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Intracellular Localization of the Bacterial Endosymbiont *Cardinium* in the Ostracod *Heterocypris spadix*

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Symbiosis is a key driver of evolution in life-history traits and reproductive strategies. Some symbiotic microorganisms manipulate host reproduction to enhance their own transmission, a phenomenon well studied in insects but less understood in crustaceans. Among these microorganisms, *Cardinium* manipulates host reproductive systems, such as parthenogenesis, cytoplasmic incompatibility, and male killing in arthropods. However, its role in ostracods, small bivalve-shelled crustaceans, remains unclear. Some ostracod species reproduce via parthenogenesis, and high *Cardinium* infection rates in these lineages suggest a potential link between the symbiont and asexual reproduction. To investigate this relationship, we examined *Cardinium* localization in the parthenogenetic ostracod *Heterocypris spadix* from Japan. Using tissue clearing and fluorescence in situ hybridization (FISH), we visualized *Cardinium* within the ovaries. FISH observations revealed a widespread infection across the germarium, nurse cells, and oocytes. In early-stage oocytes, bacteria were evenly dispersed throughout the cytoplasm, whereas in more-developed oocytes, they clustered around the nucleus. Additionally, *Cardinium* was also detected in the hepatopancreas, indicating infection of both the reproductive and digestive systems. The presence of *Cardinium* in host reproductive structures, particularly the germarium, nurse cells, and developing oocytes, suggests its role in reproductive manipulation. To our knowledge, this study provides the first detailed localization of *Cardinium* in ostracods, reinforcing its potential influence on reproduction. Future research using antibiotics and genomic analysis will be crucial to confirm *Cardinium*'s role in parthenogenesis induction.

Key words: FISH, ovariole, parthenogenesis, reproductive manipulation, symbiosis

INTRODUCTION

Symbiosis plays a key role in major evolutionary transitions by inducing changes in life history and cycle, promoting evolution and diversification in organisms (Margulis and Fester, 1991; Szathmáry and Smith, 1995; Sachs et al., 2011; West et al., 2015). Some symbiotic microorganisms markedly impact host growth, survival, and reproduction by providing essential nutrients (Moran et al., 2008; Douglas, 2009), aiding digestion (Brune, 2014; Li et al., 2021), and defending against enemies (Oliver et al., 2014; Flórez et al., 2015). Notably, reproductive manipulation occurs when certain symbionts alter host reproductive systems to maximize their own transmission (Hurst and Frost, 2015; Kaur et al., 2021). Symbionts such as *Wolbachia*, *Spiroplasma*, *Rickettsia*, and *Cardinium* enhance the production of infected female progeny through various mechanisms,

including parthenogenesis, feminization, cytoplasmic incompatibility, and male killing, thereby skewing host sex ratios and altering the host life cycle (Hurst and Frost, 2015; Ma and Schwander, 2017). Therefore, understanding where and how these symbionts induce reproductive manipulations is crucial. However, research on reproductive manipulation has primarily focused on insects, with crustaceans remaining understudied.

Ostracoda, a diverse group of small (typically 1–2 mm) crustaceans with distinctive bivalve-like shells (“valves”), is found in freshwater and marine environments (Karanovic, 2012). Over 8000 ostracod species have been described (Horne et al., 2002; Schön and Martens, 2016), with more than 2420 species inhabiting brackish or freshwater environments (Meisch et al., 2024). Sexual and asexual reproduction occur in nonmarine species, with certain species or populations within species reproducing parthenogenetically or through combined sexual–asexual reproduction (Schön and Martens, 2020). Notably, high infection rates of the intracellular symbiont *Cardinium* have been reported in parthe-

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nogenetic ostracod species and populations (e.g., Çelen et al., 2019; Schön et al., 2019; Schön and Martens, 2020).

Cardinium, a genus within the Cytophagales (Zeng et al., 2018), forms symbiotic or parasitic relationships with freshwater mussels, plant-parasitic nematodes, and arthropods such as insects, mites, and crustaceans (Giorgini et al., 2009; Harris et al., 2010; Hurst and Frost, 2015; Ma and Schwander, 2017; Tarlachkov et al., 2023). It is known that several strains of *Cardinium* manipulate host reproduction through cytoplasmic incompatibility, parthenogenesis induction, and male killing (Giorgini et al., 2009; Ma and Schwander, 2017). Studies involving antibiotic treatments and bacterial community analyses in mites and insects suggest that *Cardinium* induces parthenogenesis (e.g., Groot and Breeuwer, 2006; Matalon et al., 2007; Giorgini et al., 2009). Moreover, significant *Cardinium* infection has been reported in females of ostracod species exhibiting asexual, sexual, or mixed reproductive strategies (Schön and Martens, 2020). Consequently, *Cardinium* is hypothesized to play a similar role in parthenogenesis in ostracods (Schön and Martens, 2020). However, owing to ostracods' small size, the precise localization of *Cardinium*, its infection dynamics, and the mechanisms underlying parthenogenesis remain unclear.

In this study, we analyzed the ostracod species *Heterocypris spadix*, with specimens collected in Okinawa Prefecture, Japan (Munakata et al., 2021). This species reproduces parthenogenetically and is subject to *Cardinium* infection, as confirmed via 16S rRNA gene sequencing (Munakata et al., 2021). Therefore, we investigated *Cardinium* localization in *H. spadix* via dissection and subsequent symbiont visualization through fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Taxonomic status, collection, and maintenance of studied ostracods

Although Meisch et al. (2024) dealt with *H. spadix* as a junior synonym of *Heterocypris salina*, we retain *H. spadix* as valid for the following reasons. Kilikowska et al. (2024), the source for the synonymization in Meisch et al. (2024), revealed that *H. salina* contains four species distinguished by the difference of cytochrome *c* oxidase subunit I (COI) sequences. No detailed morphological comparison was conducted among the four species. *Heterocypris spadix* was molecularly identified as one of the four. Kilikowska et al. (2024), however, lacked sequences from topotypic specimens of *H. salina*. It is thus unknown which one of the four corresponds to the true *H. salina*, with the possibility that none of four would be *H. salina* remaining.

We used captive-bred *H. spadix* females that hatched in an aquarium and were the descendants of individuals collected from the type locality on 21 November 2018 (for details, see Munakata et al., 2021).

Autofluorescence observations with transparency treatment

The internal structures of *H. spadix* were examined by observing autofluorescence in transparency-treated specimens. For tissue clearing, whole individuals were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (4%PFA/PBS) and dehydrated through a graded methanol series, followed by immersion in benzyl alcohol and benzyl benzoate solution (BABB) (1:2 benzyl alcohol:benzyl benzoate ratio) overnight at room temperature. Cleared samples were observed under a confocal laser scanning

microscope (CLSM; LSM700, LSM980; Carl Zeiss) with excitation at four wavelengths: 405, 488, 561, and 640 nm.

Whole-mount FISH

To investigate the localization of *Cardinium* in *H. spadix*, whole-mount FISH (wFISH) targeting 16S rRNA was performed as described by Oguchi et al. (2024). To visualize *Cardinium*, individuals exceeding 800 µm in body length—large enough to allow observation of eggs under a stereomicroscope—were used. Dissections were performed in PBS under a stereomicroscope using two fine needles (Insect pin No. 00, Shiga Konchu). Briefly, one needle was inserted into the gap between the two valves, and the valves were gently pried apart using both needles. The ovarioles and hepatopancreas, attached laterally to the body, were then carefully extracted using the needles. Dissected tissues were fixed in 4% PFA/PBS for 3 h, washed three times with 0.3% Triton X-100 in PBS (PBT), and washed twice with hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, and 30% formamide). Based on the 16S rRNA sequence of *Cardinium* in *H. spadix* (Munakata et al., 2021) and referring to previously designed *Cardinium* 16S rRNA-targeting probes (e.g., Brown et al., 2018), we designed the *Cardinium*-specific probe “Card622” (5'-TAT CAA TCG CAG TTC TAA CG-3'), labeled with Alexa Fluor 555 at the 5' end. In addition to Card622, the universal bacterial probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3'; Stahl et al., 1989; Amann et al., 1990) labeled with Alexa Fluor 555 or 647 at the 5' end was used. For the negative control, the sense probe targeting EUB338 (5'-ACT CCT ACG GGA GGC AGC-3') labeled with Alexa Fluor 555 at the 5' end was used. Samples were incubated overnight at room temperature in hybridization buffer containing 50 nM probe. After two PBT washes, DNA and filamentous actin were stained using 4.5 µM 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) and 1.5 µM Alexa Fluor 488-labeled phalloidin (Thermo Fisher Scientific), respectively, for 1 h at room temperature, followed by additional PBT washes (Koga et al., 2012). Finally, the samples were washed twice with PBT, mounted in 50% glycerol in PBS, and observed under the LSM700 and LSM980 ($n = 5$ in each probe).

RESULTS AND DISCUSSION

Anatomical observations

Following tissue clearing, morphological and anatomical observations revealed that mature *H. spadix* individuals possessed an ovariole filled with oocytes and a hepatopancreas inside each valve (Fig. 1A, B). Through dissection, ovarioles containing germ cells at various developmental stages were isolated (Fig. 2). DAPI and phalloidin staining of DNA and actin filaments, respectively, enhanced visualization of nuclei, cell boundaries, and cellular morphology (Fig. 2B). At the ovariole's distal end, a tightly clustered cellular region, the germarium, was observed (Fig. 2A, B). In the proximal region, large oocytes with extensive cytoplasm and sizable nuclei were identified alongside adjacent nurse cells (Fig. 2A, B). In the most-developed oocytes, yolk accumulation was evident, as indicated by the presence of slightly yellowish granules and reduced nuclear visibility, and nurse cell DNA signals were stronger (Fig. 2A, B). The germarium, vitellarium, nurse cells, and oocytes resembled those of other species described previously (e.g., *Herpetocypris reptans* in Woltereck [1898]; *Cyprinotus uenoi* in Ikuta et al. [2007]).

Cardinium localization

Bacterial signals detected via wFISH using the EUB338 probe were distributed throughout the ovarioles (Fig. 2B). To

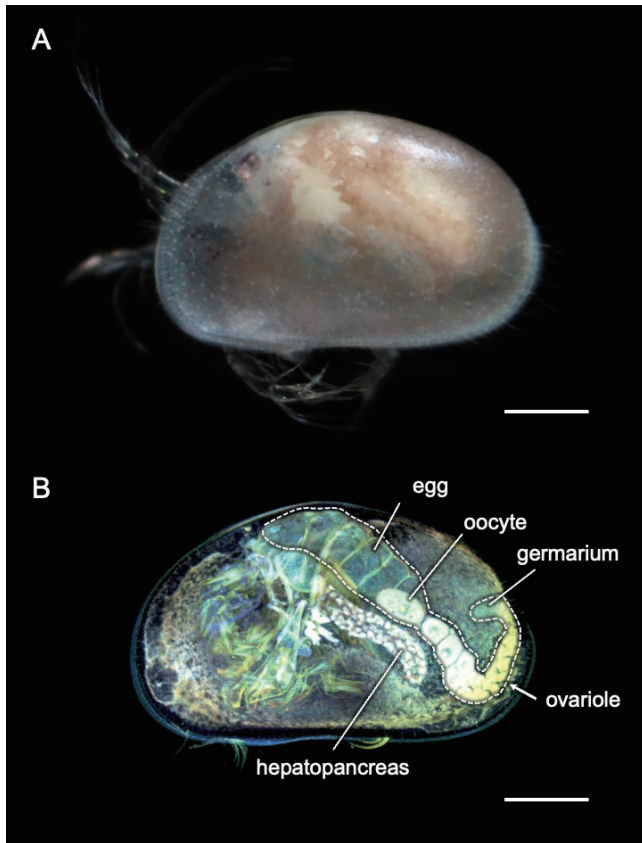


Fig. 1. Morphological and anatomical observations of *Heterocypris spadix*. **(A)** Left view of a live *H. spadix* specimen. **(B)** Autofluorescence imaging after tissue clearing. Clearing treatment made internal structures, including the eggs, left ovary, and left hepatopancreas, visible through the left valve. Dotted line marks the ovariole. Scale bars: 200 µm.

confirm probe specificity, dual-staining was performed with the Card622 (*Cardinium*-specific) and EUB338 (universal bacterial) probes (Fig. 3). Card622 signals almost entirely overlapped with EUB338 signals (Fig. 3), whereas negative controls using the EUB338 sense probe showed no signals (see Supplementary Figure S1). These results validate Card622 as a reliable probe for detecting *Cardinium* and suggest that *Cardinium* is the dominant bacterial species infecting *H. spadix*.

Further analysis of *Cardinium* localization within the ovariole (Fig. 4; and see Supplementary Figures S2, S4) revealed that Card622 and EUB338 signals were distributed throughout the germarium, indicating dense bacterial colonization (Figs. 3, 4A; and see Supplementary Figures S2, S4A). The cells in the germarium were relatively small, and strong bacterial signals were observed, likely due to the localization of bacteria within the limited cytoplasmic space (Figs. 3, 4A; and see Supplementary Figures S2, S4A). In the vitellarium, containing multiple oocytes, bacterial signals were evenly distributed throughout the oocyte cytoplasm (Fig. 2B; and see Supplementary Figures S2 and S4B, C). In contrast to bacterial signals in the germarium, those in the oocyte appeared dispersed, likely due to the increased size of the cytoplasm (Fig. 2B; and see Supplementary Figures S2 and S4B, C). However, in more mature oocytes, located

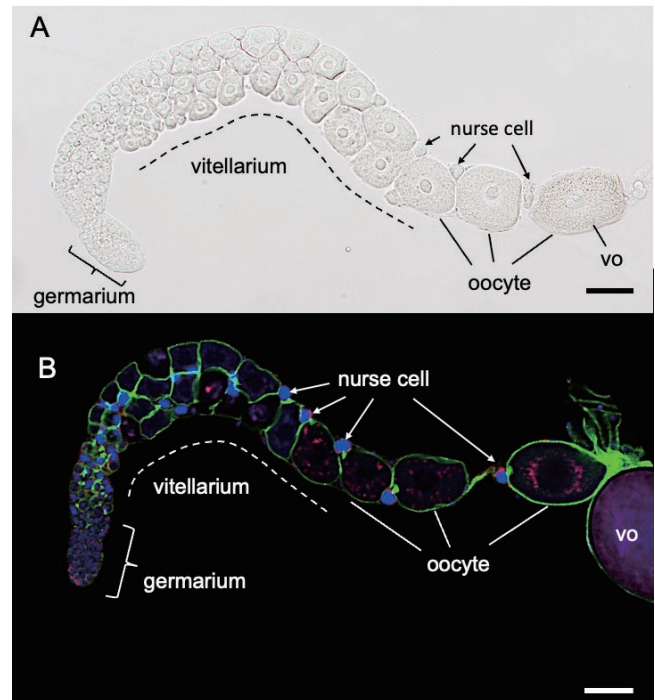


Fig. 2. Structures of the ovariole. **(A)** Bright-field image of an ovariole captured using differential interference contrast microscope. **(B)** FISH image of an ovariole obtained via confocal laser scanning microscope. Red signals indicate endosymbiotic bacteria labeled with the Alexa555-EUB338 probe. Blue signals show DNA stained with DAPI. Green signals highlight F-actin stained with phalloidin. Vo: vitellogenic oocyte. Scale bars: 50 µm.

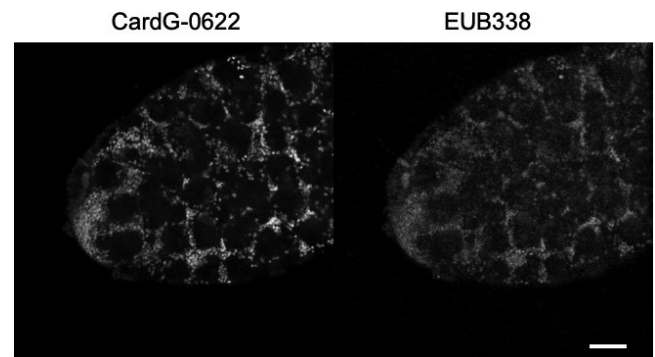


Fig. 3. High-magnification image of the germarium dual-stained with *Cardinium*-specific probe Alexa555-Card622 and universal bacterial probe Alexa647-EUB338. White signals largely overlap. Scale bar: 10 µm.

at the proximal part of the ovariole, bacterial signals were instead concentrated around the germinal vesicle (Fig. 4B). *Cardinium* localization in the germarium and oocytes may suggest its involvement in reproductive manipulation. Similar nuclear-associated bacterial localization has been observed in *Wolbachia* infections in crustaceans (Genty et al., 2014). As *Wolbachia* uses host cells' cytoskeletons to move through the cytoplasm (Albertson et al., 2009), *Cardinium* may also utilize the cytoskeleton to establish its position near the nucleolus.

Dense bacterial signals were observed in the narrow

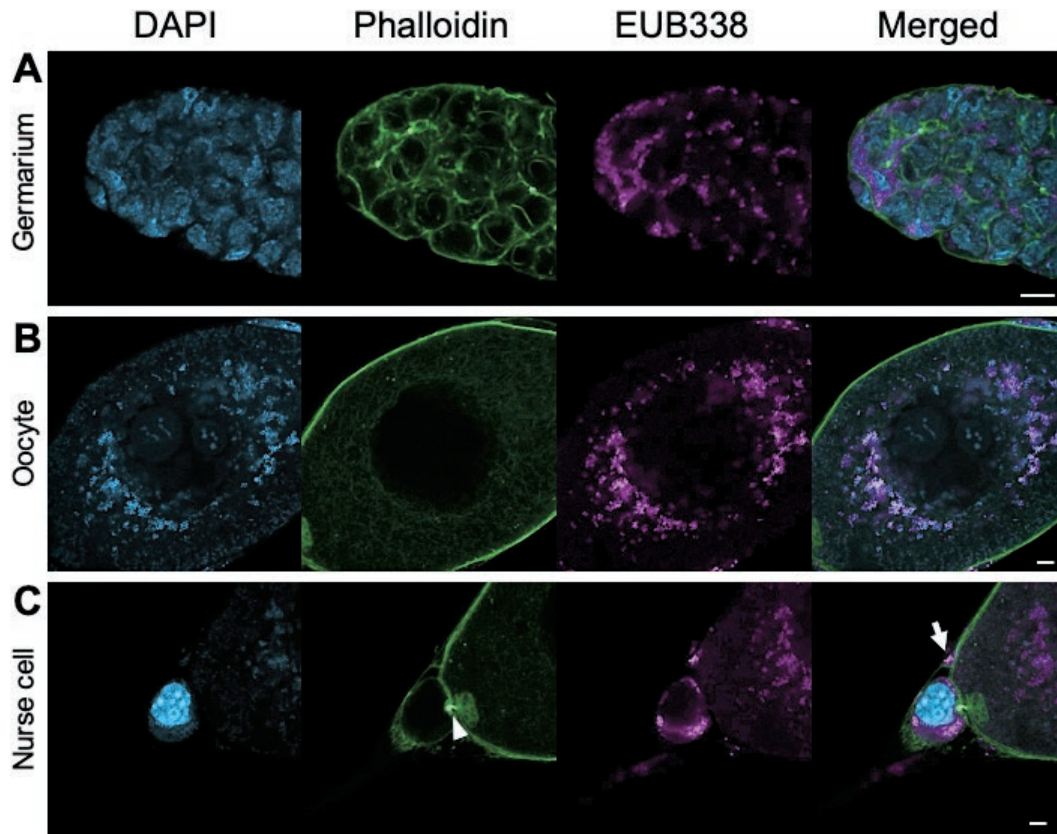


Fig. 4. Detailed structures of the ovariole. **(A)** Germarium, **(B)** oocyte, **(C)** nurse cell. Arrows indicate extracellular bacterial signals. Arrow-heads mark intercellular bridges connecting oocytes and nurse cells. Light blue signals show DNA staining with DAPI, green signals represent F-actin (forming cell membranes) labeled with phalloidin, and magenta signals indicate bacteria stained with the Alexa555-EUB338 probe. Scale bars: 5 μ m.

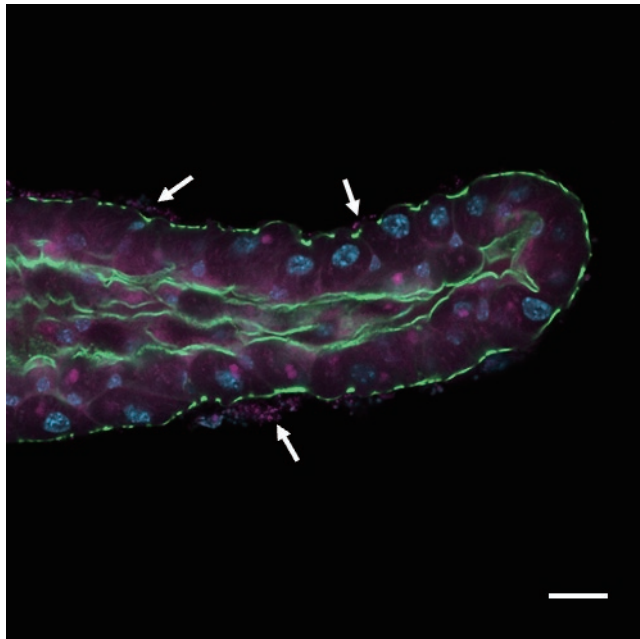


Fig. 5. Enlarged FISH image of the hepatopancreas. DNA stained with DAPI (light blue), cell membranes visualized with phalloidin (green), and bacterial signals detected using the Alexa555-EUB338 probe (magenta). Arrows indicate extracellular bacterial signals. Scale bar: 20 μ m.

cytoplasmic regions of nurse cells, with some extracellular signals were also detected (Fig. 4C, and see Supplementary Figure S3). At the boundary between nurse cells and oocytes, “intercellular bridges” composed of bundled actin filaments were observed (Fig. 4C, and see Supplementary Figure S3), as previously reported in ostracods and insects (Büning, 1994; Ikuta et al., 2007). These bridges connect the nurse cell nuclei to the distal side of the oocyte cytoplasm; however, no bacterial signals were detected within them (Fig. 4C). This suggests that *Cardinium* infects nurse cells and oocytes during germ cell division but does not use the bridges for transmission.

In addition to the ovariole, bacterial signals were detected inside and outside hepatopancreas cells (Fig. 5, and see Supplementary Figure S4D). Intracellular signals in the hepatopancreas were generally less abundant than those in the ovarioles, suggesting that *Cardinium* may preferentially infect germ cells (Fig. 5, and see Supplementary Figure S4D). *Cardinium* has been observed infecting epithelial tissues in mites (Nakamura et al., 2009), and *Wolbachia* is known to infect not only the germ cells of insects and nematodes but also various somatic tissues (e.g., Pietri et al., 2016). Given that other tissues were not examined, the full extent of *Cardinium* infection remains unclear, but at minimum, the bacteria colonize the ovaries and digestive organs (e.g., the hepatopancreas) in ostracods.

CONCLUSION AND PERSPECTIVES

This study revealed: 1) the first detailed localization of *Cardinium* in ostracods, with the following key findings: 2) *Cardinium* inhabits all intracellular regions of the ovariole, including the germarium, nurse cells, and oocytes; and 3) *Cardinium* is present intracellularly and extracellularly, infecting multiple tissues. Although bacterial depletion has been studied in insects and mites, there are few studies examining its effects in aquatic crustaceans (Edlund et al., 2012), and research in this area has not advanced significantly. However, by referencing studies such as Weeks et al. (2001) and Chigira and Miura (2005), which induced masculinization in mites, it is necessary to investigate whether masculinization can be induced in parthenogenetic ostracods through bacterial depletion operations such as heat treatment or antibiotic treatment. Furthermore, by analyzing the whole genome of *Cardinium* and performing gene prediction, it will be possible to explore the molecular mechanisms underlying reproductive manipulation.

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COMPETING INTERESTS

The authors declare that there are no competing interests.

AUTHOR CONTRIBUTIONS

KO, MM, and KK designed the study outline and performed the anatomical survey. KK collected and maintained ostracods. MM identified specimens. CH and KK designed specific probes. KO, MM, and KK performed FISH analyses. KO, MM, CH, and KK wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: <https://doi.org/10.2108/zs250018>)

Supplementary Figure S1. Negative control for FISH. Enlarged FISH images of ovariole obtained using EUB338 sense probe. (A) DNA stained with DAPI, (B) F-actin and cell membranes labeled with phalloidin, (C) FISH image visualized using Alexa555-EUB338 sense probe, (D) merged image of (A, B, C).

Supplementary Figure S2. Detailed FISH images of oocytes and nurse cells in vitellarium. (A) DNA stained with DAPI, (B) F-actin and cell membranes labeled with phalloidin, (C) FISH image visualized using Alexa647-EUB338 probe, (D) FISH image visualized using Alexa555-Card622 probe, (E) merged image of (A, B, C, D).

Supplementary Figure S3. High-magnification FISH images of nurse cell. (A) DNA stained with DAPI, (B) F-actin and cell membranes labeled with phalloidin, (C) FISH image visualized with Alexa647-EUB338 probe, (D) FISH image visualized with Alexa555-Card622 probe, (E) merged image of (A, B, C, D).

Supplementary Figure S4. Infection patterns across multiple individuals. (A) germarium, (B) vitellarium, (C) oocyte and nurse cells, (D) hepatopancreas. DNA stained with DAPI (light blue), cell membranes visualized with phalloidin (green), bacterial signals

detected using Alexa647-EUB338 probe (red) and Alexa555-Card622 probe (magenta).

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