The Extended Ecology of the Sharknose Goby Cleaner Fish

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

By

Kathryn Ellen Whittey (BSc)

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“….Mae cariad fel y moroedd

Sydd yn troi'n gymylau glaw

Yna'n disgyn ar fynyddoedd

Ac yn casglu yn y baw

Cyn llifo'i lawr y creigiau

Ar hyd llwybrau unig iawn

Ond bob un ddaw'n ôl i'r tonnau

Rhaid i'r gylchred fod yn llawn”

Tonnau, Bwncath, 2020

That is to say….

”*Learn how to see.*

Realise that everything connects to everything else”

Leonardo Da Vinci

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Statement of contributions

Where colleagues, students and research assistants have assisted with work presented in this thesis, their contributions are explicitly detailed below:

CHAPTER 2

Kathryn E. Whittey designed the experiment, formulised research hypotheses, collected in field behavioural data, collected video footage for 3D reconstructions of corals, standardised methods, created 3D models, analysed 3D models for data collection, analysed data (statistical analysis), trained and supervised project students and wrote the chapter. Dr Katie Dunkley, and Dr Amy Ellison assisted with *in situ* data collection. Patricia Turpin (President of Environment Tobago), Dr Ryan Mohammed and Environment Research Institute Charlotteville (ERIC) provided field facilities and field support. Grace C. Young and undergraduate placement students (Stephen Cheung, Molly Fairclough and Therese McAlister) helped with *in silico* data collection. Katie Dunkley assisted with statistical analysis.

CHAPTER 3

Kathryn E. Whittey designed the structures, designed the experiment, formulised research hypothesis, sought additional funds through crowdfunding, both oversaw and took part in all construction and deployment, trained and supervised volunteers, collected and seeded the structures with coral, designed data collection protocols, collected data *in silico,* analysed the data and wrote the chapter. Research assistant Lois Wynne-Williams provided field support including construction and deployment of artificial reef structures and assisted with *in silico* data collection. Dr Katie Dunkley assisted in procuring additional funds through a kickstarter crowdfunding campaign. Fish Hive design and prototype construction was supported by Fish Hive team (Robert Whittey, David Whittey, Lois Wynne-Williams and Wil Gritten), in field construction was supported by Lois Wynne-Williams, NEST (Devon Eastman and Andel Mackenzie) along with volunteers: Neil C. Cook, Amy Ludlow, Molly Fairclough, Tess McAlister and 2019 Cardiff University field course undergraduate students. Fish Hive deployment supported by Lois Wynne-Williams, NEST (Devon Eastman and Andel Mackenzie), captain Curtis Antione and ERIC (Environmental Research Institute Charlotteville - Weldon Mapp, Lanya Fanovich and Ryan Mohammed). In field collection of video footage was ERIC. Steve Cheung and Lois Wynne-Williams participated in *in silico* data collection.

CHAPTER 4

Kathryn E. Whittey designed the experiment, formulised research hypotheses, designed data collection protocols, collected samples, conducted bioinformatics, analysed the data and wrote the chapter. Dr Amy Ellison assisted with both fish collection in-field and *in silico* data analysis. Dr Sarah Christofoides provided support in statistical analysis. ERIC (Environmental Research Institute Charlotteville - Weldon Mapp, Lanya Fanovich and Ryan Mohammed) provided field support. Dr Daniela Rosado assisted with DNA extractions, Dr Raquel Xavier assisted with DNA library preparation and data analysis.

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List of Definitions

Cleaning behaviour – The act of a cleaner interacting with a client.

Mutualism – Mutually beneficial relationships (symbiosis) in which both partners gain some benefit from the interaction.

Cleaning stations - Localities on the reef in which cleaners carry out their cleaning service.

Third-party species - Other organisms not taking part in a mutualistic relationship but present to the interaction.

Microbiota – The collective term given to the microscopic biota inhabiting a space.

Microbial mutualisms – Mutually beneficial relationships between microbial organisms.

Photogrammetry - Using photography to obtain information about physical objects and the environment.

Structure-from-motion – The process of estimating the three-dimensional structure of a scene from two-dimensional images.

Rugosity – Measure of variations of amplitude in the height of a surface.

Chain and tape rugosity – A measurement derived from an in-situ method of evaluating terrain heterogeneity (rugosity).

Vector dispersion – A measure of uniformity of a surface by estimating the vector variance for all normal vectors of individual planar surfaces.

Fractal dimension – A complexity metric to assess surface complexity by the irregularity (or regularity) of geometries at different scales.

Coral heads – A protrusion of coralline material on a coral reef.

Fringing reef – A coral reef which fringes the shoreline. One of three main types of coral reefs (atoll, barrier and fringing).

Refuge size category – A measure of the size of refugia available for marine organisms. See Gratwicke and Speight (2004) for more detail.

Coral bleaching event – When the water temperature rises significantly causing corals to expel their symbiotic zooxanthellae leaving the corals to turn white i.e. bleaching.

Degraded reef – A coral reef which has undergone significant degradation (through bleaching or pollution for example) and has undergone a range shift and is no longer dominated by live corals.

Coral ecosphere – the “near-coral” seawater environment, distinct from the surrounding seawater.

Benthic-pelagic coupling – The exchange of energy, mass or nutrients between benthic and pelagic habitats.

Contents

Thesis Summary

Far from being specialised occurrences, symbiotic relationships including mutualisms, are ubiquitous. The coral reef ecosystem is the product of a mutualistic symbiosis between corals and their endosymbiotic zooxanthellae. The coral polyp creates a sheltered space for the zooxanthellae, while the zooxanthellae provide nutrients to the host through photosynthetic activity. This mutualism has allowed a diverse range of coral animals to evolve and together multiple species of corals create a highly complex 3-dimensional habitat. The plethora of available habitat space has facilitated the evolution of multiple species and, as such, coral reefs are one of the most biodiverse habitats on Earth. In addition, the diversity on coral reefs has facilitated the evolution of a multitude of behavioural niches. Cleaner fish mutualisms are perhaps one of the best-studied interactions on coral reefs. However, the role that the coral habitat plays in facilitating the cleaner-client mutualism is often overlooked and how specific habitat traits affect cleaning behaviour is unknown. This thesis firstly, quantifies the structural components of the cleaner fish's (*Elacatinus evelynae*) coral habitat and demonstrates that cleaners more frequently inhabit tall corals and that structural complexity increases cleaning interactions (**Chapter 2**).

Unfortunately, due to anthropogenic global change, coral habitat is threatened and corals are disappearing. It is therefore timely that we discover what important attributes corals may contribute to marine inhabitants. Further to this, since the extent of the loss is so vast, multiple studies have sought to artificially replicate coral structure. Although artificial reefs are being widely deployed it is not fully understood how fish respond to these artificial structures at an individual level. Thus, this thesis investigates the behavioural interactions of fish species with novel artificial structures, finding that damselfish associate with artificial structures more than natural corals, potentially due to the increased space available for algae farming (**Chapter 3**). In addition to the structural element, corals harbour microbial communities that are also threatened by global change. Commensal microbial communities are integral to host health, yet there is little known about these communities in wildlife populations. Fish harbour a diverse microbiota on their skin, and this mucosal layer is in constant contact with organisms in the marine environment; as such it is possible that microbes may be shared between closely interacting individuals. This is particularly relevant to the sharknose goby (*E. evelynae*), which spends the majority of its time in direct contact with the coral cleaner station. Therefore, the microbial communities of the sharknose goby and its habitat were characterized and intriguingly, cleaners share bacterial genera with *Palythoa caribaeorum*, a common but toxic

benthic constituent of cleaner fish stations (**Chapter 4**). Finally, to further knowledge of microbial communities of the sharknose goby, the gut and skin communities are described and using microbial gene predictions several genes associated with toxicity in other fish species are identified (**Chapter 5**).

In summary, this thesis investigates a range of species interactions at multiple scales, from fish and coral habitat interactions to the association of fish with coral bacteria. This thesis furthers our knowledge of how individual fish respond to artificial reefs and demonstrates key aspects of *E. evelynae* ecology.

Chapter 1

General introduction

Mutualisms and species interactions

Organisms tend to live in complex interconnected relationships (McFall-Ngai et al. 2013; Bronstein 2015). Mutually beneficial relationships – mutualisms (relationships between two or more organisms in which all parties benefit from the interaction) - enable organisms to exist in a range of oligotrophic environments such as deserts, tundra or tropical waters and exist in every kingdom (Alexander et al. 1978; Bordeleau and Prévost 1994; Dillon and Charnley 2002; Friesen 2012; Roth 2014). For example, the nutritional mutualism that exists between a cnidarian polyp and its zooxanthellae represents the fundamental origin of the coral animal and has allowed coral reefs to evolve in oligotrophic waters (Roth 2014; Bronstein 2015). The coral polyp cannot survive without its symbiotic photosynthesising zooxanthellae, which provide up to 90% of the coral's energy (Iluz and Dubinsky 2015). In turn, these polyps grow into huge colonies forming complex structures, which provide further habitat space for a diversity of marine species (Reaka-Kudla 1997). Many other organisms live in close proximity, even direct contact, with corals which can confer a range of benefits to their coral hosts including nutrient provision (e.g. ammonium, nitrogen and phosphorus, Hasegawa et al. 1982), sediment removal to prevent smothering (Stewart et al. 2006) and enhancement of growth (Holbrook et al. 2008). Branching corals (*Pocillopora genera*) which host damselfish, for example, grow significantly more than non-hosting corals, and the bigger the damselfish, the greater the hosting coral growth rate (Holbrook et al. 2008).

One of the most well-studied mutualisms on coral reefs is that of the cleaner fish. These brightly coloured fish are highly conspicuous on coral reefs and their mutualistic cleaning behaviour has been studied extensively (e.g. Limbaugh 1961; Cheney et al. 2009; Vaughan et al. 2017; Dunkley et al. 2020; Caves 2021). Cleaners remove parasites from the body of visiting client fish (Arnal and Côté 2000) at such a high rate that they lower parasite numbers on reefs (Grutter et al. 2018). This behaviour lessens the adverse effects of infections in clients whilst in return provides nutrition to the cleaners (Soares et al. 2011; Oliva-Teles 2012; de Jesus et al. 2018).

Coral reef habitats

Under suitable environmental conditions, corals are able to grow prolifically, forming massive aggregations (Knowlton et al. 2010; Swart 2013) which create highly productive ecosystems (Reaka-Kudla 1997). Numerous coral species growing in close proximity create a plethora of 3-dimensional habitat spaces, which are essential for the survival of reef-inhabiting species, thus creating biodiversity hotspots (Kostylev et al. 2005; Graham and Nash 2013). Despite providing a habitat for 25% of marine fish, globally, coral reefs cover as little as 0.2% of oceanic area, primarily existing in the warm shallow waters of the tropics (Reaka-Kudla 1997). Anthropogenic climate change, industrialisation such as urbanisation of coastlines, pollution and overfishing, have led to reductions in the ecological margins in which these coral species thrive (Carpenter et al. 2008; Foley et al. 2010; Anthony et al. 2015). Corals are experiencing increased frequencies of epidemics, even pandemics (Li and Reidenbach 2014). Bleaching events, which cause corals to lose their photosymbiotic algae and eventual colony mortality, are predicted to become an annual phenomenon (Maynard et al. 2015; Putnam et al. 2017). An estimated 27% of global coral cover has been lost since the mid 1980's (Sutherland et al. 2004) and in the Caribbean, mass coral mortality events over the last 30 years have significantly reduced live coral cover by approximately 80% (Gardner et al. 2003).

Microbial mutualists

The advent of Next Generation Sequencing has provided great insights into microbial mutualisms and uncovered interconnectivities between a variety of microbes (including bacteria, archaea, fungi) and their multicellular eukaryotic hosts (Vanwonterghem and Webster 2020). All animals have evolved both from, and in the presence of, a plethora of microorganisms (McFall-Ngai 2013) and consequently are colonised both externally (skin, scales, fur etc) and internally (digestive system, reproductive system, mucosal layers) by communities of them. Although more and more evidence is demonstrating the importance of the many groups of microorganisms, the focus here in this thesis is on the interactions between one group of microorganisms (bacteria) and animal hosts. In their animal hosts, bacteria facilitate and control many host functions ranging from nutrient acquisition, immunity, fighting parasitic infections, and even behaviour (Gómez and Balcázar 2007; Hayes et al. 2010; Chung et al. 2012; Ezenwa et al. 2012; Tarnecki et al. 2017). These microbes are acquired over time, throughout an individual's lifespan from external and internal sources, including vertical transfer from parents to offspring, although transmission routes are not yet fully understood (Browne et al. 2017; Quigley et al. 2019). Microbes can also be shared between individuals occupying the same habitat (Burns et al. 2017; Vanwonterghem and

Webster 2020), for example, humans and their pets (Song et al. 2013), baboons and their social partners (Tung et al. 2015) and anemone fish and their anemones (Pratte et al. 2018). Thus, mutualisms exist at different spatial scales from conspicuous relationships between coral reef fishes, to microbes inhabiting the mucosal layers of their hosts.

Broad objectives of the thesis:

The mutualistic, dedicated cleaner fish, the sharknose goby (*Elacatinus evelynae*) (Fig.1.1) uses coral habitat to advertise its cleaning behaviour at locations termed cleaning stations (Vaughan et al. 2017) and is therefore dependent on the coral habitat for its nutrition. Understanding the relationship this species has with the structural elements of the coral habitat can further our understanding of the importance of reef structure in the broader context of coral reef ecology. Further, knowing what geometric aspects of the habitat are useful to coral reef fish can help inform artificial reef design (Belhassen et al. 2017). Given that reefs are threatened, exploration of the interactions of coral reef fish with their habitat, including artificial structures will be key to conservation efforts (Paxton et al. 2020b). To investigate the importance of coral structure for coral reef fish, this thesis first investigates the relationship between cleaners (including cleaning behaviour) and the structural elements of their natural coral cleaning stations. Secondly, using artificial reef structures and behavioural observations, the interaction of coral reef fish with natural corals versus artificial structures are explored to provide insights for future conservation efforts using artificial reefs.

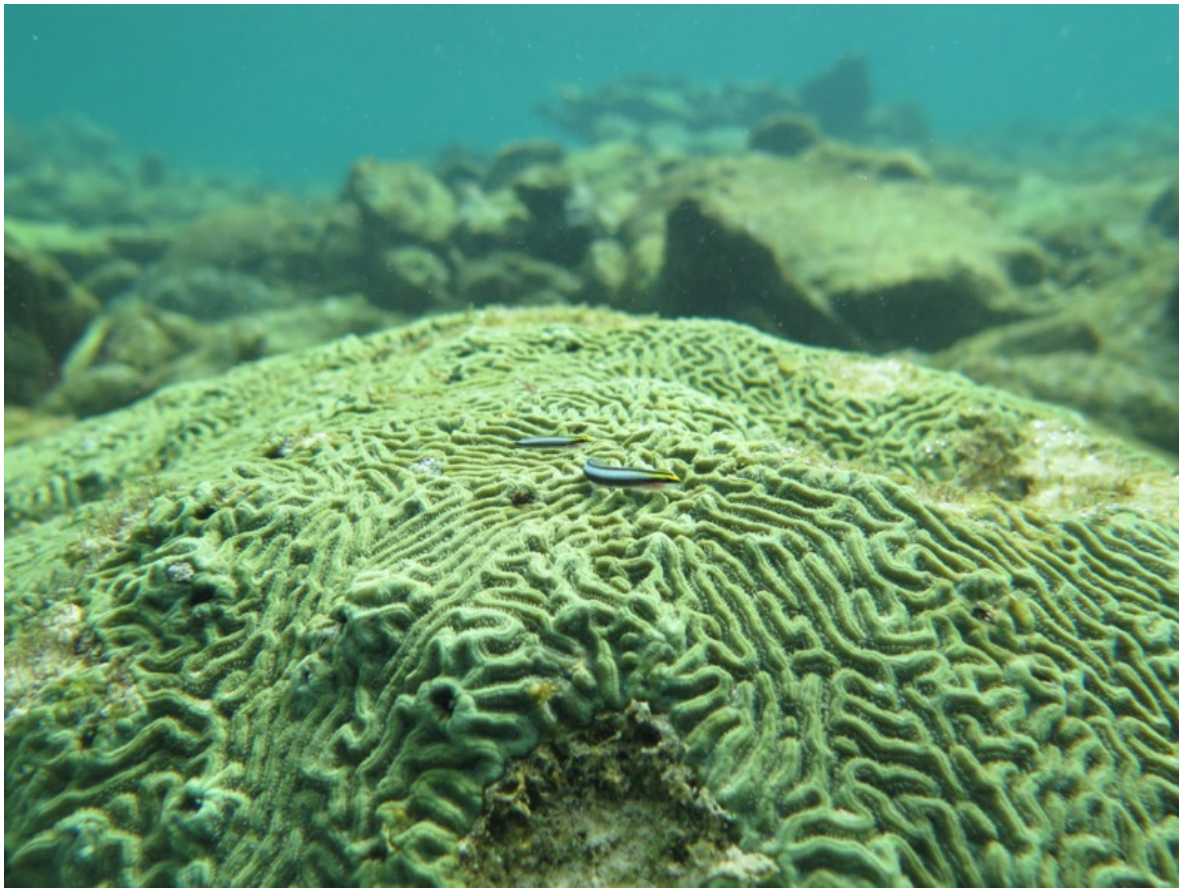

Figure 1.1: Sharknose goby cleaner fish (*Elacatinus evelynae*) on their ‘ cleaner station’: here a brain coral (Faviidae spp.) cleaner station on Booby Reef Man O’War Bay, Tobago. Sharknose goby cleaner fish will wait at their stations in direct contact with the station awaiting the arrival of clients, other reef organisms, which visit to be cleaned.

Corals and their associated fish inhabitants harbour their own microbial communities, but the microbiota of many corals and coral reef fish are still undefined (Chiarello et al. 2020; Vanwonterghem and Webster 2020), and given that it is widely accepted that microbes play an important role in host health (Egerton et al. 2018; Gomez and Primm 2021) this thesis also investigates the microbial communities of the cleaner. Cleaner fish gut and skin microbiota are characterized here and using functional analysis, the inferred gene functions of the microbial communities are explored. To further our understanding of cleaners’ connectivity to their habitat, this thesis also investigates the bacterial communities of the cleaners’ coral habitat, specifically the constituents of the cleaner station.

Coral reef habitat structural complexity, digital corals and artificial reefs (Chapters 2 and 3)

Corals are divided broadly into soft corals (Alcyonacea) such as sea fans (Gorgonians) and hard corals (Scleractinia) (Won et al. 2001). Scleractinians are the most important group of reef building species and, as such, described as principal ecosystem engineers (Feary et al. 2007; Harper 2008; Weil and Vargas 2010; Wild et al. 2011; Wallace et al. 2017). Scleractinians grow by depositing calcareous material creating hard exoskeletons which form the rocky framework of the reef (Chamberland et al. 2017). The structural complexity of corals is linked to species diversity and abundance (Gratwicke and Speight 2005; Graham and Nash 2013; Gonzalez-Rivero et al. 2017; Sánchez-Caballero et al. 2017). Consequently, coral reefs and their associated fauna are of significant socio-economic value for food, coastal protection, and tourism (Moberg and Ronnback 2003), with an estimated value of over USA$375 billion per annum (Pandolfi 2005).

Structural complexity has previously been monitored using 2-dimensional tools to study species assemblages in association with the shape of reefs (Gratwicke and Speight 2005). Previously, collecting 3-dimensional (3D) data in the field was, at best, time consuming and frequently unfeasible (Goatley and Bellwood 2011; Dustan et al. 2013). Thus, methods such as Habitat Assessment Scores (HAS), a visual score of structural variables (Gratwicke and Speight 2005), and chain-and-tape rugosity (a measure of small-scale variations of amplitude in the height of a surface measured, by laying a chain or tape over the reef topography) are frequently used to capture structural complexity (Luckhurst and Luckhurst 1978). These simplistic, methods have revealed positive correlations between habitat complexity and fish assemblages where diversity increases with increasing structural complexity (Gratwicke and Speight 2005). However, simplistic methods are not suitable for addressing finer-scale ecological questions (McCormick 1994; Harborne et al. 2012), such as relationships between corals small-scale crevices which provide space for nesting, foraging and spawning (Robertson and Sheldon 1979) at a reef-wide scale. The development of 3D digitalised models offers a means of measuring habitat complexity at a fine scale over a large area with far less investment in time (Storlazzi et al. 2016; Young et al. 2017). In addition, advances in computer vision and specialist 3D software have led to the development of the Structure-from-Motion (SfM) technique (photogrammetry), whereby video footage collected in the field can be used to render (create) 3D digital reconstructions of corals, here-on referred to as ‘digital corals’. These digital corals can then be used to derive structural complexity metrics such as rugosity (small-scale variations of amplitude in the height of a surface) , vector dispersion and fractal dimension(complexity measures – uniformity in angles of a surface) at

a far finer scale with a marked reduction in *in situ* dive time requirements (Raoult et al. 2017; Young et al. 2017).

Due to anthropogenic stressors, coral structural complexity is being lost, and thus the habitat space for associated marine fauna including fishes and benthic organisms (Moberg and Folke 1999; Magel et al. 2019; Seraphim et al. 2020). Replacing natural complexity through artificial reefs may mitigate some of the habitat loss. Furthermore, incorporating artificial structures that replicate natural structural complexity into marine infrastructure may offset some of the damage caused by coastal urbanisation (Morris et al. 2018). Such eco-engineering projects to replace lost corals are gaining in popularity (Dafforn et al. 2015; Riera et al. 2018), and the ability to accurately replicate coral structure through 3D modelling led to the proposal of 3D printing corals (Ruhl and Dixson 2019). Despite increased deployment and use of artificial reefs in conservation, many ecological functions of these structures remain unknown (Spieler et al. 2001; Paxton et al. 2020b; Paxton et al. 2020a; Seraphim et al. 2020). Expansion of marine infrastructure is inevitable due to increasing coastal populations and an increasing pressure on marine systems to provide food resources (Hinrichsen 1999; Bulleri and Chapman 2010; Hernandez-Delgado 2015; Morris et al. 2018). If marine infrastructure is to be tailored to meet human demands whilst also providing habitat space for wildlife, much more research needs to be done to understand what key features of the habitat are important to marine life (Brochier et al. 2021). Research on the impacts of artificial reefs have largely focused on their effects on species assemblages with little focus on ecological processes (Chapman and Underwood 2011).

Importance of microbial mutualisms in coral reef fish (Chapters 4 and 5)

The field of teleost microbiota is expanding, particularly bacterial microbiota (Llewellyn et al. 2014; Soares et al. 2018) and in addition to the gut community, understanding the microbial communities of other organs such as the skin and gills, and what drives their composition, can provide useful insights into the functioning of an organ which is a primary barrier against pathogens (Pérez et al. 2010; Legrand et al. 2017; Legrand et al. 2019). This is particularly relevant for marine organisms such as fish which share the ocean with a huge array of microorganisms with which they are in direct and constant contact (Eakins and Sharman 2010; Apprill 2017). The composition of fish skin microbiota, although less studied than the gut microbiota, is known to be species-specific (Larsen et al. 2013), exhibits differences based on host location (Wilson, 2008) and can be altered by parasitic infection (Zhang et al. 2018), as well as an array of environmental factors including exposure to light (Ellison et al. 2021), water pH (Sylvain et al. 2016) and environmental physiochemistry and bacterioplankton community structure (Sylvain et al. 2020).

Wild animals offer an opportunity to examine natural host functions such as microbial communities, immunology and behaviours, in an interplay with the natural environment (Pascoe et al. 2017). On coral reefs, cleaner fish maintain a mutualistic relationship with many different species of clients (*Elacatinus evelynae;* Dunkley et al. 2019), removing parasites in addition to mucus and diseased skin (fish and shrimp species; Cote 2000; Vaughan et al. 2017). During these cleaning interactions the cleaner is in direct contact with potentially diseased or parasitised individuals (multiple species Soares et al. 2018; (*Elacatinus prochilos*) Xavier et al. 2019). Previously, cleaning behaviour has been overlooked as a mechanism for parasitic and microbial transfer and reports of cleaner fish harbouring parasites themselves are rare ((*Labroides dimidiatus*) Jones et al. 2004; Narvaez et al. 2021). However, a new appreciation of cleaners as potential vectors of disease has shown that some species of cleaners such as Indo-Pacific cleaner wrasse (*Labroides dimidiatu*s), are themselves parasitised (Narvaez et al. 2021) and others (broadstripe cleaning goby, *Elacatinus prochilos*) may potentially harbour greater abundances of potential pathogenic bacteria (Xavier et al. 2019).

Specific objectives:

Mutualistic relationships span life at all spatial scales from microbes to reefs (McFall-Ngai et al. 2013; Bronstein 2015). Using a multidisciplinary approach, this thesis investigates mutualistic relationships on coral reefs at these different spatial scales. The cleaning behaviour of the sharknose cleaner fish *E. evelynae,* its relationship with the habitat and its microbiota are explored in this thesis. As such there is a certain degree of overlap of information regarding *E. evelynae* ecology throughout the thesis. Each chapter is therefore self-contained.

Within this thesis I aim to:

i) Quantify coral habitat metrics of the sharknose goby (*E. evelynae*) cleaner station using photogrammetry and test whether these attributes affect cleaning behaviour (Chapter 2).

ii) Investigate the local behaviour of coral reef fish around newly deployed artificial structures (fish hives, novel structures, designed and constructed for this thesis) compared to established natural corals to further our understanding of species interactions with their habitat (Chapter 3).

iii) Using metataxonomics, characterize the microbial communities of the sharknose goby (*E. evelynae*) skin microbiota and compare these to those of the common benthic constituents of the cleaner station (including corals, sea water and other benthic organisms associated with the station such as zoanthids) (Chapter 4).

iv) Using metataxonomics, characterize and compare the bacterial communities of sharknose goby (*E. evelynae*) gut and skin microbiota and infer the functions associated with these communities (Chapter 5).

Chapter 2

Microhabitats of sharknose goby (*Elacatinus evelynae*) cleaning stations and their links with cleaning behaviour

Abstract

Coral reefs are renowned for the complexity of their habitat structures and their ability to host more species per unit area than any another marine system. Dedicated cleaner fish, which acquire all their food resources through client interactions, rely on both the habitat structures (by using topological cleaning stations) and the wide diversity of fish species available on coral reefs, to survive. As a result of natural and anthropogenic threats, coral reef habitat structures and their complexity are being lost, yet despite this threat it is unclear how important reef geometry is to key ecological interactions, like cleaning. Using an established Caribbean reef study site, three-dimensional constructions of discrete coral heads were used to investigate how fine-scale structural complexity traits (structural complexity - measured by rugosity and vector dispersion - height, volume, surface area, percentage live coral cover and refuge availability) relate to cleaner occupancy, abundance and their cleaning interactions with clients. Coral height was a particularly important trait for cleaning, correlating with both the occurrence of cleaning stations on a reef, and with increased cleaning durations and reduced cleaning frequencies/rates. Cleaning stations were also more structurally complex than non-cleaning coral heads, and the increased availability of uneven surfaces (creating cracks and crevices) and refuge availability linked with increased cleaning durations/rates. By understanding habitat features important to cleaner fish on a typical Caribbean fringing reef, we can gain a better understanding of how important reef geometry might be for governing the occurrence and dynamics of such mutualisms.

Introduction

Coral reefs are renowned for their complex physical three-dimensional structure (termed ‘structural complexity’; Graham and Nash 2013). Scleractinia stony corals, which produce hard exoskeletons, are ecosystem engineers and are largely responsible for the structural complexity of coral reefs (Jones et al. 1994; Wild et al. 2011). Corals’ high structural

complexity provides a plethora of different microhabitats (as the different structures interlock, they create space) (Crowder and Cooper 1982), ultimately creating one of the most diverse and abundant habitats in the world (on par with rainforests; Reaka-Kudla 1997). Within a reef environment, different microhabitats influence the spatial distribution of species (*Stegastes planifrons* Tolimieri 1995) since many species show specific microhabitat preferences (review of multiple species see Booth and Wellington 1998; *Elacatinus lori* Majoris et al. 2018a), exhibit high site fidelity (*Pomacentrus moluccensi* Streit and Bellwood 2018) and form close associations with certain microhabitat types (*Pomacentrus moluccensi* Boström-Einarsson et al. 2018). However, finer scale variations in individual coral morphology (e.g. height and substrate heterogeneity) may also promote differences in fish species spatial distributions as a result of altered microhabitat – a concept which so far has received little attention. Since coral structural complexity is under threat from anthropogenic climate change and industrialism (Munday 2004), it is vital to determine the importance of small-scale variation in coral morphology for reef species. It is particularly important to determine species interactions in Caribbean reefs (as opposed to Pacific reefs) were a higher proportion of reefs are on a trajectory to collapse from various anthropogenic factors (Bellwood et al 2004).

Structural complexity can influence the outcomes of ecological interactions (Grabowski and Powers 2004; Vergés et al. 2011) (e.g. predation: Crowder and Cooper 1982; Grabowski and Powers 2004; competition: Petren and Case 1998), and herbivory: Vergés et al. 2011), with the magnitude of the effect potentially varying with the degree of complexity (Grabowski and Powers 2004). This is intriguing and may help further knowledge on the dynamic nature of a classic mutualistic relationship; cleaner-client interactions, which are ubiquitous on coral reefs (White et al. 2007). Cleaning involves a cleaner removing parasites and debris from the body of another species, termed a client (Feder 1966). Dedicated cleaner fish (formerly termed obligate; Vaughan et al. 2017) gain all their nutrition from client derived material, associate strongly with cleaning stations. Cleaners wait at their cleaning stations for clients to visit them, and it has been shown that associating with a cleaning station, rather than wandering across a reef, promotes increased cleaning interactions (Oates et al. 2010; Dunkley et al. 2018). However, despite a wealth of knowledge on the ecology of cleaner-client interactions, microhabitat characteristics of cleaning stations are poorly defined. Stations can be cryptic and have been referred to as ‘particular ecological situations’ (Limbaugh 1961; Youngbluth 1968), which may include corals, anemones or sponges, collection of rocks, and or depressions in the benthos (Limbaugh 1961; Losey 1974; Johnson and Ruben 1988; Sazima et al. 1999; Cheney and Côté 2001; Huebner and Chadwick 2012). Since substrate type can influence the frequency and duration of cleaning interactions (e.g. coral versus sponge; Whiteman and Côté 2002), in addition to the fine-scale distribution, movement, density and diversity of potential clients (Ferreira et al. 2001; Graham and Nash 2013; Ferrari et al. 2018),

localised variation in coral morphology may also be expected to influence localised variations in cleaning dynamics.

Traditional methods for quantifying structural complexity, like the chain/tape transect method and Habitat Assessment Scores (Gratwicke and Speight 2005; Wilson et al. 2007) are now being replaced by digital three-dimensional modelling. Such modelling allows for the *in silico* quantification of habitat complexity traits (e.g. substrate heterogeneity, measured as rugosity and vector dispersion; (Storlazzi et al. 2016; Gonzalez-Rivero et al. 2017; Young et al. 2017); volume and surface area (Ferrari et al. 2017; Raoult et al. 2017); coral cover; (Gonzalez-Rivero et al. 2017), and coral growth; (Lange and Perry 2020), which provides finer scale measurements for addressing ecological questions (Storlazzi et al. 2016). Indeed, these techniques have already advanced our understanding of the relationships between varying structural complexity traits and reef fish assemblages (Price et al. 2019), and identified microhabitat types that promote invasive lionfish (*Pterois volitans*) aggregations in the Caribbean (Hunt et al. 2019). The high resolution of such techniques facilitates quantification of finer scale variations in coral morphology within a reef environment.

Here, how different coral morphologies promote variation in the occupancy and cleaning patterns of the predominant dedicated Caribbean cleaner, the sharknose goby (*Elacatinus evelynae*) were investigated. Using a structure-from-motion approach (Reichert et al. 2016; Ferrari et al. 2017; Young et al. 2017), three-dimensional models of discrete Faviidae coral heads on a reef in Tobago were constructed and microhabitat traits (e.g. rugosity, height and volume) were quantified. It was then determined whether these traits distinguished coral heads (individual discrete coral colonies) utilised as cleaning stations versus those that have never been observed as cleaning stations across 8 years of long-term study (see Dunkley et al. 2019). Subsequently, for cleaning station coral heads, the hypotheses that microhabitat features link to cleaner occupancy distributions and cleaning behaviours (in terms of frequencies, durations and rates) were tested. Together, this study aimed to quantify which microhabitat features define a cleaning station.

Materials and methods

Study site, occupancy, and behavioural observations

The study was conducted on Booby Reef in Man O' War Bay, Tobago (11°19.344′N, 060°33.484′W). The site constitutes a fringing reef dominated by non-branching brain coral species (Faviidae), areas of patchy sand, remnants of dead elkhorn (*Acropora palmata*) and staghorn (*Acropora cervicornis*) corals. For this study, sharknose goby (*Elacatinus evelynae*) cleaning stations were defined as specific localities on the reef used by cleaners for performing their cleaning activities: all cleaning stations were based upon Faviidae coral heads. Corals

were not identified to a species level due to the difficulties associated with visual species-level identification (Todd 2008; Forsman et al. 2009). Within a 70 m by 60 m section of the reef, known cleaning station coral heads (from 8 years of long-term study; (Dunkley et al. 2019a), n = 55 cleaning stations) were marked, along with an additional 12 control Faviidae corals. These control corals have never been observed to be occupied by cleaners across eight years of fieldwork at this site (long-term study detailed in Dunkley et al. 2019a).

Cleaner abundance at cleaning stations was quantified using presence/absence surveys (n **=** 1549 surveys, mean ± S.E. surveys per cleaning station = 28.16 ± 1.34, S.E.) over a 6-week period in May to July 2016 by daily snorkelling between the hours of 0830 and 1730 hrs. For each survey, trained observers (n = 6) searched for cleaners at a marked coral head, and in the close vicinity (~ 2 m^3 area), for up to 2 minutes – individual cleaners show strong site fidelity to their cleaning stations (Whiteman and Côté 2002; Harding et al. 2003). A cleaner occupancy value was subsequently assigned to each cleaning station, where cleaner occupancy was defined as the proportion of observations where one or more cleaners were present at the station (range: 0 – 1).

Between presence/absence surveys, data were also collected on the cleaners' cleaning behaviour using 10-minute focal observations (n = 223 observations, mean number observations per cleaning station across 34 cleaning stations ± S.E. = 6.56 ± 0.52, range: 3 – 13 observations per station). For each observation, stations were randomly sampled throughout the day and one cleaner was randomly selected from their coral head, and the duration and frequency of cleaning interactions with clients was recorded. Cleaning frequencies, durations and cleaning rate were thus used as a measure of cleaning behaviour. The frequencies and durations represent the total effort in cleaning whilst rates represent this effort per cleaning time (i.e. total cleaning frequency/total cleaning duration). Although clients tolerate closer human approaches when being cleaned (Giglio et al. 2020), snorkelers maintained a 1 m distance from the cleaners during observations.

Three-dimensional digital coral data collection

To create three-dimensional models of the cleaning stations (n = 55) and control corals (n = 12), video footage was collected with underwater cameras (Olympus GT-4) mounted on monopods, using 1080p resolution and medium sharpness. The physical boundaries of a station were defined as discrete coral head(s) that were not connected to other reef sections (Fig. 2.1). Filming occurred under ambient light, whilst snorkelling at depths of 1 – 3 m. At each cleaner station, a cube (6.4 cm^3) was placed adjacent to the coral to serve as a scale. The filming process (adapted from Gutierrez-Heredia et al. 2016) was carried out by swimming slowly, in a spiral motion, starting from the top of the coral and moving down towards the base at the seabed whilst changing the camera angle from (i) top-down (parallel to the seabed), (ii)

at 45° to the coral and seabed, and (iii) planar to the coral. To capture fine-scale spatial features of the coral the filming procedure was repeated for each coral head (station and controls) at two different distances: firstly, with the whole coral in full frame, and then, secondly, moving closer (~ 50 cm from the coral). This videoing process was repeated three times for each coral to obtain clear, un-obstructed frames in 360°, thus accounting for error in videos from obstruction from floating debris and marine life. The duration of each video correlated with the size of the coral head: larger corals were filmed for longer. This created more images for model reconstruction to ensure quality was not lost as a result of increased coral size. Together, our video recording and processing methods created a standardized approach (e.g. across different coral head sizes and light conditions (Raoult et al. 2017). Video footage was converted to still images using QuickTime™ Player 7.6.6 at an extraction rate of three images per second, resulting in 100 – 500 images per station with a resolution of 1920×1080p. Image sequences were then imported into PhotoScan Standard (Agisoft 2021). Coral models were rendered following the standard workflow sequence in PhotoScan: alignment, dense point cloud generation, mesh building and texture building. Each step was set to medium quality except in the mesh building step where 'meshes maximum face count' was adjusted to 3,000,000 (previously shown to render high resolution models by Young et al. 2017). Final models were compared to still images of corals taken with an Olympus GT-4 camera at four different angles to control for geometric distortion.

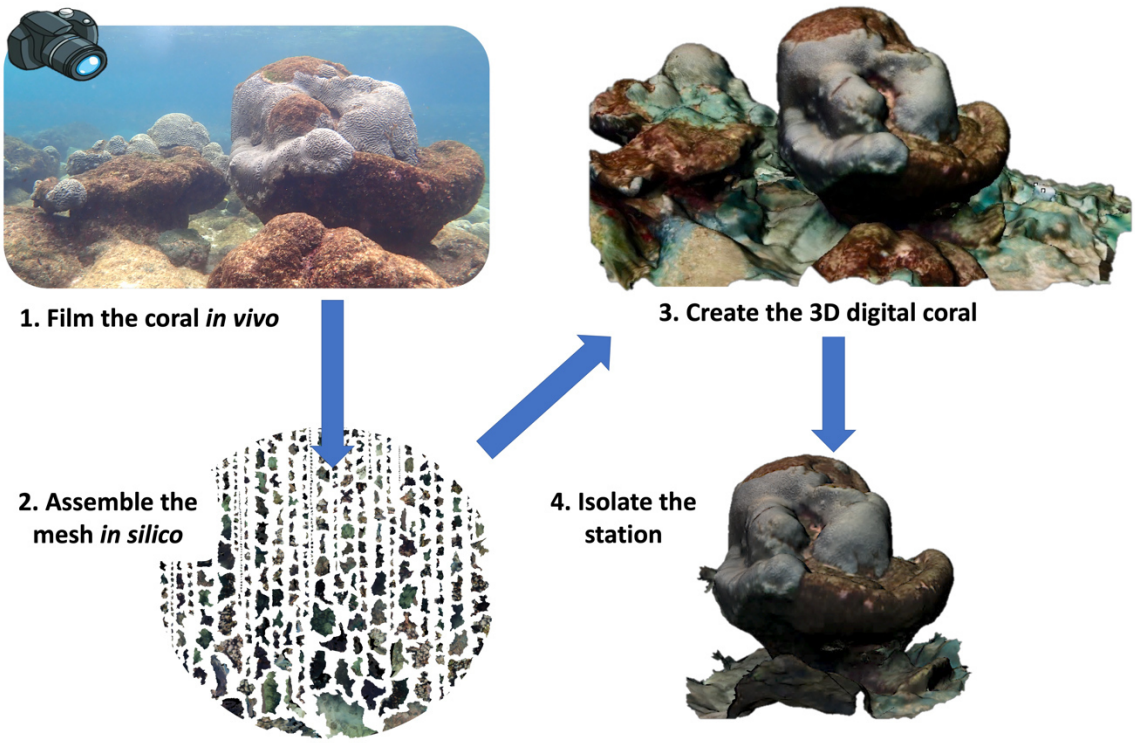

Figure 2.1: 3D digital workflow to show quantification of a control and sharknose goby (*Elacatinus evelynae*) cleaning station coral microhabitat traits on Booby Reef, Man O' War Bay, Tobago. Workflow: (1) *In vivo* filming , (2), assembled into mesh chunks in PhotoScan Standard using default settings (3) 3D model creation in Rhinoceros 3D (see: https://youtu.be/Hy1e0D4USdU), and (4) trim to cleaning station only, here a discrete coral head (one individual colony) by excluding the surrounding reef.

Quantifying habitat traits of digital corals

For each coral head (cleaning stations and control coral), the following metrics were quantified: height (cm), volume (cm^3), surface area (cm^2), linear rugosity (surface roughness/heterogeneity), vector dispersion (another measure of structural complexity; (Young et al. 2017), percentage live coral cover and refuge size category (the approximate measure of the size of spaces available for marine organisms to use as refugia) following Gratwick and Speight (2005). All *in silico* measurements were recorded using Rhinoceros 3D (Robert McNeel & Associates). Coral dimensions (height, width and depth) were obtained from three-dimensional models using the 'Line' function: to generate distance measures straight lines were drawn (i) down through the centre of the highest point of the coral, (ii) across the diameter of the coral, (iii) at the widest point and (iv) at the narrowest point (using 'DimAligned' function). These measurements were subsequently used to calculate the volume and surface area for each coral, under the assumption that corals represent an elliptical shape (Adam 2011). Structural complexity was measured with two metrics: linear rugosity and vector dispersion. Linear rugosity chains (2 cm chain link length) were created with a mesh grid with 10 cm spacing using a custom Python script (https://github.com/gracecalvertyoung/Rhino-Python-3D-Coral-Reefs). Using a consistent spacing allowed a standardisation of the number of chains, as all corals were different sizes. Vector dispersion was calculated at a 1 cm resolution following Young et al. (2017). Finally, percentage live cover and refuge size was quantified *in situ* during video collection using the habitat assessment score (defined in Gratwicke and Speight 2005). For each model, habitat that did not constitute the station (sandy seabed, adjacent rocky outcrops etc) were excluded using a circumference of 10 cm from the base of the coral (Fig 2.1, steps 3 and 4).

Data analysis

Data were analysed in R version 3.4.3 (Team 2013) using Generalized Linear Models (GLMs), Generalised Linear Mixed Models (GLMM, using 'lme4'; Bates et al. 2015) and generalized additive models for location, scale and shape (GAMLSS, package "gamlss"; Rigby and Stasinopoulos 2005). Model assumptions and fits were assessed using residual plots (as

specified by Bolker et al. 2009) and all continuous predictors were scaled and centred around zero to facilitate model convergence. Best fitting model selection was based on Akaike Information Criterion (AIC) using a backward elimination approach (with delta < 2). The significance of fixed effects was assessed using likelihood ratio tests comparing models with and without the main effect. The presence of potential influential points on model outcomes were checked for (using Cook's D and leverage), and sensitivity analyses were carried out on identified points (Chatterjee and Hadi 2009): the robustness of results was assessed when identified outlier values were temporarily excluded from models. Significant effects that were sensitive to the presence of influential points are stated in the results.

To determine whether cleaning station corals (n = 55) versus control corals (n = 12) differed in their microhabitat traits; rugosity, vector dispersion, height, percentage live cover and refuge size category were specified as fixed effects in a binomial logistic GLM (with a probit link). Due to collinearity (identified by Variance Inflation Factor values > 3) between height, volume and surface area, surface area to volume ratio was specified as a main effect (replacing volume and surface area, height still included): this removed any issue with collinearity between variables. It was not suitable to remove any one of these variables from all models or carry out a PCA, as the effects of all traits are of interest.

To determine whether microhabitat traits link with cleaner occupancy (range: 0 – 1) and abundance (range: 0 – 9 gobies per presence/absence survey), and cleaning behaviours (frequency, duration and rate), only data from cleaning stations (n = 55) were used: this removed false zeros from control corals. Due to further issues with collinearity between height and surface area to volume ratio, sequential regression was first carried out using these two variables. This method involves regressing the less important variable (in this case specified as surface area to volume ratio) against the other (height) and replacing the less important variable with the residuals from the regression – this disentangles unique from shared contributions from the two variables (Graham 2003). Following sequential analysis there was no significant correlation between the variable 'height' and the sequential model residuals. This residual variable was independently calculated for and specified as, a main effect in all further models.

To test for a link between microhabitat traits and occupancy (one value per station); rugosity, vector dispersion, height, surface area to volume ratio residuals, percentage live cover and refuge size category were specified as fixed effects in a Gaussian GLM (with identity link). Prior to analysis, occupancy values were logit transformed since other methods for analysing proportion data (e.g., binomial and beta models) produced poor fitting and overdispersed models (assessed using residual plots). To test for a link between microhabitat traits and cleaner abundance, the same microhabitat traits (including surface area to volume residuals) were specified as fixed effects in a negative binomial GLM. The negative binomial

family replaced an overdispersed Poisson model. For this GLM, multiple cleaner abundance counts were aggregated to total amounts per station (following Kratschmer et al. 2018), and an offset was specified, with a log transformation, which accounted for the number of presence/absence surveys per station.

To determine whether microhabitat traits link with cleaning behaviour (frequency, duration and rate) one GAMLSS (for frequency, replacing an overdispersed GLMM) and two GLMMs were specified all with the following fixed effects: rugosity, vector dispersion, height, surface area to volume ratio residuals, percentage live cover and refuge size. Since more than one observer collected behavioural data (n = 6), "Observer ID" was included as a random effect in all three models. Data were used on stations (n = 34) for which multiple observations (min n = 3) were carried out. For cleaning frequencies, all observation data were included (n = 223, contained zeros) whilst for rate and duration, only data containing observations where cleaning events occurred, were included in analyses (n = 132 observation, contained no zeros). Cleaning frequency (modelled using beta-binomial GAMLSS, replacing an overdispersed binomial GLMM) and rate (modelled using an inverse Gaussian family with an inverse link) represent the summed interaction frequency/duration for each cleaning interaction within each observation (single value per observation), whilst cleaning duration data (modelled using Gamma family and log link) represented each single individual cleaning event and its respective interaction length (multiple values per observation). Thus, for duration, ObservationID (a unique value assigned to each observation) was also specified as a random effect. The total time for each focal observation accounted for the amount of time a cleaner was out of view and thus varied across observations: for cleaning frequency and rate , values were therefore weighted by observation length. This correction was not necessary for cleaning duration models since their values were independent from observation length. Prior to analysis, cleaning rate values (range: 0.03 – 1.00) were rescaled from one to ten using the "scales" package (Wickham 2018): this method does not remove the variability between values, but simply transforms data to aid model fit. Finally, to determine whether significant relationships between microhabitat traits and cleaning behaviours were mediated and/or moderated by occupancy/abundance values, station occupancy and cleaner abundance (number of cleaners on the station for each observation) were added to all three final models as individual and interaction terms (occupancy/abundance separately interacted with trait terms). Across some studies of Caribbean cleaning interactions, cleaning patterns have been shown to vary with time of day (Côté and Molloy 2003; Sikkel et al. 2004; Sikkel et al. 2005). However, across 8 years of long-term data collected from the same study reef (including data used in this study), (Dunkley et al. 2020) consistently found no effect of time of day on cleaning frequencies and durations (as also shown by (Grutter et al. 2002; Whiteman and Côté 2002).

To avoid overfitting our already complex models, time of day in was not included in behavioural analyses.

Results

Do cleaning stations show specific microhabitat traits?

Cleaning stations (n = 55) were significantly taller than control corals (n = 12) and had more structurally complex surfaces (Fig. 2.2, GLM, $_{model}R^2$ = 39.0%, height: β = 1.00, χ^2_1 = 11.00, p < 0.001, vector dispersion (uniformity in angles of a surface; Young et al. 2017): β = 0.54, χ^2_1 = 4.13, p = 0.042). Cleaning stations also had lower surface area to volume ratios compared to control corals (GLM, β = -0.62, χ^2_1 = 6.27, p = 0.012) although this result became non-significant when an influential point (relating to a control coral) was temporarily removed (p > 0.20). There were no other significant differences between stations and control coral habitat traits (GLM, p > 0.05).

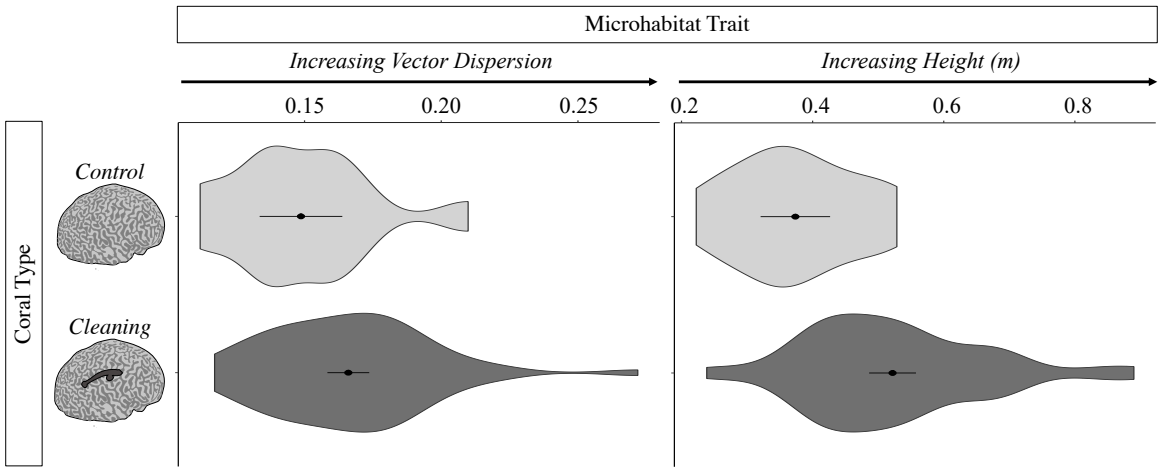

Figure 2.2: Microhabitat traits of sharknose goby (*Elacatinus evelynae*) cleaning stations. The outer shapes of the violin plot represent the range of vector dispersions (complexity measure – uniformity in angles of a surface; Young et al. 2017) and heights (m) across cleaning and control corals, while shape thickness shows how frequently these data values occurred. Point and lines show mean ± 95% CI.

Do microhabitat traits link with cleaner occupancy patterns?

Generally, cleaning station microhabitat traits did not predict how frequently cleaning stations were occupied (mean ± S.E. occupancy across stations = 0.56 ± 0.04, GLM, all predictors p > 0.05). However, cleaner occupancy tended to increase with the complexity of

the coral surface (GLM $_{model}R^2$ = 6.1%, vector dispersion: β = 0.44, $F_{1, 53}$ = 3.42, p = 0.070, p = 0.033 when one influential point (station) removed: influential station vector dispersion value = 0.27, mean ± S.E. dispersion value across stations = 0.17 ± 0.004, influential station occupancy = 0.55). Microhabitat traits did not significantly predict the variable abundance of cleaners on stations (GLM, all traits p > 0.05, up to nine cleaners occupied an individual station across time, mean cleaner abundance across presence/absence surveys ± S.E. = 0.97 ± 0.03).

Do microhabitat traits link with cleaning behaviour?

Out of 223 observations across 34 cleaning stations, cleaning was observed 308 times across 132 observations. Cleaning occurred less frequently, and bouts were longer, at taller cleaning stations (Fig 2.3 (A), cleaning frequency: GAMLSS, $_{model}R^2$ = 5.8%, χ^2_1 = 5.46, p = 0.019, Fig 2.3 (B) cleaning duration: GLMM, $_{model}R^2$ = 22.9%, χ^2_1 = 4.58, p = 0.032). Cleaning durations also increased with refuge size category (Fig 2.3 (B): GLMM, χ^2_1 = 4.10, p = 0.043, p = 0.053 when one influential cleaning event removed). Cleaning rates, which averaged 0.26 cleaning events per second (± 0.02, S.E.), were lower at taller cleaning stations but increased with structural complexity (Fig 2.3 (C), GLMM, $_{model}R^2$ = 24.4%, height = χ^2_1 = 5.97, p = 0.015, vector dispersion = 6.71, p = 0.010). Links between cleaning behaviours and microhabitat traits were not mediated/moderated by cleaner presence: generally, cleaning behaviours were not predicted by cleaning station occupancy or the abundance of cleaners, although cleaning frequencies tended to negatively link with occupancy (GAMLSS, β = -0.17, χ^2_1 = 3.45, p = 0.063, height remained significant when occupancy and cleaner abundance main effects included in model).

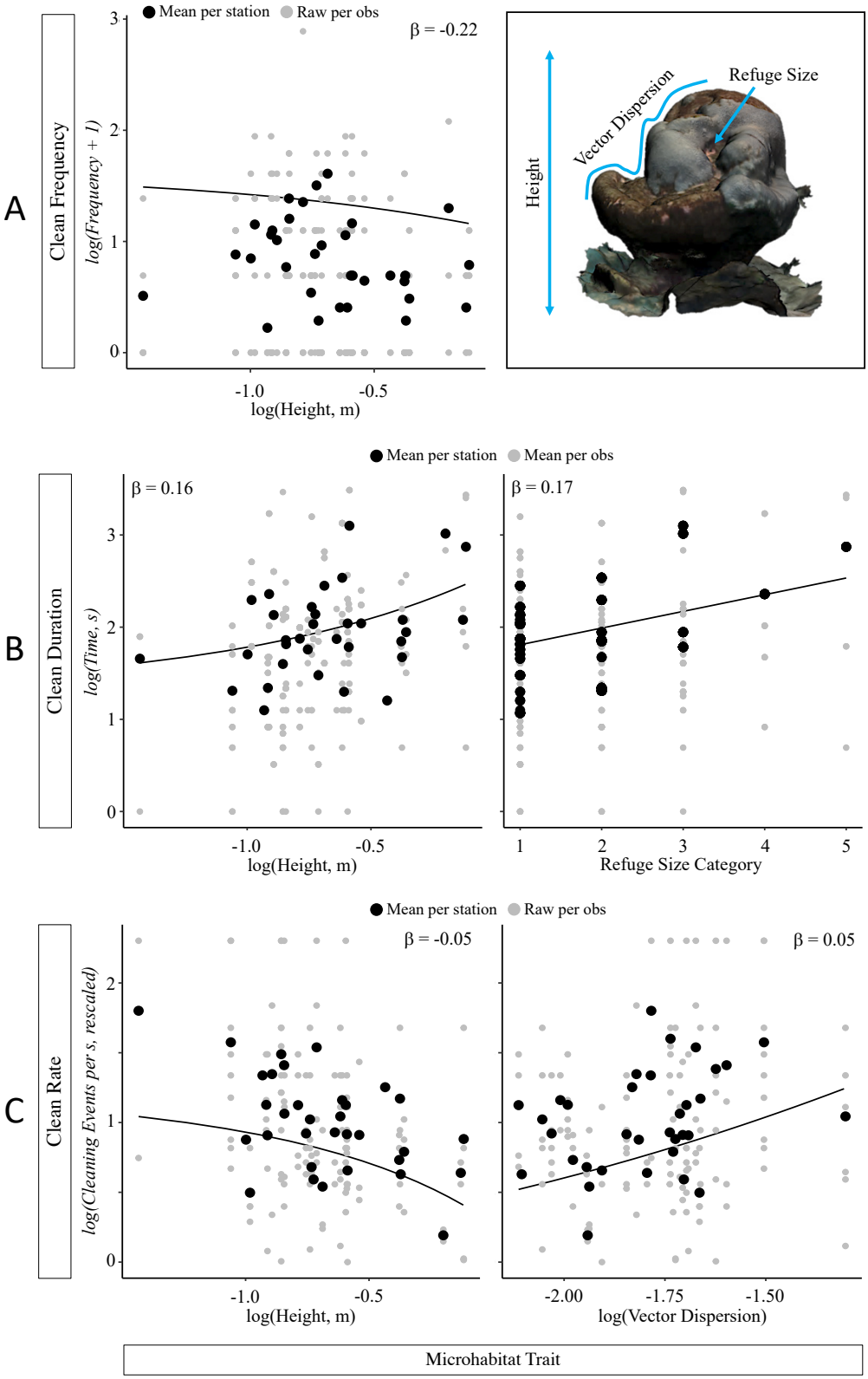

Figure 2.3: Sharknose goby (*Elacatinus evelynae*) cleaning station microhabitat traits that predicted the following cleaning behaviours; (A) cleaning frequencies, (B) durations and (C)

rates. Lines are based on model coefficients (GAMLSS or GLMM) while points represent raw or mean averaged data. Cleaning event values were rescaled from one to 10 to aid model fit. Log transformations were performed for figure clarity. Height, refuge size and vector dispersion are illustrated on a three-dimensional model of one cleaning station from Booby Reef, Man O' War Bay, Tobago.

Discussion

Here, for the first time, the cleaning stations of a predominant Caribbean cleaner, the sharknose goby (*Elacatinus evelynae*), are distinguished from non-station corals by habitat metrics including increased height and structural complexity (vector dispersion). Although microhabitat trait variation predicted the occurrence of cleaning stations, they did not predict cleaner occupancy nor abundance patterns of occupied cleaning stations. Variations in coral morphology, however, in terms of height, vector dispersion and refuge size did promote variations in cleaning frequencies, durations and rates. Cleaning events were longer but occurred at a lower frequency and rate at taller corals. Events were also longer when refuge sizes were larger, whilst rates increased with structural complexity (vector dispersion). Together, this study highlights the importance of variation in coral morphology for local cleaner distribution and thus its potential role in moderating the dynamics of cleaning interactions on a larger scale.

Cleaning gobies show strong site fidelity to their cleaning stations (Whiteman and Côté 2002; Harding et al. 2003), are assumed to have short lifespans (mean age documented < 50 days in White et al. 2007) and remain in direct contact with their coral (apart from when cleaning, and the occasional competition-induced move to adjacent coral (Whiteman and Côté 2002; Côté and Soares 2011). Adult distribution patterns of cleaners may thus be, in-part, governed by their larval post-settlement success/settlement site. *Elacatinus* gobies form monogamous pairs (Harding et al. 2003), regularly spawn (*E. evelynae* spawning interval: 9 – 20 days) and produce large clutch sizes (*E. evelynae*: 200 – 250 eggs clutch^{-1}, 10 – 50% survival to settlement). Importantly, these larvae have lengthy pelagic larval periods (*E. evelynae:* settle 30 – 40 days post hatching; (Colin 1975; Olivotto et al. 2005; Majoris et al. 2018b) and for a closely related species (*E. lori*), larvae have been documented to travel ~ 2 km from their parent site (D'Aloia et al. 2015). Taller corals which stand above others, may thus 'catch' pre-settlement larvae, whilst increased surface complexity can reduce larvae/adult predation risk (Beukers and Jones 1998; Almany 2004) and alter larval density-dependent mortality once settled (Johnson 2007), together promoting the formation of cleaning stations. However, if these results were simply down to random larvae settlement patterns mediated by their post-settlement survival success, it would also be expected that coral heads with larger

surface areas also function as cleaning stations (similar to Losey 1974), which was not the case (non-station corals were consistently observed to be unoccupied across 8 years of long-term study; Dunkley et al. 2019a). In addition, although gobies do generally stay affiliated with their coral heads, some localised movements by adults are observed between neighbouring heads (up to ~ 5 m distance; Dunkley et al. 2019b). Thus, by flexibly moving between corals, adult cleaners may more efficiently increase their fitness by benefitting from differential resources from different coral heads. Choice experiments would help decipher the absolute habitat preferences for this species.

Settlement of coral reef fish in their habitat is complicated, and it is very unlikely that finer scale settlement patterns within an environment are a matter of chance (Victor 1986). Many coral reef fish larvae rely on a combination of cues to control their settlement site selection, including visual, olfactory and acoustic stimuli (Montgomery et al. 2001; Lecchini et al. 2005). It is not clear, however, how sensitive such cues are to finer scale variations (i.e. between corals of the same family as investigated here). Some fish also base settlement cues on the presence of conspecifics and not on the coral's characteristics, since the presence of conspecifics could be an indicator of habitat quality (Öhman et al. 1998; Lecchini et al. 2005) as occurs with *E. prochilos* (see Whiteman and Côté 2004). However, since density dependence can influence settlement mortality (Johnson 2007), in the current study, it would perhaps be expected that occupancy/abundance patterns may correlate with microhabitat traits, which was not observed (in parallel with (Wilson and Osenberg 2002), assuming corals are at full carrying capacity, with coral heads assumed to be saturated at very low densities (maximum n = 23 per coral) (Cote and Whiteman 2004). Additionally, the number of cleaners were not found to affect cleaning in an 8-year study on Booby Reef (Dunkley et al. 2020), thus number of cleaners per station may not play a role in confering cleaner goby fitness. However the number of cleaners observed at a station over the 8-year study did not excede 9 per station (Dunkley et al. 2020), higher densities on other reefs therefore may affect cleaning, however this is not known.

Taller corals also played an important role in influencing the dynamics of cleaning interactions. Investments in cleaning interactions are governed by risk: clients give up foraging time (Grutter et al. 2002) and may be more vulnerable to predators during cleaning (although both cleaners and clients are thought to be afforded protection to some extent as predators visit stations to be cleaned rather than for nutritional needs (Cheney et al. 2008; Soares et al. 2012). Cleaning at a taller coral may provide both cleaners and clients with a visual advantage by creating a greater field of view, lowering predation risk (Nemeth 1998) and facilitating longer cleaning events. Indeed, for a common client of cleaning gobies, *Stegastes partitus* (see Dunkley et al. 2019b), a limited field of view around their territorial site altered their risk-taking behaviour (Rilov et al. 2007). Further exploration of this idea will rely on knowledge of the

visual acuity of cleaner fish and their clients, which is currently unknown (although is likely to correlate with eye size; (Caves et al. 2017). The assumption that station habitat traits that reduce risk to predation and are beneficial for cleaning patterns, can also be supported by our result that longer cleaning bouts were observed at stations with increase refuge size, and that cleaning rates were higher with increased complexity (creating crevices and cracks). Similar to cleaner wrasse (species unknown; Ferrari et al. 2018), here cleaning gobies were associated with cleaning stations that showed an increased variation in slopes. The availability of refuges and fine-scale variations in structural complexity (1 cm vector dispersion) may provide a 'safety net' for small bodied cleaners (max 4.5 cm fork length; Cheney and Côté 2003) minimising their risk to threats by reducing access/mobility options for larger predators. Indeed, Ferrari et al. (2018) hypothesized that a strong association with sponges by the sponge-dwelling facultative-cleaning ecotype of sharknose gobies could be explained by the shelter these benthic organisms provide. However, if taller corals are more preferable resources (compared to other coral geometries), and habitat space is limited, only gobies expressing beneficial and competitive phenotypes may be expected to dominate such environments, with variation in phenotypes also leading to variations in cleaning behaviour (Dunkley et al. 2019a). Correlating the occurrence of different cleaner behavioural phenotypes (e.g. personality traits) with station microhabitat traits, and their spatial locations may thus be beneficial for future study.

Cleaning patterns can also be goverened by the feedback behaviours of their clients (Dunkley et al. 2019a), and clients may use the "landmark feature" of cleaning stations to locate cleaners (Losey 1974; Kulbicki and Arnal 1999) with taller or larger features being easier for clients to locate (Braithwaite and de Perera 2006). In turn, clients may then learn to associate these specific features (e.g. "large spherical corals") with the cleaners (Losey 1974). Indeed, in sparser, heterogeneous environments, organisms tend to aggregate around habitat structures (García-Charton and Pérez-Ruzafa 2001) and thus taller stations may promote enhanced client numbers/diversity visiting the location – creating foraging choice options for the cleaner. Higher energy gains can be obtained through consuming higher quality foods, feeding for longer and increasing diet breadth (Toscano et al. 2016). Since different clients host species-specific parasite assemblages (Grutter 1994), cleaners could maximise their energy loss versus gains by selectively cleaning different client types to optimize their nutrition/energy through fewer interactions. Some client types will be restricted in their spatial distribution on the reef by the microhabitat features (Tolimieri 1995) and their reef-use behaviour (e.g. territorial species) however. Taller stations may thus also allow a greater range of 'favourable client types', hosting higher parasite burdens/diversity (e.g. predators, larger body sizes; (Poulin and Rohde 1997) to access the cleaner. Incorporation of client functional traits, abundance, diversity, and behaviour data should thus be considered in future studies.

To conclude, the high structural complexity of coral reefs, which is a defining and vital component of a healthy environment, is under threat from a suite of natural and anthropogenic disturbances (Magel et al. 2019). These results demonstrate that the prevalence and dynamics of cleaning interactions on a local scale, which are also thought to be a vital component of a healthy reef (Clague et al. 2011; Waldie et al. 2011; Demairé et al. 2020), may be vulnerable to even fine-scale changes in microhabitat structure, especially with regards to coral height. Through their large number of interactions with a diversity of client species, cleaning interactions can also drive patterns of fish diversity themselves (Bronstein 2015), playing an important role in the ecological community structure (Floeter et al. 2007; Quimbayo and Zapata 2018). Changes in the dynamics of cleaning interactions could thus imply significant consequences for the associated reef fish community. It is important to note however, that like all mutualisms, cleaning interactions are highly context dependent: interaction outcomes vary temporally (Côté and Molloy 2003; Sikkel et al. 2004; Sikkel et al. 2005; Dunkley et al. 2019b; Dunkley et al. 2020) and spatially (Cote 2000; Sikkel et al. 2000; Dunkley et al. 2020; Romain et al. 2020). Whilst microhabitat traits play a role in governing local interaction patterns, additional interlinked contextual factors can influence interaction outcomes (e.g. client identity and abundance; Dunkley et al. 2020). It is therefore difficult to determine at this stage, what our findings mean under wide-scale ecosystem degradation scenarios. Compared to the Indo-Pacific, Caribbean reef communities naturally exhibit lower species diversity meaning they are already less resilient to decline and degradation (Bellwood et al. 2004). It is therefore vital that we gain further knowledge of the finer scale habitat requirements of such keystone species in the Caribbean to determine how habitat losses/changes to the reef geometry may both directly and indirectly impact reef communities.

Chapter 3

Behavioural interactions of reef fish in response to artificial reef structures, Fish Hives

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Abstract

The structural complexity of corals creates a diversity of habitats which host a plethora of marine life. Due to numerous anthropogenic and natural threats, coral reefs are being degraded and this structure lost. Global conservation efforts to mitigate against habitat loss have focused on replacing or enhancing corals using artificial reefs. Despite being widely deployed, few studies have assessed how fish species interact with these artificial structures. Here, novel artificial reef structures were created and deployed, termed 'fish hives', designed to be replicated by local community or conservation groups with minimal resources and specialised equipment, and ideal for studying fish behaviour. Given the scope of reef degradation and the need to facilitate coral reef conservation in a range of ecological contexts, hives were tested at two sites with different ecologies (an existing reef and a sandy area in close reef proximity). The fish hives were seeded with the critically endangered Elkhorn coral (*Acropora palmata*) and the behaviour of reef fish at fish hives and control corals was recorded using point observations from video footage over a 16-month period. Fish hives harboured a similar community of reef fish to corals, provided a successful nursery site for elkhorn coral, a settlement site for benthic organisms, and suitable habitat for residential damselfish. Indeed, algae farming damselfish associated and exhibited territorial behaviour significantly more at hives than natural brain corals. While it remains to be seen whether fish hives can provide reef habitats in the longer term, they do offer insight into the importance of damselfish territorial and farming behaviour on artificial reef succession.

Introduction

Habitat structural complexity drives the high levels of biodiversity on coral reefs (Graham and Nash 2013). Along with benthic live cover and coral species diversity, the variety of different shaped structures plays a key role in determining local fish abundance and diversity by providing habitat space (Gratwicke and Speight 2005; Kostylev et al. 2005; Harborne et al. 2012; Gonzalez-Rivero et al. 2017). The calcium depositing corals, which create these essential structures, are threatened by a range of anthropogenic driven stressors (Bozec et al. 2013; Kennedy et al. 2013). These range from direct habitat loss due to industrialisation, to the indirect consequences of global anthropogenic change (ocean acidification, warming sea water, altered currents, infectious disease and invasive species etc; Foster et al. 1994; Pratt 1994; van Hooidonk et al. 2014; Li and Reidenbach 2014; Sigl and Laforsch 2016; Williams et al. 2019a). The extent to which these stressors alter coral structure affects the severity of damage and recovery potential of the reef ecosystem (Graham 2014; Rogers et al. 2014; Magel et al. 2019). Often coral skeletons persist after sustaining damage from phenomena, such as a coral bleaching event, keeping the structural element intact for 3-10 years (Pratchett et al. 2009). If corals do not recover and habitat loss occurs, fish communities diminish (Garpe et al. 2006; Graham et al. 2006). Modelling surface water heating and water flow patterns in the Caribbean suggests large-scale coral bleaching events and die offs will increase in the near future (Li and Reidenbach 2014) with coral adaptations to increase bleaching thresholds only delaying simulations by ~10 years (Logan et al. 2014). Thus, one objective of coral reef management is maintenance or enhancement of this structural complexity (Graham and Nash 2013). A promising and increasingly popular mitigation approach is the introduction of artificial structural modules, which replicate natural coral structures and create artificial habitat space (Paxton et al. 2020b). These structures also provide space for coral nurseries and natural coral settlement (Fitzhardinge and Bailey-Brock 1989).

Artificial reefs, used for centuries to enhance fisheries (Stone et al. 1991; Castelló Y Tickell et al. 2019), have more recently been used as a conservation tool (Seaman 2007; Paxton et al. 2020b). The array of substrates used ranges from waste materials (Barnabé et al. 2000), including shipwrecks (Simon et al. 2013) to purpose-built structures (Komyakova et al. 2019). Recently, eco-engineered artificial structures have been designed to replicate coral structural complexity (Chapman and Underwood 2011; Dafforn et al. 2015; Morris et al. 2018; Komyakova et al. 2019). A range of designs and materials have been used to emulate naturally occurring structures (wire, steel, rope, stone gravel etc. Baine 2001; Komyakova et al. 2019), with concrete structures being the most popular and effective (Spieler et al. 2001; Komyakova et al. 2019), to provide services such as coral nursery space, habitat provision for

marine life and alternative dive sites to reduce pressures on natural reefs (Schaffer and Lawley 2012; Becker et al. 2018; Paxton et al. 2020b).

Artificial reef efficacy is varied: some harbour fish assemblages similar to those on natural reefs (Granneman and Steele 2015), others show higher or lower species abundance and diversity (Carr and Hixon 1997), or they may favour particular species (Fowler and Booth 2012). Other artificial structures, particularly marine infrastructure (e.g. harbours, causeways, dikes, piers and breakwaters) not built specifically for conservation, can have negative effects, for example by increasing available habitat for invasive non-native fish (Airoldi et al. 2015; Hunt et al. 2019) and benthic colonisers (Tanasovici et al. 2020), which can increase biotic homogenisation (Dafforn et al. 2015). Thus, it seems that artificial reef efficacy is largely context dependent (Paxton et al. 2020b) and there is a lack of knowledge about their specific ecological functions (Spieler et al. 2001) and how they affect fish species assemblages (Seraphim et al. 2020).

Despite widespread use and in addition to the ecological unknowns, there are socio-economic concerns surrounding artificial reefs, ranging from cost of building and deploying to aesthetics. Artificial reefs often require expensive moulds, building expertise and sophisticated equipment for deployment due to their weight. The varied outcome of artificial reefs could also be a function of experimental design, including monitoring procedures, as the goals and specific objectives of many reef projects are not clear (Edwards and Gomez 2007; Becker et al. 2018). Fish assemblages undergo long periods of stochasticity prior to reaching stable communities after artificial deployment (Coll et al. 1998; Relini et al. 2002) therefore the duration of programmes likely affects the perceived success of artificial reef projects (Becker et al. 2017). Previously reef restoration research predominantly focused on the success of coral growth (Young et al. 2012; Schopmeyer and Lirman 2015; Seraphim et al. 2020), community assemblages (Opel et al. 2017; Higgins et al. 2019) or recruitment (Kawasaki et al. 2003) whereas studies on behavioural responses of marine fish to artificial structures are limited. For example Jamieson et al. (2006) observed differences in behavioural responses (exploring, feeding and “indifference”, defined as no interaction with the baited lander used in the experiment) of *Coryphaenoides armatus* to different baited lander structures. This is an oversight, as how species interact with their habitat can influence behaviour and therefore outcomes of community dynamics and ecological interactions (Almany 2004). For example, foraging rates and cleaning behaviour of fish are tightly linked with their habitat (Grabowski and Powers 2004; Vergés et al. 2011; Whittey et al. 2021). Thus, as fish respond differently to a variety of structures, which structure is used for monitoring fish may affect their behaviour and yield inconsistent results, such as underestimating population sizes (Jamieson et al. 2006). Increasing structural complexity from an artificial reef, for example, disrupted the visual

field of *Stegastes partitus*, which was thought to compromise feeding and mating behaviour (Rilov et al. 2007).

How fish interact with their habitat can affect fish behaviour, which in turn can affect ecological functioning of coral reefs (O'Brien et al. 2018; Mitchell and Harborne 2020). Thus, facilitating or ensuring that fish behaviour on natural reefs in replicated on artificial reefs will allow fish to carry out their trophic interactions within their ecological niche. On natural reefs, herbivorous grazing behaviour is an important ecological process as it prevents overgrowth from dominant algal species, which outcompete other species for space (Edwards and Gomez 2010). Smaller corals, including coral cuttings used in restoration projects are particularly vulnerable to algae overgrowth (Ferrari et al. 2012). Many coral transplantations and restoration projects require dive teams to clean corals of algae (Frias-Torres and van de Geer 2015). Fish species that farm algae, such as damselfish, promote the growth of algae by pecking away other settling competitors (Kaufman 1977; White and O'Donnell 2010). Farming damselfish are often the first to colonise restored reefs (Schopmeyer and Lirman 2015) and present a potential threat in reef rehabilitation due to their negative impacts on immature corals (Frias-Torres and van de Geer 2015). Damselfish, however, can also exclude corallivores from their territory preventing coral predation and increasing growth and diversity (Gochfeld 2009; White and O'Donnell 2010).

How species interact directly with artificial structures may help us understand the disparity in reported fish community assemblages at artificial reef structures. Given that artificial reefs are widespread in the ocean (an estimated 300,000 km^2 of the ocean has been altered by human structures; Halpern et al. 2008), it is now timely to move beyond comparisons with natural reefs, to consider artificial reefs in their own right and consider species interactions with artificial structures (Castelló et al. 2019), including specific behavioural observations. Here, a new affordable, hollow structure – called a 'fish hive' – was designed and built by hand (by the thesis author) from a blend of concrete and vermiculite (a light mineral) to artificially replicate dome-shaped brain corals. The blend of concrete containing vermiculite results in a lighter mix than traditional concrete mix. These lighter structures were manoeuvrable without the use of heavy machinery. Fish hives were deployed in two different environmental contexts: a reef with live coral, and a sandy area adjacent to an existing reef. Firstly, whether fish hives were an appropriate substrate for seeding and growing coral was determined; and secondly, how the fish community and fish behaviour around fish hives changed over time was studied. The behaviour of damselfish spp. was of primary focus as they are often initial colonisers of artificial reefs.

Materials and methods

Description of study sites

Two fringing reefs were used for the current study, Booby Reef and Pirates Reef in Man O' War Bay, Tobago (11°19.344′N, 060°33.484′W; Fig. 3.1), which have been used as a long-term monitoring site for >10 years (2010-2020) (Dunkley et al. 2018; Dunkley et al. 2019a; Dunkley et al. 2019b; Dunkley et al. 2020; Whittey et al. 2021). Booby Reef is dominated by non-branching brain coral species (Faviidae), areas of patchy sand, remnants of dead elkhorn (*Acropora palmata*) and staghorn (*A. cervicornis*) corals (Ramsaroop 1991) thus representing a relatively degraded reef. Pirates Reef is primarily composed of rocky sandy substrate, the encrusting zoanthid (*Palythoa caribaeorum*) and living hard coral (*Siderastrea* spp. and *Montastraea* spp.). All work was carried out in collaboration with local NGO Environmental Research Institute Charlotteville under permit numbers #001/2016 and #002/2020 issued by the Tobago House of Assembly. Both sites are easily accessible by small vessels and are therefore well suited for the deployment of artificial structures, and easy access facilitates long-term monitoring.

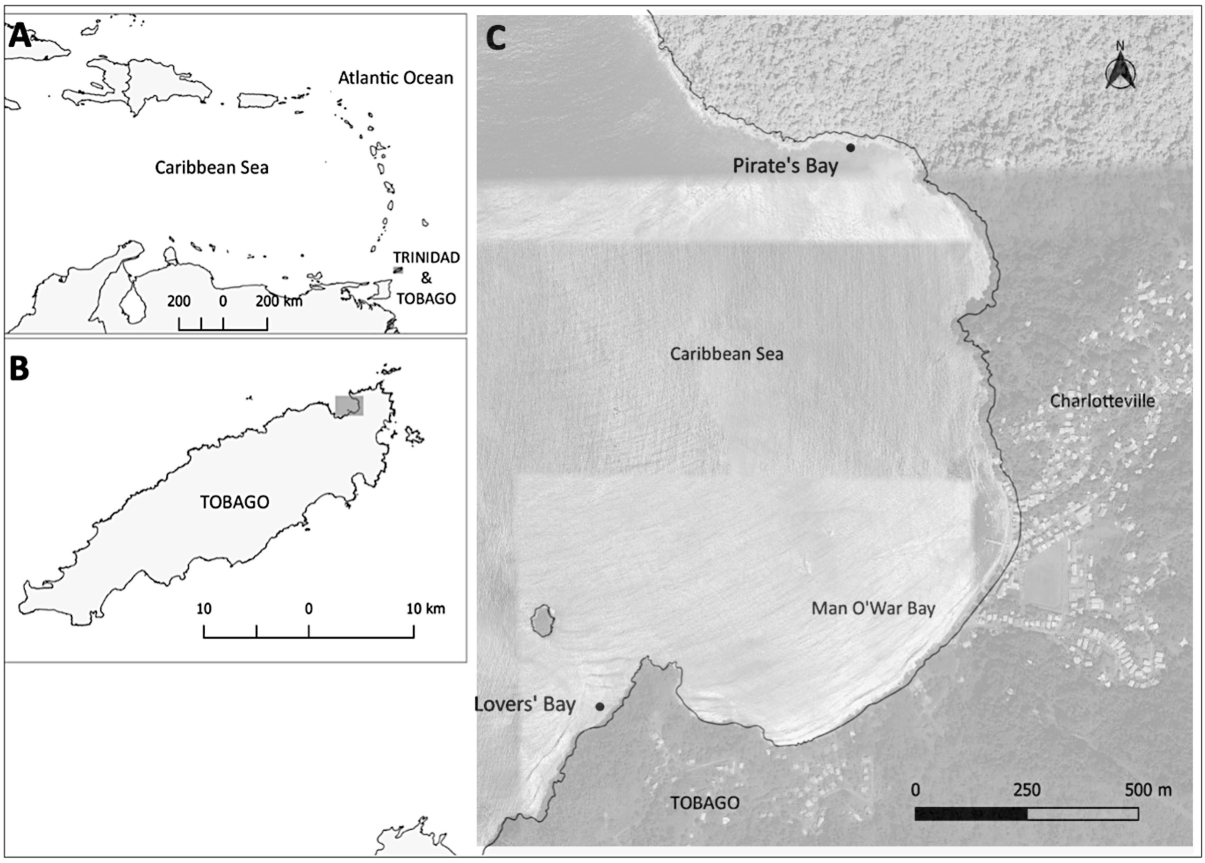


Figure 3.1: Location of sites of artificial reef structure (fish hive) deployment (black dots): Lovers Bay (Booby Reef) and Pirates Bay (Pirates Reef) in Man O'War Bay, Northeast

Tobago; Caribbean (A), Tobago (B) and local scale showing the extent of fish hive deployment and satellite image of Charlotteville town (C).

Fish Hive design, construction, and deployment

An artificial reef structure, hereafter termed 'fish hive' (Fig. 3.2), was designed to mimic the height and shape of existing brain corals (Faviidae spp.) on Booby Reef (Whittey et al. 2021). Fish hives were designed to be affordable and used as many reusable materials as possible (costs approx. £120 per unit). Each fish hive was approximately 100 cm in height and 80-100 cm diameter, conical-spherical in shape with a flat base, large top hole (diameter ~20 cm) and 9-12 smaller (diameter ~10 cm) holes arranged around the hive allowing fish to use the structure for shelter, an important feature of man-made reefs (Frazer and Lindberg 1994; Hackradt et al. 2011). A mixture of Portland cement, vermiculite, coarse sand and gravel at a ratio of 1:1:2:1 was mixed by hand and layered over an inflatable rubber sphere (60-80 cm diameter – which was deflated and re-used after construction) on to which a template was drawn so that 15 cm long segments of bamboo (9-12 per hive) could be added to create the hive entrances. Once a base of sufficient thickness (15 cm in height and 15 cm thickness) was reached, a 5 cm wire mesh was placed around the circumference of the structure onto which a complete concrete layer was added of 10 cm thickness. To facilitate curing and prevent slumping a 'skirt' of aluminium mesh covered in clingfilm was attached to the base of each hive and removed (and reused) within 24 h of curing. During curing for a minimum of 48 h in the sun, hives were kept moist by spraying periodically with fresh water.

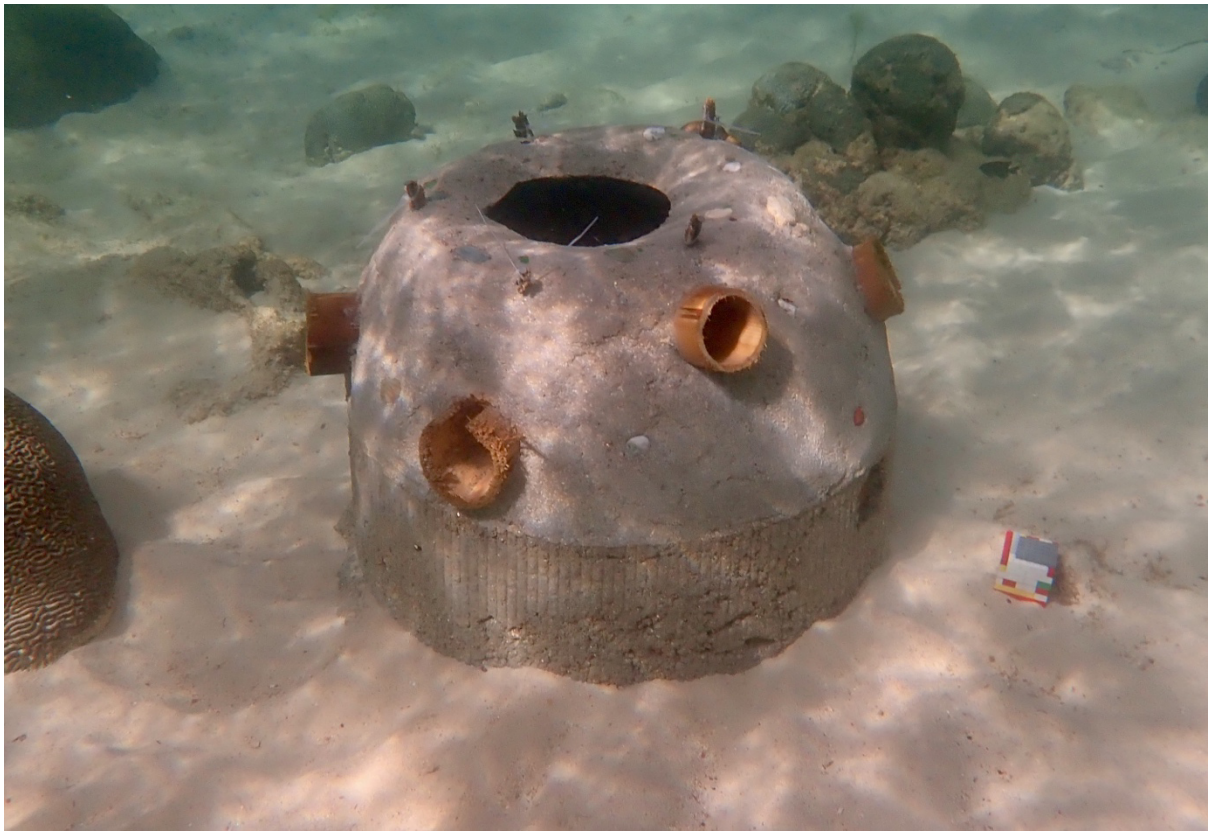


Figure 3.2: A newly deployed artificial reef structure (fish hive) seeded with five Elkhorn coral (*Acropora palmata*) cuttings. Hive is located at Lovers Bay (Booby Reef) in Man O'War Bay, Northeast Tobago; The bamboo segments used to create holes are left in the structures. Lego cube on far right for scale is 5 cm^3.

In June 2019, a total of 12 fish hives were deployed, six placed at Booby Reef and six at Pirates Reef (Fig. 3.1), two at the latter site were subsequently lost due to sedimentation after 8 months, and so only four hives from Pirates were included in the analysis (total: n = 10). The hives on Booby Reef were placed (on the north side of our long-term study site), up to 5 m away from a brain coral at ~1 m depth. At Pirates Reef, all hives were placed approximately 30 m away from existing corals in a sandy area at approximately 3-5 m depth (Fig. 3.2). Cuttings of elkhorn coral (*Acropora palmata*), approximately 5 cm in height (surface area 1200 cm^3), were taken from adjacent alive, healthy corals and attached to each hive (5 per hive). Two hives at Pirates were not seeded with Elkhorn as controls.

Monitoring fish hives and control corals

During 2019-2020, video footage and still photographs of all fish hives (including those seeded and not seeded) and controls were collected by a snorkeler or scuba diver using GoPro cameras (GoPro, Inc., San Mateo, CA, USA) at five time points 1-5 months apart over

16 months: 29 August 2019, 8 January 2020, 8 February 2020, 10 June 2020 and 11 October 2020 (scheduled sampling events at three-month intervals were disrupted by weather and local lockdowns due to the SARS-cov-2 pandemic). At each sampling event, photographs with a scale bar were taken from directly above each hive to monitor coral growth and mortality. Length, width, and surface area of seeded corals on the hives were measured using Image J software (Schneider et al. 2012) and settlement by other benthic organisms were recorded and identified to family level using the reef guide website (*https://reefguide.org/carib/index1.html*) and the Reef Fish Identification for Florida, Caribbean and Bahamas (Humman and DeLoach 2003). Brain corals (n = 6) in the vicinity of hives on Booby Reef served as controls, with 2 - 4 used at any given sampling event (n = 2 - 4).

Video footage was recorded using GoPro cameras positioned approximately 1.5 m in front of each fish hive/control coral facing offshore and left to record for ~ 20 minutes. The first minute of the video was not analysed to allow for any disturbance created by the diver to settle. Due to obstructions to the camera view (e.g., poor visibility) observations ranged from 19.3 to 22.6 minutes (mean observation duration 20.04 ± 0.38, S.E.). From each video fish species and phase were recorded where possible, otherwise fish were identified to family level. Due to cryptic morphologies, five species of damselfish (*Stegastes adustus, S. diencaeus, S. leucostictus, S. planifrons* and *S. variabilis*) were combined together as 'Dark Damsels' (*Stegastes* spp.) and referred to hereafter collectively as damselfish. For all fish within two body lengths of each hive/control coral, the duration per unit time (seconds) of three behaviours was recorded: (1) Associating - the fish associates with the structure by swimming in and/or around it (within two fish lengths); (2) Pecking - the fish pecks the structures; (3) Territorial - the fish exhibits defensive behaviour of the structure. Duration of behaviour was weighted by observation duration during statistical analysis using the 'cbind' function to create a proportion of duration weighted by observation length account for uneven timings of video recording. The frequency of the behaviours was recorded as a per observation value.

Fish count data was recorded using a MaxN approach, where the maximum number of individual fish of a particular species occurring in a single video frame were recorded (Ellis and DeMartini 1995; Campbell et al. 2015) for all fish within two body lengths of the hives/control coral. Abundance, richness and Shannon and Simpson Diversity Indices using the R package '*vegan*' (Dixon 2003) were calculated based on MaxN data.

Statistical analysis

Data were analysed in R version 1.4.1103 (R Core Team 2017) using General Generalized Linear Mixed Models (GLMMs). Model assumptions and fits were assessed using residual plots (as specified by Bolker et al. 2009) and, when needed, continuous predictors were log transformed to facilitate model convergence. In addition, where suitable, the

presence of potential influential points on model outcomes were checked for using Cook's D and leverage, and sensitivity analyses were carried out on identified points (Chatterjee and Hadi 2009): the robustness of results was assessed when identified outlier values were temporarily excluded from models. Significant effects that were sensitive to the presence of influential points are stated in the results. Type 2 ANOVA was used to test for significance of terms in GLMMs and Tukey's post-hoc method was used to run pairwise tests to decipher significance of interacting terms (from the GLMMs) using the 'emmeans' function (Russell 2019).

Reef fish community at hives and corals over time

To determine whether fish abundance, richness and diversity differed between corals and hives over the sampling period separate GLMMs with a Gamma family link were run including site and individual structure ID as random terms and the following fixed terms: structure type and months since deployment. Using separate GLMMs with a Gamma family link, abundance, richness and Shannon and Simpsons diversity indices were included as response variables to assess difference in fish communities at hives over the 16-month monitoring period in relation to the differences in coral growth at the hives. Given the apparent high abundance and widespread presence of dark damsels at sites (at hives and corals), both Simpsons and Shannon diversity were included to ensure high abundance of damsels did not overshadow the biodiversity results. Simpson diversity measure gives more weighting to a common or dominant species and was there for used in addition to Shannon. Included in the models were: months since deployment, coral growth, number of corals alive and the interaction between coral growth (measured as increased surface area cm^3) and months since deployment as dependent terms, and site (reef location) and hive ID were included as random terms to account for location differences. Coral growth was recorded as total surface area and rescaled before including in GLMM using the *scales* package in R to aid model convergence. Finally, to test between seeded coral growth on hives between sites, coral growth was included with site as fixed term and month of sampling and hive ID included as random terms in a GLMM with a Poisson family link.

Behaviour of reef fish at hives and corals over time

The time each fish species spent exhibiting each behaviour (associating, pecking and territorial) was weighted by observation time using the '*cbind*' function, creating a proportional value. The frequency of the three behaviours was calculated per observation and separate GLMMs were used to test associations between behaviours and structures over time. One taxon, damselfish (*Stegastes* spp.) was highly dominant and therefore, damselfish activity was included in a separate GLMM which included site and individual structure ID as random terms

and the following fixed terms: structure type and months since deployment. All other fish species were recorded in a separate GLMM with the same terms, apart from territorial behaviour as there were no observed territorial displays by non-damselfish around control corals. Therefore, the model was as follows: site and individual structure ID as random terms and months since deployment as a fixed term.

Results

Benthic settlement on fish hives and Elkhorn (Acropora palmata) coral growth

All hives at both Booby Reef and Pirates Reef were colonised naturally by brown algae (*Phaeophyta* spp.) and coralline algae (*Rhodophyta* spp.) within two months of deployment (Fig.3.3). In addition, at 16 months the hives at Pirates Reef were colonised by fire coral (*Capitata* spp.) and tube sponges (*Agelas widenmyeri*). After 16 months, 58% of seeded corals had survived, with 76% of coral mortalities occurring within the first 6 months (Fig. 3.4). Collective coral surface area on hives increased over ten-fold during the sampling period, from an average of 387.9 cm^3 (±124 $cm^{3,}$ S.E.) per hive at two months post deployment, to 4,709.6 cm^3 (± 4,015.1 $cm^{3,}$ S.E.) per hive 16-month post deployment (Fig. 3.4). The growth of seeded Elkhorn corals varied significantly at each individual hive (GLMM: χ57.64, $p < 0.001$).

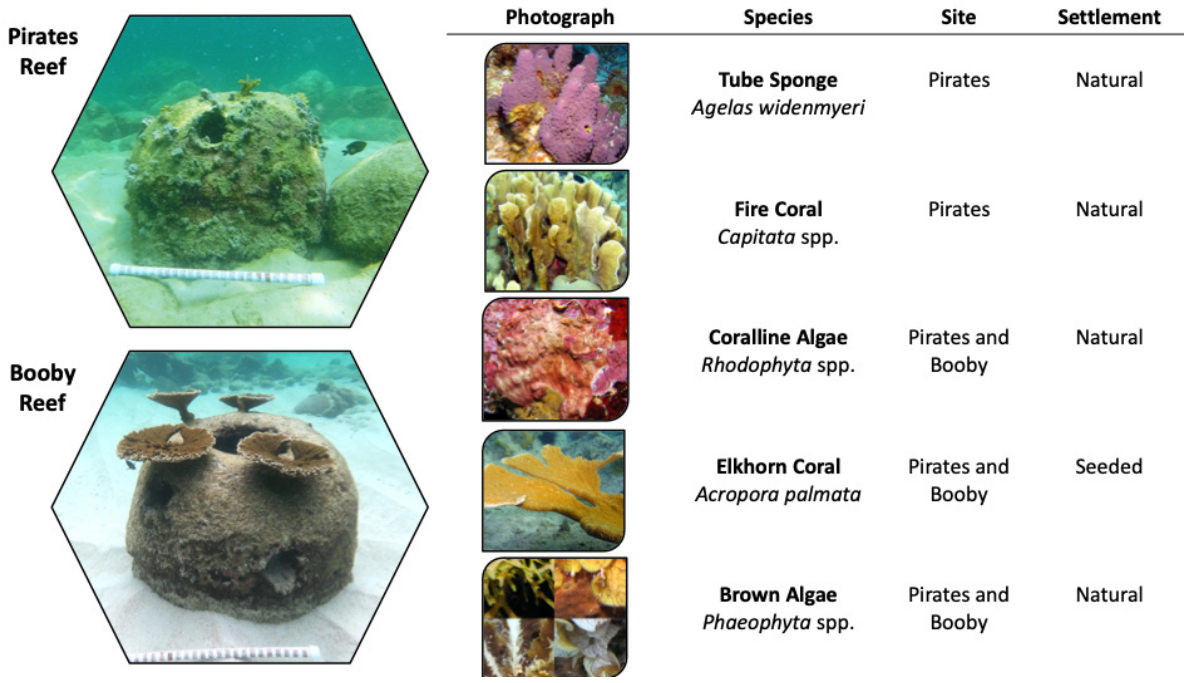


Figure 3.3: Species of benthic organism that settled on artificial reef structures (fish hives) at Pirates Reef and Booby Reef, Man O'War Bay Tobago during a 16-month post deployment. Photographs from https://reefguide.org/carib/index1.html, common name and species names, site that species in tables were observed at, mechanism of species settlement (natural –

naturally occurring settlement with no experimental intervention, seeded – cuttings added by researchers).

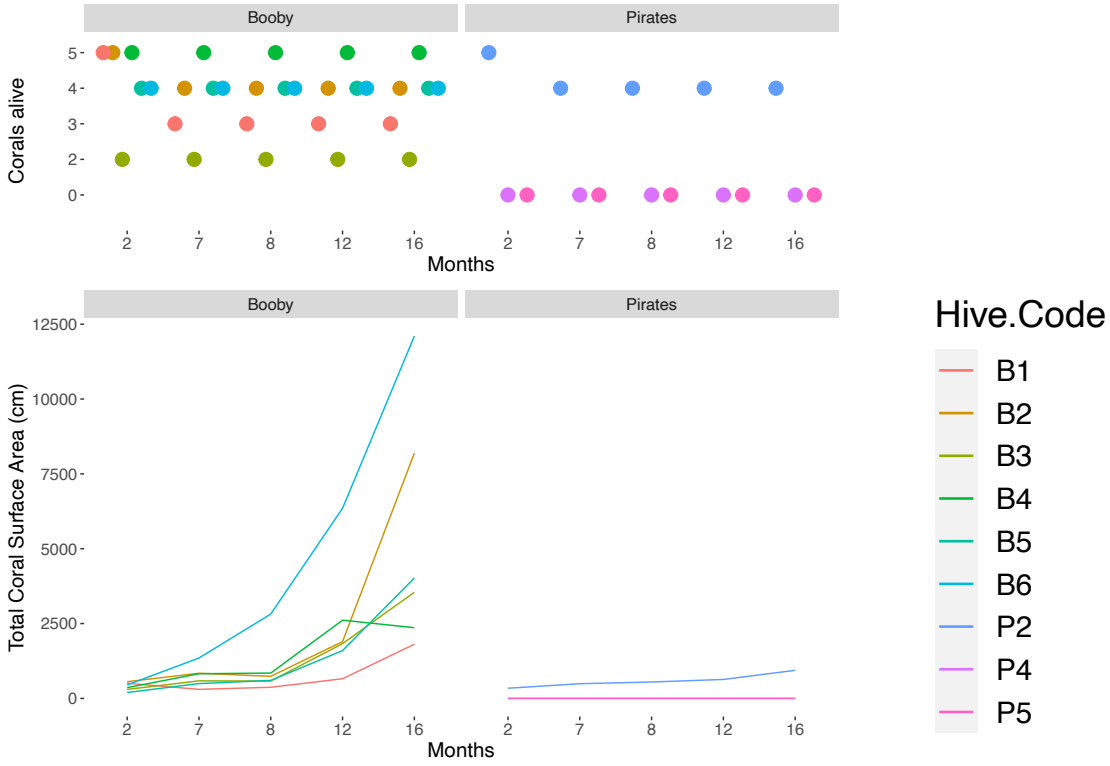


Figure 3.4: Growth and survival of seeded Elkhorn corals (*Acropora palmata)* over a 16-month monitoring period (see: https://youtu.be/pVo_pPHKJa4) in Man O'War Bay, Tobago. Corals were measured as combined total surface area of all cuttings on each artificial reef structure (fish hive) giving one value per hive for coral growth. All Elkhorn coral cuttings at initial seeding were approximately 5 cm in length, 1200 cm^3 total surface area. Top: number of corals per hive that were alive at sampling points over 16-month period, Bottom: growth of Elkhorn corals as measured by surface area.

Reef fish community at hives and control corals over time

A total of 40 species of reef fish were found in the vicinity of the hives (Supplementary Materials Table 1) and 28 species at corals. Fish abundance and richness at corals and hives did not significantly differ from each other, but did increase (see β for direction of association) at both over time (GLMM: Abundance: $\beta = 0.0017$, $\chi 21 = 7.443$, $p < 0.01$, richness: $\beta = 0.002$, $\chi 21 = 3.512$, $p < 0.05$). Fish diversity measures (Shannon and Simpson) did not differ between hives and corals, nor over time. Coral growth at hives was not significant as an individual term for any reef fish community measures. However, the interaction of time and coral growth was

significant to fish richness (GLMM: $\chi 21 = 12.843$, $p < 0.05$) and Simpson diversity (GLMM: $\chi 21 = 10.260$, $p < 0.05$), with richness and Simpson diversity increasing over time as coral size increased. Tukey tests of predicted marginal means from the GLMMs comparing coral growth at each time point only predicted the 8 month vs 12 month to be significant for richness (Tukey: $\beta = 0.2557$, $p < 0.05$) and no comparisons were significant for Simpsons diversity. Thus, the minor spike of increase in richness and Simpsons Diversity observed at the 8th month was likely a reflection of reef stochasticity rather than associated with coral growth and are therefore not discussed further.

Behaviour of damselfish at hives and corals measured by duration and frequency over time

Damselfish were responsible for 52% of all 4029 fish interactions with hives and 34% of 563 interactions with corals. They spent significantly longer associating with hives than corals (GLMM: $\beta = -0.224$, $\chi^2_1 = 4.799$, $p < 0.05$; Fig. 4) and at a greater frequency (GLMM: $\beta = 0.762$, $\chi^2_1 = 3.758$, $p = 0.052$). Territorial behaviour by damsels was also significantly more frequent at hives than corals (GLMM: $B= 0.678$, $\chi^2_1 = 12.067$, $p < 0.001$). Damselfish pecked at hives at a significantly higher frequency than corals (GLMM: $\beta = 1.430$, $\chi^2_1 = 11.329$, $p < 0.001$), a trend which significantly increased over time (GLMM: $\beta = 0.069$, $\chi^2_1 = 39.275$, $p < 0.001$). The duration of damselfish pecking and territorial displays did not differ between control corals and hives, and were not affected by time.

Behaviour of other reef fish at hives and corals measured by duration and frequency over time

A total of 4612 fish behavioural interactions (associating, pecking or territorial behaviour) were observed at the hives and control corals (4049 interactions at hives (n = 10) and 563 interactions at the control corals (n = 6)). At hives and corals, the mean number of interactions per observation was 69.9 (± 53.1, S.E.), ranging from 2 to 336. At each hive, there was an average of 81 ± 56.1(S.E.) interactions (range 2-336), and at corals 35.5 ± 15.4(S.E.) (range 10-59). The time spent associating with structures was significantly greater at hives than corals (GLMM: $\beta = -0.581$, $\chi^2_1 = 12.652$, $p < 0.001$) and did not change over the sampling period. Time spent pecking did not differ between hives and corals and was not affected by time since deployment. There was no difference in frequency of fish associating and pecking with the hives and corals, but the frequency of both associating and pecking decreased over the sampling period (Associating: GLMM: $\beta = -0.0411$, $\chi^2_1 = 65.195$, $p < 0.001$, Pecking: GLMM: $\beta = -0.110$, $\chi^2_1 = 69.6366$, $p < 0.001$).

Territorial activity (duration and frequency) did not differ over time at hives and there were no observed territorial displays by non-damselfish species around control corals. There was a total of 50 territorial records at hives by non-damsels, only four of these were recorded at Booby Reef all by Sergeant Major *(Abudefduf saxatilis).* The other 46 territorial events were recorded at Pirates hives were by the following species: Sergeant Major (*A. saxatilis*) (n = 34 instances at 5 hives), smallmouth grunt (*Brachygenys chrysargyreum*) (n = 5 instances at one hive during one observation) and Brown Chromis (*Chromis multilineata*) (n = 7 instances at one hive during one observation).

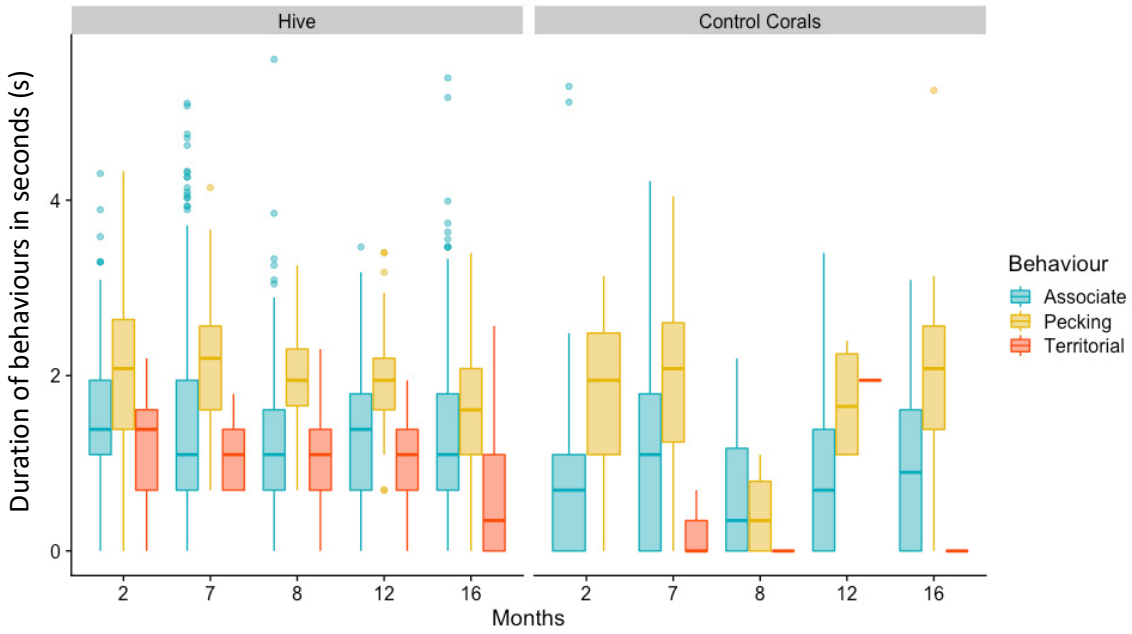


Figure 3.5: Duration of fish behaviour (in seconds) at corals (control corals) and artificial reef structures (fish hives) over a 16-month monitoring period. Three behaviours were monitored associating with the structures (within 2 fish lengths), pecking the structures (including the seeded corals on hives) and displaying territorial behaviour. Legend shows three behaviours: Blue - “Associate”, yellow - “Pecking” and red - “Territorial”.

Discussion

Here, fish hives, produced and deployed at low cost, proved to be effective artificial reef structures. They provide a nursery site for seeded Elkhorn corals and a natural settlement site for benthic settlers in different environmental contexts (demonstrated here by using two sites: Booby Reef a natural reef and a sandy area devoid of corals adjacent to Pirates Reef). Fish assemblages did not differ between hives and corals, and overall there was more damselfish activity at hives than corals. The time fish spent around the substrates did not

change over time and damselfish associated and displayed territorial behaviour around hives significantly more than natural corals.

Damselfish are commonly the first fish to colonise new reef habitat (Schopmeyer and Lirman 2015) and typically occupy the same territory over time (Itzkowitz et al. 1995) thus provide an ideal system for observing behavioural interactions with artificial reefs over time. Here, damselfish successfully established territories around hives within two months of deployment and showed relatively consistent behaviour at hives over the duration of the 16-month study. Damselfish spent longer associating with hives compared to corals, defended hives at a higher frequency and over time, the frequency at which damselfish pecked at the hives increased. Given that damselfish are ubiquitous on Caribbean reefs (Lieske and Myers 2001), it is likely that many hives were placed within, or near to damselfish territory, potentially aiding damselfish colonisation. The locations in which the hives were placed were not surveyed for damselfish territories prior to hive deployment. The footprint of an individual hive is 0.79 m^2 and the addition of a hive to an area increases the available outer surface area to 3.14 m^2. Given that almost the entire surface of the hives became covered in brown algae (Phaeophyta spp.) (Fig. 3), it is likely that damselfish favoured hives over live corals due to the increased area available for algal farming.

Reports of damselfish behaviour in terms of coral rehabilitation projects are conflicting (Seraphim et al. 2020) and their effects on reef rehabilitation are largely context dependent (Ladd et al. 2018). Damselfish territorial behaviour often deters corallivores from nurseries (Gochfeld 2009; White and O'Donnell 2010), which can aid coral growth (Glynn and Colgan 1988) and recruitment (Gleason 1996) and the size of damsels can directly enhance coral growth (Holbrook et al. 2008). But damselfish will themselves remove coral settlers whilst farming their algae patches (Ogden and Lobel 1978; Arnold et al. 2010) and can reduce coral growth rates of existing colonies (Schopmeyer and Lirman 2015). By pecking existing corals, damselfish can cause bite-lesions on corals, which promote further brown algae growth and can lead to corals being outcompeted (White and O'Donnell 2010; Hata et al. 2020). Damselfish removal prior to coral rehabilitation has been proposed as a means for overcoming their negative impact (Williams et al. 2019b); however at hives established here, seeded corals successfully established and grew. On the hive algal lawns though, at least at Booby, during this study there was no evidence of natural coral colonisation, potentially due to high populations of damselfish, since at Pirates, hives were newly colonised by fire coral and sponges and there were fewer farming damselfish compared to Booby. Addition of a large surface area colonised with brown algae, may have 'diluted' damselfish pecking activity, drawing them away from the seeded and colonizing corals and allowing all corals to grow. The addition of space for algal lawns may mitigate the need for damselfish removal when planning coral nurseries and should be further tested.

Corals successfully colonise many materials artificially deployed in the sea, including rock (Blakeway et al. 2013), seawalls (Tan et al. 2012), marble tiles (Bramanti et al. 2007), and it is well established that corals can settle on concrete structures (Coles 1984), which is commonly used in artificial reef construction (Baine 2001; Komyakova et al. 2013; Plumlee et al. 2020). Recruitment of organisms is crucial for ensuring longevity in success of artificial reefs (Mumby and Steneck 2008). Thus, it is useful to know that concrete containing vermiculite used for our hives is a viable substrate for naturally occurring marine species to colonise (brown algae *Phaeophyta* spp., coralline algae *Rhodophyta* spp., Fire coral *Capitata* spp. and tube sponges *Agela widenmyeri*) in addition to supporting growth of seeded Elkhorn corals. Coralline algae (as opposed to brown algae see Fig. 3.3) has been demonstrated as an essential precursor for natural coral settlement (Harrington et al. 2004; Ritson-Williams et al. 2010; Jorissen et al. 2021), this natural settlement of coralline algae at hives could therefore facilitate natural coral settlement in the future. Fish associating with the hives also came into close contact with the hives and a few species, (primarily surgeonfish) were seen displaying scraping behaviour demonstrating that the composition of concrete did not deter reef fish.

The territorial behaviour of some damselfish species effectively deters other reef fish from their territories through aggressive displays, biting and chasing (Bay et al. 2001). While damselfish territorial behaviour did not increase over time, pecking frequency did. Simultaneously, the frequency at which other non-territorial reef fish associated and pecked hives decreased over the 16-month study period, perhaps indicating the established dominance of the dark damselfish territory over time. At Booby Reef, damselfish may have successfully created a homogenous algal lawn on the hives over this time, which became less attractive to other reef fish. Likewise, at Pirates the coverage of sponge and fire coral may have been less attractive to grazing herbivorous species.

Many artificial reef designs have not incorporated the requirements of reef inhabitants (Baine 2001), however the shape of the coral habitat can affect specific ecological interactions (Whittey et al. 2021) and how fish use the habitat (Rilov et al. 2007). Laboratory studies testing coral-associated damselfish showed no discrimination in behaviour towards coral skeletons and 3D printed coral replicates (Ruhl and Dixson 2019) demonstrating that such structures can be effectively replicated artificially. The relationship between marine species and reef complexity is rarely a simple linear trend where increasing complexity results in an increased fish abundance (Bozec et al. 2013; Gonzalez-Rivero et al. 2017). The bicolor damselfish *Stegastes partitus* for example prefer less complex areas for foraging as it enables them to survey their surroundings for threats (Rilov et al. 2007). Thus, assessing the ecological requirements for specific species and species interactions is imperative for artificial reef design (Ladd et al. 2019) to facilitate restoration in several environmental contexts.

It is difficult to disentangle whether artificial reefs are actively promoting fish recruitment and enhancing fisheries as opposed to redistributing fish away from natural reefs (Carr and Hixon 1997). Here fish abundance at both fish hives and control corals increased during the sampling period, though in this short-term study, this trend probably reflects the natural variation in fish diversity and abundance at the reef (previously documented by (Dunkley et al. 2019a). As fish abundance was similar between hives and corals, this does indicate that addition of hives is not causing redistribution of reef fish within the reef. The data collection for this study was conducted during the COVID-19 pandemic and changes to local fishing activities around our study site are unknown. The study site Booby Reef is a popular destination for tourism and there was a significant decrease in tourism throughout the Caribbean (Sheller 2020). The observed increase in abundance therefore could reflect reduced anthropogenic pressure. Conversely, fishing activity might have increased during this period (Higgs 2021) including illegal, unreported, and unregulated fishing (Bennett et al. 2020). The COVID-19 pandemic also caused several study limitations including the sporadic video data collection time points and a lack of field data collection by our research team. In addition, two of our hives were lost due to sedimentation, indicating that these hives are only suitable for deployment in areas where there is less sand movement. However, this was probably a flaw in the selection of the deployment site rather than the design of the hive itself. Also, it is unknown how they would tolerate poor weather conditions.

Reefs provide an array of ecosystem services (Woodhead et al. 2019), and loss of coral habitat and restoration will require a range of rehabilitation efforts (Moberg and Ronnback 2003), which include all aspects of reef functions, such as providing structure and live polyps for corallivores (Cox 1994; Cole et al. 2008). The damage to reefs ranges from total destruction creating a rubble-like wasteland to a phase shift of a sponge dominated reef (Rasser and Riegl 2002; Norström et al. 2009; Dudgeon et al. 2010; Fung et al. 2011). Thus, restoration projects must span the need of these different environmental scenarios. Fish hives were tested in two different habitats, one relatively stable reef and another sandy area where there is no natural living coral. At both sites, hives attracted fish that interacted with hives and provided settlement sites for benthic organisms. Further, this study showed that relatively large (100 cm height, approximately the height of natural structures at the sites; Whittey et. al. 2021) artificial reef modules can successfully be created affordably without the use of specially made moulds, and using vermiculite made the structures lighter, which could therefore be deployed without heavy machinery. In addition, Elkhorn corals can successfully establish in the presence of farming damselfish when there is also additional habitat space provided. After 16 months of deployment, fish behaviour was relatively consistent at fish hives as with coral, but further monitoring is required as corals continue to grow and as natural settlement occurs. Indeed, future research of artificial reefs needs to include longer-term, multi-year studies

(Baine 2001). Hives provided habitat. Without intervention, coral reefs could take decades to re-establish (Williams et al. 2019a) and effectively harnessing ecological functions can facilitate reef rehabilitation (Ladd et al. 2018).

Chapter 4

Microbiota sharing between sharknose cleaner fish and their cleaner station

Abstract

Animals support beneficial microbial communities that play important roles in host functions. Fish skin contains a mucosal microbiota that is typically dominated by *Proteobacteria* along with other bacterial constituents, presumably from the surrounding water. The extent to which commensal bacteria are acquired from the environment, however, is poorly understood. The sharknose goby cleaner fish (*Elacatinus evelynae*) occupy specific corals on reefs where they advertise their cleaning behaviour. When these gobies are not cleaning they spend the majority of their time in direct physical contact (fish are resting on the coral so that fish skin is in direct contact with coral mucus and polyp tentacles) with their cleaning stations. These station substrates are often living corals or zoanthids, and each harbour their own microbiota. We predicted that there would be significant sharing of microbial communities between cleaner fish and their cleaning stations. To test this, we sampled cleaners (skin mucous), different cleaner station substrates and water samples from three different reefs in Tobago and sequenced their microbial communities. We found that alpha diversity of fish microbiota and all benthic organism microbiota where significantly distinct from one another and the surrounding sea water, suggesting high interspecies specificity of microbial communities. Despite this high specificity, however, cleaner fish shared 34 bacterial genera with one benthic organism, often found on cleaner stations, the yellow encrusting zoanthid (*Palythoa caribaeorum).* Given the high specificity between fish and benthic communities, the shared bacterial genera *E. evelynae* and *P. caribaeorum* are unlikely to be random and these microbes may offer unknown benefits to either organism.

Introduction

Corals create habitat for a diversity of marine life (Graham and Nash 2013) and for many reef mutualists, such as cleaners, the habitat can facilitate their cleaning behaviour (Huebner and Chadwick 2012; Whittey et al. 2021). Cleaners play an important role on the reef by removing parasites from the bodies of visiting reef fish known as clients (Cote 2000). Not only do corals provide shelter to these important mutualistic species, but specific structural features enhance cleaning behaviour through facilitating advertising and assisting clients spatial mapping to navigate to cleaner stations (Kulbicki and Arnal 1999; Braithwaite and de Perera 2006; Whittey et al. 2021). The benefits of the habitat may even extend beyond structural attributes. It is well known, for example, that an animal's bacterial community provides many benefits to immunity, metabolism and health (Bergman 1990; Hooper et al. 2012; Bengmark 2013; Bourne et al. 2016) and the coral habitat may be a living source of these microbes (Vanwonterghem and Webster 2020).

While establishment and functions of human microbial communities have been studied extensively (Eggesbø et al. 2011; Li et al. 2016), less well studied are the microbiota of wildlife (Pascoe et al. 2017), particularly those of corals and coral reef fish (Chiarello et al. 2020). Despite the paucity of studies, it is still well accepted that fish microbial communities, in particular their mucosal communities, are essential for host functioning (Gomez et al. 2008; Salinas et al. 2011; Xu et al. 2013) and that they are impacted by host diet, phylogeny, circadian rhythms and the environment (Miyake et al. 2015; Pollock et al. 2018; Le and Wang 2020; Ellison et al. 2021). For reef fish, the latter constitutes reef substrates, such as corals and the surrounding water. Corals depend on their symbiotic zooxanthellae for energy production (Titlyanov and Titlyanova 2020), but also rely on microbes for nutrient acquisition to survive in oligotrophic waters (Cardini et al. 2014; Rädecker et al. 2015; Peixoto et al. 2017) and the coral microbiota is important for maintaining health (reproductive capacity, disease resistance etc.) (Rosenberg et al. 2007; Bourne et al. 2016; Vanwonterghem and Webster 2020). Corals harbour unique microbial communities distinct from the surrounding seawater (Weber et al. 2019; Chiarello et al. 2020), and distinct microbial communities are also found in the mucous, tissue, skeleton and gastric cavity of corals (Pollock et al. 2018; Wada et al. 2019). Additionally, the water in the immediate surroundings of the coral, harbours its own unique microbial community. This space directly surrounding the corals is named the coral 'ecosphere' (Weber et al. 2019) and the release of organic matter from coral mucous can increase bacterial production by 50 times in this ecosphere zone (Silveira et al. 2017). Therefore, corals and coral reef inhabitants are in direct contact with an ever-changing

external environment, the sea, which hosts a diverse microbial community (Hernandez-Agreda et al. 2018).

The outer mucosal layers for both corals and fish are the interface zones between substrates and the surrounding aqueous environment, which facilitates exchange of microbes between habitat and animal mucosal layer(Gomez 2021). In addition to free-living organisms in the water, coral reef fish are exposed to a range of benthic organisms which each harbour unique (Chiarello et al. 2020) and dynamic microbial communities (Van Oppen and Blackall 2019). Animals inhabiting corals are strongly interlinked through exchange of energy via benthic-pelagic coupling which suggests that microbial communities could also be shared (Vanwonterghem and Webster 2020; Bourne and Webster 2013), however this is not well understood.

Despite the depth of knowledge on the importance of the microbiota, how and from where hosts acquire their microbial counterparts is not clear, particularly in wildlife contexts. However, it is assumed that microbes are likely acquired, by some mechanism, from the immediate surrounding of the host (Browne et al. 2017). Multiple factors such as host diet, genetics, behaviour and the environment will affect which microbes the hosts are exposed to and which are able to successfully colonise the host (Tung et al. 2015; Browne et al. 2017; Sylvain et al. 2020). In laboratory settings, individuals closely associated with each other share commensal microbes, for example, zebra fish, *Danio rerio,* shared microbial communities with each other when introduced to the same tanks (Burns et al. 2017). Microbiota sharing between individuals has also been documented in a few terrestrial species. The microbial composition driven by social contact between conspecifics (Baboons, *Papio* spp. see Tung et al 2015). Microbe sharing even occurs between different species, for example humans cohabiting with pets (Song et al. 2012) and anemone fish altering the community of their anemone hosts (Pratte et al. 2018). Commensal bacteria likely follow the same routes of transmission as those for pathogenic bacteria and 'infect' individuals during direct contact although much about microbial transmission is not known (Browne et al. 2017). Thus, interspecies microbe sharing should be a widespread phenomenon. Further, given that individuals harbour highly specific and unique microbial communities (Chiarello et al. 2020), this also suggests that hosts are somehow able to regulate their microbiota.

Cleaner fish, such as the sharknose goby (*Elacatinus evelynae*), occupy stations and exhibit strong site fidelity (Waldie et al. 2011). Sharknose gobies remain in direct contact with their habitat while they wait for visits from client fish (Bshary and Schäffer 2002; Dunkley et al. 2018). Stations usually consist of coral (which itself is often a matrix of healthy and diseased coral), along with zoanthids and algae (Whittey et al. 2021). These gobies are theoretically exposed to a diversity of microbial communities from these different habitat substrates, as well as the surrounding seawater, and the microbial community of their clients (Pereira et al. in

press). These cleaners therefore offer an interesting system for exploring the extent to which the diversity and composition of bacterial communities are shared. Given that cleaners are in constant contact with their habitat, it is hypothesised that a level of microbe sharing will exist between cleaners and cleaner station. Here, the skin microbiota of the sharknose goby cleaner fish is characterised using metataxonomics and Illumina technology as well as the benthic organisms forming the immediate substrate of the cleaner station, including coral, zoanthid and algae, as well as the coral 'ecosphere'. Using co-occurrence network analysis, which bacterial genera are shared between cleaners and their cleaner stations is explored.

Materials and Methods

Sampling the microbial community of cleaner fish and their associated habitats

All sampling was carried out at Man O' War Bay, Tobago (11°19.344′N, 060°33.484′W) during May-June 2019. Microbial community samples associated with the mucosal surface of the dedicated Caribbean cleaner fish the sharknose goby, *Elacatinus evelynae,* (n = 33) were taken from three different coral reefs (Booby n = 15, Turpin's n = 10 and Pirates Reef n = 8), all within 1.5 km of each other in the Man O' War Bay but representing non-contiguous reefs. At each reef individual gobies were captured from discrete 'cleaning stations'; defined as a location that the cleaner is tightly associated with, shows site fidelity to, and typically consists of coral (Whiteman and Cote, 2002). Each station was labelled and the sharknose goby occupancy monitored for a minimum of 3 consecutive days prior to fish collection. Occupancy monitoring occurred between 8:30 am and 5:30 pm by random swim surveys, which involved two observers snorkelling over the study site and searching for cleaners at the given station and immediate vicinity (~ 2 m^3 area), spending up to 2 minutes at each station searching for a cleaner. In addition, 8 control corals, were identified as not being occupied by cleaners, using the methods above.

Fish were live-captured, using a hand net and transferred into a container of fresh seawater, and then transferred to the field laboratory. Here, each fish within 3 minutes of its capture, was placed on a sterile glass petri dish and swabbed over the entire length of the body on both sides using an individually packaged sterile cotton swab (Tubed Sterile Dryswab™ Tip, company, country). Cleaner gobies were returned to their original point of capture within 10 minutes. Each sample swab was placed in a sterile 1.5 ml Eppendorf tube with molecular biology grade ethanol (99.9%) and stored at 4 °C for subsequent sequencing.

To assess the extent of microbial community sharing between cleaners and their cleaning stations, we collected samples of the associated cleaning station reef substrates. Each cleaning station in this study consisted predominantly of brain coral (Faviidae spp.), with

a mix of healthy and diseased coral, zoanthids and algae. At each cleaning station, a swab was taken by inverting a tube underwater before opening and held vertically to retain an air bubble during swabbing. A swab was taken from each substrate to capture the station microbial diversity (see Table 1, Appendix 1 for full details of samples taken at each station). For each brain coral investigated, samples of healthy coral (n = 31), diseased areas (coral that exhibited bleaching, or discolouration suggesting the beginning of bleaching n =10), yellow encrusting zoanthid, *Palythoa caribaeorum* (n = 7), green mat zoanthid, *Zoanthus pulchellus* (n = 27), and algae (n = 31) were included. In addition, we sampled the microbial community of 8 control brain corals and the associated benthic organisms. This included a single swab of healthy tissue, and diseased coral (n = 7), *P. caribaeorum* (n = 1), *Z. pulchellus,* (n = 6)*,* and algae (n = 8). See Tables 1 and 2 (and Appendix 1) for full details of samples taken from each control.

For all corals (station and non-stations; n = 39), the ecosphere was sampled by collecting one litre of seawater within 15 cm of the coral. In addition, as a seawater control four one litre seawater samples were taken in randomly selected locations from each of the three reef locations (Booby, Turpin's and Pirates Reef), which were subsequently pooled giving one sample per reef. All reef water samples were collected by inverting water bottles in the middle of the water column whilst swimming parallel to the shore. Negative controls consisted of one litre of bottled water from the commercial drinking water bottles used during sampling, one swab exposed to the air during processing in the field, and one swab exposed to seawater. All water samples were filtered through 250 ml Analytical test Filter Funnels. Once filtered the filter from the funnel was collected using sterile tweezers and placed into a sterile 28 g aluminium tin. To ensure samples were kept sterile, filters were not cut up and stored in Eppendorfs as this would have required further handing in the field.

All samples were collected between 8 am – 12 am during 29 May – 21June 2019. All animal capture and sampling was carried out under licence number #001/2016, from the Tobago House Assembly, Department of Natural Resources and the Environment, and approved by Cardiff University's animal ethical committee.

DNA extraction

Ethanol was evaporated off from the swab samples (n = 177, see Supplementary Table 2 and 3) under aseptic laboratory conditions in a heated block at 42°C for 6-12 h contained within a laminar-air flow cabinet. DNA was extracted from 31 fish skin swabs, 31 coral mucus swabs, 10 diseased patches on corals, 27 green zoanthids, 7 yellow zoanthids, and 31 algae-covered dead coral using DNeasy® PowerSoil® Kits (QIAGEN©, Milan, Italy) according to the manufacturer's instructions as recommended by Rosado et al. (2019).

Water filters (n = 40) were removed from their aluminium tins under aseptic conditions, folded 4 times and cut 5 times allowing them to be placed straight into Lysing Matrix E Tubes (MP Biomedicals) from the FastDNA® Spin kit for Soil (MP Biomedicals), which was used for DNA extractions. Multiple filters containing sea water samples from each site (Booby Reef: n = 7, Pirates Reef: n = 5 and Turpin's Reef: n = 4) were pooled into one water filter sample per site.

Different kits were used for water samples and mucosal substrates (benthic organisms) due to the different nature of the samples. Genetic material from water samples are harder to capture due to degradation in the water, filters were used to capture free DNA and optimally represent the diversity present. FastDNA® Spin kit for Soil (MP Biomedicals) can be used to capture genetic material from environmental samples efficiently (Eichmiller et al. 2016). DNeasy® PowerSoil® Kits can be used with minimal material to account for inhibitors present in the samples (Rosado et al. 2019)

16S rRNA gene sequencing of microbial communities

A total 245 samples were sequenced, including 4 'blank' samples free of sample material as negative controls. DNA concentration was measured using a Qubit 4.0 fluorometer (Thermo Fisher Scientific Inc.). Using 16S rRNA gene primers F515/R806 (forward: 5' GTGCCAGCMGCCGCGGTAA 3', reverse: 5' GGACTACHVGGGTWTCTAAT 3'; (Caporaso et al. 2011) with Illumina adaptors, each DNA sample was amplified for the V4 region of the 16S rRNA gene (~250 bp). These F515/R806 primers have been used previously to characterize bacterial communities in coral reef fish (Parris et al. 2016; Chiarello et al. 2018) including in a closely related species of cleaner fish (*Elacatinus prochilos*, see Xavier et al. 2019). Samples were prepared on plates, randomly assigning samples to plates.

To create the library, samples where multiplexed using the dual-indexing sequencing strategy of Kozich et al. (2013) and PCR was performed: 95 °C for 2 min (1 cycle), 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min (30 cycles), and 72 °C for 10 min (1 cycle). The PCR products were visualised using an E-Gel with SYBR safe DNA Gel Stain 2% (Life Technologies). The library was normalised using SequalPrep Normalisation Plate Kit (Life Technologies) following the manufacture's protocol for sequential elution. The concentration of pooled samples in the library was determined using Kapa Biosystems Library Quantification kit for Illumina® platform. The Agilent Bioanalyzer High Sensitivity DNA analysis kit (Agilent®) was used to determine the sizes of the amplicons and the final library was normalised to equal molar concentrations. Sequencing was performed in a single run on the Illumina® MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina® MS 102-2003) targeting a depth of 46,000 to 51,000 reads per sample. Two positive controls (ZymoBIOMICS™ Microbial

Community Standard) and four negative controls (nuclease free water) were processed through the entire DNA extraction and PCR amplification stages alongside the test samples.

Bioinformatic pipeline processing 16S rRNA gene data

Raw FASTQ files were analysed using QIIME2 (release 2020.2). Reads were denoised trimmed to 230 ensuring a minimum quality score of 25. A frequency table of Amplicon Sequence Variants (ASVs) was built using Dada2 (Callahan *et al*., 2016). Taxonomy was assigned using the SILVA 132 database (https://www.arb-silva.de/download/archive/ qiime; Silva_132) using a 99% similarity threshold. The resulting representative sequences were used to create a phylogenetic tree using SEPP (Mirarab *et al.* 2012) for downstream phylogenetic analyses. Processing of samples was carried out in RStudio 1.3.1056 (R Core Team 2018) using the phyloseq package (McMurdie and Holmes, 2013). Using phyloseq, samples with fewer than 5000 reads were filtered and removed (two skin samples and five gut samples). Mock microbial libraries were also removed and non-bacterial reads were filtered out by keeping all 'bacteria' (Kingdom level) and removing 'mitochondria' (Family level) and 'Chloroplasts' (Class level). Singletons were removed, and samples were rarefied to 90% of the minimum sequence depth based on rarefaction curves leaving a minimum of 5396 reads per sample. The most abundant ASVs were plotted by selecting sequences which represented >1% of all sequences.

Statistical analysis

All statistical analysis was carried out in RStudio version 1.3.1056 (R Core Team 2018). Measures of microbial taxonomic alpha-diversity (Shannon and Inverse Simpson) were calculated using the phyloseq package (McMurdie and Holmes, 2013) and data were analysed using General Linear Models (GLMs) with appropriate error distribution. Model assumptions and fit were assessed using residual plots (as specified by Bolker et al. 2009). Best fitting model selection was based on Akaike Information Criterion (AIC) using a backward elimination approach and the presence of potential influential points on model outcomes were checked for (using Cook's D and leverage). To test the relationship between alpha diversity measures (Shannon [Inverse Gaussian family and log link], Inverse Simpson [Inverse Gaussian family and identity link] where included as response variables and cleaner fish station metadata (individual station identity, site and sample type) as explanatory variables. Date of sampling was included in the initial GLMs to test for links between diversity measures and sampling points. No effect of sampling date was found on diversity measures and so was removed from the final models. Prior to analysis alpha diversity measures were rescaled from zero to one using the "scales" package (Wickham 2017): this method does not remove the variability between values, but simply transforms data to aid model fit. Beta diversity was assessed using PERMANOVA, based

on unweighted UniFrac indices, using the 'adonis' function in the R package 'vegan' (Dixon 2003).

Building network relationships of shared bacteria between substrates

Bipartite networks were used to visualise the association of bacteria genera within each substrate at site level; these graphs represent interconnectedness (edges) between entities (nodes). Nodes, here represent substrates, which included (ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae genera), Coral Ecosphere water – water from the immediate surrounding of the coral, Diseased Coral – brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green – green mat zoanthid, *(Zoanthus pulchellus),* Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid*, Palythoa caribaeorum*) grouped by site. Nodes were coloured by substrate type and generated using a median average proportion ASVs at genera level within each site. Edges represent shared bacterial genera between substrates. Networks depicting microbe sharing between fish, water and substrates were constructed using genera instead of using ASV level as using individual ASVs introduced considerable noise. These networks were calculated in R following the graph constructor workflow by Sedlar et al. (2016) using a 0.001 threshold of abundance of Genus level ASVs nodes and edges were created. Output from the graph constructor workflow were then visualised using Gephi (gephi.org, accessed November, 2021) (Bastian et al. 2009). Using Gephi, modularity was used to detect community structure in the network. A modularity matrix is built by determining the number of edges that fall within groups (by measure of eigenvalues – metrics of distance) in the network, compared to a theoretical standard number of edges in an equivalent network where edges are placed at random (Newman 2006).

Results

Microbial communities of the cleaner fish station constituents

The microbiota of 212 samples of cleaner fish and cleaner fish station benthic organisms comprised 48,531 amplicon sequence variants (ASVs) (fish skin n = 3167, coral n = 6308, diseased coral n = 4408, green zoanthid n = 12040, yellow zoanthid n = 1137, algae covered dead coral (ACDC) n = 17723, coral ecosphere n = 22520 and reef water n = 4266). Note n = 33 samples did not contain sufficient reads for detailed analysis, thus were not included in analyses (see Supplementary Materials Table 5 for full list of samples). All samples were dominated by the common top four phyla Protobacteria, Cyanobacteria, Bacteroidetes and Planctomycetes (Fig. 4.1). Proteobacteria had the highest relative abundance in all substrates

(range from 30%-82%) apart from diseased coral for which Cyanobacteria had the highest abundance at 31%.

Fish skin and yellow zoanthid had the most similar community profiles in terms of abundance (Fig. 4.1). Fish skin were dominated by Proteobacteria (75%) and the top ten phyla made up 98.6% of all ASVs (Actinobacteria (5.2%), Bacteroidetes (4.9%), Cyanobacteria (3.3%), Tenericutes (2.9%), Planctomycetes (2.9%), Firmicutes (2.0%), Acidobacteria (1.6%), Chloroflexi (0.7%), Verrucomicrobia (0.3%) and Omnitrophicaeota. Yellow zoanthid was also dominated by Proteobacteria (82.1%) and the top ten phyla made up 99% of all ASVs (Bacteroidetes (7.3%), Cyanobacteria (5.5%), Actinobacteria (1.4%), Planctomycetes (1.2%), Epsilonbacteraeota (0.5%), Omnitrophicaeota (0.3%), Verrucomicrobia (0.3%), Acidobacteria (0.2%), Patescibacteria (0.2%)).

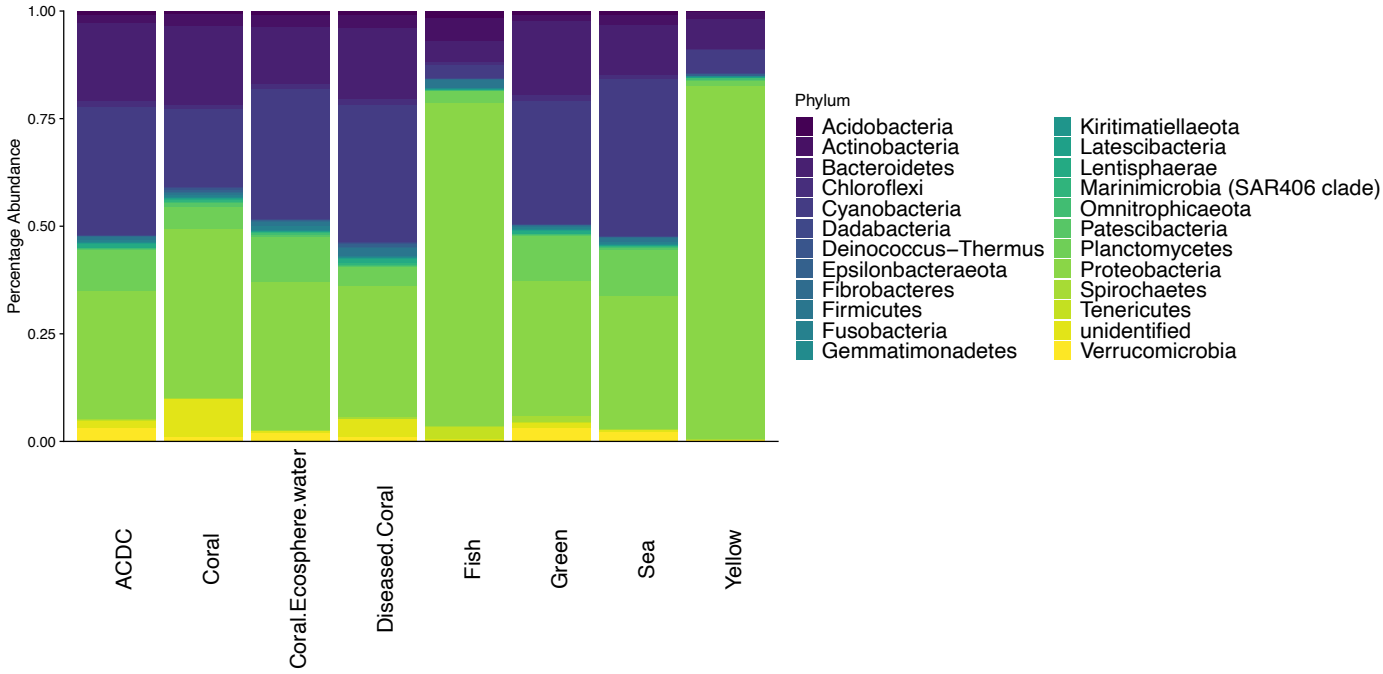


Figure 4.1: Proportional abundance of top ten bacterial phyla found in each sample type: Sharknose goby cleaner fish (*Elacatinus evelynae*) and associated benthic constituents of each cleaner fish station from three reefs in Man O'War Bay, Tobago. ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae, genera), Coral Water (Coral Ecosphere Water) – water from the immediate surrounding of the coral, Diseased Coral – areas of brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green – green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoonathid (*Palythoa caribaeorum*).

All substrates significantly separated from each other by measures of beta diversity (PERMANOVA;$p<0.05$) see Supplementary Materials Table 4, Supplementary Fig 1 and Fig. 4.1. demonstrating that the microbial communities of all samples were significantly different from each other. Measures of alpha diversity, Shannon and Inverse Simpson, were included in GLMs with the following variables: date of sampling, station number, reef site (Booby, Pirates or Turpin's) and sample type. Both Simpson and Shannon measures were significantly different between sample types (Inverse Simpson GLM: $F = 7.3683$, $p<0.0001$; Shannon GLM: $F = 31.017$, $p<0.0001$), while date of sampling, station number and reef site were not significant. This difference in samples is illustrated in nMDS analysis (Fig. 4.2) using Bray Curtis ordination and 95% confidence ellipses. Fish and coral particularly clustered separately and showed no overlap in ellipses. ACDC and green zoanthids also showed close connections while coral and yellow zoanthid ellipses seemed to span all substrates. Substrates were separated to site in ordination plots using Bray Curtis, there were no clear trends associated with site, however the coral ecosphere water from Booby Reef clustered away from Turpin's and Pirates Reef. Despite this, coral mucus, which was predicted to correlate with ecosphere water, did not show the same clustering.

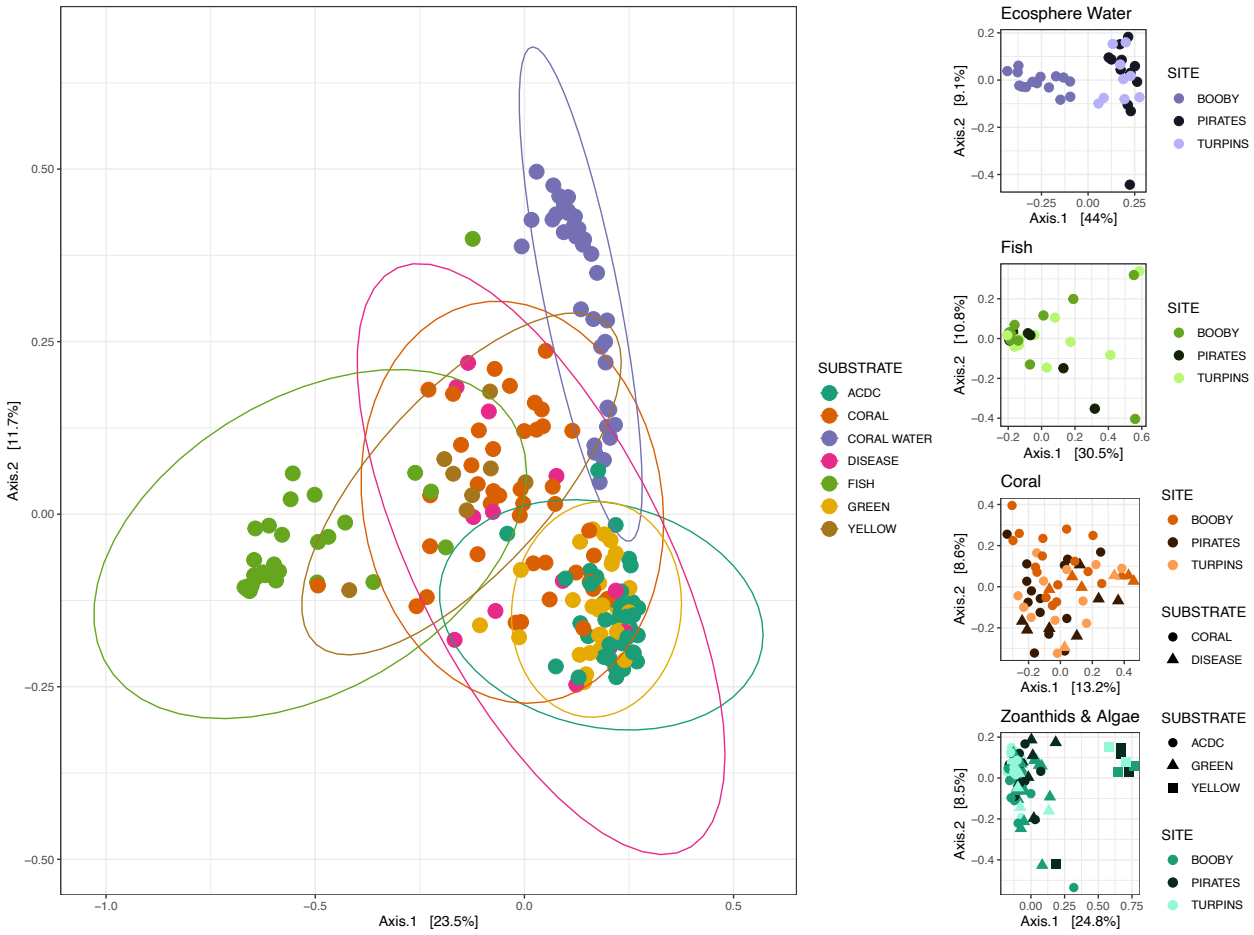


Figure 4.2: NMDS plots of microbial communities from sharknose cleaner fish (*Elacatinus evelynae*) and the substrate constituents of their cleaner stations. Left: All substrates (ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae), Coral Water (Coral Ecosphere Water) – water from the immediate surrounding of the coral, Disease – brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green – green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid, (*Palythoa caribaeorum*) with 95% confidence ellipses. Right: Three groups of benthic constituents (Ecosphere water, Coral and Diseased Coral, and ACDC and yellow or green zoanthids) separated by site.

Network relationships of shared bacteria between substrates

A bipartite network was used to demonstrate the relatedness between the microbial communities with nodes represented by different substrates (Fig. 4.3 A). Modularity analysis of this network revealed five distinct communities, firstly - fish cluster with yellow zoanthids (Fig. 4.3 B). Within the fish-yellow zoanthid cluster, 34 bacterial genera were shared with fish from the following phyla: Proteobacteria (41%), Actinobacteria and Bacteroidetes (12%) followed by: Planctomycetes, Acidobacteria, Cyanobacteria, Verrucomicrobia, Acetothermia,

Omnitrophicaeota and Patescibacteria (between 3% and 9% in descending order). The second community cluster consisted of coral and coral water. The third community cluster consisted to algae covered dead coral (ACDC) and green zoanthid. However, there was crossover between some coral and diseased coral nodes representing more sharing of genera with green zoanthid than each other. One water sample from Booby Reef and one from a diseased coral clustered into independent communities.

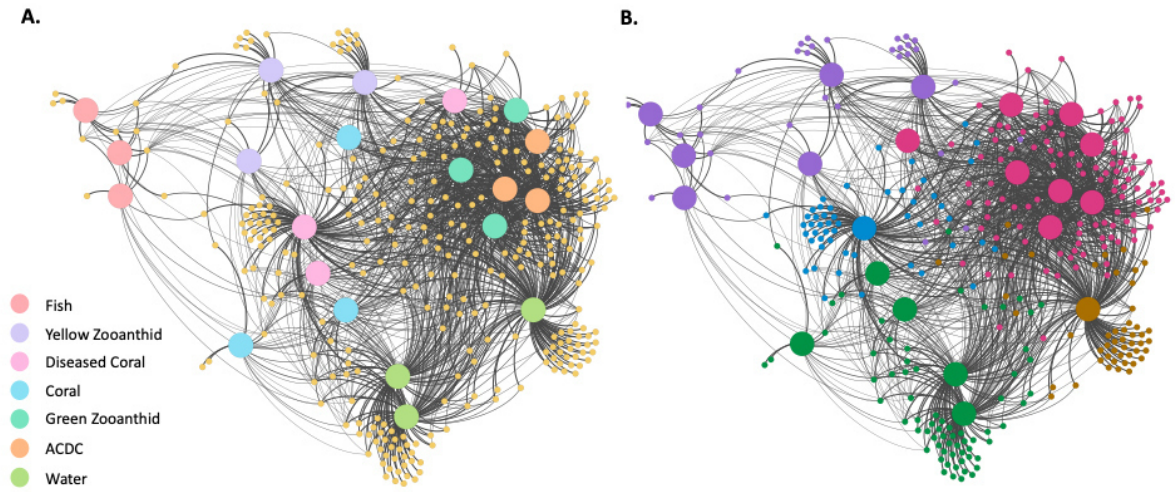


Figure 4.3: Bipartate networks of shared microbial genera substrates including sharknose goby cleaner fish mucosal samples (*Elacatinus evelynae*) and cleaner fish station substrates from three different reefs in the Man O'War Bay, Tobago. Station substrates include; (ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae), Coral Water (Coral Ecosphere Water) – water from the immediate surrounding of the coral, Disease – brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green zoanthid– green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid, (*Palythoa caribaeorum*) A. Network showing shared bacterial genera among substrate type. B. Modularity network depicting the substrates separating into five distinct modules, colours represent the distinct modules allocated to the nodes.

Discussion

Together our results provide evidence for microbial sharing between sharknose goby cleaner fish (*Elacatinus evelynae*) and a benthic zoanthid associated with their cleaner

stations. To our knowledge this is the first study to test whether there are any shared genera of bacteria between sharknose goby cleaner fish and their immediate habitat. Overall, there was a distinct, separation between all station constituents in terms of microbial communities: coral, diseased coral, algae covered dead coral, yellow zoanthid, green zoanthid and coral 'ecosphere' water. Although all microbial communities of the station were distinct, we found shared genera between cleaners and the yellow zoanthid *Palythoa caribaeorum.* This result is intriguing given that cleaners are most often observed on live corals and previously it has been shown that fish skin microbiota is distinct from their surrounding communities (Sylvain et al. 2020). Together, this study highlights the potential for microbe sharing between cleaner and live benthic organisms of the cleaner station, thus furthering the importance of the coral reef habitats microbes. Bacteria may be of functional importance to organisms and bacteria may be shared between individuals thus conveying these functional traits..

Sharknose cleaner fish spend most of their time on their cleaning stations waiting for clients (Whiteman and Côté 2002; Côté and Soares 2011). During this time they are mostly found on corals, in direct contact with the coral's mucosal layer and within the coral ecosphere, therefore we predicted shared microbes between these three substrates. Yet shared microbes only occurred between fish skin and the yellow zoanthid, *Palythoa caribaeorum*. Fish and yellow zoanthid shared 34 genera, from 10 phyla, primarily Proteobacteria, Actinobacteria and Bacteroidetes (for full list see Supplementary Table 6). Further, modularity clustering identified cleaners and yellow zoanthids in the same module, independent from other substrates including ecosphere water (see Fig. 4.3 B).

P. caribaeorum is a common mat zoanthid in the Caribbean (Kemp et al. 2006; Durante et al. 2018), and sharknose gobies are occasionally seen resting on top of *P. caribaeorum* (pers. observ.). Passive contact does not usually correlate with shared microbes: marine animals harbour distinct communities from the surrounding water (Bik et al. 2016; Sylvain et al. 2020) and amphibian species co-inhabiting ponds also do not share microbes (McKenzie et al. 2012). This suggests that skin-associated microbes are not simply a reflection of the available microbiota within the immediate surroundings of the host and that there may be a selective mechanism for microbial acquisition. Contact between individuals can elicit microbial changes; for example, anemone fish reared in laboratory settings showed altered microbial compositions when fish were introduced to anemones (Pratte et al. 2018). Anemones sampled in the wild were also found to have converged microbial functions with their anemone fish symbiont (Titus et al. 2020). The microbial coupling and convergent microbiomes seen in the anemone-anemone fish mutualism however is not likely driven by passive contact (Titus et al. 2020). These two mutualists have co-evolved and benefits each other through a range of behavioural and chemical attributes; the anemone fish protects the anemone from predators,

and in return the anemone gains a variety of nutrients (Porat and Chadwick-Furman 2005; Cleveland et al. 2011).

P. caribaeorum is known to harbour high levels of the marine toxin palytoxin (Béress et al. 1983; Guppy et al. 2019). Palytoxin is a highly complex and potent toxin found in several marine species (Sharma et al. 2014; Patocka et al. 2015) though the origin of the toxin is unknown and how *Palythoa* spp. acquire, or make, the toxin is still not understood (Aratake et al. 2016). However, there is evidence for a bacterial origin, Seemann et al. (2009) found several strains of bacteria, isolated from *P. caribaeorum*, which demonstrated palytoxin-like hemolysis suggesting the ability to produce palytoxin. Additionally free-living Cyanobacteria, *Trichodesmium,* also produce palytoxin (Patocka et al. 2015). In *P. caribaeorum,* palytoxin is thought to provide protection defending the colony from predation (Guppy et al. 2019). Intriguingly, there is some evidence that palytoxins could bioaccumulate up the food chain, posing a risk to reef ecology and human consumption (Guppy et al. 2019). Sharknose gobies are not known to consume *P. caribaeorum,* however *Elacatinus prochilos,* a close relative of the sharknose do consume sponges and coral polyps (Arnal and Côté 2000). Therefore, consumption of *P. caribaeorum* by sharknose gobies cannot be ruled out as a potential mechanism for these shared microbes.

During cleaning behaviour, cleaner fish spend a significant amount of time in direct contact with potentially diseased clientele removing their ectoparasites, dead or damaged tissue and mucous (Cote 2000; Grutter and Bshary 2004). Horizontal transfer of microbes between fish has been demonstrated (Burns et al. 2017) thus contact with clients could facilitate transfer of both pathogenic microbes and beneficial ones. *E. prochilos,* is thought to exist as two ecotypes; cleaning and non-cleaning (sponge-eaters), those that clean were found to have a significantly increased prevalence of potentially pathogenic bacteria genera in their gut and skin microbial communities (Xavier et al. 2019). Yet despite this increased exposure, the cleaning mutualism persists, suggesting the benefits of cleaning outweigh the negatives of acquiring potential pathogens.

In addition to increased infection potential, some clients could predate the cleaner (Darcy et al. 1974; Cote 2000). Cleaners are small fish with very little physical protection and although their mutualistic cleaning behaviour affords them protection from predation to some extent (Cheney et al. 2008; Cheney 2013), it has also been proposed that sharknose gobies may be toxic, similar to their close relatives (Colin 1975; Lettieri and Streelman 2010). Toxins have been found in Gobiodon spp. (see Noguchi and Hashimoto 1973) and are thought to potentially be secreted from cells in the skin (Hashimoto et al. 1974), although this is not confirmed. Their striped colouration may both signal cleaning behaviour and be an aposematic cue (Lettieri & Streelman, 2010). Toxins such as tetrodotoxins cause paralysis through disruption of nerve to muscle signalling (Narahashi 2008). Though the chemical nature of

Elactinus spp. are unknown, *Elacatinus* individuals proved to be “distasteful” to predators and caused hyperventilation after consumption (Tuttle et al. 2021). The origin of toxins in many species is unresolved, however microbial origins have been proposed for tetrodotoxin synthesis in many species (Chau et al. 2011; Li et al. 2020). Further, some animals can sequester chemical defences through consuming organisms which harbour toxins (Hay and Fenical 1996). For example, the Spanish dancer nudibranch *Hexabranchus sanguineus* consumes sponge (*Halichondria* spp.) which contains oxazole macrolides. The nudibranch is then able to modify these macrolides and concentrate them into its dorsal mantel and eggs where they act as defence against predators (Hay and Fenical 1996; Hines and Pawlik 2012). If cleaners were able to sequester palytoxin, or microbes which produce palytoxin, during their contact (or potential consumption) with *P. caribaeorum* the toxin could be the source of the cleaners “distastefulness”.

Evidence for a host’s ability to preferentially select their bacterial communities through genetics and immunity is increasing (Browne et al. 2017). Further, there is surprising evidence that hosts may also actively control their symbiont populations (Ezenwa et al. 2012), thus suggesting that sharknose gobys may be able to actively play a role in acquiring specific microbes. Several squid species (Sepiolidae and Cephalopoda spp.) harbour bioluminescent bacteria (*Vibrio* and *Photobacterium*) in a specialised cavity lined with epithelial cells called a light organ (Herring 1977; Jones and Nishiguchi 2004). The bioluminescent capacity of the Hawaiian bobtail squid *Euprymna scolopes* and its commensal bacteria, *Vibrio fischeri,* has been extensively studied (McFall-Ngai and Ruby 1991). Upon hatching, the squid ventilates seawater through pores into their light organs where only *V. fischeri* can establish (Nyholm and Nishiguchi 2008). The bioluminescent activity of the symbiont follows cyclic fluctuations in accordance with circadian rhythms of the squid host which may be regulated by oxygen flow (which is needed to catalyse the reaction producing the luminescence, and regulation in leiognathid fishes has been previously suggested by McFall-Ngai (1991) to the symbionts. This suggests that the squid host may be able to regulate the activity of the symbiont (Boettcher et al. 1996). Few examples of such microbial acquisitions pathways exist, more research should focus on microbe acquisition, including the pathways relevant to the sharknose goby.

Access to mutualistic symbionts is an underappreciated benefit to group living (Lombardo 2008). Social insects living together offer a unique demonstration of how shared microbes can be beneficial to hosts (Lombardo 2008; Koch and Schmid-Hempel 2011). *Apis mellifera* worker bees initially lack gut bacteria and acquire their microbes in part from other members of the hive, faecal material and exposure to hive components (Powell *et al.* 2014). Without this microbial transmission bumblebees are more susceptible to *Crithidia bombi*, a virulent gut parasite. Affording such protection, the microbiota may be considered as a host’s

extended immune phenotype (Koch and Schmid-Hempel 2011). Sharknose gobies can be found living individually on corals or in groups, up to 9 cleaners were found on one station used in this study (Dunkley et al. 2020; Whittey et al. 2021). In an eight-year study, cleaner local abundance (number of cleaners at a station) was only significant in predicting cleaning during one of these years of sampling (Dunkley et al. 2020). Further, overall cleaner local abundance was not as significant as other variables relating to client identity and abundance. This suggests that cleaner number in cleaning behaviour may be context dependent, however is not likely to be a fundamental predictor of cleaning behaviour (Dunkley et al. 2020). Thus, group living in cleaners is likely not essential to cleaning behaviour, but effects regarding microbiota has not been considered and need further exploration.

Mutualistic relationships are ubiquitous in nature and have facilitated numerous ecological functions and inter-species symbioses (Meyer-Abich 1943; Aanen and Hoekstra 2007; Bronstein 2015). Indeed, symbiotic relationships are fundamental to biological systems (Gilbert et al. 2012) and microorganisms are emerging as key players in maintaining reef health (Vanwonterghem et al. 2020). Meta-organisms, such as coral or zoanthid holobionts do not exist in isolation but are nested within communities of other holobionts and are therefore a part of a larger marine environment (McFall-Ngai et al. 2013) therefore impacts on corals and their microbiota may affect the system at reef scale (Hoegh-Guldberg et al. 2019; Vanwonterghem and Webster 2020). Here, we show that the cleaner fish microbiota although distinct from the microbial communities found at the station, do share some microbial taxa with the yellow mat zoanthid. This study has provided evidence for potential inter-species horizontal transfer of microbes however full sequencing of microbial taxa is needed to confirm the same strains are shared between individuals. Although the transmission route, and the benefits for this microbe sharing is unclear we suggest that cleaners may actively seek out beneficial microbes for protection. We suggest further research into the association of cleaners with the yellow mat zoanthid and to investigate the nature of cleaner fish ‘distastefullness’ as proposed by Tuttle et al. (2021). Given this highly complex and integrated system it is timely to investigate these nested microbial ecosystems using metagenomics to understand their functions so that we might predict not only how they will function in future but also how we then might be able to harness these functions to help coral reefs (Vanwonterghem and Webster 2020; Bell et al. 2018).

Chapter 5

Microbiota of the Caribbean cleaner fish, the sharknose goby (*Elacatinus evelynae*)

Abstract

The sharknose goby (*Elacatinus evelynae)* is a dedicated cleaner fish, gaining all its nutrition from cleaning interactions with its clients. During these cleaning events, the cleaner is inadvertently exposed to numerous potentially parasitised individuals in the form of client fish species. Despite the benefits of this feeding strategy, the cleaner fish potentially risks high transmission potential from the parasites and pathogens of the client fish. Given the importance of skin microbiota in fish as the first barrier of immune defence it is possible that cleaners may harbour a diversity and composition of microbes that facilitate their protection from parasites. Here, we quantify bacterial communities of the sharknose gut and skin, and identify functional gene groups in these commensal communities. As in closely related *Elacatinus* spp. previously studied, the skin communities were dominated by proteobacteria, while Tenericutes and Proteobacteria dominated the gut community. We identified genes with predicted functions involved in argine biosynthesis, which are potentially linked to the creation of toxin precursors**.** Our study reveals potential further importance of the microbial communities of cleaner fish. The diversity and function of many reef fish microbiota is unknown and given the current threat to coral reefs there is an urgent need to further our understanding of the complex microbial interactions on reefs.

Introduction

Coevolution between microbes and vertebrates has resulted in a highly interdependent system where microbes live in symbiosis with these hosts (Shapira 2016). The diverse microbial communities that live within and on their multicellular hosts provide major health benefits (Gómez and Balcázar 2007; Hayes et al. 2010; Chung et al. 2012; Ezenwa et al. 2012; Tarnecki et al. 2017). Although it is widely accepted that microbiota are important to all living organisms, studies of non-human animal microbiota, particularly wild animals lag behind those of humans (Pascoe et al. 2017). Incorporating the complex environment of wild animals

into microbial studies will help us understand the function of microbial communities in a natural context, allowing us to further our understanding of the microbiota.

The gut microbiota has received increased attention for its association with host health, fitness, development, nutrition acquisition and behavioural attributes (Ley et al. 2005; Ley et al. 2008; Backhed 2011; Bäckhed et al. 2015; Li et al. 2016; Hajjo and Geva-Zatorsky 2020). An animal's skin is a unique interface affected by both host factors (mobility, cellular functions, immunity, mucous secretion etc) and the surrounding environment (water, air, soil, other multicellular organisms) (Chiarello et al. 2015). The skin barrier is especially relevant for organisms inhabiting aquatic environments. Marine mammals, fish, amphibians and other aquatic animals share their environment with a huge array of microorganisms, which they are in direct and constant contact with (Eakins and Sharman 2010; Apprill 2017). Whereas the outer layer of a terrestrial animals' skin is typically dead epithelial cells (stratum corneum in mammals; Prausnitz et al. 1993), fish skin is an immunologically active mucosal surface (Magnadottir 2010; Salinas et al. 2011; Gomez and Primm 2021), which hosts a diverse microbial community (Legrand et al. 2017). The skin-environment interface has high potential for exchange of microbes, particularly in marine ecosystems, making it a dynamic and complex environment (Gomez and Primm 2021).

Coral reefs are one the most diverse and rich aquatic environments, as hotspots of biodiversity they support 25% of all marine species (Reaka-Kudla 1997). Coral reefs are also rich in microbial diversity and the functions of these microbes are only just being appreciated (Somboonna et al. 2014; Silveira et al. 2017; Somboonna et al. 2017; Vanwonterghem and Webster 2020). Globally, millions of people rely on reefs as nutritional and socio-economic resources (Reaka-Kudla 1997; Moberg and Folke 1999; Hoegh-Guldberg et al. 2019). Coral fisheries are an important food source for many coastal communities and support an estimated 6 million reef fishers globally (Teh et al. 2013). Cleaner fish play a critical role in coral reef ecosystems by providing a cleaning service in which they remove ectoparasites and dead or damaged tissue from visiting marine organisms, termed clients (Cote 2000). Increased access of clients to cleaners has been linked to higher body condition (Ros et al. 2011) and stress reduction (Soares et al. 2011). 'Dedicated' cleaners gain nutrition exclusively from cleaning (Vaughan et al. 2017) and therefore come into direct contact with a number of different client species (e.g. Dunkley et al. 2019; Cote 2004). Few cleaners have been reported as being parasitised, with the exception of *Labroides dimidiatus* (see Narvaez et al. 2021), despite clients being potential vectors of disease (Grutter 2002). Frequent contact with clients creates the potential for microbial exchange, therefore cleaners offer a unique model system to explore the microbial communities in a species which engages in multiple social interactions in a wild and diverse system (Soares et al. 2019; Pereira et al. in press.).

In other vertebrate species, a high diversity of bacteria has been associated with increased resilience to pathogenic bacteria (van der Waaij et al. 1971; Girvan et al. 2005) and disruptions to communities, which often lead to a loss in diversity, can cause infections by opportunistic pathogens (Antwis et al. 2014; Bates et al. 2019). Further, specific bacteria have been directly linked to providing protection against pathogens, for example blocking arboviruses in mosquitoes (Moreira et al. 2009) and deterring *Crithidia bombi,* a virulent eukaryotic parasite, in bumble bees *Bombus terrestris* (see Koch and Schmid-Hempel 2011). Thus, the host microbiota can be considered as an extended immune phenotype (Koch et al 2011). Advances in functional gene prediction software packages, such as Picrust2, means we can now infer gene functions of microbial communities (Douglas et al. 2020; Laroche et al. 2021). The use of inference approaches such as Picrust2 is increasing, Picrust2 has been used to quantify core microbial communities on salamander skin (*Plethodon cinereus*) (see Loudon et al. 2014), identify a potential microbial origin for toxicity in fish (li et al 2020) and has uncovered different functional profiles in healthy and unhealthy fish (Ma et al. 2019; Ellison et al. 2021). Further, Titus et al. (2020) demonstrated association of functional genes from microbial communities in anemones which hosted mutualistic anemone-fish (*Amphiprion* spp.), suggesting functional roles of microbes in this mutualistic relationship.

The co-evolution between wild vertebrate hosts and their microbial communities is an emerging area of interest in wildlife ecology (Gillman 2020), given that reefs are under considerable threats from climate change (Garpe et al. 2006; Graham et al. 2006) it is timely to uncover these interactions, particularly in ecologically important species such as cleaner fish. Here we compare the skin and gut microbiota from sharknose cleaner gobies from two long-term study sites in Tobago. We predict distinct communities in skin and gut microbiota. In line with other microbial investigations of fish microbial communities, we predict that fish skin will harbour increased diversity of microbial constituents compared to the gut (Chiarello et al. 2015) and that this may also be reflected in the predicted functionality of the microbes. Additionally we predict some differences in microbial communities between the geographical sites sampled.

Materials and Methods

Study site and sample collection

During June 2017, sharknose gobies (*Elacatinus evelynae*) were collected using hand nets from two spatially distinct reefs: Booby Reef (n=12) and Pirates' Reef (n=12) in Man O' War Bay, Tobago (11°19.344′N, 060°33.484′W). All fish were collected from live brain (Faviidae spp.) or star coral (Montastraea spp.) cleaner stations, each inhabited by 1-13

individual sharknose gobies. Immediately following capture within the sea, individual fish (one per location – station) were transferred from a net into a container of seawater *in situ*. On transfer to the field laboratory, within 2 hours of capture, each fish was euthanized by immersion in a lethal dose of MS-222 (tricaine methanesulfonate). Skin mucous samples were collected immediately by swabbing the skin with a sterile cotton swab, which were placed into individual Eppendorfs containing 95% molecular grade ethanol. The fish were then transferred into individual Eppendorfs containing RNAlater®. MS-222 is known to inhibit bacterial growth (Fedewa and Lindell 2005), however as samples were stored immediately in RNAlater® we suggest no interference in microbial community composition. Swabs and whole fish samples were stored at 3 °C for 3 weeks before being transported to the UK where whole fish were frozen at -18°C and swabs kept at room temperature before processing, eight months later. Swabs kept in ethanol were not frozen due to stability of samples when submerged in ethanol. Prior to DNA extraction, ethanol from the skin swabs was evaporated using miVacTM. Using a dissecting microscope, each fish was screened for macroparasites on the skin, gills and buccal cavity, and in the gut and body cavity. Fish were photographed with a scale bar and from these fish standard lengths were measured using Image J (Schneider *et al*. 2012). During dissection, the entire gut and contents were removed for DNA extraction.

Bacterial 16S rRNA amplification and sequencing

DNA was extracted from the contents of 24 skin swabs and 24 gut samples using DNeasy® PowerSoil® Kit (QIAGEN©, Milan, Italy) according to the manufacturer's instructions. DNA concentration and quality was measured in a NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, United States). Using 16S rRNA primers F515/R806 (forward: 5' GTGCCAGCMGCCGCGGTAA 3', reverse: 5' GGACTACHVGGGTWTCTAAT 3'; (Caporaso et al. 2011) and Illumina adaptors, each DNA sample was amplified for the V4 region of the 16S rRNA gene (~250 bp). These F515/R806 primers have been used previously to characterize bacterial communities in coral reef fish (Parris et al. 2016; Chiarello et al. 2018) including in a closely related species of cleaner fish (*Elacatinus prochilos*; see Xavier et al. 2019). Two positive controls (ZymoBIOMICS™ Microbial Community Standard) and four negative controls (nuclease free water) were processed through the entire DNA extraction and PCR amplification stages alongside the test samples. To create the library, samples where multiplexed using the dual-indexing sequencing strategy of Kozich et al. (2013) and PCR was performed: 95 °C for 2 min (one cycle), 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min (30 cycles), and 72 °C for 10 min (1 cycle). The PCR products were visualised using an E-Gel with SYBR safe DNA Gel Stain 2% (Life Technologies). The library was normalised using SequalPrep Normalisation Plate Kit (Life Technologies) following the manufacture's protocol for sequential elution. The concentration

of pooled samples in the library was determined using Kapa Biosystems Library Quantification kit for Illumina® platform. The Agilent Bioanalyzer High Sensitivity DNA analysis kit (Agilent®) was used to determine the sizes of the amplicons and the final library was normalised to equal molar concentrations. Sequencing was performed in a single run on the Illumina® MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina® MS 102-2003) targeting a depth of 46,000 to 51,000 reads per sample.

Bioinformatic pipeline processing 16S data

Raw FASTQ files were analysed using QIIME2 (release 2018.11). Reads were denoised trimmed to 230 ensuring a minimum quality score of 25. A frequency table of Amplicon Sequence Variants (ASVs) was built using Dada2 (Callahan *et al*., 2016). Taxonomy was assigned using the SILVA 132 database using a 99% similarity threshold. The resulting representative sequences were used to create a phylogenetic tree using SEPP (Mirarab *et al.* 2012) for downstream phylogenetic analyses. Processing of samples was carried out in RStudio 1.1.456 (R Core Team 2018) using the phyloseq package (McMurdie and Holmes, 2013). Using phyloseq, samples with fewer than 5000 reads were filtered and removed (two skin samples and five gut samples). Note, n = 7 samples (n = 5 gut and n = 2 skin) did not contain sufficient reads for detailed analysis. Mock microbial communities were removed and non-bacterial reads were filtered out by keeping all 'bacteria' (Kingdom level) and removing 'mitochondria' (Family level) and 'Chloroplasts' (Class level). Singletons were removed, and samples were rarefied to 90% of the minimum sequence depth based on rarefaction curves leaving a minimum of 5396 reads per sample. The most abundant ASVs were plotted by selecting sequences which represented >1% of all sequences.

Statistical analysis

All statistical analysis was carried out in R version 1.1.456 (R Core Team 2018). PERMANOVA was used to test the significance of alpha diversity metrics between site and tissue type. The R package mvabund (Wang et al. 2012) was used to compare the relative abundance of bacterial phyla and classes between the two sites. To compare the phylogenetic distance profiles of microbial communities between tissue type, and geographically distinct reefs ordination plots were created using weighted and unweighted UniFrac measures. Multivariate analyses (PCoA) of microbial community structure between sample type and site were assessed using PERMANOVA based on UniFrac indices (weighted and unweighted) and Bray-Curtis dissimilarity, using the 'adonis' function in the R package 'vegan'. Data were visualised using R packaged ggplot2 (Ginestet et al. 2011).

Measures of microbial taxonomic alpha-diversity (Shannon, Inverse Simpson and Faiths phylogenetic diversity) were calculated using the phyloseq package (McMurdie and

Holmes, 2013) and data were analysed using General Linear Models (GLMs) with appropriate error distribution. Model assumptions and fit were assessed using residual plots (as specified by Bolker et al. 2009). Best fitting model selection was based on Akaike Information Criterion (AIC) using a backward elimination approach and the presence of potential influential points on model outcomes were checked for (using Cook's D and leverage). To test the relationship between alpha diversity measures (Shannon [Gamma family and identity link], Inverse Simpson [Gamma family and log link] and Faith's Phylogenetic Distance measures [Gamma family and identity link]) and sharknose metadata (individual station location, body length, site and body sample type).

Microbial gene function prediction using Picrust2

Picrust2 was used to predict the functional profiles of gut and skin bacterial communities (Douglas et al. 2020). Through inferences based on reference genomes, gene functions of the predicted genes were inferred from MetaCyc pathways (using the online database https://metacyc.org/). DESeq2 was used to test differential gene abundance between in skin and gut communities (Love et al. 2014). Abundances with a difference of $P < 0.05$ were considered significant.

Results

Comparison of gut and skin microbial communities

No macroparasites were detected on the skin, gills nor buccal cavity of sharknose goby cleaner fish (*Elacatinus evelynae*; n = 24). A total of 474,208 raw reads were generated from microbiota 16S sequencing (97,128 for gut and 377,080 for skin). The gut microbiota of 18 individual sharknose gobies consisted of 293 amplicon sequence variants (ASVs) and 1113 ASVs from 22 skin swab samples, 103 (7.3%). of which were unclassified at genus level and not included in downstream analysis. Tenericutes and Proteobacteria dominated the gut samples making up 47.3% and 46.8% of the total reads, respectively, followed by Spirochaetes (3.4%), Firmicutes (1.3%), Fusobacteria (0.7%) and Cyanobacteria (0.2%) (Fig. 5.1). In contrast, for the skin microbiota 87.6% of reads comprised Proteobacteria and the next top 5 phyla Actinobacteria, Bacterioidetes, Tenericutes, Firmicutes and Cyanobacteria comprising between 0.5 and 3% of the total reads (Fig. 5.1).

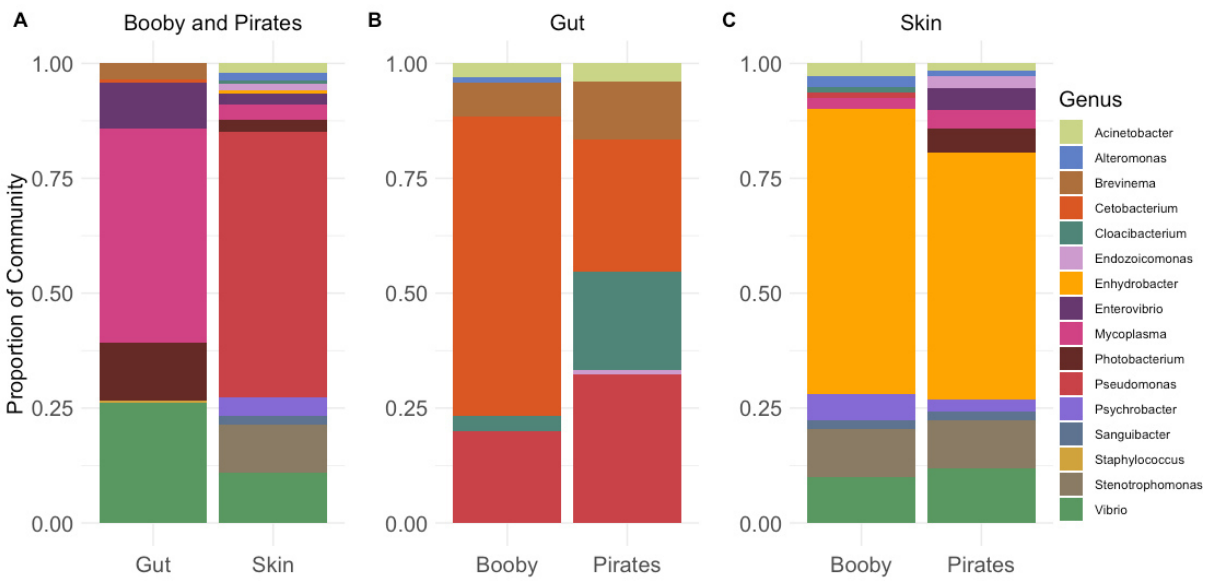


Figure 5.1: Proportional abundance of the most abundant bacterial communities present (>1%) at genera level in gut and skin of Sharknose goby cleaner fish (*Elacatinus evelynae*) from two different reefs Booby Reef and Pirates Reef collected from the Man O'War Bay, Tobago. 7.3% of ASVs were unclassified to genus level and were removed from plots.

All alpha diversity of skin was significantly higher than gut communities for all tested measures (Inverse Simpson GLM: ß = 0.7118, F = 17.52, p<0.001; Shannon GLM: ß = 0.2369, F = 36.118, p<0.0001; Faith's Phylogenetic Distance, GLM: ß = 1.5918, F = 23.384, p<0.0001). Skin and gut microbial communities were distinct from one another for all measures (weighted UniFrac adjusted R2 = 0.40, $F_{1,39}$ = 6.10, P = 0.001), unweighted UniFrac (adjusted R2 = 0.12, $F_{1,39}$ = 5.2, P = 0.001) and Bray-Curtis (adjusted R2 = 0.36, $F_{1,39}$ = 21.34, P = 0.001;), see Supplementary Materials Fig. 2.

Comparison of skin and gut communities between the two reef sites

There were no differences in alpha diversity associated with site (Booby vs Pirates reefs), locations within the site nor fish body length (PERMANOVA P>0.05). No significant differences were found in beta diversity between sites for skin and gut when using weighted UniFrac (adjusted R2 = 0.06, $F_{1,39}$ = 2.57, P = 0.08) unweighted UniFrac (adjusted R2 = 0.03, $F_{1,39}$ = 0.98, P = 0.44) and Bray-Curtis (adjusted R2 = 0.03, $F_{1,39}$ = 1.16, P = 0.29), see Supplementary Fig. 2. Abundances of Proteobacteria, however, were higher in cleaners gut samples from Pirates than Booby and Tenericutes were higher in gut samples from Booby Reef than Pirates Reef (Fig. 5.2 and Supplementary Fig 3).

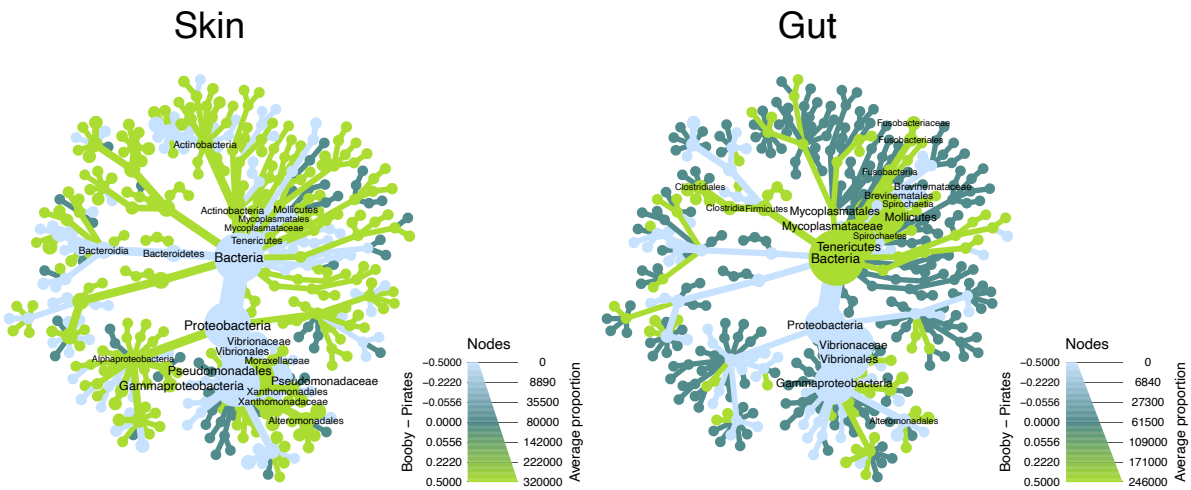


Figure 5.2: Metacoder heatmaps of read abundances from skin and gut microbial samples of sharknose goby cleaner fish (*Elacatinus evelynae*) collected from two different reefs Booby Reef and Pirates Reef in the Man O'War Bay, Tobago to Family level. Left; differences in read abundance in skin samples from Booby and Pirates sites combined. Right; differences in read abundance in gut samples from Booby and Pirates sites combined.

Functional analysis of microbial genes

We identified four main classes of genes that differed significantly between skin and gut microbial communities: "Biosynthesis", "Degradation/Utilization/Assimilation", "Generation of Precursor Metabolites" and "Macromolecule Modification". In total, 423 genes were identified and 207 genes varied significantly between skin and gut samples, suggesting that these two organs have functionally distinct microbial communities. In the skin, 126 microbial genes were increased in abundance compared to gut and in 81 genes were at greater abundance in the gut (Fig. 5.3). Of the 126 genes increased in skin communities 46% were "Biosynthesis" and "Degradation/Utilization/Assimilation", 6% were "Generation of Precursor Metabolites" and 2% "Macromolecule Modification". For gut microbial communities, most genes increased were "Biosynthesis" at 73%, followed by "Degradation/Utilization/Assimilation" (17%), Generation of Precursor Metabolites" (9%) and "Macromolecule Modification" (1%). The average logfold2 increase in gene abundance in skin microbes was 3.63 (± 4.23, S.E.), whereas gut genes were only increased by 1.21 (±1.04, S.E.), suggesting an overall increased microbial activity in the skin communities as opposed to gut (Fig. 5.3). Finer scale classifications were noted for three "Biosynthesis" genes associated with arginine synthesis (ARGORNPROST-PWY, ARGSYN-PWY, ARGSYNBSUB-PWY) (n = 2 increased abundance in the skin, n = 1 increased abundance in gut) and one "Degradation" gene (increased abundance in the skin, PWY-7616) associated with detoxification pathways.

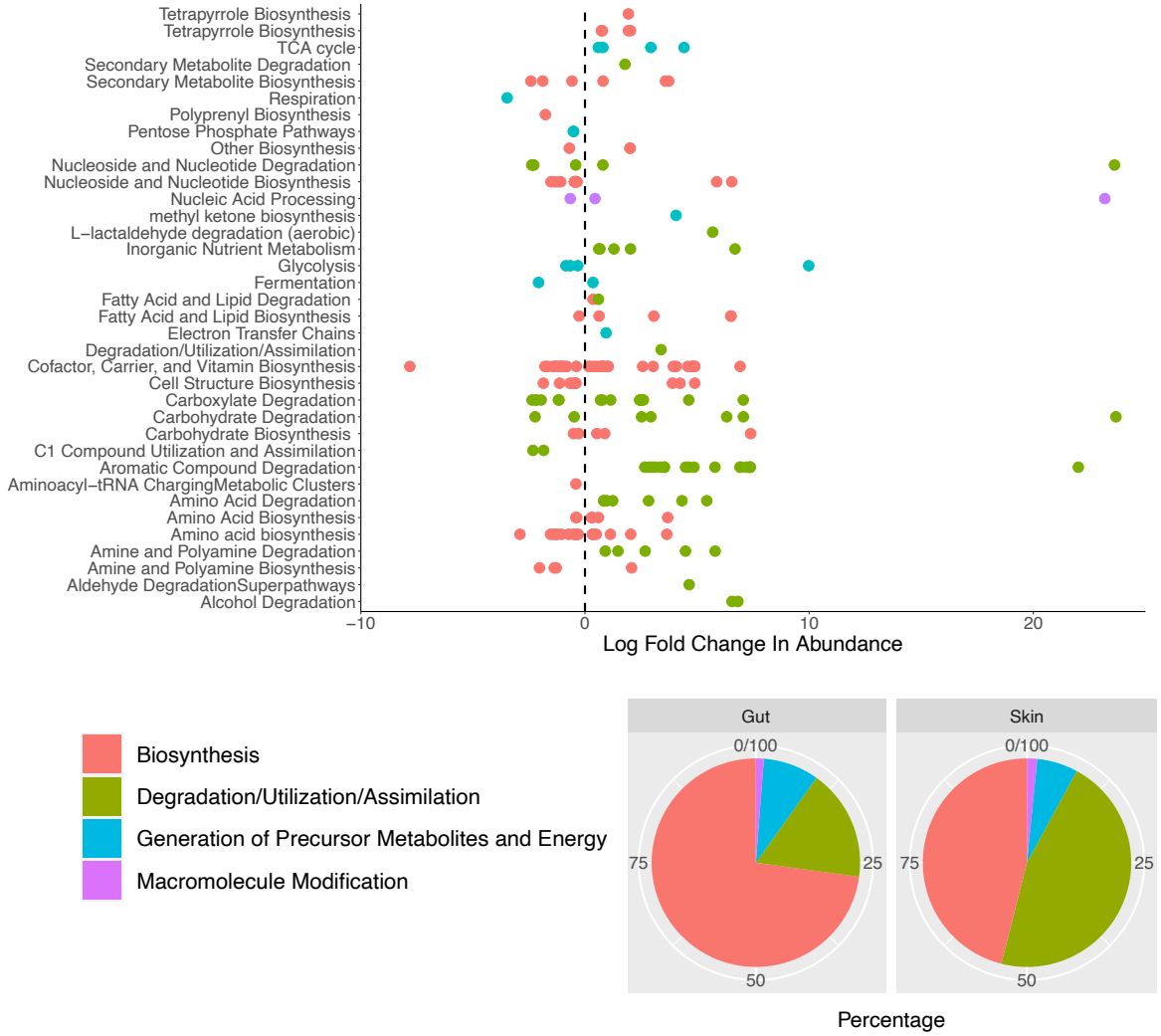


Figure 5.3: Plots showing the relative abundance of significantly different MetaCycle pathways associated with microbial communitites of the skin and gut from Sharknose goby cleaner fish (*Elacatinus evelynae*). Genes to the right of the dashed line are significantly increased skin communitites, while genes to the left are significantly increased in gut communities. Genes were identified via Picrust2 and significance between samples were determined using DEseq2.

Discussion

This study demonstrates the phylogenetic differences in microbial communities in the gut and skin of sharknose goby (*Elacatinus evelynae*) cleaner fish and shows that these communities were consistent across two different reef localities. Our results support previous findings that the skin and gut microbiota of wild sharknose goby cleaner fish are distinct (Xavier et al. 2019; Pereira et al. preprint). Tenericutes and Proteobacteria dominated the gut

communities, while the skin communities were dominated by Proteobacteria only. Further, here for the first time, we identified 423 genes associated with microbial functions and found that the skin microbial communities were richer in functional genes than the gut (Fig. 5.3).

Overall, the gut and skin profiles matched those found in *Elacatinus* spp. previously studied in the Caribbean (Xavier et al. 2019; Pereira et al. preprint). This is unsurprising as many vertebrate species exhibit phylosymbiosis, the phenomenon whereby the phylogeny of the host is a key driver of microbial community composition (Boutin et al. 2014; Chiarello et al. 2018). Phylosymbiosis suggests that there are genetically driven host factors which select for and against particular microbial species. The environment can be an equal driver of microbiota diversity and composition (Pérez et al. 2010; Kueneman et al. 2014; Chiarello et al. 2019). Additionally, both environmental and genetic factors may affect the communities associated with particular organs in different ways (Sylvain et al. 2016) and a change in one may elicit change in another (Legrand et al. 2017). Investigating the microbiota of 114 wild fish, Sylvain et al. (2020) identified that species-specific factors of the host significantly modulated gut communities, while skin microbiota were primarily associated with environmental factors including the bacterioplankton community (Sylvain et al. 2020).

Although not significant, in this study we observed higher abundance of Tenericutes in cleaners gut communities at Booby Reef as opposed to Pirates Reef (Fig. 5.2). Further investigation of results would include full genome comparisons of bacterial communities from the different sites. Given the difference in site ecologies we would expect some significant differences in microbial communities. However as reef fish abundance is stochastic, cleaner fish microbiota may reflect the different species that cleaner fish make contact with during cleaning behaviour. This observation is intriguing as this is similar to the community assemblages in the non-cleaning ecotype of the broadstripe cleaner fish (*Elacatinus prochilos*) sampled in Barbados (Xavier et al. 2019). The non-cleaning ecotype of *E. prochilos,* which are sponge dwellers also harboured higher abundance of Tenericutes compared to the cleaning ecotype (Xavier et al. 2019). Certainly, the sharknose gobies (*E. evelynae*) sampled in this study are prolific cleaners (Dunkley et al. 2019a; Dunkley et al. 2019b; Dunkley et al. 2020; Whittey et al. 2021). However, whether the observed difference in tenericutes abundance between sites is the result of host genetics or ecology, including behaviour and diet, is not clear. The cleaning broadstripe cleaning goby ecotype was found to harbour a greater prevalence of potentially pathogenic bacteria than its non-cleaning ecotype (Xavier et al. 2019), but genus-level identification does not necessarily identify pathogenicity (Gomez and Primm 2021). In the current study, we found no evidence for virulent nor pathogenic genes in the microbial community functional profiling of gut and skin communities of the sharknose goby.

During cleaning interactions, cleaners can remove ectoparasites such as gnathiids from clients at a rate that significantly decreases gnathiid abundance (Grutter 1996). Although an essential food source to cleaners, clients are thought to be potential vectors for disease transmission, including parasites (Xavier et al. 2019). Although rarely reported, cleaners can indeed become infected with parasites themselves (Jones et al. 2004). In aquaculture, cleaners became infected by pathogenic *Aeromonas salmonicida* demonstrating the transmission potential of parasites during close contact (Treasurer and Leider 1994). Wild cleaner wrasse (*Labroides dimidiatus*) collected from the Great Barrier Reef were parasitised by 12 species of parasites from eight groups; five ectoparasites and three endoparasites (Narvaez 2021). In our study, however we found no evidence of either ecto- or endoparasites. The fish in both studies were caught using similar methods, with nets; however, in our study fish were euthanised with MS222 prior to being preserved in RNAlater, although there is currently no evidence to support that this would affect parasite detection, the level of fish handling does and therefore could have affected the lack of parasites found on the samples. Surprisingly, macroparasites are often overlooked (Poulin et al. 2016), despite making up over 40% of all biodiversity (Hatcher and Dunn 2011) and are thought to be highly prevalent on coral reefs (Justine 2010; Bernal et al. 2015; Sikkel et al. 2019). They play active roles in food webs (Kuris et al. 2008) and cause significant adverse effects to their hosts (Jones and Grutter 2005; Grutter et al. 2018) ranging from increased stress (Triki et al. 2016) and wounds (Honma and Chiba 1991) to host death (Mugridge and Stallybrass 1983). Thus, sharknose goby consuming parasites whilst maintaining close contact with infected individuals but not themselves being heavily parasitised suggests that they have some specialised mechanism of withstanding infection.

Fish skin is a highly active immune organ which elicits immune responses similar to the gut of other vertebrates (Xu et al. 2013). The immune response of vertebrates is to a large extent orchestrated by microbial commensals (Gomez et al. 2008; Gomez et al. 2013; Xu et al. 2013). Indeed, the skin microbiota of fish is often considered an extended immune phenotype (Koch and Schmid-Hempel 2011). Thus, the cleaner fish skin microbiota may play a functional role in resistance to parasitism. All measures of microbial diversity were higher in the skin of cleaner fish than gut and the functional analysis of the microbial communities identified 126 bacterial genes which were increased in the skin community of fish compared to gut communities, providing evidence of a highly productive and metabolically important environment. In addition, using Picrust2, Li et al. (2020) identified pathways relating to the production of arginine were significantly enhanced in toxic puffer fish as opposed to non-toxic puffer fish. Although not confirmed, arginine has been proposed as a precursor in the synthesis of tetrodotoxin. Here we found three arginine biosynthesis genes, two of which were present at significantly higher rates in the gut and another at a higher rate in the skin.

Additionally, we found one gene with a high abundance in the skin as opposed to the gut, associated with detoxification pathways. The presence of detoxification pathways may be an indication of a 'coping' mechanisms by bacteria to co-habit toxic forming bacteria. The advantages of harbouring toxins are not known for sharknose goby, toxins could deter predation by clients or third-party species, but may also deter parasites. Further research on sharknose goby parasites including the use of molecular detection (e.g. 18s metataxonomics) should be used to further understand the prevalence of parasites in this species.

The role of the microbiota in host behaviour can range from social signalling and mate preference (*Dicentrarchus labrax)* (Sharon et al. 2010), (*Crocuta crocuta)* (Theis et al. 2012), metamorphosis (*Hydroides elegans*) (Huang et al. 2012), regulating emotional behaviour (*Mus musculus)* (Bravo et al. 2011; Heijtz et al. 2011), attractiveness to parasites (*Mus musculus* (Verhulst et al. 2011) and pathogen defence (*Apis mellifera*) (Engel et al. 2012). Given this interaction between the microbiota and host behaviour (for review of multiple species see Ezenwa et al. 2012) and knowing that cleaning interactions can be affected by behavioural traits such as personality (*Elacatinus evelynae*) (Dunkley et al. 2019b), there is the potential for microbes to in turn influence cleaning behaviour of sharknose gobies. Understanding the microbiota of cleaner fish may help us understand the role microbes may play in this mutualism and in social interactions (Soares et al. 2018).

Most microbial communities in coral reef fish have not been described (Parris et al. 2016). Coral reefs are currently threatened by multiple anthropogenic stressors and the consequences of human-mediated perturbations on microbial communities are largely understudied (de Melo and Sarmento 2019). Further, warm water bleaching events, which are increasing due to climate change (Hughes et al. 2018), can disrupt host-parasite numbers on reefs and the effects of rebounding parasite numbers are unclear, thus cleaner species, and their parasite removal service, may prove even more valuable on future reefs (Sikkel et al. 2019). Knowledge of host microbiota, including which microbes are present and what functionality the microbial community may confer to hosts could provide a valuable tool for conservation and monitoring species health (Hauffe and Barelli 2019) thus microbiota research is an important area of research. We need to further our understanding of how these microbes function within fish communities and what is their relevance in terms of immunity and nutritional relevance so that we might potentially be able to apply this knowledge for conservation or for enhancing aquaculture (Tarnecki et al. 2017).

Chapter 6

General Discussion

Summary of Chapters

The aim of this thesis was to further our understanding of species interactions on reefs at different spatial scales, from within host to across reefs. **Chapters 2** and **3** review coral reef fish interactions with natural coral and artificial structures on reefs, while **Chapter 4** and **5** investigate interactions at a microbial level by investigating the microbiota of cleaner fish (*Elacatinus evelynae*) within host, and with their surrounding habitat. The importance of the structural complexity of the cleaner fish station to cleaning behaviour was hitherto unexplored. **Chapter 2** shows that taller and more structurally complex (by fine scale vector dispersion measures) corals are inhabited more frequently by cleaner fish than short, uniform corals. These same morphologies of cleaning stations also promoted higher rates, durations and frequencies of cleaning events. Cleaning events were longer at taller stations and at stations with larger refuge sizes. Thus, **Chapter 2** highlights the need for heterogeneity at a microscale (at the level of a single coral head) to facilitate particular ecological functions such as cleaning interactions.

Due to anthropogenic losses of coral structure, artificial reefs are being increasingly used to replace these lost structures (Seaman 2007; Paxton et al. 2020b), however behavioural observations of individual coral reef fish interactions with artificial structures are scarce. Most studies which have observed fish behaviour around artificial structures focus on existing structures: wrecks, wind or tidal turbines, cables, and oil and gas structures (Priyadarshana et al. 2001; Jamieson et al. 2006; Williamson et al. 2021; Yoshida et al. 2021). In these studies, 'behaviours' usually refer to the broader elements of fish ecology such as fish movement including avoidance of structures (Priyadarshana et al. 2001; Jamieson et al. 2006; Williamson et al. 2021; Yoshida et al. 2021) or species distribution and presence (Hammar et al. 2013; Viehman et al. 2015). Additionally, some studies have focused on predation, for example coastal jetties provide a refuge for fish predators of flatback turtle (*Natator depressus*) hatchlings and jetties had significantly increased predation rates compared to those without (Wilson et al. 2019). How species interact with their habitat, and their consequential behaviour can affect outcomes of community dynamics and ecological

interactions (Almany 2004). Therefore, understanding the intricacies of reef fish interactions with artificial habitat will have conservation implications. To further knowledge of fish responses to artificial structures at a local scale, **Chapter 3** investigates the interactions of individual coral reef fish with artificial structures (fish hives). Interactions of fish with fish hives were studied in two differing ecological settings, one existing reef and another sandy area, reef adjacent. Fish assemblages were similar around hives and corals, but damselfish dominated the interactions around hives and defended hives at a higher rate than natural corals. As fish hives became covered in algae **Chapter 3** suggests hives were defended at a higher rate than live corals due to the increases surface area available for farming algae.

Chapter 4 presents a novel link between the microbial communities of *E. evelynae* and the yellow encrusting zoanthid (*Palythoa caribaeorum*) which were shown to share 34 genera of bacteria while all other microbial communities of the cleaner station organisms were distinct. Finally, **Chapter 5** further investigates the microbiota of *E. evelynae* in the gut and skin and identifies 423 genes associated with their microbial communities. Together, **Chapters 2** and **4** demonstrate the importance of the habitat to *E. evelynae* both structurally and as living sources of microbial communities, while **Chapter 5** suggests potential sharing of *E. evelynae* skin microbiota with the microbiota associated with the habitat.

Mutualisms on coral reefs

The common theme throughout the thesis was that of species interactions and mutualistic relationships. In **Chapter 1** the importance of the habitat to the mutualistic cleaner fish was demonstrated, showing that particular features of the coral habitat affect this mutualism. In any mutualism, there is always outside influence from other independent variables than constitute the context of the mutualism (Chamberlain et al. 2014; Hoeksema 2015). Context includes environmental factors and other species that may directly or indirectly affect the mutualism (Bronstein 2015). Mutualisms are integral interactions in a much broader web of interactions (Fontaine et al. 2011) and as such take place in the presence of other species, referred to as third-party species (Bronstein 2015; Dunkley et al. 2020). Sharknose goby cleaning events are largely driven by the abundance of clients and the presence of third-party species (other reef inhabitants not taking part in the cleaning interaction)(Dunkley et al. 2020). Corals can certainly be thought of as playing a role in the environmental context of cleaning interactions as corals shape reef fish assemblages (Kawasaki et al. 2003; Almany 2004; Gratwicke and Speight 2005; Darling et al. 2017; Ferrari et al. 2018). Corals therefore are linked to this mutualism by providing the sharknose goby with an array of clients. However, given the importance of the cleaner station in facilitating cleaning behaviour (**Chapter 2**), coral species could also be considered as a third-party influencer in cleaning mutualisms.

Coral structure may influence behaviour of clients and affect personality types

Different levels of complexity within habitats can affect species behaviours (Danley 2011; Church and Grant 2018). Complex habitats provide space for refuge (Höjesjö et al. 2004; Gratwicke and Speight 2005; Graham and Nash 2013) and typically support a greater diversity of biota (Ferrari et al. 2018) thus creating different ecological contexts and altering the costs and benefits of particular behaviours (Grant 1993; Adams 2001). For example, the physical construct of complex habitats can restrict an individuals' field of view, this renders aggressive behaviours less effective and territorial animals will avoid habitats where structures inhibit visibilities (Eason and Stamps 1992). Additionally, aggressive behaviour of zebra fish (*Danio rerio*) facilitated food acquisition more in "open", less complex habitats as opposed to more complex habitats (Basquill and Grant 1998). Consequently, aggressive behaviours occur less in habitats with greater complexity (Eason and Stamps 1992; Basquill and Grant 1998). Further, in less complex and more open habitats, bold behaviour can be rewarded through gaining access to food or mates (Ward et al. 2004; Myhre et al. 2013). However, there may also be less need for aggression in complex habitats due to increased resources, and in open habitats increased aggression may also increase the likelihood of encountering predators (Grabowski 2004; Barley and Coleman 2010; Kobler et al. 2011). Intriguingly Church and Grant (2018) propose evidence of personalities of Atlantic salmon (*Salmo salar*) based on behavioural observations of salmon in habitats of varying complexity. Salmon were captured from habitats of varying degrees in complexity and placed in artificial open or more complex habitats (in semi-natural environments). Salmon were more active and aggressive in open habitats (contrary to findings from Eason and Stamps 1992; Basquill and Grant 1998), however the original habitat of the salmon did not then predict personality types (Church and Grant 2018). Thus the habitat heterogeneity, along with other variables such as diversity and abundance of other species (i.e. the context), may elicit a variety of behaviours and ultimately behavioural strategies and personality (Höjesjö et al. 2004; Brockmark et al. 2007; Church and Grant 2018).

Sharknose goby cleaner fish exhibit a range of personality traits including activity, boldness and exploration (Dunkley et al. 2019b). Sharknose gobies which are bolder experience an increased rate of posing by clients whereas more active cleaners clean a lower diversity at a lower rate (Dunkley et al. 2019b). Given that the habitat can directly determine personality traits and behaviour (Church and Grant 2018), and that personality effects cleaning (Dunkley et al. 2019b), it is unsurprising then that cleaners and cleaning behaviour are associated with given coral shapes (tall and complex in shape); **Chapter 2.** To further understand this interaction of habitat, behaviour and personality, we must consider structural complexity at a reef-wide scale and suggest this be an area of future research. Additionally,

given that mutualisms are affected by third-party species, we must also consider how the habitat affects third party species and their behaviour.

Corals are threatened – can we artificially reproduce this habitat?

In the Caribbean, live coral cover has significantly decreased (Gladfelter 1982; Aronson and Precht 2001) and many reefs are also overfished, resulting in a reduction of fish stocks (Paddack et al. 2009; Vermeij et al. 2019). The extent of damage on reefs is preventing normal ecological functioning (Graham et al. 2007; Newton et al. 2007) therefore there is an urgent need for conservation efforts (Edwards and Gomez 2007; Kennedy et al. 2013). Consequently, artificial structures are being deployed to enhance fisheries (Paxton et al. 2020b) and there are huge global efforts to replant corals, Boström-Einarsson et al. (2020), estimates there are over 229 coral species are currently being replanted.

Some reef conservation projects, particularly artificial reef projects (as opposed to coral nurseries) have yielded inconsistent results with regards to their success (Paxton et al. 2020b). Some artificial structures have facilitated invasive species (Airoldi et al. 2015; Castro et al. 2021) and tyres which were widely used as an artificial reef substrate (Jensen et al. 2001), have been found to release toxic chemicals into the aquatic environment (Fenner and Member 2006). Some of the inconsistencies regarding the overall outcome of artificial reef projects stems from the lack of cohesiveness, such as goals of projects are often not set (Edwards and Gomez 2007; Becker et al. 2018). The duration of monitoring artificial reefs is also problematic as it takes years for assemblages to settle yet monitoring efforts tend to last on average 18 months (Bostrom-Einarsson et al. 2020). In **Chapter 3** the behavioural responses of coral reef fish to fish hives were studied for over a 16-month period. Therefore, continued monitoring of hives in future years will be necessary to further understand the long-term responses to artificial structures.

The coral loss on reefs often results in a phase-shift, where reefs change from being coral dominated ecosystems to algae and sponge dominated along with a change in community structure of associated reef fauna (McManus and Polsenberg 2004; Cheal et al. 2010; Cruz et al. 2015). Farming damselfish may further perpetuate the issue by farming algae which can hinder coral settlement, growth and survival of seeded corals in conservation projects (Ogden and Lobel 1978; Arnold et al. 2010; White and O'Donnell 2010; Schopmeyer and Lirman 2015; Hata et al. 2020). Effects of farming damselfish are so detrimental that their removal has been suggested as a means of enhancing coral restoration success (Williams et al. 2019b). However, it is important to understand that artificial reefs and their success or failure are context dependent (Paxton et al. 2020b), and by appreciating that damsels do not always hinder coral growth (Seraphim et al. 2020), it should be noted that removal of

damselfish prior to nursery or artificial reef deployment may be unnecessary. In **Chapter 3,** seeded elkhorn corals (*Acropora palmata*) established and grew despite being colonised by farming damselfish. **Chapter 3** suggests therefore that a combination of additional damselfish habitat space when setting up coral nurseries should be considered in future conservation efforts as opposed to damselfish removal.

Live corals offer structure, harbour essential microbes and predate parasites

Coral structure is important for many species and influences ecological process such as predator-prey interactions (Catano et al. 2016; Pereira and Munday 2016; Richardson et al. 2017). However, there are attributes of living corals which cannot be replicated artificially. Corals have chemical cues which not only encourage the settlement of other corals but have also been found to attract reef fish (Lecchini et al. 2005; Lecchini and Nakamura 2013; Soeparno et al. 2013). Corals have been observed predating gnathiid parasites in laboratory feeding experiments and the abundance of gnathiids were reduced on live, hard corals compared to dead corals on the Great Barrier Reef (Paula et al. 2021). Additionally, corals harbour an array of diverse microorganisms distinct from the surrounding sea water microbiota (Vanwonterghem and Webster 2020), which may confer advantages to reef fish (**Chapter 4**). Thus, while replication of structure is a promising mitigation strategy for coral reefs (Paxton et al. 2020b), artificial structures alone cannot replicate corals.

There is a growing appreciation for the importance of microorganisms in maintaining animal health (Peixoto et al. 2017; Egerton et al. 2018) and loss of corals will also result in a loss or disruption of their associated microbiota (Vanwonterghem and Webster 2020). When commensal microbial populations are disrupted, often causing severe negative affects to the host (Gomez and Primm 2021). Causes of dysbiosis include antibiotic treatments (He et al. 2017; Kim et al. 2019), thermal stress (Ezzat et al. 2021), changes in diet (Tomasello et al. 2016) and ingestion of microplastics (Jin et al. 2018). With this knowledge there is an understanding that conservation efforts must also include microorganisms (McFall-Ngai et al. 2013; Hutchins and Fu 2017; Hauffe and Barelli 2019). Given that the hosts microbiota is dynamic and susceptible to change, how they may be affected by dysbiosis and potential anthropogenic drivers is complex (Hernandez-Gomez 2020). Therefore, a comprehensive baseline of microbial functions and community assemblages in functional coral reef systems must be established. In **Chapters 4** & **5** of this thesis the microbial communities of *E. evelynae* and its associated habitat are quantified. While **Chapter 4** reveals an intriguing relationship between the microbial communities of *E. evelynae* and *Palythoa caribaeorum,* demonstrating shared genera between the two, **Chapter 5** reveals, for the first time microbial genes which in other species of marine fishes are associated with the production of toxic chemicals. Further

research needs to be undertaken to first investigate the origin of palytoxin in *P. caribaeorum* and whether microbes which produce the toxin are present in cleaner fish microbiota. Many marine organisms including *Cyanobacteria* produce the palytoxin and given that it is a large and highly complex molecule, it is highly unlikely to be a chemical by-product (Seemann et al. 2009; Patocka et al. 2015; Patocka et al. 2018).

Conclusions

Anthropogenic stressors are negatively affecting biological communities at a range of scales, from microbial communities to reef wide coral and fish assemblages (Greenspan et al. 2020). The research presented in this thesis has extended our knowledge of coral reef fish behaviour in relation to the structural elements of their habitat both natural and artificial (**Chapter 2** & **3**). Additionally, this work has furthered our appreciation of the microbial community of the cleaner fish *E. evelynae* (**Chapter 4**) and our understanding of the connectivity of microbial communities between reef species (**Chapter 5**). This research has highlighted areas for future research for conservation interest including fish behaviour with reef scale complexity and to pursue more robust knowledge of the functions of microbial communities in *E. evelynae.*

Supplementary Information

Supplementary Table 1: Species observed within two fish lengths of corals and artificial reef structures (hives) and further subdivided into species observed around hives at Pirates Reef and Booby Reef, Man O'War bay Tobago.

Species	*Booby Coral*	*All hives*	*Pirates hive*	*Booby hive*
Baitfish	x	x	x	x
Banded Butterflyfish	x	x	x	x
Barracuda		x		x
Blueheaded Wrasse	x	x	x	x
Blue Tang	x	x	x	x
Brown Chromis		x	x	
Caeser Grunt	x	x	x	x
Clown Wrasse		x	x	x
Dark Damselfish	x	x	x	x
Doctorfish	x	x	x	x
Four Eyed Butterflyfish	x	x	x	x
French Grunt	x	x	x	x
Graysby Grouper	x	x	x	x
Gray Snapper		x	x	
Honeycomb Cowfish	x	x	x	x
Jacknife Fish		x	x	
Mahogany Snapper		x	x	x
Ocean Surgeon	x	x	x	x
Orange Spotted Filefish		x	x	
Porcupine Fish		x	x	
Princess Parrotfish	x	x	x	x
Puddingwife	x	x	x	x
Queen Parrotfish	x	x	x	x
Redband Parrotfish	x	x	x	x
Sand Diver	x	x	x	x
Scrawlled Filefish	x	x		x
Scorpion fish		x		x
Sergeant Major	x	x	x	x

Sharknose goby	x	x		x
Slippery Dick	x	x	x	x
Spanish Hogfish		x	x	x
Stoplight Parrotfish	x	x	x	x
Striped Parrotfish	x	x	x	x
Squirrel fish		x	x	
Tomtate	x	x		x
Trumpetfish	x	x	x	x
White Spotted Filefish		x	x	x
Yellowfin Mojarra		x	x	
Yellowtail Goatfish	x	x	x	x
Yellowtail Parrotfish	x	x	x	x

Supplementary Table 2. Substrate sampling location and details of brain corals that constituted cleaning stations for the sharknose goby (*Elacatinus evelynae*). Zero indicates no sample, and one a sample taken.

Brain coral number	**Reef location**	**Healthy brain coral**	**Diseased brain coral**	**White encrusting zoanthid,** *Palythoa caribaeorum*	**Mat zoanthid,** *Zoanthus pulchellus*	**Algae**	**Coral water**	**Total samples per cleaning station**
1	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
2	**Booby**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
3	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
4	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
5	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
6	**Booby**	**1**	**0**	**0**	**0**	**1**	**1**	**3**
7	**Booby**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
8	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
9	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
10	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
11	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
12	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**

13	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
14	**Booby**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
15	**Turpins**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
16	**Turpins**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
17	**Turpins**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
18	**Turpins**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
19	**Turpins**	**1**	**0**	**0**	**0**	**1**	**1**	**3**
20	**Turpins**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
21	**Turpins**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
22	**Turpins**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
23	**Turpins**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
24	**Pirates**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
25	**Pirates**	**1**	**0**	**1**	**1**	**1**	**1**	**5**
26	**Pirates**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
27	**Pirates**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
28	**Pirates**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
29	**Pirates**	**1**	**1**	**0**	**0**	**1**	**1**	**4**
30	**Pirates**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
31	**Pirates**	**1**	**1**	**1**	**0**	**1**	**1**	**5**
Total samples per substrate		**31**	**10**	**7**	**27**	**31**	**31**	**137**

Supplementary Table 3. Substrate sampling details of 'control' brain corals with no associated cleaner fish. Zero indicates no sample, and one a sample taken.

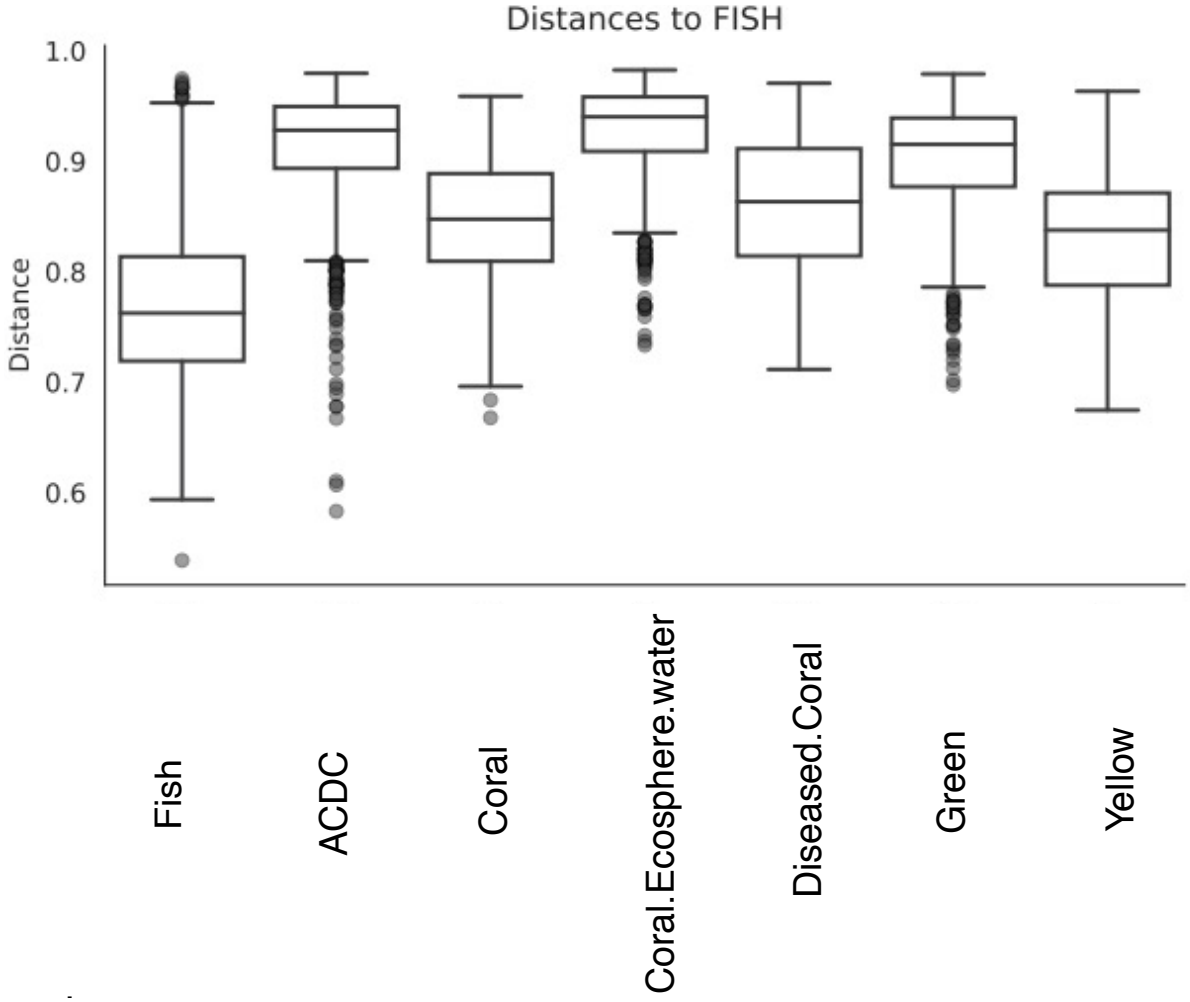
Brain coral number	**Reef location**	**Healthy brain coral**	**Diseased brain coral**	**White encrusting zoanthid,** *Palythoa caribaeorum*	**Mat zonathid,** *Zoanthus pulchellus*	**Algae**	**Coral water**	**Total samples per cleaning station**
1	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
2	**Booby**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
3	**Turpins**	**1**	**1**	**0**	**1**	**1**	**1**	**5**

4	**Pirates**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
5	**Pirates**	**1**	**1**	**1**	**0**	**1**	**1**	**5**
6	**Pirates**	**1**	**1**	**0**	**1**	**1**	**1**	**5**
7	**Pirates**	**1**	**0**	**0**	**1**	**1**	**1**	**4**
8	**Pirates**	**1**	**1**	**0**	**0**	**1**	**1**	**4**
Total samples per substrate		**8**	**7**	**1**	**6**	**8**	**8**	**38**

Supplementary Table 4. PERMANOVA results between beta of diversity microbial communities of different substrates Sharknose goby cleaner fish (*Elacatinus evelynae*) and associated benthic constituents of each cleaner fish station from three reefs in Man O'War Bay, Tobago. ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae), Coral Ecosphere water – water from the immediate surrounding of the coral, Diseased Coral – brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green – green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid, *Palythoa caribaeorum*.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
ACDC	CORAL	84	999	7.54233778	0.001	0.00110526
ACDC	CORAL WATER	79	999	5.18348491	0.001	0.00110526
ACDC	DISEASE	57	999	2.96419273	0.001	0.00110526
ACDC	FISH	75	999	14.0688407	0.001	0.00110526
ACDC	GREEN	76	999	1.76690432	0.001	0.00110526
ACDC	YELLOW	50	999	4.07932836	0.001	0.00110526
CORAL	CORAL WATER	81	999	9.7191176	0.001	0.00110526
CORAL	DISEASE	59	999	1.18539748	0.08	0.08
CORAL	FISH	77	999	7.01578476	0.001	0.00110526
CORAL	GREEN	78	999	5.91975984	0.001	0.00110526
CORAL	YELLOW	52	999	1.73467338	0.001	0.00110526
CORAL WATER	DISEASE	54	999	4.55997744	0.001	0.00110526
CORAL WATER	FISH	72	999	16.4598511	0.001	0.00110526

CORAL WATER	GREEN	73	999	5.95602112	0.001	0.00110526
CORAL WATER	YELLOW	47	999	5.11389304	0.001	0.00110526
DISEASE	FISH	50	999	4.44260805	0.001	0.00110526
DISEASE	GREEN	51	999	2.53358436	0.001	0.00110526
DISEASE	YELLOW	25	999	1.51326524	0.019	0.01995
FISH	GREEN	69	999	12.6038724	0.001	0.00110526
FISH	YELLOW	43	999	2.79680676	0.001	0.00110526
GREEN	YELLOW	44	999	3.61379183	0.001	0.00110526



Supplementary Figure 1: Beta diversity of microbial communities of different substrates Sharknose goby cleaner fish (*Elacatinus evelynae*) and associated benthic constituents of each cleaner fish station from three reefs in Man O'War Bay, Tobago. ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae), Coral Ecosphere water – water from the immediate surrounding of the coral, Diseased Coral – brain corals exhibiting disease, **Fish** - Sharknose

goby cleaner fish skin mucus, Green – green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid, *Palythoa caribaeorum*.

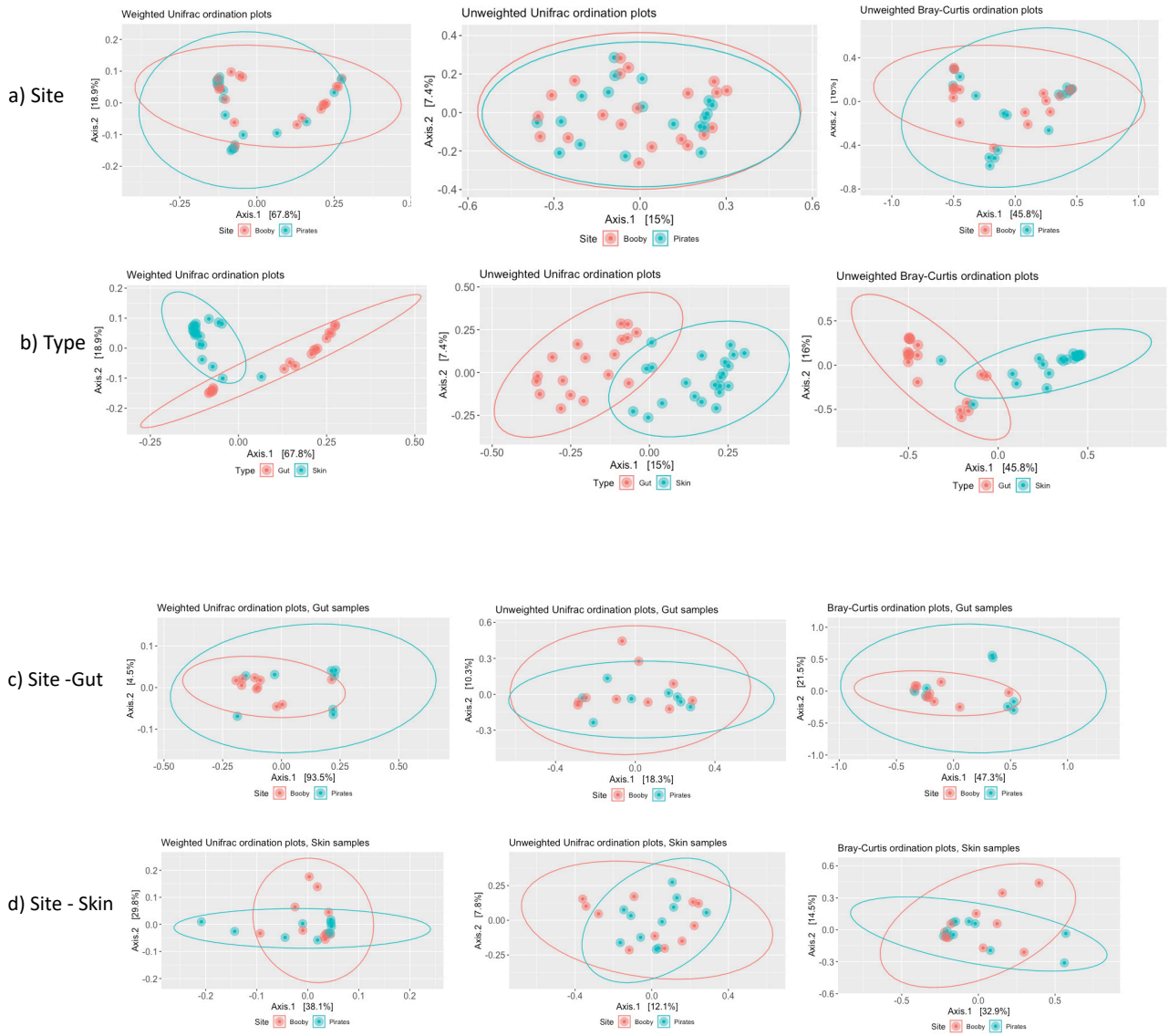
Supplementary Table 5: Top ten bacterial phyla found in each sample type, Sharknose goby cleaner fish (*Elacatinus evelynae*) and associated benthic constituents of each cleaner fish station from three reefs in Man O'War Bay, Tobago. ACDC – Algae Covered Dead Coral, Coral – brain coral (Faviidae), Coral Ecosphere water – water from the immediate surrounding of the coral, Diseased Coral – brain corals exhibiting disease, Fish - Sharknose goby cleaner fish skin mucus, Green – green mat zoanthid, (*Zoanthus pulchellus*), Sea – sea water from the three reef sites and Yellow - yellow encrusting zoanthid, *Palythoa caribaeorum*.

Sample	Top five phyla in descending order				
Cleaner fish skin mucus (*Elacatinus evelynae*)	Proteobacteria	Actinobacteria	Bacteroidetes	Cyanobacteria	Planctomycetes
Brain coral mucus	Proteobacteria	Cyanobacteria	Bacteroidetes	Planctomycetes	Actinobacteria
Brain coral water	Proteobacteria	Cyanobacteria	Bacteroidetes	Planctomycetes	Actinobacteria
Diseased brain coral	Cyanobacteria	Proteobacteria	Bacteroidetes	Planctomycetes	Actinobacteria
Palythoa caribaeorum	Proteobacteria	Bacteroidetes	Cyanobacteria	Actinobacteria	Planctomycetes
Zoanthus pulchellus	Proteobacteria	Cyanobacteria	Bacteroidetes	Planctomycetes	Verrucomicrobia
Algae covered dead coral	Proteobacteria	Cyanobacteria	Bacteroidetes	Planctomycetes	Verrucomicrobia

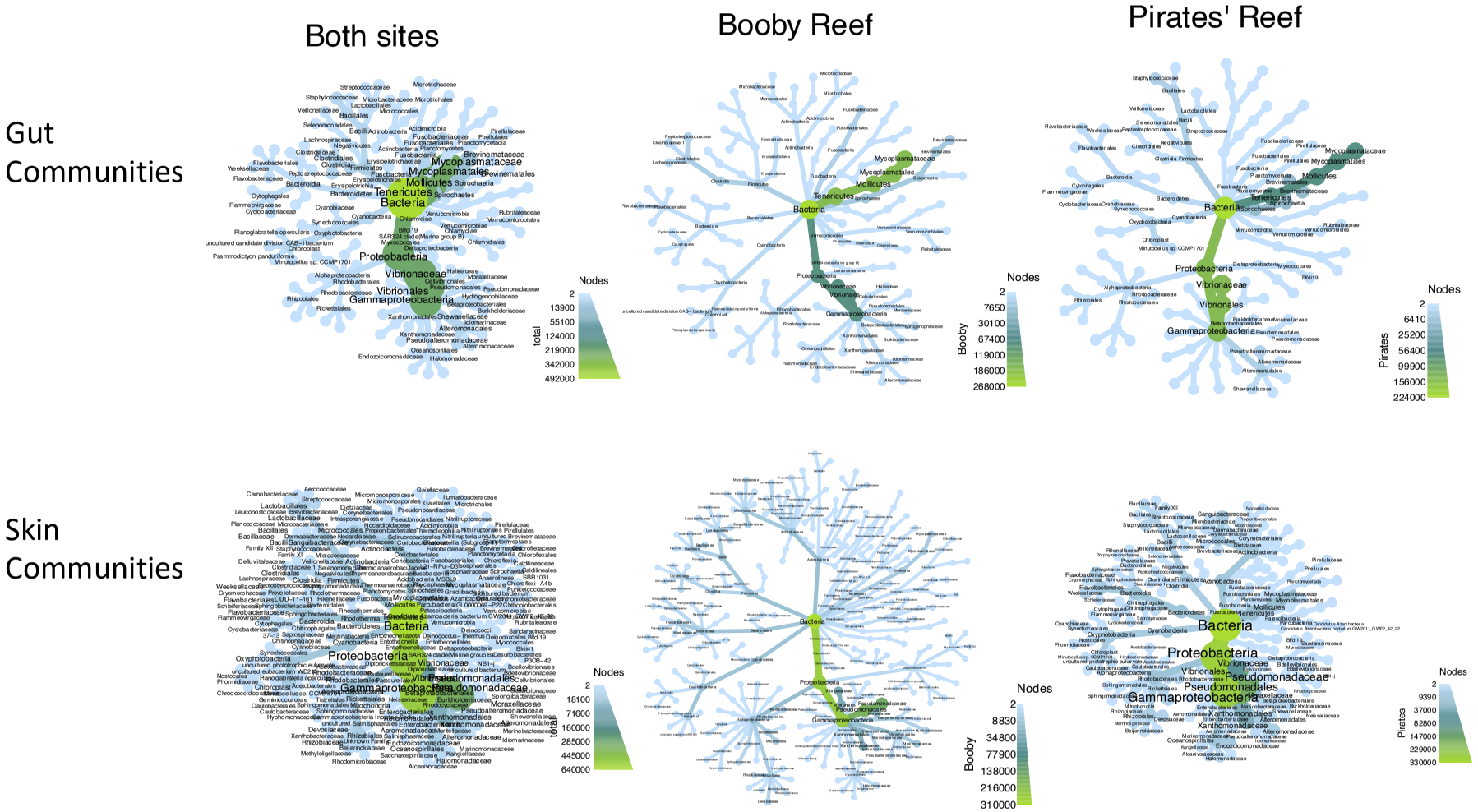
Supplementary Table 6: Percentage of shared bacteria of 10 genera of bacteria shared between cleaner fish (*Elacatinus evelynae*) and yellow zoanthid (*Palythoa caribaeorum).*

Bacteria at genera level	Percentage shared between Fish and Yellow Zoanthid
Proteobacteria	41.18
Actinobacteria	11.76
Bacteroidetes	11.76
Planctomycetes	8.82
Acidobacteria	5.88
Cyanobacteria	5.88
Verrucomicrobia	5.88

Acetothermia	2.94
Omnitrophicaeota	2.94
Patescibacteria	2.94



Supplementary Figure 2: PCoA plots showing beta diversity of cleaner fish (*Elacatinus evelynae)* microbiota for the variables a) site; b) sample type; c) gut samples between site; d) skin samples between site; using the weighted UniFrac, unweighted UniFrac and Bray-Curtis beta diversity index with 95% confidence interval (CI) ellipses.



Supplementary Figure 3: *Metacoder heatmaps of read abundances from skin and gut microbial samples of sharknose goby cleaner fish* (*Elacatinus evelynae*) *from two different reefs Booby Reef and Pirates Reef collected from the Man O War Bay, Tobago to Family level. Top; differences in read abundance in gut samples from Booby and Pirates separately, Bottom; differences in read abundance in gut samples from Booby and Pirates both*

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