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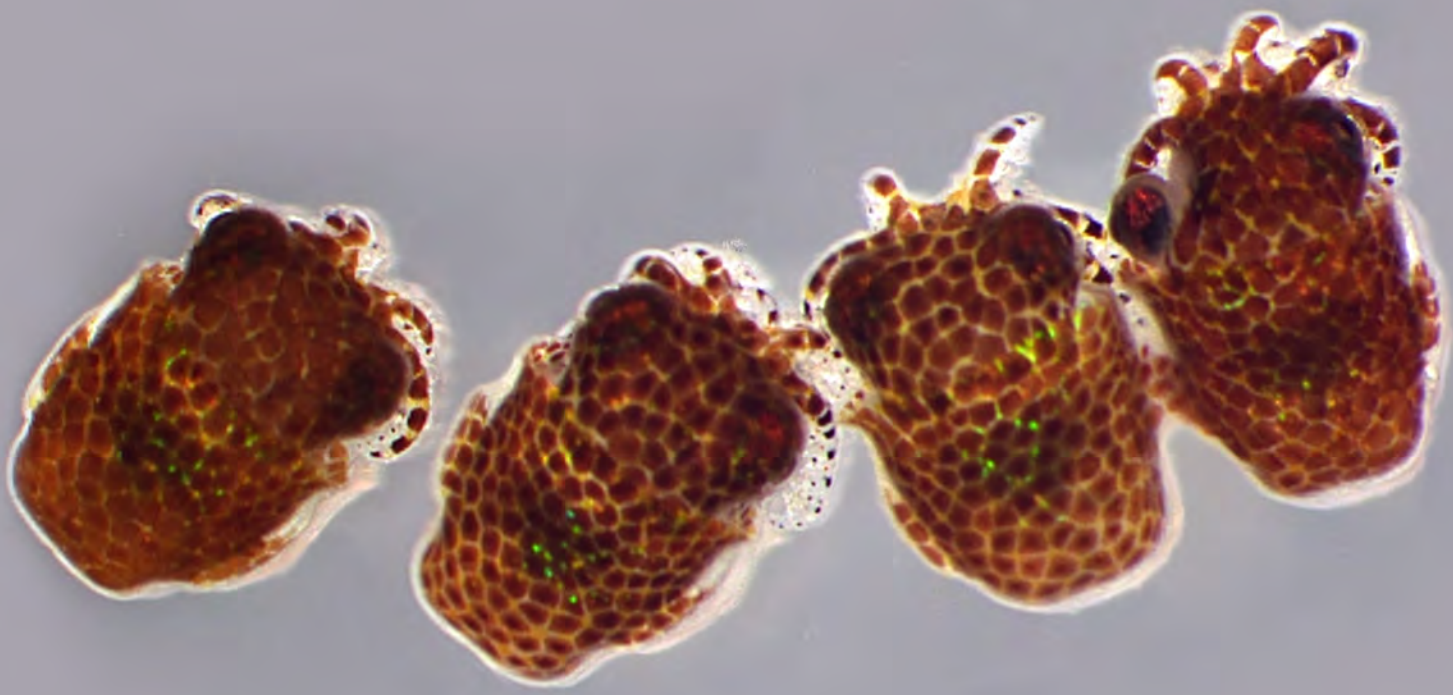

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Short Communication

Potential of the *Euprymna/Vibrio* Symbiosis as a Model to Assess the Impact of Microgravity on Bacteria-Induced Animal Development

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Long-duration spaceflight imposes numerous physiological challenges to astronauts working in the space environment. Some of these challenges have been well-documented including bone and tissue loss (Smith and Heer, 2002), dysregulation of the immune system (Crucian et al., 2008), and increased risk to infectious diseases (Wilson et al., 2007). However, relatively little is known regarding the impact of the space environment, specifically microgravity, on those mutualistic bacteria that interact directly with animal cells. To understand the effects of microgravity on beneficial microbes we propose using the model system between the bobtail squid *Euprymna scolopes* and the luminescent bacterium *Vibrio fischeri* strain ES114. For over 20 years the *Euprymna/Vibrio* system has provided key insight into the role that symbiotic bacteria play in the normal development of animal tissues (McFall-Ngai and Ruby, 1991; Weir et al., 2010). This mutualistic association has several advantages that render it highly amenable to experimental manipulation in the space environment. First, the symbiosis is monospecific with only one host and one symbiont making it easier to discern the effects of the symbiont on host cells. Second, both partners can be cultured independently in the laboratory and the symbiosis can be initiated at any point after the juvenile animal hatches. Lastly, the symbiosis can be activated and monitored using currently available flight hardware such as the Fluid Processing Apparatus (FPA; BioServe Space Technologies, Boulder, CO). Here, we present evidence from a pilot study that demonstrates the overall potential of the *Euprymna/Vibrio* model system to examine the impact of microgravity on bacteria-induced development of host animal tissues.

In the *Euprymna/Vibrio* model system the mutualism is initiated when symbiosis-competent strains of *V. fischeri* colonize a specialized host structure called the light organ (Figure 1A). On either side of the incipient light organ there are two appendage-like structures comprised of ciliated epithelial cells (Figure 1B). The function of these ciliated cells is to entrain bacteria from the environment into the vicinity of pores found on the surface of the light organ (Figure 1C). If the symbiosis-competent strain of *V. fischeri* is not present in the environment then these ciliated epithelial appendages are retained and the light organ does not undergo morphogenesis (Figure 1C, D).

However, if symbiosis-competent *V. fischeri* is present then the bacteria enter the light organ through one of the surface pores and travel to an epithelial-lined crypt space where the bacteria then begin to divide. Upon reaching a critical cell density the bacteria begin to luminesce within the light organ. A few hours after initial colonization the bacteria trigger a series of developmental remodeling events that includes an apoptotic cell death event of the ciliated epithelial appendages (Figure 1E). Over a period of 4 d the light organ undergoes complete morphogenesis resulting in the loss of the ciliated appendages (Figure 1F). Several of these bacteria-induced events, such as the onset of luminescence, occur at discreet time intervals within the first 24 h after the initiation of symbiosis. These early developmental events can be readily monitored to assess the progression of the natural symbiosis. To assess the tractability of the *Euprymna/Vibrio* symbiosis in the space environment, we first determined whether the animals could survive under microgravity conditions. To simulate microgravity we used eight 10-ml volume high aspect ratio vessels with a rotary culture system (HARV; Synthecon, Houston, TX) at 13 rpm. These HARV have been successfully used in numerous microgravity studies to reproduce space-like conditions (Hammond and Hammond, 2001) and have been shown to be directly comparable to real

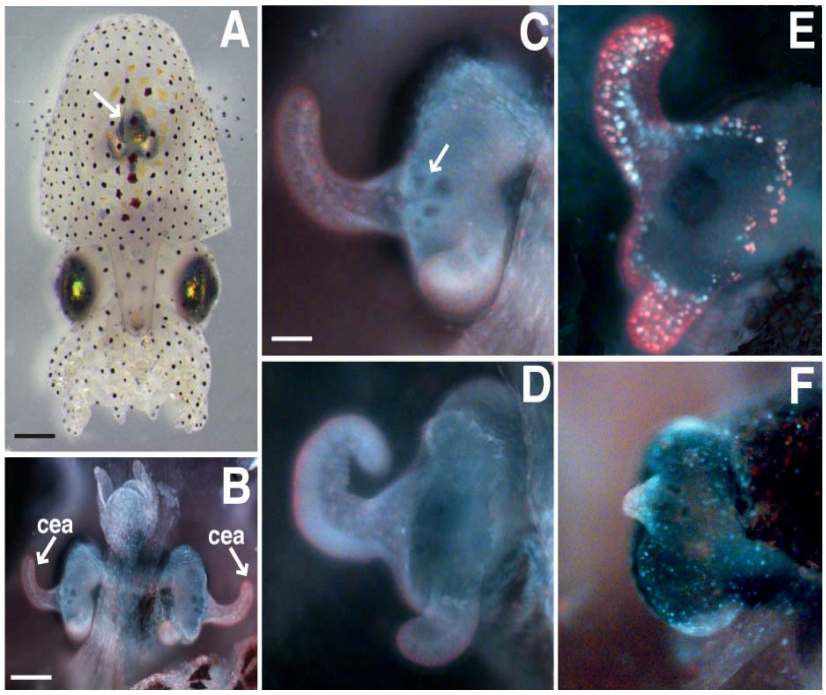

Figure 1. The morphology of the *Euprymna scolopes* light organ. A. Juvenile animal with light organ (arrow) in the center of mantle cavity. Bar = 0.5 mm. B. Micrograph of nascent light organ depicting fields of ciliated epithelial appendages (cea) Bar = 100 μm. C. One half of 24 h aposymbiotic light organ with arrow pointing to pores. Bar = 50 μm. D. Micrograph of 96 h aposymbiotic light organ showing no signs of morphogenesis in the absence of competent bacteria. E. Symbiotic light organ at 14 h stained with acridine orange, a fluorescent molecule that intercalates into cells undergoing cell death. F. Symbiotic animal at 96 h showing full morphogenesis of the light organ and the loss of the cea.

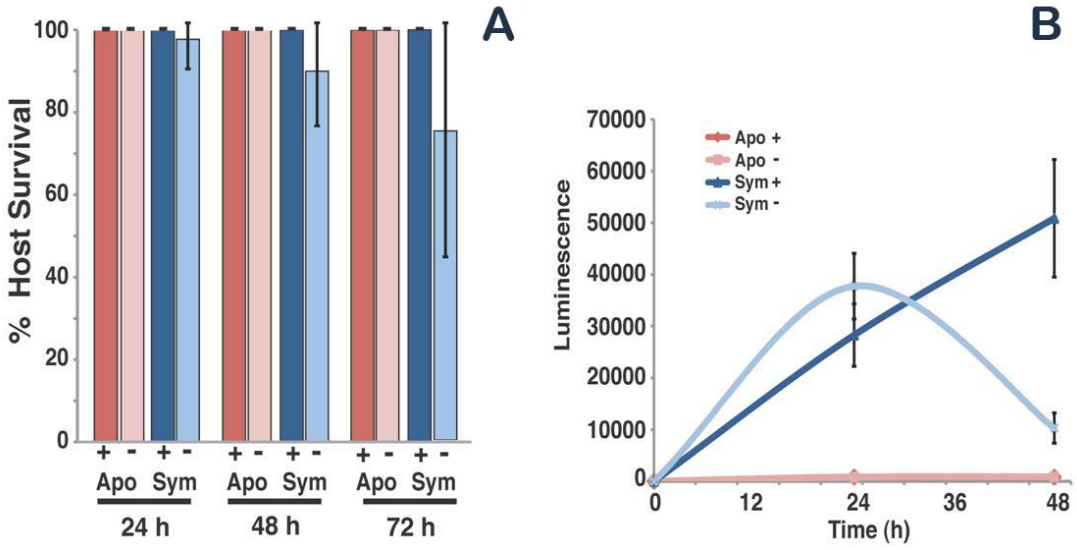

Figure 2. Effects of simulated microgravity on *Euprymna/Vibrio* symbiosis. A. Comparison of the survivability of aposymbiotic (Apo) and symbiotic (Sym) host squid under gravity (+) and simulated microgravity (-). B. Impact of microgravity (-) on the luminescence of host squid compared to gravity (+) conditions.

microgravity conditions (for review Nickerson et al., 2004). Animals were collected within 1 h of hatching, rinsed in filtered seawater (FSW) and either maintained aposymbiotically (i.e., without *V. fischeri* ES114) or were rendered symbiotic by inoculating with *V. fischeri* at a concentration of 1 x 10^5 cells per ml of aerated FSW. Animals were placed in the HARV chambers and incubated for up to 72 h at 23°C. A subset of control animals was maintained under normal gravity conditions in 5 ml of aerated FSW in borosilicate vials at 23°C. The results of five replicate experiments (n = 8 animals per treatment) are depicted in Figure 2A. All of the animals maintained aposymbiotically under both normal gravity (n = 40) and simulated microgravity (n = 40) conditions survived to 72 h. However, in the symbiotic animals there was a difference in the survival rate between gravity and simulated microgravity treatments. In normal gravity conditions all symbiotic animals survived throughout the experiment, whereas in symbiotic animals exposed to simulated microgravity there was a gradual increase in animal death rate over the course of the three-day experiment. At 24 h, an average of 98% of the animals exposed to symbiotic bacteria survived in each of the five replicate experiments. However, the mean survival rate decreased to 90% and 75% by 48 and 72 h, respectively. The variability between experiments also increased over time (Figure 2A) and may be the result of differences between host squid size (2-3 mm), or may reflect changes in the symbiont under microgravity conditions. Despite the gradual decrease in survival, most symbiotic squid survived the HARV experiments during the critical time window (0 - 24 h) when many of the early symbiosis phenotypes are being initiated. Additional testing of symbiotic animals using the HARV chambers perpendicular to the axis of rotation in the microgravity treatments showed that the survival rate was 100% (n = 15) after 72 h. These results coupled with the 100% survival of the aposymbiotic controls in the HARV chambers under microgravity conditions indicate that the HARV chamber itself has a minimal impact on the squid survival. Lastly, animals tested with flight approved FPA hardware under normal gravity conditions indicated that the animals survived up to five days in aerated FSW (data not shown); well beyond the four-day time window of light organ morphogenesis.

In addition to basic survival under microgravity conditions we examined the onset of luminescence, an early phenotype of the symbiosis and bacterial growth rate in the HARV chambers. In the experiments described above, the luminescence of the host squid was checked by temporarily removing the aposymbiotic and symbiotic animals from the HARV chamber for ~5 min, placing them in borosilicate vials and inserting the vial into a luminometer to measure luminescence (GloMax 20/20, Promega, WI). The results indicated that at 24 h there was no statistical differences between luminescence levels between symbiotic animals maintained under normal gravity and simulated microgravity (Figure 2B). However, by 48 h there was a significant decrease in the luminescence output of microgravity-exposed symbiotic animals. In addition to luminescence there were also differences detected in the growth rate of the symbiont under microgravity conditions. *V. fischeri* cultures within the HARV were examined and shown to have a 2-fold increase in growth rate at 24 h compared to gravity controls (data not shown). These results indicate that although microgravity does not impact the initiation of the symbiosis, microgravity does affect the persistence and maintenance of the symbiosis compared to the normal gravity controls. These results may reflect the health of the host organism, or the flow of oxygen into the light organ crypt spaces, as luminescence requires aerobic conditions. However, the bacteria were viable despite the loss of luminescence. Plating of dissected, homogenized light organs on sea water tryptone agar plates revealed no significant loss in cell numbers between animals exposed to microgravity and gravity conditions (data not shown).

Together these results suggest that the *Euprymna/Vibrio* model system can be successfully manipulated under simulated microgravity conditions and that the symbiotic association may be negatively impacted by microgravity. Further work is required to elucidate the precise effects that microgravity has on the symbiotic partners and the overall developmental time line of the host squid. Understanding the effects that spaceflight has on the animal/human microbiome may provide critical insight into maintaining the health of crew members and decrease their potential risk during the exploration of space.

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