The cytokine MIF controls daily rhythms of symbiont nutrition in an animal–bacterial association

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The recent recognition that many symbioses exhibit daily rhythms has encouraged research into the partner dialogue that drives these biological oscillations. Here we characterized the pivotal role of the versatile cytokine macrophage migration inhibitory factor (MIF) in regulating a metabolic rhythm in the model light-organ symbiosis between *Euprymna scolopes* and *Vibrio fischeri*. As the juvenile host matures, it develops complex daily rhythms characterized by profound changes in the association, from gene expression to behavior. One such rhythm is a diurnal shift in symbiont metabolism triggered by the periodic provision of a specific nutrient by the mature host: each night the symbionts catabolize chitin released from hemocytes (phagocytic immune cells) that traffic into the light-organ crypts, where the population of *V. fischeri* cells resides. Nocturnal migration of these macrophage-like cells, together with identification of an *E. scolopes* MIF (EsMIF) in the light-organ transcriptome, led us to ask whether EsMIF might be the gatekeeper controlling the periodic movement of the hemocytes. Western blots, ELISAs, and confocal immunocytochemistry showed EsMIF was at highest abundance in the light organ. Its concentration there was lowest at night, when hemocytes entered the crypts. EsMIF inhibited migration of isolated hemocytes, whereas exported bacterial products, including peptidoglycan derivatives and secreted chitin catabolites, induced migration. These results provide evidence that the nocturnal decrease in EsMIF concentration permits the hemocytes to be drawn into the crypts, delivering chitin. This nutritional function for a cytokine offers the basis for the diurnal rhythms underlying a dynamic symbiotic conversation.

symbiosis | daily cycling | *Euprymna scolopes* | *Vibrio fischeri* | symbiont metabolism

Recent research has demonstrated that host-associated microbes are strongly integrated into, and often control, fundamental features of host biology. These effects on the host span such broad influences as the shaping of development, maturation, physiology, behavior, ecology, and even their evolution (1, 2). One of the most significant conceptual shifts resulting from these new findings has been in our view of the form and function of the immune system. The vertebrate immune system is now broadly recognized as simultaneously mitigating pathogenesis and maintaining healthy interactions with a complex microbiota (3–5). These two diverse functions demand cellular and molecular flexibility in the components of the immune system to mediate a broad array of physiological processes.

One such flexible element is the population of phagocytic macrophages, which participate in tissue remodeling, brain homeostasis, and wound healing, in addition to their role in innate immunity (6). Diverse mechanisms control the movement of macrophages through the body, including macrophage migration inhibitory factor, or MIF, which is a functionally versatile regulator of the immune system in animals (7, 8). Initially described in 1966 (9), it was one of the first biomolecules to be recognized as a cytokine, with the foundational studies showing that the protein inhibits the migration of macrophages toward a chemoattractant (10). The sequence and structure of MIF proteins are highly evolutionarily conserved, with representatives of the protein recognizable in all domains of life (11, 12). Its enzymatic properties, which require evolutionary conservation of specific amino acids, include tautomerase and oxidoreductase activities (13, 14). Studies in mammals showed that MIF also has potent proinflammatory properties and occurs in numerous human-tissue cell types, including epithelial cells (15).

A recent study linking MIF activity to the mammalian microbiota demonstrated that MIF both influences the integrity of the epithelial barrier (16) and contributes to the regulation of host–microbiota signaling pathways (17). However, overproduction of MIF has been implicated in inflammatory conditions, such as ulcerative colitis and inflammatory bowel disease (18, 19), and compelling evidence has shown that MIF plays a role in metabolic disorders, including diabetes and obesity (20). All of these pathological conditions have been convincingly linked to a

Significance

Daily biological rhythms are fundamental, evolutionarily conserved features of many symbioses. Here we use the binary squid–vibrio association, the simplicity of which has offered the resolution to explore strategies of symbiosis that are shared with more complex systems, such as those of mammals. We demonstrate a pivotal role for an evolutionarily conserved cytokine, macrophage migration inhibitory factor, or MIF, which is abundant in epithelia supporting the symbionts of both the squid light organ and the mammalian gut. In the mature squid association, MIF regulates the cyclic metabolic dialogue that underlies oscillations in host-provided symbiont nutrients. As such, this study integrates the role of MIF across two distinct time scales: the host's life history and its daily rhythms.

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The authors declare no competing interest.

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dysbiotic microbiota (21). Thus, evidence to date suggests that keeping the correct balance of MIF is important for normal host tissue form and function, particularly for tissues in contact with microbes.

In this study, we explored the role of MIF in the maintenance of the mature symbiosis between the squid, *Euprymna scolopes*, and its luminous bacterial partner, *Vibrio fischeri* (22, 23). In this binary model system, *V. fischeri* lives extracellularly in epithelium-lined crypt spaces within a specialized light-emitting organ (Fig. 1*A*). A prominent feature of the association is a profound daily rhythmicity (24–27), a character that is widespread in symbiotic systems, from corals (28) to humans (29, 30). In the squid–vibrio system, each day at dawn, ∼95% of the symbiont population is vented from the light organ into the surrounding environment; the remaining 5% of the population then grows to repopulate the organ, providing the host with a cohort of symbionts, the luminescence of which it uses during its nocturnal activities (31) (Fig. 1*B*). While this rhythmic venting behavior begins in the juveniles (24), other daily rhythms take longer to develop, appearing only after 3 to 4 wk. This maturation includes the onset of oscillations in symbiont nutrition and changes in the cycling of host gene expression (22, 26, 32). These changes in the light-organ rhythms are concomitant with the host assuming its mature behavior of quiescence during the day and foraging and mating in the water column at night (22).

Associated with adult daily rhythms are extensive alterations both in the ultrastructure of the epithelia and in the physiology of the bacteria, changes that are also reflected in the oscillations of gene expression by both partners (27) (Fig. 1*B*). Analyses of these patterns revealed that, when symbionts are vented at dawn, the cell junctions of the crypt epithelium are disturbed and the apical membrane surfaces are shed into the lumina, indicating a breakdown in barrier function (27). Transcriptomic data and biochemical analyses have provided evidence that, in the mature light organ, the small population of symbionts that remains in the crypts after the dawn venting responds to this disturbance by upregulating genes associated with the use of sloughed membranes as a lipid source for metabolism, which occurs through the pH-neutral process of anaerobic respiration (27). By noon, the symbiont population has repopulated the crypts and ceased growing, and the host epithelium has repolarized. Subsequently, during the night, the symbionts up-regulate genes required for chitin fermentation, a process that acidifies the crypt spaces (26). Imaging of the light organ and genetic studies of the symbiont have revealed that macrophage-like hemocytes migrate into the crypt spaces at night where they lyse, releasing chitin and providing the bacteria with a fermentable nocturnal nutrient (33) (Fig. 1*B*). This behavior of the host hemocytes occurs in addition to their role in immune surveillance throughout the body, during which they phagocytose and/or encapsulate potential pathogens (34). Here we provide evidence that daily cycling of the host cytokine MIF in the light organ regulates the nocturnal delivery of metabolites to bacterial symbionts, repurposing its immunological role to one of rhythmically providing a specific nutrient.

Results

***E. scolopes* MIF Is Biochemically Similar to Other MIF Proteins and Is Phylogenetically Related to MIF Proteins of Invertebrate Relatives.** The transcript for *E. scolopes* MIF (EsMIF) was identified from a previous RNA sequencing (RNAseq) analysis of the adult light organ (35). To examine MIF in *E. scolopes*, the full-length amino acid sequence was derived from the gene sequence (36) (*SI Appendix*, Fig. S1*A* and *SI Materials and Methods*). The monomer of EsMIF is 114 amino acids with a predicted molecular mass of 12.5 kDa and a predicted pI of 7.8, which are within the ranges reported for other MIF monomers (37). The amino acid sequence of EsMIF has 39% and 65% identity to those of human and *Octopus bimaculoides* MIF, respectively. MIF proteins of other species are often exported (38), yet have no predicted signal peptide to mediate transport, which is also true of EsMIF. EsMIF contains the highly conserved amino acid residues associated with the phenylpyruvate tautomerase activity of the MIF protein, i.e., the N-terminal proline at position 2, lysine at position 36, isoleucine at 69, and tyrosine at 101 (11, 39). However, similar to the MIF of some other invertebrates (12), the CxxC

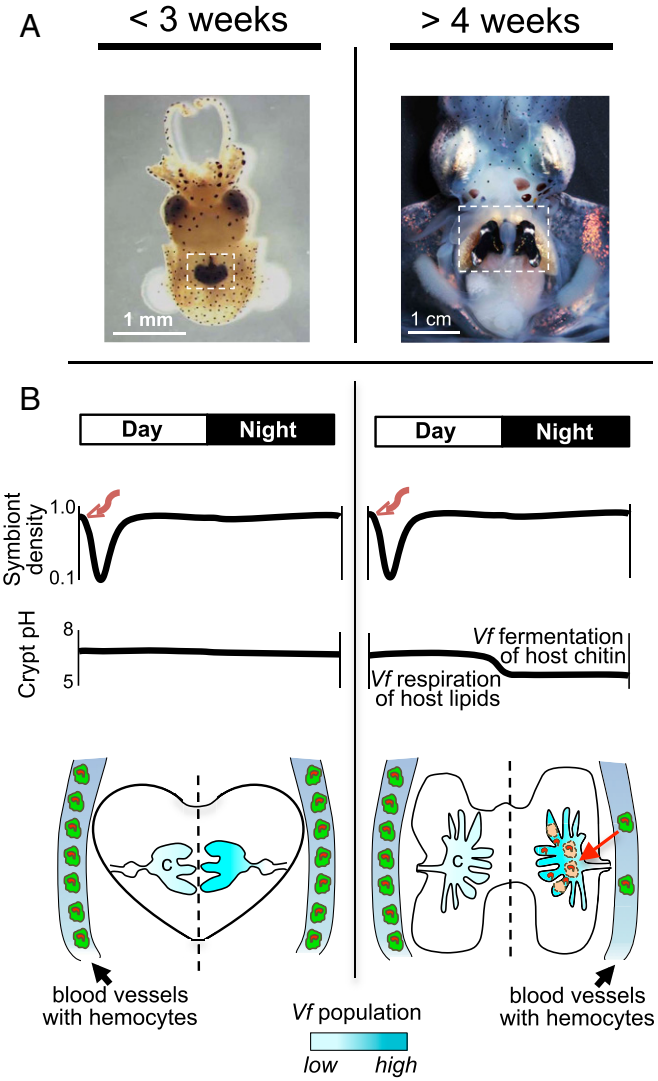

Fig. 1. Maturation of the daily rhythm of the squid–vibrio symbiosis with animals maintained on a 12L:12D cycle. (*Left*) The immature symbiosis; (*Right*) the mature symbiosis. (*A*) The light organ (white boxes), where the symbionts are housed, is located in the center of the body cavity of juvenile and adult animals. (*B*) Variation in characters over the day–night cycle in the juvenile and mature symbioses. (*Top*) Symbiont density. In both life stages, the majority of the symbiont population is vented from the crypts into the surrounding seawater in response to the dawn light cue (orange arrows), which is followed by rapid regrowth of the remaining cells. (*Middle*) Crypt pH. The pH of the light-organ crypts in the immature symbiosis remains constant throughout the day–night cycle, whereas the crypts in the mature symbiosis acidify as a result of chitin fermentation. (*Bottom*) Hemocyte behavior. *V. fischeri* populations reside extracellularly in epithelium-lined crypts (c). In both juvenile and adult animals, symbiont luminescence is attenuated during the day (pale blue, *Left* crypts) and brightest at night (vivid blue, *Right* crypts). The light organ is heavily vascularized (flanking regions) and contains circulating hemocytes (green) with chitin stores (red). Only in the mature symbiosis do the hemocytes migrate in large numbers at night into the crypts (red arrow), where they die and release stores of chitin.

motif at residue positions 61 to 64 that is critical for the protein-thiol oxidoreductase activity of mammalian MIF (40) is not conserved in EsMIF; the cysteine at position 64 is replaced by a leucine. In addition, the residues associated with the binding of vertebrate MIF to its well-described receptors, CD 74, which mediates MIF proinflammatory functions, and CXCR2 and CXCR4, which are G protein-coupled membrane receptors of chemokines (11), are not conserved in the EsMIF sequence. Furthermore, orthologs of the genes encoding these MIF receptors were not detected in analysis of the *E. scolopes* genome (41), suggesting that EsMIF binds to different receptors.

A three-dimensional (3D) structural model of EsMIF (*SI Appendix*, Fig. S1*B*) was constructed using human MIF as a template. A qualitative model energy analysis score (42) of 0.61 for EsMIF predicts high structural similarity between the squid and human proteins, with EsMIF forming the typical MIF monomer consisting of two alpha-helices flanking four beta-sheets with a βαββαβ order (43). The structural similarity with human MIF also predicts that the biologically active form of EsMIF is a homotrimer.

Using Bayesian analysis and the same parameters as an available phylogeny of MIF proteins (12), a phylogenetic tree was constructed to predict the evolutionary relationships among 44 selected MIF proteins from all domains of life, with an emphasis on animals (*SI Appendix*, Fig. S1*C*). In our phylogenetic tree, EsMIF grouped with proteins of other mollusks.

EsMIF Displayed Characteristic Inhibition of Immune-Cell Migration and Was Abundant in the Central Core of Adult Light Organs. We sought to determine whether EsMIF inhibits hemocyte migration using a Boyden chamber assay. Hemocytes that were preincubated with central-core lysate exhibited a ∼50% decrease in migration when compared with control cells that were incubated without lysate (P value <0.1) (Fig. 2*A*). Next, we asked whether the inhibition was specific to the activity of EsMIF in the lysate. Because two MIF-specific inhibitors (ISO-1 and 4-IPP) were toxic to the hemocytes, we instead applied an antibody-interference method that has been used to attenuate MIF activity in other systems (44–46). Introduction of EsMIF antiserum into the lysate restored migration to an uninhibited level. As a control, lysate to which EsMIF preimmune serum was added decreased migration levels similarly to the lysate-only addition (Fig. 2*A*). These results provided evidence that EsMIF inhibits migration of host hemocytes.

ELISA was used to quantify EsMIF in host tissues or cells (Fig. 2*B*), including: 1) the central core of the light organ, the epithelial tissue that supports the symbiont population; 2) the light-organ tissue remaining after central core removal; 3) the gills and 4) the hemocytes, two components of the host immune system (47, 48); 5) the accessory nidamental gland (ANG), a symbiotic organ exclusive to the female reproductive tract (49); 6) the eye lens, where MIF occurs in mammals (50); and (7) the retina; eye tissues show strong convergent properties with the light organ (51, 52). All tissues were sampled at ZT5 (Zeitgeber time, with the dawn light cue being ZT0). The protein was ∼10× more abundant in the light-organ central core than in other tissues tested; the ANG and eye lens had lower levels of EsMIF, while the remaining tissues had even lower or undetectable levels.

Immunocytochemistry (ICC), using recently developed methods for tissue clearing (DEEP-Clear) or for refractive-index matching (for details, see *Materials and Methods*), revealed that light-organ EsMIF localized to the cytoplasm of the central-core epithelia and to the crypt lumina, often as puncta (Fig. 2*C* and *SI Appendix*, Fig. S2, preimmune). Because other immune cells, including those of mollusks, have detectable MIF protein (12), we also performed ICC on isolated hemocytes of the host squid. Consistent with the low levels of antibody cross-reactivity of the hemocytes observed by ELISA, EsMIF was not detected in the hemocytes by ICC (Fig. 2 *B*, *Upper Right*; *SI Appendix*, Fig. S2, preimmune). In addition, no cross-reactivity above background to the EsMIF antibody was detected in *V. fischeri* (*SI Appendix*, Fig. S3).

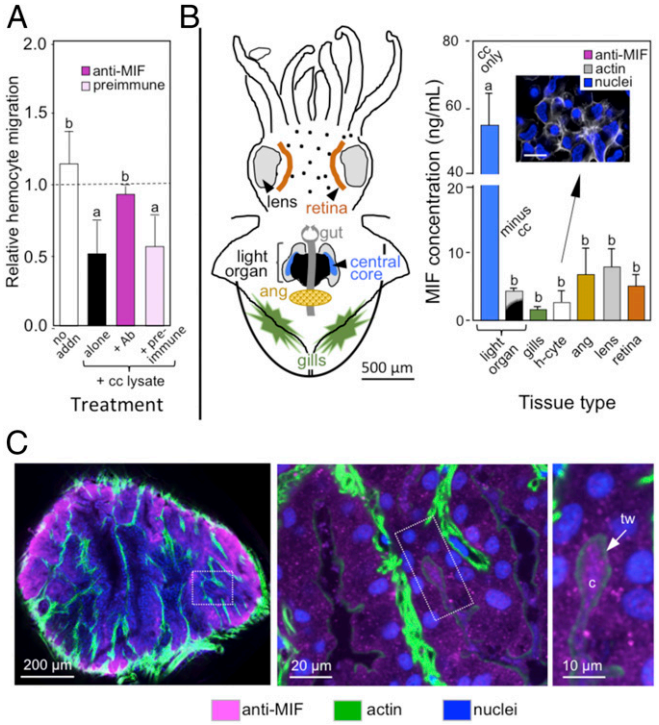

Fig. 2. Characteristics of EsMIF. (*A*) Hemocyte migration toward the chemoattractant 10% bovine serum albumin in the absence (no addn) and presence (alone) of central core lysate; addition of EsMIF antiserum (+Ab) or EsMIF preimmune serum (+preimmune). The dashed line represents the control by which samples were normalized, i.e., where no chemoattractant was added. (*B*, *Left*) Diagram of adult squid, indicating tissues that were sampled for ELISA. (*B*, *Right*) ELISA of extracts of an array of adult cells, tissues, and organs, and ICC of hemocytes showing undetectable EsMIF in the immune cells. Calculation of MIF concentration was based on a standard curve of rMIF. ang, accessory nidamental gland; cc, light-organ central core; h-cyte, hemocytes. (*C*) Immunocytochemistry showing EsMIF protein in symbiotic light-organ tissues. EsMIF was abundant in the central core (*Left*) and localized to the epithelium surrounding the symbiont-containing crypts and the crypt spaces (c) (*Middle* and *Right*); terminal web (tw). Statistical significance was determined by a one-way ANOVA with multiple comparisons; different letters indicate adjusted P value <0.1; differing letters indicate statistically significantly different results; similar letters, no statistical difference; error bars, SD (n = 3).

Both EsMIF Transcript and Protein Had a Strong Daily Rhythm in Light Organs of the Adult Symbiotic Squid. To examine transcript and EsMIF protein levels over the day–night cycle, light organs of mature squid were sampled at three time points, ZT5, ZT13, and ZT22 (Fig. 3 *A* and *B*). Using three housekeeping genes for normalization, qRT-PCR revealed a reproducible rhythm of lower *esmif* expression in mature light organs at night (Fig. 3*A*). Western blot analysis of the soluble protein extract revealed a strong and specific cross-reactivity to the EsMIF antibody at all three time points, confined to bands appearing at ∼12.5 kDa, the predicted molecular mass of EsMIF (Fig. 3 *B*, *Left* and *Middle*). A comparison of the intensity of cross-reactivity across the three time points, while controlling for differential protein loading, indicated that EsMIF was most abundant at ZT5, relative to ZT13 and ZT22. Quantification of MIF concentrations of central core samples by ELISA confirmed these variations of EsMIF protein over the day–night cycle (Fig. 3 *B*, *Right*). Accordingly,

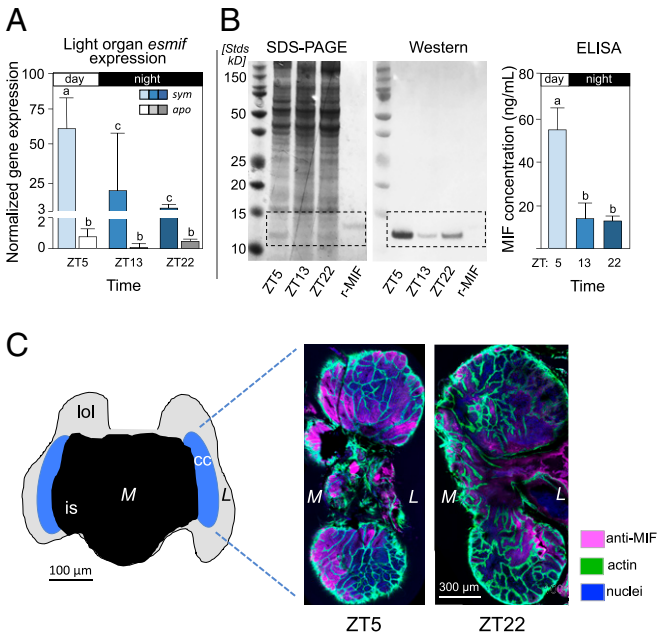

Fig. 3. Gene expression and protein analyses in the adult light organ. (*A* and *B*) Light organs from symbiotic and aposymbiotic adults were sampled at three time points throughout the day: ZT5, ZT13, or ZT22. (*A*) *esmif* gene expression in adult light organs. Statistical significance was determined by a two-way ANOVA with multiple comparisons; different letters indicate adjusted *P* value <0.1; differing letters indicate statistically significantly different means; similar letters, no statistical difference; error bars, SD ($n = 5$ to 6) for each column. (*B*) Characterization of EsMIF in total soluble extracts of adult central cores. (*Left* and *Middle*) SDS-PAGE with Western blot (30 µg/lane); rMIF, recombinant MIF; dashed boxes, predicted EsMIF subunit molecular mass. (*Right*) ELISA. Statistical significance was determined by a one-way ANOVA with multiple comparisons (letter designations same as above); SD ($n = 3$) for each column. (*C*) Confocal ICC of the light-organ central core. (*Left*) Diagram of the light organ, showing the location of the central core. (*Right*) Confocal images of regions of EsMIF antibody cross-reactivity in isolated central cores at day and night time points. Scale is the same for both confocal images. cc, central core; is, ink sac; L, lateral; lol, light organ lens; and M, medial.

ICC detected lower overall EsMIF levels in central cores at night (Fig. 3*C* and *SI Appendix*, Fig. S4, preimmune). Taken together, these findings provide evidence that EsMIF is reduced in the light organ during the night, relative to the day, a pattern similar to that observed with *esmif* expression.

The Daily Rhythm of EsMIF Begins with Maturation of the Symbiosis. With the knowledge that, in the adult light organ, EsMIF is more abundant during the day than at night, we sought to determine when this cycling begins during the development of the symbiosis. Specifically, we asked whether the EsMIF cycling begins with 1) the onset of the mature rhythms of hemocyte migration and symbiont metabolism; and 2) the nightly acidification of the crypt spaces, all of which begin to show a day–night rhythm after 3 to 4 wk of development (26) (Fig. 1). ICC analysis at 2 and 4 weeks detected EsMIF in the crypts and associated tissues throughout the day and night (Fig. 4, *SI Appendix*, Fig. S5, preimmune, and Movie S1). However, at 2 wk, when the symbiosis is still immature (26), EsMIF levels were relatively constant over time (Fig. 4, *Left*). In contrast, after 4 wk, when the symbiosis has matured (26), the abundance of EsMIF cycled within the light organ (Fig. 4, *Right*). While at midday (ZT5), ICC labeling of EsMIF was high, indicating an abundance of EsMIF, at night (ZT13 and ZT22), ICC labeling of EsMIF was diminished. Because these 4-wk samples at ZT13 were variable and sometimes harder to distinguish by ICC from those at ZT5, it appeared that at this stage of development the animals were still transitioning to the mature diurnal/nocturnal behaviors and cycling of symbiont metabolism. Nonetheless, these results support the conclusion that the period 2 to 4 wk following the initiation of symbiosis represents a key point in the development of the symbiosis, one in which the levels of EsMIF begin to change with a timing that is consistent with maturation of other rhythms associated with the adult partnership.

Hemocyte Migration Was Stimulated by Symbiont Products. We sought to determine whether products of the *V. fischeri* population in the light organ induce migration of chitin-rich hemocytes, which could facilitate delivery of this nutrient at night, when the symbiont population is dense and EsMIF levels are low. Symbionts release several molecules, including derivatives of two microbe-associated molecular patterns (MAMPs), the lipid A of lipopolysaccharide (LPS) and the monomer of peptidoglycan (or tracheal cytotoxin, TCT), which are already known to be key biomolecules of the host–symbiont dialogue occurring in this association (53). Molecules exported by the symbiont, at concentrations that induce squid–host developmental phenotypes (54, 55), enhanced hemocyte migration (Fig. 5*A*). When growing on chitin breakdown products, *V. fischeri* cells also release millimolar levels of organic acids (56), which are likely to accumulate in the host crypts and provide additional molecules that could induce hemocyte migration. To approximate the nutritional conditions experienced by the symbionts at night (26, 27), we grew *V. fischeri* in a medium containing *N*-acetyl-D-glucosamine (GlcNAc), the chitin monomer, as the primary source of carbon, nitrogen, and energy. Because of the limiting oxygen concentrations believed to exist in the crypts, these cultures were grown under both aerobic and anaerobic conditions, and the spent medium was analyzed by gas chromatography-mass spectrometry (GC-MS) for the presence of small-molecule metabolic products. While the relative proportions of each metabolite varied between anaerobic and aerobic growth conditions, the

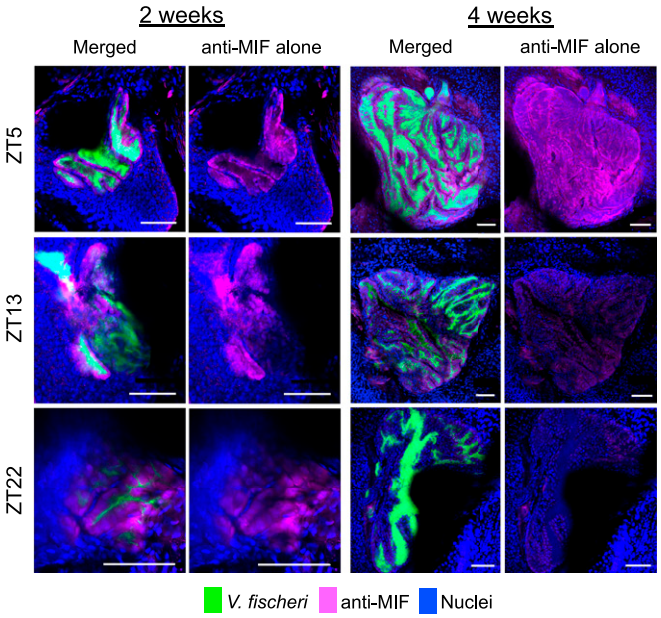

Fig. 4. Maturation of the day–night cycling of EsMIF in the light organ of the host. Immunocytochemistry showing localization of EsMIF to the light-organ crypts. The images are shown with all channels (Merged) and with only channels showing the EsMIF antibody cross-reactivity and its relationship to host nuclei (anti-MIF). (Scale bars, 100 µm.) All images were taken with the same laser settings within each developmental stage.

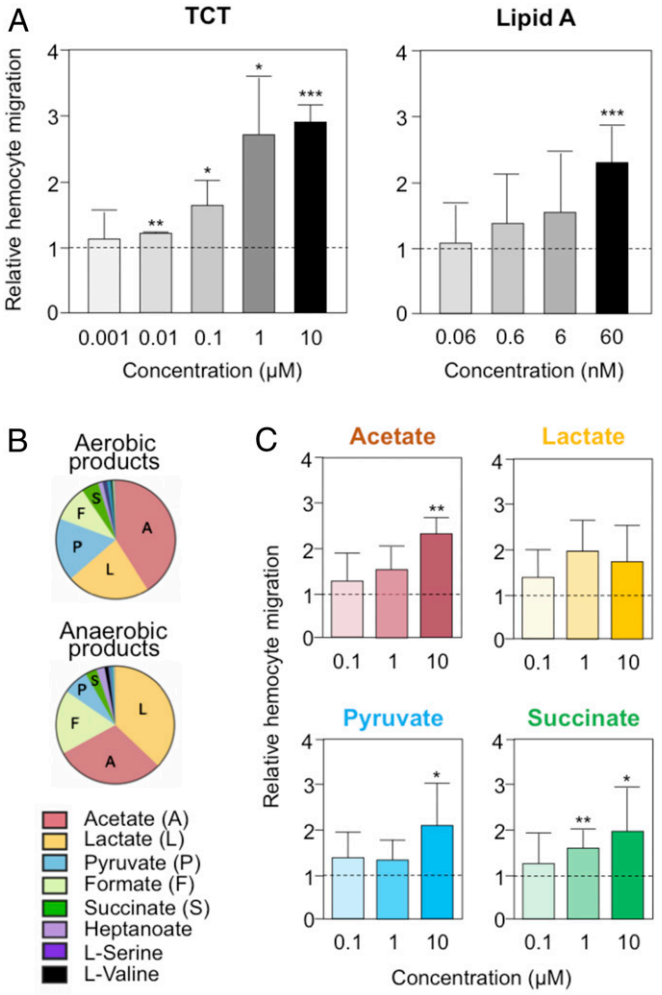

Fig. 5. Chemotaxis of hemocytes to *V. fischeri* products. (*A*) Hemocyte migration toward the MAMPs TCT (*Left*) and lipid A (*Right*). (*B*) Average relative abundance of short-chain fatty acid metabolites in spent media from *V. fischeri* cultures grown under aerobic or anaerobic conditions, as measured by GC-MS (n = 3 biological replicates). (*C*) Hemocyte migration toward prominent *V. fischeri* fermentation metabolites. Statistical difference for each treatment relative to the control (dashed line) was determined by a one-sample t test (*, **, *** = P value <0.1, 0.05, and 0.01, respectively). Error bars, SD (n = 4) for each column.

same five major products of GlcNAc catabolism were released: acetate, lactate, pyruvate, formate, and succinate (Fig. 5*B*). To determine whether the accumulation of these catabolites might draw hemocytes into the crypts, we examined four of them for their ability to influence migration behavior (Fig. 5*C*); because of its high acidity and toxicity to animal cells (57), formate was not tested. The catabolites acetate, lactate, pyruvate, and succinate all enhanced hemocyte migration, although the increase was not statistically significant for lactate at our cutoff of P value <0.1. These results provided evidence that hemocytes are drawn toward both *V. fischeri* MAMPs and fermentation products.

To distinguish whether the enhanced hemocyte movement to *V. fischeri* products was due to chemotaxis (directed migration) or chemokinesis (random motility) (58, 59), we performed further migration analyses using a "checkerboard" protocol (59). We selected TCT and acetate, as these were the two most potent inducers of hemocyte migration among the tested *V. fischeri* MAMPs and catabolites, respectively. The pattern of response indicated that, while acetate was a chemoattractant for the hemocytes, the increase in migration induced by TCT was due to a combination of chemotaxis and chemokinesis behaviors (*SI Appendix*, Fig. S6). These data show that the hemocytes of *E. scolopes* can respond to bacterial molecules through an enhancement of both chemokinesis and chemotaxis.

Discussion

The discoveries presented here allow for the synthesis of an integrated view of the key features, from behavioral traits to molecular mechanisms, that underlie the maintenance of a mature symbiotic relationship. Specifically, this work reports the discovery of the daily cycling of EsMIF transcript and protein abundance, a rhythm that is found only in the mature symbiotic light organ and that drives the nightly provision of a particular nutrient to the symbiont population (Fig. 6).

This report describes an MIF homolog in the phylum Mollusca, class Cephalopoda (i.e., octopus, squid, and their relatives). MIF serves to control immune-cell migration and, in many mollusks, it is associated with responses to pathogen challenge. However, nonpathogenic symbioses are widespread in mollusks (60–62), so it is not surprising to find MIF as a regulator of host responses to beneficial microbes as well. The results presented here expand the scope of biological functions for this evolutionarily conserved cytokine, specifically its key role in host management of bacterial metabolism in a symbiosis.

EsMIF, an Immunomodulator, Regulates the Complex Daily Rhythms of the Mature Symbiosis. The sequence, 3D structure, and phylogenetic position of EsMIF provide strong evidence that it is an ortholog of other MIF proteins. However, as in other invertebrates, the EsMIF sequence lacks key residues for certain enzyme activities and receptor binding that are present in vertebrate orthologs (*SI Appendix*, Fig. S1*A*), and the *E. scolopes* genome does not encode the well-described MIF receptors present in vertebrates (36). These data support the idea that MIF proteins have different functions and regulatory mechanisms in vertebrates and invertebrates (63–65).

The discovery of a high relative abundance of EsMIF in the host light organ compared to other regions of the body, and confirmation of the protein as a potent inhibitor of hemocyte migration, adds this cytokine to a growing set of proteins demonstrated to have immune function in the squid–vibrio symbiosis. Among these proteins are four others that are secreted into the crypt lumen: a squid ortholog of the mammalian antimicrobial protein myeloperoxidase (MPO) (66, 67); a peptidoglycan-recognition protein (EsPGRP2) (68) and alkaline phosphatase (AP) (69), both of which degrade MAMPs; and complement C3 (70). Studies of the squid–vibrio system have provided transcriptomic and/or ICC evidence that, while the levels of AP, EsPGRP2, and complement C3 oscillate throughout the day even in juveniles (27, 68, 69), only in the mature symbiosis, when EsMIF is cycling as well, does the overall set of host rhythms become more complex. During the night, when the mature animal is actively foraging and using luminescence for camouflaging, and when EsMIF levels are low in the light organ, hemocytes migrate to the crypts, where they release chitin. The symbionts release organic acids from the fermentation of chitin, driving the pH down, resulting in at least three consequences: 1) in a classic Bohr effect, the acidic conditions promote the offloading of oxygen from the blood pigment hemocyanin (69) which, in turn, enhances luminescence production (24); 2) at a crypt pH of 5, the activities of the MAMP-degrading enzymes AP and EsPGRP2, which have pH optima around 8 (68, 69), are suppressed; and 3) the acidic conditions promote the antibacterial activity of the MPO-like enzyme, which has a pH optimum of 3.5 (66). Taken together, the daily rhythm of the mature symbiosis has a pattern suggestive of inflammatory conditions being low during the day, when the animal is quiescent, and increased at

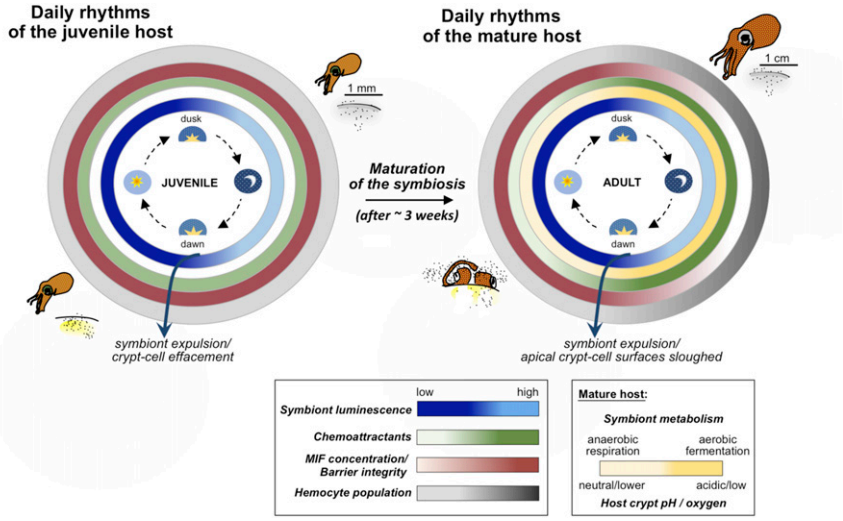

Fig. 6. A model of the maturation of a series of integrated daily rhythms in a symbiotic system, with the cytokine MIF as "maestro." (*Left*) In the first weeks of symbiosis, the juvenile squid does not display distinct behavioral patterns over the day–night cycle, and the developing symbiosis displays relatively few rhythms. After colonization is initiated, the per-cell level of symbiont luminescence becomes elevated every night, possibly due to changes in blood flow to the light organ (24). Each dawn, ~95% of the symbiont population is expelled from the crypts of the organ, accompanied by an effacement of the sparse crypt-cell microvilli (87, 107) and followed by a rapid regrowth of the remaining symbionts (31). In this early period of the partnership, the symbiont population derives nutrients from host amino acids and membrane lipids released into the crypt space (27, 31). Throughout the daily cycle (white), the crypt environment is both at a neutral pH (26) and oxic (108). Reflecting the relatively stable environment of the host crypts, EsMIF remains abundant within the light-organ epithelium throughout the day and night. (*Right*) After 3 to 4 wk of development, the squid assumes its mature behavior of burying in the sand during the day and actively hunting at night (22) while, concurrently, the symbiosis initiates additional daily rhythms. Each dawn brings a pronounced shedding of the elaborate microvilli and apical surfaces of the crypt epithelium, providing the symbiont population with host lipids that are catabolized to pH-neutral products (27). Concomitant with a down-regulation in EsMIF at night, the hemocytes are allowed to migrate into the symbiont-containing crypts and offload chitin, which drives a metabolic shift in *V. fischeri* toward aerobic fermentation (26). The fermentation of chitin acidifies the crypts, which encourages release of oxygen from the host blood pigment, hemocyanin (109). The increased availability of oxygen drives a higher intensity of symbiont luminescence for the nocturnal foraging behavior of the host. Thus, the daily cycling of EsMIF levels within the light organ acts as a pivotal regulator for the rhythms underlying the mature light-organ symbiosis.

night when the animal is active and the symbionts are in a brightly luminous, metabolically active, stationary phase (22).

Hemocyte Migration Controls the Daily Nutritional Regime of the Bacterial Symbionts. One characteristic differentiating EsMIF from MIF proteins in other animals is its absence from the squid macrophage-like hemocytes (7), a condition not reported for the macrophage of any other organism known to produce MIF (see, e.g., refs. 71–73). These results are supported by proteomes and transcriptomes of *E. scolopes* hemocytes in which MIF was also near or below detection levels (Fig. 2*B*) (74, 75). Furthermore, analysis of a transcriptome of *Octopus vulgaris* hemocytes (47) showed a similarly low expression of the MIF homolog in this species. This finding indicates that unusually low levels of this protein in hemocytes may be a shared characteristic of cephalopods that may have been exploited by squid species to manage mutualistic interactions with microbes.

The absence of EsMIF in circulating hemocytes and its abundance in the light organ (Fig. 2*B*) suggest that an important role of EsMIF is its regulation of host interactions with the symbiotic population of *V. fischeri*. While *E. scolopes* hemocytes are unusual in their lack of detectable MIF, they have an endogenous chitin synthase and share with many other invertebrates the ability to carry substantial stores of chitin (76). This chitin is an important nutrient for symbionts in the mature host: a *V. fischeri* mutant defective in chitin utilization is unable to persist normally once the light organ reaches maturity (26). Further, if EsMIF were to be present within hemocytes, it would be expected to compromise their function in providing chitin to the symbionts: the release of EsMIF into the crypts by the lysing of the hemocytes would inhibit additional hemocytes from migrating into this region to feed the symbionts.

While the nightly decrease in EsMIF would remove this key barrier to hemocyte migration, the finding that MAMPs and *V. fischeri* metabolic products acted as stimulators of migration would provide evidence for a corresponding positive stimulus for hemocyte trafficking into the crypts, adding to the model of day–night regulation of the symbiosis (Fig. 6). Constitutively produced LPS and its derivatives are well-studied chemoattractants of immune-cell migration that act through both chemokinesis and chemotaxis in vertebrates and invertebrates (7, 77–80). By inhibiting this attraction, the role of EsMIF would be to prevent hemocytes from migrating into the crypts during the day. In contrast, the peptidoglycan monomer, or TCT, is a MAMP exported by only a few gram-negative bacteria (see, e.g., refs. 81, 82) including *V. fischeri* (55). Studies of the daily rhythms of the symbiont transcriptome in mature *E. scolopes* have demonstrated that a *V. fischeri* gene responsible for TCT production (83) is up-regulated in the late afternoon and stays high throughout the night (27). These data together with the results reported here suggest a two-stage process: The lowering of EsMIF levels and release of MAMPs at night may serve as initial catalysts for hemocyte migration, through chemokinesis and potentially chemotaxis, into the crypts. Once hemocytes have begun to migrate into the crypts, symbiont catabolism of the delivered chitin into organic acids (26) would be expected to result in a positive feedback loop. Specifically, the transition to chitin fermentation by *V. fischeri* results in the release of metabolic byproducts, e.g., acetate, lactate, succinate, and pyruvate, that would attract an increasing number of hemocytes for the continuing delivery of chitin stores directly to the crypts through chemotaxis and/or chemokinesis. These observations are consistent with patterns of mammalian immune-cell migration

toward short-chain fatty acids (SCFAs) as a response both to potential pathogens and beneficial microbiota (84, 85).

Findings to Date on the Squid–Vibrio Association Provide Evidence for Multiple EsMIF Functions in Symbiosis. To the authors' knowledge, the symbiotic systems of the mammalian gut and squid light organ are the only ones in which MIF has been reported to promote maintenance of the normal microbiota. Within the light organ, EsMIF localizes primarily to the epithelial lining of the crypts, with lower amounts being secreted into the lumen. This pattern is similar to that seen in mouse and human intestinal epithelial cells, where MIF is secreted into the gut lumen (16, 86). In mammalian gut epithelia, MIF is not known to play a nutritional role in symbiosis, but rather it serves to regulate and organize barrier function and has been implicated in control of the microbiota. Previous work in the squid–vibrio system suggests that EsMIF is also important in maintaining the integrity of the epithelial barrier within the light organ. In the mature symbiosis, conditions in the light organ during the night, when EsMIF levels are low, lead to an eventual sloughing around dawn of the apical portions of the light-organ epithelial cells (Fig. 6, *Right*) (27). This perturbation is accompanied by hallmark features of loss of barrier function, most notably degradation of the cell–cell junctional complexes. After dawn, barrier function is restored and maintained throughout the day, concomitant with sustained high levels of EsMIF. In contrast, EsMIF in the immature light organ is essentially constant throughout the day and, while effacement of microvilli takes place each day at dawn (Fig. 6, *Left*), electron microscopy revealed no apical cell shedding or loss of junctional complexes (87). The occurrence of EsMIF in both the crypt lumina and the surrounding epithelia provides evidence for two distinct functions: inhibiting hemocyte trafficking and maintaining barrier function, respectively. It should be noted that these functions may be interrelated; i.e., with barrier function impaired, the access to the crypts by the hemocytes may be enhanced.

Possible Conservation of MIF Regulation of the Daily Oscillations of Symbiotic Animal–Microbe Systems. We present here evidence for daily rhythms of the MIF protein in the symbiotic tissues of animals. Reports in the literature hint that daily cycling of MIF in such tissues may be a conserved feature of animal–microbe symbioses, although these findings have not been brought together thus far into a coherent picture. The presence of MIF in the gut epithelium of vertebrates ranging from fish to mammals (88–91) suggests that, like the carriage of a complex consortium in the gut, MIF activities there may be a shared character of vertebrates. In addition, the importance of circadian rhythms in the mammalian gut–microbiota associations is well documented (5, 92–95), particularly in the regulation of the immune system (30, 96, 97). Further, a disruption of the circadian rhythm that compromises gut barrier integrity, a function of MIF, has been linked to changes in the gut microbiota (30, 92, 94, 98). Finally, while MIF rhythms have not been explored in the gut epithelium, levels of MIF in other mammalian tissues fluctuate on a pronounced circadian rhythm, notably in the pancreas and blood of humans (99, 100).

Conclusions and Future Directions

The data presented here demonstrate that, by modulating the amount of EsMIF present in the surrounding epithelium over the course of the day, the squid host controls the trafficking of hemocytes into the crypts. This migration of hemocytes, in turn, drives a metabolic transition in *V. fischeri* from respiration to fermentation, which has cascading effects on the molecular and cellular behavior of the entire symbiotic organ. Future research will be focused upon determining factors responsible for the cycling of the MIF itself. Regardless of the underlying drivers of these oscillations, the ability of MIF to regulate multiple biological rhythms shows how a single protein can simultaneously foster a beneficial symbiosis while also maintaining host physiology. With the other functions described for MIF across the vertebrates and invertebrates, the protein seems to offer a classic example of "gene sharing," i.e., highly divergent functions of a protein being encoded by the same gene (101). Taken together, the data suggest that the recruitment of MIF as a regulator of animal microbiomes may be a trait that occurs across the animal kingdom, and traits that are broadly conserved are typically critical.

Materials and Methods

Rearing of the Symbiotic and Aposymbiotic Animal Hosts. To produce samples for either experiments or rearing, adult *E. scolopes* were collected from the field and maintained under laboratory conditions (22). For all experiments, animals were anesthetized in 2% ethanol in seawater. Hatchling squid from egg clutches of mated females were either 1) colonized with wild-type *V. fischeri* strain ES114 as previously described (102), or 2) maintained separately as aposymbiotic, i.e., in natural seawater with no *V. fischeri* cells present. For experiments with reared animals, maintenance as either symbiotic or aposymbiotic was monitored over the months of rearing by periodically assessing the presence or absence, respectively, of dawn-vented *V. fischeri* in the surrounding seawater or luminescence output from the host (22). All animals were maintained on a 12:12 L:D cycle, with ZT0 at dawn.

Analyses of *esmif* Expression and EsMIF Production. Tissues were stored in RNAlater until use and then total RNA was extracted from light organs as previously described (103) (*SI Appendix, Materials and Methods*). Host gene expression was measured by qRT-PCR (103). The housekeeping genes used to normalize qRT values across samples were the ribosomal protein S19 of the 40S ribosome subunit, serine hydroxymethyl transferase, and heat-shock protein 90 (for primer sequences, see *SI Appendix, Materials and Methods*).

For studies of EsMIF, polyclonal antiserum was generated using recombinant MIF (rMIF, the full-length amino acid sequence with an N-terminal histidine tag of six residues), which was expressed in and purified from *Escherichia coli* (Biologics International Corp). Polyclonal antiserum was then raised against rMIF in rabbit (Covance Inc.). Sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE), ELISA, Western blots, and confocal ICC were performed by standard methods applied to the squid–vibrio system (104). As this was the first use of ICC on deeper tissues of the light organ, two methods for ICC were used: immersion in SlowFade Glass, which is a refractive-index matching method (Thermo Fisher Scientific), and a tissue clearing method, DEEP-Clear (105).

Determining the Ability of EsMIF and Microbial Products to Affect Hemocyte Migration. To examine how EsMIF and *V. fischeri* products affect hemocyte migration, in vitro assays were performed using a modified Boyden chamber assay (86, 106) and blind-well chemotaxis chambers with 200 μL upper and lower well capacity and 5 μm polycarbonate membranes (Neuro Probe). Briefly, hemocytes from adult *E. scolopes* were either exposed to central core lysate, which contains high concentrations of EsMIF, or left untreated. The bottom well was filled with a potential chemoattractant, and the hemocytes were added to the upper well with the membrane separating the two wells. To determine whether migration was due to chemotaxis (directed migration) or chemokinesis (random motility), a checkerboard protocol was used where potential chemoattractants were added only to the bottom well and to both the bottom and upper wells of separate chambers (59). After 3 h of incubation at room temperature, the membranes were fixed, stained, and imaged. To quantify hemocyte migration, five representative images were taken of the bottom side of each membrane, and fluorescence from hemocyte nuclei was derived using ImageJ.

See *SI Appendix, Materials and Methods* for additional experimental details.

Data Availability. The sequence for EsMIF reported in this paper has been deposited in the publicly accessible GenBank database, the National Center for Biotechnology Information (Accession No. MT843322).

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