indicated by the horizontal line and the dotted line represents the initial inoculation ratio of 1:1.

onized after losing their first symbionts. Nonsymbiotic animals could be colonized at any time during the first 28 days following hatching; however, did establishment of a previous colonization affect the host's ability to initiate a subsequent one? In these experiments, we colonized juvenile squids with either the wild-type or the Δ*lux* strain (Table 2). After 1, 3 or 5 days, the animals were cured with antibiotics and re-exposed to an inoculum of wild-type *V. fischeri*. Juveniles colonized with wild type for 1 day and then cured could uniformly be recolonized, although their development of full symbiont populations (as indicated by the appearance of luminescence) was delayed (Table 2). Curing after 5 or 10 days of wild-type colonization resulted in squid that no longer could be recolonized under the inoculation conditions used. In contrast, 80–95% of the animals colonized by Δ*lux* for 5 or 10 days could become recolonized. These outcomes indicate that when colonized by wild-type symbionts, but not nonluminous ones, by 5 days, the development of *E. scolopes* has progressed to a point at which it can no longer be secondarily colonized, even if the primary colonization is lost. The data presented in Table 2 are from a representative experiment that was repeated three separate times with the same outcome.

We next determined whether this difference in the ability to become recolonized correlates with differences in the morphological development of light organs of nonsymbiotic, wild-type colonized and Δ*lux*-colonized animals. Colonization by symbionts typically triggers the loss of the juvenile-specific ciliated fields on the organ surface that potentiate colonization (Montgomery & McFall-Ngai 1994). One possible reason that animals initially colonized by the Δ*lux* strain retain the capacity for secondary colonization by the wild-type strain is that Δ*lux* is defective in inducing the regression of these fields. A previous study demonstrated that light organs colonized by the Δ*lux* strain (Koropatnick *et al.* 2007) are delayed in morphogenesis in the first 3 days. In the present study, however, by 7-days postinoculation, the light organs of animals colonized by either wild type or Δ*lux* strains had undergone the typical regression of the ciliated field, that is, they were indistinguishable (Fig. 4). This finding suggests that the extent of ciliated-field regression can't explain the resistance of animals previously infected by wild-type *V. fischeri* for 5 days to a secondary colonization. Nonsymbiotic light organs exhibited little change over this time period (Fig. 4). Between 8 and 28 day, light organs colonized by the Δ*lux* strain continued to retain a small region of the ciliated fields surrounding the pores (Fig. 4, white arrows), while those animals colonized by the wild-type strain progressively lost this feature, taking on the morphology of the fully mature organ. Nonsymbiotic animals exhibited only a very gradual change in the ciliated surface, likely due to their exposure to the small amounts of morphogens (lipopolysaccharide and peptidoglycan derivatives; Koropatnick *et al.* 2004) in the ambient seawater. By 28-days posthatch, the light organs of Δ*lux*-colonized squid had a level of ciliated-field regression more similar to that of nonsymbiotic animals.

Discussion

The squid–vibrio symbiosis undergoes an important maturation process within the first 4 weeks after colonization (Montgomery & McFall-Ngai 1998). However, because newly hatched squid survive for only 4–5 days without access to food, direct, experiment-based analysis of later stages in this process requires raising juvenile *E. scolopes* into adults (Hanlon *et al.* 1997). In this study, we combined the ability to rear juvenile *E. scolopes*, with a capacity to colonize the newly hatched animals with a genetically modified strain of their symbiont, *V. fischeri*. In this way, the long-term impacts of a bacterial mutation could be assessed as the symbiosis matures.

During the first few weeks of the association, the nascent light organ progresses from a morphology that facilitates colonization (Nyholm & McFall-Ngai 2004) to one that both fosters persistence of the symbiont and mediates the nocturnal bioluminescent behaviour of the host (Jones & Nishiguchi 2004). This maturation not only involves distinct morphological changes in the organ (Fig. 4; Hanlon *et al.* 1997; Montgomery & McFall-Ngai 1998), but also includes critical changes in the nature of the interaction between the partners (Ruby 2008; McFall-Ngai *et al.* 2012). By monitoring the impact of specific bacterial mutations on the maturing symbiosis, we hope to discover signalling and metabolic interactions between the host and its symbiont population as the partnership develops (Dunn 2012; McFall-Ngai *et al.* 2012). The importance of such developmental changes has become increasingly evident in recent years: for instance, while transcriptional analyses of the adult symbiosis have suggested that the host provides chitin oligosaccharides as a nutrient for their symbionts (Wier *et al.* 2010), juvenile squid do not (Miyashiro *et al.* 2011).

In the present study, we chose to examine how the absence of a major product of the association, the symbiont's bioluminescence, affects both the persistence of the bacterial partner and the host's response to it. While it was previously reported that nonluminous mutants of *V. fischeri* initially colonize normally, but had declined to 1/4 of wild-type levels within 48 h (Visick *et al.* 2000; Bose *et al.* 2008), it was not known if or how the population level of such a 'dark' strain changed as

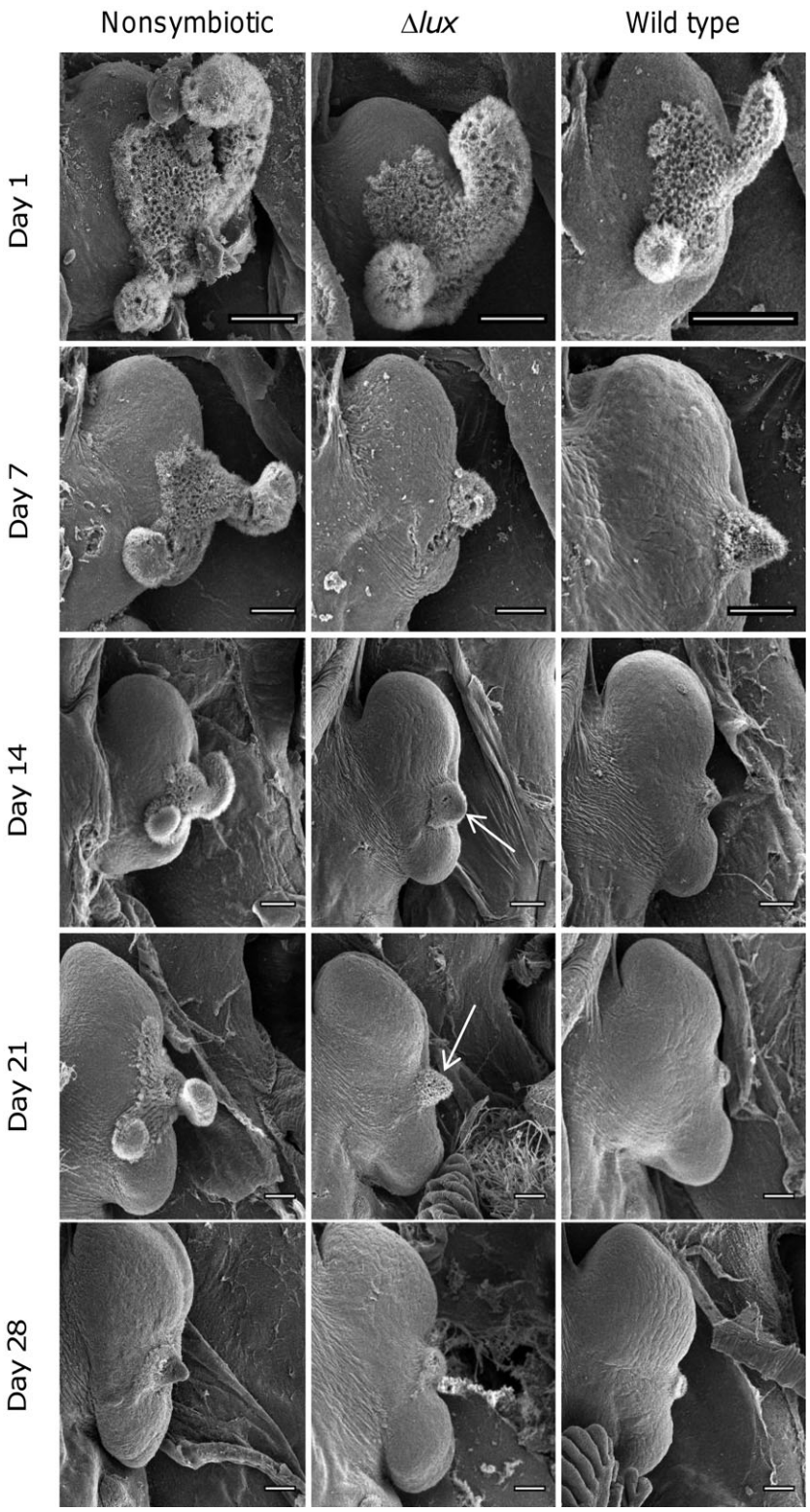

Fig. 4 Scanning electron micrographs (SEMs) of the right half of *E. scolopes* light organs at different stages of maturation over the first 4 weeks posthatch. *Left column:* nonsymbiotic animals; *middle column:* Δ*lux*-colonized animals; *right column:* wild-type colonized animals. The ciliated epithelial fields, seen on all organs at day 1, regress at different rates. The white arrows indicate remnants of the ciliated field that are retained longer by Δ*lux*-colonized light organs. Scale bars are 100 μm.

the symbiosis continued to mature. Here, we show that this decline continues at an exponential rate over the next 3–4 weeks, with a Δ*luxCDABEG* mutant eventually reaching undetectable levels, that is, fewer than 10^{-5} the level of the wild-type population (Fig. 2). Such a remarkably effective apparent selection against a

nonluminous symbiont by the host suggests an ability to perceive their light emission (Tong *et al.* 2009; McFall-Ngai *et al.* 2012) and/or the creation of physiological conditions that punish 'cheaters' (Ruby & McFall-Ngai 1999; Stabb 2005).

A similarly dramatic example of sanctioning has been reported in the legume–rhizobia symbioses (Kiers *et al.* 2003) and is believed to be the result of an evolutionary selection for mutualism called 'partner choice' (Foster & Wenseleers 2006). Beyond the extensive studies of this system and the related plant–arbuscular mycorrhizal symbioses (Sanders 2003; Engelmoer *et al.* 2013), little experimental work on cheating by either partner has been reported for other host–microbe mutualisms (Palmer *et al.* 2010; Sachs *et al.* 2011), especially animal–bacteria ones. Nevertheless, the opportunity exists to discover how symbioses like those between nematodes (Chaston *et al.* 2013) or ants (Caldera & Currie 2012) and their microbiota have evolved to resist the appearance and persistence of cheaters.

The squid host's ability to select against dark symbionts is even more remarkable because it occurs with equivalent efficiency in either single or mixed (*i.e.* Δ*lux* + wild-type) colonizations (Fig. S1, Supporting information). That is, the increasingly diminished number of nonluminous cheaters fails to persist even amongst a vast preponderance of luminous cooperators; such high specificity is rarely observed in other symbioses (Oono *et al.* 2011). While the actual mechanism of any host-sanctioning in the light organ remains mostly speculative (Stabb 2005), our data help limit the possibilities. For instance, in our mixed-colonization experiments, we expect that, on average, at least one of the six crypts in a light organ will be colonized by both a wild-type and a Δ*lux* strain (Wollenberg & Ruby 2009). If the host sanctions cheaters by simply restricting the growth of symbionts within dark (*i.e.* colonized by Δ*lux* alone) crypts (Lee & Ruby 1994), we would expect Δ*lux* cells to persist only in the one co-colonized crypt. However, if this were the sole mechanism for sanctioning, the population of dark symbionts could theoretically remain at approximately 8% of the total. However, the specificity and effectiveness of the sanctioning (>99.9% of the Δ*lux* cells in a co-colonization are lost; Fig. 3b) suggest the targeting of individual dark (cheater) bacteria, even within a mixed population.

Evolutionary theory suggests that it benefits the host to restrict the number of different strains that colonize a symbiotic organ (Wollenberg & Ruby 2009); thus, it is not surprising that once colonized by a wild-type strain, the light organ soon becomes refractory to secondary colonization (Table 2). However, If colonized by an uncooperative (*i.e.* nonluminous) symbiont, the host must not only be able to completely rid itself of the 'cheater', but also have a mechanism in place to allow recolonization. In fact, unless a functionally cooperative relationship is formed, the squid allows additional reinitiations of the symbiosis with different strains of *V. fischeri* to occur, perhaps indefinitely, until a stable association forms (Table 2). The mechanism underlying the decision of whether to 'commit' to a symbiont population is not known; however, it appears to function within the first few days after colonization and is not dependent on delaying the regression of the light organ's ciliated field (Fig. 4; Doino & McFall-Ngai 1995).

In summary, all horizontally transmitted symbioses face two fundamental challenges: effective initiation of the association by symbionts from an environmental reservoir and successful maintenance of a functionally cooperative symbiont population. We are now in a position to bring to bear animal husbandry, imaging, metabolic and genetic approaches to determine how both these critical steps are managed with such efficiency and fidelity in the squid–vibrio association.

Acknowledgements

We thank the staff of the Biological and Bio-materials Preparation, Imaging, and Characterization Laboratory of UW-Madison for assistance with drying, coating and mounting of samples for scanning electron microscopy. This work was supported by NIH R01 Grants AI50661 (to M.M.-N.) GM099507 (to E.G.R.) and OD11024 (to E.G.R. & M.M.-N.) and NSF Grant IOS 0817232 (to M.M.-N. & E.G.R.). T.M. was supported during this project by NIH K99 Grant GM097032.

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