Coral Reefs
https://doi.org/10.1007/s00338-018-01750-z

NOTE

Association with a sea anemone alters the skin microbiome of clownfish

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Received: 3 July 2018 / Accepted: 26 October 2018

Abstract Host-associated microbes play important roles in animal health. Clownfish and anemones form a mutualistic relationship where external surfaces are in constant contact with one another. The effects of this mutualism on the skin mucosal microbiome of clownfish are unknown. We monitored the mucosal microbiome of the clownfish *Amphiprion clarkii* before, during and after association (hosting) with the anemone *Entacmaea quadricolor*. Microbiome composition differed significantly between hosting and non-hosting clownfish. Upon separation of clownfish from anemones, the microbiome of post-hosting fish converged to resemble that of non-hosting fish. Bacterial sequence variants that significantly distinguished hosting from non-hosting clownfish were more abundant in the anemone microbiome and that of hosting fish, compared to non-hosting fish, suggesting transfer from or enrichment by the anemone. These results show that direct contact mutualism results in significant but reversible microbiome shifts, raising questions about a potential microbial role in mediating the fish–anemone interaction.

Keywords *Amphiprion clarkii* · *Entacmaea quadricolor* · Symbiosis · Clownfish · Anemone · Mutualism

Introduction

Animals benefit from associations with microbes (Cantley and Clardy 2015; Gilbert et al. 2016), which can provide nutrients and vitamins to the host (Kau et al. 2011), defend the host against potential pathogens (Buffie and Pamer 2013) and alter host behavior (Ezenwa et al. 2012). Although the majority of microbe–host interactions are thought to occur in the gastrointestinal tract, microbes on non-GI mucosal surfaces also influence host health and behavior. For example, surface microbes can affect host chemical signaling (Verhulst et al. 2010; Theis et al. 2013), which may have important implications for initiating or sustaining mutualistic associations between animals.

The clownfish–anemone relationship is among the most emblematic mutualisms in nature. Unlike that of other fish, the mucus coating of the clownfish does not trigger the stinging cells (nematocysts) of the anemone's tentacles (Mebs 2009), allowing the fish to shelter (host) among the tentacles for protection. In return for protection, the clownfish fends off potential predators (Godwin and Fautin 1992; Porat and Chadwick-Furman 2004) and provides the anemone with excreted nutrients such as ammonia, sulfur and phosphorus, which are utilized by the anemone's endosymbiotic zooxanthellae algae and subsequently incorporated by the anemone host (Porat and Chadwick-Furman 2005; Cleveland et al. 2011). Recently, it has been shown that clownfish also obtain carbon and nutrients from the anemone, suggesting a chemical recycling loop between the fish and its host (Verde et al. 2015).

While the mechanism(s) of clownfish immunity to anemone nematocysts is under debate, it has been shown that components of anemone mucus, such as antigens, can be transferred to clownfish mucus upon contact (Elliott et al. 1994). It is therefore reasonable to predict that close

Topic Editor Morgan S. Pratchett

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Published online: 02 November 2018

contact between fish and anemone may also involve, and potentially be influenced by, shifts in the mucosal microbiome of the interacting partners, either by transfer of microbes or differential microbial growth in response to shifts in the mucus environment. Considering that the skin mucosa and associated microbiome of teleost fish are the first line of defense against potential pathogens (Ángeles Esteban 2012; Schmidt et al. 2017), mucosal microbiome shifts may also directly impact the health of the clownfish. This study therefore tested the hypothesis that the skin mucosal microbiome of clownfish changes upon contact with an anemone host.

Materials and methods

We conducted an experiment to test for changes in the skin microbiome of clownfish (*Amphiprion clarkii*) before, during and after being hosted (direct contact) by an anemone (*Entacmaea quadricolor*) and compared these patterns to microbiomes of fish that were never allowed direct contact, according to the design in Fig. 1. The experiment was conducted in a closed seawater system that recirculates 562 gallons of artificial seawater (Instant Ocean) among 8 interconnected 29-gallon glass aquariums (system turnover time: ~ 20 min). The system was housed in a temperature-controlled vivarium in the Physiological Research Laboratory (PRL) at Georgia Tech, with water quality maintained by mechanical and biological filtration (live rock), UV sterilization and weekly 10% volume water changes. Ammonia, nitrate and nitrite were monitored weekly by PRL technicians (data not shown). Water parameters were monitored daily and maintained at pH 8.2, temperature 25.5 °C and salinity 35 ppt. Lighting was supplied via full-spectrum LED units (Ecoxotic Panorama) with a 9/15 h day–night cycle.

For the experiment, we distributed 23 anemones (10–20 cm diameter, supplied by Vivid Aquariums in October, 2017) among three aquariums, and 12 clownfish (5 cm in length, supplied by Sustainable Aquatics in October, 2017) among three other aquariums (the remaining two aquariums in the system housed healthy fish of the same species but not used in the study). Anemones were introduced to the system two weeks before fish were added and allowed to acclimate for a three-week period. Our prior work showed this timeframe to be sufficient for stabilization of a cnidarian (coral) microbiome (Pratte et al. 2015). During acclimation, lights were adjusted to less than full capacity based on responses of the anemones, i.e., to a level promoting what appeared to be a healthy animal (full tentacle extension, normal coloration, limited repositioning); we did not measure photosynthetically active radiation available to the anemones. Anemones were fed frozen brine shrimp once per week (after microbiome sampling) during acclimation and throughout the experiment. Clownfish were added to the system at 2 weeks into the anemone acclimation period (week 0 in Fig. 1) and allowed to acclimate for 1 week. Fish were fed twice daily during acclimation and throughout the experiment; fish feed consisted of dry pellets (0.8 mm, produced by Sustainable Aquatics) of krill meal, fish meal, squid meal, wheat gluten, potato starch, fish oil, spirulina, astaxanthin and garlic oil.

Fish mucus microbiomes were sampled at various times throughout the experiment, beginning at the end of week 1 (Fig. 1). Each individual was removed from the water with a sterile net and swabbed three to four times from gill to tail with a sterile cotton swab presoaked in sterile seawater; no individual was out of the water for more than 15 s. Anemones were sampled in week 1 only (see "Results and discussion" section); all individuals were sampled in the aquaria (anemones not removed) by swabbing the tentacles and oral disk. Immediately after the initial sampling (week 1), three pairs of clownfish were moved into the three aquariums containing anemones (two fish per tank), while the remaining three pairs remained in aquariums without anemones (Fig. 1). Hosting, defined as direct contact between anemone and clownfish partners, was observed within minutes. Hosting and non-hosting clownfish were sampled weekly for 4 weeks (weeks 2–5), after which

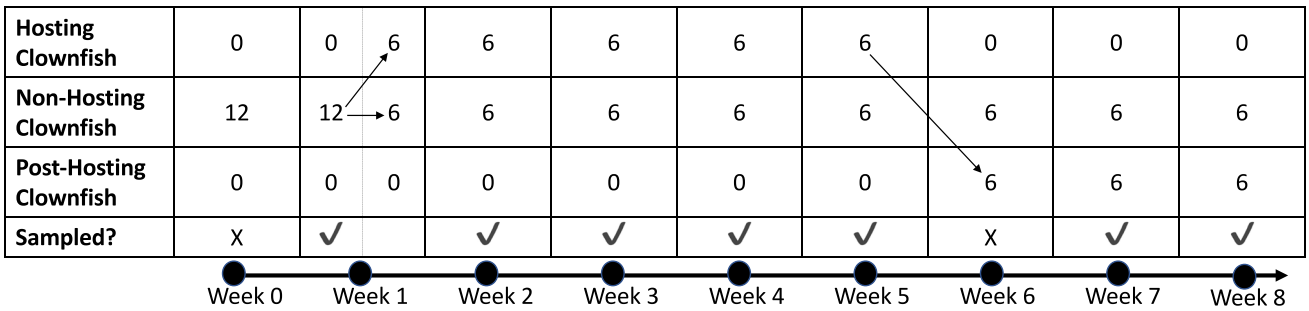

Fig. 1 Experimental design and sampling timeline. The first sampling occurred at week 1, when both clownfish (*A. clarkii*) and anemones (*E. quadricolor*) were sampled prior to the hosting period. See Methods for details

hosting clownfish were placed back into aquariums without anemones and resampled 2 and 3 weeks later (weeks 7–8). Samples of tank water microbiomes were collected at each time point via filtration through a 0.2-µm Isopore membrane filter (Millipore), as in Pratte et al. (2017). Care was taken to minimize stress to all animals during sampling to the extent possible (anemones retracted during sampling, indicating some amount of stress). All procedures were approved by the Georgia Institute of Technology IACUC committee (A100024), and all animals were adopted out at the end of the experiment.

Microbiome taxonomic composition was assessed by Illumina sequencing of the 16S rRNA gene (V4 region) following procedures of the Earth Microbiome Project, as done routinely in our laboratory. See Pratte et al. (2017) for detailed descriptions of DNA extraction, PCR and sequencing. Seventeen of 23 anemone samples, 79 of 84 clownfish samples and 9 of 14 water samples amplified successfully. The resulting amplicons were sequenced on an Illumina MiSeq using a V2 500 cycle kit (250 × 250 bp) with 10% PhiX to increase read diversity. Using QIIME2, reads were quality filtered and trimmed to the recommended 120 bp, and sequence variants (SVs) were identified using Deblur (Amir et al. 2017). Taxonomy was assigned using the SILVA pre-trained classifier (silva-119-99-515-806-nb-classifier). All chloroplast, mitochondria or unidentified sequences (at the domain level) were removed. Microbiome alpha diversity (observed SV richness, Chao1-estimated richness, Shannon diversity) was calculated in Phyloseq (McMurdie and Holmes 2013) and beta diversity (Bray–Curtis dissimilarity) was calculated in PRIMER7 (PRIMER-E Ltd.) using a uniform depth of 2000 reads per sample. Statistically significant variation in microbiome taxonomic composition was assessed via analysis of similarity (ANOSIM), and non-metric multi-dimensional scaling (nMDS) based upon Bray–Curtis dissimilarities using PRIMER7 (PRIMER-E Ltd). Detection of SVs predictive of distinct microbiome states (hosting or non-hosting) was done via Random Forest analysis in QIIME2. Raw sequences are available in NCBI's Sequence Read Archive (BioProject PRJNA448853).

Results and discussion

The mucosal microbiome of captive *A. clarkii* in our experiment was distinct from that of the tank water and of co-occurring *E. quadricolor* anemones, but resembled that of marine fish from the natural environment. We detected 2 930 SVs across all *A. clarkii* rarefied datasets, compared to 939 and 1 532 SVs from the smaller water and anemone sample sets, respectively. Regardless of hosting state (hosting, non-hosting, post-hosting), the taxonomic composition of the *A. clarkii* microbiome differed significantly from that of the anemone microbiome (sampled at week 1 only) and the surrounding water (sampled weekly; $P < 0.05$, ANOSIM), although some overlap between water and animal microbiomes was evident (Fig. 2). The clownfish microbiome was dominated by Gammaproteobacteria (26–62% of sequences), Alphaproteobacteria (8–15%) and Flavobacteria (2–19%) (Fig. 3). Other microbial groups common to marine host-associated communities, including Clostridia, Bacteroidetes, Cytophagia, Cyanobacteria, Betaproteobacteria and nitrifying Thaumarchaeota, were also consistently represented, but at lower proportions (Fig. 3). Recent surveys of the skin or gill microbiomes of reef fish from Moorea (Pratte et al. 2018) and the Western Indian Ocean (Chiarello et al. 2018), including the clownfish *A. akallopisos,* reported highly similar community structure at broad taxonomic levels. While we anticipate that microbiome composition likely varies between field and laboratory conditions when evaluated at finer taxonomic resolution, the broad similarity between the microbiomes of our captive fish and those of fish on reefs suggests that the community shifts observed in our experiment are environmentally relevant.

We observed a significant shift in the *A. clarkii* microbiome in response to association with an anemone (Fig. 4). Within one week of association with anemones (week 2), microbiome composition of hosting fish diverged significantly from that of non-hosting fish ($P < 0.05$, ANOSIM). This divergence is illustrated by separation of hosting and non-hosting microbiomes in nMDS analysis (Fig. 4), and a shift in the ANOSIM R value from negative (-0.065) to positive (0.413) upon hosting, with a positive R indicating less variation within a sample type than between sample types. This difference remained through weeks 2–4 of the hosting period ($R = 0.413$–0.587, $P = 0.02$–0.06), although microbiomes became more similar by week 5 ($R = 0.087$, $P = 0.119$). Two weeks after the hosting period, the microbiomes of fish that had been hosted no longer differed from those of non-hosting fish ($R < 0$, $P > 0.59$). Hosting-associated changes in microbiome composition were accompanied by a general decline in alpha diversity in hosting versus non-hosting fish (Table 1).

Differences between hosting vs. non-hosting microbiomes were verified by Random Forest analysis, which showed that microbiome composition could be used to correctly predict hosting status with an accuracy of 80%. Two SVs were critical for correctly categorizing hosting status. One could be classified only as belonging to the domain bacteria, while the other was assigned to the genus *Rubritalea* (phylum Verrucomicrobia), members of which have been observed in the microbiomes of bull kelp and

Fig. 2 Relatedness of clownfish, anemone and aquarium water microbiomes. The plot reflects a non-metric multi-dimensional scaling (nMDS) analysis based on Bray–Curtis dissimilarities (square-root transformed). Water microbiome data for week 2 are missing. Low 2D stress indicates accurate visual representation

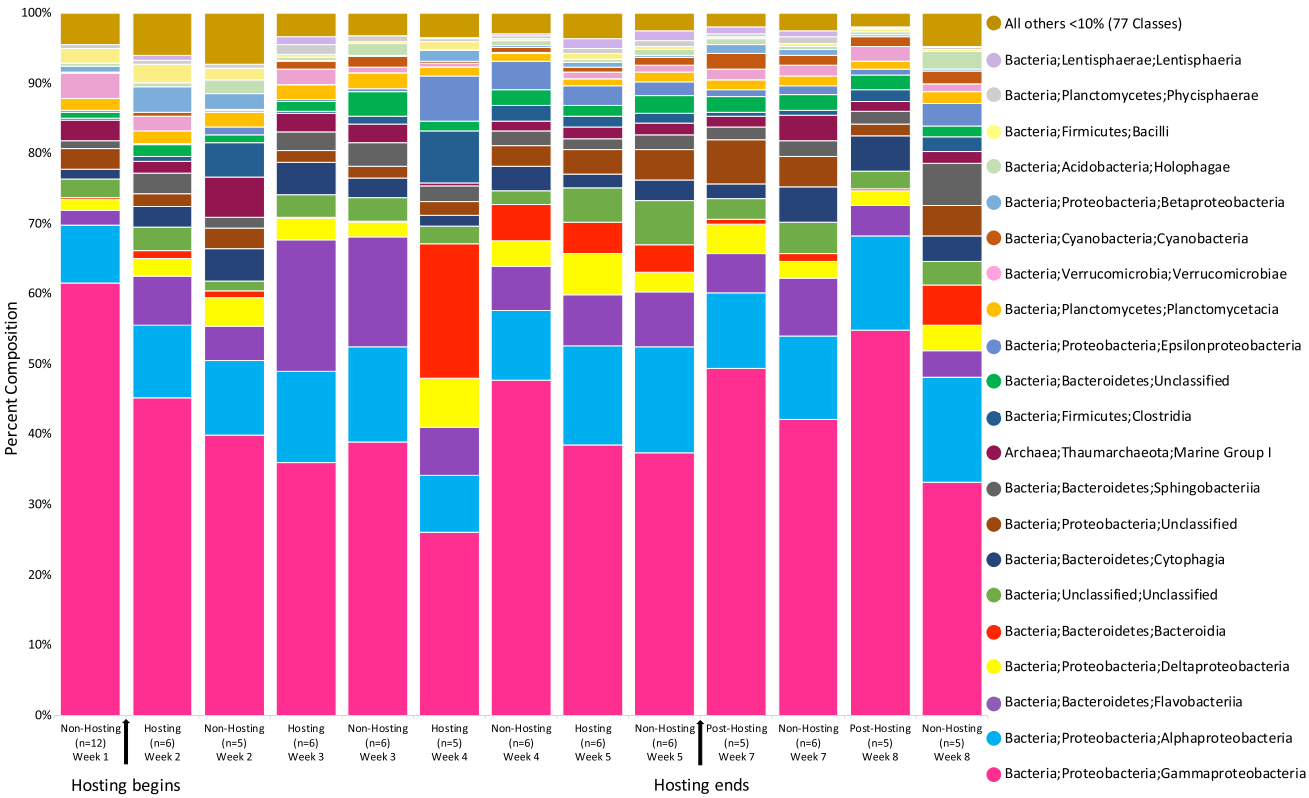

Fig. 3 Representation (mean percentage of total sequences) of major taxonomic groups in clownfish microbiomes

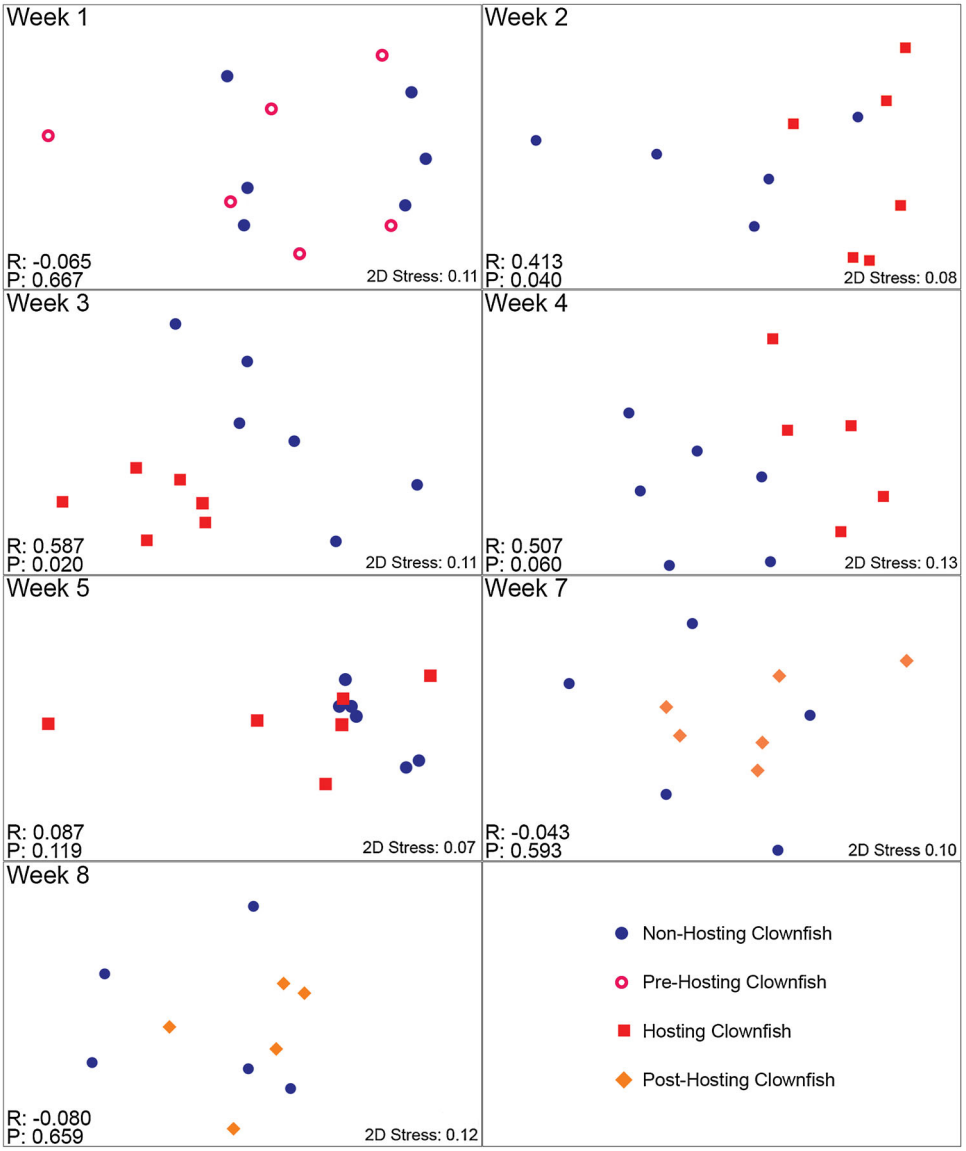

Fig. 4 Association with an anemone (hosting) significantly changes the mucus microbiome of the clownfish *Amphiprion clarkii*. Plots show microbiome samples clustered by non-metric multi-dimensional scaling (NMDS) based upon Bray-Curtis dissimilarities. One-way ANOSIM results comparing microbiomes of pre-hosting, hosting, non-hosting and post-hosting *A. clarkii* are found in the bottom left of each panel. A separate one-way analysis was conducted for each week. Low 2D stress indicates accurate visual representation

Table 1 Average alpha diversity values (standard deviation in parentheses), and results of comparisons of diversity

	Averages				Water (All weeks)	*t* test *p*-value	
	Hosting (weeks 2–5)	Non-hosting (weeks 2–5)	Post-hosting (weeks 7–8)	Non-hosting (weeks 7–8)		Hosting versus non-hosting (weeks 2–5)	Post-hosting versus non-hosting (weeks 7–8)
Observed	323 (± 76)	354 (± 78)	262 (± 64)	287 (± 58)	265 (± 55)	0.198	0.369
Chao1	418 (± 131)	490 (± 153)	330 (± 94)	336 (± 86)	245 (± 98)	0.095*	0.871
Shannon	4.55 (± 0.59)	4.61 (± 0.54)	4.07 (± 0.67)	4.56 (± 0.51)	4.10 (± .49)	0.694	0.073*

Comparisons indicating strong differences ($p < 0.1$) are shown with an asterisk

sponges (Dobson et al. 2015; Chen and Parfrey 2018); to our knowledge, neither SV has been reported in microbiomes of marine fish or cnidarians. These SVs were almost undetectable in non-hosting fish but enriched > tenfold in hosting individuals and 5- to 26-fold in the week 1 microbiome of anemones (Fig. 5), suggesting either direct transfer from the anemone or an anemone-induced change in growth conditions to favor these taxa. It is unclear what functional roles these two SVs may play in the anemone–clownfish relationship.

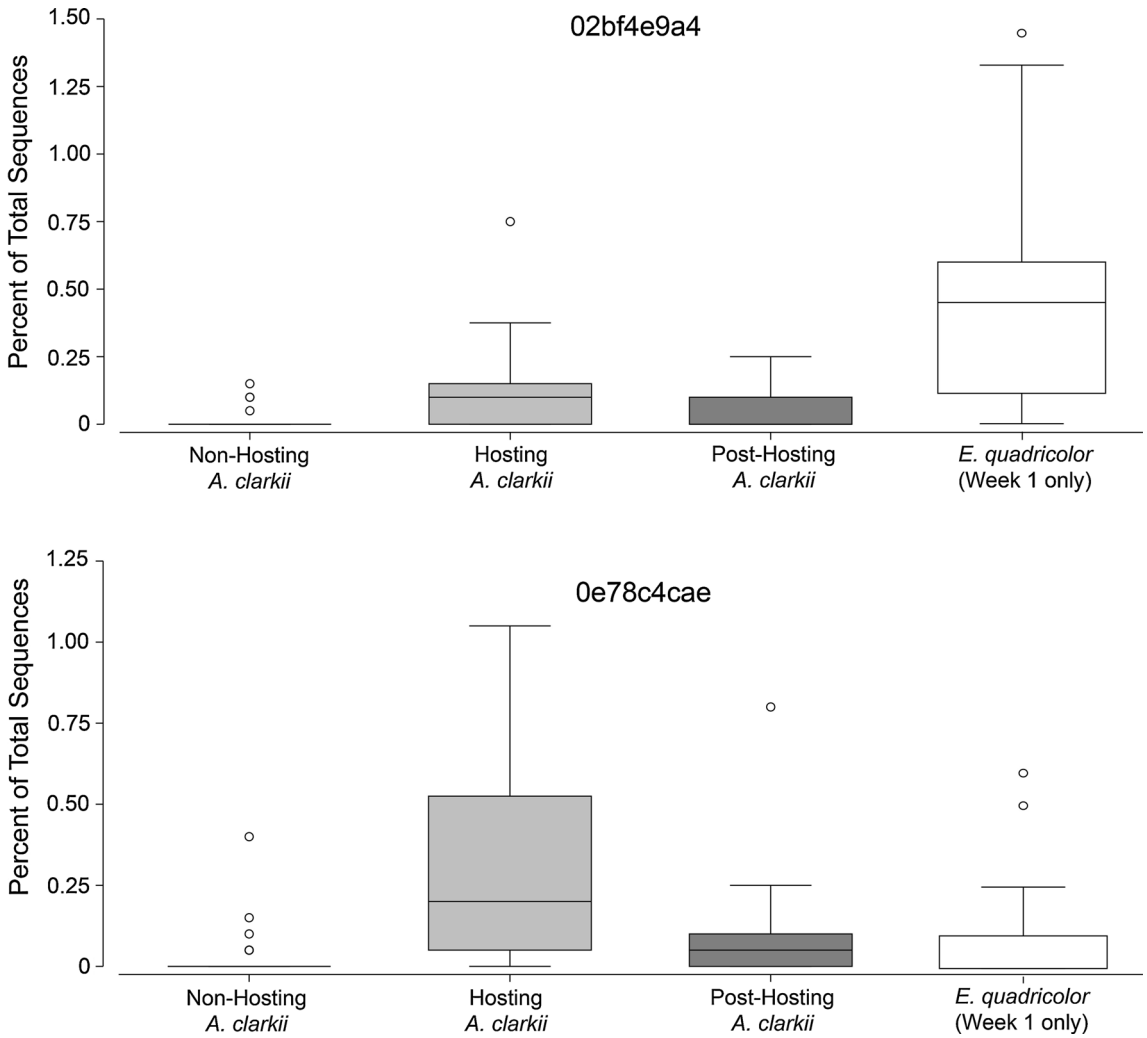

Fig. 5 Relative abundance of two 16S rRNA gene sequence variants with relatively high GINI importance, indicating they are critical in distinguishing hosting from non-hosting *Amphiprion clarkii* mucosal microbiomes through Random Forest analysis in QIIME2. Center lines of box plots indicate median values. SV 02bf4e9a4 was classified as *Rubritalea* sp.; SV 0e78c4cae was classified only as "Bacteria"

Our results show that contact with anemones can significantly reshape the clownfish microbiome. This effect, however, is dynamic, as the microbiome reverts back to a non-hosting state soon after contact with an anemone stops. The mechanism by which the clownfish microbiome changes during hosting is not clear. Microbes could be transferred directly from anemone to fish. Alternatively, the resident microbiome could shift in response to changes in the environment, such as changes in chemical substrate availability in the mucus or surrounding water. Indeed, while the mucus layer of clownfish differs in thickness from that of non-anemone fish (Lubbock 1980), the potential for mucus composition to change during hosting is not well understood. It has been hypothesized that clownfish tolerate exposure to anemones due to a lack of mucus-associated components that would, in other fish, stimulate anemone nematocysts (Lubbock 1980; Fautin 1991).

It remains unknown if hosting induces a similar change in the anemone's microbiome. Our study did not assess changes in anemone microbiome composition over time due to hosting, as fish moved frequently among anemones during hosting, preventing us from determining an anemone's history/status of fish contact. Given that an individual anemone was only intermittently in contact with a fish, while a hosted fish was in near constant contact with an anemone (albeit different anemones), it is possible that hosting only minimally affected the anemone microbiome. However, the situation may be very different in nature if multiple clownfish stably associate with the same anemone. Further work is needed to test these predictions under natural conditions.

Our findings confirm that microbial components of the clownfish mucus change with hosting, raising the hypothesis that these changes may be ecologically important to the fish–microbiome and fish–anemone interactions. Verde et al. (2015) hypothesize that microbes mediate the exchange of nutrients between anemone and clownfish, and changes in microbial community structure may therefore influence carbon and nitrogen cycling. Further work should test whether such changes represent only a benign and transitory restructuring of the fish microbiome or are instead linked to chemical transformations that affect fish health and help sustain this charismatic marine mutualism.

Acknowledgements This work was supported by Simons Foundation (award 346253 of FJS) and the Teasley Endowment to the Georgia Institute of Technology. We are grateful to the Physiological Research Laboratory staff at the Georgia Institute of Technology for the dedicated care of the research animals, and to Sustainable Aquatics for supplying animals. Research was conducted under IACUC permit A100024. ZAP, FJS, NVP and DJP developed the experimental design. ZAP, NVP, MEM and AMC performed the experimental procedures. ZAP, NVP and FJS contributed to data analysis, and ZAP and FJS wrote the manuscript with editorial assistance from all authors.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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