Microbiomes of clownfish and their symbiotic host anemone converge before their first physical contact.

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Research

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Abstract

Background One of the most charismatic, and yet mostly unexplained example of mutualistic interaction is the partnership of clownfish and its symbiotic sea anemone. The mechanism explaining this tolerance currently relies on the molecular mimicry of clownfish epithelial mucus, which could serve as camouflage, preventing the anemone's nematocysts' discharge. Resident bacteria are known as key drivers of epithelial mucus chemical signature in vertebrates. A recent study has proposed a restructuration of the skin microbiota in a generalist clown fish when first contacting its symbiotic anemone. We explored a novel hypothesis by testing the effect of remote interaction on epithelial microbiota restructuration in both partners.

Methods With metataxonomics, we investigated epithelial microbiota dynamics of 18 pairs of percula clownfish (*Amphiprion percula*) and their symbiotic anemone *Heteractis magnifica* in remote interaction, physical interaction and control groups for both partners during a four weeks trial.

Results Physical and Remote Interaction groups' results evidence epithelial microbiota convergence between both partners as soon as 15 minutes after fish and anemone have been placed in the same water system. This convergence occurred preceding any physical contact between partners, and was maintained during the two-weeks interaction period in both contact groups. After the interaction period, community structure of test anemones gradually shifted back to the state of their controls, whereas skin community structure of test clownfish maintained the interaction signature two weeks after fish-anemone pairs separation. Furthermore, the interaction signature persistence was observed both in Physical and Remote Interaction group fishes, thus suggesting that water-mediated chemical communication between symbiotic partners was strong enough to shift the skin microbiota durably, even after fish-anemone pairs separation. Finally, our results suggest that fish-anemone convergent microbiota restructuration was increasingly associated with the parallel recruitment of three *Flavobacteriaceae* strains closely related to a tyrosinase-producing *Cellulophaga tyrosinoxydans*.

Conclusions Our study shows that bacterial community restructuration, in the acclimation process, does not only rely on direct physical contact. Furthermore, our results challenge, for the first time, the traditional unidirectional chemical camouflage hypothesis, as we argue that convergence of the epithelial microbiota of both partners may play essential roles in establishing mutual acceptance.

Background

The interaction of anemones and clownfish is a charismatic example of mutualistic partnership [1], in which the anemone protects the clownfish against predators [2], while the clownfish provides the anemone's endosymbiotic zooxanthellae algae with excreted nutrients (ammonia, sulfur, and phosphorus) [3]. This mutualism is contingent upon a protective mechanism for clownfish against the anemone's nematocyst discharge. Numerous studies and reviews that attempted to identify the protective mechanism in different clownfish species, have highlighted two main non-exclusive hypotheses: either

clownfishes beneficiate from an innate protective mechanism in their skin mucus, and/or they need to coat their body with anemone mucus [4, 5]. Interestingly, during clownfish-anemone acclimation (i.e. prior to first physical contact), the epithelial mucus immunological profile of clownfish changes to mimic that of the anemone [6-8]. Thus, clownfish epithelial mucus is suspected to act as camouflage preventing "not-self" recognition associated with nematocysts' discharge [5]. Given that skin microbial communities are important drivers of the chemical and immunological profiles of vertebrates' epithelia, which modulate host-parasite interactions [9], it is relevant to investigate to which extent clownfish anemone symbiosis translates into epithelial microbiome shifts in both partners. In addition, as clownfish are known to hover near/above their partner anemone, before first contacting its tentacles, it is relevant to test whether fish and anemone microbiome shifts may precede their physical contact. To date, the structure of clownfish skin microbiome after contact with their symbiotic anemone has only been investigated partly, without anemone control groups during the contact phase [10, 11]. Therefore, the general mechanism underlying the microbial community changes observed in the initiation of the mutualist partnership is still unknown. First, it is essential to characterize the dynamic of anemone microbiome to assess its involvement in anemone-clownfish mutual acceptance. Then, the study of control groups with remote interaction (i.e. absence of physical contact) between both hosts has also never been done, and yet, this data is essential to determine if the putative restructuring of the transient epithelial microbiome of both partners actually participates in establishing mutual acceptance, or if it is merely an artifact of the physical contact between both hosts. Our objective was to test two hypotheses: (1) is anemone-clownfish mutualistic partnership associated with a significant restructuration of epithelial microbiotas in both partners during acclimation? and (2) does this skin microbiota restructuration precedes physical contact between partners? Then, we aimed to characterize the microbial taxa driving the observed community dynamics. In order to detect evolutionary relevant microbiota changes regarding mutualism, we focused on *Amphiprion percula*, a clownfish species exhibiting narrow host specificity, which is mainly associated with the *Heteractis magnifica sea anemone*.

To achieve our goal, we acclimated 18 naïve *A. percula* juveniles (i.e. no prior contact with anemone) and 18 *H. magnifica* during three weeks in four six-tanks experimental systems with separated water flows to avoid any chemical communication between experimental groups. 30% of the water was changed daily to minimize taxonomic drift in water microbial communities. Four experimental groups were tested: anemone control, fish control, physical interaction (i.e. fish and anemone in the same tank), and remote interaction (i.e. fish and anemone in different tanks, both connected to the same water flow). Overall, our study showed that there was a convergent skin mucus microbiota restructuration between both symbiotic partners during the interaction period, which did not only rely on physical contact. Furthermore, separation between symbiotic partners revealed contrasting microbiota resilience dynamics: the interaction signature in skin microbiota persisted in clownfish only, up to two weeks after separation with their anemone for the physical interaction experimental group.

Methods

Clownfish and sea anemone rearing

Our objective was to test whether anemone-clownfish mutualistic partnership is associated with a significant restructuration of epithelial surface microbiotas during remote and physical interactions. Then, we aimed to characterize the microbial taxa driving the community dynamics observed. To reach our objective, we acclimated 18 *H. magnifica* and 18 naïve *A. percula* juveniles (no prior contact with anemone) during three weeks in 20 L tanks with separated water flow to avoid any chemical contact prior to the experiment. Tanks were illuminated 12h/24h with bright lightning provided by pairs of Fluval Sea Marine 2.0 LED Light Fixture 48" ramps. Nitrates were maintained below 5-10 mg/L. In each 20 L tank, water was pulsed with a 180 L/h water pump, in addition to the intake water from the recirculated system. Clownfish were fed daily, and anemones were fed three days a week with mysis shrimps, directly pipetted on the oral disc. Food waste was retrieved daily by syphoning water.

There were four experimental groups, each containing with six biological replicates: anemone control (AC), fish control (FC), physical interaction (i.e. fish and anemone in the same tank, PI), and remote interaction (i.e. fish and anemone in different tanks, all being connected to the same water flow, RI) (Fig. 1). To minimize bacterioplankton taxonomic drift due to independent water flow in each experimental group, 30% water changes were conducted each day in both interaction groups with a water mix from both control groups. To prevent any induction of “remote interaction” between anemone and clownfish control groups, we did not add the water mix (control anemone and control fish) in the control tanks. Following the acclimation period (Fig. 1a), the interaction period between clownfish and anemone for physical and remote interaction groups lasted two weeks (Fig. 1b), after which clownfish individuals and anemone were separated for a two weeks resilience period (Fig. 1c).

Host microbiota and water sampling

Seven sampling steps were as follow: T0, at the end of a three weeks acclimation period; T1, after the first 15 min of physical interaction between clownfish and anemone (PI), 15 min after transfer of fish to the recirculation system containing anemones (RI), and immediately in both control groups; T2 and T3, respectively one and two weeks after initial interaction (T1); T4 and T5, respectively one and two weeks after fish-anemone pairs separation from physical and remote interaction groups (T3). Mucus surface of both clownfish and anemone was sampled with sterile cotton swabs outside of the water [as described in 37]. To characterize the bacterioplankton community of each group, 2 L of tank water was collected and filtered on 0.22 mm membranes at every sampling time. We used four bacterioplankton replicates per experimental group.

DNA extraction, libraries preparation, and 16S amplicons sequencing

DNA extraction of epithelial mucus from clownfish and sea anemone, as well as 0.2-µm membranes from water samples was performed using the Qiagen® Blood and Tissue Kit from QIAGEN according to the manufacturer's instructions. Amplicon libraries of the V3 region of the rDNA 16S gene were sequenced on Illumina MiSeq (San Diego, CA, USA), including control samples.

Bioinformatics and biostatistics analyses

Bioinformatics' processing was undertaken as reported in [38]. 4,770,388 raw reads were quality filtered with a truncation to 270 base pairs and allowing a maximum of 2 expected errors (maxEE) and then processed using the rest of the dada2 pipeline for ASV construction with default parameters, except for the "dada" step where all samples were pooled for ASV inference [39]. Taxonomic assignation of amplicon sequence variants (ASV) was performed by using blastn matches NCBI 16S Microbial database. Matches above 99% identity were assigned the reported taxonomic identity. Sequences with no matches above the identity threshold were assigned taxonomy using a lowest common ancestor method generated on the top 50 blastn matches obtained.

Statistical analyses and graphs were generated with libraries from R/Bioconductor: ggplot2, Phyloseq, Vegan and DESeq2. Thetayc dissimilarity analyses were performed with the following sample description: Control/Interaction groups and six times (T0, T1, T2, T3, T4, T5). Differential abundance analysis (DESeq2) was performed with Control/Interaction groups at six times (T0, T1, T2, T3, T4, T5). Thresholds used were a Bonferroni corrected p-value of 0.05 and a fold change of 1. Differential abundance analyses (DESeq2) performed with the three ASVs related to *Cellulophaga tyrosinoxydans* were performed for bacterioplankton, sea anemone and clownfish, at six times (T0, T1, T2, T3, T4, T5). Thresholds used were a FDR adjusted p-value of 0.0001 and a fold change of 1. PERMDISP (betadisper) and ANOSIM analyses were performed on unweighted UniFrac distances with the R vegan package.

Faith PD and Simpson indexes were calculated using the "picante" and "vegan" R packages. For the Faith PD index, a phylogenetic tree was built (JC69 substitution model and neighbor joining) using the "ape" package. Pairwise Kruskal-Wallis tests for diversity index values were performed with the R "stats" package using FDR adjusted p-value of 0.05.

Results

We first assessed the dynamics of the bacterioplankton community, a factor known to covariate with fish skin mucus communities [12-14]. PERMDISP and ANOSIM tests performed on unweighted UniFrac distances (Table 1), and Kruskal-Wallis tests performed on Simpson index (Supplementary data, Figure S1, Tables S2, S3) showed that phylogenetic structure and alpha diversity of bacterioplankton did not exhibit any time or treatment-specific pattern. This result suggests that bacterioplankton was not significantly associated to the microbial community restructuring observed in clownfish and anemones from physical (PI) and remote (RI) interaction groups from T1 to T3, as detailed below.

Thetayc dissimilarity analyses. Analysis of the anemones' epithelial microbiota (Figure 2a) shows that prior to contact with clownfish, dissimilarity between test and control groups was minimum (0.12 ± 0.01) (T0: after three weeks of acclimation). At T1, 15 minutes after the clownfish test individuals were transferred from the fish control tank system into their respective two-tanks systems for remote interaction (RI) (i.e. six biological replicates of one anemone tank connected with one fish tank), and after the first 15 min of physical contact between physical interaction (PI) clownfish individuals with their respective anemone (i.e. six biological replicates of physical interaction), dissimilarity between test

(physical and remote interaction) and control anemones was significantly higher (0.39 ± 0.05) (Student T test, $p < 0.001$ ***) relatively to that of T0. Then, the dissimilarity between test and control anemones remained high during the interaction period (0.43 ± 0.03) (T1 to T3). From T4 (one week after PI / RI clownfish individuals were retrieved), to T5 (two weeks after PI / RI clownfish individuals were retrieved), dissimilarity between test and control anemones was significantly lower (0.36 ± 0.03) compared to that of the contact period (Student T test, $p < 0.009$ ***).

Regarding clownfish skin microbiota (Fig. 2b), the same pattern as that of the anemones occurred from T0 to T3: dissimilarity at T0 between PI / RI clownfish test and control groups was minimum (0.029 ± 0.005) prior to fish contact with their respective anemone. At T1, as soon as PI and RI clownfish test individuals were placed into their respective tank systems, dissimilarity between PI / RI test and control clownfish was significantly higher (0.61 ± 0.08, Student T test, $p < 0.001$ ***) relatively to that of T0. Then, the dissimilarity between PI / RI test and control clownfish remained high (0.46 ± 0.04) during the interaction period (T1 to T3). From T4 (one week after PI / RI clownfish individuals were retrieved and moved back to the control clownfish water system), to T5 (two weeks after), dissimilarity between PI / RI test and control clownfish groups remained stable (0.47 ± 0.03) and not significantly lower compared to that of the contact period (T1-T2-T3) (Student T test, $p = 0.6$).

Finally, regarding dissimilarity between fish and anemone microbiota (Fig. 2c), it was similar at T0 (0.41 ± 0.02) in all groups. Then, at T1, T2 and T3, the dissimilarity dropped to 0.16 ± 0.04 in PI and 0.11 ± 0.02 in RI test groups, significantly below the stable dissimilarity (0.56 ± 0.04) observed between fish and anemone control groups (Student T test, $p < 0.001$***). At T4 and T5, after fish-anemone pairs' separation, the dissimilarity values increased in PI (0.44 ± 0.03) and RI (0.30 ± 0.04) contact groups, but without reaching that of their respective controls (0.50 ± 0.04). This partial recovery is most likely explained by the stability of the dissimilarity between PI / RI test and control clownfish groups from T3 to T5 (Fig. 2b). In addition, the dissimilarity between the host microbiota and the bacterioplankton (Fig. S2) was never significantly different between the test and control groups.

Differential abundance analysis in clownfish and anemone skin microbiota between contact and control groups. *De novo* ASV abundances in clownfish and anemone were monitored during the whole experiment (from T0 to T5) using differential abundance analysis (DESeq2) to identify bacterial taxa that were mostly associated to fish-anemone epithelial microbiota convergence. To reach this goal, *de novo* ASV abundances of clownfish and anemone were combined, PI and RI groups were combined as an interaction group, as well as clownfish and anemone controls were combined as a control group. ASVs with log2-normalized fold-change over 1 and Bonferroni corrected p-value < 0.05 were kept (Table S1). At T0, there were only 5 differentially abundant taxa between interaction and control fish-anemone pairs. At T1, after the first 15 minutes of clownfish-anemone interaction, differentially abundant taxa increased to 10 ASVs. At T2, after one week of interaction, differentially abundant taxa doubled to reach 21 ASVs. At T3, after two weeks of interaction, differentially abundant taxa peaked at 30 ASVs. At T4, one week after separation of interaction fish-anemone pairs, the number of differentially abundant ASVs remained at 30. At T5, two weeks after separation of interaction fish-anemone pairs, the number of differentially

abundant taxa dropped to 17 ASVs. From T2 to the end of the experiment (T5), three ASVs (2, 49, 177) matching to *Cellulophaga tyrosinoxydans* strain EM41 (95% identity, 100% coverage, 1.31E-119 to 2.81E-121 e-values) exhibited an interesting dynamic: they peaked at T3, with the three highest Bonferroni corrected p-values, with a fold change ranging from 9 to 12, then decreased gradually after separation of fish-anemone pairs: fold change ranging from 6 to 11 at T4, and from 3 to 8 at T5, relatively to fish-anemone control group. Therefore, these three ASVs related to *Cellulophaga tyrosinoxydans* were further analyzed in terms of abundance dynamics in water, sea anemone and clownfish mucus.

Differential abundance analysis (DESeq2) on *Cellulophaga sp.* in water, sea anemone and clownfish. The monitoring of the three ASVs (2, 49, 177) related to *C. tyrosinoxydans*, which were differentially abundant from T2 to T5 (DSeq2, Fig. 3, Table 3) was decomposed in terms of host community (sea anemone, clownfish, water), experimental groups and time. At T0, *Cellulophaga sp.* counts were both low and variable across experimental groups and host communities. ASVs with log2-normalized fold-change over 1 and FDR corrected p-values < 0.0001 were kept (Table 2).

Tank system water: From T0 to T1, *Cellulophaga sp.* dropped in all experimental groups to become undetectable except for FC, where only ASV 2 was significantly higher to PI (8.9 fold change). At T2, *Cellulophaga sp.* was still undetectable in anemone control group, and dropped under the detection threshold in clownfish control group. On the contrary, *Cellulophaga sp.* counts increased for the three ASVs both in PI and RI tank water to become statistically higher than in AC and FC groups (8.6 to 13.6 fold changes). At T3, the three ASVs were still undetectable in both control group water, whereas peaking in both PI and RI groups (9.7 to 13.7 fold changes). From T4 to T5, one and two weeks after clownfish retrieving from PI and RI tank systems, the three ASVs counts decreased gradually (5.2 to 13.7 fold changes at T5) in both PI and RI tank system water.

Sea anemone epithelium. From T0 to T1, *Cellulophaga sp.* counts dropped under the detection threshold in the three experimental groups hosting anemones (AC, PI, RI). At T2, *Cellulophaga sp.* counts increased for ASVs 49 and 177 in PI and RI to become significantly higher than in control (7.7 and 9.5 fold changes). At T3, the counts of the three ASVs peaked in PI and RI groups, and were still significantly higher than in control (8 and 13 fold changes). From T4 to T5, one and two weeks after clownfish retrieving from PI and RI tank systems, *Cellulophaga sp.* counts decreased quickly: ASV 2 was no more differentially abundant, and ASVs 49 and 177 dropped from 5.8 to 10.2 fold changes at T4, and were no more significantly different from their control at T5.

Clownfish skin mucus.

At T0, the three *Cellulophaga sp.* related ASVs counts were low and comparable between the three clownfish groups, which had shared the same tank system water for the three weeks acclimation period. From T0 to T1, *Cellulophaga sp.* counts remained low and comparable between the three clownfish groups, despite the transfer of PI and RI individuals to their respective PI and RI tank systems hosting anemones. At T2, *Cellulophaga sp.* counts of ASVs 49 and 177 increased in PI and RI to become significantly higher than in control (7.7 to 9.4 fold changes). At T3, the counts of the three ASVs peaked in

PI and RI groups, and were still significantly higher than in control (10.7 to 13.3 fold changes). At T4, one week after PI and RI clownfish were reintroduced into the fish control water system, the counts of the three ASVs remained high and significantly higher than in control fish (7.1 to 12.9 fold change), despite sharing the same tank system water. At T5, two week after PI and RI clownfish reintroduction into the fish control water system, the three ASVs counts remained high only in PI clownfish (5 to 10.6 fold changes), whereas ASVs 2 and 49 dropped drastically in RI clownfish, both of them being no more significantly higher than in control fish.

Discussion

Remote interaction between naïve clownfish and sea anemone triggered epithelial microbiota convergence. Our results from remote and physical interaction groups revealed that prior to the first physical contact, both clownfish and anemone epithelial microbiotas converged from T1. This convergence was not accompanied with a shift of the bacterioplankton profile according to alpha diversity dynamics between T0 and T1 (Supplementary results, Tables S2, S3). Contrastingly, an increase of the Simpson index in both PI and RI fish mucus (Table S3) suggests therefore that the interaction between symbiotic partners involved a quick restructuration of clownfish microbiota in terms of richness and evenness. Furthermore, after the first 15 minutes of physical interaction, PI anemones exhibited a restructuration of their mucus microbiota (Supplementary data, Tables S2, S3). Interestingly, this restructuration in terms of richness and evenness was delayed for remote interaction as RI anemones exhibited a shift of their mucus microbiota composition at T2 (Supplementary data, Figure S1). Moreover, the occurrence and persistence of convergent restructuration in the remote interaction (RI) group from T1 to T3 (Fig. 2c) suggests that community restructuration does not only rely on physical interaction and occurs before such direct contact. Therefore, the convergent restructuration between symbiotic partners starts very likely as soon the clownfish skin mucus is exposed to sea anemone chemical compounds that are released into the surrounding water, as observed by Schlichter (1975, 1976) [15, 16]. In addition, this clownfish / anemone epithelial microbiota convergence has to be paralleled with the observation of Mariscal (1971) [6], which reported that the fish skin mucus composition changed during acclimation to resemble that of the anemone. In a more recent survey focusing on clownfish / anemone epithelial microbiota, Roux *et al*. (2019) [11] observed in a closely related clownfish species (*A. ocellaris*) that taxonomical composition of fish skin microbiota in physical contact with sea anemone (*H. magnifica*) was closer to those of the anemone when compared to control clownfish. However, as there was neither control anemone nor replication of experimental groups, their observations need further validation.

Separation between symbiotic partners revealed contrasting microbiota resilience dynamics. After separation of symbiotic partners (T4-T5), clownfish and sea anemone interaction group microbiota exhibited a contrasting response. Both physical and remote interaction anemone microbiota started converging to that of their control, despite remaining in different water systems. As such, this result shows that neither environmental water nor time, two factors that are known to drive bacterial community shifts [12-14], did play any major rule in reshaping the sea anemone microbiota in interaction groups. Contrastingly, one and two weeks (T4-T5) after interaction period, when clownfish individuals were

separated from their respective anemone tank system, the dissimilarity index with the control clownfish group remained high, and not significantly different from that observed during the contact period (T1-T3). This result is even more striking because interaction groups individuals were reintroduced into the control clownfish water system during the resilience period (Fig. 1c). Therefore, the lack of convergence between ex-interaction PI and RI fish with control fish during the resilience period, despite sharing the same water system, confirms further that the water did not play a major rule in reshaping the clownfish skin microbiota. Furthermore, the fish skin microbiota signature of physical/remote interaction with sea anemone, remained detectable two weeks after clownfish-anemone pairs separation (Fig. 1c, 2c). This observation can be paralleled with what was observed in previous experiments focusing on anemone mucus proteins and antigens: those molecules persisted in clownfish skin mucus after clownfish-anemone pairs' separation [8].

***Cellulophaga sp*. are the main mucus symbionts involved in sea anemone clown fish contact.** The microbiota convergence observed between fish and anemone during the contact period starting at T1 was followed from T2 by the gradual parallel recruitment of three initially rare *Flavobacteriaceae* symbionts closely related to *Cellulophaga tyrosinoxydans*. This parallel recruitment of *Cellulophaga sp.* peaked at T3, two weeks after fish-anemone pair contact and fade out with contrasting dynamics in fish and anemones from their separation (T4-T5). In PI and RI anemones, two out of three ASVs were still significantly more abundant than in controls at T4, but not at T5, whereas being significantly more abundant in both PI and RI water than in AC water at the same time. Contrastingly, all the three ASVs were still significantly more abundant in both PI and RI clownfish than in controls at T4, as well as at T5 for PI clownfish only, despite sharing the same FC water. Moreover, the same three ASVs were significantly less abundant in FC water than in PI and RI tank systems hosting anemones only since T4. Therefore, these results suggest that physical interaction during two weeks (T1-T3) exerted a more sustainable imprinting in fish skin microbiota than remote interaction, where two out of three *C. tyrosinoxydans* related ASVs counts converged to that of control fish. Given that anemone mucus proteins and antigens were transferred to clownfish skin mucus and persisted after clownfish-anemone pairs' separation [8], the persistence of *C. tyrosinoxydans* strains two weeks after clownfish-anemone pairs' separation at least demonstrates their relationship with the biochemical imprinting of the clownfish-anemone mutualistic association, and possibly suggests that those ASVs are tightly involved in the remote communication between clownfish and its anemone host.

A complex network of inter-kingdom interactions

Our study aimed to investigate the microbial population dynamics during the initiation of the intricate partnership between clownfish and sea anemones, which both host complex bacterial communities. One of our most salient results is the epithelium microbiota convergence between symbiotic partners, which could be linked to an important driver in the evolution of symbioses: the use of a shared signaling pathway as a common 'language' [17] between symbiotic partners. Furthermore, the persistence of the clownfish-anemone interaction signal in fish microbiota after symbiotic partner separation, and the potential direct or indirect link with the biochemical imprinting, needs further discussion. Overall, results

from other model species showed that there are multiple ways in which the dialogue between two eukaryotic hosts can involve microbial communities via: host-microbiota interactions, microbiota-microbiota interactions, and host-host interactions.

(1) Host-microbiota interactions. A complex bidirectional communication is taking place between microorganisms and their host via chemical signals. A report [18] showed that host immune signaling molecules are recognized by bacteria, suggesting that the lexicon of inter-kingdom languages is considerable. The interaction between the symbiotic microbiota and the eukaryotic host brain was extensively documented [19-22]. One of the most studied host-microbiota communication tools is the catecholamine hormones, a group of neuromediators derived from tyrosine and other dietary sources [23]. Dopamine, noradrenaline, and adrenaline are catecholamines ("stress hormones"), produced by the eukaryotic host and by several microbial symbionts (reviewed in [24]). In the host-associated microbiome, these hormones are known to drive the structure of symbiotic microbial communities [24]. Prokaryote responsiveness to eukaryotic catecholamine hormones is widespread, and bacteria associated with animal surface epithelia are especially stress hormone responsive [25-28]. Thus, the catecholamines potentially produced by the clownfish brain during the initial phase of mutual acceptance could affect the taxonomic and functional profile of the epithelial microbiome of both eukaryotic partners (clownfish and anemone). In a similar manner, symbiotic bacteria can also influence host physiology. In our experiment, we observed that three ASVs related to *Cellulophaga tyrosinoxydans*, a tyrosinase producer, were especially associated to the convergence of microbiomes during the interaction period. These taxa might play significant roles in the chemical signaling convergence. For instance, melanin synthetized by bacterial tyrosinases are immunologically active compounds, known to bind diverse chemicals [29], and have many pharmaceutical applications including host skin protection against radiation, antioxidants, antiviral agents, or immunogens [30]. The metabolites repertory of the three *C. tyrosinoxydans* ASVs merits further investigations to highlight its functional importance in clownfish-anemone mutual acceptance.

(2) Microbiota-microbiota interactions. An important communication system between bacterial communities relies on quorum sensing, which regulates a wide variety of bacterial population density-dependent processes. An increasing population density results in increased concentrations of bacterial autoinducers released by bacterial cells, which then eliciting specific responses. For instance, in gram-negative bacteria, *N*-acylated homoserine lactones are common autoinducers [24]. These lactones activate expression of operons associated to the development of biofilms, antibiotic synthesis, persister cell formation, bioluminescence, and the synthesis of the autoinducer per se [31, 32]. In our experiment, it is possible that the proximity of clownfish and anemones in physical and remote interaction groups induced the detection of an autoinducer shared in the epithelial microbiome of both eukaryotic host species. Then, density-dependent quorum sensing processes could have modulated the phylogenetic structure of bacterial communities from both hosts, potentially leading to their convergence (as observed in this study).

(3) Host-host interactions. Numerous studies have shown that symbiotic interactions between eukaryotic hosts can perturb the host-associated microbial habitats, which translates in a restructuring of the microbial communities involved [33]. For instance, in epithelial surface membranes, the initial colonization of a host by a parasite markedly alters the host's epithelial barrier by affecting mucus production and composition, tight junctions, and epithelial cell turnover [34]. Indeed, infection by *Toxoplasma gondii* causes an increase in the number of goblet cells, one of the main constituents of animal mucus. Several microbial taxa, notably *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia* [35], use mucus carbohydrates as a carbon source and thus gain a competitive advantage following an increased mucus production [34]. Analogously to a parasite colonization, the colonization of a sea anemone by a clownfish may modify the physical characteristics of the epithelial mucus habitat, and thus indirectly perturb the microbiomes of both symbiotic partners. However, as observed with the remote interaction (i.e. no physical contact), our study suggests that most of the epithelial microbiota restructuring relies on remote biochemical communication.

Conclusions

Whether the restructuration of the microbiome of both hosts when in physical/remote interaction is a prerequisite for mutual acceptance (i.e. the "camouflage hypothesis"), or whether this community remodeling is an indirect consequence of an unknown remote biochemical detection system between partners is yet to be completely resolved. Here, we provide salient insights supporting the multilayered model of microbiome structuring from Shapira (2016) [36], which proposes that the variable environmentally modulated flexible microbial pool, termed as transient microbiota, allows instant adaptation of holobionts to changing environments. We propose that a remote interaction between both symbiotic partners triggers a convergence of the transient epithelial microbiome of both partners, which may participate in establishing mutual acceptance. As such, our results suggest that the protective mechanism of *A. percula* clownfish, which has a narrow spectra of host sea anemone species, not only involves the coating of fish skin with components of the host anemone mucus [4, 5], but also implicates a chemical dialog between symbiotic partners prior to the first physical contact. Then, the present work provides additional insights regarding the hypothesis that epithelial mucus immunological profile of clownfish changes to mimic that of the anemone prior to first contact [6-8]: the convergence of the transient epithelial microbiota of both partners in remote interaction reveals that the chemical dialog might also trigger a restructuring of the sea anemone epithelial mucus. As such, our results may challenge the traditional unidirectional chemical camouflage hypothesis [4]. In other words, both symbiotic partners each take a step towards the other to establish their mutualistic relationship. We hope that our study serves as a foundation for the design of other mechanistic studies to unravel this complex inter-kingdom interaction network between clownfish, anemones, and their bacterial symbionts.

List Of Abbreviations

AC: anemone control group

FC: fish control group

PI: physical interaction group

RI: remote interaction group

ASV: amplicon sequence variant

Declarations

Ethics approval and consent to participate

We abided by the protocol approved by the Comité de Protection des Animaux de l'Université Laval (CPAUL protocol # 2017-023-1).

Consent for publication

NA

Availability of data and material

All sequences are freely available in the SRA database (BioProject #: PRJNA532435).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EAG, FES and ND planned the experiment. EAG, FES and ND collected the samples. EAG built the rRNA amplicon librairies. EAG and SB did the bioinformatic processing of the rRNA sequences. EAG, FES and SB did the statistical analyses. EAG, FES, SB and ND wrote and revised the manuscript.

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Tables

Table 1: Pairwise ANOSIM and PERMDISP (betadisper) analyses results performed with unweighted UniFrac distances on bacterioplankton samples. All time points were used and grouped by either Anemone control water (ACW), Clownfish control water (FCW), Physical interaction water (PIW) or Remote interaction water (RIW).

ANOSIM:						
		Sample size	Permutations	R	p-value	q-value
Group 1	Group 2					
ACW	FCW	48	999	0.317494	0.001	0.001
	PIW	48	999	0.211762	0.001	0.001
	RIW	48	999	0.28269	0.001	0.001
FCW	PIW	48	999	0.232607	0.001	0.001
	RIW	48	999	0.349701	0.001	0.001
PIW	RIW	48	999	0.172127	0.001	0.001
PERMDISP:						
		Sample size	Permutations	F-value	p-value	q-value
Group 1	Group 2					
ACW	FCW	48	999	0.646777	0.408	0.612
	PIW	48	999	0.025679	0.884	0.884
	RIW	48	999	0.78066	0.363	0.612
FCW	PIW	48	999	0.414063	0.537	0.6444
	RIW	48	999	2.14882	0.141	0.612
PIW	RIW	48	999	0.979428	0.333	0.612

Table 2: DESeq2 analysis results performed on the normalized abundances of *C. thyrosinoxydans* related ASVs: For each time point (T0 to T5), all conditions/niches were compared pairwise. Differences with FDR adjusted q-values < 0.0001 were deemed as significant.

Niche	Comparison	ASV	T0	T1	T2	T3	T4	T5
			log2FC	log2FC	log2FC	log2FC	log2FC	log2FC
Anemone	AC vs API	ASV 2	-	-	-	-9,0	-	-
		ASV 49	-	-	-7,7	-13,0	-5,8	-
		ASV 177	-	-	-9,2	-10,7	-9,6	-
	AC vs ARI	ASV 2	-	-	-	-9,5	-8,1	-
		ASV 49	-	-	-8,0	-8,0	-7,0	-
		ASV 177	-	-	-9,5	-12,0	-10,2	-
Clownfish	FC vs FPI	ASV 2	-	-	-	-11,0	-11,1	-11,0
		ASV 49	-	-	-8,1	-11,8	-7,1	-4,9
		ASV 177	-	-	-9,4	-13,3	-12,7	-5,0
	FC vs FRI	ASV 2	-	-	-	-10,7	-11,3	-
		ASV 49	-	-	-7,7	-11,8	-7,3	-
		ASV 177	-	-	-8,8	-13,1	-12,9	-
Bacterioplankton	ACW vs APIW	ASV 2	-	-	-9,8	-13,7	-12,3	-12,4
		ASV 49	-	-	-9,1	-10,7	-12,3	-11,9
		ASV 177	-	-	-11,5	-10,7	-13,6	-13,7
	ACW vs ARIW	ASV 2	-	-	-11,8	-13,0	-12,8	-11,7
		ASV 49	-	-	-11,7	-10,0	-12,4	-11,2
		ASV 177	-	-	-13,6	-9,7	-14,1	-13,0
	FCW vs FPIW	ASV 2	-	-	-10,5	-	-12,6	-11,8
		ASV 49	-	-	-8,6	-	-12,6	-5,2
		ASV 177	-	-	-12,1	-	-13,9	-13,2
	FCW vs FRIW	ASV 2	-	8,9	-11,6	-	-	-11,9
		ASV 49	-	-	-10,1	-	-	-5,2
		ASV 177	-	-	-13,4	-	-	-13,3

Figures

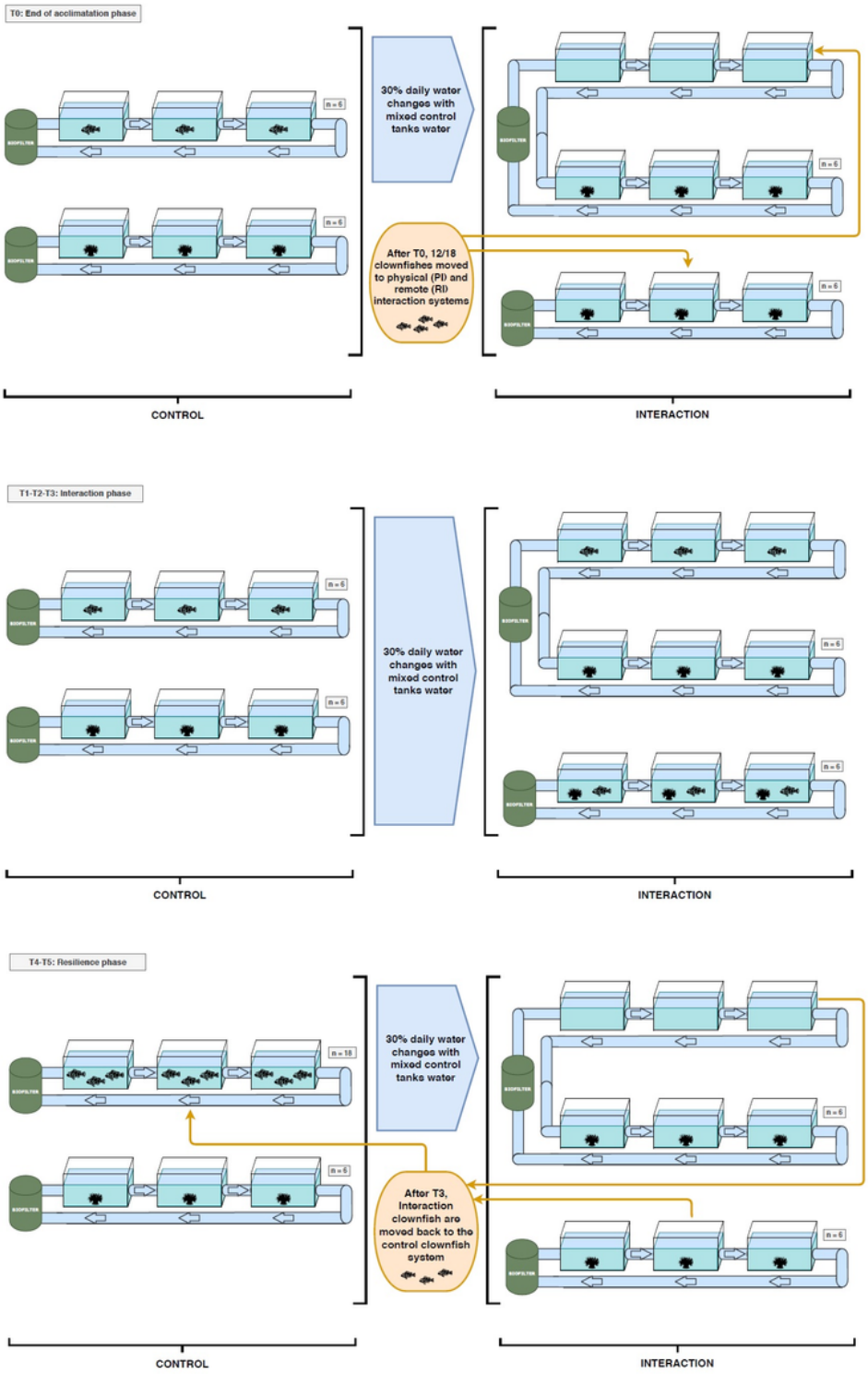

Figure 1

Experimental design: each experimental group, anemone control (AC, top left), fish control (FC, bottom left), physical interaction (PI, bottom right) and remote interaction (RI, top right), was replicated six times. a) Acclimation, three weeks until T0. b) Interaction, two weeks (from T1 to T3). c) Resilience, two weeks (from T4 to T5).

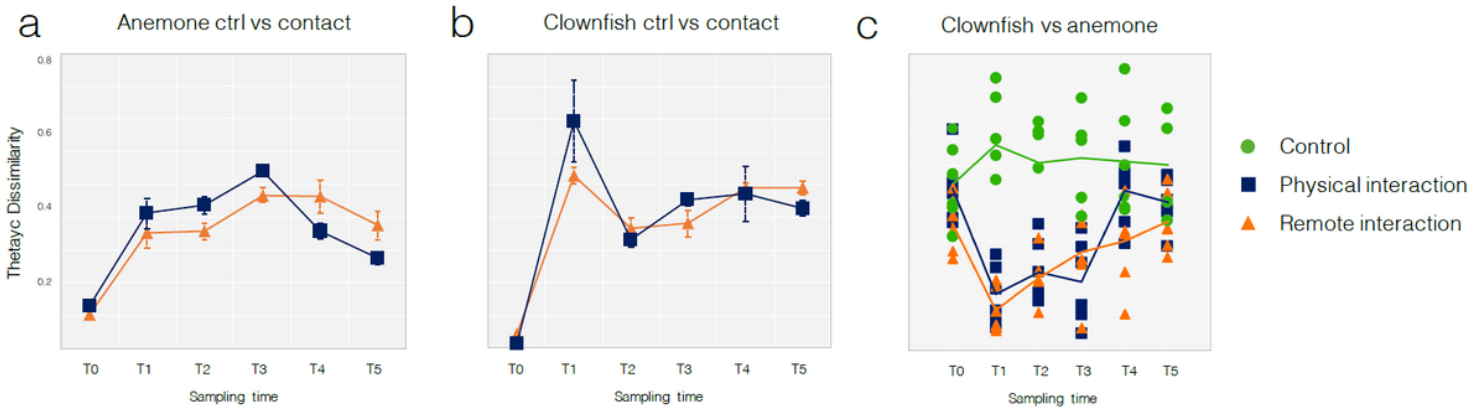

Figure 2

(a) Thetayc dissimilarity time plots between the epithelial microbiota of: (a) control anemones versus interaction (PI and RI) anemones, (b) control clownfish versus interaction (PI and RI) clownfish, (c) all clownfish and their associated anemone.

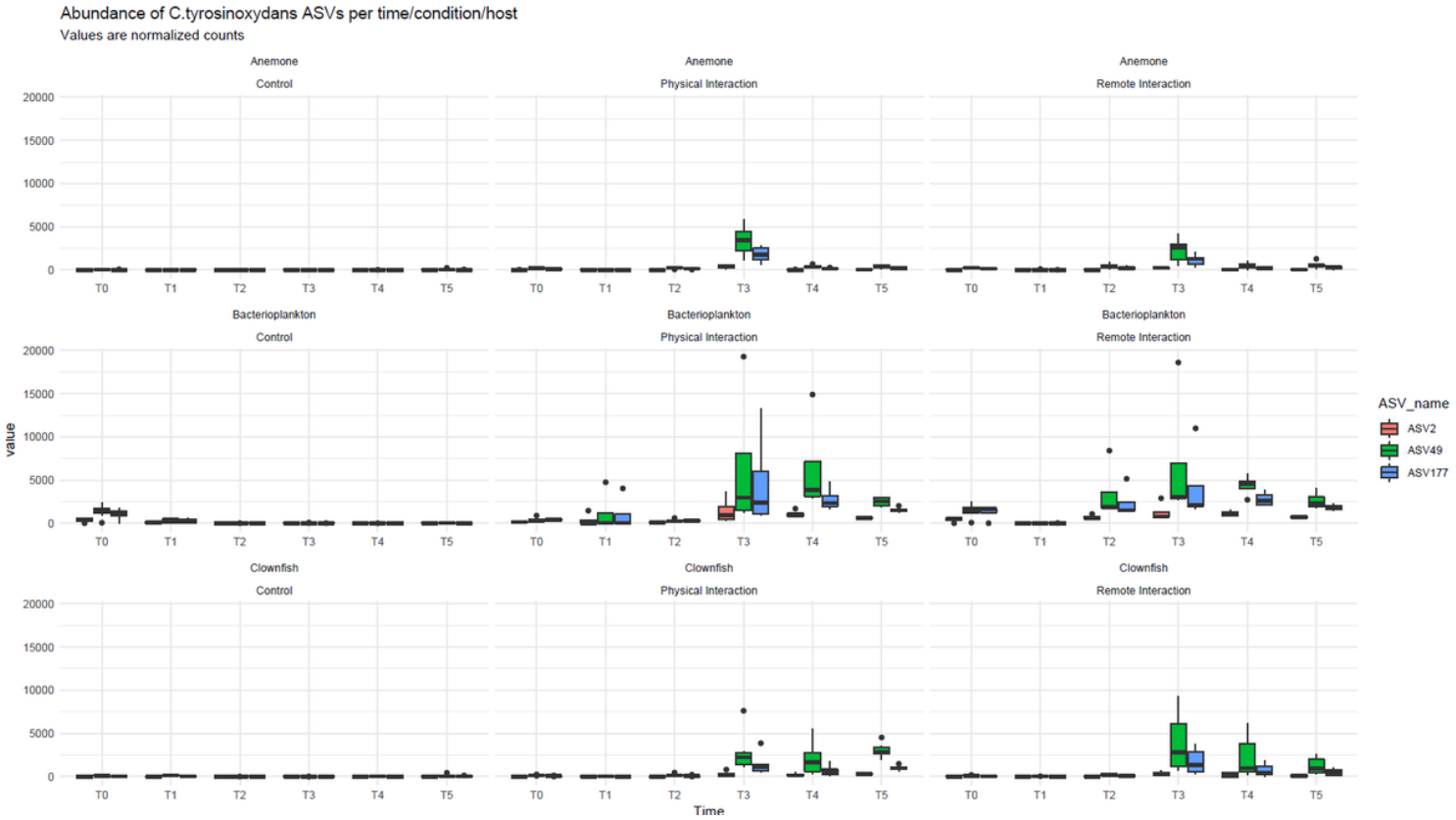

Figure 3

Abundance boxplots of C. tyrosinoxydans related ASVs per time point (T0 to T5). Titles above each boxplot specify the host (Clownfish/Anemone/Bacterioplankton) and the conditions (Control/Physical Interaction/Remote Interaction).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.pdf
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