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Mixed mode bacterial transmission in the common brooding coral *Pocillopora acuta*

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Originality-Significance Statement

Corals form critical associations with microorganisms, which exert numerous beneficial functions for the host and are indispensable for its survival. Due to their ability to evolve rapidly, microbes could also mediate intra- and transgenerational acclimatisation of corals in the face of current climate change. In order to gain insight into the biological mechanisms that underlie the functioning of the holobiont and its persistence, it is important to understand how microbes are transmitted and maintained across generations. Only a handful of studies have investigated the transmission mode of bacteria in corals and this process remains unclear. Here, we combined amplicon sequencing with fluorescence *in situ* hybridisation (FISH) microscopy in order to investigate the patterns of microbiome acquisition in asexually brooded offspring. This study analysed the microbial community composition at the amplicon sequence variant level and in several coral life stages (parental colonies, newly released larvae and early recruits), in conjunction with FISH to address this question. Through DNA metabarcoding, we were able to capture the identity and abundance of bacteria that were either horizontally taken up from the environment or vertically inherited from the parents. Microscopy examinations enabled us to confirm the presence of maternally transmitted bacterial aggregates inside coral larvae. Our findings provide important information towards a better understanding of bacterial dynamics and transmission patterns in the coral holobiont.

Summary

Reef-building corals form associations with a huge diversity of microorganisms, which are essential for the survival and well-being of their host. While the acquisition patterns of Symbiodiniaceae microalgal endosymbionts are strongly linked to the coral's reproductive strategy, few studies have investigated the transmission mode of bacteria, especially in brooding species. Here, we relied on 16S rRNA gene and ITS2 marker metabarcoding in conjunction with fluorescence *in situ* hybridisation microscopy to describe the onset of microbial associations in the common brooding coral *Pocillopora acuta*. We analysed the bacterial and Symbiodiniaceae community composition in five adult colonies, their larvae, and 4-day old recruits. Larvae and recruits inherited Symbiodiniaceae, as well as a small number of bacterial strains, from their parents. Rhodobacteraceae and *Endozoicomonas* were among the most abundant taxa that were likely maternally transmitted to the offspring. The presence of bacterial aggregates in newly released larvae was observed with confocal microscopy, confirming the occurrence of vertical transmission of bacteria in *P. acuta*. We concluded that host factors as well as the environmental bacterial pool influenced the microbiome of *P. acuta.*

Keywords: Coral microbiome, Symbiodiniaceae, bacteria, early life stages, *Pocillopora acuta*, horizontal acquisition, vertical transmission, fluorescence *in situ* hybridisation, amplicon sequencing

Introduction

Tropical coral reefs are among the most productive and biodiverse ecosystems on the planet (Harrison and Booth, 2007). Stony corals secrete a calcium carbonate skeleton, thus building the three-dimensional structure of the reef and providing habitat and resources for ~25% of all marine species (Spalding et al., 2001). To obtain sufficient energy required to calcify, reproduce and function in oligotrophic waters, corals rely on intracellular microalgae in the family Symbiodiniaceae with which they establish a mutualistic symbiosis (Dubinsky and Falkowski, 2011). In addition to these microalgae, corals form associations with a multitude of other microorganisms, including Bacteria, Archaea, fungi and viruses (Blackall et al., 2015). Together, the coral and its microbial partners form a functional entity called the holobiont (Rohwer et al., 2002). Bacteria are reported to exert numerous beneficial roles for coral health (reviewed in Bourne et al. (2016)). For example, by participating in carbon, nitrogen, sulfur and phosphorus cycling, bacteria supply the host with essential nutrients and contribute to waste removal (Lesser et al., 2007; Raina et al., 2009; Zhang et al., 2015). Moreover, coral-associated bacteria may prevent the colonisation of the mucus by invasive pathogens by inhibiting their growth through the production of antimicrobial peptides and by occupying entry niches (Ritchie, 2006; Nissimov et al., 2009; Shnit-Orland and Kushmaro, 2009; Raina et al., 2016). Similar to other marine animals, the well-being of the coral holobiont is contingent on its microbial communities, as diseased and stressed states are usually associated with dysbiotic microbiomes (reviewed in Egan and Gardiner (2016)).

Given the fundamental roles of the microbiome in corals and other organisms (McFall-Ngai et al., 2013), it is important to understand how these relationships are established and maintained. Microbial symbionts can be acquired horizontally from the environment, transmitted vertically from parents, or acquired via a

combination of both (Bright and Bulgheresi, 2010). While vertical transmission enables the maintenance of specific mutualistic partners and does not rely on a potentially unpredictable symbiont source, horizontal acquisition provides the advantage of selecting microbes that could confer a local advantage (Byler et al., 2013; Hartmann et al., 2017).

Corals can reproduce by broadcast spawning (the release of eggs and sperm in the water column, where fertilisation occurs) or by brooding planula larvae within their polyps and releasing mature larvae into the water column (Harrison, 2011). The transmission mode of Symbiodiniaceae is closely linked to the coral's reproductive strategy, with the majority of broadcast spawners acquiring Symbiodiniaceae from the environment, while most brooders transmit them from parent to offspring by seeding the planulae with symbiotic algae (Baird et al., 2009). A mixed transmission mode has been documented in brooders, as vertical acquisition of Symbiodiniaceae is accompanied by some degree of environmental uptake (Byler et al., 2013; Quigley et al., 2018). Knowledge about the transmission of bacterial symbionts is more limited, but several broadcast spawners have been shown to acquire bacteria horizontally (Apprill et al., 2009; Sharp et al., 2010). Additionally, metabarcoding of the 16S rRNA gene suggests that some bacteria may be vertically transmitted to the gametes via the mucus layer (Leite et al., 2017; Zhou et al., 2017; Bernasconi et al., 2019; Damjanovic et al., 2019b). One study used microbial community profiling and fluorescence *in situ* hybridisation (FISH) microscopy in the brooder *Porites astreoides* to show that at least some bacteria are vertically transmitted (Sharp et al., 2012), and 16S rRNA gene metabarcoding of field-collected parents and larvae of the brooder *Pocillopora damicornis* implied that larvae mostly acquire bacteria from the surrounding water column but that a small number of taxa may be inherited from the parent colony (Epstein et al., 2019).

In this study, we investigated the bacterial and Symbiodiniaceae transmission mode in the brooder, *Pocillopora acuta,* using three coral colonies collected from Orpheus Island (OI) and two colonies collected from Magnetic Island (MI), Australia. We analysed the microbial community composition in adult corals, newly released larvae and early recruits with metabarcoding of the 16S rRNA gene and the Internal Transcribed Spacer 2 (ITS2) region (Fig. 1). To localize bacteria in the coral larvae, we employed FISH and confocal laser-scanning microscopy (CLSM).

Results and discussion

Vertical transmission of Symbiodiniaceae in *P. acuta*

After quality control and filtering of rare sequences in QIIME2 (QIIME 2 Development Team, 2017b), 1,896,627 ITS2 reads were recovered from the 71 coral samples (Fig. S1). The ITS2 dataset was rarefied to the lowest number of reads across samples (16,014 reads) and a total of 15 Symbiodiniaceae sequence variants were detected (Fig. S2). It is unclear whether the Symbiodiniaceae genetic variation was due to intraspecific or interspecific diversity. Processing the same ITS2 dataset with the SymPortal workflow (Hume et al., 2019a) resolved three distinct Symbiodiniaceae genotypes (Fig. S3), all belonging to the genus *Cladocopium* (formerly known as clade C (LaJeunesse et al., 2018)). Two colonies from OI harboured type C1d−C1−C42.2 and the third colony from OI type C1d−C42.2−C1−C1bm−C1b, while the two adult coral colonies from MI harboured type C1d−C1bl−C42.2−C1 (Fig. S3). All larvae and recruits harboured the same

Symbiodiniaceae type as their parent colony, which confirms vertical transmission of algal endosymbionts in *P. acuta* (Baird et al., 2009; Schmidt-Roach et al., 2014). *P. acuta* offspring may also acquire Symbiodiniaceae from the environment but we were unable to assess this, as the present experiment was conducted in captivity with 0.4 µm filtered seawater (FSW), which minimised the pool of microorganisms available in the environment. In the field, a mixed mode of transmission for Symbiodiniaceae has been described in the brooders *Seriatopora hystrix* (Quigley et al., 2018) and *P. damicornis* (Epstein et al., 2019), where larvae and one-week old recruits respectively hosted symbiont types that were absent from their parents.

***P. acuta* early life stages associate with diverse bacteria**

The 16S rRNA gene dataset consisted of 2,152,907 reads obtained from the 84 samples (i.e. 71 coral, 8 water and 5 negative control samples), representing 3,116 amplicon sequence variants (ASVs). After quality filtering, the number of reads per sample ranged between 4,477 and 38,463, with an average of 25,630 reads per sample. ASVs representing reagent or laboratory contamination (Salter et al., 2014) were identified based on their relative abundances in the negative control samples (Lee et al., 2015) and removed from the dataset (see Supporting Information and Table S1). The five negative controls were also removed from the original dataset, which was filtered and rarefied anew to 4,225 reads (the lowest read count across all processed samples, Figs S4 and S5).

Bacteria in the genus *Endozoicomonas* (Endozoicomonadaceae) dominated the communities associated with adults from OI, but were present at lower abundances in the adult colonies from MI and in all larvae and recruits (Fig. 2A). Alteromonadaceae, Flammeovirgaceae and Rhodobacteraceae were among the most

abundant bacterial families in all corals, as well as Flavobacteriaceae, which were more prevalent in larvae and recruits compared to adults (Fig. 2A). The most prevalent bacterial families detected in corals occurred at lower proportions in the surrounding water. Campylobacteraceae, Cellvibrionaceae, Clostridiaceae, Marinilabiaceae and Vibrionaceae comprised the most abundant bacterial families in the large planulation tank (Fig. 2A). Water samples collected from the recruit rearing containers were dominated by Halieaceae, Planctomycetaceae, Rhodospirillaceae and Rhodobacteraceae (Fig. 2A). Overall, coral early life stages had higher α-diversity indices than adult colonies (Table S2). All larvae and recruits were associated with a richer bacterial community than their respective parents, even though the difference was not statistically significant for the two MI colonies (Fig. 2B, Table S3). Higher α-diversity in juvenile corals compared to adults has previously been described in other coral species, including the broadcast spawners *Acropora tenuis* and *A. millepora* (Littman et al., 2009) and the brooder *P. damicornis* (Epstein et al., 2019). Given the dynamic nature that often characterises bacterial assemblages in coral early life stages (Littman et al., 2009; Lema et al., 2014; Zhou et al., 2017), it is hypothesised that a winnowing process takes place throughout ontogeny (Nyholm and McFall-Ngai, 2004), during which the microbiome is progressively fine-tuned until a more stable and more restricted community is established.

Patterns of horizontal and vertical transmission of bacteria in *P. acuta*

So far, few studies have undertaken a detailed comparison of bacterial assemblages between adult coral colonies and their progeny. In a recent field experiment, it was suggested that the brooder *P. damicornis* vertically transmits a small proportion of bacterial ASVs and that young recruits acquire the majority of their bacterial associates from the envrionment (Epstein et al., 2019). While the latter study did not analyse larval-associated bacterial communities, these patterns were confirmed for a congener in our experiment,

in which we investigated the bacterial assemblages present in adults, larvae and recruits. Only a minority of ASVs were shared between adults and their respective offspring, which associated with numerous ASVs not present in the parents (Fig. 2C). Contrary to the vertical transmission of Symbiodiniaceae, this suggests that a large proportion of bacterial ASVs comprising the early life stage microbiome are acquired from the environment. Given that the bacterial community composition in the water column differed from those associated with coral samples (Fig. 2A), it is likely that *P. acuta* adult and early life stages formed non-random associations with bacteria from the surroundings. Such patterns have also been documented for various coral species in natural settings (Sunagawa et al., 2010; Tremblay et al., 2010; Sweet et al., 2011). It is possible that a fraction of ASVs found to be unique to offspring were also present as rare members in adults or were not detected due to limited sampling from each parent colony (i.e., one small branch tip) (Fig. S6). The coral microbiome varies across coral compartments (Sweet et al., 2011; Pollock et al., 2018) and across different locations within individual colonies (Rohwer et al., 2002; Daniels et al., 2011). Thus, restricting sampling to branch tips, done so to minimise stress, might have precluded the detection of bacteria situated in other regions of the coral. Additionally, bioinformatics processing of the sequencing data may have removed some ASVs during the filtering step of low-quality sequences, as well as during rarefaction.

The number of ASVs shared between each adult and its offspring represented a minority of the larval and recruit ASVs, but the relative abundance of these ASVs was larger in the early life stages. On average, larvae from OI and MI shared 5% and 26% of ASVs with their parents, respectively, and these ASVs represented between 10% and 37% of the bacteria in OI larvae, and between 42% and 81% of the bacteria in MI larvae (Table S4). Since the ASVs shared between parents and offspring had a relatively high abundance within

individual samples (Fig. 2C and Fig. S7), we hypothesise that these bacteria likely perform relevant functions during coral reproduction or in other stages of the coral life cycle.

Importance of specific bacterial ASVs in *P. acuta* early life stages

Among the total 3,094 ASVs detected across all coral samples, only 70 ASVs occurred in all life stages (i.e. parent, larvae and recruits) of at least one colony (Fig. 3). Since some of these shared ASVs were not detected in water samples, it is likely that they were vertically transferred from parent to the offspring during the brooding period (Fig. 3). Among the 70 shared ASVs, 16 were identified as members of Rhodobacteraceae, four as Alteromonadaceae and four as *Endozoicomonas* (Endozoicomonadaceae). Bacteria belonging to these families are common members of the bacterial communities associated with adult and early life stages of other coral species. For instance, members of Rhodobacteraceae and Alteromonadales (Alteromonadaceae) were abundantly present in larvae and juveniles of *A. millepora* (Lema et al., 2014). *Roseobacter* sp. (Rhodobacteraceae) were associated with planulae of the broadcast spawner *P. meandrina* (Apprill et al., 2009), and together with *Marinobacter* (Alteromonadaceae), consistently occurred in early life stages of the brooder *P. astreoides* (Sharp et al., 2012). In *P. damicornis*, a close relative of *P. acuta* (Schmidt-Roach et al., 2014), several Rhodobacteraceae, Alteromonadaceae and *Endozoicomonas* (Endozoicomonadaceae) ASVs were reported to likely be vertically transmitted from parent to offspring (Epstein et al., 2019).

Indicator value analysis (De Cáceres and Legendre, 2009) identified 28 bacterial ASVs as indicators for particular life stages within OI and MI coral colonies (Fig. S8). Several Rhodobacteraceae and Alteromonadaceae were significantly associated with larvae and recruits of OI or MI. Five indicator ASVs

(i.e. two Rhodobacteraceae, one *Alteromonas* (Alteromonadaceae), one *Endozoicomonas* (Endozoicomonadaceae), and one *Alcanivorax* (Alcanivoracaceae)) belonged to the aforementioned 70 ASVs shared between parents and their offspring (Fig. 3 and Fig. S8). The high relative abundance of these bacterial families in our samples, as well as in adult and early life stages of other coral species, suggest that they might be exerting important roles for the holobiont. These bacteria could be involved in nutrient cycling, as one *Alteromonas* strain has previously been shown to provide nitrogen to *P. damicornis* larvae (Ceh et al., 2013), while *Roseobacter* and some *Alteromonas* strains are known to participate in sulfur cycling (Raina et al., 2009). *Alteromonas* are sometimes able to produce antimicrobial peptides and could therefore help against pathogenic infections (Ritchie, 2006). Bacteria in the genus *Endozoicomonas* are generally accepted to be important coral symbionts due to their abundance and widespread associations with different coral species (Neave et al., 2016a; Neave et al., 2016b) and due to their putative beneficial roles, such as carbohydrate and protein provisioning to the host (Neave et al., 2017) or protection from heat (Pantos et al., 2015) and other stressors (Ding et al., 2016). Further investigations are required to decipher whether these bacteria play a role in ontogenetic development.

Bacterial aggregates in *P. acuta* larvae provide evidence for vertical transmission

Microscopy examinations of haematoxylin and eosin (H&E) stained larval sections showed that newly released *P. acuta* larvae were fully developed, as they possessed differentiated cells and dense Symbiodiniaceae communities occupied their endodermal region (Fig. 4A-B, Fig. S9). In addition to 16S rRNA gene metabarcoding of *P. damicornis* adult and recruit samples (Epstein et al., 2019), FISH on *P. acuta* larvae followed by CLSM observations was undertaken in order to examine whether some bacteria were vertically transmitted. The universal bacterial probe EUB338 was applied on 63 sections from nine larvae,

with successive sections treated with the non-EUB probe and no probe as negative controls. Altogether, features resembling bacterial aggregates were observed in four of the nine larvae. Sixteen out of the corresponding 26 sections treated with EUB338 probes had aggregates (Table S5), suggesting that the latter did not span the whole depth of the samples. Bacterial clusters were primarily situated within tissues of the newly released larvae (Fig. 4C, E-F). Several aggregates were visible per section and they were slightly larger than 10 µm in size (Fig. 4D, G-H). At high magnification we were able to distinguish cells resembling bacteria more clearly (Fig. 4D). When simultaneously applying the EUB338 and non-EUB probes labelled with different fluorophores, the aggregates were only visible with the EUB338 probe (Fig. 4E, Fig. S10C, G-H), providing strong support that these signals were from bacterial cells. Figs S10 and S11 display representative images of larval sections treated with the EUB338 and/or non-EUB probes, also confirming that when these probes were applied on successive sections, the aggregates visible with the EUB338 probe were not present in the negative controls. Bacterial aggregates imaged with CLSM did not correspond to any morphological feature of the host on the H&E stained sections (Fig. S10I-L, Fig. S11A, D-E).

Host tissue autofluorescence and nonspecific binding of the probes are major challenges when applying FISH to coral samples (Wada et al., 2016). To address the first issue, we recorded the emission spectra of the oligonucleotide probes when applied to bacterial cultures, as well as the natural autofluorescence of the host by treating sections using the FISH protocol without any probe. Spectral scanning with CLSM followed by linear unmixing allowed discrimination of the probe from the coral emission spectrum. To address the second issue and ensure that the observed aggregates represented bacteria rather than nonspecific binding of the probes to host tissue, we compared numerous pairs of successive sections treated with the EUB338 and non-EUB probes, as well as sections treated with both probes simultaneously. When applied to coral samples, FISH probes are known to stick nonspecifically to granular gland cells and

nematocysts (Sharp et al., 2012; Lema et al., 2016; Wada et al., 2016). In addition to those patterns (Fig. S11C), we also observed a high level of nonspecific binding occurring on and around Symbiodiniaceae cells (Fig. 4E, Figs S10C and S11C). Even though other studies have reported similar signals as algal-associated bacteria (Ainsworth et al., 2015), the consistent binding of non-EUB probes that we recorded around Symbiodiniaceae suggests that this represents nonspecific binding in our *P. acuta* samples. Bacterial aggregates were only associated with EUB-338 probes, confirming they are bacteria. The reports of other authors describing similar features as bacterial aggregates in adult corals support our findings (Ainsworth et al., 2006; Ainsworth and Hoegh-Guldberg, 2009; Wada et al., 2016).

As *P. acuta* larvae were sampled within a few hours after planulation, bacteria from the environmental pool could have bound to the surface mucus layer, and therefore become part of the horizontally acquired microbiome. However, we believe the time frame between larval release and sampling (maximum 8 h) was too short for bacteria from the seawater enter the larvae and grow into aggregates within the larval tissue. We therefore conclude that these aggregated bacteria were seeded by the parental colonies into developing larvae during the brooding period. Oligonucleotide probes targeting specific bacterial taxa should be used in the future to identify at higher taxonomic levels which bacteria constitute the aggregates. This was done in one study that reported the clusters to be Gammaproteobacteria (Ainsworth et al., 2006). In the present work, selecting probes particularly for *Endozoicomonas* and *Alteromonas* (both Gammaproteobacteria) would be a first approach, but probes for Rhodobacteraceae (Alphaproteobacteria) and Flavobacteriaceae (Bacteroidetes) would also be relevant, as all these bacteria were highly abundant in larvae (Fig. 2A) and likely to be vertically transmitted (Fig. 3). In broadcast spawners, microscopic examinations of embryos and larvae across multiple coral species did not reveal evidence for vertical transmission of internalised bacteria (Apprill et al., 2009; Sharp et al., 2010; Apprill et al., 2012; Leite et al.,

2017). So far, only one study on *P. astreoides* demonstrated that brooders are capable of vertically transmitting at least some bacteria to their offspring (Sharp et al., 2012). Contrasting with our observations of bacterial aggregates within larval tissues, Sharp et al. (2012) identified bacterial cells in the ectoderm and on the surface of newly released larvae. Our work corroborates the occurrence of vertical transmission of bacteria in a brooder and provides insights into the proportion and abundance of ASVs that are shared between parental corals and their offspring. Future histological analyses using H&E staining and bacterial stains such as Giemsa or Gram will help determine whether bacterial aggregates are intracellular, which could point to an obligate symbiosis. Work on adult corals suggests that ultrastructural microscopy may also be necessary to discriminate whether bacteria are intra- or extracellular (Work and Aeby, 2014).

A complex combination of factors shape the coral microbiome

For most symbioses, disentangling the drivers of microbiome composition is challenging, as numerous host and environmental factors play a role and the composition can change in time and space (Wullaert et al., 2018). In corals, host species and genotype (Rohwer et al., 2002; Glasl et al., 2019), as well as reef habitat and geographic location (Pantos et al., 2015; Hernandez-Agreda et al., 2016b) have been shown at multiple instances to modulate the composition of the microbiome (summarised in Hester et al. (2016)). Numerous abiotic factors such as salinity, pH, temperature or nutrient levels influence the coral microbiome (Hernandez-Agreda et al., 2016a), in addition to season (Li et al., 2014), age (Williams et al., 2015) and Symbiodiniaceae type (Littman et al., 2010). Some of these complex patterns were reflected in our study, with the bacterial associates of *P. acuta* corals being contingent on life stage, collection location of the parental colony, host factors and Symbiodiniaceae.

Among samples from each collection location, life stage had a strong influence on the bacterial associates, with adults, larvae, and recruits having significantly different bacterial communities (Fig. 5A, PERMANOVA with 999 permutations: OI: $F_{Life\ stage}$ = 17.74, p = 0.001, MI: $F_{Life\ stage}$ = 12.31, p = 0.001). Further, OI samples had significantly different bacterial communities from MI samples independently of life stage, as assessed by permutational analysis of variance (PERMANOVA, with 999 permutations: $F_{Location}$ = 6.11, p = 0.001). One example is the higher relative abundance of Endozoicomonadaceae in OI compared to MI adult corals (Fig. 2A). Endozoicomonadaceae had a low relative abundance in the water column of the planulation tank (0.13%) and were undetected in the water sampled from recruit rearing containers. We hypothesise that parental colonies maternally transmitted *Endozoicomonas* sp. with which they had formed associations in their native habitat.

The five adult colonies were maintained in the same tank before and during the experiment and were thus exposed to the same microbial environment. Host specific factors such as genotype or environmentally induced phenotypic variation might therefore have driven differences in the microbial community structure. Although not confirmed by this study, it is likely that the five *P. acuta* colonies were genotypically distinct, especially when considering corals were collected from separate reefs. Host taxonomy has been shown to influence the microbiome in the closely related coral *P. damicornis* (Brener-Raffalli et al., 2018). Alternatively, the local environments in which the *P. acuta* colonies grew on the reef may have induced phenotypic divergences, which may have led to variations in the microbiome. It is known that corals of the same genotype exposed to distinct conditions can exhibit phenotypic plasticity, such as differences in growth and survival (Drury et al., 2017) or contrasting patterns of thermal stress tolerance (Durante et al., 2019).

Considering each life stage separately, principal coordinates analysis (PCoA) visualisation of β-diversity illustrates that microbiomes of different coral genotypes clustered separately from each other (Fig. 5B, PERMANOVA with 999 permutations: Adults: F_{Colony} = 4.86, p = 0.001, Larvae: F_{Colony} = 5.32, p = 0.001, Recruits: F_{Colony} = 5.66, p = 0.001). Pairwise comparisons within each life stage yielded significant differences in bacterial communities between most groups (Table S6), further suggesting an effect of host genotype even among colonies from the same location. The segregation retained by larvae and recruits according to their parental origin could have resulted from a combination of the vertically transmitted bacteria and host genotypic effects (Fig. 5B). Symbiodiniaceae type explained some of the variance in bacterial community composition (Fig. 5B), which could suggest an association between endosymbiotic algae and bacterial assemblages. For example, colonies OI1 and OI3 possessed the same algal type and clustered closely to each other and further away from OI2, which had a different algal type (Fig. 5B). However, it is possible that host genotype influenced both algal and bacterial communities and that Symbiodiniaceae did not play a role in shaping the bacterial microbiome. The absence of a link between coral-associated bacteria and Symbiodiniaceae types during early ontogeny in *A. tenuis* and *A. millepora* is in agreement with this hypothesis (Littman et al., 2009).

Concluding remarks

In cnidarians and other animals, including humans, interactions with symbiotic microorganisms guide fundamental biological processes such as ontogenetic development, growth, immunity, physiological functions, behaviour and life span (Bosch, 2013; Rees et al., 2018). Host health and survival is thus contingent on the equilibrium maintained with its symbionts. In this study, we combined metabarcoding

and microscopy approaches to show that the brooding coral *P. acuta* vertically transmits Symbiodiniaceae and some of its bacterial associates. A reliable transfer of selected symbionts across generations may be essential to maintain these potentially beneficial microbial partners. Coupled with the ability to acquire particular microbes from the environment, this strategy could facilitate transgenerational acclimatisation of corals to changing conditions. Future studies should investigate the identity of bacteria constituting the dense aggregates situated in newly released larvae, as well as the function and temporal sequence of all vertically transmitted and horizontally acquired bacterial associates. Tackling these questions will provide important insights into the potential of microbial communities to assist coral tolerance to stress and into the biological mechanisms that govern the complex entity defined as holobiont.

Experimental procedures

Field collection, planulation, settlement and rearing

Three colonies of the coral *P. acuta* were collected from OI (S -18°60 E 146°48), on February 17th 2017 and two colonies were collected from MI (S -19°15 E 146°86), on March 18th 2017, all between 3-5m depth. The coral colonies were transported to the Australian Institute of Marine Science (AIMS) and deployed in one 3000 l tank with 0.4 µm filtered flow-through FSW at 60 l min^{-1}. Three days after the new moon of March 28th 2017, the corals were isolated in individual vessels within the same 3000 l tank. The vessel rims were above the 3000 l tank water level and the vessels contained harvesters on their outlets to collect the larvae

released overnight. Planulation peaked throughout the night on April 2nd 2017 (day 1), and larvae from each colony were collected the following morning at 8:00 am for metabarcoding, microscopy and settlement (Fig. 1).

Five larval samples of five larvae each, and five small branch tips from each parent colony were collected for DNA extraction. Samples were rinsed with 0.22 µm FSW to remove loosely associated microorganisms, snap-frozen in liquid nitrogen and stored at -80°C. Several larvae from each colony were also fixed in 4% paraformaldehyde (PFA) for subsequent FISH microscopy and left at 4°C for ~ 10 h. These samples were then washed in PBS and stored at -20°C in 50% ethanol-PBS (Lema et al., 2016). Most larvae subsequently observed with FISH microscopy had already adopted a rounded shape, as the stress of sampling likely triggered their metamorphosis. Aliquots of approximately 200 living larvae from each parent colony were placed into individual 2 l plastic containers filled with 0.4 µm FSW and operated with flowing FSW (0.1 l min^{-1}) and gentle aeration. To induce larval settlement, each container had a PVC tray with aragonite plugs (20 mm diameter) pre-conditioned with crustose coralline algae and a biofilm, then sterilised by autoclaving. The larvae attached to the plugs and metamorphosed into recruits within a day. The recruits were reared under a 12 h light/dark illumination cycle reaching a maximum light intensity of 10 µmol m^{-2} s^{-1} after 2 h ramping. On April 6th (day 4), five samples of 4-day old recruits (with each replicate sample containing 5 individual recruits) were removed from the plugs with a sterile scalpel blade, washed with 0.22 µm FSW and snap-frozen for DNA extraction.

Five 500 ml replicate seawater samples were collected from the 3000 l tank on day 1 (Fig. 1). Following recruit sampling on day 4, 500 ml of seawater were collected from each of the five containers. Water

samples were filtered through a 0.22-µm Sterivex filter with a peristaltic pump and the filters were snap frozen for subsequent DNA extraction.

DNA extraction and amplification

DNA was extracted from the samples following the protocol reported in (Damjanovic et al., 2019a). To provide enough bacterial biomass for downstream PCR amplification of the 16S rRNA gene, five to seven larvae and five recruits per replicate sample were pooled for DNA extraction. Approximately 60 mg of adult fragments were used for each replicate. Samples were placed in 1.5 ml sterile microcentrifuge tubes containing 250 µl of extraction buffer (100 mM Tris pH 9.0, 100 mM EDTA, 1% SDS, 100 mM NaCl). Ten µl of lysozyme at 10 mg ml^{-1} were added to all tubes and tubes were incubated at 37°C for 30 min. About 30 mg of sterile glass beads and 10 µl of Proteinase K at 20 mg ml^{-1} were added to the tubes. The samples were bead-beaten at 4 m s^{-1} for 20 sec and incubated at 55°C for 2 h, followed by 65°C for 15 min. After this step, 62.5 µl of KOAc at 5 M were pipetted into the tubes and incubated on ice for 30 min. After spinning the tubes at 25,000 *g* for 15 min at room temperature, the supernatant was transferred into new 1.5 ml sterile microcentrifuge tubes and 0.8 vol. isopropanol was added to precipitate DNA. The solutions were left at room temperature for 15 min and centrifuged again at 25,000 *g* for 15 min. After removing the supernatant, the precipitate was washed with 100 µl of 70% ethanol, centrifuged at 25,000 *g* for 3 min, air-dried and resuspended overnight in 25 µl Milli-Q water.

The same DNA suspensions were used to amplify the bacterial 16S rRNA gene and the Symbiodiniaceae ITS2 marker. Regions V5 to V6 of the 16S rRNA gene were amplified with the primer pair 784F [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGATTAGATACCCTGGTA-3']

and 1061R [5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCRRCACGAGCTGACGAC-3'] (Andersson et al., 2008). The ITS2 region was amplified with the forward primer ITS-DINO [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATTGCAGAACTCCGTG-3'] (Pochon et al., 2001) and reverse primer ITS2Rev2 [5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTCCGCTTACTTATATGCTT-3'] (Stat et al., 2009). The underlined segments represent Illumina adapter overhangs (Illumina, San Diego, CA, USA). The PCRs were conducted in 10 µl triplicates using the AmpliTaq Gold 360 Master Mix and 0.4 µM of each primer. The amplification cycles were: 95°C for 10 min; 30 cycles each at 95°C for 30 sec, 57°C for 1 min, 72°C for 30 sec; a final extension at 72°C for 7 min. PCR triplicates for each template were pooled and sent to Ramaciotti Centre for Genomics (UNSW, Sydney) for library preparation and sequencing on the Illumina MiSeq system with 2 x 300bp paired-end reads.

In addition to processing the samples, four blank DNA extractions (i.e. DNA extraction conducted as explained above but without any sample, then amplified by PCR) and one no-template PCR (i.e. PCR reaction with Milli-Q instead of DNA extract) were processed and sequenced to check for laboratory contamination. No PCR products were visible by agarose gel electrophoresis in any of these negative controls.

Bioinformatics

The amplified 16S rRNA gene sequences were processed using the QIIME 2 pipeline version 2017.10 (Caporaso et al., 2010; QIIME 2 Development Team, 2017b). Plugin demux (QIIME 2 Development Team, 2017a) was used to visualise interactive quality plots and check read quality. Plugin DADA2 (Callahan et al.,

2016) was subsequently applied to remove primers, truncate poor-quality bases based on the interactive plots, dereplicate, identify chimeras and to merge paired-end reads. Commands included in plugin feature-table (McDonald et al., 2012) enabled generation of summary statistics of sequences associated with the samples. A Naïve Bayes Classifier was trained with the feature-classifier plugin (QIIME 2 Development Team, 2017c) using the 16S rRNA gene database at 99% similarity of the SILVA 128 QIIME release (Quast et al., 2013) and based on the 784F/1061R primer pair. The taxa plugin (QIIME 2 Development Team, 2017d) allowed us to filter mitochondria and chloroplast sequences, as well as to visualise taxonomic bar plots and generate tables with absolute read counts of all taxa for each sample. The biom tables containing taxonomic counts and metadata were imported into R (R Core Team, 2018) for statistical analyses.

The ITS2 marker of Symbiodiniaceae is present in multiple copies and commonly characterised by intragenomic variation (Wilkinson et al., 2015). Hence, it is challenging to identify whether the genetic diversity of a sample originates from intragenomic variation or from the presence of multiple Symbiodiniaceae genotypes (Sampayo et al., 2009). As the standard processing of ITS2 marker sequences with QIIME 2 did not allow the discrimination between intragenomic and intergenomic diversity (see Supporting Information), ITS2 data were analysed with the new SymPortal tool, which is able to resolve Symbiodiniaceae taxa (Hume et al., 2019b). Decomplexed paired-end sequences were submitted onto SymPortal.org for the workflow to be conducted remotely, as described in (Hume et al., 2019b, a) (using standard parameters).

Statistical analyses of bacterial communities

Exploratory and statistical data analyses were performed at the ASV level, a higher-resolution equivalent of the operational taxonomic unit (OTU), as ASVs are delineated by 100% sequence similarity (Callahan et al., 2017; Porter and Hajibabaei, 2018). ASVs for which the overall relative abundance was lower than 10^{-5} were filtered from the dataset as these may represent sequencing errors (Bokulich et al., 2013). Results in negative control samples were examined to identify potential reagent and laboratory contaminants (Salter et al., 2014), which were removed from the test dataset based on their relative abundances (Lee et al., 2015). To account for the variability in sequencing effort, data counts were rarefied to an even depth, corresponding to the lowest number of reads across samples (Hughes and Hellmann, 2005; Weiss et al., 2017).

The α-diversity was measured using richness (Lande, 1996; Legendre and Legendre, 1998), to which generalised linear models with negative binomial distributions were fitted to understand the differences in richness according to coral life stage and host genotype. Linear contrasts were then used to test differences in richness between pairs of samples. Differences in community composition (β-diversity, (Anderson et al., 2006)) were computed using Bray-Curtis dissimilarity matrices and tested via permutational multivariate analysis of variance (PERMANOVA, (Anderson, 2001)). Variation in community composition among samples was visualised with principal coordinates analysis (PCoA) (Legendre and Legendre, 1998). Analysis of multivariate homogeneity of group dispersions was carried out (PERMDISP, (Anderson, 2006)) to check for homogeneity of variances, and pairwise comparisons were performed between groups using the Benjamin and Hochberg (Benjamini and Hochberg, 1995) correction for multiple testing. Indicator value analysis (De Cáceres and Legendre, 2009) was applied to detect ASVs that were significantly associated with different coral groups when both specificity and fidelity had probabilities greater than 0.8 (i.e. respectively, the

probability of finding the ASV in samples belonging to the target group and the probability of a sample belonging to the group given that the ASV has been detected).

All statistical analyses were conducted using R version 3.3.2 (R Core Team, 2018) and packages phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2016), DESeq2 (Love et al., 2014), tidyverse (Wickham, 2017), ggplot2 (Wickham, 2009), RVAideMemoire (Herve, 2018), multcomp (Hothorn et al., 2008), indicspecies (De Cáceres and Legendre, 2009) and ggplot 2 (Wickham, 2009).

Fluorescence *in situ* Hybridisation (FISH) microscopy

Two larval samples from each coral colony were blocked in paraffin wax and sectioned (4 µm thickness) at the University of Melbourne Biomedical Sciences Histology Facility. FISH followed a protocol similar to the ones described in (Hugenholtz et al., 2002; Wada et al., 2016). Slides with tissue sections were incubated in 100% xylene (2x 3 min) to remove wax. Deparaffinised sections were then dehydrated in 100% ethanol (3x 5 min). After air-drying, a hydrophobic pen was used to create a barrier around the sections. The latter were subsequently washed in 0.2 M HCl for 12 min, followed by a wash in 20 mM Tris-HCl (pH 7.4) for 10 min. Once the sections dried, 18 µl of hybridisation buffer (30% v/v formamide, 0.9M NaCl, 0.01% sodium dodecyl sulphate (SDS), 20 mM Tris-HCl pH 7.4 – Table S7) and 2 µl of oligonucleotide probe were added over the tissue to yield a final probe concentration of 25 ng μl^{-1}. The universal bacterial probe EUB338 (5'-GCW GCC WCC CGT AGG WGT-3', (Amann et al., 1990)) and the negative control probe non-EUB (5'-ACA TCC TAC GGG AGG C-3', (Wallner et al., 1993)), were labelled either with fluorophore Atto550 (excitation 553 nm, emission 576 nm) or Atto647N (excitation 645 nm, emission 663 nm) (biomers.net). When applied on the same sections, EUB338-Atto647N was used in conjunction with non-EUB-Atto550. The slides were

incubated in an oven at 46°C for 2 h in the dark, and then placed for 10 min in a 50 ml pre-heated (48°C) washing buffer (0.1M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.4 – Table S7) at 48°C. Finally, slides were rinsed in ice-cold reverse osmosis (RO) water for 10 s, dried with pressurised air and mounted with ProLong™ Glass Antifade Mountant (ThermoFisher P36984) and glass coverslips. The slides were stored at 4°C in a light-proof box until visualisation, which was conducted within 24 h. Each sample type was also prepared without probe to assess tissue autofluorescence.

Slides were examined with a Zeiss710 laser-scanning confocal microscope (LSCM; Zeiss, Germany) and the Zeiss Zen 2009 software. A combination of the laser lines 405 nm, 561 nm and 633 nm was used to excite the samples. Linear unmixing of the recorded emission spectra was undertaken in order to distinguish autofluorescence of coral tissue and Symbiodiniaceae from the signal emitted by the probes (see Supporting Information for acquisition details).

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The authors have no conflict of interest to declare.

Availability of data and material

The sequence data generated and analysed in this study are available at NCBI under http://www.ncbi.nlm.nih.gov/bioproject/525296, BioProject accession PRJNA525296.

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Figure legends

Figure 1 Experimental design for coral rearing, planulation and sampling. A) Three *P. acuta* adult colonies collected from OI and two colonies collected from MI were maintained in a single 3000 l tank operated with flowing FSW. Before planulation, all colonies were placed into individual vessels within the 3000 l tank. Harvesters attached to the outlet of the five aquaria allowed collecting the larvae released overnight. In the morning of the first planulation day, larvae from each colony as well as five replicate adult branch tips per colony were sampled. The remaining larvae were settled on aragonite plugs pre-conditioned with a microbial biofilm and crustose coralline algae. The plugs were sterilised by autoclaving before settlement trials. Recruits from each colony were reared in five separate 2 l tanks with flow-through FSW and a small level of aeration. Using a sterile scalpel blade, five replicate recruit samples were taken per colony after four days. B) Photographs of adult polyps, larvae and recruits. Seawater was also collected from the adult and recruit rearing tanks to analyse its microbial community composition. OI: Orpheus Island; MI: Magnetic Island; Ad: Adult; Lar: Larvae; Rec: Recruit. Symbols for diagrams were modified from the Integration and Application Network (accessed 2018) and from (Jones et al., 2015).

Figure 2 Overview of bacterial community composition and diversity in coral samples. A) Relative abundance of the 15 most prevalent bacterial families across all coral samples (left, colour) and across all water samples (right, grey scale). The top bacterial families in the water that also occurred in coral samples are shown in the same colour and highlighted in bold. The average of the five replicates is shown for each group. B) Boxplots representing richness as measured by the Chao index for all coral samples. Asterisks indicate that offspring have a significantly higher richness than their respective parent. Open circles indicate a significantly different richness in recruits compared to larvae. C) Number of shared and unique

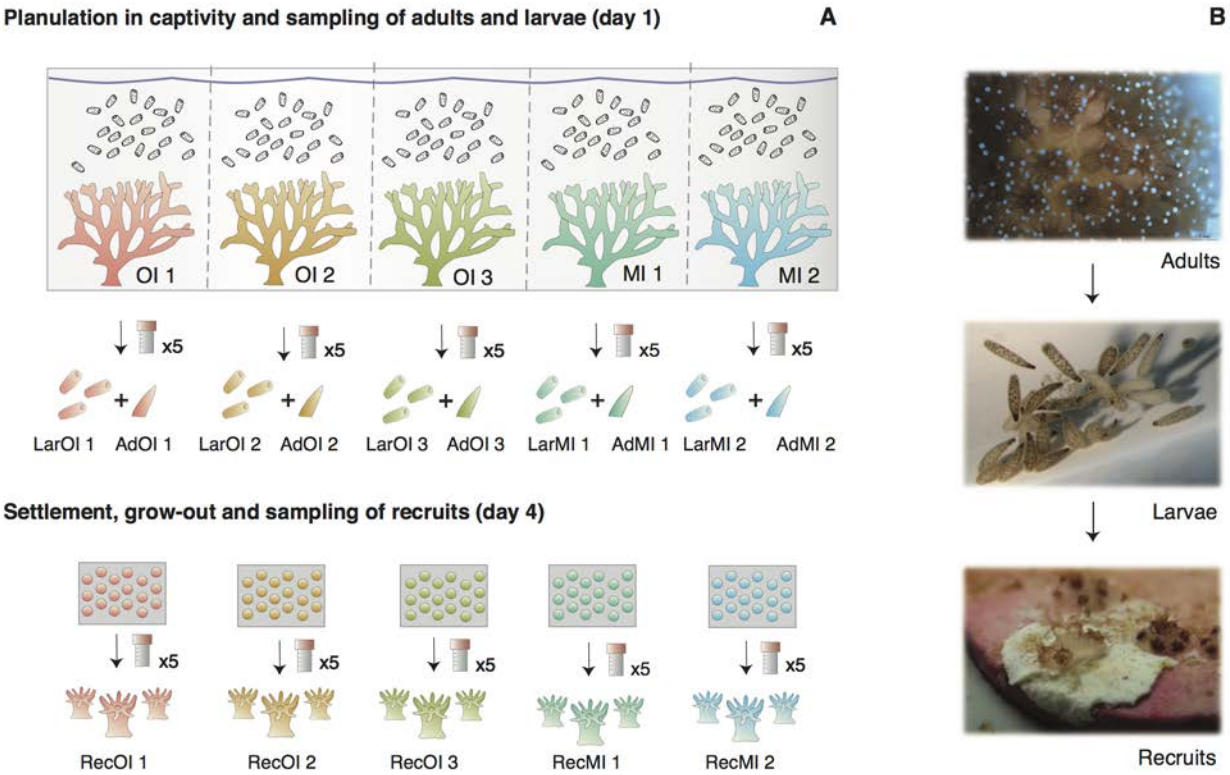
bacterial ASVs within each life stage across all replicates for each colony. Ad: Adults; Lar: Larvae; Rec: Recruits; OI: Orpheus Island; MI: Magnetic Island.

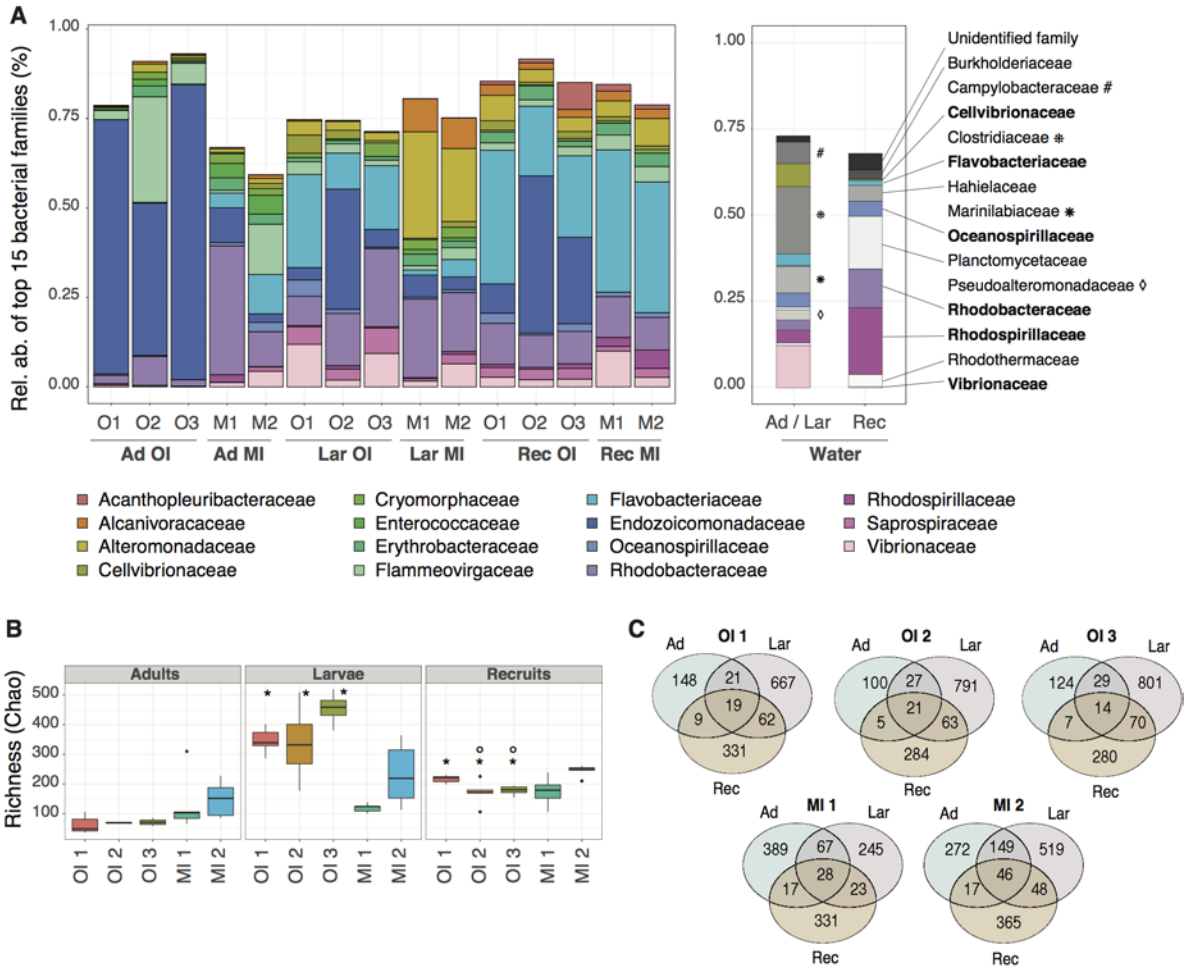
Figure 3 Potentially vertically transmitted ASVs between at least one parental colony and its offspring. This graph represents the relative abundance across all coral samples and within water samples of the 33 most abundant ASVs (out of 70) that were found to be shared between the adult, larvae and recruits of at least one coral colony. Some ASVs were not detected in water samples and were thus most likely transmitted vertically from parent to offspring. ASVs are identified at the family level, but all Endozoicomonadaceae sequences in this study belong to the genus *Endozoicomonas*. Families highlighted in bold occurred at higher abundance and their presence in other studies suggests that they could be exerting relevant functions for the holobiont. Ad: Adults; Lar: Larvae; Rec: Recruits; OI: Orpheus Island; MI: Magnetic Island.

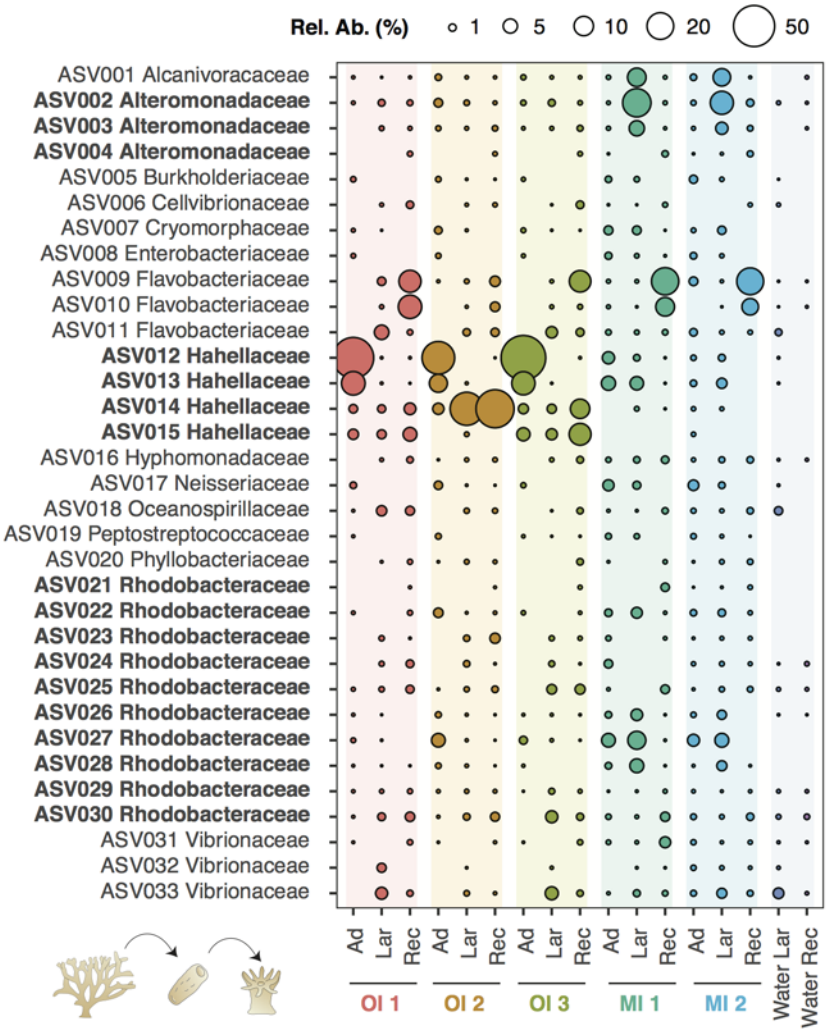
Figure 4 Representative histological section of a newly released *P. acuta* larva (A-B) and detection of bacterial aggregates by confocal microscopy following probe hybridisation (C-H). The larvae started metamorphosing in the water column upon sampling, hence their rounded shape. Larval sections shown in A, E and F originated from the same individual, while the section shown in C came from a different larva. A) H&E stained section of a whole larva (and photograph in the inset). B) H&E stained close-up of the larval tissue layers separated by the mesoglea, where several Symbiodiniaceae cells (S) are visible in the endoderm and cnidocytes containing nematocysts (N) in the ectoderm. C and F) Confocal images of whole larval sections treated with the EUB338 probe labelled with Atto550 fluorophore (yellow-boxed zones – enlarged in D, G-H). D, G-H) Close-ups of the bacterial aggregates (yellow) identified in C and F. Images were obtained from a maximum intensity projection of z-stacks. E) Larval section simulteanously treated using the EUB338 probe labelled with Atto647N (red – arrowed Ag) and the non-EUB probe labelled with

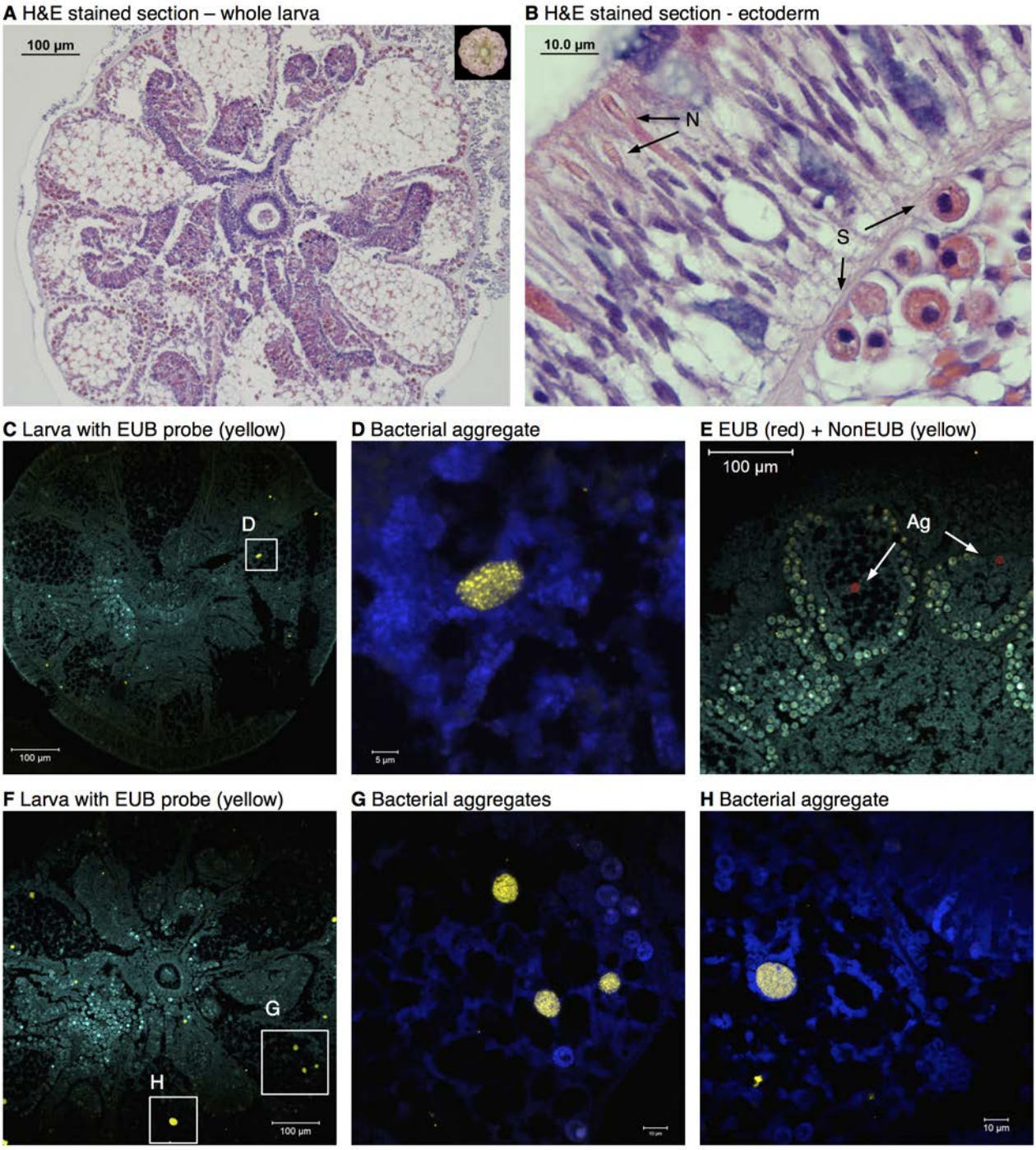
Atto550 (yellow) to discriminate signals due to unspecific binding. Bacterial aggregates (Ag) were only visible with the general bacterial probe. Autofluorescence of coral tissue is shown in cyan or blue.

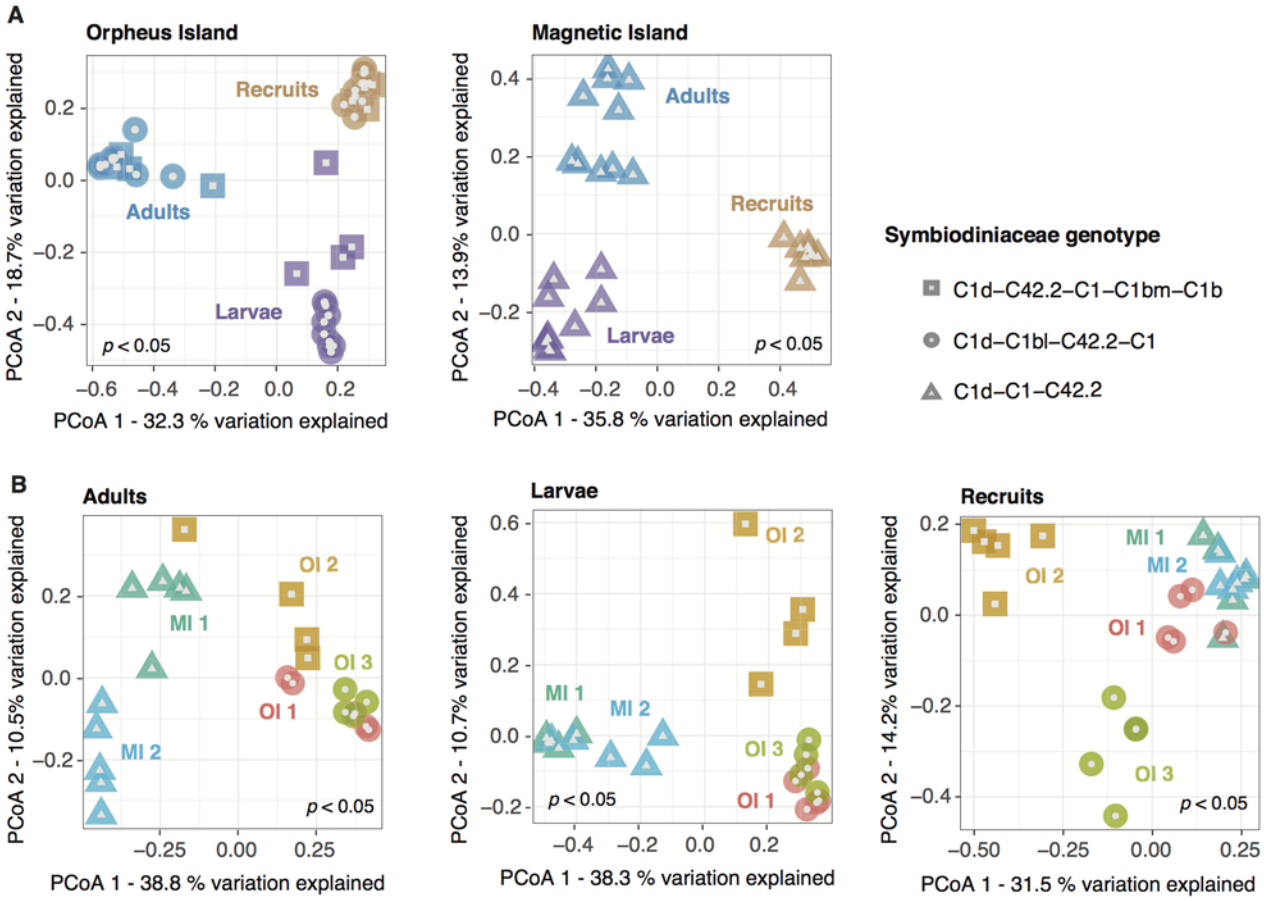
Figure 5 PCoA using the Bray–Curtis dissimilarity index to represent the clustering of coral samples according to their bacterial community composition. A) Life stage explains most of the variance in bacterial communities at both sampling locations. B) Further structuring among bacterial microbiomes within each life stage is driven by host factors. In A and B, the three Symbiodiniaceae genotypes that were resolved are represented by different symbols. OI: Orpheus Island; MI: Magnetic Island.

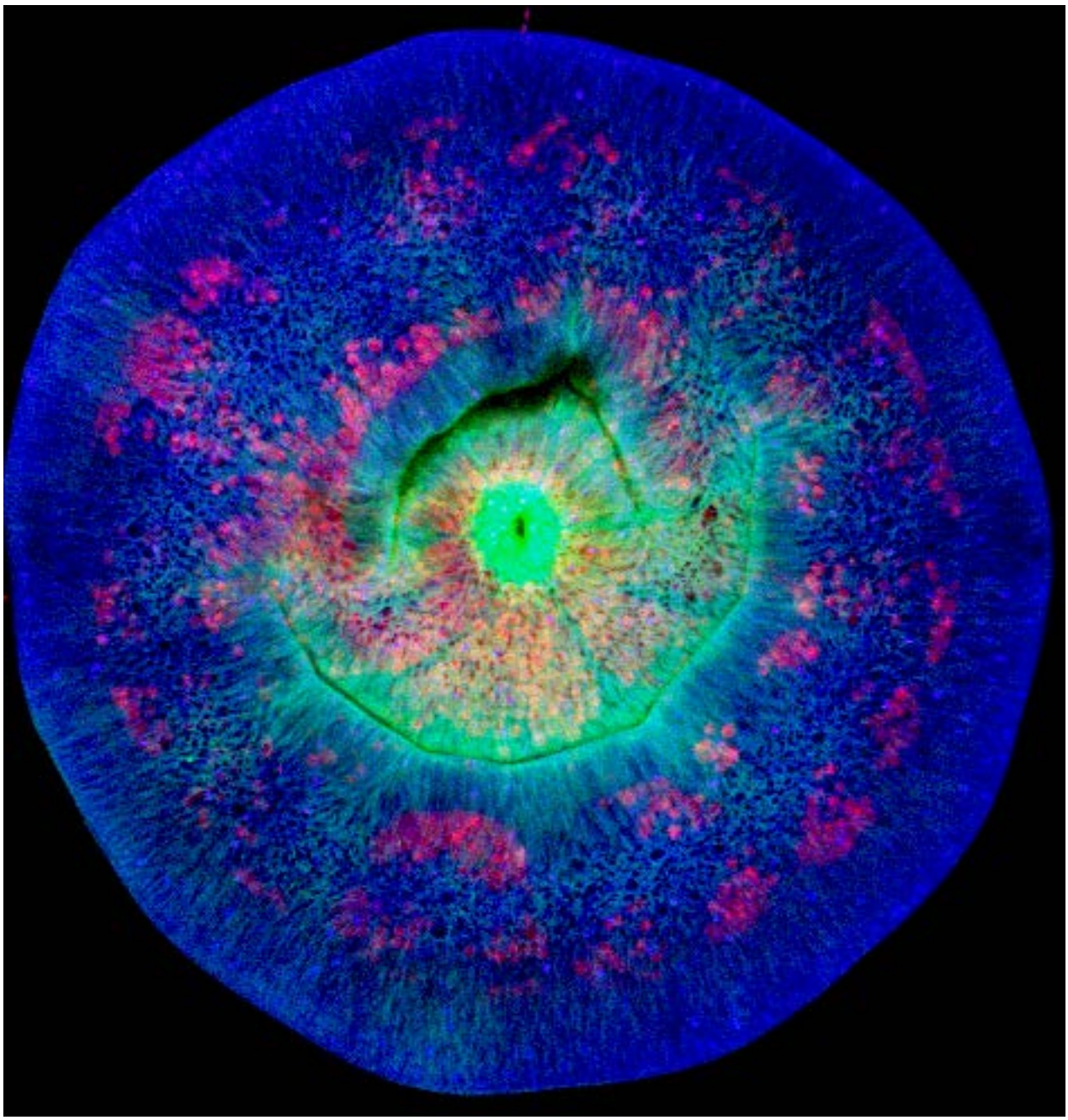


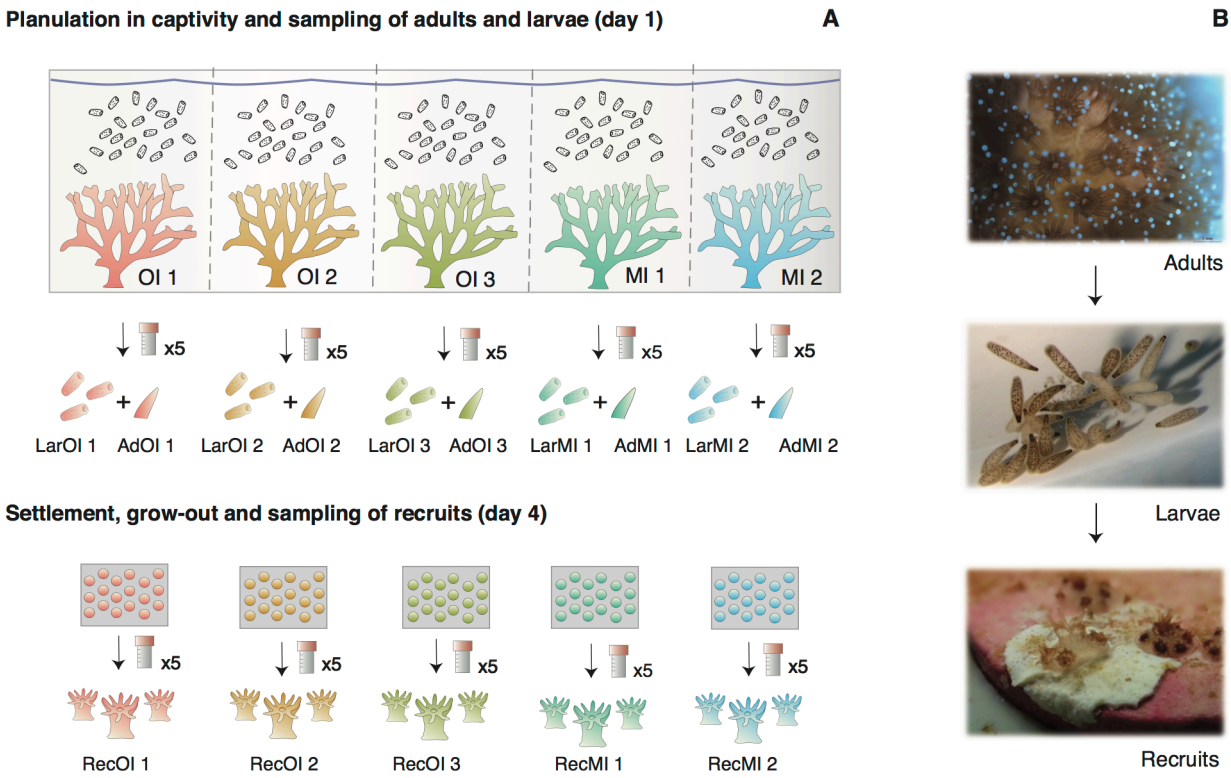












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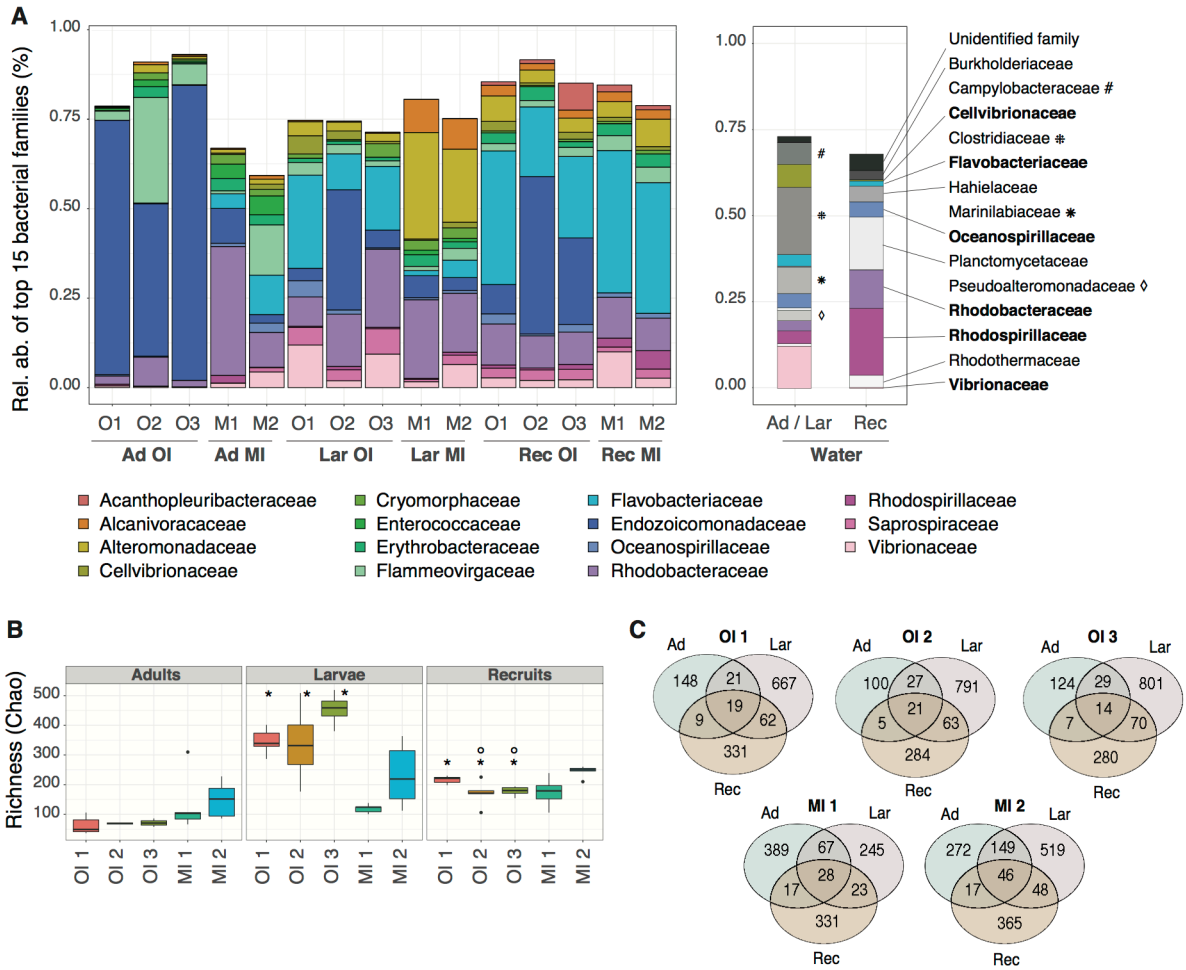

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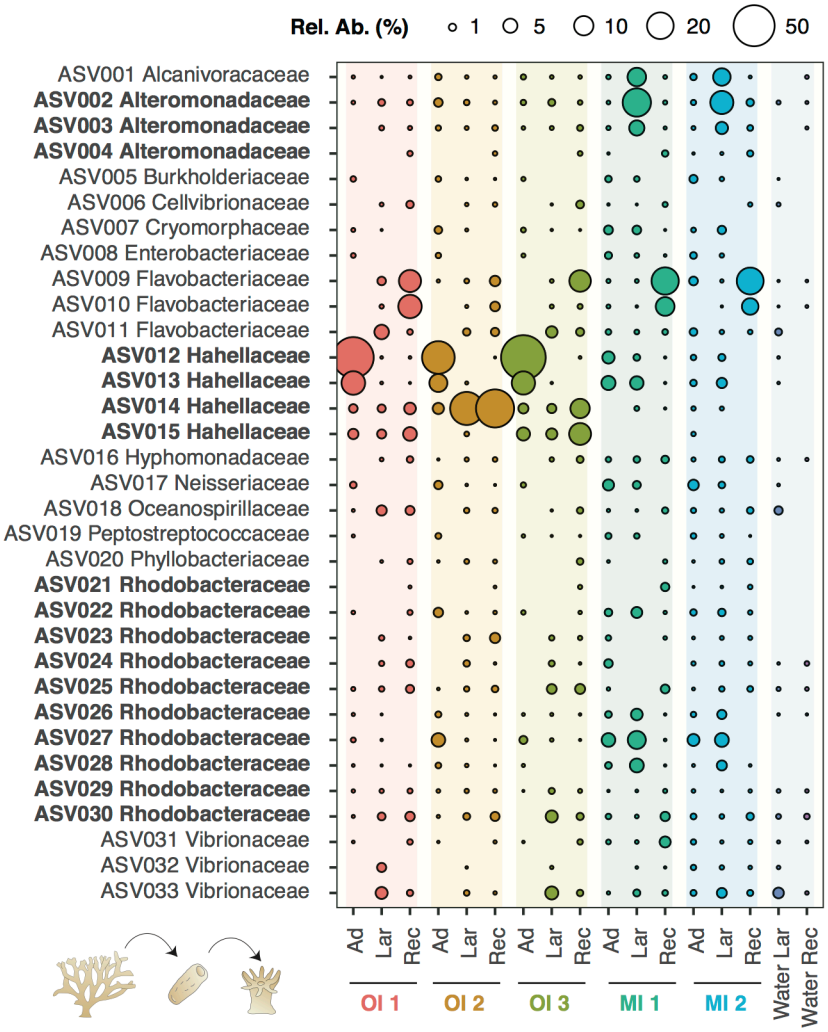

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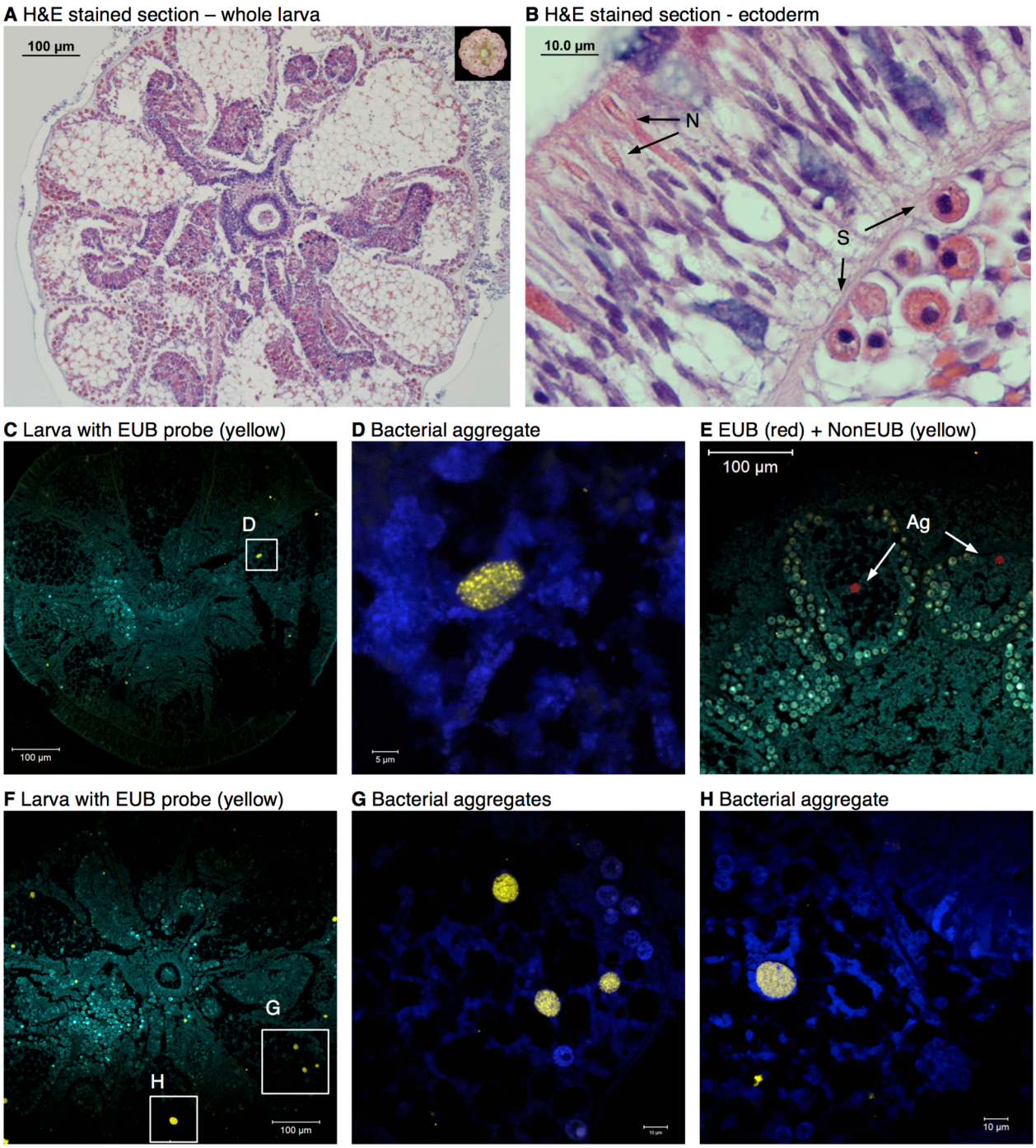

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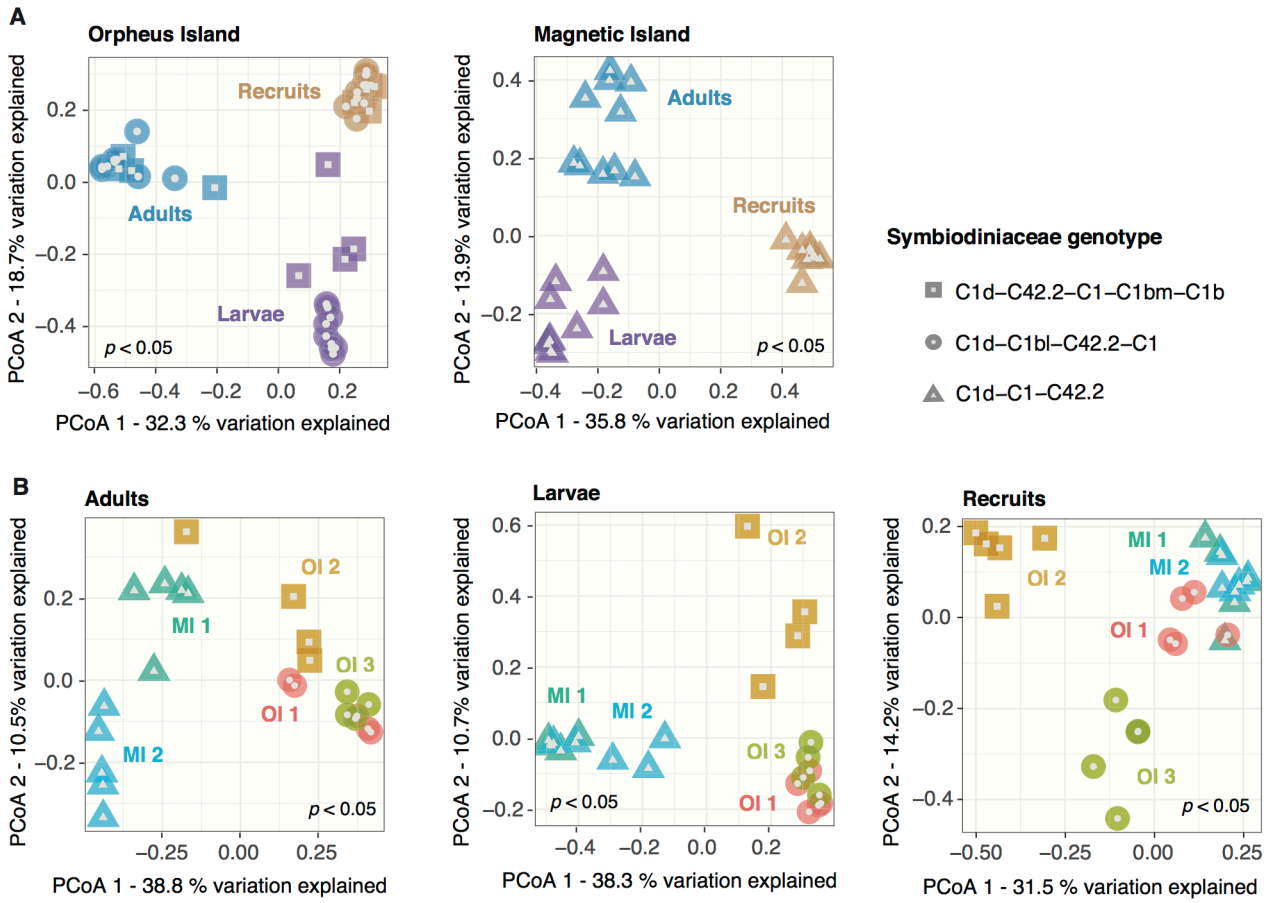

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Mixed mode bacterial transmission in the common brooding coral *Pocillopora acuta*

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Running title: Microbiome transmission in brooding coral

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Originality-Significance Statement

Corals form critical associations with microorganisms, which exert numerous beneficial functions for the host and are indispensable for its survival. Due to their ability to evolve rapidly, microbes could also mediate intra- and transgenerational acclimatisation of corals in the face of current climate change. In order to gain insight into the biological mechanisms that underlie the functioning of the holobiont and its persistence, it is important to understand how microbes are transmitted and maintained across generations. Only a handful of studies have investigated the transmission mode of bacteria in corals and this process remains unclear. Here, we combined amplicon sequencing with fluorescence *in situ* hybridisation (FISH) microscopy in order to investigate the patterns of microbiome acquisition in asexually brooded offspring. This study analysed the microbial community composition at the amplicon sequence variant level and in several coral life stages (parental colonies, newly released larvae and early recruits), in conjunction with FISH to address this question. Through DNA metabarcoding, we were able to capture the identity and abundance of bacteria that were either horizontally taken up from the environment or vertically inherited from the parents. Microscopy examinations enabled us to confirm the presence of maternally transmitted bacterial aggregates inside coral larvae. Our findings provide important information towards a better understanding of bacterial dynamics and transmission patterns in the coral holobiont.

Summary

Reef-building corals form associations with a huge diversity of microorganisms, which are essential for the survival and well-being of their host. While the acquisition patterns of Symbiodiniaceae microalgal endosymbionts are strongly linked to the coral's reproductive strategy, few studies have investigated the transmission mode of bacteria, especially in brooding species. Here, we relied on 16S rRNA gene and ITS2 marker metabarcoding in conjunction with fluorescence *in situ* hybridisation microscopy to describe the onset of microbial associations in the common brooding coral *Pocillopora acuta*. We analysed the bacterial and Symbiodiniaceae community composition in five adult colonies, their larvae, and 4-day old recruits. Larvae and recruits inherited Symbiodiniaceae, as well as a small number of bacterial strains, from their parents. Rhodobacteraceae and *Endozoicomonas* were among the most abundant taxa that were likely maternally transmitted to the offspring. The presence of bacterial aggregates in newly released larvae was observed with confocal microscopy, confirming the occurrence of vertical transmission of bacteria in *P. acuta*. We concluded that host factors as well as the environmental bacterial pool influenced the microbiome of *P. acuta.*

Keywords: Coral microbiome, Symbiodiniaceae, bacteria, early life stages, *Pocillopora acuta*, horizontal acquisition, vertical transmission, fluorescence *in situ* hybridisation, amplicon sequencing

Introduction

Tropical coral reefs are among the most productive and biodiverse ecosystems on the planet (Harrison and Booth, 2007). Stony corals secrete a calcium carbonate skeleton, thus building the three-dimensional structure of the reef and providing habitat and resources for ~25% of all marine species (Spalding et al., 2001). To obtain sufficient energy required to calcify, reproduce and function in oligotrophic waters, corals rely on intracellular microalgae in the family Symbiodiniaceae with which they establish a mutualistic symbiosis (Dubinsky and Falkowski, 2011). In addition to these microalgae, corals form associations with a multitude of other microorganisms, including Bacteria, Archaea, fungi and viruses (Blackall et al., 2015). Together, the coral and its microbial partners form a functional entity called the holobiont (Rohwer et al., 2002). Bacteria are reported to exert numerous beneficial roles for coral health (reviewed in Bourne et al. (2016)). For example, by participating in carbon, nitrogen, sulfur and phosphorus cycling, bacteria supply the host with essential nutrients and contribute to waste removal (Lesser et al., 2007; Raina et al., 2009; Zhang et al., 2015). Moreover, coral-associated bacteria may prevent the colonisation of the mucus by invasive pathogens by inhibiting their growth through the production of antimicrobial peptides and by occupying entry niches (Ritchie, 2006; Nissimov et al., 2009; Shnit-Orland and Kushmaro, 2009; Raina et al., 2016). Similar to other marine animals, the well-being of the coral holobiont is contingent on its microbial communities, as diseased and stressed states are usually associated with dysbiotic microbiomes (reviewed in Egan and Gardiner (2016)).

Given the fundamental roles of the microbiome in corals and other organisms (McFall-Ngai et al., 2013), it is important to understand how these relationships are established and maintained. Microbial symbionts can be acquired horizontally from the environment, transmitted vertically from parents, or acquired via a combination of both (Bright and Bulgheresi, 2010). While vertical transmission enables the maintenance of specific mutualistic partners and does not rely on a potentially unpredictable symbiont source, horizontal acquisition provides the advantage of selecting microbes that could confer a local advantage (Byler et al., 2013; Hartmann et al., 2017).

Corals can reproduce by broadcast spawning (the release of eggs and sperm in the water column, where fertilisation occurs) or by brooding planula larvae within their polyps and releasing mature larvae into the water column (Harrison, 2011). The transmission mode of Symbiodiniaceae is closely linked to the coral's reproductive strategy, with the majority of broadcast spawners acquiring Symbiodiniaceae from the environment, while most brooders transmit them from parent to offspring by seeding the planulae with symbiotic algae (Baird et al., 2009). A mixed transmission mode has been documented in brooders, as vertical acquisition of Symbiodiniaceae is accompanied by some degree of environmental uptake (Byler et al., 2013; Quigley et al., 2018). Knowledge about the transmission of bacterial symbionts is more limited, but several broadcast spawners have been shown to acquire bacteria horizontally (Apprill et al., 2009; Sharp et al., 2010). Additionally, metabarcoding of the 16S rRNA gene suggests that some bacteria may be vertically transmitted to the gametes via the mucus layer (Leite et al., 2017; Zhou et al., 2017; Bernasconi et al., 2019; Damjanovic et al., 2019b). One study used microbial community profiling and fluorescence *in situ* hybridisation (FISH) microscopy in the brooder *Porites astreoides* to show that at least some bacteria are vertically transmitted (Sharp et al., 2012), and 16S rRNA gene metabarcoding of field-collected parents and larvae of the brooder *Pocillopora damicornis* implied that larvae mostly acquire bacteria from the surrounding water column but that a small number of taxa may be inherited from the parent colony (Epstein et al., 2019).

In this study, we investigated the bacterial and Symbiodiniaceae transmission mode in the brooder, *Pocillopora acuta,* using three coral colonies collected from Orpheus Island (OI) and two colonies collected from Magnetic Island (MI), Australia. We analysed the microbial community composition in adult corals, newly released larvae and early recruits with metabarcoding of the 16S rRNA gene and the Internal Transcribed Spacer 2 (ITS2) region (Fig. 1). To localize bacteria in the coral larvae, we employed FISH and confocal laser-scanning microscopy (CLSM).

Results and discussion

Vertical transmission of Symbiodiniaceae in *P. acuta*

After quality control and filtering of rare sequences in QIIME2 (QIIME 2 Development Team, 2017b), 1,896,627 ITS2 reads were recovered from the 71 coral samples (Fig. S1). The ITS2 dataset was rarefied to the lowest number of reads across samples (16,014 reads) and a total of 15 Symbiodiniaceae sequence variants were detected (Fig. S2). It is unclear whether the Symbiodiniaceae genetic variation was due to intraspecific or interspecific diversity. Processing the same ITS2 dataset with the SymPortal workflow (Hume et al., 2019a) resolved three distinct Symbiodiniaceae genotypes (Fig. S3), all belonging to the genus *Cladocopium* (formerly known as clade C (LaJeunesse et al., 2018)). Two colonies from OI harboured type C1d−C1−C42.2 and the third colony from OI type C1d−C42.2−C1−C1bm−C1b, while the two adult coral colonies from MI harboured type C1d−C1bl−C42.2−C1 (Fig. S3). All larvae and recruits harboured the same Symbiodiniaceae type as their parent colony, which confirms vertical transmission of algal endosymbionts in *P. acuta* (Baird et al., 2009; Schmidt-Roach et al., 2014). *P. acuta* offspring may also acquire Symbiodiniaceae from the environment but we were unable to assess this, as the present experiment was conducted in captivity with 0.4 µm filtered seawater (FSW), which minimised the pool of microorganisms available in the environment. In the field, a mixed mode of transmission for Symbiodiniaceae has been described in the brooders *Seriatopora hystrix* (Quigley et al., 2018) and *P. damicornis* (Epstein et al., 2019), where larvae and one-week old recruits respectively hosted symbiont types that were absent from their parents.

P. acuta early life stages associate with diverse bacteria

The 16S rRNA gene dataset consisted of 2,152,907 reads obtained from the 84 samples (i.e. 71 coral, 8 water and 5 negative control samples), representing 3,116 amplicon sequence variants (ASVs). After quality filtering, the number of reads per sample ranged between 4,477 and 38,463, with an average of 25,630 reads per sample. ASVs representing reagent or laboratory contamination (Salter et al., 2014) were identified based on their relative abundances in the negative control samples (Lee et al., 2015) and removed from the dataset (see Supporting Information and Table S1). The five negative controls were also removed from the original dataset, which was filtered and rarefied anew to 4,225 reads (the lowest read count across all processed samples, Figs S4 and S5).

Bacteria in the genus *Endozoicomonas* (Endozoicomonadaceae) dominated the communities associated with adults from OI, but were present at lower abundances in the adult colonies from MI and in all larvae and recruits (Fig. 2A). Alteromonadaceae, Flammeovirgaceae and Rhodobacteraceae were among the most abundant bacterial families in all corals, as well as Flavobacteriaceae, which were more prevalent in larvae and recruits compared to adults (Fig. 2A). The most prevalent bacterial families detected in corals occurred at lower proportions in the surrounding water. Campylobacteraceae, Cellvibrionaceae, Clostridiaceae, Marinilabiaceae and Vibrionaceae comprised the most abundant bacterial families in the large planulation tank (Fig. 2A). Water samples collected from the recruit rearing containers were dominated by Halieaceae, Planctomycetaceae, Rhodospirillaceae and Rhodobacteraceae (Fig. 2A). Overall, coral early life stages had higher α-diversity indices than adult colonies (Table S2). All larvae and recruits were associated with a richer bacterial community than their respective parents, even though the difference was not statistically significant for the two MI colonies (Fig. 2B, Table S3). Higher α-diversity in juvenile corals compared to adults has previously been described in other coral species, including the broadcast spawners *Acropora tenuis* and *A. millepora* (Littman et al., 2009) and the brooder *P. damicornis* (Epstein et al., 2019). Given the dynamic nature that often characterises bacterial assemblages in coral early life stages (Littman et al., 2009; Lema et al., 2014; Zhou et al., 2017), it is hypothesised that a winnowing process takes place throughout ontogeny (Nyholm and McFall-Ngai, 2004), during which the microbiome is progressively fine-tuned until a more stable and more restricted community is established.

Patterns of horizontal and vertical transmission of bacteria in *P. acuta*

So far, few studies have undertaken a detailed comparison of bacterial assemblages between adult coral colonies and their progeny. In a recent field experiment, it was suggested that the brooder *P. damicornis* vertically transmits a small proportion of bacterial ASVs and that young recruits acquire the majority of their bacterial associates from the envrionment (Epstein et al., 2019). While the latter study did not analyse larval-associated bacterial communities, these patterns were confirmed for a congener in our experiment, in which we investigated the bacterial assemblages present in adults, larvae and recruits. Only a minority of ASVs were shared between adults and their respective offspring, which associated with numerous ASVs not present in the parents (Fig. 2C). Contrary to the vertical transmission of Symbiodiniaceae, this suggests that a large proportion of bacterial ASVs comprising the early life stage microbiome are acquired from the environment. Given that the bacterial community composition in the water column differed from those associated with coral samples (Fig. 2A), it is likely that *P. acuta* adult and early life stages formed non-random associations with bacteria from the surroundings. Such patterns have also been documented for various coral species in natural settings (Sunagawa et al., 2010; Tremblay et al., 2010; Sweet et al., 2011). It is possible that a fraction of ASVs found to be unique to offspring were also present as rare members in adults or were not detected due to limited sampling from each parent colony (i.e., one small branch tip) (Fig. S6). The coral microbiome varies across coral compartments (Sweet et al., 2011; Pollock et al., 2018) and across different locations within individual colonies (Rohwer et al., 2002; Daniels et al., 2011). Thus, restricting sampling to branch tips, done so to minimise stress, might have precluded the detection of bacteria situated in other regions of the coral. Additionally, bioinformatics processing of the sequencing data may have removed some ASVs during the filtering step of low-quality sequences, as well as during rarefaction.

The number of ASVs shared between each adult and its offspring represented a minority of the larval and recruit ASVs, but the relative abundance of these ASVs was larger in the early life stages. On average, larvae

from OI and MI shared 5% and 26% of ASVs with their parents, respectively, and these ASVs represented between 10% and 37% of the bacteria in OI larvae, and between 42% and 81% of the bacteria in MI larvae (Table S4). Since the ASVs shared between parents and offspring had a relatively high abundance within individual samples (Fig. 2C and Fig. S7), we hypothesise that these bacteria likely perform relevant functions during coral reproduction or in other stages of the coral life cycle.

Importance of specific bacterial ASVs in *P. acuta* early life stages

Among the total 3,094 ASVs detected across all coral samples, only 70 ASVs occurred in all life stages (i.e. parent, larvae and recruits) of at least one colony (Fig. 3). Since some of these shared ASVs were not detected in water samples, it is likely that they were vertically transferred from parent to the offspring during the brooding period (Fig. 3). Among the 70 shared ASVs, 16 were identified as members of Rhodobacteraceae, four as Alteromonadaceae and four as *Endozoicomonas* (Endozoicomonadaceae). Bacteria belonging to these families are common members of the bacterial communities associated with adult and early life stages of other coral species. For instance, members of Rhodobacteraceae and Alteromonadales (Alteromonadaceae) were abundantly present in larvae and juveniles of *A. millepora* (Lema et al., 2014). *Roseobacter* sp. (Rhodobacteraceae) were associated with planulae of the broadcast spawner *P. meandrina* (Apprill et al., 2009), and together with *Marinobacter* (Alteromonadaceae), consistently occurred in early life stages of the brooder *P. astreoides* (Sharp et al., 2012). In *P. damicornis*, a close relative of *P. acuta* (Schmidt-Roach et al., 2014), several Rhodobacteraceae, Alteromonadaceae and *Endozoicomonas* (Endozoicomonadaceae) ASVs were reported to likely be vertically transmitted from parent to offspring (Epstein et al., 2019).

Indicator value analysis (De Cáceres and Legendre, 2009) identified 28 bacterial ASVs as indicators for particular life stages within OI and MI coral colonies (Fig. S8). Several Rhodobacteraceae and Alteromonadaceae were significantly associated with larvae and recruits of OI or MI. Five indicator ASVs (i.e. two Rhodobacteraceae, one *Alteromonas* (Alteromonadaceae), one *Endozoicomonas*

(Endozoicomonadaceae), and one *Alcanivorax* (Alcanivoracaceae)) belonged to the aforementioned 70 ASVs shared between parents and their offspring (Fig. 3 and Fig. S8). The high relative abundance of these bacterial families in our samples, as well as in adult and early life stages of other coral species, suggest that they might be exerting important roles for the holobiont. These bacteria could be involved in nutrient cycling, as one *Alteromonas* strain has previously been shown to provide nitrogen to *P. damicornis* larvae (Ceh et al., 2013), while *Roseobacter* and some *Alteromonas* strains are known to participate in sulfur cycling (Raina et al., 2009). *Alteromonas* are sometimes able to produce antimicrobial peptides and could therefore help against pathogenic infections (Ritchie, 2006). Bacteria in the genus *Endozoicomonas* are generally accepted to be important coral symbionts due to their abundance and widespread associations with different coral species (Neave et al., 2016a; Neave et al., 2016b) and due to their putative beneficial roles, such as carbohydrate and protein provisioning to the host (Neave et al., 2017) or protection from heat (Pantos et al., 2015) and other stressors (Ding et al., 2016). Further investigations are required to decipher whether these bacteria play a role in ontogenetic development.

Bacterial aggregates in *P. acuta* larvae provide evidence for vertical transmission

Microscopy examinations of haematoxylin and eosin (H&E) stained larval sections showed that newly released *P. acuta* larvae were fully developed, as they possessed differentiated cells and dense Symbiodiniaceae communities occupied their endodermal region (Fig. 4A-B, Fig. S9). In addition to 16S rRNA gene metabarcoding of *P. damicornis* adult and recruit samples (Epstein et al., 2019), FISH on *P. acuta* larvae followed by CLSM observations was undertaken in order to examine whether some bacteria were vertically transmitted. The universal bacterial probe EUB338 was applied on 63 sections from nine larvae, with successive sections treated with the non-EUB probe and no probe as negative controls. Altogether, features resembling bacterial aggregates were observed in four of the nine larvae. Sixteen out of the corresponding 26 sections treated with EUB338 probes had aggregates (Table S5), suggesting that the latter did not span the whole depth of the samples. Bacterial clusters were primarily situated within tissues of the newly released larvae (Fig. 4C, E-F). Several aggregates were visible per section and they were slightly larger

than 10 µm in size (Fig. 4D, G-H). At high magnification we were able to distinguish cells resembling bacteria more clearly (Fig. 4D). When simultaneously applying the EUB338 and non-EUB probes labelled with different fluorophores, the aggregates were only visible with the EUB338 probe (Fig. 4E, Fig. S10C, G-H), providing strong support that these signals were from bacterial cells. Figs S10 and S11 display representative images of larval sections treated with the EUB338 and/or non-EUB probes, also confirming that when these probes were applied on successive sections, the aggregates visible with the EUB338 probe were not present in the negative controls. Bacterial aggregates imaged with CLSM did not correspond to any morphological feature of the host on the H&E stained sections (Fig. S10I-L, Fig. S11A, D-E).

Host tissue autofluorescence and nonspecific binding of the probes are major challenges when applying FISH to coral samples (Wada et al., 2016). To address the first issue, we recorded the emission spectra of the oligonucleotide probes when applied to bacterial cultures, as well as the natural autofluorescence of the host by treating sections using the FISH protocol without any probe. Spectral scanning with CLSM followed by linear unmixing allowed discrimination of the probe from the coral emission spectrum. To address the second issue and ensure that the observed aggregates represented bacteria rather than nonspecific binding of the probes to host tissue, we compared numerous pairs of successive sections treated with the EUB338 and non-EUB probes, as well as sections treated with both probes simultaneously. When applied to coral samples, FISH probes are known to stick nonspecifically to granular gland cells and nematocysts (Sharp et al., 2012; Lema et al., 2016; Wada et al., 2016). In addition to those patterns (Fig. S11C), we also observed a high level of nonspecific binding occurring on and around Symbiodiniaceae cells (Fig. 4E, Figs S10C and S11C). Even though other studies have reported similar signals as algal-associated bacteria (Ainsworth et al., 2015), the consistent binding of non-EUB probes that we recorded around Symbiodiniaceae suggests that this represents nonspecific binding in our *P. acuta* samples. Bacterial aggregates were only associated with EUB-338 probes, confirming they are bacteria. The reports of other authors describing similar features as bacterial aggregates in adult corals support our findings (Ainsworth et al., 2006; Ainsworth and Hoegh-Guldberg, 2009; Wada et al., 2016).

As *P. acuta* larvae were sampled within a few hours after planulation, bacteria from the environmental pool could have bound to the surface mucus layer, and therefore become part of the horizontally acquired microbiome. However, we believe the time frame between larval release and sampling (maximum 8 h) was too short for bacteria from the seawater enter the larvae and grow into aggregates within the larval tissue. We therefore conclude that these aggregated bacteria were seeded by the parental colonies into developing larvae during the brooding period. Oligonucleotide probes targeting specific bacterial taxa should be used in the future to identify at higher taxonomic levels which bacteria constitute the aggregates. This was done in one study that reported the clusters to be Gammaproteobacteria (Ainsworth et al., 2006). In the present work, selecting probes particularly for *Endozoicomonas* and *Alteromonas* (both Gammaproteobacteria) would be a first approach, but probes for Rhodobacteraceae (Alphaproteobacteria) and Flavobacteriaceae (Bacteroidetes) would also be relevant, as all these bacteria were highly abundant in larvae (Fig. 2A) and likely to be vertically transmitted (Fig. 3). In broadcast spawners, microscopic examinations of embryos and larvae across multiple coral species did not reveal evidence for vertical transmission of internalised bacteria (Apprill et al., 2009; Sharp et al., 2010; Apprill et al., 2012; Leite et al., 2017). So far, only one study on *P. astreoides* demonstrated that brooders are capable of vertically transmitting at least some bacteria to their offspring (Sharp et al., 2012). Contrasting with our observations of bacterial aggregates within larval tissues, Sharp et al. (2012) identified bacterial cells in the ectoderm and on the surface of newly released larvae. Our work corroborates the occurrence of vertical transmission of bacteria in a brooder and provides insights into the proportion and abundance of ASVs that are shared between parental corals and their offspring. Future histological analyses using H&E staining and bacterial stains such as Giemsa or Gram will help determine whether bacterial aggregates are intracellular, which could point to an obligate symbiosis. Work on adult corals suggests that ultrastructural microscopy may also be necessary to discriminate whether bacteria are intra- or extracellular (Work and Aeby, 2014).

A complex combination of factors shape the coral microbiome

For most symbioses, disentangling the drivers of microbiome composition is challenging, as numerous host and environmental factors play a role and the composition can change in time and space (Wullaert et al., 2018). In corals, host species and genotype (Rohwer et al., 2002; Glasl et al., 2019), as well as reef habitat and geographic location (Pantos et al., 2015; Hernandez-Agreda et al., 2016b) have been shown at multiple instances to modulate the composition of the microbiome (summarised in Hester et al. (2016)). Numerous abiotic factors such as salinity, pH, temperature or nutrient levels influence the coral microbiome (Hernandez-Agreda et al., 2016a), in addition to season (Li et al., 2014), age (Williams et al., 2015) and Symbiodiniaceae type (Littman et al., 2010). Some of these complex patterns were reflected in our study, with the bacterial associates of *P. acuta* corals being contingent on life stage, collection location of the parental colony, host factors and Symbiodiniaceae.

Among samples from each collection location, life stage had a strong influence on the bacterial associates, with adults, larvae, and recruits having significantly different bacterial communities (Fig. 5A, PERMANOVA with 999 permutations: OI: $F_{Life\ stage} = 17.74$, $p = 0.001$, MI: $F_{Life\ stage} = 12.31$, $p = 0.001$). Further, OI samples had significantly different bacterial communities from MI samples independently of life stage, as assessed by permutational analysis of variance (PERMANOVA, with 999 permutations: $F_{Location} = 6.11$, $p = 0.001$). One example is the higher relative abundance of Endozoicomonadaceae in OI compared to MI adult corals (Fig. 2A). Endozoicomonadaceae had a low relative abundance in the water column of the planulation tank (0.13%) and were undetected in the water sampled from recruit rearing containers. We hypothesise that parental colonies maternally transmitted *Endozoicomonas* sp. with which they had formed associations in their native habitat.

The five adult colonies were maintained in the same tank before and during the experiment and were thus exposed to the same microbial environment. Host specific factors such as genotype or environmentally induced phenotypic variation might therefore have driven differences in the microbial community structure. Although not confirmed by this study, it is likely that the five *P. acuta* colonies were genotypically distinct, especially when considering corals were collected from separate reefs. Host taxonomy has been

shown to influence the microbiome in the closely related coral *P. damicornis* (Brener-Raffalli et al., 2018). Alternatively, the local environments in which the *P. acuta* colonies grew on the reef may have induced phenotypic divergences, which may have led to variations in the microbiome. It is known that corals of the same genotype exposed to distinct conditions can exhibit phenotypic plasticity, such as differences in growth and survival (Drury et al., 2017) or contrasting patterns of thermal stress tolerance (Durante et al., 2019).

Considering each life stage separately, principal coordinates analysis (PCoA) visualisation of β-diversity illustrates that microbiomes of different coral genotypes clustered separately from each other (Fig. 5B, PERMANOVA with 999 permutations: Adults: F_{Colony} = 4.86, p = 0.001, Larvae: F_{Colony} = 5.32, p = 0.001, Recruits: F_{Colony} = 5.66, p = 0.001). Pairwise comparisons within each life stage yielded significant differences in bacterial communities between most groups (Table S6), further suggesting an effect of host genotype even among colonies from the same location. The segregation retained by larvae and recruits according to their parental origin could have resulted from a combination of the vertically transmitted bacteria and host genotypic effects (Fig. 5B). Symbiodiniaceae type explained some of the variance in bacterial community composition (Fig. 5B), which could suggest an association between endosymbiotic algae and bacterial assemblages. For example, colonies OI1 and OI3 possessed the same algal type and clustered closely to each other and further away from OI2, which had a different algal type (Fig. 5B). However, it is possible that host genotype influenced both algal and bacterial communities and that Symbiodiniaceae did not play a role in shaping the bacterial microbiome. The absence of a link between coral-associated bacteria and Symbiodiniaceae types during early ontogeny in *A. tenuis* and *A. millepora* is in agreement with this hypothesis (Littman et al., 2009).

Concluding remarks

In cnidarians and other animals, including humans, interactions with symbiotic microorganisms guide fundamental biological processes such as ontogenetic development, growth, immunity, physiological functions, behaviour and life span (Bosch, 2013; Rees et al., 2018). Host health and survival is thus contingent on the equilibrium maintained with its symbionts. In this study, we combined metabarcoding and microscopy approaches to show that the brooding coral *P. acuta* vertically transmits Symbiodiniaceae and some of its bacterial associates. A reliable transfer of selected symbionts across generations may be essential to maintain these potentially beneficial microbial partners. Coupled with the ability to acquire particular microbes from the environment, this strategy could facilitate transgenerational acclimatisation of corals to changing conditions. Future studies should investigate the identity of bacteria constituting the dense aggregates situated in newly released larvae, as well as the function and temporal sequence of all vertically transmitted and horizontally acquired bacterial associates. Tackling these questions will provide important insights into the potential of microbial communities to assist coral tolerance to stress and into the biological mechanisms that govern the complex entity defined as holobiont.

Experimental procedures

Field collection, planulation, settlement and rearing

Three colonies of the coral *P. acuta* were collected from OI (S -18°60 E 146°48), on February 17th 2017 and two colonies were collected from MI (S -19°15 E 146°86), on March 18th 2017, all between 3-5m depth. The coral colonies were transported to the Australian Institute of Marine Science (AIMS) and deployed in one 3000 l tank with 0.4 µm filtered flow-through FSW at 60 l min^{-1}. Three days after the new moon of March 28th 2017, the corals were isolated in individual vessels within the same 3000 l tank. The vessel rims were above the 3000 l tank water level and the vessels contained harvesters on their outlets to collect the larvae

released overnight. Planulation peaked throughout the night on April 2nd 2017 (day 1), and larvae from each colony were collected the following morning at 8:00 am for metabarcoding, microscopy and settlement (Fig. 1).

Five larval samples of five larvae each, and five small branch tips from each parent colony were collected for DNA extraction. Samples were rinsed with 0.22 µm FSW to remove loosely associated microorganisms, snap-frozen in liquid nitrogen and stored at -80°C. Several larvae from each colony were also fixed in 4% paraformaldehyde (PFA) for subsequent FISH microscopy and left at 4°C for ~ 10 h. These samples were then washed in PBS and stored at -20°C in 50% ethanol-PBS (Lema et al., 2016). Most larvae subsequently observed with FISH microscopy had already adopted a rounded shape, as the stress of sampling likely triggered their metamorphosis. Aliquots of approximately 200 living larvae from each parent colony were placed into individual 2 l plastic containers filled with 0.4 µm FSW and operated with flowing FSW (0.1 l min^{-1}) and gentle aeration. To induce larval settlement, each container had a PVC tray with aragonite plugs (20 mm diameter) pre-conditioned with crustose coralline algae and a biofilm, then sterilised by autoclaving. The larvae attached to the plugs and metamorphosed into recruits within a day. The recruits were reared under a 12 h light/dark illumination cycle reaching a maximum light intensity of 10 µmol m^{-2} s^{-1} after 2 h ramping. On April 6th (day 4), five samples of 4-day old recruits (with each replicate sample containing 5 individual recruits) were removed from the plugs with a sterile scalpel blade, washed with 0.22 µm FSW and snap-frozen for DNA extraction.

Five 500 ml replicate seawater samples were collected from the 3000 l tank on day 1 (Fig. 1). Following recruit sampling on day 4, 500 ml of seawater were collected from each of the five containers. Water samples were filtered through a 0.22-µm Sterivex filter with a peristaltic pump and the filters were snap frozen for subsequent DNA extraction.

DNA extraction and amplification

DNA was extracted from the samples following the protocol reported in (Damjanovic et al., 2019a). To provide enough bacterial biomass for downstream PCR amplification of the 16S rRNA gene, five to seven larvae and five recruits per replicate sample were pooled for DNA extraction. Approximately 60 mg of adult fragments were used for each replicate. Samples were placed in 1.5 ml sterile microcentrifuge tubes containing 250 µl of extraction buffer (100 mM Tris pH 9.0, 100 mM EDTA, 1% SDS, 100 mM NaCl). Ten µl of lysozyme at 10 mg ml^{-1} were added to all tubes and tubes were incubated at 37°C for 30 min. About 30 mg of sterile glass beads and 10 µl of Proteinase K at 20 mg ml^{-1} were added to the tubes. The samples were bead-beaten at 4 m s^{-1} for 20 sec and incubated at 55°C for 2 h, followed by 65°C for 15 min. After this step, 62.5 µl of KOAc at 5 M were pipetted into the tubes and incubated on ice for 30 min. After spinning the tubes at 25,000 *g* for 15 min at room temperature, the supernatant was transferred into new 1.5 ml sterile microcentrifuge tubes and 0.8 vol. isopropanol was added to precipitate DNA. The solutions were left at room temperature for 15 min and centrifuged again at 25,000 *g* for 15 min. After removing the supernatant, the precipitate was washed with 100 µl of 70% ethanol, centrifuged at 25,000 *g* for 3 min, air-dried and resuspended overnight in 25 µl Milli-Q water.

The same DNA suspensions were used to amplify the bacterial 16S rRNA gene and the Symbiodiniaceae ITS2 marker. Regions V5 to V6 of the 16S rRNA gene were amplified with the primer pair 784F [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGATTAGATACCCTGGTA-3'] and 1061R [5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCRRCACGAGCTGACGAC-3'] (Andersson et al., 2008). The ITS2 region was amplified with the forward primer ITS-DINO [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATTGCAGAACTCCGTG-3'] (Pochon et al., 2001) and reverse primer ITS2Rev2 [5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTCCGCTTACTTATATGCTT-3'] (Stat et al., 2009). The underlined segments represent Illumina adapter overhangs (Illumina, San Diego, CA, USA). The PCRs were conducted in 10 µl triplicates using the AmpliTaq Gold 360 Master Mix and 0.4 µM of each primer. The amplification cycles were: 95°C for 10 min; 30 cycles each at 95°C for 30 sec, 57°C for 1 min, 72°C for 30 sec; a final extension at 72°C for 7 min. PCR triplicates for each template were pooled and

sent to Ramaciotti Centre for Genomics (UNSW, Sydney) for library preparation and sequencing on the Illumina MiSeq system with 2 x 300bp paired-end reads.

In addition to processing the samples, four blank DNA extractions (i.e. DNA extraction conducted as explained above but without any sample, then amplified by PCR) and one no-template PCR (i.e. PCR reaction with Milli-Q instead of DNA extract) were processed and sequenced to check for laboratory contamination. No PCR products were visible by agarose gel electrophoresis in any of these negative controls.

Bioinformatics

The amplified 16S rRNA gene sequences were processed using the QIIME 2 pipeline version 2017.10 (Caporaso et al., 2010; QIIME 2 Development Team, 2017b). Plugin demux (QIIME 2 Development Team, 2017a) was used to visualise interactive quality plots and check read quality. Plugin DADA2 (Callahan et al., 2016) was subsequently applied to remove primers, truncate poor-quality bases based on the interactive plots, dereplicate, identify chimeras and to merge paired-end reads. Commands included in plugin feature-table (McDonald et al., 2012) enabled generation of summary statistics of sequences associated with the samples. A Naïve Bayes Classifier was trained with the feature-classifier plugin (QIIME 2 Development Team, 2017c) using the 16S rRNA gene database at 99% similarity of the SILVA 128 QIIME release (Quast et al., 2013) and based on the 784F/1061R primer pair. The taxa plugin (QIIME 2 Development Team, 2017d) allowed us to filter mitochondria and chloroplast sequences, as well as to visualise taxonomic bar plots and generate tables with absolute read counts of all taxa for each sample. The biom tables containing taxonomic counts and metadata were imported into R (R Core Team, 2018) for statistical analyses.

The ITS2 marker of Symbiodiniaceae is present in multiple copies and commonly characterised by intragenomic variation (Wilkinson et al., 2015). Hence, it is challenging to identify whether the genetic

diversity of a sample originates from intragenomic variation or from the presence of multiple Symbiodiniaceae genotypes (Sampayo et al., 2009). As the standard processing of ITS2 marker sequences with QIIME 2 did not allow the discrimination between intragenomic and intergenomic diversity (see Supporting Information), ITS2 data were analysed with the new SymPortal tool, which is able to resolve Symbiodiniaceae taxa (Hume et al., 2019b). Decomplexed paired-end sequences were submitted onto SymPortal.org for the workflow to be conducted remotely, as described in (Hume et al., 2019b, a) (using standard parameters).

Statistical analyses of bacterial communities

Exploratory and statistical data analyses were performed at the ASV level, a higher-resolution equivalent of the operational taxonomic unit (OTU), as ASVs are delineated by 100% sequence similarity (Callahan et al., 2017; Porter and Hajibabaei, 2018). ASVs for which the overall relative abundance was lower than 10^{-5} were filtered from the dataset as these may represent sequencing errors (Bokulich et al., 2013). Results in negative control samples were examined to identify potential reagent and laboratory contaminants (Salter et al., 2014), which were removed from the test dataset based on their relative abundances (Lee et al., 2015). To account for the variability in sequencing effort, data counts were rarefied to an even depth, corresponding to the lowest number of reads across samples (Hughes and Hellmann, 2005; Weiss et al., 2017).

The α-diversity was measured using richness (Lande, 1996; Legendre and Legendre, 1998), to which generalised linear models with negative binomial distributions were fitted to understand the differences in richness according to coral life stage and host genotype. Linear contrasts were then used to test differences in richness between pairs of samples. Differences in community composition (β-diversity, (Anderson et al., 2006)) were computed using Bray-Curtis dissimilarity matrices and tested via permutational multivariate

analysis of variance (PERMANOVA, (Anderson, 2001)). Variation in community composition among samples was visualised with principal coordinates analysis (PCoA) (Legendre and Legendre, 1998). Analysis of multivariate homogeneity of group dispersions was carried out (PERMDISP, (Anderson, 2006)) to check for homogeneity of variances, and pairwise comparisons were performed between groups using the Benjamin and Hochberg (Benjamini and Hochberg, 1995) correction for multiple testing. Indicator value analysis (De Cáceres and Legendre, 2009) was applied to detect ASVs that were significantly associated with different coral groups when both specificity and fidelity had probabilities greater than 0.8 (i.e. respectively, the probability of finding the ASV in samples belonging to the target group and the probability of a sample belonging to the group given that the ASV has been detected).

All statistical analyses were conducted using R version 3.3.2 (R Core Team, 2018) and packages phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2016), DESeq2 (Love et al., 2014), tidyverse (Wickham, 2017), ggplot2 (Wickham, 2009), RVAideMemoire (Herve, 2018), multcomp (Hothorn et al., 2008), indicspecies (De Cáceres and Legendre, 2009) and ggplot 2 (Wickham, 2009).

Fluorescence *in situ* Hybridisation (FISH) microscopy

Two larval samples from each coral colony were blocked in paraffin wax and sectioned (4 µm thickness) at the University of Melbourne Biomedical Sciences Histology Facility. FISH followed a protocol similar to the ones described in (Hugenholtz et al., 2002; Wada et al., 2016). Slides with tissue sections were incubated in 100% xylene (2x 3 min) to remove wax. Deparaffinised sections were then dehydrated in 100% ethanol (3x 5 min). After air-drying, a hydrophobic pen was used to create a barrier around the sections. The latter were subsequently washed in 0.2 M HCl for 12 min, followed by a wash in 20 mM Tris-HCl (pH 7.4) for 10 min. Once the sections dried, 18 µl of hybridisation buffer (30% v/v formamide, 0.9M NaCl, 0.01% sodium dodecyl sulphate (SDS), 20 mM Tris-HCl pH 7.4 – Table S7) and 2 µl of oligonucleotide probe were added over the tissue to yield a final probe concentration of 25 ng μl^{-1}. The universal bacterial probe EUB338 (5'-GCW GCC WCC CGT AGG WGT-3', (Amann et al., 1990)) and the negative control probe non-EUB (5'-ACA

TCC TAC GGG AGG C-3', (Wallner et al., 1993)), were labelled either with fluorophore Atto550 (excitation 553 nm, emission 576 nm) or Atto647N (excitation 645 nm, emission 663 nm) (biomers.net). When applied on the same sections, EUB338-Atto647N was used in conjunction with non-EUB-Atto550. The slides were incubated in an oven at 46°C for 2 h in the dark, and then placed for 10 min in a 50 ml pre-heated (48°C) washing buffer (0.1M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.4 – Table S7) at 48°C. Finally, slides were rinsed in ice-cold reverse osmosis (RO) water for 10 s, dried with pressurised air and mounted with ProLong™ Glass Antifade Mountant (ThermoFisher P36984) and glass coverslips. The slides were stored at 4°C in a light-proof box until visualisation, which was conducted within 24 h. Each sample type was also prepared without probe to assess tissue autofluorescence.

Slides were examined with a Zeiss710 laser-scanning confocal microscope (LSCM; Zeiss, Germany) and the Zeiss Zen 2009 software. A combination of the laser lines 405 nm, 561 nm and 633 nm was used to excite the samples. Linear unmixing of the recorded emission spectra was undertaken in order to distinguish autofluorescence of coral tissue and Symbiodiniaceae from the signal emitted by the probes (see Supporting Information for acquisition details).

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The authors have no conflict of interest to declare.

Availability of data and material

The sequence data generated and analysed in this study are available at NCBI under http://www.ncbi.nlm.nih.gov/bioproject/525296, BioProject accession PRJNA525296.

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Figure legends

Figure 1 Experimental design for coral rearing, planulation and sampling. A) Three *P. acuta* adult colonies collected from OI and two colonies collected from MI were maintained in a single 3000 l tank operated with flowing FSW. Before planulation, all colonies were placed into individual vessels within the 3000 l tank. Harvesters attached to the outlet of the five aquaria allowed collecting the larvae released overnight. In the morning of the first planulation day, larvae from each colony as well as five replicate adult branch tips per colony were sampled. The remaining larvae were settled on aragonite plugs pre-conditioned with a microbial biofilm and crustose coralline algae. The plugs were sterilised by autoclaving before settlement trials. Recruits from each colony were reared in five separate 2 l tanks with flow-through FSW and a small level of aeration. Using a sterile scalpel blade, five replicate recruit samples were taken per colony after four days. B) Photographs of adult polyps, larvae and recruits. Seawater was also collected from the adult and recruit rearing tanks to analyse its microbial community composition. OI: Orpheus Island; MI: Magnetic Island; Ad: Adult; Lar: Larvae; Rec: Recruit. Symbols for diagrams were modified from the Integration and Application Network (accessed 2018) and from (Jones et al., 2015).

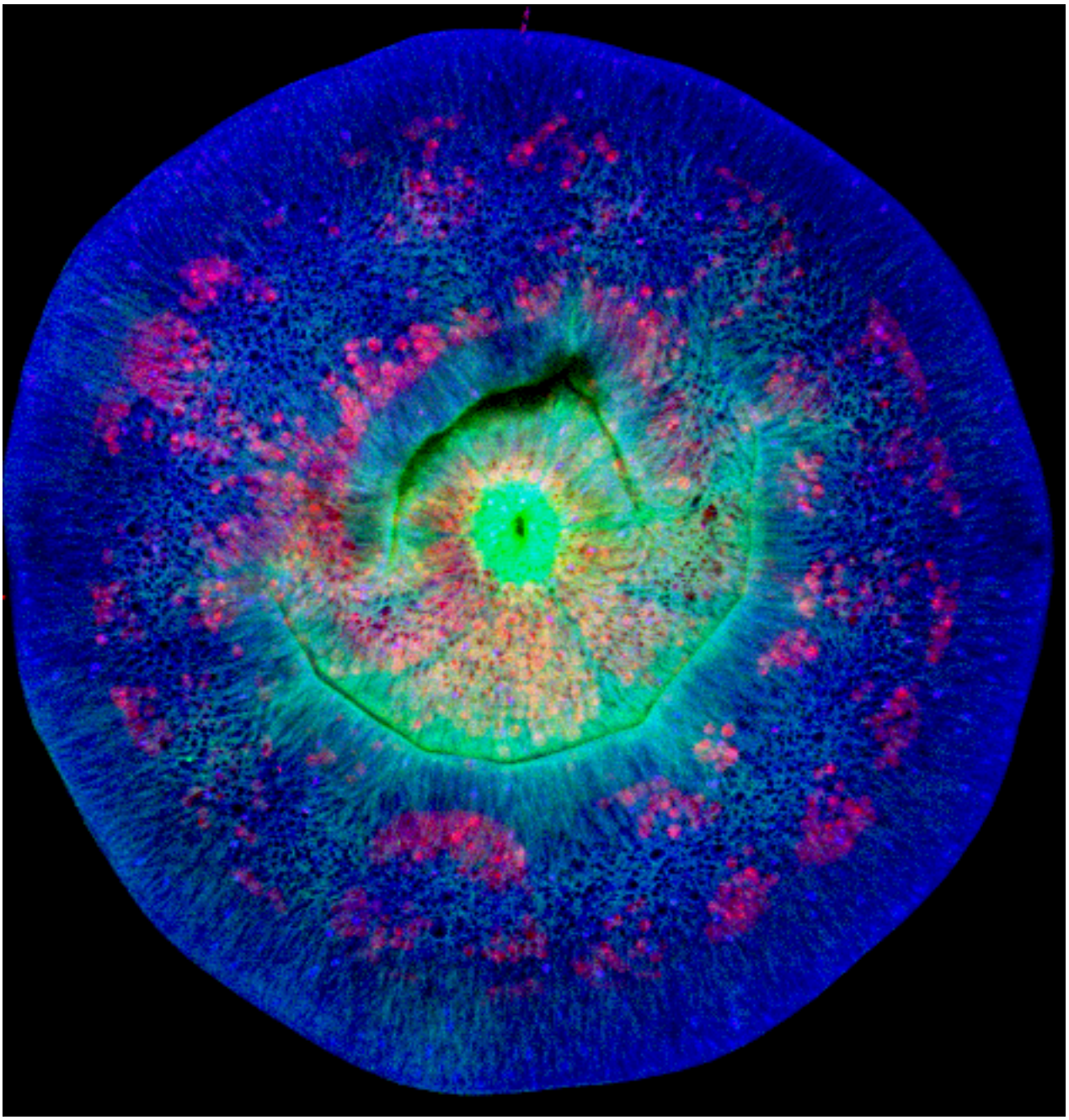
Figure 2 Overview of bacterial community composition and diversity in coral samples. A) Relative abundance of the 15 most prevalent bacterial families across all coral samples (left, colour) and across all water samples (right, grey scale). The top bacterial families in the water that also occurred in coral samples are shown in the same colour and highlighted in bold. The average of the five replicates is shown for each group. B) Boxplots representing richness as measured by the Chao index for all coral samples. Asterisks indicate that offspring have a significantly higher richness than their respective parent. Open circles indicate a significantly different richness in recruits compared to larvae. C) Number of shared and unique bacterial ASVs within each life stage across all replicates for each colony. Ad: Adults; Lar: Larvae; Rec: Recruits; OI: Orpheus Island; MI: Magnetic Island.

Figure 3 Potentially vertically transmitted ASVs between at least one parental colony and its offspring. This graph represents the relative abundance across all coral samples and within water samples of the 33 most abundant ASVs (out of 70) that were found to be shared between the adult, larvae and recruits of at least one coral colony. Some ASVs were not detected in water samples and were thus most likely transmitted vertically from parent to offspring. ASVs are identified at the family level, but all Endozoicomonadaceae sequences in this study belong to the genus *Endozoicomonas*. Families highlighted in bold occurred at higher abundance and their presence in other studies suggests that they could be exerting relevant functions for the holobiont. Ad: Adults; Lar: Larvae; Rec: Recruits; OI: Orpheus Island; MI: Magnetic Island.

Figure 4 Representative histological section of a newly released *P. acuta* larva (A-B) and detection of bacterial aggregates by confocal microscopy following probe hybridisation (C-H). The larvae started metamorphosing in the water column upon sampling, hence their rounded shape. Larval sections shown in A, E and F originated from the same individual, while the section shown in C came from a different larva. A) H&E stained section of a whole larva (and photograph in the inset). B) H&E stained close-up of the larval tissue layers separated by the mesoglea, where several Symbiodiniaceae cells (S) are visible in the endoderm and cnidocytes containing nematocysts (N) in the ectoderm. C and F) Confocal images of whole larval sections treated with the EUB338 probe labelled with Atto550 fluorophore (yellow-boxed zones – enlarged in D, G-H). D, G-H) Close-ups of the bacterial aggregates (yellow) identified in C and F. Images were obtained from a maximum intensity projection of z-stacks. E) Larval section simulteanously treated using the EUB338 probe labelled with Atto647N (red – arrowed Ag) and the non-EUB probe labelled with Atto550 (yellow) to discriminate signals due to unspecific binding. Bacterial aggregates (Ag) were only visible with the general bacterial probe. Autofluorescence of coral tissue is shown in cyan or blue.

Figure 5 PCoA using the Bray–Curtis dissimilarity index to represent the clustering of coral samples according to their bacterial community composition. A) Life stage explains most of the variance in bacterial communities at both sampling locations. B) Further structuring among bacterial microbiomes within each

life stage is driven by host factors. In A and B, the three Symbiodiniaceae genotypes that were resolved are represented by different symbols. OI: Orpheus Island; MI: Magnetic Island.



EMI_14856_P acuta max intensity projection z stack no probe.tif