

Critical symbiont signals drive both local and systemic changes in diel and developmental host gene expression

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The colonization of an animal's tissues by its microbial partners creates networks of communication across the host's body. We used the natural binary light-organ symbiosis between the squid *Euprymna scolopes* and its luminous bacterial partner, *Vibrio fischeri*, to define the impact of colonization on transcriptomic networks in the host. A night-active predator, *E. scolopes* coordinates the bioluminescence of its symbiont with visual cues from the environment to camouflage against moon and starlight. Like mammals, this symbiosis has a complex developmental program and a strong day/night rhythm. We determined how symbiont colonization impacted gene expression in the light organ itself, as well as in two anatomically remote organs: the eye and gill. While the overall transcriptional signature of light organ and gill were more alike, the impact of symbiosis was most pronounced and similar in light organ and eye, both in juvenile and adult animals. Furthermore, the presence of a symbiosis drove daily rhythms of transcription within all three organs. Finally, a single mutation in *V. fischeri*—specifically, deletion of the *lux* operon, which abrogates symbiont luminescence—reduced the symbiosis-dependent transcriptome of the light organ by two-thirds. In addition, while the gills responded similarly to light-organ colonization by either the wild-type or mutant, luminescence was required for all of the colonization-associated transcriptional responses in the juvenile eye. This study defines not only the impact of symbiont colonization on the coordination of animal transcriptomes, but also provides insight into how such changes might impact the behavior and ecology of the host.

squid-vibrio | symbiosis | development | daily rhythm | bioluminescence

Recent studies of animal and plant microbiomes have demonstrated that they can have far reaching effects, influencing both the internal and external environments of the host (1, 2). For example, the human microbiota impacts both tissues with which it directly interacts and more remote tissues of the body (3), as well as the built and natural environment in which the human host resides (4, 5). These complex microbial networks profoundly influence host development, from embryogenesis through senescence, while maintaining physiological homeostasis along this trajectory (2).

The best-studied nexus of these complex interactions is the mammalian gut microbiota (6), which affects not only the gut tissues themselves, but also the immune system (7), brain (8), liver (9), heart (10, 11), kidney (12), lung (13, 14), and eye (15–17). The microbiota also helps coordinate the activities of these tissues and organs: for example, the strong association of the gut microbiota with the control of host circadian rhythms (18, 19). Furthermore, axes of influence between the gut and other organs have revealed that dysbiosis of the microbiota is a critical driver of seemingly unrelated diseases (3).

Thus far, the mechanisms underlying these wide-ranging effects remain poorly studied. The integration of the gut microbiota into host biology is reflected in the transcriptomic regulation of genes in tissues both in direct contact with (20–22) and distant from (23,

24) the microbial assemblage. Available data suggest that the metabolomes of the blood, sweat, and urine carry products of the gut microbiota, such as short-chain fatty acids and microbe-associated molecular patterns (25, 26), to which these remote tissues respond. The complexity of the mammalian gut microbiota, however, renders it difficult to investigate the impact of a particular microbe on host biology under natural conditions, because the responses of adjacent and remote host tissues are the result of the cumulative effects of microbe–microbe and host–microbe interactions with hundreds to thousands of microbial phylotypes. In contrast, here we use the binary light-organ symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and its luminous bacterial partner, *Vibrio fischeri* (27, 28), to define the impact of a single symbiotic partner on the transcriptomic responses of host tissues, both those housing the symbiont population and those remote from the symbionts.

Significance

Biologists now recognize that animal microbiomes have strong impacts not only on the organs with which they associate, but also anatomically remote tissues; however, the precise triggers underlying these impacts remain unknown. Here, using the squid-vibrio light-organ association, which affords unparalleled resolution of a natural binary partnership, we report both near-field (light organ) and far-field (eye and gill) symbiont-driven effects on host gene expression. Colonization by the symbiont results in unique transcriptional signatures for each organ. Furthermore, distinct organ-specific patterns arise over the day/night cycle, and across the host's developmental trajectory. Most strikingly, the loss of a single genetic locus in the symbiont, that encoding bioluminescence, triggers a dominant and biologically relevant change of gene expression across the host's body.

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The squid host acquires its symbiont each generation from the surrounding environment and, similar to the mammalian gut microbiota, the bacteria reside extracellularly along the apical surfaces of epithelium-lined crypts (29). Also analogous to the mammalian gut microbiota, the squid–vibrio symbiosis undergoes significant development and maturation. Within hours following initial colonization of the juvenile animal, the symbionts trigger the regression of superficial ciliated fields of cells that promote light-organ inoculation (28). The symbionts also induce development of the crypt cells with which they directly associate throughout the animal's life, notably an increase in microvillar density and a swelling of the cells lining the crypts (Fig. 1A). A dark mutant derivative of *V. fischeri* (Δlux), defective in light production, the principal “currency” of the symbiosis, is also defective in the induction of this latter hallmark event of early light-organ development (30–32).

Development of the light organ also involves the onset of diel cycles. Beginning during the first day of colonization and thereafter, ~90% of the symbiont population is vented each day at dawn into the surrounding environment (33). Furthermore, in response to luminous (but not Δlux) symbionts, the organ's cryptochrome-encoding clock gene, *escr1*, begins a day/night cycling, and the host concomitantly imposes a cycling of the symbiont's luminescence levels, which peak in the hours of the early evening (1900–2000 hours), when the nocturnal squid host begins to forage. Then, after 3–4 wk of colonization, the symbiosis undergoes a final mat-

uration, with the onset of a strong daily rhythm of metabolic processes (34, 35), not unlike the circadian rhythms of metabolism described in the mammalian gut symbioses (18, 36). Specifically, the animal becomes fully nocturnal, and the symbiont metabolism begins a day/night fluctuation between respiration and fermentation in response to a change in nutrients provided by the host.

Here we compared the transcriptomes of three highly vascularized organs of the squid host: the light organ itself and the eye and gill, manipulating both their symbiotic state and the genetics of the bacterial partner, in both juvenile and adult animals, and over the day/night cycle (Fig. 1A). The eye was chosen because, like the symbiotic organ, it is a light-sensing organ, and shows convergence in morphology, biochemistry (37), molecular biology (38, 39), and developmental pathways (40). As an immune organ, the gill, like the light organ, responds to bacterial colonization (41). Here, we present evidence that both light-organ colonization and luminescence influence gene regulation of not only symbiotic tissue, but also host organs remote from the symbionts.

Results

De Novo Transcriptome Assembly, Annotation, and Validation Provide the Resources for Analyses of Symbiosis Effects on Host Gene Expression. To determine the extent to which symbiotic colonization impacts host gene expression, we sequenced transcripts isolated from the squid light organ, eye, and gill (Fig. 1A and B). Samples were collected for RNA-sequencing (RNA-seq) analysis from both juvenile (24-h

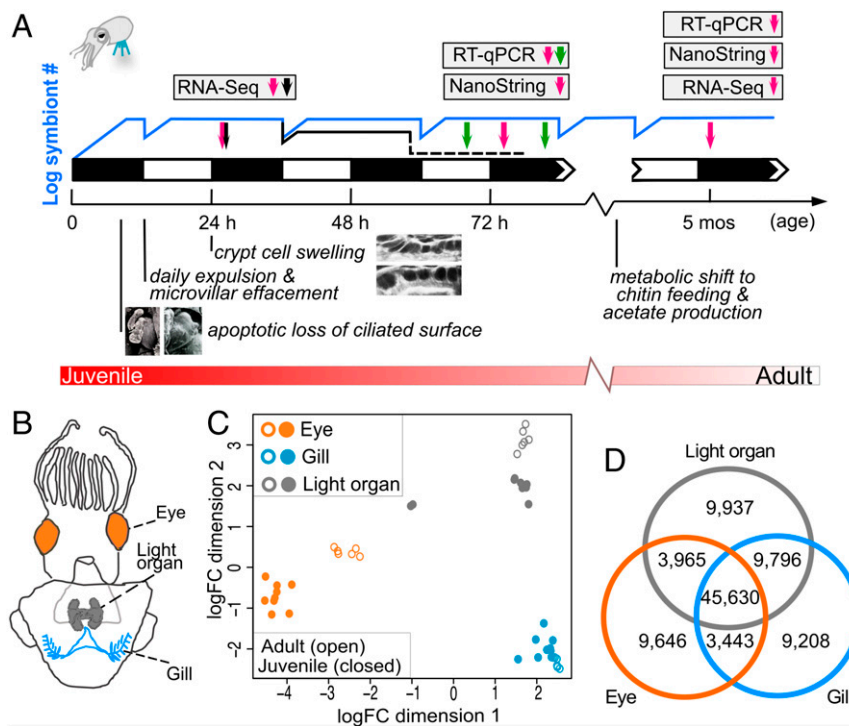


Fig. 1. Transcriptome profiling of *E. scolopes* organs. (A) Transcriptome sampling scheme during symbiotic development. On the night (black squares) that they hatch, juvenile squid become inoculated by *V. fischeri* cells, which proliferate, (blue line), filling the light-organ crypts and producing bioluminescence. Colonization triggers developmental events in the light organ's tissues, including apoptosis of the surface epithelium (Left Inset, APO; Right Inset, SYM), and edematous swelling of the crypt epithelial cells (Upper Inset, APO; Lower Inset, SYM). Each dawn, the nocturnally active host effaces the crypt-cell microvilli and expels most of its symbiont population, which grows back up by noon. A dark mutant (Δlux) colonizes normally, but is unable to persist in the organ (dotted black line), and doesn't induce normal crypt cell swelling. After 1 mo, the host begins providing chitin to the symbionts, which ferment it to acetate. Transcriptomes of light organ, eye, and gill were constructed by RNA-seq or NanoString from organs sampled at 2000 hours (magenta arrows) in APO and SYM hosts of juvenile and adult animals. SYM-dark hosts, colonized by a dark-mutant strain, were also sampled (black arrow). For day/night comparisons, APO and SYM organs were sampled at 1600 and 0400 hours (green arrows) as well. Levels of transcripts of interest were confirmed by NanoString and qRT-PCR. (B) Schematic drawing of *E. scolopes* indicating tissue types collected from both juvenile and adult squid. (C) Multidimensional scaling plot of gene expression for the *E. scolopes* reference transcriptome. (D) Venn diagram of the number of shared and specific genes, expressed by tissue type. A gene is considered expressed when FPKM (fragments per kilobase million) > 0.5 in at least two samples per tissue.

posthatch) and mature adult (5-mo-old) animals, under two colonization states: symbiont-free (i.e., aposymbiotic, or APO) or colonized by the wild-type *V. fischeri* light-organ isolate ES114 (i.e., symbiotic, or SYM). An additional condition that was analyzed in juvenile animals was colonization by an isogenic, non-luminous (Δlux) mutant of ES114 (i.e., SYM-dark) (42) (see *SI Appendix, SI Results* for details). The 2.2 billion paired-end reads obtained by Illumina sequencing, were de novo assembled to create a reference transcriptome (*SI Appendix, Fig. S1* and *Dataset S1*). The large number of assembled transcripts is a common trait found in other de novo assembled transcriptomes of *E. scolopes* (43, 44). Cephalopods are known to expand certain gene families (45, 46). This feature, together with the high levels of heterozygosity and transcript editing (47) that are known to challenge assembly software (48), contributed to the high number of observed expressed transcripts. Transcriptomic profiles clustered by tissue type and, within each tissue type, by developmental stage, with a higher degree of variation among juvenile replicates (Fig. 1C and *SI Appendix, Fig. S2*). Irrespective of developmental stage, eye-derived samples showed the most divergent transcriptional profile. The light organ and gill displayed a more highly correlated expression pattern (Fig. 1C and *SI Appendix, Fig. S2A*), and shared more total expressed genes than either did with the eye (Fig. 1D), perhaps because they are both predominantly composed of epithelial tissue.

When considering the total number of genes that are expressed in each organ, depending on the host developmental stage and symbiotic state (*SI Appendix, Fig. S3A*), on average juvenile samples expressed 20% more detectable genes than their adult counterparts. Only 7,464 genes were expressed in all three organs, from both juveniles and adults, and in both the SYM and APO state (*SI Appendix, Fig. S3B*), suggesting that these genes encode core or “housekeeping” functions (for more details of transcriptomic patterns, see *Dataset S2*). In contrast, when we determined tissue-specific genes (i.e., those that were expressed at least eightfold higher in one organ relative to the other two), a total of 5,587 genes were identified (*SI Appendix, Fig. S2E* and *Dataset S2*). Not surprisingly, gene ontology (GO) terms enriched for the eye were related to visual perception or synaptic signaling, while for the gill were linked to gas exchange or pH regulation; similarly, the light organ was enriched in GO terms related to the expected activities of oxidative stress (49, 50) and chitin-associated processes (51) (*SI Appendix, Fig. S2B–D* and *Dataset S3*). Furthermore, we validated the RNA-seq dataset by two methods, qRT-PCR, and the NanoString nCounter XT platform (*SI Appendix, Fig. S4* and *Dataset S4*), which all had a high degree of congruity. These results provide strong evidence that the transcriptional patterns in response to colonization are robust, and clearly differentiate the three tissue types and their developmental states.

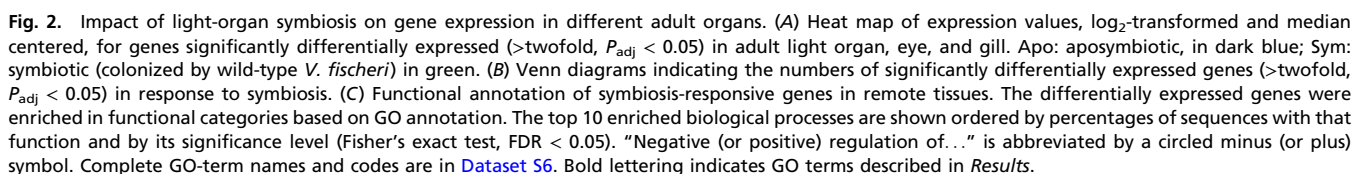
Adult Gene Expression in the Light Organ and Remote Tissues Responded Uniquely to Symbiont Colonization. To identify whether and how the light organ, eye, and gill responded to colonization of the light organ, we compared the gene-expression patterns of these three organs when sampled from adult APO and SYM squid (Fig. 1A). According to the values for the differentially expressed transcripts, the samples clustered by condition (APO or SYM) within each organ (Fig. 2A). Transcripts having expression levels that differed significantly between APO and SYM were identified as up- or down-regulated by symbiosis (Fig. 2B). Unsurprisingly, the light organ, which harbors the symbionts, had the strongest transcriptional response to its colonization, with a total of 206 genes significantly differentially regulated, which clustered into five distinct expression profiles (Fig. 2 and *SI Appendix, Fig. S5*). Although they are in anatomically remote organs (Fig. 1B), the transcriptomes of both eye and gill also responded to colonization of the light organ. Because of the greater similarity between the number of total transcripts in the

light organ and gill (Fig. 1C and D and *SI Appendix, Fig. S2A*), and because both of these organs respond to bacteria as part of their normal function, we anticipated that, compared with the eye, more symbiotically responsive genes would be detected in the gill, and they would overlap more significantly with the light organ. However, the eye had twice as many symbiotically regulated genes as the gill (84 vs. 42) (Fig. 2B, *SI Appendix, Fig. S5*, and *Dataset S5*). Furthermore, each organ had a distinctive transcriptional response to light-organ colonization: only one gene (annotated as angiotensin-converting enzyme or ACE) was up-regulated in two of the organs (eye and gill).

To further analyze not only the possible functions of these symbiosis-responsive genes, but also whether there were shared functions (if not genes) among the three organs, we conducted a GO-enrichment analysis for all of the differentially expressed genes. This analysis identified overrepresented terms in each organ, using the entire transcriptome as the background for the enrichment analysis. In APO animals, we found 40, 32, and 29 overrepresented functions in eye, gill, and light organ, respectively. In contrast, overrepresented functions in response to symbiosis were highest in the light organ, followed by gill and eye (*Dataset S6*). In addition, each of the three organs expressed genes within a unique set of top 10 enriched biological processes, in a symbiosis-dependent manner (Fig. 2C), a further indication of the distinct ways in which they react to the presence of bacteria in the light organ. For example, in the light organ itself, the three major responses to symbiosis, encompassing 9 of the 20 enriched biological functions (Fig. 2C), could be associated with: (i) vascularization and an increased oxygen demand driven by the symbiont’s bioluminescence; (ii) tissue stress from the presence of the symbionts; and (iii) an easing of innate immune responses once the organ is colonized. All of these functions are consistent with previous studies (40, 52, 53). In contrast, in the eye, light-organ colonization resulted in an up-regulation of genes encoding structural proteins, and down-regulation of genes encoding elements of sensory perception and oxidative stress, while the gill exhibited an increased expression of genes encoding stress responses and transcriptional regulation.

Because a robust systemic response to colonization was observed that included functions associated with light-perception in the squid eye (Fig. 2C), an organ convergent in form and function with the mammalian eye (39), we asked whether and how eyes of another well-studied symbiosis model, the mouse, respond to host colonization; to our knowledge, the impact of the gut microbiota on the transcriptomic profile of the mouse eye has not been reported. We compared, by RNA sequencing, the expression profile of the eye of conventionalized mice (i.e., mice in which the gut microbiota was present) to that of germ-free mice. Adult stage mice and squid were compared to minimize any effects due to differences in their developmental rates. Applying the same level of stringency as used for the squid eye (i.e., an adjusted $P < 0.05$) only five genes were detected as differentially expressed in the mouse eye in response to conventionalization (*Dataset S7*). One predicted gene was down-regulated, and four genes were up-regulated, including lactotransferrin, which was previously reported as present in the transcriptome of mouse eye (54), IFN-activated gene 205, a mitochondrial tRNA, and a noncoding RNA of the RIKEN family. Although the evolutionarily convergent eyes of cephalopods and vertebrates share a large number of conserved genes with similar expression levels (55, 56), we detected no shared symbiosis-regulated genes in the eyes of these two organisms.

In summary, in the mature squid symbiosis: (i) functionally distinct and anatomically distant tissues are influenced by the presence of symbiotic bacteria; (ii) unlike the total expression profile for each of the three organs, the transcriptional responses to symbiosis, and their functional annotations, were specific and



Colonization of Juvenile Hosts Had a Rapid Impact on Gene Expression, Even in Remote Tissues. In the adult host, transcriptional responses to symbiosis are evident both locally and systemically (Figs. 2 and 3), but how quickly during symbiotic development does this outcome appear? The transcriptional response of the light organ has been reported to occur as early as 3 h following exposure to environmental *V. fischeri* (52). To determine the manner and timing of symbiosis-specific responses in other organs, more distant from the light-organ symbionts, we compared the RNA-seq gene-expression data of light organ, eye, and gill 24 h after the initiation of symbiosis, when the bacteria have fully colonized and are brightly luminous (28). At this point, we found that the light organ

As expected, a smaller number of symbiosis-responsive genes (44 in the eye and 184 in the gill) were detected (*SI Appendix, Figs. S6 and S7*), and there was essentially no overlap with the adult response in either of these organs. Nevertheless, a trend in

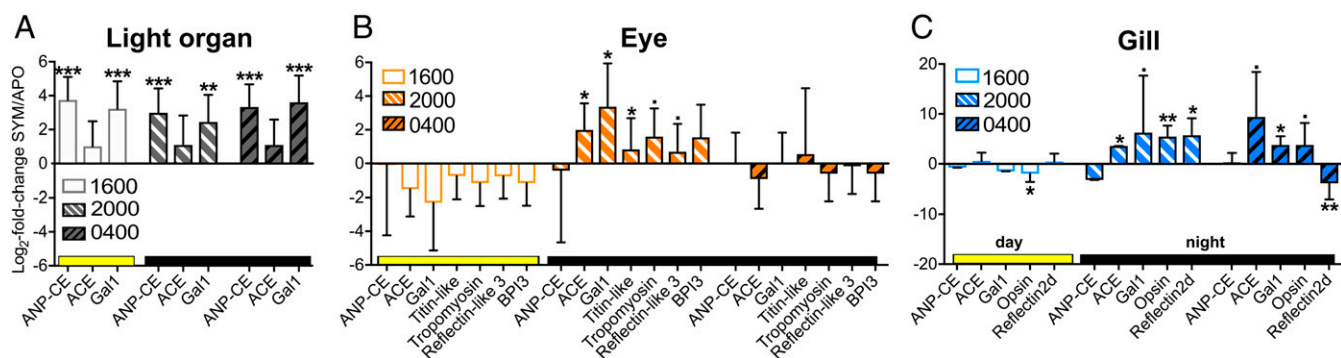


Fig. 3. Variation of symbiosis-responsive gene expression over the day/night cycle. Gene expression changes in SYM (compared with APO) light-organ (A), eye (B), and gill (C) tissue at different times of day: 0400 hours = 2 h before dawn; 1600 hours = 2 h before dusk; 2000 hours = 2 h after dusk. Juvenile squid were maintained for 3 d under a 12–12 light-dark schedule. Candidate genes were chosen for qRT-PCR based on expression changes observed at 72 h (SI Appendix, Fig. S9 and Table S1 and Dataset S4). BPI3, bactericidal/permeability-increasing protein 3. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; $P < 0.1$ (SYM vs. APO).

which eye (but not gill) genes clustered with colonization state was detected (SI Appendix, Fig. S7). Unlike the light organ, the responses of eye and gill at 24 h were highly variable between samples; thus, we hypothesized that many genes that were differentially regulated in these organs in adults may have not yet become apparent in 24-h juveniles (SI Appendix, Fig. S84). Thus, we used the NanoString platform to determine whether the patterns of a selected set of 23 genes that were not significantly regulated at 24 h (Dataset S4), but were either trending toward induction at 24 h or would become induced in adult eye and gill had, by 72 h, become significantly differentially regulated by symbiosis. While such a temporal comparison can be made with juvenile eye or gill tissues, changes in the light organ transcriptome are confounded by this organ's substantial morphogenic transformation between 24 and 72 h (28).

Of the 13 selected adult eye-specific, symbiosis-responsive genes, 4 became clearly differentially regulated in juvenile eye tissue between 24 and 72 h postcolonization (e.g., SI Appendix, Figs. S84' and S9), indicating that during this period much of the transcriptional signature of the adult eye was still developing. For the gill, we chose two groups of genes: six that were significantly up-regulated in adults and four that were not, but were trending toward significance in 24-h juveniles. Of the first group, only one (ACE) had become differentially regulated by 72 h, while all four of the 24-h trending genes had. Thus, the data suggest that the gill has a more juvenile-specific response that is not retained in adults. In summary, the analysis of juvenile organs indicates that: (i) a robust transcriptional response to symbiosis appeared in the light organ within 24 h postcolonization, (ii) a smaller systemic response by eye and gill also became apparent, and (iii) by 24 h, the juvenile eye began to show an adult-like response, which became more significant at 72 h.

Expression of Some Symbiosis-Responsive Genes Was Regulated over the Day/Night Cycle. Because the light organ has a well-described daily rhythm of transcriptional regulation (35) that is reflected in crypt-cell ultrastructural remodeling, symbiont luminescence, and metabolic activity in both partners (34, 35), we asked whether the symbiosis-regulated gene expression detected in remote organs also changed over the day (Fig. 14). Based on the NanoString data for 72-h juveniles at 2000 hours (SI Appendix, Fig. S9), we characterized symbiosis-responsive gene expression from juvenile organs by qRT-PCR at three times: 2 h before dusk (1600 hours, at ~70-h postcolonization) and 2 h before dawn (0400 hours), both times when the host is quiescent and symbiont luminescence is reduced, compared with 2 h after dusk (2000 hours, at ~74-h postcolonization) (Fig. 14), when the host is active and the symbionts are brightly luminous (34).

Expression levels of three genes [atrial natriuretic peptide-converting enzyme (ANP-CE), ACE, and galaxin 1 (Gal1)] were determined across all of the organs. In the light organ, while ACE was not significantly regulated by symbiosis, ANP-CE and Gal1 remained up-regulated in SYM relative to APO at all times tested (Fig. 3A). In contrast, Gal1 and ACE were up-regulated by symbiosis in the eye only at 2000 hours although, in gill, ACE was up at both 2000 and 0400 hours (Fig. 3B and C). Thus, ANP-CE is specifically regulated in the light organ, as is ACE regulated only in the eye and gill.

Expression of an additional four eye-specific and two gill-specific genes that were symbiosis-regulated at 2000 hours were likewise dependent on time of day. While there are trends of down-regulation of these genes in the eye at 1600 hours, no significant differences appeared beyond 2000 hours (Fig. 3B). Similarly, in gill, opsin is up-regulated at 2000 relative to 1600 hours, while reflectin 2 d becomes down-regulated (relative to APO) at 0400 hours (Fig. 3C). Cephalopods are noted for extraocular photoreceptors (57), but these structures are associated with the surface of the animal, and not with internal organs, such as the gills (58).

In summary, symbiosis-responsive genes that were regulated in one organ at one time of day can be differentially expressed in other organs at a different time, emphasizing the time- and context-dependency of the response. In addition, among the genes examined here, the symbiosis-dependent up-regulation of expression in gill, and especially in eye, was generally most prominent early in the evening (2000 hours), when the host is ecologically active. In contrast, in the light organ, no pattern was observed for these genes (Fig. 3A), although other genes show strong patterns of temporal regulation (35).

Symbiont Luminescence Was the Principal Driver of Transcriptomic Patterns in both the Light Organ and the Eye. Because symbiosis-induced up-regulation of gene expression occurred at night, coincident with high levels of symbiont luminescence, we asked whether light-emission itself is a factor driving gene expression. To this end, the gene-expression profiles of the juvenile light organ, eye, and gill were compared when the light organ was colonized by either a wild-type, light-producing strain (SYM) or a nonluminous Δlux mutant derivative (SYM-dark). Because such dark mutants can only maintain normal levels of colonization for the first day postinoculation (32), we focused our analyses on 24 h after symbiosis had initiated.

At this time, under normal conditions of SYM colonization, 1,919 genes are regulated in the light organ compared with APO (Fig. 4A). Comparison of the SYM expression profile with that of the SYM-dark animals revealed that at 24 h the light organ has a strong transcriptional response, independent of light production.

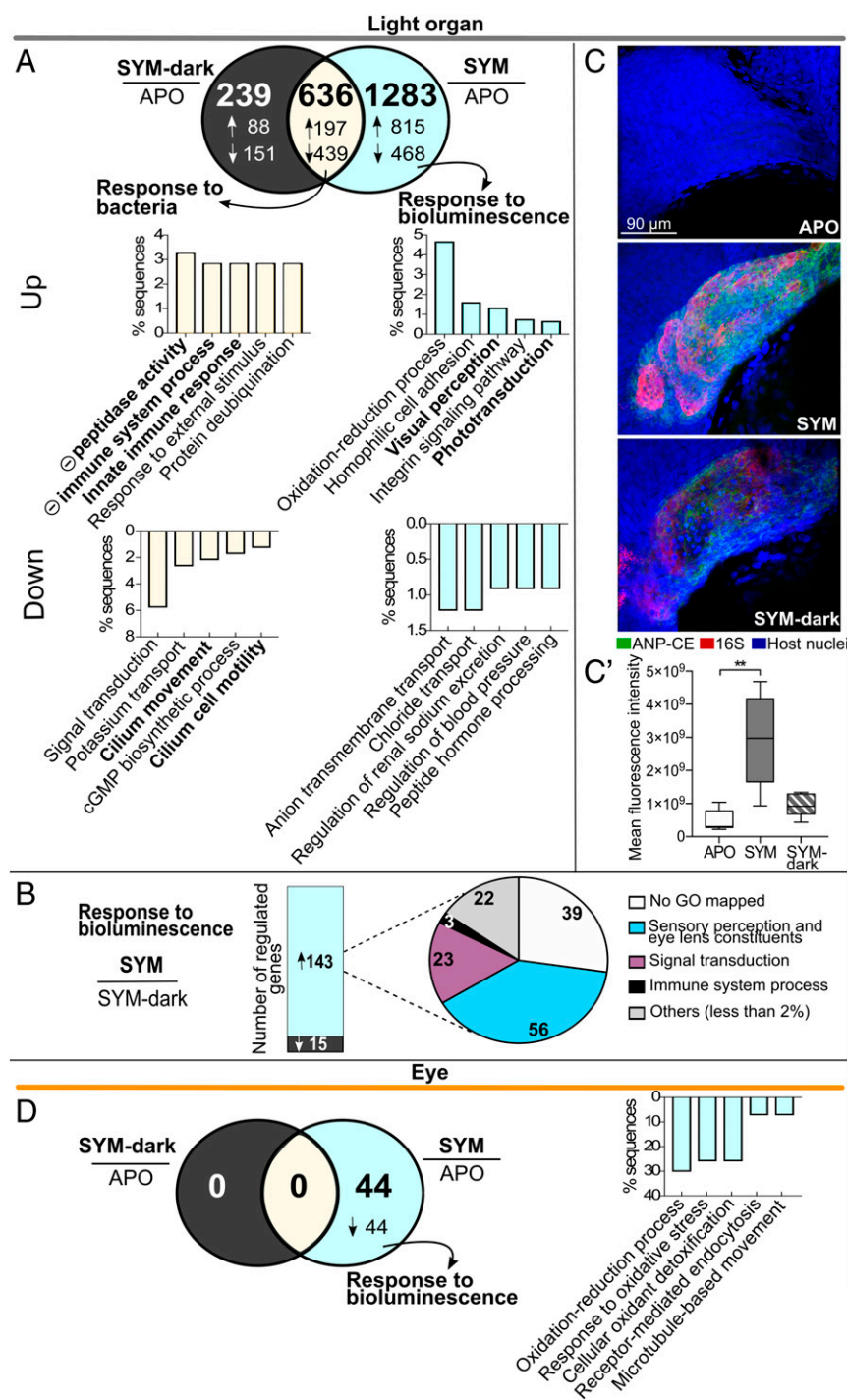


Fig. 4. Impact of symbiont bioluminescence on juvenile gene expression. (A) Venn diagram of numbers of differentially expressed genes in the light organ, 24 h after colonization by either SYM (wild-type) or SYM-dark (Δlux) strains, compared with APO (>twofold, $P_{adj} < 0.05$). Arrows indicate either up (\uparrow) or down (\downarrow) regulation. Bar graphs: functional enrichment of genes significantly up-regulated and down-regulated with symbiosis. For each set, the top five biological process terms are represented (Fisher's exact test, FDR < 0.01). Notations as in Fig. 2. (B, Left) number of genes significantly up- or down-regulated in SYM compared with SYM-dark colonized light organs; (Right) proportion of annotated biological processes accounting for >2% of up-regulated genes. (C) Visualization of ANP-CE transcript in whole-mount light organs 24 h after colonization. Representative confocal images showing ANP-CE expression in crypt epithelium of APO, SYM, or SYM-dark colonized juvenile squid; merged mid-section of z-stack of crypt #1; ANP-CE (green), 16S RNA (symbionts, red), and host nuclei (TOPRO, blue) (*SI Appendix, Fig. S10 and Movies S1–S3*). (C) Quantification of ANP-CE signal by fluorescence intensity from z-stacks of crypt #1 in five light organs. P values were calculated using Kruskal-Wallis test and Dunn's multiple comparison test. Error bar: SD (** $P < 0.01$). (D) Venn diagram of differentially expressed genes in the eye, 24 h after colonization by either SYM (wild-type) or SYM-dark (Δlux) strains, compared with APO (>twofold, $P_{adj} < 0.05$). Arrow indicates down (\downarrow) regulation. Bar graph: notations as in A (*Datasets S8 and S9*).

Specifically, a total of 636 genes were regulated by both strains, over two-thirds of which were down-regulated. The functional annotation of >25% of these down-regulated genes was dominated by GO categories associated with maintenance of ciliary structure and function (Fig. 4A), which is not surprising because both SYM and SYM-dark bacteria induce the cell death and loss of the ciliated surface that mediates initial colonization (28). Also enriched in this shared set were genes up-regulated in immune response and stress (Fig. 4A), which is also not unexpected, as the morphogenesis of the ciliated surface is driven largely by symbiont microbe-associated molecular patterns.

However, it is most striking that over two-thirds of the 1,919 genes regulated by colonization required that the symbionts be

luminescent, underscoring how critical a role *V. fischeri* bioluminescence plays in shaping the symbiosis. In direct contrast to the luminescence-independent response, nearly 70% of the genes of the luminescence-specific response were up-regulated. Notably, the 39 GO categories of up-regulated genes included “visual perception,” “phototransduction,” “photoreceptor activity,” and “structural constituent of eye lens,” all involved with light perception or modulation, as well as homophilic cell adhesion and oxidative-reduction processes (Fig. 4A and *Dataset S9*). The light organs that were colonized by the dark mutant not only failed to regulate these luminescence-specific genes, but also had an expression signature of their own. The dark mutant

regulated 875 genes, only 46% the number regulated by the wild-type strain (1,919), with just over one-quarter of their regulated genes specific to the SYM-dark colonization, compared with the two-thirds of genes specific to the luminous SYM colonization. Furthermore, unlike the SYM animals, the SYM-dark condition resulted principally in down-regulated genes, with no significant functional enrichment in any GO category (Fig. 4A). Finally, when gene regulation was compared directly between SYM and SYM-dark animals, 143 annotated genes were up-regulated in SYM, and only 15 down-regulated (Fig. 4B). Thirty-nine percent of these up-regulated genes were associated with sensory perception of light stimulus or modulation of light (e.g., lens proteins) (Dataset S9).

A particularly interesting difference between colonization conditions was the ~sevenfold up-regulation in SYM compared with SYM-dark of ANP-CE, which regulates cell volume and inflammation in a variety of systems (59). One of the key developmental features of the light organ is the SYM-induced swelling of the crypt cells with which the bacteria directly associate (Fig. 1A); however, the dark mutant is defective in inducing this phenotype (32). If ANP-CE were involved in such a cell-swelling and inflammation phenotype, we would predict that the transcript for this protein would specifically localize to the crypt epithelium in the SYM host, and be at a higher abundance than in SYM-dark-colonized animals. Using hybridization chain reaction-FISH, we compared the localization of the ANP-CE transcript in light organs at 24-h postinoculation. Abundant transcripts localized specifically to the cytoplasm of the crypt cells in SYM-colonized animals (SI Appendix and Movies S1–S3). In contrast, only low levels were detected in either APO or SYM-dark animals, with no significant difference between these conditions (Fig. 4C and C').

Unlike the light organ, no difference in colonization-dependent gene expression was detected between gill tissue of SYM and SYM-dark juveniles at 24 h, perhaps due to this organ's high variability in development at this time point. However, the eye showed a uniform, down-regulation in the expression of all 44 of the genes that responded in any way to colonization by the luminous symbiont (Fig. 4D); in contrast, no significant change in expression of any of those genes was detected when the symbiont was the dark mutant. These data suggest that, like the light organ, the eye's principal reaction to symbiotic colonization is mediated by the presence of light production. Interestingly, eye genes down-regulated by symbiont colonization were enriched in biological processes related to oxidation state or tissue reorganization (Fig. 4D and Dataset S9). Only 4 of the 44 genes down-regulated in the eye were shared with the light-organ's response: specifically, ANP-CE, MAM/LDL-receptor class A domain-containing 2-like, dynein heavy-chain axonemal, as well as the hypothetical protein KGM_03810, which is also regulated in the adult light organ. However, unlike with the juvenile light organ, the first three of these eye genes are up- (not down-) regulated in response to symbiosis. In summary, symbiosis-dependent gene expression in both the light organ and eye was more dependent on the existence of bacterium's luminescence than on the presence of the bacteria themselves.

Discussion

The data presented here demonstrate that, beginning within hours of the onset of the *E. scolopes* light-organ symbiosis, localized colonization of tissues by the specific symbiont *V. fischeri* creates a network of communication across the host's body that reprograms transcription system-wide. The coordination of this network persists throughout the developmental trajectory of the association, beginning with early symbiosis-induced changes in light-organ form and function, and continuing well into the maturation of the partnership. Furthermore, the network reprograms remote organs to respond to the daily rhythms set by the *V. fischeri* population within the light organ. Finally, genetic

manipulation of the symbiosis revealed that bioluminescence, the principal currency of the symbiont, while having no effect on the gill response, is not only the major symbiosis-dependent driver of transcriptional regulation in the light organ, but also the only driver in the eye.

Transcriptomes of the Host's Organs Reflect Their Biological Functions Through Development.

The transcriptomes examined in this study, which segregated both by organ and by life stage, reflect known functions of the light organ, eye, and gill: that is, control of symbiont luminescence, vision, and respiration, respectively (SI Appendix, Fig. S3 and Dataset S3). The light organ, eye, and gill transcriptomes clustered separately between juveniles and adults (Fig. 1C and SI Appendix, Fig. S24), a finding that may reflect the dramatic change in the animal's ecology upon its maturation. Briefly, from hatching to ~4-wk postcolonization, the juvenile's behavior is not controlled by a daily rhythm, being either active or quiescent at all times of day and night; however, by about 1 mo, the animal has assumed a profound diel rhythm of burying in the substrate during the day and coming out to forage at night, a behavior that will persist throughout its ~1-y life (60). This change in lifestyle coincides with a dramatic shift in the daily cycling of host and symbiont metabolism (34), a shift that promotes a brighter luminescence of the bacteria in the evening, when the squid is active and using the light emission of the symbionts to camouflage itself by "counterillumination" (28, 61). We hypothesize that the eye may be responding transcriptionally not only to its commitment to a diel rhythm of environmental light, but also to the requirement that the eye coordinate its function with the light organ's emission. Specifically, during counterillumination, the light organ modulates its luminescence in response to the intensity of down-welling moonlight and starlight, which is monitored by the eye, through as yet undefined mechanisms. With such developmental changes in day/night behavior, it is not surprising that the transcriptomes of both the light organ and eye adopt new patterns as these organs mature. Similarly, the respiratory and immune functions of gill tissue, like its transcriptome (Fig. 1C), change between juvenile and adults as the animal begins to bury in the substrate each day by 4 wk of age.

Symbiosis-Induced Changes in Squid–Host Gene Expression Show Similarities with Those Reported for the Mammalian Microbiota.

As with the global patterns of squid gene expression (Fig. 1C), both the light organ and remote tissues reacted robustly to colonization by *V. fischeri*, and several features of this life-stage and organ-specific transcriptomic response appear to be evolutionarily conserved between the squid light-organ and mammalian-gut symbioses. For example, a comparison of intestinal epithelial cell transcriptomes from germ-free and conventionalized mice found that the response of intestinal epithelial cells to the presence of the microbiota was only a fraction of the genes expressed in these cells and, as in squid, little overlap in this response occurs between juvenile and adult mice (62). In the squid, such largely stage-specific patterns of symbiosis reprogramming apply not only to the colonized tissue (i.e., the light organ), but also to the anatomically remote eye and gill. As yet, we know little about how remote tissues receive information about the colonization state or activity of symbiotic organs, but two modes are possible. The first is a chemical signal, such as a bacterial metabolite, delivered through the circulation (63, 64); one such metabolite, acetate, is generated in the light organ as byproduct of symbiont metabolism (34). For example, the presence of *V. fischeri* in the light organ has a systemic effect on hemocyte signaling (65). The second mode is neural: cephalopods, in particular, produce both targeted and systemic responses via their nervous system, similar to mammals, where the vagus nerve conveys information about the gut microbiota to the brain (66). However they are delivered, the transcriptional

changes appearing during postnatal development in the mouse and squid organs reveal only part of the impact of symbiosis. In fact, many of these effects are deployed during embryogenesis, when symbionts are not yet present: that is, both in the squid–vibrio system and in mammals, the developing host creates specific target-tissue conditions that are poised to respond to the eventual arrival of their symbiotic partners (28). Conversely, in a synoptic study comparing digestive system transcriptomes of four regions of the mouse intestine and liver (67), as with the squid, very little overlap in the transcriptional response to symbiosis occurred across the body.

To our knowledge, an integrated comparison both across tissues and through development has not yet been addressed in the mouse; thus, whether the trajectory of the robust mammalian transcriptional response to symbiont colonization over early development varies among organs, as it did in the squid (i.e., first the light organ, then eye, then gill) (Datasets S4 and S8) remains to be determined. However, taken together, these similarities in life-stage and organ/tissue-specific responses to symbiosis in the two distantly related animal taxa suggest that, rather than having a generalized response to bacteria or their products, as they develop each tissue or organ near to or distant from sites of colonization integrates the partnership into its specific function. For example, in the squid association, we observed an up-regulation of genes involved in vascular development of tissues (Fig. 2C and Dataset S6), which has also been reported for the nutrition-based gut symbioses of mammals (68). The finding that the squid system, wherein bioluminescence and not nutrition is the principal benefit to the host, suggests, not surprisingly, that increased vascularization is important for other aspects of this symbiosis, such as facilitating the support of the bacterial population and their dialogue with host tissues (34, 53).

Symbiotic Regulation of Genes Encoding Specific Functions Occurs on a Diel Cycle. In addition to sharing life-stage and tissue-specific responses to symbiosis, in both mammalian (69) and squid hosts, there exist diel transcriptomic rhythms in colonized and remote tissues that are influenced by their bacterial partners. For example, although the overall transcriptomes of the three squid organs examined here differed greatly (Fig. 1C), the juvenile light organ shared with eye and gill a symbiosis-dependent diel regulation of two genes: ACE and Gal1. ACE occurs widely among animals, controlling blood pressure and electrolyte balance, although the conserved function of this protein family is immune modulation in both vertebrates (70, 71) and invertebrates (59, 72). Immunity is also likely to be its ancestral role because ACE occurs even in taxa without a closed circulatory system; however, in the squid, whose circulation is closed, this protein may serve both an immune and a vascular function. In contrast, galaxins are invertebrate-specific proteins, first identified in corals; in *E. scolopes*, Gal1 is an antimicrobial peptide present in the light organ (73). Although the genes encoding ACE and Gal1 were both regulated (either up or down) by symbiosis, each had a different daily rhythm of expression depending on the host organ (Fig. 3).

What biological purposes might underlie the diel regulation of these genes? At all times analyzed—that is, 0400, 1600, and 2000 hours—the light organs were fully colonized (74); however, at 1600 and 0400 hours, the per cell luminescence of the symbionts is relatively low, compared with its maximum at 2000 hours (75). The light organ expressed ACE and Gal1 constitutively throughout the day, whereas ACE and Gal1 expression was up-regulated in the eye only when luminescence was highest (2000 hours). In addition, the light organ had symbiosis-induced expression of ANP-CE, which transforms a propeptide to the edema-related peptide ANP (76). Because ANP-CE and ACE often offset one another's functions in immunity and vascular homeostasis (77), these findings suggest that an ACE-driven

modulation of ANP-CE activity in the light organ releases ANP into the host blood stream, modulating the biochemistry of the eye and gill during the day/night cycle. Taken together, the transcriptional data are consistent with an organ-specific expression of an increased immune potential at particular times of day.

Light Organ and Eye Are More Similar in Their Responses to Symbiosis. The repertoire of gene expression characteristic of light organ and gill tissues overlapped more in gene identity and number (Fig. 1D), consistent with both their similar relationship to the environment (i.e., both are bathed with bacteria-rich seawater during ventilation) and their shared immune function. In contrast, the interior portions of the eye examined here are protected from any direct exposure to environmental microbes. However, compared with the gill, the differential gene expression in symbiosis responses of the squid's eye and light organ were more similar in magnitude (Fig. 2B), reinforcing the hypothesis proposed above, that a coordination between these organs facilitates the host's counterillumination behavior within the ambient light field (61).

The squid eye also had a stronger relative response to light-organ colonization (84 genes) (Fig. 2B) than the mouse eye did to colonization of the gut by the microbiota (five genes) (Dataset S7). This difference in the scale of transcriptional response was unexpected because, while the mice were an inbred strain, the squid were genetically diverse, reared from wild-caught parents, and such genetic variation should lead to an underestimation of significant transcriptomic differences. However, the relatively weak reprogramming of the mouse by the presence of its gut microbiota may reflect the vertebrate eye's status as a site of immune privilege (17). Nevertheless, colonization has been reported to influence the mouse eye's lipid content (16), and metabolomics studies have revealed a gut–retina axis that correlates with a proclivity toward development of macular degeneration (15, 78). In short, the difference between squid and mouse responses may be either due to differences in how the immune system interacts with the eye, or because of a greater need for light-organ/eye coordination in bioluminescent symbiotic associations. The latter hypothesis could be tested by a study of fishes with light-organ symbioses, which have both the vertebrate immune privilege of the eye, and the need to modulate luminescence by the symbiotic organ (79, 80).

Symbiont Luminescence Has a Disproportionately Large Transcriptomic Effect. A striking feature of symbiont-induced gene expression was discovered during a comparison of the organ transcriptomes of squid colonized with either the wild-type or the Δlux mutant symbiont. The results highlight the remarkable dominance of luminescence in reprogramming gene regulation in not only the symbiont-containing light organ (Fig. 4A and B), but also the anatomically remote eye (Fig. 4D). In many other associations, studies of such systemic consequences of losing the symbiont's principal activity have been clouded by the resultant physiological effects on the host. For example, in the rhizobium-legume symbiosis, bacterial mutants defective in nitrogen fixation similarly have a differential affect on transcription in both nodule tissue (81) and other plant organs (82); however, it was difficult to separate functions within the nodule from their general nutritional role. Similarly, animal–microbe interactions within gut tracts and bacteriomes also revolve around the symbiont's provision of an important nutrition function (83). That is, unlike bioluminescence, these functions directly impact the host's general physiology and health. Because the light-organ symbiosis plays an ecological role for the squid (i.e., antipredation), under laboratory conditions, the physiology of the host is not negatively affected by carriage of a dark mutant (32). As such, this system serves as a paradigm for studies of the reprogramming of remote-tissue by other microbiota assemblages with nonnutritional functions [e.g., an antibiotic

or defensive toxin (84, 85)], like those of the skin and urogenital tract of humans (86).

ANP-CE is one of the most abundant and highly regulated genes in the squid transcriptomes; specifically, it is overexpressed in SYM relative to APO tissues (Fig. 4C), its level fluctuates on a day/night cycle in eye and gill (Fig. 3 B and C), and in light organ and eye its expression is induced by the symbiont's luminescence (Dataset S8). Because ANP-CE plays a role in inducing cell edema (76), and the epithelia lining the symbiont-containing crypts swell when colonized by wild-type, but not dark-mutant, *V. fischeri* (32, 87), we predicted that ANP-CE expression would be reduced in the epithelium when the symbionts are dark mutants (Fig. 4C). This reduction suggested a direct link between symbiont function and host-tissue response, and may indicate a mechanism by which dark mutants are sanctioned (32, 88), possibly by withholding the nutrients typically delivered by the crypt's edematous epithelium.

The data presented here demonstrate that different tissues can have different transcriptional drivers in response to symbiosis, and future work will address the array of symbiont features that trigger the systemic response to light-organ colonization. Here we demonstrate that, among several effects on remote tissues, the most remarkable was the role of the symbiont's product (luminescence), rather than the presence of the symbiont itself, as the sole driver reprogramming the eye's transcriptome (Fig. 4D). As yet, it is unclear how the eye recognizes the presence of a luminous symbiont: perhaps a signal is delivered indirectly through a humoral or neural signal from the light organ (39). In any case, determining the mechanisms underlying this response presents a rich horizon for discovering shared principles of microbe–organ communication. The transcriptomic responses described here not only document symbiosis-induced molecular networks across the host, but also reveal how these networks may influence the behavior and ecology of the host: for example, mediating the counterillumination antipredatory strategy.

In conclusion, we determined three types of organ-specific transcriptional responses to symbiont colonization over the trajectory of development and over the day/night cycle: (i) a strong reaction to both the microbe and its primary product, luminescence (light organ); (ii) a response to the presence of the symbionts, independent of their luminescence (gill); and (iii) a response triggered solely by the luminescence product (eye). Determining the presence and mechanistic basis of the in-

terorgan network that connects symbiotic and remote tissues, enabling a coordinated response, is a critical area of exploration, and will eventually reveal the degree to which symbioses can influence host health and homeostasis throughout life.

Materials and Methods

Sample Collection. Animals were collected 24 and 72 h after hatching (juveniles), or ~5 mo (adults) (Fig. 1A), and anesthetized in seawater containing 2% ethanol; juveniles were stored whole in RNAlater (Sigma-Aldrich) as previously described (52), while adult tissues were dissected before storage. With the exception of day/night cycle studies, all samples were collected at 2000 hours, 2 h after dusk.

RNA-Seq Assembly and Analysis. A total of 2.2 billion paired-end reads were de novo assembled using the Trinity-v2.4.0 RNA-Seq assembler (89) (Dataset S1), and annotated by BLASTx against the National Center for Biotechnology Information nonredundant protein database. For functional annotation of the reference transcriptome, GO mapping of the transcripts was performed with Blast2GO software (90). To estimate the relative expression value for transcripts, RSEM software (91) was used in combination with the R package edgeR (92) to identify the significantly differentially expressed transcripts. Statistical enrichment of GO terms for differentially expressed genes was performed in Blast2Go using the Fisher's exact test with a false-discovery rate (FDR) < 0.01. In addition, gene set-weighted enrichment analysis with 500 permutations and FDR < 0.1 was performed on the differentially expressed transcripts (SI Appendix and Dataset S10).

Transcript Quantification by qRT-PCR or NanoString nCounter Analysis. Changes in host gene expression were measured by qRT-PCR using Light-Cycler 480 SYBR Green I Master Mix (Roche). Ribosomal protein 19L, serine hydroxymethyl transferase, and heat-shock protein 90 were used to normalize the transcript level (SI Appendix, Table S1). The nCounter Custom CodeSet Kit (NanoString Technologies) was used to detect changes in gene expression (Dataset S4). Assay and spike-in controls were used for normalization based on identical amounts of input RNA. Analysis was performed with nSolverAnalysis software v3.0.

See SI Appendix, SI Materials and Methods for additional experimental details. All experiments involving mice were performed using protocols approved by the University of Wisconsin–Madison Animal Care and Use Committee.

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