The roles of age, parentage and environment on bacterial and algal endosymbiont communities in *Acropora* corals

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Running title

The microbiome of *Acropora* corals

Abstract

The bacterial and microalgal endosymbiont (Symbiodiniaceae spp.) communities associated with corals have important roles in their health and resilience, yet little is known about the factors driving their succession during early coral life stages. Using 16S rRNA gene and ITS2 metabarcoding, we compared these communities in four *Acropora* coral species and their hybrids obtained from two laboratory crosses (*Acropora tenuis* x *Acropora loripes*, *Acropora sarmentosa* x *Acropora florida*) across the parental, recruit (seven months old) and juvenile (two years old) life stages. We tested whether microbiomes differed between 1) life stages, 2) hybrids and purebreds, and 3) treatment conditions (ambient/elevated temperature and $p\text{CO}_2$). Microbial communities of early life stage corals were highly diverse, lacked host specificity and were primarily determined by treatment conditions. Over time, a winnowing process occurred, and distinct microbial communities developed between the two species pair crosses by two years of age, irrespective of hybrid or purebred status. These findings suggest that the microbial communities of corals have a period of flexibility prior to adulthood, which can be valuable to future research aimed at the manipulation of coral microbial communities.

Keywords

Bacteria, Symbiodiniaceae, microbial community, *Acropora* corals, holobiont

Introduction

Reef-building corals associate with diverse microbial communities including dinoflagellates (Symbiodiniaceae), prokaryotes (bacteria, archaea), fungi, and viruses (Blackall, Wilson, & van Oppen, 2015; Rosenberg, Koren, Reshef, Efrony, & Zilber-Rosenberg, 2007). Particularly important members of this consortium, which is called the coral holobiont, are the Symbiodiniaceae and bacteria. Symbiodiniaceae algae live inside the coral gastrodermal cells and provide their host with fixed organic carbon from photosynthesis; in return the algae gain protection and inorganic waste metabolites (Muscattine, 1967). Bacteria occupy the surface mucus layer, tissue, gastrovascular cavity and skeleton of corals (Bourne & Munn, 2005). While research on the functional roles of bacteria to the coral holobiont is still in its infancy, several putative functions have been suggested. These include the potential of nutrient scavenging (Knowlton & Rohwer, 2003; Zhang et al., 2015), carbon, nitrogen, sulfur and phosphorus cycling (Lesser et al., 2007; Raina, Dinsdale, Willis, & Bourne, 2010; Zhang et al., 2015), as well as defence against predation and pathogens through secondary metabolites and antimicrobial compounds (Castillo, Lodeiros, Núñez, & Campos, 2001; Nissimov, Rosenberg, & Munn, 2009).

Symbiodiniaceae and bacterial symbionts can be transmitted vertically (from parent to offspring) and/or horizontally (acquired from the environment) (Ceh, van Keulen, & Bourne, 2013; Quigley, Baker, Coffroth, Willis, & van Oppen, 2018). Multiple Symbiodiniaceae and bacterial taxa can co-exist within a coral host, with up to 10^2 - 10^3 bacterial strains and ~ 15 Symbiodiniaceae operational taxonomic units (OTUs based on 97 % ITS2 sequence similarity cut-off) found within a coral host (Blackall et al., 2015). Temporal changes and spatial differences in bacterial communities associated with adult coral colonies are thought to be driven by a variety of factors,

including temperature, pH/pCO₂, coral health status, light intensity, water depth and nutrient level (Morrow, Muller, & Lesser, 2018; van Oppen & Blackall, 2019). Conversely, bacterial communities of conspecific corals may remain stable despite seasonal environmental variations (Littman, Willis, Pfeffer, & Bourne, 2009b) or exposure to different pH conditions (Meron et al., 2012; Webster et al., 2016; Zhou et al., 2016). While the succession of coral-associated bacterial communities is poorly studied, community winnowing during coral development into adulthood is an emerging pattern (Epstein, Torda, Munday, & van Oppen, 2019; Lema, Bourne, & Willis, 2014; Littman et al., 2009b).

Symbiodiniaceae communities of corals may vary between reef sites that differ in light (Iglesias-Prieto, Beltrán, LaJeunesse, Reyes-Bonilla, & Thomé, 2004) and temperature (Oliver & Palumbi, 2011). However, stable communities over time and with changing temperature conditions have also been observed (McGinley et al., 2012; Pettay, Wham, Pinzón, & Lajeunesse, 2011). Symbiodiniaceae genera and strains can differ in their thermal tolerance and physiological optima, and this can affect the thermal tolerance of the coral holobiont (Boulotte et al., 2016; Stat & Gates, 2011). The loss of Symbiodiniaceae (i.e., coral bleaching) as a consequence of thermal stress often results in coral mortality (Douglas, 2003; Hoegh-Guldberg, 1999). A common finding is that corals dominated by *Durussdinium* spp. have higher thermal tolerance compared to those dominated by *Cladocopium* spp. (Berkelmans & van Oppen, 2006; Stat & Gates, 2011), although the opposite has also been documented (Abrego, Ulstrup, Willis, & van Oppen, 2008). Young coral recruits generally harbour a higher diversity of Symbiodiniaceae strains, but these communities can go through a winnowing process as the corals age (Abrego, van Oppen, & Willis, 2009; Gómez-Cabrera, Ortiz, Loh, Ward, & Hoegh-Guldberg, 2008; Little, van Oppen, & Willis, 2004).

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84 The aim of this study was to investigate the composition and succession of bacterial and
85 Symbiodiniaceae communities in hybrid and purebred *Acropora* corals. Hybrid *Acropora* corals
86 naturally occur in the Caribbean (Fogarty, 2012; Vollmer & Palumbi, 2002) and Indo-Pacific
87 (Willis, van Oppen, Miller, Vollmer, & Ayre, 2006), and they have been shown in some studies to
88 have comparatively high fitness in the laboratory (Chan, Peplow, Menéndez, Hoffmann, & van
89 Oppen, 2018; Isomura, Iwao, & Fukami, 2013; Willis et al., 2006) and in the wild (Fogarty, 2012).
90 However, information about their bacterial and Symbiodiniaceae communities is lacking. Access
91 to purebred and interspecific hybrid offspring from four *Acropora* species bred in the laboratory
92 and exposed to ambient and elevated conditions (temperature and $p\text{CO}_2$) for seven months,
93 followed by a 13 month grow-out period under ambient conditions, allowed us to examine the
94 influence of coral age, parentage and treatment conditions on the composition of the microbiome.
95 A second aim was to examine whether the coral-associated microbial communities contributed to
96 the differences in holobiont fitness previously reported in Chan et al. (2018).

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98 **Materials and methods**

99 ***Experimental design and sampling***

100 Adult parental corals were collected from Trunk Reef (18°35'S, 146°80'E), central Great Barrier
101 Reef in November 2015 and maintained in flow-through aquaria of the National Sea Simulator
102 (SeaSim) at the Australian Institute of Marine Science (AIMS). The adults were bred to form 1)
103 an *Acropora tenuis* x *Acropora loripes* cross, and 2) an *Acropora sarmentosa* x *Acropora florida*
104 cross, each resulting in two purebred and two hybrid offspring groups. Details of the crossing
105 protocols and the experimental design are in Chan et al. (2018). Coral hybridization was completed

within ten days of arrival of the parental colonies to the SeaSim. After successful fertilization was confirmed, a small branch tip of each adult parents was removed and stored in absolute ethanol (EtOH) for microbial community analyses (n = 5 for *A. tenuis*, n = 2 for *A. loripes*, n = 5 for *A. sarmentosa*, and n = 2 for *A. florida*). Eight offspring groups (four hybrid and four purebred groups) resulted from the two crosses: TT, TL, LT and LL for the *A. tenuis* x *A. loripes* cross, and SS, SF, FS and FF for the *A. sarmentosa* x *A. florida* cross. The abbreviation of the offspring groups is that the first letter represents the origin of the eggs and the second letter the origin of sperm (e.g., TL is a hybrid crossed with *A. tenuis* (T) eggs with *A. loripes* (L) sperm). During larval settlement, Symbiodiniaceae cells isolated from the parents were added at a density of 2×10^6 cells mL⁻¹ to encourage Symbiodiniaceae uptake (see Chan et al. (2018)). Recruits settled onto ceramic plugs were randomly distributed and reared under ambient conditions (27°C and 415 ppm *p*CO₂) or elevated conditions (ambient +1 °C and 685 ppm *p*CO₂) for seven months in filtered seawater (0.5 µm) (n = 12 tanks per treatment, and n = 20 plugs per offspring group per tank). The ambient conditions applied in this study are those of Davies Reef (18°35'S, 147°63'E), which is a reef proximal to Trunk Reef and from which long-term temperature records are available via an AIMS weather station. Light was provided at $\sim 120 \mu\text{E m}^{-2} \text{s}^{-1}$ using Aquillumination Hydra following the natural light/dark cycle. Fitness comparisons (e.g., survival, growth) between hybrids and purebreds are reported in Chan et al. (2018). At the end of the seven-month experiment (Jul 2016), three recruits from three randomly selected tanks of each treatment were sampled and stored in absolute ethanol (EtOH) (n = 3 per offspring group per tank per treatment, 144 samples in total).

To accommodate the higher light and feeding demand as the recruits grew, they were moved to two grow-out tanks at one year of age. Light was gradually increased to $250 \mu\text{E m}^{-2} \text{ s}^{-1}$ over two weeks, and the juveniles were maintained under ambient conditions with unfiltered seawater. To assess possible changes in microbial communities between the recruit and juvenile life stages, a branch tip was sampled from two-year-old juveniles in November 2017 and stored in absolute ethanol (EtOH). A total of 33 samples were collected (Tables S1, S2). Three individuals per offspring group per treatment (previous treatment) were sampled whenever possible, however, certain offspring groups/treatment did not have three samples due to mortality (see Tables S1, S2). Three 1 L seawater samples were also collected from each rearing tank and filtered on a $0.22 \mu\text{m}$ Sterivex filter using a peristaltic pump. A total of 198 samples was collected for each of the 16S bacterial and ITS2 Symbiodiniaceae datasets ($n = 14$ for adult parents, $n = 143$ for seven-month-old recruits, $n = 33$ for two-year-old juveniles, $n = 6$ for seawater, $n = 1$ for mock community (16S/ITS2) and $n = 1$ for negative controls (16S/ITS2) – see Tables S1, S2).

DNA extraction, PCR amplification and library preparation

Detailed protocols of DNA extraction, PCR amplification and library preparation can be found in the Supplemental Materials and Methods. Briefly, DNA was extracted from the entire recruit for the small seven-month-old samples, and from a ~ 20 mg fragment collected from the coral branch tip for the adult and two-year-old juvenile samples. The extraction was conducted using a salting-out method with bead beating and additional enzymatic digestion steps with Lysozyme and Proteinase K. A tissue-free extraction was also conducted as a contamination control. To assess the accuracy of sequencing and bioinformatic analyses of the bacterial 16S rRNA gene samples, a reference mock community was constructed using pure cultures of seven bacteria species

previously isolated from marine invertebrates (i.e., *Acinetobacter*: MH744724, *Bacterioplanes*: MH744725, *Marinobacter*: MK088251, *Paracoccus*: MH744726, *Pseudoalteromonas*: MK088250, *Pseudovibrio*: KX198136 and *Vibrio*: X56578). These bacteria species were selected as they were marine in origin and their identities have been confirmed previously by sequencing. The mock community was made using equal quantities of PCR-amplified cell lysate of the pure cultures (see Supplemental Materials and Methods on how the amplicons were quantified and pooled), and. The Primers 515fB [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGT GYCAGCMGCCGCGGTAA-3'] (Apprill, McNally, Parsons, & Weber, 2015) and 806rB [5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3'] (Parada, Needham, & Fuhrman, 2016) with Illumina adapter sequences (underlined) (Illumina, San Diego, California, USA) added at the 5-prime ends were used to amplify the hypervariable V4 region of the bacterial 16S rRNA gene.

A reference mock community was also established for Symbiodiniaceae ITS2 amplicon sequencing using equal quantities of PCR products from 10 Symbiodiniaceae cultures, representing eight sequence types. These included sequence type A (MK007295, MK007303), A2 (MK007324), A3 (MK007259, MK007296), C1 (MK007304), D1a (MH229352), F1(AF427462) and F5.1(MK007305), G3(MH229354) (see Supplemental Materials and Methods for details). Symbiodiniaceae ITS2 primers itsD [5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGAATTGCAGAACTCCGTG-3'] and Its2rev2 [5'-GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGCCTCCGCTTACTTATATGCTT-3'] (Pochon, Pawlowski, Zaninetti, & Rowan, 2001) with Illumina adapter sequences (underlined) added at the 5-prime ends to amplify the partial 5.8S, entire ITS2 and partial 28S rDNA genes.

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175 For both loci, triplicate 15 μ L PCR reactions were set up for each sample, the mock community,
176 tissue-free extraction control and no-template PCR control. PCR cycling conditions included an
177 initial denaturation at 95°C for 10 min followed by 28 cycles at 95°C for 30 s, 55°C for 30 s, and
178 72°C for 30 s; and a final extension at 72°C for 10 min. Triplicate PCR products for each sample
179 were pooled, purified and visualized on agarose gels. A single Indexing PCR reaction was carried
180 out on each purified sample amplicon pool, following the Illumina Metagenome Sequencing
181 library preparation guide. Indexing PCR products were bead purified and quantified. Sample
182 concentrations were normalized to 25 nM, pooled in equal volumes and sent to Ramaciotti Centre
183 for Genomics (UNSW, Sydney, Australia) for MiSeq v3 sequencing.

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185 *Sequence data processing*

186 Demultiplexed FASTQ files of all samples were processed in the same run and read quality was
187 visualized using FastQC (Andrews, 2018). Conservative quality trimming was conducted using
188 Trimmomatic and sequence reads were joined with PandaSeq (Masella, Bartram, Truszkowski,
189 Brown, & Neufeld, 2012). The bacterial 16S data were processed via Qiime2 (2019.4.0) with a
190 custom pipeline. Denoising, chimera removal and the construction of amplicon sequence variants
191 (ASVs) were conducted using Deblur (Amir et al., 2017). Taxonomic classification of the ASVs
192 was carried out using a classifier trained on the SILVA 132 database (Glöckner et al., 2017) and
193 with the primers used in this study. Two samples with less than two reads were removed by Deblur
194 during the denoising step (Tables S1, S2). Sequences that were classified as mitochondria and
195 chloroplasts were removed. All sequences that were classified as bacteria with an ASV were
196 retained in the dataset. The ITS2 Symbiodiniaceae data were processed via Qiime1 (v1.9.1).

Chimeras were identified and removed using Usearch61, and closed reference OTU picking was conducted at a 97% similarity cut-off based on a Symbiodiniaceae reference database (Arif et al., 2014). Symbiodiniaceae OTUs are hereafter called sequence types. Raw counts of the 16S bacterial data and ITS2 Symbiodiniaceae data were outputted to R.

Statistical analyses

Library sizes of the samples were rarefied at a rarefaction level of 886 reads and 1719 reads for the bacterial and Symbiodiniaceae data, respectively. Of the 198 samples, eleven were removed from the bacterial data and seven from the Symbiodiniaceae data due to low read counts (Tables S1 and S2). The samples that were removed also had difficulties in the DNA extraction and/or PCR amplification step. The 16S bacterial negative control only contained two reads from the same ASVs in the family Mycoplasmataceae, and the sequence was not considered further (i.e., the sequence was not removed from the samples). The relationship between read depths and alpha-diversity was visually checked and this confirmed that alpha-diversity plateaued at around the selected rarefaction level (Figures S1, S2). Bacterial ASVs and Symbiodiniaceae sequence types with relative abundance of $< 0.005\%$ were removed following the recommendation in Bokulich et al. (2013). The subsequent statistical analyses, multivariate ordination analyses and visualization figures (i.e., alpha-diversity plot, non-metric multidimensional scaling (nMDS) plots, and Permutational Multivariate Analyses of Variance (PERMANOVA)) were conducted using the rarefied, filtered data at the ASV level for the bacterial data, and at sequence type level for the Symbiodiniaceae data (see below for exceptions).

The full dataset (including adult parents, seven-month-old recruits and two-year-old juveniles) was used to compare the bacterial and Symbiodiniaceae communities between life stages. Cross-sectional analyses were then carried out individually for offspring of the *A. tenuis* x *A. loripes* and *A. sarmentosa* x *A. florida* crosses that were seven months old and two years old to investigate the effects of hybridization (hybrid vs. purebred) and treatment (ambient vs. elevated) on the microbiome within each subset. Bray-Curtis distance (Legendre & Legendre, 1998) was used to compare bacterial and Symbiodiniaceae communities between samples for the full dataset and each of the subsets. Ordination analyses using nMDS (Legendre & Legendre, 1998) based on the same distance were then conducted. Multivariate homogeneity of variances was tested using *betadisper*, a multivariate analogue of Levene's test (Anderson, Ellingsen, & McArdle, 2006). Differences in microbial communities between 1) life stages, 2) species pair crosses, 3) hybrid vs. purebred corals and 4) ambient vs. elevated conditions were tested with PERMANOVA (Oksanen et al., 2016), with 999 permutations and each factor evaluated separately. Pairwise comparisons were carried out with p-values corrected using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). The possibility of tank effects was tested by comparing the microbial communities of each offspring group in the three tanks within a treatment. These analyses confirmed the absence of a tank effect, hence it was not considered further in the analyses. The nMDS plots and alpha-diversity plots among samples from the six experiment tanks are shown in Figures S3-S5.

Differential analyses (Love, Huber, & Anders, 2014) based on generalized linear models assuming negative binomial distributions for the raw abundance counts were used to identify bacterial taxa that were significantly different in relative abundance between the three life stages and between

treatments. For visualization, compositional plots and the results of the differential analyses are shown at the family level to highlight certain bacterial families of interest. Microbial communities of the seawater and mock community samples were visualized as compositional plots for comparisons with coral samples but were excluded from the nMDS ordination plots and statistical analyses. All the above analyses were conducted using R (3.5.0) (R Core Team, 2018) and the packages *ggplot* (Wickham, 2016), *phyloseq* (McMurdie & Holmes, 2013), *DESeq2* (Love et al., 2014) and *vegan* (Oksanen et al., 2016).

Results

Coral life stages differed in their microbial communities

Coral life stages differed significantly in their bacterial communities (PERMANOVA $p < 0.001$, Tables 1) (Figures 1, S6); pairwise comparisons indicated that the adult parents, the seven-month-old recruits, and the two-year-old juveniles within the same cross were significantly different from each other ($p < 0.001$ for all pairs). See Table S3 for results of homogeneity of variances. Further, the parents and two-year-old juveniles of the *A. tenuis* x *A. loripes* cross differed in their bacterial communities from those of the *A. sarmentosa* x *A. florida* cross (PERMANOVA $p < 0.001$ for both pairs, Table 1) (Figure S7, S8). Pairwise comparisons between individual offspring groups were not conducted for the above analyses due to low sample size ($n = 2$) for some offspring and parental groups. The bacterial 16S mock community sample was classified accurately at the genus level and had approximately equal proportions of the seven expected taxa (Figure 1, Table S4). Alpha-diversity of seven-month-old recruits was higher than that of two-year-old juveniles and adult parents (Figure 2), with no taxa occurring at a relative abundance of more than 12% (Figure 1). When combining all offspring groups of the same life stage from both crosses, a total of 1448

bacterial ASVs were found in the seven-month-old recruits, whereas a total of 744 and 277 ASVs were found in two-year-old juveniles and adult parents respectively (see Table S5 for the number of ASVs of each specific offspring group). Adult parents shared a total of 213 and 172 bacterial ASVs with seven-month-old recruits and two-year-old juveniles respectively, whereas seven-month-old recruits and two-year-old juveniles shared a total of 506 bacterial ASVs (based on all offspring groups at that life stage, Figure S9). A total of 193 and 184 bacterial ASVs found in seawater samples was also present in seven-month-old recruits and two-year-old juveniles (based on all offspring groups at that life stage (Figure S9).

Figure 1. Compositional plots showing the averaged relative abundances of the bacterial taxa at the family level of parental and offspring groups at different life stages (Parent, 7M: seven months old, 2Y: two years old). Bacterial communities of seawater samples (SW) and a 16S mock communities (16S_mock) are also shown. A similar colour between families in the legend indicates that they belong to the same order. For illustration purpose, only taxa with relative abundance of > 0.2 % are display.

Table 1. Summary of results (p-values) from PERMANOVA based on Bray-Curtis dissimilarity matrices to test the differences in bacterial or Symbiodiniaceae communities between 1) life stages, 2) the *A. tenuis* x *A. loripes* (AT x AL) and the *A. sarmentosa* x *A. florida* (AS x AF) crosses, 3) hybrid vs. purebred corals, and 4) ambient vs. elevated temperature and *p*CO₂ conditions. The analyses were conducted at ASV level and each factor was tested independently. Homogeneity of variances and R² of each comparison are shown in Table S3.

Comparison	Life stage	Cross	No. of samples [†]	Permanova-Bacteria	No. of samples [†]	Permanova-Symbiodiniaceae
Parent vs. 7 months vs. 2 years	All	AT x AL	n = 7, 67, 21	≤ 0.001 ***	n = 7, 65, 21	≤ 0.001 ***
Parent vs. 7 months vs. 2 years	All	AS x AF	n = 7, 65, 12	≤ 0.001 ***	n = 7, 71, 12	≤ 0.001 ***
AT x AL vs. AS x AF cross	Parent	N/A [‡]	n = 7, 7	≤ 0.001 ***	n = 7, 7	0.014 *
AT x AL vs. AS x AF cross	7 months (ambient)	N/A [‡]	n = 32, 33	0.098	n = 31, 36	0.571
AT x AL vs. AS x AF cross	7 months (elevated)	N/A [‡]	n = 35, 32	0.005 **	n = 34, 35	0.739
AT x AL vs. AS x AF cross	2 years	N/A [‡]	n = 21, 12	≤ 0.001 ***	n = 21, 12	≤ 0.001 ***
Hybrid vs. Purebred	7 months	AT x AL	n = 35, 32	0.307	n = 30, 35	0.548
Hybrid vs. Purebred	7 months	AS x AF	n = 36, 29	0.358	n = 36, 35	0.068
Hybrid vs. Purebred	2 years	AT x AL	n = 12, 9	0.814	n = 12, 9	0.494
Hybrid vs. Purebred	2 years	AS x AF	n = 7, 5	0.957	n = 7, 5	0.496
Ambient vs. Elevated	7 months	AT x AL	n = 32, 35	≤ 0.001 ***	n = 31, 34	≤ 0.001 ***
Ambient vs. Elevated	7 months	AS x AF	n = 33, 32	≤ 0.001 ***	n = 36, 35	≤ 0.001 ***
Once Ambient vs. Once Elevated [§]	2 years	AT x AL	n = 15, 6	0.321	n = 15, 6	0.209
Once Ambient vs. Once Elevated [§]	2 years	AS x AF	n = 6, 3	0.333	n = 7, 5	0.103

[†] The no. of samples refers to the final number of samples used in the analyses. The order presented here follows the order in the “Comparison” column. See Tables S1 and S2 for full details.

* Indicates statistical significance. * *p* < 0.05, ** *p* < 0.01, *** *p* ≤ 0.001.

[‡] This part compares between crosses, therefore subdivision of cross is not applicable (N/A) here.

[§] The two-year-old juveniles were all under ambient condition. ‘Once ambient’ or ‘once elevated’ indicates whether they were previously under ambient or elevated temperature and *p*CO₂ conditions during the first seven months of their life.

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Figure 2. Alpha-diversity (based on Shannon index) of the bacterial communities for offspring groups at different life stages (Parent (red), 7M: seven-month-old recruits (green), 2Y: two-year-old juveniles (blue)). The number of samples (n) of each offspring group is displayed in brackets.

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In the later life stages, stronger dominance by one or a few bacterial taxa was observed (Figures 1, 3). For both crosses, adult parents were dominated by the family Endozoicomonadaceae (Bartz et al., 2018) (25-73%) (Figure 3). Endozoicomonadaceae also occurred in moderate abundance in two-year-old juveniles (2-16%), but not in seven-month-old recruits (< 0.05%) (Figures 3). The family Oceanospirillaceae occurred only in parents and two-year-old juveniles (Figure 3). At two years of age, all offspring groups of the *A. tenuis* x *A. loripes* cross were dominated by Simkaniaceae (~50%) (Figure 3). However, dominance by a single taxon was not observed in the two-year-old juveniles of the *A. sarmentosa* x *A. florida* cross (Figure 3). Simkaniaceae also had limited abundance in parents and seven-month-old recruits (Figure 3). Conversely, the families Cyclobacteriaceae, Flavobacteriaceae, Pirellulaceae and Rhodobacteraceae were relatively abundant in all seven-month-old recruits (4-8 %) but not in parents or two-year-old juveniles (< 1% in most cases) (Figure 3). Since the top 10 bacterial families that were significantly different

between seven months vs. two years and seven months vs. parents were similar, only the top 10 taxa of seven months vs. two years were displayed using bubble plots (Figure 3).

Figure 3. The top 10 taxa at family level that were significantly different in relative abundance between the three life stages for the *A. tenuis* x *A. loripes* and *A. sarmentosa* x *A. florida* cross (7M: seven months old, 2Y: two years old).

As for the Symbiodiniaceae communities, significant differences between life stages were observed (PERMANOVA $p < 0.001$ for both crosses, Table 1) (Figures 4, S10). Pairwise comparisons showed that the parents, the seven-month-old recruits and the two-year-old juveniles within a cross were significantly different from each other ($p < 0.001$ for all pairs; see Table S3 for results of homogeneity of variance). The only exception was that there was no difference between the parents and the two-year-old juveniles of the *A. sarmentosa* x *A. florida* cross ($p = 0.247$). Between crosses, Symbiodiniaceae communities did not differ at the early life stage of seven months (Table 1, Figure 4). At the later life stages, however, Symbiodiniaceae communities of the *A. tenuis* x *A. loripes* cross were significantly different from those of the *A. sarmentosa* x *A. florida* cross (PERMANOVA parents: $p = 0.014$, two years old $p < 0.0001$, Table 1). The ITS2 mock community was classified accurately at the sequence type level (Table S6). The relative

abundance of the majority of the sequence types of the mock community was close to expectation. The only exception was that sequence type A had a lower relative abundance than expected, which may be a consequence of the presence of intragenomic variants (Table S6).

Figure 4. Compositional plots showing the averaged relative abundances of different Symbiodiniaceae sequence types for parent and offspring groups at different life stages. Symbiodiniaceae communities of seawater samples (SW) and an ITS mock communities (ITS_mock) are also shown.

Adult parents had fewer Symbiodiniaceae sequence types (18) compared to seven-month-old recruits and two-year-old juveniles (23 for both). A change in dominant Symbiodiniaceae sequence types was also observed with age (Figure 4). At seven months of age, all recruits were dominated by sequence type Cspc (72-84%), with C33 (type 1) being the second most abundant type (10-23%). Two-year-old juveniles of the *A. tenuis* x *A. loripes* cross were dominated by sequence types A1 (26-44%) and Cspc (53-66%), with low abundance of D1 (0.03-5%) (Figure 4). Juveniles of the *A. sarmentosa* x *A. florida* cross, in contrast, were dominated by sequence types C3k (9-40%)

and Cspc (29-94%), with low to moderate abundance of D1 (5-17%) (Figure 4). Sequence types C33 (type 1), A1 and D1 were absent in all but one parent (and only occurred at < 0.02% in that parent). Adult parents were dominated by sequence type C3k (15-83%) and Cspc (12-79%) (Figure 4).

Microbial communities were similar between hybrid and purebred *Acropora* corals

At both seven months and two years of age, bacterial and Symbiodiniaceae communities did not differ significantly between purebred and hybrid corals within both the *A. tenuis* x *A. loripes* and *A. sarmentosa* x *A. florida* crosses (Table 1) (seven months: Figures 5, 6; two years: Figures S11, S12).

Figure 5. nMDS plots of the bacterial communities in hybrid vs. purebred recruits at seven months of age for the *A. tenuis* x *A. loripes* and *A. sarmentosa* x *A. florida* cross based on an analysis of Bray-Curtis dissimilarity matrices.

Figure 6. nMDS plots of the Symbiodiniaceae communities in hybrid vs. purebred recruits at seven months of age for the *A. tenuis* x *A. loripes* cross and *A. sarmentosa* x *A. florida* cross based on an analysis of Bray-Curtis dissimilarity matrices.

Elevated temperature and $p\text{CO}_2$ conditions affected microbial communities

There was a significant difference in bacterial and Symbiodiniaceae communities between seven-month-old recruits reared under ambient versus elevated temperature and $p\text{CO}_2$ conditions for both the *A. tenuis* x *A. loripes* and *A. sarmentosa* x *A. florida* crosses ($p < 0.001$ for each comparison, Table 1) (Figures 7, S13). For example, Blattabacteriaceae occurred at 1.2-4.5% under ambient conditions, but only occurred at 0.3-1.9% under elevated conditions (Figure S14). For Symbiodiniaceae communities, sequence type C33 (type 1) occurred in moderate to high abundance in all offspring groups under ambient conditions (15- 42%), but only occurred in 2-5% under elevated conditions (Figure S15). Sequence types in the genus *Durisdinium* (D1, D1a, D2) ranged from 0-1.5% under ambient conditions and from 0-12% under elevated conditions (Figure S15). Sequence type Cspc remained the most abundant type under both ambient (54- 78%) and elevated (75- 92%) conditions. At two years of age (after 17 months at ambient conditions), bacterial and Symbiodiniaceae communities of the juveniles that were under ambient and elevated

conditions during the first seven months of their life were indistinguishable from one another (Table 1, Figures S16, S17).

Figure 7. nMDS plots based on an analysis of Bray-Curtis dissimilarity matrices of the Symbiodiniaceae communities of seven months old recruits reared under ambient vs. elevated temperature and $p\text{CO}_2$ conditions.

Discussion

Winnowing of microbial communities

DNA metabarcoding data obtained here showed that coral-associated microbial communities go through a winnowing process as the coral recruits develop into adults. At seven months of age, the bacterial communities of recruits had higher alpha-diversity than both the two-year-old juveniles and the adult parents. Winnowing of bacterial communities is known from other cnidarian species. *Hydra*, for instance, has highly diverse and variable bacterial communities in early life stages but diversity is drastically decreased in the adult stage (Franzenburg et al., 2013). Several previous studies on corals are also in agreement with our findings (Epstein et al., 2019; Lema et al., 2014; Littman, Willis, & Bourne, 2009a). Lema et al. (2014) found distinct bacterial communities in the larval stage, early recruit stage (one and two weeks old) and recruit/juvenile stages (three, six, 12

months old) of *A. millepora*. The highest number of OTUs (590 OTUs) was observed in the three months old recruits but this declined to 423 OTUs by 12 months of age (Lema et al., 2014). Similarly, Littman et al. (2009a) observed two to almost three times more bacterial OTUs in nine months old recruits of *A. millepora* and *A. tenuis* compared to adult conspecifics. In contrast, higher bacterial diversity was observed in larger (and presumably older) than in smaller (and presumably younger) colonies of the massive coral, *Coelastrea aspera*, (Williams, Brown, Putschim, & Sweet, 2015). However, older *C. aspera* colonies in that study were exposed to the air during low tide while younger colonies were not exposed, and this could have an effect on their microbiome.

In addition to the change in bacterial diversity, the dominant bacterial taxa associated with corals also shifted with age. The moderate to high abundance of members in the families Endozoicomonadaceae and Simkaniaceae in the two-year-old juveniles and adult parents suggests that these families may play an important role beyond the earliest life stages. While the exact function of these bacterial taxa in the coral holobiont is unknown, certain strains of *Endozoicomonas* may have the potential for dimethylsulfoniopropionate (DMSP) degradation (Raina, Tapiolas, Willis, & Bourne, 2009; Shiu & Tang, 2019). A high abundance of *Endozoicomonas* spp. has been found in adult corals across geographical regions (Epstein et al., 2019; Morrow, Moss, Chadwick, & Liles, 2012; Pollock et al., 2018). For example, this taxon accounted for > 70 % of the bacterial communities of *Pocillopora damicornis* from Australia (van Oppen et al., 2018) and the Red Sea (Bayer et al., 2013). In contrast, the families that were relatively abundant in the seven-month-old recruits (Cyclobacteriaceae, Flavobacteriaceae, Pirellulaceae and Rhodobacteraceae) were rare in older life stages. Similar observations have been

made in *C. aspera*, where Rhodobacteraceae occurred at higher abundance in younger corals, but occurred to a lesser extent or was undetected in older conspecifics from the same reef (Williams et al., 2015). Unfortunately, little is known about the possible roles of these bacteria in holobiont functioning. The change in bacterial communities with age may be due to changing needs at different life stages and/or availability of new niches as the coral grows. The coral host may actively select for bacteria suitable for its state of development and newly available niches.

It is well established that Symbiodiniaceae taxa may change in relative abundance during ontogeny of corals (Abrego et al., 2009; Gómez-Cabrera et al., 2008; Little et al., 2004). For instance, recruits of *A. tenuis* gradually shifted from codominance by *Cladocopium* and *Durusdinium* spp. to dominance by *Durusdinium* spp. from four to 37 weeks of age (Little et al., 2004). In contrast, conspecific adults from the same reef were dominated by *Cladocopium* spp. (Little et al., 2004). Similarly, adults of *Acropora longicyathus* showed dominance by *Cladocopium* spp., while 10-day and three-month-old conspecific recruits were dominated by *Symbiodinium* spp., or a mix of *Symbiodinium*, *Cladocopium* and *Durusdinium* spp. (Gómez-Cabrera et al., 2008). Possible explanations for the change in dominant Symbiodiniaceae taxa with age include the change in growth form (Abrego et al., 2009) and coral pigmentation (Gómez-Cabrera et al., 2008). In the present study, vertical growth was absent in the seven-month-old recruits while the two-year-old juveniles had simple vertical growth and adult parents showed complex 3D structures. Vertical growth may alter light and other environmental conditions inside the host tissues, favoring certain types of Symbiodiniaceae (Abrego et al., 2009). In addition, the increase in tissue thickness and pigmentation with age may modify the irradiance level that the algae experience (Salih, Larkum, Cox, Köhl, & Hoegh-Guldberg, 2000). It is also possible that the coral host actively selects

Symbiodiniaceae taxa to maximize their effectiveness to suit ontogenetic changes in physiological needs (Little et al., 2004), resulting in the succession pattern observed in this study.

One potential limitation of this study on the interpretation of microbial community winnowing with age is that there were temporal changes in the environment. The adult parental colonies were collected from the field and sampled for microbiome analyses after ~10 days in aquaria containing a diversity of reef organisms, whereas the seven-month-old recruits were maintained in filtered seawater (0.5 μ m) and the two-year-old juveniles were reared in unfiltered seawater from the age of one year onwards. The change from filtered to unfiltered seawater was necessary due to the increasing feeding demand as the coral grew. Since coral microbial communities can be sensitive to their environments, the different conditions experienced by corals at the three different life stages may confound the effect of age. Adult colonies from the field are expected to be exposed to a diverse range of microbes in the field, whereas the seven-month-old recruits are likely to have the least diverse microbial community as they were reared in filtered seawater. For example, ~824 bacterial ASVs were found in seawater samples collected from field (Heron Island, Australia) (Epstein et al., 2019), while only ~220 to 440 ASVs were found in filtered seawater samples from the SeaSim (K. Damjanovic, personal communication, Jun 24, 2019). This is opposite to the diversity pattern observed in this study, suggesting that factors other than the environment (e.g., life stage) were likely drivers of the observed pattern.

Distinct microbiome succession patterns between species pair crosses

The succession patterns of bacterial and Symbiodiniaceae communities differed between the two species pair crosses. While offspring groups of the *A. tenuis* x *A. loripes* cross showed strong

dominance of one bacterial taxon by two years of age, offspring groups of the *A. sarmentosa* x *A. florida* cross at the same age did not. Similarly, the rate of change to adult-like Symbiodiniaceae communities also differed between the two species pair crosses. The *A. sarmentosa* x *A. florida* cross established parent-like Symbiodiniaceae communities by two years of age. All offspring groups of this cross were dominated by sequence types of the genera *Cladocopium* and *Diusdurnum* while all offspring groups of the *A. tenuis* x *A. loripes* cross were dominated by *Cladocopium* and *Symbiodinium*. Since all juveniles were under the same treatment conditions and randomly distributed in the same tanks, the observed differences in microbial communities between the crosses indicate coral host specificity. The dominance of *Symbiodinium* spp., as observed in all offspring groups of the *A. tenuis* x *A. loripes* cross at two years of age, is unusual in adult populations (Abrego et al., 2009; Gómez-Cabrera et al., 2008; Little et al., 2004). *Symbiodinium* is the only Symbiodiniaceae genus known to synthesize mycosporine-like amino acids, a compound that may act as a shield against ultraviolet radiation (Banaszak, LaJeunesse, & Trench, 2000). Members of *Symbiodinium* are more commonly found in shallow water corals (Baker, 2003; LaJeunesse, 2001) and are tolerant to high irradiance (Rowan, 1998). The dominance of *Symbiodinium* in these offspring groups may be a response to the increased light levels (from 120 to 250 $\mu\text{E m}^{-2} \text{s}^{-1}$) from the age of one year.

The bacterial communities harboured by adult corals have been showed to be species-specific in several studies (Bourne & Munn, 2005; Frias-Lopez, Zerkle, Bonheyo, & Fouke, 2002; Rohwer, Breitbart, Jara, Azam, & Knowlton, 2001), although it is unknown at what age this specificity develops. For Symbiodiniaceae communities, it is known that the onset of a species-specific pattern can vary between *Acropora* species (Abrego et al., 2009); *A. tenuis* harboured similar

Symbiodiniaceae communities to that of the conspecific adults by ~3.5 year of age, but *A. millepora* from the same locations did not (Abrego et al., 2009). The present study showed that the onset of a species-specific pattern in bacterial and Symbiodiniaceae communities occurred in multiple *Acropora* species by two years of age. Interestingly, the difference in the extent and taxa of specialization in bacterial and Symbiodiniaceae communities occurred only between the two crosses. Within a cross, the hybrid and purebred offspring groups had similar microbial communities, suggesting that hybrid and purebred corals underwent a similar succession and winnowing process as they grew.

Parental species within a cross shared similar bacterial communities

Bacterial communities of the adult parents *A. tenuis* and *A. loripes* differed from those of *A. sarmentosa* and *A. florida*. Species-specific bacterial communities are commonly observed in adult corals (van Oppen & Blackall, 2019). However, Littman et al. (2009b) found closely related *Acropora* species from the same reef shared similar bacterial communities, yet these communities were distinct from conspecifics from a different reef. Given that *A. sarmentosa* and *A. florida* are closely related species (Fukami, Omori, & Hatta, 2000; Márquez, van Oppen, Willis, Reyes, & Miller, 2002) and they were collected from the same reef, it is not surprising that they harboured similar bacterial communities. However, *A. tenuis* and *A. loripes* are phylogenetically divergent and fall within different clades (van Oppen, 2001; Fukami et al., 2000; Márquez et al., 2002). It is unclear why *A. tenuis* and *A. loripes* in this study shared similar bacterial communities (that were distinct from *A. sarmentosa* and *A. florida*), and whether the similar bacterial communities between the two species pair crosses contributed to their successful hybridization (Brucker & Bordenstein, 2012). For future coral hybridization studies, investigating the bacterial communities

of parental species from successful and unsuccessful crosses will provide valuable insights into this question

Hybridization did not affect microbe association

Bacterial and Symbiodiniaceae communities were the same in hybrid and purebred *Acropora* offspring within each cross, indicating that parentage plays a minor role in shaping these communities at the early coral life stages. At seven months of age, hybrids showed ~16-34% higher survival than purebred *A. tenuis* in the *A. tenuis* x *A. loripes* cross, and ~14-22% higher survival than both purebred offspring groups in the *A. sarmentosa* x *A. florida* cross (Chan et al., 2018). Our findings suggest these early life stage fitness differences are unlikely due to the microbial communities, unless the same communities expressed different genes in hybrid and purebred corals (which was not tested).

Environmental condition was a primary driver of microbial communities in early life stage corals

Treatment conditions had a major impact on the bacterial and Symbiodiniaceae communities harboured by early life stage recruits. However, our results cannot resolve whether the change in microbial communities was a passive response to treatment conditions or a consequence of active selection by the coral host (Webster et al., 2016). Changes in bacterial communities have previously been observed when adult conspecific corals were exposed to different temperatures (Bourne, Iida, Uthicke, & Smith-Keune, 2007; Lins-de-Barros et al., 2013; Santos et al., 2014; Webster et al., 2016) and/or pH/pCO₂ (Meron et al., 2011; Morrow et al., 2015; Webster et al., 2013) treatments. Conversely, bacterial communities of adult conspecific corals were unaffected

by changing temperature or pH/pCO₂ conditions is other studies (Littman et al., 2009b; Meron et al., 2012; Webster et al., 2016; Zhou et al., 2016). This discrepancy might be due to differences in study durations, the extent of the stress, the origin, species and age of the corals studied. For adult *A. millepora*, abundance of *Endozoicomonas* spp. declined significantly when subjected to low pH conditions (Morrow et al., 2015; Webster et al., 2016), while *Chlorobi* spp. increased when exposed to elevated temperatures (Webster et al., 2016). However, *Endozoicomonas* and *Chlorobi* spp. were either absent or barely present in the seven-month-old recruits of this study. Bacterial families that were suppressed under elevated conditions in this study (e.g., Blattabacteriaceae) have not been previously reported from corals, perhaps due to the comparatively small number of studies on microbiomes from early life stage corals. An additional consideration is that the bacterial communities can differ between corals that were raised in filtered seawater in the laboratory (the present study) and those that were raised in the field (the other studies) (Lema et al., 2014).

For the Symbiodiniaceae communities, the increase in occurrence and abundance of *Durisdinium* spp. in recruits under elevated temperature and pCO₂ conditions can be a mechanism of acclimatization under these challenging conditions (Quigley et al., 2018; Stat & Gates, 2011). While members of *Durisdinium* generally account for < 1% of a corals' Symbiodiniaceae community (Boulotte et al., 2016), they tend to occur in higher abundance in corals experiencing environmental stress. Examples include corals from the Persian Gulf where seawater temperatures typically exceeded 33°C (Baker, Starger, McClanahan, & Glynn, 2004; Mostafavi, Fatemi, Shahhosseiny, Hoegh-Guldberg, & Loh, 2007), corals from the back-reef environments in American Samoa (Oliver & Palumbi, 2009) and from the lagoonal environment in Palau where

diurnal variations in temperature and pH were extreme (Fabricius, Mieog, Colin, Idip, & van Oppen, 2004); as well as corals recovered from a bleaching event (Bay, Doyle, Logan, & Berkelmans, 2016; Boulotte et al., 2016).

Summary and recommendations to future studies

The lack of host specificity and the occurrence of a strong treatment effect on microbial communities of the seven-month-old recruits suggest environmental factors play a more important role than host factors at the early life stage. The importance of host factors increased over time, resulting in unique microbial communities between the two species pair crosses by two years of age. This in combination with the highly diverse bacterial communities in early life stages suggest a period of flexibility before adult assemblages are established in corals. In contrast to corals that have comparatively long generation times, microbes are capable of more rapid changes and their roles in assisting host climate change adaptation and acclimatization within the host's reproductive lifetime are increasingly being recognized (Rosenberg et al., 2007; Theis et al., 2016; van Oppen et al., 2017; van Oppen, Oliver, Putnam, & Gates, 2015). The manipulation of microbial communities in corals via inoculation with resilient microbial strains may serve as a tool to increase coral stress tolerance (Damjanovic, Blackall, Webster, & van Oppen, 2017; Rosado et al., 2019; van Oppen et al., 2017). Our findings point toward early recruits as the life stages where such interventions may be most successful, and testing this is an important new avenue of research.

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Data Citation

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Data Accessibility

Raw sequences of the 16S bacterial and ITS2 Symbiodiniaceae datasets are available in Genbank (16S dataset: SRR9308575- SRR9308772; ITS dataset: SRR9317148- SRR9317344; project accession number: PRJNA549088). Raw sequences of the 16S bacterial and ITS2 Symbiodiniaceae data, as well as sample biological Sequences of the reference mock communities are available on GenBank with the accession numbers provided in the Materials and methods section. Bioinformatics scripts used in this study are available as supporting information.

Author Contributions

W.Y.C., M.vO., L.P., and A.H. designed the experiment. W.Y.C. and L.P. performed the experiment. L.P. carried out the laboratory work. P.M., W.Y.C. and A.H. undertook statistical analyses. W.Y.C. and M.vO wrote much of the manuscript and all authors contributed to the final edited version of the manuscript.

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