Cleaning symbiosis and the disease triangle

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March 2022

For the Degree of Doctor of Philosophy

ARC Centre of Excellence for Coral Reef Studies

College of Science and Engineering

Centre for Sustainable Tropical Fisheries and Aquaculture

James Cook University

Je dédicace cette thèse à mes parents

Acknowledgements

First, I want to thank Dr Kate Hutson, my principal advisor for the first part of my thesis for her support and guidance. Since the first day of my PhD, you have been always very positive, receptive and open minded with many of my ideas for this thesis. Your enthusiasm with this project was crucial for me to be able to progress in the right direction. Thank you, Kate, for your constant positivity and support and I hope we will be able to meet again soon!

I am extremely grateful to Dr Jennifer Donelson for her incredible support as my principal advisor for the remainder of my thesis. The beginning of 2020 was a challenging time for my PhD candidature, but you jumped into this role without any hesitation, helped me with many conundrums, and have been such an amazing mentor. I am very glad to have been part of your research lab for the last two years of my PhD. Thank you for everything Jenni.

I also want to thank Dr Cecilia Villacorta-Rath, my co-advisor. Cecilia, I feel so lucky to have been working with you. I started this PhD with no molecular background, and you managed to make me love it! You are an excellent mentor. I could not imagine having better support than what you provided me. I hope I will have many opportunities to work with you again in the future. Thank you, Cecilia!

I want to thank Dr David Vaughan, my external advisor. David, I started to work in the Marine Parasitology Lab as your volunteer for your PhD, and then I became your PhD colleague and then you became my advisor! I am very thankful for the knowledge I have acquired from you. You are such a dedicated (as the cleaner fish!) scientist and I could listen to your conceptual deliberations on parasites/fish taxonomy and ecology for hours! I am extremely grateful to have you as advisor. Thank you so much for your support, your trust, your patience, and your teaching skills.

Finally, I want to thank Dr Mark McCormick, for his constant assistance and support in the field and for his feedback on many of my drafts. Mark, thank you for being always available when I needed! I really appreciate your help.

This thesis would not have been possible without the support from Dr Lexa Grutter. Lexa, I want to thank you for your enthusiasm with this thesis. Thank you for your time, your patience and your mentorship. Your suggestions are always extremely significant, and I really hope there will be many other collaborations in the future. I also want to thank you for all the opportunities you gave me by working with you and your team at Lizard Island. I am extremely grateful to have you as a part of this thesis.

I want to thank my PhD lab mates from the Marine Parasitology Lab. I feel extremely lucky to have spent the first years with all of you! Thank you, David and Alejandro, for your extremely wise advice for fieldwork, lab work, paperwork and for your positiveness all the time! Thank you JB and Julian for bringing so much joy around you! Thank you, Dylan, for your wonderful support with the parasite transfer experiment! Thank you, Katie, for being in this with me until the end. We went through a lot together and I am so thankful to have you as colleague and friend.

I want to thank Alejandro Trujillo González, Jordan Casey, Russell Yong, Renato Morais, and Roger Huerlimann for your collaboration, support and pertinent advice. I feel very fortunate to have the opportunity to work with all of you.

I want to thank all the amazing people that helped me with my lab work and fieldwork at Lizard Island during this PhD. Huge thanks to these incredible humans, my friends Alexia Dubuc, Ale Siqueira, Chris Hemingson, Renato Morais, Veronica Radice, and Victor Huertas. Thank you for your dedication and your support. You brought me so much happiness and laughter during fieldtrips.

I want to thank my PhD fellows and fantastic friends from the Bellwood lab. Huge thanks to Ale, Chris, Mike, Renato, Robert, Sterling, and Victor (and all the 2020 to 2022 Reef Function Hub cohort of course!). You supported me no matter what and this PhD would not have been the same without all of you. Thank you. And thank you Orpha and David Bellwood for accepting me within your fish family!

I want to thank Anne Hogget and Lyle Vail from Lizard Island Research Station for their overwhelming support. I feel very lucky to have done my PhD fieldwork and lab work at LIRS. Your constant assistance for researchers makes such a big difference. Thank you so much Anne and Lyle! Thank you

to Marianne and John, Marianne and Lance, Snow and Rennie, Arthur and Ruth as well as all the other volunteers working at the station to keep this place remarkable and functioning so well. I want to thank the McCormick lab for their support during the 2018 fieldwork trip. Thank you Noa and Zohar for spending many hours discussing ideas with me and helping me to release fish.

A huge thank to Johan Larson, Rhys Cornish and Rob Edwards from Orpheus Island Research Station for making possible the use of the underwater live camera for research. These data did not make it in time for this thesis, but I am working on it, and this will be a huge and awesome project, and this is all thank to your technical support and availability. Thank you very much!

A huge thank to these legends from James Cook University that made everything possible: Greg Napier, Craig Godfrey, Greg Suosaari, the MARFU team and Andrew Norton. Thank all of you for your technical support! Thank you, Carolyn Smith-Keune and Nikolaos Andreakis, for taking such a good care of the MEEL lab and being always available. I also want to thank Denise Blake, Liz Tynan, Vivian Doherty, Janet Swanson, Olga Bazaka and Alana Grech for their assistance in so many different ways. Thank you Sandra Infante-Villamil for your support with the DNA metabarcoding methods. Thanks to the Centre for Sustainable Tropical Fisheries and Aquaculture for support. Finally, thank you Carolyn, my office mate, to be such a nice person and let me discover your physio and shark world!

Thanks to Geoffrey Boxshall, Jean-Lou Justine and Marlies Monnens for support with parasite identification for Chapter 3. Gabriela Muñoz for sharing data for comparison with other labrids for Chapter 3. And finally, Miwa Takahashi and Mathilde Scheifler for discussion about DNA metabarcoding methods for Chapter 5.

A special thanks to Marta Soares, my Master thesis advisor for being a wonderful mentor since 2012. You always believed in me, and I really hope we will be able to work again in the near future. Thank you for transmitting your passion for cleaner fish!

I want to thank all my friends here in Townsville and from many other places in Australia and all over the world. A special thanks to my lovely friends: Alexia, Ale, Charlie, Daizinha, Gus, Hanaka, Isabear, Juju, Kate, Lyn-Lyn, Pouya, Roque and Veronica for your amazing friendship during this PhD and

beyond. You have a really special place in my heart. Your incredible support made this journey easier. Merci aussi à tous mes ami(e)s incroyables de France qui suivent mes aventures et m'encouragent depuis tellement d'années.

A huge thank to my family. Merci de tout mon cœur Papa, Maman, Réré et Floflote. Être si loin de vous a été si difficile mais votre bonne humeur, votre soutien a été crucial et m'a fait tenir le coup. Je ne vous remercierai jamais assez Papa et Maman pour m'avoir soutenu depuis toujours, sans vous, je n'y serais jamais arrivé. Mille merci. Merci Floflote et Rémi pour m'avoir donné tellement de bonheur avec Pablo. L'année 2020 a été difficile et vous m'avez tellement manqué mais Pablo a été mon rayon de soleil et le sera toujours. Je vous aime. Merci à tout le reste de ma famille pour le soutient même en étant loin. Une forte pensée pour mon papi Charly et ma mamie Manuela qui malheureusement nous ont quitté pendant cette thèse.

Finally, I will finish these acknowledgements with the main pillar in my life. Renato, I could write several paragraphs about your incredible personal and professional support, but I will try to be succinct. You have been the one encouraging me since the beginning and even before my PhD started. You have been the one coming to JCU in the lab during the weekends and holidays to take care of fish and parasites. You have been the one waking up at 2 am to look for parasites attacking fish. You have been teaching me new skills with R, Illustrator and so many other things. You are an incredible human. Merci chéri and I cannot wait for the next amazing adventures ahead of us.

Acknowledgement of Traditional Owners

In the spirit of reconciliation, I acknowledge the Traditional Custodians of this land where I deliver this thesis, in Townsville, the Wulgurukaba and the Bindal People. I also acknowledge the Dingaal and Quandamooka people, the traditional owners and custodians of the land and country upon which this research was carried out. I pay my respect to their Elders past and present and extend that respect to all Aboriginal and Torres Strait Islander peoples today.

Statement of the Contribution of Others

This thesis was supported by the James Cook University Postgraduate Research Scholarship, the Michael Hall Student Innovation Award (Australian Society for Fish Biology), the Holsworth Wildlife Research Endowment (Ecological Society of Australia), the HDR Competitive Research Training Grant (James Cook University), and the David Yellowee Grant (ARC CoE Centre for Coral Reef Studies). This thesis was also supported by funds provided to Kate Hutson through a James Cook University Development Grant, and also funds provided to Mark McCormick by The Australian Research Council.

This thesis was conducted under the supervision of Kate Hutson, Jennifer Donelson, Cecilia Villacorta-Rath, David Vaughan and Mark McCormick. In **Chapter 2**, Kate Hutson from James Cook University, David Vaughan from Central Queensland University and Alexandra Grutter from The University of Queensland participated during the conceptual phase and editorial assistance. In **Chapter 3**, Russell Yong from The University of Queensland, Kate Hutson and Alexandra Grutter participated in the conceptualisation. Russell Yong participated in the data collection and all authors provided editorial assistance. In **Chapter 4**, Renato Morais from James Cook University, David Vaughan and Kate Hutson participated in the conceptualisation. Renato Morais participated in the data collection and statistical analyses. All authors provided editorial assistance. In **Chapter 5**, Cecilia Villacorta-Rath from James Cook University participated in the conceptualisation. Cecilia Villacorta-Rath provided laboratory work assistance and guidance. All authors provided editorial assistance. In **Chapter 6**, Mark McCormick from James Cook University and Alexandra Grutter participated in the conceptualisation. Renato Morais participated in the data collection and statistical analyses. All authors provided editorial assistance.

All work reported in this thesis has been conducted in accordance with JCU Animal Ethics Committee Approval A2558 and A2457 as well as Great Barrier Reef Marine Park Authorization Permit number G19/39553.1; G18/39035.1; G18-38392.1 and G18-38392.2.

Abstract

To manage parasite infections, organisms use several behavioural techniques such as grooming, migration, and self-medication. One of the most common and effective means to remove parasites is called *cleaning symbiosis*. In this complex interaction, individuals from one species act as a "cleaner" while individual(s) from another species are "clients". Cleaning symbiosis has been reported in terrestrial systems, for example, birds act as cleaners by removing ticks from mammals. In marine environments, cleaning interactions are common and mainly involve fish and shrimp as cleaners. The cleaner wrasse *Labroides dimidiatus* and its interactions with client fish are the most studied aquatic mutualistic model globally. When considering this cleaner-client relationship, historical research has largely focused on positive aspects of the association. This includes how the clients are positively affected by the cleaners removing their parasites, and how clients, in turn, provide food for the cleaners. However, mutualism is not only shaped by benefits between species but also involves costs. While costs and trade-offs have been considered from the perspective of cleaners 'cheating', there has been no previous exploration of the potential for cleaners to facilitate disease transmission and spread in the wild. The aim of this thesis was to challenge the prevailing paradigm that cleaning symbiosis has predominantly positive impacts on fish communities and to understand the potential costs associated with it.

In this thesis, I first reviewed the knowledge on cleaning symbiosis in the marine environment and identified gaps in the literature, mainly associated with the potential costs of cleaning symbiosis, as well as how environmental changes could impact the dynamic between fish and parasites. I then expanded the concept of the disease triangle, which illustrates how the relationships between the *parasite* (or other pathogens), *host* and *environment* may predict the outcomes of a disease, to highlight several associations that have been previously overlooked when considering *cleaners* as a fourth element (**Chapter 2**). I proposed an enhanced perspective on cleaning symbiosis, by exploring new costs potentially associated with it. From this, I outlined four potential pathways previously overlooked: Pathway 1: Whether cleaner fishes are susceptible to parasite infections from their clients (**Chapter 3**); Pathway 2: Whether cleaner fishes can transmit parasite to their clients in the wild (**Chapter 4**).

Pathway 3: Mechanisms that parasites may use to evade cleaner organisms and maximise their chance of transmission; Pathway 4: Human impacts on cleaning symbiosis and parasites (**Chapter 6**).

The parasite community of *L. dimidiatus* has not been previously investigated. In **Chapter 3, I** examined the parasite community of *L. dimidiatus* in two different regions in north-eastern Australia. I discovered that *L. dimidiatus* is infected by a diversity of parasites in the natural environment including 5 ectoparasite groups (copepods, isopods, protozoans, monogeneans and turbellarians) and 3 endoparasite groups (myxozoans, trematodes and cestodes) totalling at least 12 species. Comparison of the parasite community of *L. dimidiatus* with other wrasses from the same region showed that *L. dimidiatus* had similar abundance and prevalence of most parasitic groups. However, the presence of adult bucephalid digenean endoparasites was found to be a unique feature to the *L. dimidiatus* endoparasite community. This presents an intriguing finding given adult bucephalids typically mature in piscivorous hosts. Bucephalids that infect cleaners likely exploit cleaning symbiosis (i.e., cleaners grazing on infected client skin and mucus) as a mechanism for transmission.

Based on the hypothesis that cleaner fish could act as parasite transmitters, I developed laboratory experiments to test whether *L. dimidiatus* is susceptible to generalist parasites that could be potentially transmitted to other fish clients during cleaning interactions (**Chapter 4**). Experiments revealed that *L. dimidiatus* is susceptible to infection by their main food source – gnathiid isopod parasites, *Gnathia aureamaculosa* – while exhibiting some degree of resistance to one species of ciliate protozoan, *Cryptocaryon irritans*, and one species of monogenean flatworm, *Neobenedenia girellae*. Subsequently, I performed two experiments, by manually transferring adult *N. girellae* from a donor host (susceptible to *N. girellae*) to the receiver, *L. dimidiatus*. I found that adult *N. girellae* could survive on *L. dimidiatus* for at least 2 days and produce viable eggs. Over 2 days, a wild *L. dimidiatus* can clean more than 4,000 fish individuals. Consequently, it is probable that transmission of mobile adult *N. girellae* is facilitated from cleaners to clients and *vice versa*. Parasites can survive on cleaners for sufficient duration to potentially infect other fish or release viable eggs that could hatch and find a new host in the vicinity.

Another aspect of the disease triangle that I explored when including cleaners as a fourth element was to understand which items cleaners remove from their fish clients. I used DNA metabarcoding techniques to explore the diet of *L. dimidiatus* and two species of cleaner shrimp *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* (**Chapter 5**). Two universal primers targeting the mitochondrial *COI* and *16S* regions were used, which have been previously successfully utilised to investigate the parasite community of fishes and have been also used to target a wide range of metazoan prey in fish. With the *COI* primer, I found high prevalence of fish sequences from the gut of *L. dimidiatus*. At the phylum level, *L. dimidiatus* was targeting Chordata (represented by the fish sequences), Arthropoda and Mollusca. The presence of sequences associated to fish support the hypothesis that *L. dimidiatus* removes tissues from their clients. These could represent the removal of fish mucus, fish dead tissues or parasites that ingest host tissues and therefore contain host DNA (iDNA). Ingestion of fish mucus by *L. dimidiatus* will represent an opportunistic behaviour because the loss of mucus is costly for their clients. By contrast, the removal of dead fish tissues or parasites by *L. dimidiatus* is beneficial for their clients. With the *16S* primer, for the cleaner fish *L. dimidiatus* and the two cleaner shrimp *Urocaridella antonbruunii* and *U. cyrtorhyncha*, I found sequences from parasitic organisms from three different phyla: Nematoda, Apicomplexa and Platyhelminthes, which could represent encysted parasites from the body of fish clients and/or parasites from the cleaners themselves.

The *environment* element of the disease triangle is susceptible to change. In **Chapter 2,** I identified knowledge gaps in the literature on how habitat degradation influences the dynamic between cleaner and clients, but also parasites and hosts. Therefore, in the last chapter of my thesis (**Chapter 6**), I investigated the effect of seawater associated with dead coral on the infection rate of gnathiid isopods on juvenile and adult damselfish. To do so, three different seawater treatments were used mimicking: degraded reefs (i.e., dead coral substrate covered by a variety of colonisers), non-degraded reefs (i.e., high live coral cover), and a control (with no coral). I found that juvenile damselfish were twice as susceptible to gnathiid infection in the seawater treatment exposed to dead coral compared to damselfish in the seawater treatment exposed to live coral. By contrast, the susceptibility of adult damselfish to

gnathiid infection was not affected by the different treatments. These results suggest that juvenile damselfish are more sensitive to chemical cues released from the dead coral and/or dead coral colonisers that may consequently affect their behavioural responses. Adult fish were not affected by changes in water chemistry from dead coral and this may be due to chemical cue interference avoidance but also other factors such as prior infection by wild gnathiids on adult fish (and induced immune response). This chapter provides an enhanced understanding of the dynamic between parasites and hosts at the small-scale when considering habitat disturbance in environment from the disease triangle.

Overall, this thesis considered the potential for dynamic and complex drivers in cleaning symbiosis from the perspective of the disease triangle. This thesis proposes a paradigm shift in our consideration of disease transmission ecology in wild marine environments and advances previously unexplored relationships between cleaners, parasites, clients/hosts, and the environment.

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Chapter 1: General Introduction

Parasitism and cleaning symbiosis

Parasitism is a symbiotic association between two organisms where one depends on the other to live: the parasite benefits at the expense of the host, by feeding on it (Rohde 2005). While endoparasites live inside the host's body, ectoparasites infect external surfaces (e.g., skin, gills) for a limited period (depending on the parasite's life cycle; Bellay et al. 2015). Parasites are omnipresent, occurring in a wide range of environments in both terrestrial and aquatic systems. In marine environments, ectoparasites include numerous species of protozoans, monogeneans, copepods, isopods and hirudineans, while endoparasites commonly include species of myxozoans, digeneans, cestodes and nematodes (Rohde 2005). Parasite infections compromise host health. For example, haematophagous gnathiid isopods that infect the body surface and gills of fishes can cause blood congestion, destruction, and inflammation of mucosal coat tissues (Heupel and Bennett 1999) and, in large numbers, can kill their fish hosts (Smit and Davies 2004; Jones and Grutter 2007). Parasites can also reduce host growth (Hirazawa et al. 2016), swimming performance (Wagner et al. 2003; Binning et al. 2012), and affect physiological responses such as reduced aerobic capacity and increased metabolic rate (Binning et al. 2012).

Organisms use a wide range of techniques to combat parasites and infectious diseases. Hosts may physically dislodge parasites by rubbing against hard substrates or jumping out of the water, as some fish do (Misganaw and Getu 2016), while host immunity may respond to infection (Schmid-Hempel 2009). Migration from one environment to another may indirectly allow temporary escape from infested habitats (parasite avoidance) and can reduce associated parasitic infections (Poulin et al. 2012; Binning et al. 2017). Other strategies include self-medication; as for example, when chimpanzees, lemurs and goats, control internal helminth infections by ingesting plants leaves with anthelmintic properties (Fowler et al. 2007; Villalba et al. 2014). Intraspecific behaviour may also be utilised to help congeners to rid their parasites. For example, in terrestrial environments, organisms living in social communities, such as monkeys, groom to remove parasites between conspecifics in a socially constructive, reciprocal altruism known as allogrooming (MacIntosh et al. 2012). Bees also exhibit allogrooming or auto-

grooming (e.g., individual grooming itself) when infected by parasitic mites (Bąk and Wilde 2015). Complex mutualistic interactions (interactions that are beneficial and reciprocal for the species involved; Boucher et al. 1982; Bronstein 1994), such as cleaning symbiosis have been demonstrated as effective parasite removal techniques (Grutter 1999a; Côté 2000; Vaughan et al. 2017). There are a few terrestrial examples of cleaner organisms, including birds such as the red-billed oxpecker, *Buphagus erythrorhynchus* and mammals such as the banded mongoose, *Mungos mungo* that remove ticks from mammalian clients in the African savannah (Weeks 2000; Sazima 2010, respectively). In marine environments, cleaning interactions are common and mainly involve fish or shrimp as the cleaner (Côté 2000).

Cleaning in the tropical ocean: the benefits of a specialised dedicated cleaner fish

Marine cleaning symbiosis is a mutualism that has been widely studied over the past few decades (reviewed in: Côté 2000; Grutter 2002; Vaughan et al. 2017), particularly in tropical settings such as coral reefs. Among the most dedicated tropical marine cleaners – cleaners that clean during their entire life and prey mostly on parasites that they remove from other 'clients' – are cleaner fishes, especially wrasses from the genus *Labroides* and gobies from the genus *Elacatinus* are the most studied (Côté and Soares 2011). The genus *Labroides* includes five species, all considered dedicated cleaners and restricted to the Indo-Pacific region (Vaughan et al. 2017). The bluestreak cleaner wrasse, *Labroides dimidiatus* is the most common, the most widely distributed, and the best studied of all tropical cleaners, and is the model species of this thesis (Fig. 1). This species can consume more than 1,200 gnathiid isopods per day and may spend an average of 4.5 hours inspecting up to 2,300 clients each day (Grutter 1996a).

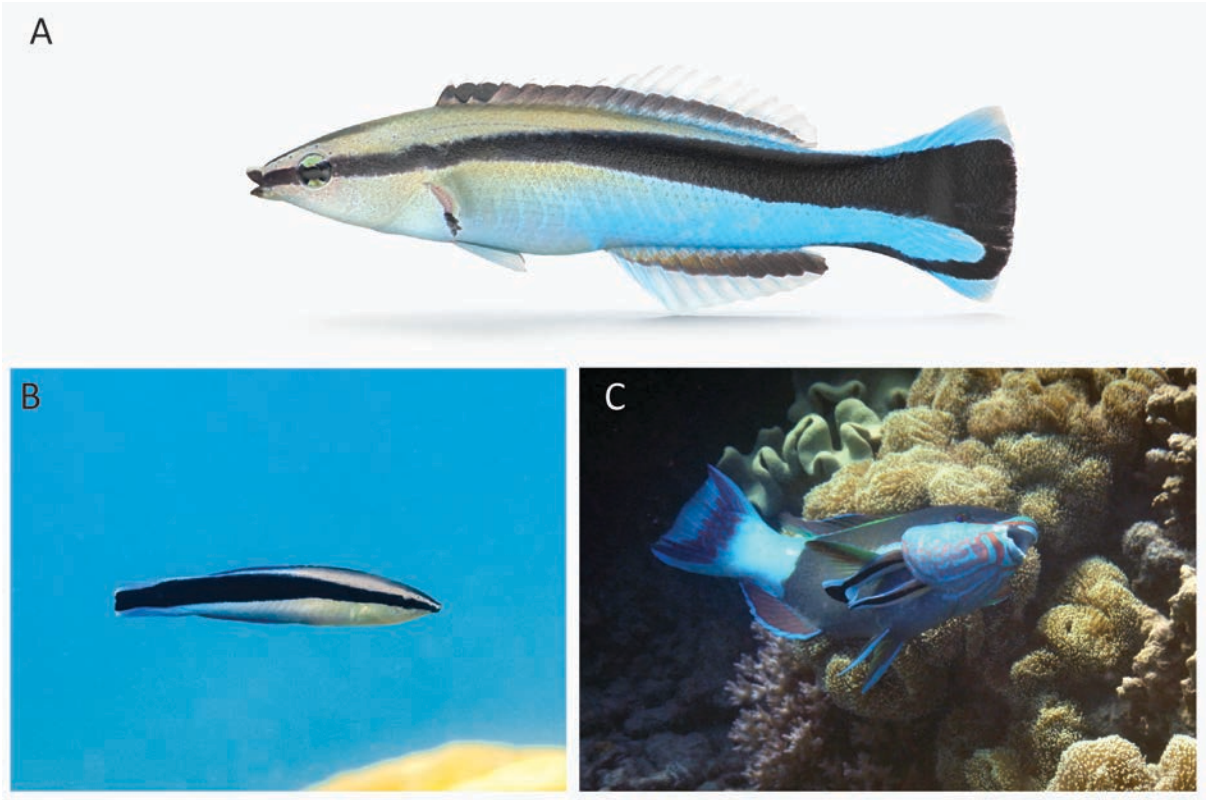

Figure 1 The cleaner wrasse, *Labroides dimidiatus* (Valenciennes, 1839), which is the focal species of this thesis is represented as an artwork (A), in the wild (B) and in pair, cleaning the parrotfish *Scarus frenatus* (C). Images: Victor Huertas and the author

Cleaning symbiosis is hypothesised to generate positive impacts for individuals, populations, and communities. Such positive effects have been most extensively investigated in *L. dimidiatus*. Since 1996, AS Grutter and subsequent researchers, have continuously removed cleaner fish *L. dimidiatus* from specific patch reefs at Lizard Island, on the northern Great Barrier Reef to investigate long-term consequences of the absence of cleaner fish on reef communities. The effects of the dedicated cleaner wrasse *L. dimidiatus*, as determined from their presence on 'control' patch reefs or absence on 'removal' patch reefs are summarised below (Fig. 2). On the removal reefs, the abundance of damselfish recruits was lower compared to the control reefs (Fig. 2B number 1; Sun et al. 2015), suggesting an enhancement of recruitment by the presence of cleaners through direct (i.e., damselfish prefer to settle near cleaner fish) or indirect mechanisms (i.e., cleaners reduce ectoparasites in the environment that could affect the success of damselfish recruitment; Sun et al. 2015). Highly mobile fishes visiting control reefs were twice as species diverse and four times more abundant than on removal reefs (Fig 2A number 2; Grutter et al. 2003). Both juvenile visiting (Fig. 2B number 3) and resident (Fig. 2A number 4) fishes were also

less abundant and species diverse on the removal compared to the control reefs (Waldie et al. 2011). Altogether, these results indicate that cleaner fish may affect movement patterns and subsequently, the spatial distribution of reef fishes (Grutter et al. 2003).

From a parasite perspective, gnathiid isopods, the most common ectoparasite prey of *L. dimidiatus*, were found to be 4.5 times more abundant on fishes on the removal reefs than on the control reefs during sunset hours (Fig. 2B number 5; Grutter 1999a). This suggested that the previously observed reduction in the abundance of gnathiids throughout the day from dawn to sunset could be a result of gnathiid predation by cleaner fish (Grutter 1999a). Parasitic copepods were less abundant on damselfish *Pomacentrus moluccensis* from control compared to removal reefs. Furthermore, *P. moluccensis* exhibited a smaller body size on the removal patch reefs than conspecifics inhabiting control reefs (Fig. 2A number 6; Clague et al. 2011a). This may be linked to observed higher growth rates of damselfishes in the presence of *L. dimidiatus* (Clague et al. 2011a). Other physiological mechanisms also seem to be affected by the presence or absence of cleaner fishes. For example, fish clients from removal reefs have been found to have lower cognitive performance and to take more time to solve experimental tasks compared to fish from control reefs (Fig. 2B number 7; Binning et al. 2018). In this case, it was suggested that, through the removal of ectoparasites, cleaner fish indirectly affect the cognitive ability of fishes (Binning et al. 2018). Finally, the body condition (represented by the variation in girth relative to length: girth TL^{-1}) of four damselfish species was found to have deteriorated on removal compared to control reefs (Fig. 2B number 8; Ros et al. 2020). These results are in line those found by Clague et al. (2011a) and suggest that the lack of access to cleaner fish increases energy expenditure in dealing with higher ectoparasite load (Ros et al. 2020).

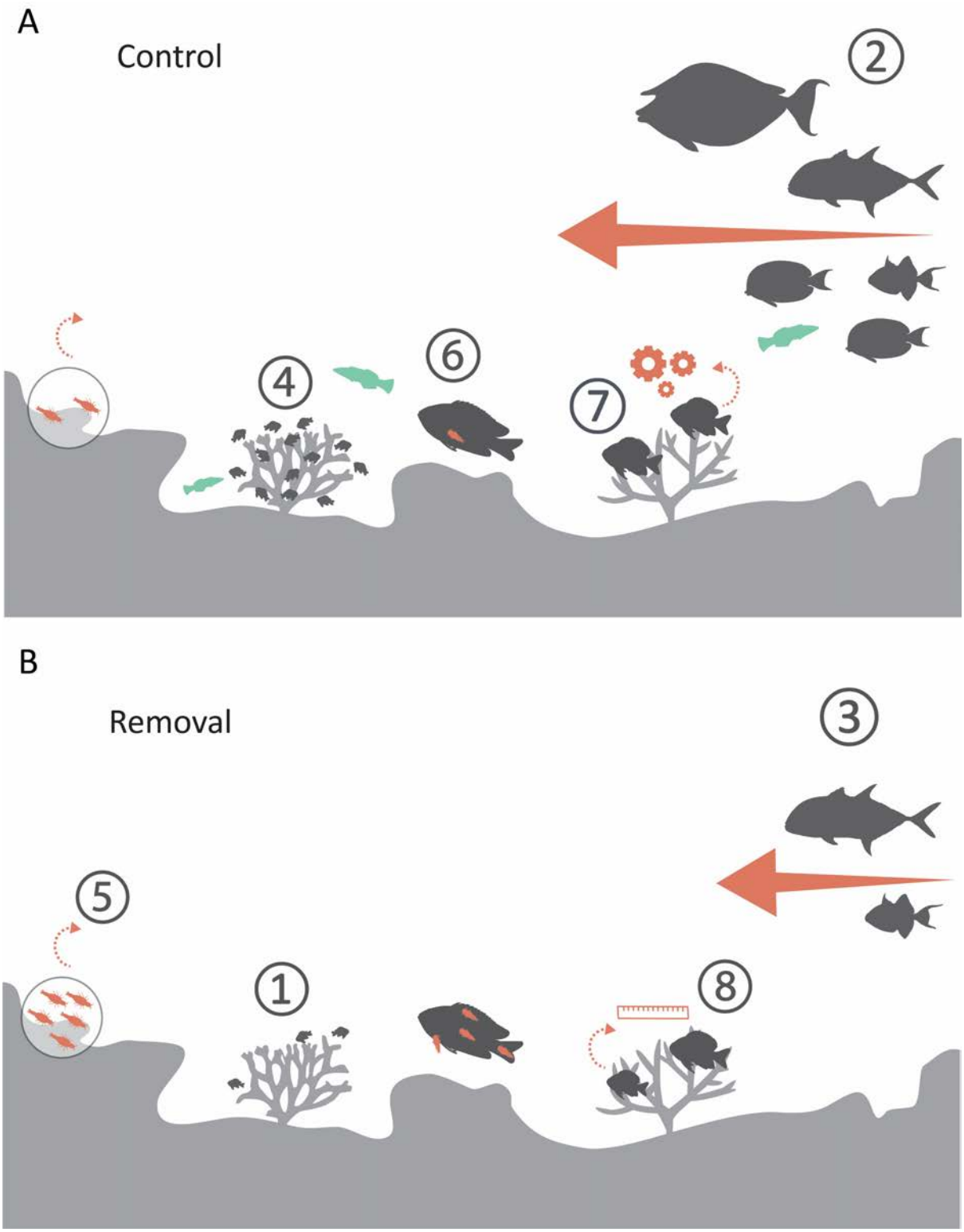

Figure 2 Representation of (A) an example control patch reef where cleaner wrasse *Labroides dimidiatus* were maintained and (B) an example removal patch reef where *L. dimidiatus* have been removed. The studied patch reefs were located at Lizard Island, Great Barrier Reef, and ranged in area from 29 to 146 m^2 (Grutter 1996b; Grutter et al. 2003). Numbers indicate a documented change between control and removal reefs, with a visual difference between panel a and b highlighting the response pattern. Cleaner wrasse (in green in A), gnathiid (number 5 in a and B) and copepod parasites (number 6, A and B) are not represented to scale

The costs associated with cleaning symbiosis

Mutualism is not only defined by benefits between species but is also shaped by costs (Bronstein 2001a). To gain from another species implicated in any given interaction, it is essential to also give in return, which has been characterised as the "biological market" (Bronstein 2001a; Noë 2001; Bshary and Bronstein 2004). For example, to disperse pollen, plants may trade nectar with a pollinator. In many cases, this trade-off is not entirely linear and can trigger side and negative effects. For example, in myrmecophyte trees' mutualistic relationship with ants, the ants eradicate potential herbivores that are harmful for the trees while the trees provide shelter and food for the ants (Stanton and Palmer 2011). In an experimental study, ants from four different species were excluded from their mutualistic trees over 4.5 years and overall, the trees with no ants were more vulnerable to herbivore attacks. However, interestingly, growth and reproduction of the trees were enhanced in the absence of ants. This suggested that the cost of maintaining ant colonies is not negligible, and may in cases even exceed the short-term benefits of herbivory protection (Stanton and Palmer 2011).

One of the main previously investigated costs of marine cleaning symbiosis is 'cheating' behaviour. Cheating normally occurs when cleaners remove healthy tissues, such as mucus, from their clients instead of ectoparasites (e.g., Grutter and Bshary 2003; Soares et al. 2008a). The cleaner fish *L. dimidiatus* favours eating fish mucus over ectoparasites because of its high nutritional value and constant availability on the skin of fish (Grutter and Bshary 2003). Clients purportedly respond to cheating behaviour with a body jolt reaction (Soares et al. 2008a), and often terminate the interaction by chasing the cleaner or swimming away (Bshary and Grutter 2002). To prevent conflict or to reconcile with clients, and consequently to maintain the mutualistic relationship, cleaners might 1) feed against their preferences, that is, consuming more ectoparasites than mucus; or 2) develop strategies and services that avoid such conflicts (e.g., Grutter and Bshary 2003; Grutter 2004). For example, tactile stimulation from 'rubbing' clients with their fins and body is often used by *L. dimidiatus* as a "pre-conflict management strategy" (Grutter 2004) focused on reducing the chance of being preyed upon by predatory fish clients (Grutter 2004).

Another cost associated with cleaning symbiosis, investigated in aquaculture, is the inadvertent transmission of pathogens from cleaner fishes to farmed fishes (see Treasurer 2012; Erkinharju et al. 2020 for review). Since the 1990s, cleaner wrasses such as *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Crenilabrus melops*, *Labrus bergylta* and *Labrus mixtus*, have been introduced to salmon farming in northern Europe as biocontrols to reduce sea lice infection (Costello 1993, 1996a). However, these cleaner wrasses are also susceptible to sea lice infection (Bron and Treasurer 1992), as well as to other infections in the wild (Costello 1996b; Treasurer 1997). More recently, the potential role of cleaner wrasses as a source of pathogen transmission to salmon has been investigated. For example, the protozoan parasite *Neoparamoeba perurans* that causes amoebic gill disease is found to infect both salmon and the temperate cleaner wrasse *L. bergylta* in captivity. As such, *L. bergylta* may become potential reservoirs of this disease (Karlsbakk et al. 2013).

Whether parasite transmission occurs between wild fishes engaging in cleaning interactions has not been investigated. Prior to this thesis, there was only a single parasite species recorded to infect *L. dimidiatus,* thus there is limited baseline information to begin to explore this question. The bucephalid digenean *Rhipidocotyle labroidei* (see Jones et al. 2003) is an unusual and exciting host-parasite record for a cleaner fish given that bucephalids usually attain sexual maturity in piscivorous hosts. This indicates potential unique adaption of bucephalids to utilise cleaning interactions to complete their life cycle (Jones et al. 2004).

Is the role of cleaners reflected in their diets?

Interest in the functional role of cleaner organisms in the marine environment was rapidly followed by studies investigating their diets. Stomach content analyses are essential to confirm the removal of parasites from the body of other fishes to support behavioural observations of a symbiotic relationship between cleaners and clients (Youngbluth 1968; Hobson 1971; Losey 1974; Grutter 1996a). In this context, visual analysis of stomach contents has been widely used as a tool to characterise diet, and to contrast it with the abundance and availability of different dietary items (Arnal and Côté 2000). Marine cleaner organisms, such as fish and shrimp, actively and selectively remove parasites from the body of

other fish both in tropical and temperate environments (e.g., Grutter 1999b; Arnal and Côté 2000; Arnal and Morand 2001; Becker and Grutter 2004; Narvaez et al. 2015; see also Vaughan et al. 2017 for a comprehensive list of prey items by cleaners), supporting prior behavioural observations and highlighting the crucial role cleaners play in their ecosystems. However, some potential prey of cleaners, such as small and/or soft-bodied ectoparasites, may be hard to visually detect and/or be quickly digested following ingestion (Vaughan et al. 2017). Recent molecular technologies, such as DNA metabarcoding, enable the amplification of small species-specific DNA sequences – called barcodes – from dietary samples retrieved directly from predators that may contain a wide assortment of prey items (Casey et al. 2019). Universal primers targeting specific gene regions that are common among species within certain broad taxonomic groups have also been developed within the last decade, allowing elevated accuracy for non-specific detection of barcodes (Leray 2013a; Parada et al. 2016). To the best of my knowledge, this innovative technique had never been used to investigate the diet of cleaner organisms prior to this thesis. Here, it will be explored as a crucial next step to enhance the accuracy with which we characterise the ecological role of cleaners.

Marine ecosystems under threat and potential impacts on cleaning symbiosis

Ecosystems are under stress globally (McCauley et al. 2015, Schulze et al. 2019). In the ocean, numerous stressors have been linked with habitat degradation, including pollution, overfishing and climate change (McCauley et al. 2015). Coral reefs are particularly vulnerable to human pressures (Okazaki et al. 2013; Hughes et al. 2018), and have recently experienced extensive periods of thermal stress globally (Hughes et al. 2017). These have led to widespread habitat degradation from severe mortality of corals, with corresponding changes from coral- to algal-dominated seascapes (De'ath and Fabricius 2010, Hughes et al. 2018). These changes brought about not only changes to the resource basis for reef consumers, but also to the composition of consumer communities (Jones et al. 2004, Graham et al. 2015). For example, coral mortality-induced habitat degradation has been found to negatively impact the olfactory information processing of juvenile coral reef fishes, leading to increasing susceptibility to predation (McCormick and Allan 2017). Thermal stress may also directly impact fishes via physiological pathways. For example, persistent thermal stress has been found to lead

to reduced breeding rates and egg production in a common damselfish species on the Great Barrier Reef (Donelson et al. 2010).

The impacts of habitat degradation on parasites and their hosts have been widely studied (e.g., Lafferty 2008; Lafferty et al. 2008, Wood et al. 2014, Lõhmus and Björklund 2015, Brunner and Eizaguirre 2016; Sikkel et al. 2019). The effect of increasing water temperature (Sikkel et al. 2019), fishing pressure (Wood and Laferty 2015), ocean acidification (Paula et al. 2020) among others revealed different trends and impacts depending on 1) the nature of the pressure, 2) the parasite studied and its response/adaptation to habitat degradation, 3) hosts responses/availability to these pressures. Conspicuous to all the evaluations, habitat degradation was found to disturb hosts, parasites, or their interactions (Brunner and Eizaguirre 2016). For example, cleaner fish consume gnathiid parasites which are common in the wild. However, during a severe thermal stress event on the Great Barrier Reef, the abundance of gnathiid isopods declined abruptly during the warmer months (Sikkel et al. 2019), leading to potential decreases in parasite infection rates and thus food availability for cleaners. Furthermore, both thermal stress and acidification were found to negatively affect behaviour and cognitive performance of *L. dimidiatus* (see Paula et al. 2019 a, b). However, knowledge on how habitat degradation may affect cleaning interactions and, in particular, the tripartite relationship between cleaners, clients and parasites in the wild remains limited.

Thesis outline

The main aim of this thesis was to develop a novel conceptual approach to cleaning symbiosis, and to use both ecological data and experimental techniques to test new hypotheses. In **Chapter 2,** I expand the concept of the disease triangle to explore how cleaning symbiosis may interact with the complex interrelationship between hosts (also called 'clients' in specific circumstances), pathogens and the environment (i.e., all components of the disease triangle). In this chapter, I first consider a new perspective on 'mutualistic' cleaning symbiosis that also includes potential negative impacts of wild cleaner fish on fish communities as vectors. I then propose the novel 'parasite hotspot hypothesis', whereby cleaning stations may inadvertently concentrate infective stages of parasites that vacate host individuals as the host is cleaned. **Chapter 2** comprises the theoretical foundation for this thesis, leading

me to explore four key questions. These four questions are the foundation for four subsequent experimental data chapters:

1) Are cleaner fish susceptible to parasitism?

2) Can cleaner fish act as parasite vectors?

3) What do cleaner organisms eat?

4) How will future climate scenarios impact parasite infection on fish?

In **Chapter 3,** I investigated the parasite community of the dedicated cleaner wrasse *L. dimidiatus* and compared its parasite community to other wrasses from the same environment. This chapter is an important first step to understand if *L. dimidiatus* is susceptible to parasites and if, in turn, this species could potentially transmit infections to other wild fishes during cleaning interactions. In **Chapter 4**, I experimentally exposed *L. dimidiatus* to common and generalist parasites to explore their susceptibility to infection. Then, I tested if *L. dimidiatus* can transport and spread viable ectoparasites to understand the potential consequences of inadvertent parasite transmission to the dynamics of cleaning symbiosis. In **Chapter 5**, I used DNA metabarcoding techniques to examine the diet of cleaner fish and cleaner shrimp that live in the same environment and often share cleaning stations or territories. This chapter employs innovative approaches to reveal aspects of cleaning symbiosis that may have been previously overlooked. Finally, in **Chapter 6**, I investigated the impacts of habitat degradation on parasite infection rates under laboratory conditions. Gnathiid isopods were used as a model; a common coral reef fish ectoparasite that is also the preferred food item of *L. dimidiatus*. This chapter was a crucial step to understand the dynamics between parasites and hosts under environmental stress.

Chapter 2: New perspectives on the role of cleaning symbiosis in the possible transmission of fish diseases

Published as: Pauline Narvaez, David Brendan Vaughan, Alexandra Sara Grutter, Kate Suzanne Hutson (2021) New perspectives on the role of cleaning symbiosis in the possible transmission of fish diseases. *Review in Fish Biology and Fisheries*, 31:233–251. https://doi.org/10.1007/s11160-021-09642-2

Abstract

For the last seven decades, cleaning symbiosis in the marine environment has been a research field of intrigue. There is substantial evidence that, by removing undesired items from their client fishes, cleaner organisms have positive ecosystem effects. These include increased fish recruitment, abundance and enhanced fish growth. However, the intimate association and high frequency of interactions between cleaners and clients potentially facilitates pathogen transmission and disease spread. In this review, I identify knowledge gaps and develop novel hypotheses on the interrelationship between parasites, hosts and the environment (disease triangle concept), with a particular emphasis on the potential role of cleaner organisms as hosts and/or transmitters of parasites. Despite evidence supporting the positive effects of cleaner organisms, I propose the *cleaners as transmitters hypothesis*; that some parasites may benefit from facilitated transmission to cleaners during cleaning interactions, or may use cleaner organisms as transmitters to infect a wider diversity and number of hosts. This cost of cleaning interactions has not been previously accounted for in cleaning theory. I also propose the *parasite hotspot hypothesis*; that parasite infection pressure may be higher around cleaning stations, thus presenting a conundrum for the infected client with respect to cleaning frequency and duration. The impact of a changing environment, particularly climate stressors on cleaners' performance and clients' cleaning demand are only beginning to be explored. It can be expected that cleaners, hosts/clients, and parasites will be impacted in different ways by anthropogenic changes which may disrupt the long-term stability of cleaning symbiosis.

Introduction

The concept of the relationship between environment, parasite and host has been extensively studied (e.g., Wolinska and King 2009; Tseng and Meyer 2014; Cai et al. 2018). In the presence of a virulent pathogen, a favourable environment, and a susceptible host, it is very likely that infectious disease will occur (Francl 2001; Scholthof 2007). This theory was initially investigated by Duggar (1909) who suggested that the rise of a disease is linked to environmental factors that can potentially affect hosts and pathogens independently, as well as the interrelations between them (Francl 2001). Later, the concept of a disease triangle was explicitly defined (McNew 1961; Stevens 1960). Currently, the disease triangle is a well-established and commonly used concept, with a series of modified versions that often include other elements such as 'time', 'vector' or 'humans' (Francl 2001; Scholthof 2007). The addition of other elements is case-specific and is used to illustrate how the original interactions among host, pathogens and environment will respond when facing changes. Cleaner organisms may be included as a fourth element in the disease triangle because they present an important and complex influence on the way disease interactions may occur. Cleaner organisms, most often fish or shrimp, typically establish a cleaning station, recognised by 'client' fishes as a location to directly seek service for the removal of external pathogens, epibionts and dead tissues (Feder 1966). There is an extensive body of work on the ecological, behavioural and physiological aspects of cleaning symbiosis presented under the premise that cleaning limits disease in fish clients and brings extended community benefits (see Côté 2000; Vaughan et al. 2017). However, cleaner fishes and shrimp exhibit close physical contact with client fishes during cleaning interactions (e.g., entering the gills and mouth; Grutter 2004), suggesting that cleaner organisms potentially risk acquiring pathogens from their clients. However, little is known about the cleaners' potential susceptibility to their clients' pathogens, or the potential for pathogen transmission from cleaners to their fish clients (e.g., Treasurer 1997; Treasurer 2012; Matejusova et al. 2016).

For this review, cleaning symbiosis was examined in the context of the disease triangle to develop hypotheses on the interrelationships between cleaners, parasites, fish hosts/clients and the environment (Fig. 3). Four elements were used in different contexts and are defined accordingly. For example, in the context of parasitism, the word "host" was used to characterise fishes infected with parasites which may

be a cleaner fish or a client fish. In the context of cleaning symbiosis, the cleaner fish seeks to remove external parasites from the client, representing a short-term relationship between fishes. In this review, most of the examples given are focused on host/parasite relationships. However, I acknowledge, through the text, that other organisms such as viruses or bacteria may also be relevant in the context of cleaning symbiosis. I define parasites as organisms that have a specific part of their life cycle dedicated to parasitism whereas pathogens are disease-causing agents, triggering negative changes in the host tissue, causing pathology. Pathogens encompass many organisms such as viruses, bacteria, and parasites.

While shrimp are important as cleaners in marine ecosystems, most of our examples focus on cleaner fish given that the majority of studies about cleaner organisms investigate cleaner fish (e.g., ecology, behaviour, physiology) and thus reflect the most evidence.

I reviewed research published on cleaning symbiosis in marine environments with a specific focus on four pathways, with the following aims:

1) *Cleaners and parasites*: to determine whether cleaner fishes are susceptible to parasite infections from their fish clients;

2) *Cleaners and clients*: to explore the potential role of cleaner fishes as parasite transmitters in the wild;

3) *Parasites and cleaners:* to evaluate mechanisms that parasites may use to evade cleaner organisms and maximise their chance of transmission;

4) *Cleaners and environment*: to examine the impact of global and local scale human impacts on cleaning symbiosis.

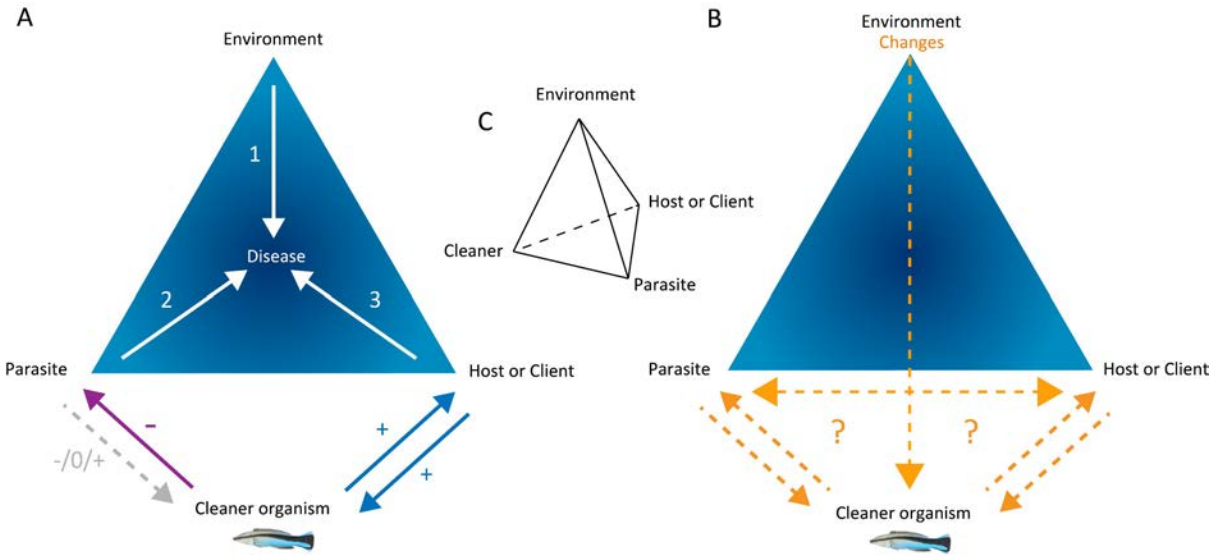

Figure 3 Cleaner organisms as the fourth element of the disease triangle and effects on environment, parasite, and host. (A) For disease to occur, a favourable environment (1), parasite virulence (2) and a susceptible host (3) are needed. With cleaners added, arrows represent potential interactions between the cleaner organism, the parasite and the host. Blue arrows represent positive interactions between hosts, which also act as clients, and cleaners through cleaning symbiosis. The purple arrow represents negative effects of cleaners on parasites through parasite removal and consumption. I propose these pathways could include parasites using cleaner fish as new hosts (- negative effect on the cleaner), and/or cleaners as transmitters to infect other fishes (0 no effect on the cleaner) or parasites being eaten by cleaner fish (+ positive effect on the cleaner) (grey dotted arrow). (B) When interactions between cleaners and the environment were considered, I identified knowledge gaps in the literature on the potential effect of these disturbances on cleaner-host or client and cleaner-parasite relationships. The resulting tetrahedron (C) encompasses the extra corner created when cleaner organisms are added as a fourth element to this system. Cleaner organisms are represented by the cleaner wrasse *Labroides dimidiatus*

Role of cleaner organisms in marine environments

Marine cleaner organisms remove external parasites and diseased or damaged tissue from clients (Feder 1966). These items compose an important food source for the cleaners (Feder 1966). Cleaners can be defined as dedicated or facultative, depending on their degree of dependence on cleaning interactions for accessing food (Vaughan et al. 2017). While dedicated cleaners rely almost exclusively on cleaning interactions as a source of food, facultative cleaners are more opportunistic cleaners. Clients of dedicated cleaners (see Bshary 2003; Côté and Soares 2011) and some facultative cleaners (see Arnal and Morand 2001; Narvaez et al. 2015) visit cleaner territories called ‘cleaning stations’ and often adopt specific poses by opening their fins and inclining their body to signal willingness to be cleaned (Randall

1958; Losey 1972; Poulin 1993; Côté et al. 1998). The main cleaner organisms in aquatic systems are teleost fishes and decapod shrimp, with a total of 208 fishes (with 16 dedicated and 192 facultative fishes) and 56 shrimp species so far reported as cleaners (Vaughan et al. 2017; Wirtz and Muller 2020). Among dedicated cleaner fishes, cleaner wrasses from the genus *Labroides* and cleaner gobies from the genus *Elacatinus* are the best studied (Vaughan et al. 2017; Côté and Soares 2011). While the genus *Labroides* includes five species, all of which are considered dedicated cleaners restricted to the Indo-Pacific region (Vaughan et al. 2017), the bluestreak cleaner wrasse, *Labroides dimidiatus* is the most common and geographically widespread. This species can consume around 1,200 ectoparasitic gnathiid isopods per day and may spend an average of 4.5 h inspecting an average 2,300 clients each day (Grutter 1996a). Facultative cleaner fishes include a wide assortment of families, including other members of Labridae (e.g., species in *Bodianus*, *Coris*, *Halichoeres* and *Thalassoma*), butterflyfishes (Chaetodontidae), damselfishes (Pomacentridae) and 23 other families, reviewed in Vaughan et al. (2017). Their impact on client health and the degree of effectiveness with which they engage in cleaning is variable and, in general, is much less well-known than their dedicated counterparts (Vaughan et al. 2017). Still, in parts of the globe where no dedicated cleaner fishes occur, these facultative cleaner fishes and shrimps comprise one of few sources of cleaning interaction for fishes (e.g., Moosleitner 1980; Sazima et al. 1999; Arnal and Morand 2001; Östlund-Nilsson 2005; Narvaez et al. 2015; Morais et al. 2017).

Cleaner shrimp have also been shown to effectively remove and eat parasites from fish clients in laboratory conditions and eat them in the wild (e.g., Bunkley-Williams and Williams 1998; Becker and Grutter 2004; Vaughan et al. 2018a, b). This includes breaking infection cycles by feeding on parasite eggs, cysts, and cocoons present in the environment (non-infective stage) in the laboratory (Vaughan et al. 2018a, b; Barton et al. 2020), a function so far only known to be performed by cleaner shrimps. The degree of reliance on cleaning interactions is largely unknown for most shrimp species, mainly due to their secretive and often nocturnal habits (Bonaldo et al. 2015; Bos and Fransen 2018; Vaughan et al. 2018a). Besides removing parasites, at least one species of tropical cleaner shrimp, *Lysmata amboinensis*, is also capable of attending to injured clients by feeding on diseased tissue in laboratory

conditions (Vaughan et al. 2018c). In doing so, *L. amboinensis* helps their clients' wounds to heal, with direct health benefits that transcend parasite removal or stress relief.

In the past decade, there has been a large body of work on the effects of cleaner fish on reef communities using large spatial scale and long-term experimental removals of cleaners. For example, in the absence of the cleaner wrasse *L. dimidiatus*, many fishes grow to smaller sizes and become less abundant, suggesting that *L. dimidiatus* affects growth rates, survivorship, recruitment success, and movement patterns of fishes (Bshary 2003; Grutter et al. 2003; Clague et al. 2011a; Waldie et al. 2011; Sun et al. 2015). The processes involved appear to include increases in ectoparasite abundances which occur over the short-term (1 to 12 days; Grutter 1999a, Grutter and Lester 2002) and long-term absence of cleaners (1.5 to 18 years, Clague et al. 2011a; Grutter 2012; Grutter et al. 2018, 2019; Sikkel et al. 2019). The negative effects fish incur may involve direct or indirect effects of cleaner presence (Grutter et al. 2018) with complex consequences to clients, such as changes in predator aggression, cognition, and various blood parameters in clients (Cheney et al. 2008; Soares et al. 2011; Binning et al. 2018; Demairé et al 2020).

Cleaning symbiosis by marine fishes: collective body of knowledge, limitations and developing fields

Overall, research interest on cleaner fishes has grown in the last three decades (Fig. 4A), with considerable disparity in the fields investigated. Between 1950 and 2020, studies on tropical species comprised 68 % of the entire literature on cleaner fishes, while temperate species studies comprised 32 % (Fig. 4B). Almost half of all temperate work (47 %) has been related to the use of temperate cleaner fishes as biocontrols in aquaculture (see Appendix A for methods and references). From the 1950s to 1980s, most research described observations of symbiotic behaviour on coral reefs, aiming to understand ecological implications (e.g., Eibl-Eibestfeldt 1955; Youngbluth 1968; Losey 1972; Losey 1979). In the 1960s, investigations were expanded to other environments, such as temperate ones (Von Wahlert and Von Wahlert 1961; McCutcheon and McCutcheon 1964; Gotshall 1967). Studies in aquaculture began in the 1980s and were the most common field of research in cleaning symbiosis in

the 1990s, mainly due to investigations on the use of facultative temperate cleaner fishes as potential biological controls of ectoparasites in aquaculture in Northern Europe (Costello 1993). This body of research also triggered interest in parasite communities and diseases of cleaner wrasses used to clean farmed fish from the 1990s (Fig. 4A). More recently, in the 2010s, research on the effects of climate change on cleaner organisms started to gain traction (Fig. 4A; Appendix A for methods and references). Ongoing global climate changes, such as global warming and ocean acidification, are known to affect physiological processes (e.g., Paula et al. 2019b). Since many behaviours involved in cleaning symbiosis are directly associated with physiological processes (Soares et al. 2012; Cardoso et al. 2015; Messias et al. 2016; Triki et al. 2017; Soares et al. 2019a; Triki et al. 2019), climate change is likely to interfere directly and indirectly with cleaning interactions. Globally, the proportion of studies investigating (i) effects of environmental changes on cleaning symbiosis and (ii) the diseases or parasites involved in cleaning symbiosis are negligible (Fig. 4). However, these fields of research will be critical to understanding the future of marine cleaning symbiosis in the next decade as they comprise intrinsic elements of the disease triangle. Below, I deduce expected trends from unlinked, yet comparable, systems for four major pathways involving cleaners and the disease triangle.

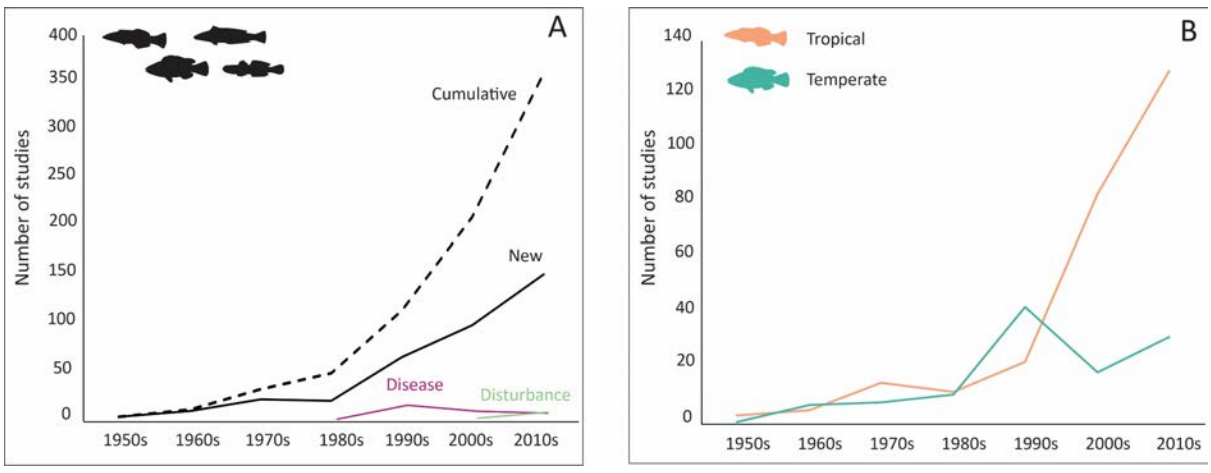

Figure 4 Compilation of research investigations (n = 359) on marine cleaner fishes from 1950 to 2020. (A) the overall cumulative number of studies (dashed line) and new studies on marine cleaner fishes each decade (solid black line). Studies investigating disease of cleaner fishes (purple line) and disturbance in cleaning interactions (green line) were added to emphasise the limited literature available regarding these two topics. (B) New studies separated by tropical vs temperate cleaner fish studies each decade

Pathway 1 - Cleaners and parasites: determining whether cleaner fishes are susceptible to parasite infections from their fish clients

During cleaning, cleaner organisms inspect the body and may enter the gills and the buccal cavity of some of their clients (Fig. 5). For example, the cleaner wrasse *L. dimidiatus* spends between 48 % and 78 % of the total time in cleaner interactions inside the gills of its client fish, *Hemigymnus melapterus* (Grutter et al. 2002a). This same cleaner species also uses its fins for tactile stimulus (i.e., massage) of clients, resulting in high rates of physical contact between cleaner and client (Soares et al. 2011). Direct contact such as skin to skin contact, bites and touching are characterised as common routes of pathogens transmission (e.g., Antonovics et al. 2017) and in the context of cleaning symbiosis, prolonged and direct physical contact could enable direct transfer of parasites between cleaners and clients. This hypothesis is indirectly supported by findings reported by Hobson (1971) who demonstrated that 70 % of the temperate facultative cleaner wrasse *Oxyjulis californica* individuals previously observed to clean other fishes, were infected with the same copepod parasite species found to infect their clients (i.e., *Lepeophtheirus* sp.; *Caligus hobsoni*; and *C. serratus*). In contrast, only 10 % of *O. californica* individuals that were not observed cleaning other fishes were infected with caligid parasites (*Lepeophtheirus* sp.; *C. hobsoni*; see Hobson 1971). It is plausible that cleaner wrasse became infected with the copepods when cleaning their clients, given that members of the Caligidae are mobile and can easily move around the surface of their host or swim short distances from one host to another (Ritchie 1997).

In the tropics, gnathiid isopods, the most frequent item (95 %) in the diet of cleaner wrasse *L. dimidiatus* (see Grutter 1997, 2000) is considered as one of the most common ectoparasites on tropical reefs. Gnathiid isopods live in the substrate, and temporarily attach to their host for a blood meal before releasing and returning to the substrate to moult to the next developmental stage (Grutter 1994; Grutter and Poulin 1998). While gnathiid isopods show a strong preference for infecting fishes of the family Labridae (Nagel and Grutter 2007), a previous study did not find them on wild *L. dimidiatus* (Smit et al. 2006). However, anecdotal reports by Grutter (2002a) indicated that *L. dimidiatus* could become infected with gnathiid isopods under laboratory conditions and they were also recently found to infect

wild *L. dimidiatus* off Lizard Island, GBR, Australia (Narvaez et al. 2021b; **Chapter 3**). Given the high susceptibility of other wrasses to gnathiid infection, it is not surprising that cleaner wrasse can also be infected with gnathiid isopods directly from the reef or also plausibly through horizontal transmission from infected client fishes. Indeed, gnathiids can easily drop off from their hosts if disturbed (Grutter 1995a) and cleaning interactions likely represent a threat for them that could trigger detachment.

To our knowledge, the endoparasitic bucephalid trematode (Platyhelminthes), *Rhipidocotyle labroidei* is the only parasite reported in *L. dimidiatus* and is likely acquired from clients through cleaning interactions (Jones et al. 2004; Gibson et al. 2005). The typical life cycle of bucephalid trematodes is complex. They are found as sporocysts in the internal organs of bivalves, the first intermediate host. Then, they develop as metacercariae in the organs of fishes as their second intermediate host (Muñoz et al. 2015). Finally, adult bucephalids are found in the digestive tract of their definitive hosts, piscivorous fishes (Jones et al. 2003). Piscivorous fishes become infected following the consumption of prey with encysted metacercariae (Jones et al. 2004). Yet, *L. dimidiatus* feeds mostly on ectoparasites of fishes (Grutter 2000). It is possible that *L. dimidiatus* becomes infected by removing encysted bucephalid metacercariae from the exterior skin surface of client fishes (Jones et al. 2004). In this scenario, the metacercariae subsequently develop, mature and produce eggs in the gastrointestinal tract (rectum) of *L. dimidiatus* (see Jones et al. 2003). This represents atypical transmission by a bucephalid trematode, and I hypothesise that this parasite species exploits cleaning behaviour as a mechanism to infect cleaner organisms. Future research into the life cycle of *R. labroidei* will enable identification of susceptible intermediate host fish and microhabitat specificity. Nevertheless, this example of infection of a dedicated cleaner fish by a bucephalid worm indicates a clear potential for cleaning interactions to select for novel parasite transmission pathways from clients to cleaners. Despite the intense research interest in cleaner organisms, there is a remarkable paucity of information on the parasite communities of wild cleaner organisms, their susceptibility to infection, and how this may influence parasite transmission dynamics in cleaner-client interactions. As suggested by Soares et al. (2019b), investigating the skin microbiome community of both cleaner fishes and clients is also crucial to understand if other organisms such as bacteria can be transmitted and/or shared between cleaner and client via cleaning symbiosis.

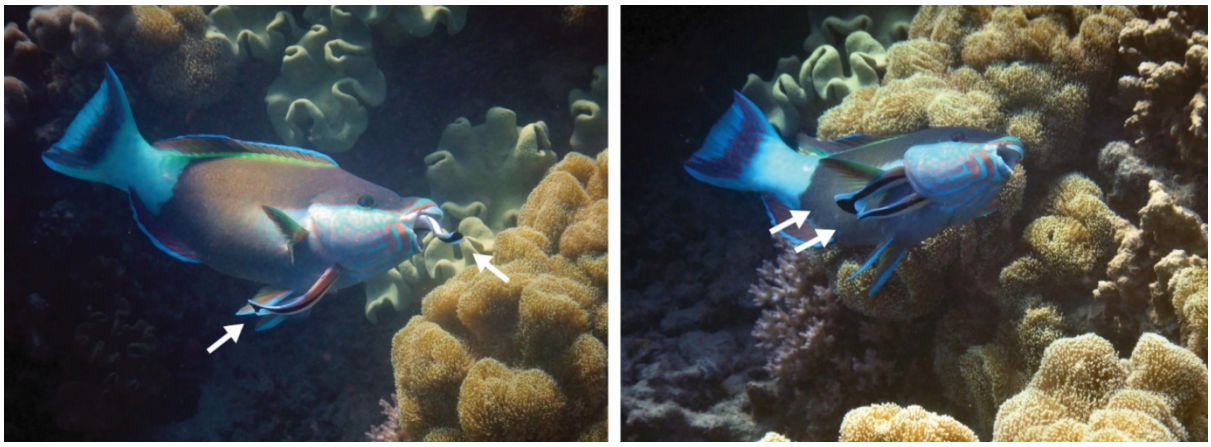

Figure 5 Cleaning symbiosis involves close physical contact which could enable the transmission of infectious taxa from the client to the cleaner and vice versa. Here, two adult bluestreak cleaner wrasse *Labroides dimidiatus* (represented by the white arrows) inspect and clean the mouth and the gills of the parrotfish *Scarus frenatus* at Lizard Island, in the northern Great Barrier Reef, Australia. This type of interaction may last for a few minutes

Pathway 2 - Cleaners and clients*: *exploring the potential role of cleaner fishes as parasite transmitters in the wild

In Northern Europe salmonid aquaculture, cleaner wrasses are commonly used as biological controls to remove copepod parasites in intensive fish production. However, many species of wrasse that act as cleaners naturally carry parasites and some are susceptible to salmonid pathogens. For example, five cleaner fish species tested in aquaculture, *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Crenilabrus melops*, *Labrus bergylta* and *Labrus mixtus* were found to be infected with between 22 to 35 parasite species each in the wild (Costello 1996b; Treasurer 1997). Moreover, some of these cleaners used as biocontrols in salmon farming are susceptible to salmon pathogens. For example, *C. rupestris* and *C. exoletus* are infected with the bacterium *Aeromonas salmonicida* and *S. melops* and *C. rupestris* are infected with *Vibrio* spp. (Laidler et al. 1999; Bergh and Samuelsen 2007). Moreover, parasitic infections such as *Paramoeba perurans* that cause amoebic gill disease, have been found to infect *L. bergylta* (see Karlsbakk et al. 2013). This situation demonstrates that in captive environments, cleaner fishes can act as heterospecific transmitters of harmful disease agents (i.e., transmitters being organisms not susceptible to disease but that can transport viable parasites; Evans et al. 2020). To the best of our

knowledge, the implications of cleaner organisms as diseases transmitters in the wild has not been previously explored.

While there is an extensive body of work suggesting that cleaner fishes have negative impact on parasites by selectively removing them (Grutter 1995b, 1996a, 1997), some parasites with low host-specificity may, in some situations, take advantage of cleaning symbiosis. If wild cleaner fishes are susceptible to generalist parasites (Fig. 6A) they may act as a temporary host or transmitter (Fig. 6B) of the wide range of infectious taxa to the large number of clients they encounter daily (Fig. 6 C, D).

Furthermore, there is emerging evidence from the study of skin microbiomes that pathogens may be shared between cleaners and their clients in the wild. Recently, Xavier et al. (2019) investigated the bacterial community from the skin of the Caribbean goby *Elacatinus prochilos*, which has two ecotypes: cleaners (coral-dwellers) and non-cleaners (sponge-dwellers). The authors found that the skin microbiome of the cleaner ecotype had higher alpha diversity (i.e., intra-sample diversity) compared to the non-cleaner ecotype. Of particular interest, significantly more Vibrionaceae bacteria, such as *Vibrio* and *Photobacterium* were found on the cleaner ecotype than on the non-cleaner ecotype (Xavier et al. 2019). Because these two bacterial genera are potential pathogens found in fish, the authors hypothesised that there is a chance of pathogen transmission from diseased clients to cleaners and vice-versa. In the absence of research on the parasite community of cleaner organisms, it is challenging to identify the diversity of pathogen species that may use cleaner organisms as transmitters in the wild and how frequently it may occur. Yet given that pathogens are known for complex evolutionary adaptations that maximise their chance of transmission (e.g., Reece et al. 2009; Binning et al. 2017), I hypothesise that some pathogens species likely exploit cleaning behaviour as a mechanism to infect a wider diversity of clients.

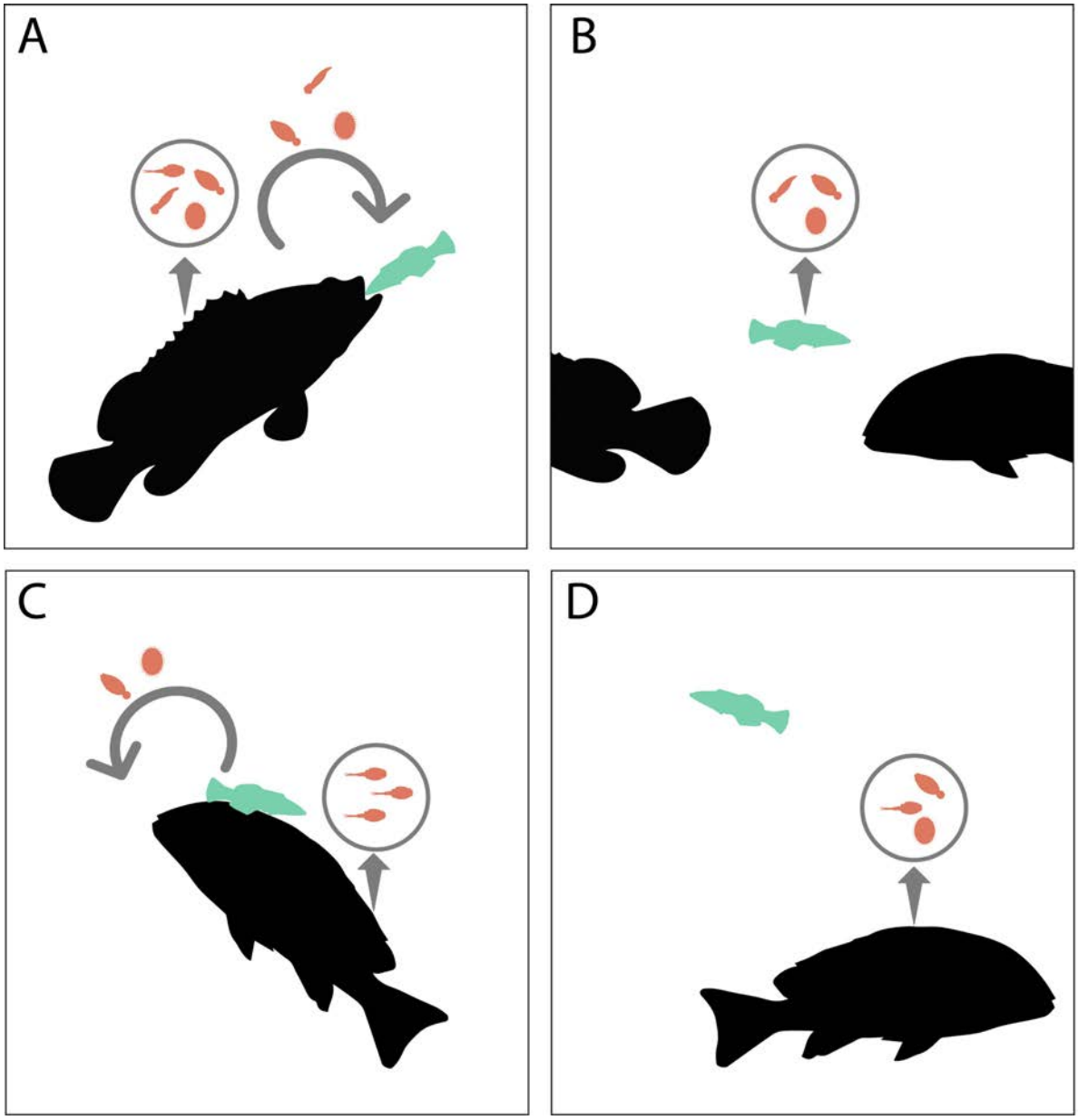

Figure 6 Cleaners as transmitters hypothesis. (A) Client fish (black) soliciting a cleaning interaction by posing and opening its mouth. The cleaner wrasse (green) becomes exposed to potential pathogen infection (orange) from the client through direct physical contact. (B) Cleaner fish may act as a transmitter, moving these pathogens to new clients. (C) When a new client visits the cleaning station, new physical contacts such as tactile stimulation (i.e., massage to the client using the pectoral and pelvic fins) may promote pathogen transmission from the cleaner to the client. (D) At the end of the cleaning interaction, the second client may leave the cleaning station with pathogens that were transmitted indirectly from the first client. In this scenario the cleaning interaction facilitates pathogen transmission from one client to another via the cleaner fish transmitter

Pathway 3 - Parasites and cleaners: evaluating mechanisms that parasites may use to evade cleaner organisms and maximise their chance of transmission

Some cleaner fishes and shrimp establish fixed cleaning stations within their territories, with high attendance by parasitised clients (Vaughan et al. 2017). These cleaning stations are analogous to a doctor's waiting room, where patients (= clients) converge and wait to be treated (Potts 1973; Bshary and Schäffer 2002; Shepherd et al. 2005; Fig. 7). Studies in human health have shown that people are more likely to get sick after visiting the doctor by being exposed to multiple routes of transmission. These routes include horizontal transmission via direct contact with healthcare workers and via indirect contact in the waiting rooms by airborne pathogens (Simmering et al. 2014; Laskowski et al. 2011; Hope et al. 2012). Nevertheless, waiting rooms are often overlooked as a potential source of disease and transmission (Botelho-Nevers et al. 2012). When considering the analogy between doctors' waiting rooms and cleaning stations, I propose that cleaning stations could act as disease 'hotspots', where clients could be exposed, as human patients, to a potential high infection pressure of infectious species while waiting at the cleaning station to interact with the cleaners. Besides the direct contact between cleaner fishes and clients, environmental transmission from water containing infection life stages and fomite transmission through contaminated objects (Antonovics et al. 2017) could affect clients waiting at the cleaning stations. To the best of our knowledge, no studies have investigated client waiting times at cleaning stations. Quantifying mean client waiting times is therefore critical to understand the potential cost-benefit for clients waiting to be cleaned.

Longer waiting times at cleaning stations may present clients with a higher risk of parasitic infection from other infected clients in close proximity. It has been shown that the abundance and the diversity of client fishes near cleaning stations is high, with an average (±SE) of 94 (±11) individual fish within 1 m radius of cleaning station of juvenile *L. dimidiatus* (n = 79 cleaning stations, D. Sun pers. comm.), with more than 100 species recorded within this same area (Sun et al. 2016). In a study comparing the abundance of fishes on patch reefs with and without cleaner fish, there were four times more fish on patch reefs with cleaner wrasses comparing to patch reefs without (Grutter et al. 2003). I propose that the diversity and density of parasites around cleaning stations is likely to be high, despite the fact that client fishes purportedly leave cleaning stations with fewer parasites than before being cleaned. In

addition to factors such as temporal and spatial scales (Buck et al. 2018), large aggregations of several organisms (both terrestrial and marine) can lead to increased parasite prevalence (e.g., Mikheev et al. 2015; Krkošek 2017; Van Schaik and Kerth 2017) suggesting transmission of parasites can be correlated with host aggregation. When considered in the context of cleaning stations, where there is a high frequency of parasitised client fish visiting the area resulting in a high concentration of fish aggregated around the station, I suggest cleaning stations could constitute hotspots of parasitic infection.

Cleaner organisms are predators of ectoparasites, and consequently, ectoparasites on clients that seek cleaning are exposed to the risk of predation. However, ectoparasites may deal with these risks in sophisticated ways (e.g., Whittington 1996). For example, ectoparasites that are not permanently attached to their host may drop-off or release eggs when disturbed by cleaner organisms. Many copepod parasites have egg strings, which readily detach when disturbed (Svensson 1996), and some monogenean also release eggs when disturbed (Whittington and Kearn 1988). Some crustaceans and monogenean species can swim short distances (Ritchie 1997; Höglund and Thulin 1988; Cable et al. 2002) or even drift in the water column and re-infect fish (Soleng et al. 1999) when disturbed. Gnathiid isopods have the capacity to detach quickly when their host is subjected to stress (Grutter 1995a). Therefore, cleaning stations could serve as disease hotspots if cleaning interactions trigger the release of parasites and/or eggs that then remain in the vicinity of the cleaning station (Fig. 7).

It is clear that parasitised fish spend significantly more time seeking cleaner fishes. In a laboratory study, parasitised *H. melapterus* with gnathiid isopods spent significantly more time around *L. dimidiatus* than unparasitised *H. melapterus* (see Grutter 2001). *In situ*, various client fishes sought *L. dimidiatus* more frequently when their species' ectoparasite load was high (Grutter 1995b). For example, wild individual rabbitfish *Siganus doliatus*, usually highly parasitised (i.e., 110 ectoparasites per individual on average), interacts with *L. dimidiatus* an average 114 times per day (Grutter 1995b). However, clients may interact differently, and spend more or less time in the interaction depending on the cleaner species/individual and *vice versa* (Soares et al. 2007; 2008b; 2013). Moreover, the availability of ectoparasites may also vary geographically and may also result in differing cleaning needs in client fish (Sikkel et al. 2004; Soares et al. 2013). An understanding of the role of the cleaning

stations as a potential source of parasitic infection is critical to clarify more subtle links in the complex interconnection between cleaners, clients (as hosts) and parasites in the wild.

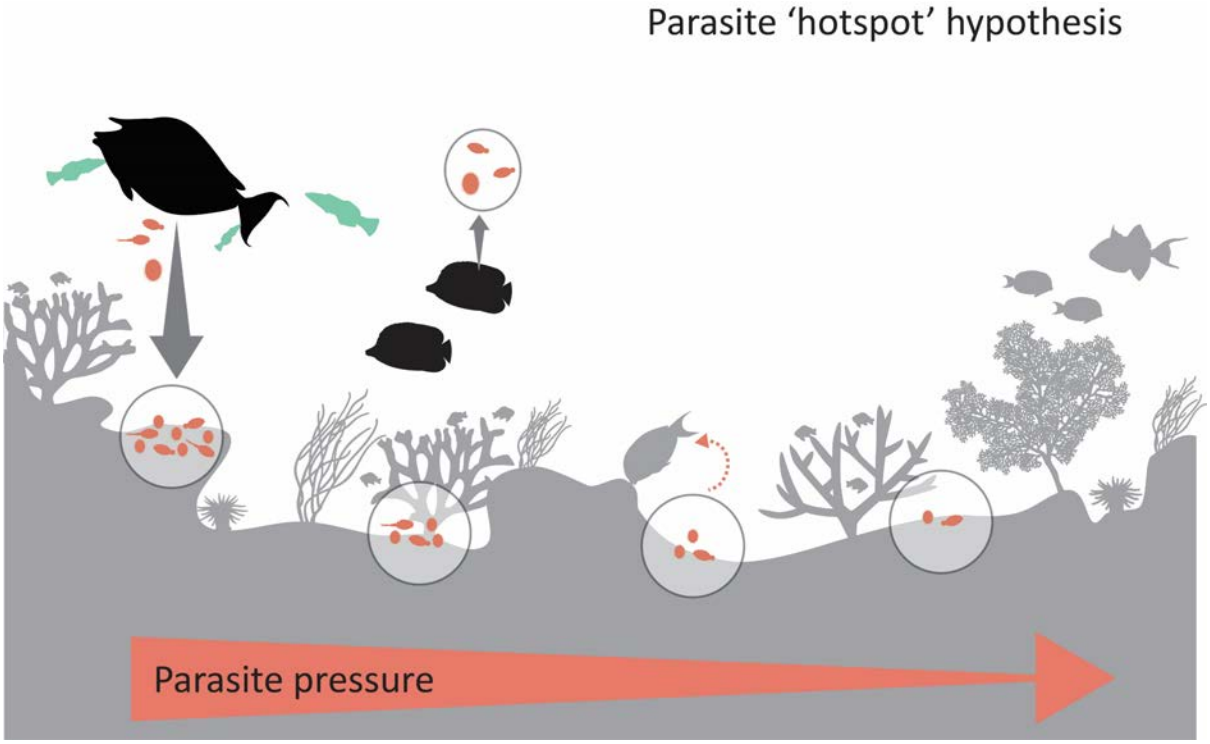

Figure 7 Parasite hotspot hypothesis: parasite infection pressure is higher around cleaning stations in marine environments. Here, a cleaner wrasse *Labroides dimidiatus* (green) cleaning a client fish (black) disturbs ectoparasites attached to the client. Ectoparasites detach and parasite eggs are released into the water column during cleaning interactions (grey arrow). Parasites in the water column find new hosts or eggs entangled on the substrate hatch and infect new hosts in the vicinity (orange dashed arrow). A gradient of parasite infection pressure is expected with the increasing distance from the cleaning station in the direction of prevailing currents (orange solid arrow)

Pathway 4 - Cleaners and environment*: *examining the impact of global and local scale human impacts on cleaning symbiosis

In this section, I examine how changes in the environment may influence other elements of the disease tetrahedron. I review the potential impacts of environmental changes on fish interactions (i.e., between cleaners and clients) and on ectoparasites.

(i) Impacts on cleaner organisms and client fishes

To the best of our knowledge, only seven studies have directly investigated the impact of human disturbances on cleaning symbiosis. These studies have been focused on 1) the effect of increasing temperature and ocean acidification on the behaviour and physiology of cleaner organisms (Rosa et al. 2014; Di Santo and Lobel 2016; Paula et al. 2019a, b); 2) the impacts of fishing on cleaning interactions (Silvano et al. 2012), 3) the impacts of severe disturbances, such as cyclones and bleaching, on cleaner fish abundance and behaviour (Triki et al. 2018) and 4) the impacts of boat noise on cleaning symbiosis (Nedelec et al. 2017). These studies suggest that human disturbances will likely have negative effects on cleaner organisms. For example, physiological responses of the cleaner shrimp *Lysmata amboinensis*, such as lactate and the activity of antioxidant enzymes, as well as heat shock responses are negatively affected by ocean warming (Rosa et al. 2014). Moreover, under laboratory conditions simulating future ocean warming and ocean acidification, the number of interactions between *L. dimidiatus* and clients declined and was physiologically translated by alterations of the dopaminergic (quality of the cleaning interaction) and serotoninergic (motivation of cleaners to interact) systems (Paula et al. 2019a). In the wild, after environmental perturbations on the Great Barrier Reef (i.e., two cyclones and one coral bleaching event) *L. dimidiatus* densities have been reported to decrease locally by up to 80 % (Triki et al. 2017). In experimental tests, this decrease in abundance was reflected by a decrease in sophisticated strategies employed by *L. dimidiatus* such as feeding against their preference to maintain a good reputation in the presence of a waiting client and prioritising certain clients over others (i.e., residents vs visitors; Triki et al. 2017). Finally, boat activity near cleaning stations has been shown to disrupt cleaning interactions (Nedelec et al. 2017). While the noise emitted by motorboats did not change the clientele composition or number, client fishes reacted negatively to cleaners more frequently and *L. dimidiatus* spent significantly more time inspecting their clients (Nedelec et al. 2017). These results suggest that acoustic disturbances can distract, confuse, and lead to a decrease in cooperation by the cleaners, affecting cleaning symbiosis (Nedelec et al. 2017). Our knowledge on parasite transmission during cleaning interactions between fishes is very limited as proposed in Pathways 1, 2 and 3. However, parasites presumably exploit cleaning symbiosis in many ways and I

propose that the transmission of potential parasites during cleaning interaction may also be affected by future global change scenarios.

In cleaning symbiosis, visual communication between cleaners and clients is one of the most crucial features resulting in mutual cooperation (Vaughan et al. 2017). This communication can be initiated by: 1) clients seeking cleaning attention by posing to signal their willingness to be cleaned, and 2) cleaners, which frequently have characteristic colouration (e.g. lateral yellow/blue body stripes, Stummer et al. 2004, Cheney et al. 2009) and sometimes display 'dancing behaviours' (Côté et al. 1998; Stummer et al. 2004) to attract clients. It seems self-evident that visual displays are a key feature of cleaning interactions, particularly true for ones involving fishes. For instance, the wide variety of colour patterns among coral reef fishes is a good indicator of the usefulness of vision to communicate and exchange information about species identity and ontogenetic phase (Rowland 1999; Marshall 2000). However, this is not exclusive to fishes, since the cleaner shrimp *Urocaridella* sp. have also been reported to display a "rocking dance" to attract clients and to advertise cleaning services (Becker et al. 2005). The cleaner shrimp *Periclimenes longicarpus* also use clapping behaviour as a signal to avoid predation (Chapuis and Bshary 2010). Since visual cues are an apparent important means of communication in cleaning interactions, one type of anthropogenic effect on marine ecosystem likely to affect cleaning interactions is the increased prevalence of suspended sediments on reefs (Wenger et al. 2012; Bainbridge et al. 2018). This is associated with increased discharge of sediments from land to the ocean (Brodie et al. 2012), increasing turbidity and generating a series of effects on a range of organisms on coral reefs. High levels of turbidity diminish the ability of some coral reef fishes to use visual cues to detect and migrate to their preferred habitat (Wenger and McCormick 2013), or to feed (Johansen and Jones 2013). These can alter prey-predator interactions (Chivers et al. 2013; Wenger et al. 2013), reduce growth rates and increase mortality in the juvenile life-phase (Wenger et al. 2012). To our knowledge, only one field-based study by Hobson (1971) commented on how cleaning interactions respond to increased turbidity, he briefly reported that cleaning activities declined considerably in turbid conditions compared to clear conditions in temperate waters off California.

On the other hand, it is important to emphasise that cleaner shrimp often engage in cleaning interactions during the night (Vaughan et al. 2018a). Cleaner shrimp species generally have vision characterised by low spatial resolution, not allowing them to distinguish colour patterns of client fishes or conspecifics (Caves et al. 2016). This poor eyesight suggests that cues other than visible cues might be more relevant to their ability to interact (Vaughan et al. 2017). Chemical cues generated by the parasite's odour could mediate cleaning interactions by cleaner shrimp. Indeed, reliance on chemical cues to recognise conspecifics, locate mates and find food and suitable habitats is widespread in crustaceans (Breithaupt and Thiel 2013). For example, it has been shown that social behaviours in crustaceans, such as mating and attraction, as well as foraging and defence, can be negatively affected by various pollutants associated with boat use (Olsén 2011). On the other hand, fishes use chemical cues to escape predators and alert conspecifics (Sorensen and Wisenden 2015). Indeed, experiments *in situ* showed that settlement stage damselfish, living among live coral, are not able to detect alarm odour cues when within up to 2 m distance from colonies of dead corals (McCormick et al. 2017b). This has been attributed to the presence of chemicals emitted by dead-coral colonisers, such as cyanobacteria, diatoms and red algae, which hinder fish perception and their ability to respond to odour alarm cues (McCormick et al. 2017b). These sensory impairments have been directly linked to decreased survivorship of common coral reef prey fishes (McCormick et al. 2017a), but, to our knowledge, interactions other than predator-prey have not been investigated for these chemicals after or during disturbance cues. Furthermore, although very little is known on how other groups of marine organisms (i.e., crustaceans) behave under these same chemically altered circumstances, their reliance on chemical cues suggests cleaner shrimp interactions could be particularly affected by chemical changes in the environment.

Given environmental changes in terms of both chemistry and visibility that follow coral mortality and increased sediment inputs (McCormick et al. 2017a; Brodie et al. 2012; Bainbridge et al. 2018), understanding the degree of reliance on visual, olfactory and other chemical cues by cleaner organisms (i.e., both fishes and shrimp) will be more important than ever. I anticipate that chemical disruption might affect mostly cleaning interactions led by shrimp, whereas suspended sediments might affect mostly those led by cleaner fishes. Nevertheless, experiments confirming the reliance (or absence

thereof) of cleaner fish and shrimp on chemical cues, and how these might be disturbed under changed odour scapes will be essential.

(ii) Impacts on ectoparasites

Ectoparasites that have a direct life cycle (i.e., that require only one fish host to complete their development), need to identify, attach to a suitable host, and find a mate (Mordue Luntz 2003; Sharma et al. 2019). Studies investigating which sensory cues are used by ectoparasites in fish farming industries, are gaining more attention (e.g., Devine et al. 2000; Ingvarsdóttir et al. 2002; Genna et al. 2005; Fields et al. 2007; Skilton et al. 2020). For example, the sea louse *Lepeophtheirus salmonis* on Atlantic salmon (*Salmo salar*) respond to light intensity due to a relatively highly developed visual system (Flamarique et al. 2000), can detect swimming hosts using water oscillation as mechanical stimuli (Heuch and Karlsen 1997), and can also react to fish odour when reattaching on new hosts (Devine et al. 2000). Other fish ectoparasites, such as *Argulus* spp., rely on visual cues to search for hosts early in their ontogeny (Mikheev et al. 2004). Gnathiid isopods, common fish ectoparasites on tropical coral reefs (Artim et al. 2017) and the main food of *L. dimidiatus* (Grutter 2000), also respond to visual cues to detect and attach to suitable hosts (Nagel et al. 2008; Sikkel et al. 2011). Despite a considerable number of studies investigating which sensory cues ectoparasites use to detect, settle and attach to a specific host, there have been few studies investigating if and how the use of sensory cues by ectoparasites might be altered by global and local scale human impacts.

To our knowledge, only two studies evaluated impacts of climate change on the survivorship or abundance patterns of tropical gnathiid ectoparasites. The first study reported that, in the wild, gnathiid isopods were lower in abundance during a marine heatwave that generated widespread coral bleaching in the Great Barrier Reef compared to cooler months (Sikkel et al. 2019). The authors suggested a mechanism whereby altered developmental rates would mediate an apparent low tolerance of gnathiids to temperature fluctuations (Sikkel et al. 2019). In a different study, gnathiids demonstrated a clear preference for dead coral rubble compared to live corals (Santos and Sikkel 2019), suggesting that physiological impacts from climate change could be offset, to some extent, by larger availability of desirable microhabitats. So far, the only study to experimentally test the effects of climate change on

gnathiids observed no impacts on their survivorship from exposition to elevated CO_2 concentrations (Paula et al. 2020). Altogether, the small available evidence indicates that impacts of climate change on marine ectoparasites could be complex and involve both positive and negative effects at different temporal and biological scales, i.e., organisms *vs* populations.

Other local impacts from human activities have also been shown to alter the ectoparasite community on coral reefs. Indeed, parasite communities have been increasingly used as biological indicators of water quality, which often reflects ecosystem health (e.g., Williams et al. 1992; Palm and Rückert 2009; Sures et al. 2017). As an example, the crustacean ectoparasite community of cardinal fishes has been shown to vary significantly according to local pollution sources and hydrodynamics in New Caledonia (Sasal et al. 2007). Overall, these ectoparasites were more abundant in locations with less detectable chemical and biological pollution (such as ammonium, nitrate, phosphate, turbidity) present in the water and higher rates of water renewal. This suggested a potential susceptibility of the ectoparasites to eutrophic and polluted conditions. However, other ectoparasites, such as trichodinid ciliates, have been shown to be positively affected by increasing organic pollution (i.e., eutrophication; Ogut and Palm 2005). This has also been found for monogenean parasites, which responded positively to eutrophication, crude oil and industrial effluents (Lafferty 1997). Thus, the differential responses of fish ectoparasites appear to be largely case-specific, with evidence for both vulnerability and facilitation relative to poor water quality.

Emerging diseases in aquaculture are another example of how human activities can impact the dynamics between fish parasite and their hosts. Several factors may affect how new diseases develop such as i) the high density of fish in a limited area generating stress and increasing the risk of infections, ii) the introduction of disease from exotic fishes, and iii) the movement of contaminated fomites (Murray and Peeler 2005). Nevertheless, investigating the effects of human impacts on ectoparasites communities (be it survivorship, infection and transmission rate or abundance) remains a challenging research field due to the varying nature of different impacts and the high morphological and functional diversity in parasites.

Conclusion and final remarks

There is a strong body of work showing the positive effects of cleaner organisms on client fishes, ranging from a reduction in parasite infection rates (Grutter 1999a; Grutter 2008; Clague et al. 2011a; Grutter et al. 2018) to affecting overall fish recruitment and abundance (Grutter et al. 2003; Clague et al. 2011a; Waldie et al. 2011; Sun et al. 2015). Despite almost seven decades of ecological and behavioural research on cleaning symbiosis, the impact of cleaner organisms on the interrelationships between clients, parasites, and environment, i.e., the disease tetrahedron (Fig. 3) are still poorly understood. Here, examples of when cleaner fishes may be susceptible to parasites while engaging in cleaning interactions were highlighted. I also hypothesised the role of cleaners as potential transmitters for disease spread in reef communities. Parasites have evolved numerous, often unique pathways to ensure the successful infection of hosts and to maximise their chance of transmission. In the cleaning symbiosis context, there exists compelling evidence that a species of digenean parasite has evolved to exploit cleaning interactions for transmission to the dedicated cleaner fish *L. dimidiatus* (see Jones et al. 2003, 2004). I hypothesise that numerous parasites could be spread through cleaning stations to infect a wide diversity of client fishes. In fact, mutualism interaction also involves costs (Bronstein 2001a) and if cleaners act as transmitters for parasite transmission, this represents a cost to the cleaning interaction that has not been accounted for in cleaning theory to date. Contrarily, if cleaners are not susceptible to generalist parasites, this suggests that cleaners could have evolved specific behavioural or physiological strategies to avoid parasite infection. In both cases, future research involving the disease tetrahedron will not only reveal insights on the evolution of this key mutualistic symbiosis, but also, be critical to predict their long-term stability in the wake of global changes.

Chapter 3: Are cleaner fish clean?

Published as: Pauline Narvaez, Russell Qi-Yung Yong, Alexandra Sara Grutter, Kate Suzanne Hutson (2021) Are cleaner fish clean? *Marine Biology* 168:59. https://doi.org/10.1007/s00227-021-03858-3

Abstract

Cleaner fish remove parasites from other organisms, called clients. While there is an extensive body of work on the positive role of cleaners for their clients and reef communities, remarkably, potential parasites hosted by specialised cleaner fishes themselves have not been explored. In this study, the parasite community of the Indo-Pacific cleaner wrasse *Labroides dimidiatus* was surveyed, and compared to other wrasses from the same region. *L. dimidiatus* was found to be infected by eight parasite groups including ectoparasites (copepods, isopods, trichodinids, monogeneans and turbellarians) and endoparasites (myxozoans, trematodes and cestodes) representing at least 12 species. The abundance and prevalence of most parasite groups was comparable to other wrasses, with the exception of bucephalid trematodes, which are not known to infect any other tropical wrasses except for Labroides species. This adds to mounting evidence that some parasite species exhibit atypical life cycles that exploit cleaning symbiosis. Particularly noteworthy was the discovery of gnathiid isopods on *L. dimidiatus*, which are generally considered the cleaner's primary food item. Our findings provide new evidence for a potential role of wild cleaner fish as vectors of parasites to new clients, which highlights potential costs associated with cleaning symbiosis.

Introduction

Mutualistic relationships are interspecific interactions that are beneficial for the species involved (Bronstein 2001a). This have been shown to be particularly true for marine cleaning symbiosis, where cleaner fish or shrimp positively affect other fish ('clients'), by removing their parasites (Grutter 1999a, Becker and Grutter 2004), dead tissues (Feder 1966) and cleaning their wounds (Vaughan et al. 2018c; Grutter et al. 2020a). In the Indo-Pacific region, the dedicated bluestreak cleaner wrasse *Labroides dimidiatus* feeds almost exclusively on food items from the skin and external orifices of client fish

(Vaughan et al. 2017). At Lizard Island, Great Barrier Reef, it consumes an average of 1,200 gnathiid isopod parasites per day, a number possible due to gnathiids' small size (1-2 mm), cleaners' high feeding rate (2,300 clients per day) and rapid gut clearance rate (3.7 h) (Grutter 1996a). Cleaning interactions have been shown to have extensive benefits for client fish and coral reef communities. Daily cleaning activities have positive impacts on fish health (Clague et al. 2011a), recruitment (Sun et al. 2015) and abundance (Waldie et al. 2011) as well as improving fish cognition abilities (Binning et al. 2018). However, mutualistic relationships are often associated with costs that are largely overlooked (Bronstein 2001a; Stanton and Palmer 2011).

In the marine cleaning symbiosis, the benefits appear to outweigh costs for cleaners and clients; cleaners benefit from a source of food and clients benefit from removal of unwanted agents. There may, however, be several costs to this relationship, including cheating behaviour (when cleaner fish remove mucus or healthy tissues from the clients instead of parasites; see Bshary and Grutter 2002), risk to the cleaner of predation (Francini-Filho et al. 2000; Messias and Soares 2015), and potentially, risk of infection from other parasites (e.g., the *parasite hotspot hypothesis* wherein parasite infection pressure may be higher around cleaning stations; **Chapter 2;** Narvaez et al. 2021a). Furthermore, while cleaners and parasites exhibit a predator-prey relationship, previous research shows that in various contexts, parasites have evolved to exploit their predator to complete their life cycle on new hosts (Lafferty 1999; Poulin 2010). It is plausible that some parasites may use cleaning symbiosis to facilitate infection of cleaner fish or as a dispersal mechanism to new host (client) fish (Grutter 2002; Jones et al. 2004**; Chapter 2**; Narvaez et al. 2021a).

One of the behavioural aspects that characterise cleaner organisms is their close and intimate physical contact with their clients, where they enter the gills and mouth in search for parasites (Grutter 2004). Furthermore, highly specialised cleaners such as *L. dimidiatus* provide tactile stimulation to some of their clients by rubbing their pelvic and pectoral fins on the body surface of clients, reducing clients' stress level (Soares et al. 2011). This frequent contact between cleaner and infected clients may increase the likelihood of parasite transmission between fish (Grutter 2002; Jones et al. 2004). There have also been a few records of intraspecific cleaning activities between *L. dimidiatus* individuals (Robertson 1974; Clague et al. 2011b; Dunkley et al. 2020). This indicates that *L. dimidiatus* likely have external

parasites, but the parasite community of this species has never been studied. So far, it remains unknown whether these intraspecific cleaning interactions are motivated by parasites or other drivers of cleaning interactions (e.g., wound healing).

Given the ubiquitous distribution of parasites in marine environments (Rohde 2005), previous parasite records on labrids (Muñoz and Cribb 2005, 2006; Muñoz et al. 2007), observations on intraspecific cleaning (Robertson 1974; Clague et al. 2011b; Dunkley et al. 2020) and the potential for endoparasitic transmission through cleaning behaviour (Jones et al. 2004), I hypothesise that *L. dimidiatus* is susceptible to parasite infection and that the parasite fauna of *L. dimidiatus* is distinct from that of other labrids. In this study, the parasite community of wild *L. dimidiatus* was investigated at two different locations in north-eastern Australia. The parasite community of *L. dimidiatus* was compared to that of 14 other wrasse species, compiled from studies in the same region.

Methods

Fish collection

Bluestreak cleaner wrasse, *L. dimidiatus*, were collected in two locations in north-eastern Australia. A total of 10 *L. dimidiatus* were collected from Welsby Light beacon in Rainbow Channel, Moreton Bay, southeast Queensland (26° 56'S, 153° 09'E) between March and May 2008. Fish were collected on SCUBA using barrier nets by a professional aquarium fish collector, packed individually and couriered to The University of Queensland St Lucia campus, where they were maintained individually or in pairs (individuals within a tank separated by opaque partitions), in a multi-tank marine aquarium system. Each fish was superficially examined for the presence of ectoparasites before being introduced to the aquarium system; no attempts were made to purge them of parasites using anthelminthic or other anti-parasite agents. Fish were fed with a mixture of frozen finely chopped squid and prawn, and dissected within a week of arriving.

A total of 30 *L. dimidiatus* were caught on SCUBA using barrier- and hand-nets from various sites off Lizard Island (14° 40'S, 154° 24'E) on the Great Barrier Reef, in November 2018. The collection sites at Lizard Island included lagoon sites (four sites and 11 fish captured) and non-lagoon sites (six sites

and 19 fish captured). Fish were transferred directly to individual hermetic plastic bags filled with seawater to minimise the risk of losing ectoparasites and returned to the Lizard Island Research Station.

Dissection and parasite examination

L. dimidiatus collected from Moreton Bay (n=10) were euthanised using cranial pithing and measured (standard length (SL), total length (TL) in cm). The entire external surface was visually inspected before the dissection commenced under a low-power dissection microscope for external parasites. The entire branchial basket and pharynx were removed, sub-sectioned and each section, along with the oropharyngeal cavity, examined for the presence of ectoparasites. For internal parasites, the entire alimentary tract was removed from the body, placed in vertebrate saline (10% seawater and 90% freshwater; one part seawater to four parts freshwater), split with a lengthwise incision and its internal surface and contents inspected. Gut wash techniques as per Cribb and Bray (2010) were used. The gut cavity and all internal organs were superficially inspected for presence of parasites. Smears and squashes were produced of body muscle, gall bladder/bile, brain, kidney, spleen, and liver tissue and inspected for presence of protozoan and myxozoan infection. Helminth parasites were fixed in near-boiling saline and preserved in 70% formalin. All other parasites were preserved in 80% ethanol. Dissected fish were not preserved.

At Lizard Island, fish were directly euthanised using an ice slurry with the fish still inside their individual bags to retain ectoparasites. Fish were measured (TL, cm), weighed (wet, g) and photographed, then immediately examined for their parasites. For external parasites, the external body surface, fins, gills and oral cavity were inspected under a low-power dissecting microscope, and a skin scrape was done. Gills were removed, separated, and examined for ectoparasites in a dish with seawater using a low-power dissecting microscope; any cysts of potentially parasitic origin were squashed under cover slip pressure and scrutinised using a compound microscope at ×20 and ×40 magnifications. Fish were then placed into a dish with vertebrate saline for 10 min to remove any potential external parasites susceptible to osmotic shift (Cribb and Bray 2010; Hutson et al. 2018; Skilton et al. 2020). Following external evaluation, the fish were dissected for internal examination. The entire alimentary tract was separated from the body, placed in another dish containing vertebrate saline. Smears were made from

eye, mouth, liver, gallbladder, spleen, kidney, stomach, intestine, gonads and body muscle tissue (1 cm^3) and examined under high-power compound microscope for the presence of protozoan and myxozoan infection. Helminth parasites were heat-fixed in near-boiling saline solution and preserved in 70% ethanol. Other parasites were directly preserved in 70% ethanol. All fluids in which the fish were immersed were filtered through a 63 μm filter and then inspected under the dissecting microscope. Dissected fish were preserved in 70% ethanol.

Given that gnathiids are the main source of food for *L. dimidiatus*, I sought to ensure that any recovered gnathiids were not an artefact or product of host faeces or regurgitation. Gnathiid isopods were only considered as parasitic on *L. dimidiatus* when they were found attached to the gills, attached to the skin, or loose but completely intact and undamaged. As such, parasitic gnathiids were readily distinguishable from masticated or partly digested gnathiids from the gut contents.

Identification of helminth parasites was facilitated by employing standard morphological staining and mounting techniques. Whole mounts were created using the technique outlined in Yong et al. (2018). Specimens were characterised under an Olympus BX-53 high-power compound microscope with mounted camera attachment and cellSens™ v 1.13 (Olympus) imaging software.

For copepod identification, temporary mounts were made by clearing individuals in lactic acid. Identification of protozoan and myxozoan parasites was based off photomicrographs taken at the time of dissection. Myxozoan and protozoan parasites were recorded as present or absent.

Comparison of parasite communities between Labroides dimidiatus and other species of wrasses

(i) Data gathering

To compare the parasite community of *L. dimidiatus* with other wrasses from the Great Barrier Reef, data from Muñoz and Cribb (2005; 2006; data of parasite abundance) and Muñoz et al. (2007; data of parasite prevalence) was compiled. First, the parasite abundance and composition between *L. dimidiatus* and two common and extensively-sampled labrid species, *Hemigymnus melapterus* and *Coris batuensis* collected at Lizard Island (see section ii below) were compared. Then parasite prevalence in 14 species of wrasses, also collected at Lizard Island (Muñoz et al. 2007), with that of *L. dimidiatus* collected in this study (see section iii below) was compared.

(ii) Comparing the mean parasite infection for three species of wrasses

To compare the proportional infection of different parasite groups between *L. dimidiatus*, *H. melapterus* and *C. batuensis*, I used data available from Muñoz and Cribb (2005, 2006) and unpublished raw data provided by G. Muñoz. Muñoz and Cribb (2005) collected *H. melapterus* (n=14), and Muñoz and Cribb (2006) collected *C. batuensis* (n=32), to analyse the community of metazoan parasites of these species. The number of parasites for each species per higher taxonomical group was calculated by summing the number of individual parasites from each group and for each fish species (*H. melapterus, C. batuensis, L. dimidiatus*). The parasite groups considered were: Turbellaria, Isopoda, Copepoda, Monogenea, Trematoda, Nematoda, Cestoda and Acanthocephala. The mean number of parasites for each group, per individual fish, was obtained by dividing the number of parasites per group by the number of fish collected (i.e., 14 *H. melapterus*, 32 *C. batuensis* and 30 *L. dimidiatus*). Finally, to account for differences in body size between fish species, the number of parasites by body mass was standardised, obtaining the density of parasites per 10 g for each parasite group and each fish species. The average fish weight (± SE) was 260.1 (± 55.3) g for *H. melapterus*, 18.6 (± 2.7) g for *C. batuensis* (Muñoz pers. comm.) and 3.9 (± 0.2) g for *L. dimidiatus.*

(iii) Comparing the prevalence and parasite community of Labroides dimidiatus *with co-occurring wrasses*

The prevalence of parasites for *L. dimidiatus* with data compiled for 14 co-occurring species of wrasses from Lizard Island was compared, collected by Muñoz et al. (2007). To visualise differences in parasite prevalence between labrid species, I grouped parasite species (from the same labrid species) by higher taxonomic ranks, i.e., family and order. For the family rank ("prevalence grouped by family"), the highest observed prevalence of any parasite species within a specific family to represent the family was used. The same was performed for the order rank ("prevalence grouped by order"). Unidentified cysts and immature copepodids found on *L. dimidiatus* from our study were excluded from this comparison because they could not be assigned unequivocally to any family or order (represented on Fig. 10). Data from Moreton Bay were excluded from the comparative study given differences in collection location (approx. 1,800 km apart) and parasite collection method.

Statistical analysis

All data analyses were performed in R version 4.0.2 (R Core Team 2020). To evaluate the parasite community of *L. dimidiatus* according to fish weight (g) and fish location (lagoon vs non-lagoon), two distinct model-based multivariate analyses with the R package "mvabund" (Wang et al. 2012; Warton et al. 2012) were performed. For this analysis, data from the fish collected at Lizard Island was used and excluded data from Moreton Bay because of the small sample size (n=10). First, I separated only quantifiable parasites [Gnathiidae, Caligidae (*Lepeophtheirus* sp.), immature copepodids, Bucephalidae, cestode cyst, Turbellarian] in a negative binomial multivariate GLM using fish weight and fish location as predictor variables. Then, the abundance was converted to presence/absence for each parasite group to allow simultaneous evaluation of both quantifiable and non-quantifiable parasites (with the quantifiable parasites mentioned above and adding Trichodinidae (*Trichodina* sp.) Myxobolidae and Ceratomyxidae (myxozoan). For that, another model-based multivariate analysis was performed with a binomial distribution, using fish weight and fish location as predictor variables. For both model-based multivariate analyses, the estimated coefficients were reported and tests of the null hypothesis of no effect of predictors on parasite abundance. The prevalence of parasites among *L. dimidiatus* and the 14 other wrasses was compared using two taxonomic levels: parasite order and family. A matrix of prevalence per group of parasites (order and family) was obtained by fish species. These matrices were fourth root transformed to level out common and rare groups, and then, subjected to Correspondence Analysis (CA) using chi-squared dissimilarity. To visualise differences in parasite composition and prevalence, the first two axes of the two CAs in ordination plots were exhibited.

Results

Parasite community of Labroides dimidiatus

The parasite community of *L. dimidiatus* comprised representatives of eight taxonomic groups, involving five ectoparasitic and three endoparasitic groups. Seven taxonomic groups of parasites were recovered from fish at Lizard Island (n=30), and four groups from Moreton Bay (n=10; Table 1). Empty, unidentified cysts were observed in the gills and muscles, but none contained morphologically

distinguishable parasitic agents and thus could not be identified further. No parasites were retrieved from five of thirty fish collected at Lizard Island and two of ten collected at Moreton Bay.

The gills of *L. dimidiatus* at Lizard Island contained the highest diversity and number of parasites (Table 1, Fig. 8). Crustacean parasites were the most prevalent parasites of the skin of *L. dimidiatus*, occurring on 30% of individuals. These comprised isopods from the family Gnathiidae, copepods from the genus *Lepeophtheirus* (family Caligidae), as well as immature (unidentified) copepodid individuals. The majority of individuals (60%) had unidentified cysts in the gills, totalling 75 cysts (maximum of 13 per individual) while 20% were infected with copepodid-stage copepods. Also in the gills, 13.3% of *L. dimidiatus* were infected by *Trichodina* sp. (Ciliophora: Trichodinidae), 13.3% by isopods (Gnathiidae), and 3.3% by turbellarians possibly of the genus *Paravortex* (Rhabdocoela: Graffillidae). The gall bladders of *L. dimidiatus* were infected by myxosporeans, with two individuals infected by species of *Henneguya* and one by *Ceratomyxa* and a further individual infected with an unidentified myxozoan species.

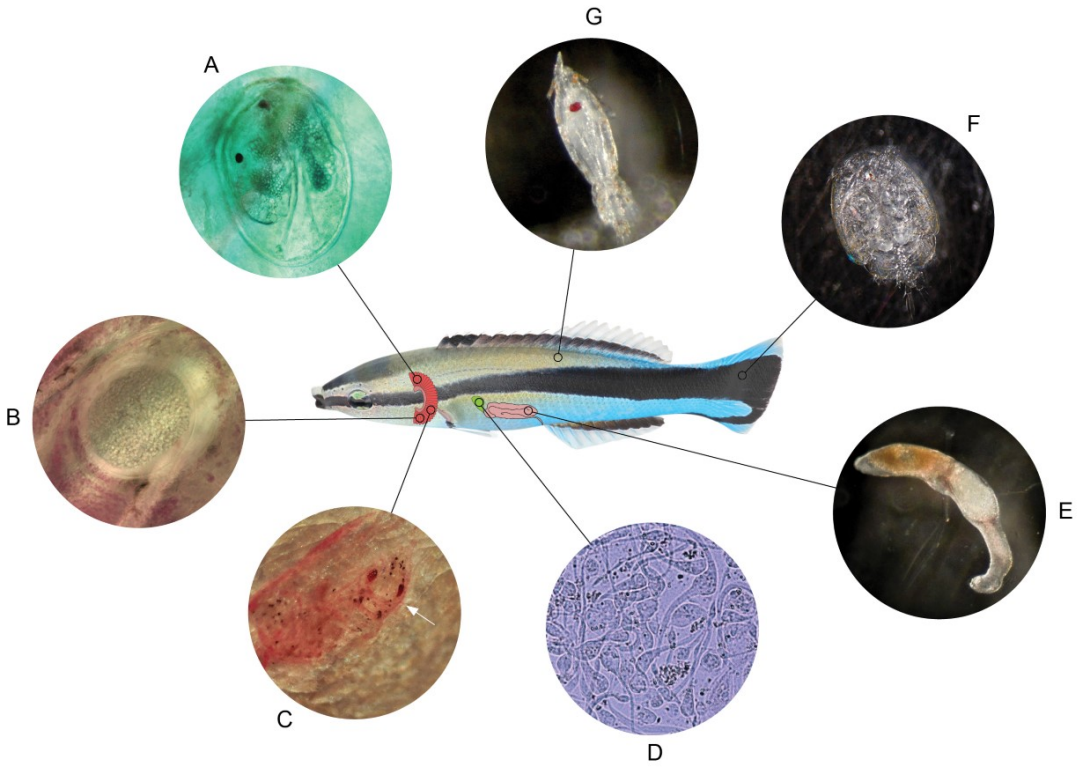

Figure 8 Representatives of the parasite community of *Labroides dimidiatus* from Lizard Island, Great Barrier Reef, Australia (n=30). In the gills (coloured red): turbellarian (A), cyst (B) and gnathiid isopod (only the head protruded) (C); in the gall bladder (coloured green): myxozoan (D); in the digestive tract (coloured pink): bucephalid (E); attached to the scale: caligid *Lepeophtheirus* sp. copepod (F); and on the skin mucus: immature copepodids (G). Monogeneans, *Trichodina* sp. and cestodes are not shown

Of all individuals of *L. dimidiatus* dissected, 33.3% were infected by bucephalid trematodes in the digestive tract. The most abundant species of bucephalid encountered conformed closely to the description of *Rhipidocotyle labroidei* although they were distinctly larger than those originally described by Jones et al. (2003). In the absence of molecular data for this species, I regard these specimens as conspecific. In addition, I also found two specimens (one immature) which closely conformed to the genus *Rhipidocotyle* (Table 1) but did not share morphological characters of *R. labroidei*. Finally, specimens were also obtained of a species of *Prosorhynchoides*. These specimens of *Rhipidocotyle* sp. and *Prosorhynchoides* sp. represent new infection records for *L. dimidiatus* and are likely new undescribed species.

Table 1 Parasite community of *Labroides dimidiatus* collected at Lizard Island (LI), Great Barrier Reef (n=30) and collected at Welsby light beacon, Moreton Bay (MB), Southeast Queensland (n=10). Number of parasites is summed across all individuals for each location. The number of fish infected is calculated per location. The prevalence represents the percentage of fish infected for each location. Finally, the abundance represents the mean (±SE) number of parasites per fish for each location

Parasite group	Species	Site	Location	Number of parasites	Number of fish infected	Prevalence (%)	Abundance (mean ±SE)
Ectoparasites							
Copepoda	cf. *Lepeophtheirus* sp.	Skin	LI	1	1	3.3	0.03 (±0.03)
	Immature copepodid	Skin, gills	LI	9	6	20	0.30 (±0.12)
	Immature copepodid	Caudal fin	MB	1	1	10	0.1 (±0.09)
Isopoda	*Gnathia* sp.	Skin, gills	LI	11	4	13.3	0.37 (±0.20)
Monogenea	Unknown species	Gills	MB	1	1	10	0.1 (±0.09)
Ciliophora	*Trichodina* sp.	Gills	LI	present	4	13.3	NA
Cestoda	Evacuated cyst	Mouth	LI	1	1	3.3	0.03 (±0.03)
Turbellaria	cf. *Paravortex* sp.	Gills	LI	1	1	3.3	0.03 (±0.03)
Other	Empty cysts	Gills	LI	75	18	60	2.5 (±0.7)
Other	Empty cysts	Fin	MB	1	1	10	0.1 (±0.09)
Endoparasites							
Trematoda	*Rhipidocotyle labroidei*	Intestine, rectum	LI	8	6	20	0.27 (±0.1)
	Rhipidocotyle labroidei	Stomach, large intestine, rectum	MB	11	6	60	1.1 (±0.36)
	Prosorhynchoides sp.	Intestine, rectum	LI	6	2	6.7	0.20 (±0.17)
	Rhipidocotyle sp.	Intestine, rectum	LI	1	1	3.3	0.03 (±0.03)
	Prosorhynchoides sp.	Large intestine	MB	1	1	10	0.1 (±0.09)
	Immature bucephalid	Intestine, rectum	LI	1	1	3.3	0.03 (±0.03)
Myxozoa	*Ceratomyxa* sp.	Gall bladder	LI	present	1	3.3	NA
	Henneguya sp.	Gall bladder	LI	present	2	6.7	NA
	Unidentified myxozoan	Gall bladder	LI	present	2	6.7	NA
Cestoda	Tetraphyllidea	Upper digestive tract	MB	34	2	20	3.4 (±3.12)
Other	Empty cysts	Muscle	LI	3	1	3.3	0.03 (±0.03)
Other	Empty cysts	Gall bladder	MB	2	1	10	0.2 (±0.19)
Other	Empty cysts	Mesentery	MB	2	2	20	0.2 (±0.13)
Total				**170**			

At Moreton Bay, 53 individual parasites were found infecting *L. dimidiatus* (n = 10; Table 1). There were similarities with the parasite community of *L. dimidiatus* from Lizard Island, with three group taxa shared between the sample locations. Similar to fish from Lizard Island, *Rhipidocotyle labroidei* was found with a high prevalence (60%) in the stomach, large intestine and rectum. These specimens were also larger than those described by Jones et al. (2003) but otherwise conformed well to the original description and are assumed to be conspecific. The specimens of *R. labroidei* found in Moreton Bay represent a new locality record and significant southward range extension for this species. Interestingly, only one parasite, a single specimen of an undetermined species of monogenean, was found in the gills of *L. dimidiatus*. Tetraphyllidean metacestodes were found in the upper digestive tract of 20% of the fish sampled, with a mean abundance of 3.4 individuals per fish. The number of ectoparasites on the skin of *L. dimidiatus* from Moreton Bay was low (10% prevalence represented by one individual immature-stage copepod).

Relationship between the parasite community of Labroides dimidiatus *and their weight and habitat at Lizard Island*

No evidence of an effect of habitat (lagoon *vs* non-lagoon) or fish weight on the parasite community of *L. dimidiatus* was found. Results were consistent when only quantifiable parasites were analysed (negative binomial mvabund, Table 2A), or when both quantifiable and non-quantifiable (e.g., myxozoans and protozoans) parasites combined were analysed via presence/absence (binomial mvabund, Table 2B).

Table 2 Summary of model-based analyses (mvabund) testing for potential multivariate relationships between parasite community of *Labroides dimidiatus* and weight and habitat at Lizard Island, Great Barrier Reef, Australia. Both (A) quantitative (negative binomial) and (B) presence/absence (binomial) mvabund outputs are presented

(A)	**Quantitative data**	
	Wald value	*Pr(>wald)*
(Intercept)	3	0.09
Location	2.17	0.15
Weight	2.48	0.36
(B)	**Presence/Absence data**	
	Wald value	*Pr(>wald)*
(Intercept)	3.24	0.11
Location	1.19	0.81
Weight	2.7	0.28

Despite the lack of clear patterns for the parasite community as a whole, bucephalids and gnathiids were the most abundant parasites found at Lizard Island (Fig. 9A), while metacestodes were the most abundant parasites found at Moreton Bay (Fig. 9A). Bucephalids were the most prevalent parasites in the two habitats at Lizard Island and also at Moreton Bay (Fig. 9A, B).

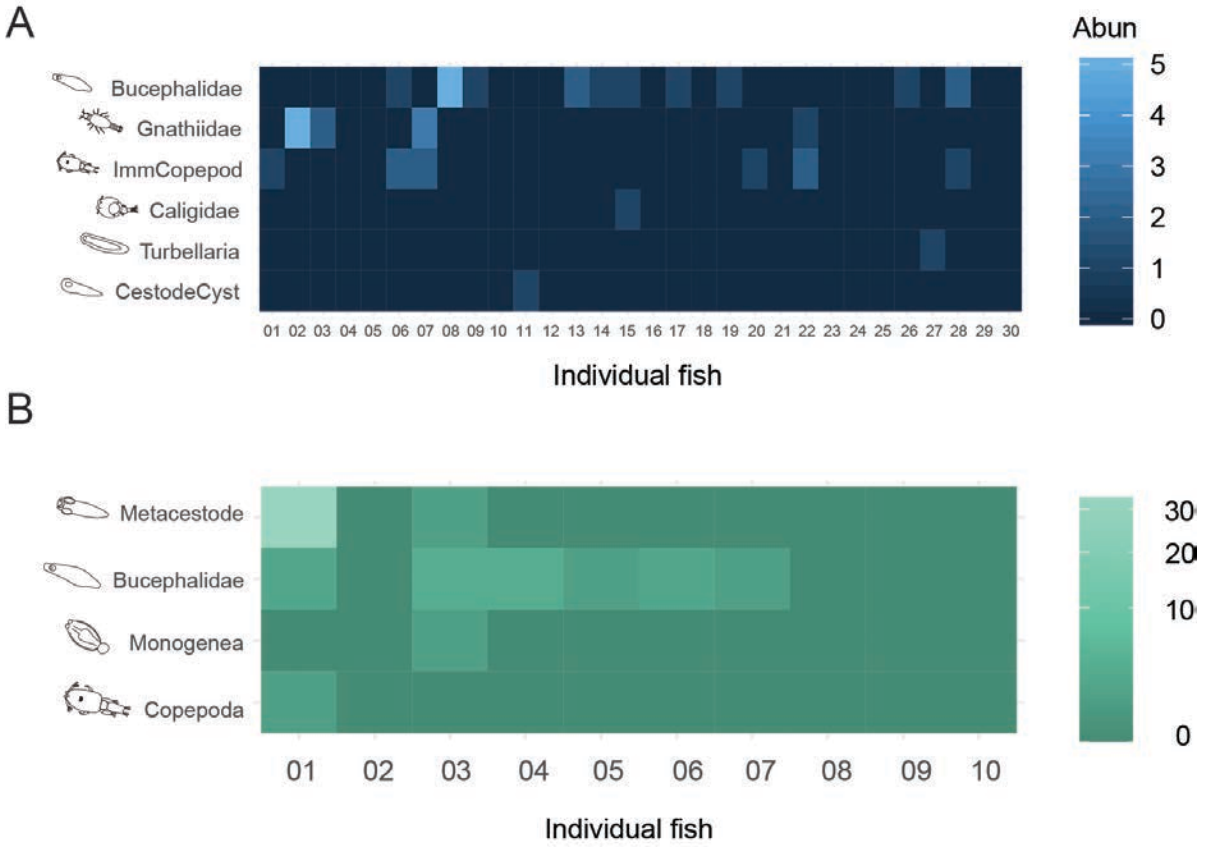

Figure 9 Parasite abundance of each individual *Labroides dimidiatus* from (A) Lizard Island (n =30) and (B) Moreton Bay (n=10)

Comparison of the parasite community of Labroides dimidiatus *with other wrasses*

All three species of wrasses analysed at Lizard Island, *H. melapterus*, *C. batuensis* and *L. dimidiatus*, were found to be infected by isopods, copepods, cestodes and trematodes (Table 3). However, *L. dimidiatus* were not found to be infected by nematodes; both *L. dimidiatus* and *C. batuensis* were not infected by acanthocephalans, and both *L. dimidiatus* and *H. melapterus* were not infected by monogeneans. Importantly, standardising by the number of parasites per individual and per gram of the body weight showed that the individual number of trematodes, isopods and turbellarians was higher for *L. dimidiatus* than for *H. melapterus* and *C. batuensis*.

Table 3 Eight groups of parasites from three species of wrasses from Lizard Island, Great Barrier Reef, Australia. Values per individual and adjusted for host weight are reported. Values in bold are for groups that have a higher mean per *Labroides dimidiatus* (per 10g) than *Hemigymnus melapterus* and *Coris batuensis*

	Hemigymnus melapterus (n=14)*		***Coris batuensis (n=32)*****		***Labroides dimidiatus (n=30)***	
	Mean per individual	**Mean per individual/10g**	**Mean per individual**	**Mean per individual/10g**	**Mean per individual**	**Mean per individual/10g**
Turbellaria	1.21	0.05	0	0	0.03	**0.09**
Isopoda	21.50	0.83	1.22	0.66	0.37	**0.94**
Copepoda	84.36	3.24	0.09	0.05	0.33	0.85
Monogenea	0	0	0.22	0.12	0	0
Trematoda	21.64	0.83	0.97	0.52	0.57	**1.45**
Cestoda	202.57	7.79	43.56	23.42	0.03	0.09
Nematoda	0.43	0.02	0.62	0.34	0	0
Acanthocephala	0.36	0.01	0	0	0	0

* Muñoz and Cribb 2005, ** Muñoz and Cribb 2006

The ordination of prevalence of distinct parasite orders from the 15 wrasse species (including *L. dimidiatus* from Lizard Island) from a Correspondence Analysis (CA) showed no particular segregation of the parasite community of *L. dimidiatus* from the other wrasses (Fig.10A). Indeed, *C. batuensis* was most dissimilar to other wrasse species, mainly due to the unique presence of monogeneans from the order Mazocraeidea. However, when categorising parasites according to family, *L. dimidiatus* was clearly separated from all other wrasses, particularly due to the presence of bucephalid trematode species, which were unique to *L. dimidiatus* (Fig. 10B).

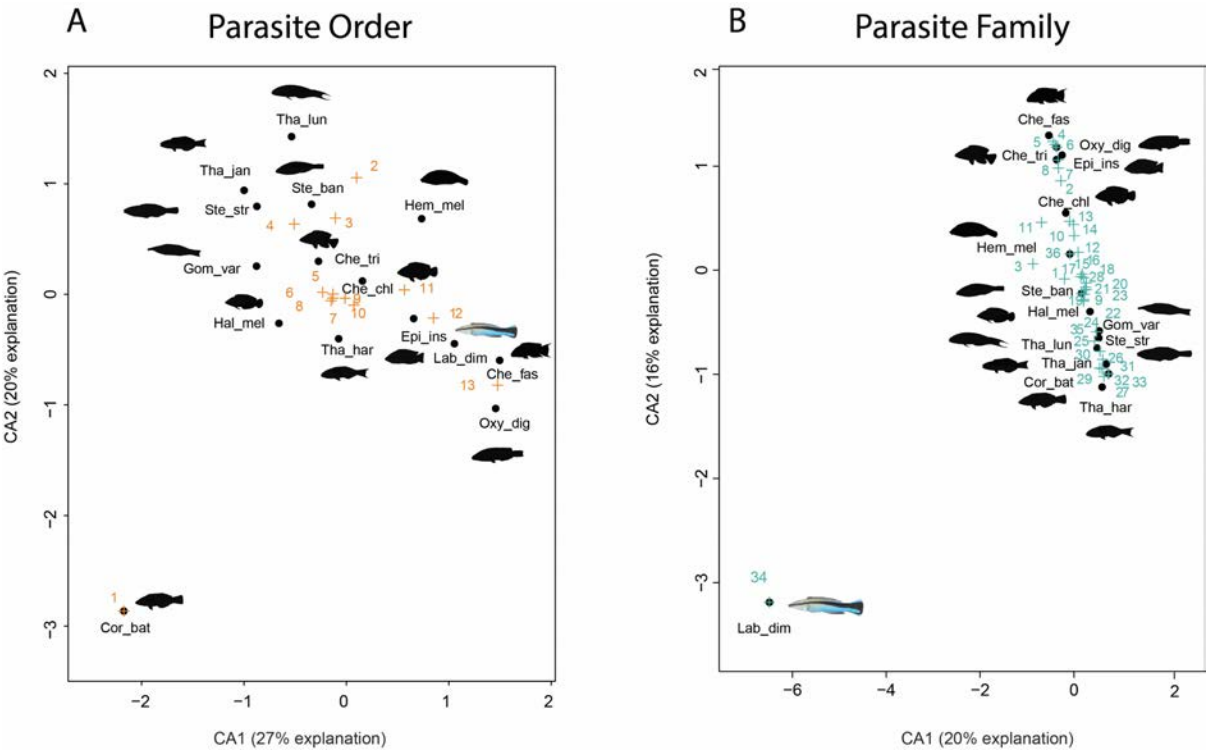

Figure 10 Parasite prevalence grouped by order (A) and family (B) (or higher taxonomic group when the order or family were not available, for example Acanthocephala or Turbellaria) for 15 species of wrasse represented with Component Analysis (CA1 and CA2). Fish species are represented in silhouette and *Labroides dimidiatus* in colour as follows: Che_fas: *Cheilinus fasciatus*, Che_tri: *Cheilinus trilobatus*, Che_chl: *Cheilinus chlorourus*, Epi_ins: *Epibulus insidiator*, Oxy_dia: *Oxycheilinus diagramma*, Gom_var: *Gomphosus varius*, Tha_lun: *Thalassoma lunare*, Tha_jan: *Thalassoma jansenii*, Tha_har: *Thalassoma hardwicke*, Ste_ban: *Stethojulis bandanensis*, Ste_str: *Stethojulis strigiventer*, Hal_mel: *Halichoeres melanurus*, Cor_bat: *Coris batuensis*, Hem_mel: *Hemigymnus melapterus*, Lab_dim: *Labroides dimidiatus*. Parasite values (contributing to the dissimilarity) are represented with "+". Parasite orders (or higher taxonomic rank available) are represented in orange colour (A) and associated with number: 1: Mazocraeidea; 2: Pseudophyllidea; 3: Acanthocephala; 4: Spirurida; 5: Trypanorhyncha; 6: Tetraphyllidea; 7: Plagiorchiida; 8: Rhabditida; 9: Isopoda; 10: Cyclopoida; 11: Siphonostomatoida; 12: Turbellaria; 13: Capsalidea. Parasite families (or higher taxonomic rank available) are represented in green colour (B) and associated with number: 1: Apocreadiidae; 2: Hatschekiidae; 3: Turbellaria; 4: Capsalidae; 5: Corallanidae; 6: Philometridae; 7: Fellodistomidae; 8: Lernanthropidae; 9: Acanthocephala; 10: Bomolochidae; 11: Caligidae; 12: Eutetrarhynchidae; 13: Raphidascarididae; 14: Cucullanidae; 15: Spirurida; 16: Tetraphyllidea; 17: Gnathiidae; 18: Opecoelidae; 19: Bivesiculidae; 20: Pterobothriidae; 21: Anisakidae; 22: Haplosplanchnidae; 23: Camallanidae; 24: Physalopteridae; 25: Zoogonidae; 26: Lacistorhynchidae; 27: Transversotrematidae; 28: Pseudophyllidea; 29: Monorchiidae; 30: Lecithasteridae; 31: Chondracanthidae; 32: Microcotylidae; 33: Paranybeliniidae; 34: Bucephalidae; 35: Trypanorhyncha; 36: Grillotiidae

Discussion

Our study shows that the cleaner wrasse, *L. dimidiatus*, harbours a diverse parasite fauna. Eight groups of endo- and ectoparasites were recorded from *L. dimidiatus* sampled at Lizard Island, Great Barrier Reef and at Moreton Bay, Queensland, Australia. *L. dimidiatus* was often infected by similar numbers of parasites when compared to other wrasses such as *H. melapterus* and *C. batuensis* (Muñoz and Cribb 2005, 2006). When comparing parasite prevalence with that of 14 other species of wrasses (Muñoz et al. 2007), I found that *L. dimidiatus* has a similar composition of parasite orders. However, at the family level, *L. dimidiatus* was distinctly separated from the other wrasses because of the presence of bucephalid trematodes, a unique feature in their endoparasite community among co-occurring labrids. Following these findings, aspects of parasite communities of *L. dimidiatus* were discussed from three different perspectives: a) parasites that are specific to *L. dimidiatus*, such as the bucephalid species that likely rely on cleaning symbiosis to reach *L. dimidiatus* as final host; b) parasites for which *L. dimidiatus* is likely an intermediate host, such as cestodes, implying predation on *L. dimidiatus* is required to complete their life cycle; and c) parasites that may possibly transmit from other host fish species (e.g., copepods) for which *L. dimidiatus* may play an important role as a vector while cleaning other fish species.

The bucephalid *R. labroidei* was described from the digestive tract of *L. dimidiatus* at Lizard Island (Jones et al. 2003). This species has also been found from New Caledonia and French Polynesia infecting *L. dimidiatus* and another species of dedicated cleaner wrasse, *L. bicolor* (Jones et al. 2004). Bucephalid trematodes typically exhibit a three-host life cycle, with first-stage intermediate infections in bivalve molluscs, a small fish used as second intermediate hosts, and adult parasites primarily infecting predatory, piscivorous fishes (Muñoz et al. 2015). Apart from species of *Labroides*, other labrid species known to exhibit cleaning behaviour, including *Symphodus tinca* and *S. mediterraneus* in the Mediterranean, are also known final hosts for bucephalids (Jones et al. 2004). Furthermore, bucephalid species have also been recorded infecting the fangblennies *Plagiotremus tapeinosoma* and *P. rhinorhynchos* (Blenniidae) (Dyer et al. 1988; Roberts-Thomson and Bott 2007). These, like cleaner fishes, feed by nipping the fins, scales and mucus of other fishes and, indeed, mimic *L. dimidiatus* and other cleaner wrasses to successfully approach client fishes (Russell et al. 1976). The presence of *R.*

labroidei and other bucephalid species in *L. dimidiatus* as well as other cleaner fish species and their mimics is indicative of host-switching by members of this trematode family, between larger piscivorous fishes and species that exploit the cleaning symbiosis dynamic. It has been hypothesised that metacercariae of cleaner-infecting bucephalids, which encyst on the external surface of client fishes may have been ingested by cleaner fish during interactions (Jones et al. 2004).

Tetraphyllidean metacestodes were found in the upper digestive tract of *L. dimidiatus* from Moreton Bay (prevalence 20%) and one metacestode cyst (presumed either long-dead or evacuated) was found in the mouth of one individual from Lizard Island. The prevalence of tetraphyllidean metacestodes in *L. dimidiatus* is relatively low compared to the other 14 wrasses analysed by Muñoz et al. (2007), which exhibited a mean (±SE) prevalence of 81.1 % (±7.2; maximum prevalence of 100% for half of the wrasses studied and a minimum prevalence of 21 % for *Epibulus insidiator*). The life cycle of tetraphyllidean cestodes is not fully understood, but cestodes typically have complex life cycles involving copepods and teleost fishes as intermediate hosts, and elasmobranchs as final hosts (Sakanari and Moser 1989; Caira and Reyda 2005; Jensen and Bullard 2010). Although cleaner organisms are usually in continuous contact with potential predators, records of cleaner fishes being predated in the wild are very rare (e.g., Lobel 1976; Francini-Filho et al. 2000; Messias and Soares 2015). The presence of metacestodes in *L. dimidiatus* raises questions about whether the cestode could manipulate cleaner behaviour in a way that encourages predation of the cleaner (e.g., Franceschi et al. 2007; Barber and Scharsack 2010; Gabagambi et al. 2019). Alternatively, the presence of these larval cestodes could represent dead-end infections, the unintended infection of an intermediate host that ultimately has little to no chance of being consumed by the target definitive host and therefore continuing the life cycle (Marcogliese 1995).

L. dimidiatus were also infected with crustacean ectoparasites on the skin and in the gills. Particularly, I found that 13.3% of *L. dimidiatus* presented infection with gnathiid isopods, which are considered one of the most common ectoparasites in tropical environments (Nagel and Grutter 2007; Sikkel et al. 2019). Importantly, these constitute the primary food source of *L. dimidiatus* (see Grutter 2002). However, the prevalence of gnathiid isopods in *L. dimidiatus* was lower than for the other 14 labrids analysed at Lizard Island (Muñoz et al. 2007). For these 14 species, gnathiid isopods had an average prevalence of

60.8 % (± 8.1 SE; min 11.1 %, max 100 %). Individuals of *L. dimidiatus* were also infected by copepods, with one individual infected by *Lepeophtheirus* sp. (Caligidae) and 20% with immature copepodids. Muñoz et al. (2007) also reported that half of their investigated species were also infected with caligid copepods, with a higher average prevalence than for *L. dimidiatus* (i.e., 22.1 % ± 8.8 SE). In general, *L. dimidiatus* appear to exhibit a lower prevalence of crustacean infection than other labrids. This observation may be related to, among other factors, intraspecific cleaning within pairs (e.g., Clague et al. 2011b), and warrants further investigation. Although the prevalence of crustacean parasites may be lower in *L. dimidiatus* compared to other wrasses, I found evidence that parasite abundance was not. This was clear when standardising parasite abundance per body size. In this context, the parasite community of *H. melapterus* (see Muñoz and Cribb 2005), *C. batuensis* (see Muñoz and Cribb 2006) and *L. dimidiatus* (this study) standardised to their weight appeared to be similar despite the major differences in body size. For example, the abundance of gnathiid in *L. dimidiatus* (group Isopoda, Table 3) was comparable to *H. melapterus* and *C. batuensis*.

The fact that *L. dimidiatus* is susceptible to several crustacean parasite taxa raises new questions on the role of cleaner fish in parasite transmission. Indeed, Hobson (1971) found that temperate-water facultative cleaner fish species that interacted with client fish shared the same species of caligid copepods, whereas cleaner fish that did not interact with clients had lower similarity in caligid infection (see also **Chapter 2**; Narvaez et al. 2021a). Caligid parasites are mobile and can easily move on the surface of their host and potentially translocate to other fishes (Ritchie 1997). It is plausible that *L. dimidiatus* may have become infected with copepods while cleaning other infected fish. Parasite species with low host-specificity may be best placed to take advantage of cleaning symbioses as a means of transmission to the cleaner or transfer to new hosts (**Chapter 2**; Narvaez et al. 2021a).

Cysts in the gills of *L. dimidiatus* were very frequently observed. The overwhelming majority of these cysts did not contain any identifiable material nor any indication of their origin. Caution is therefore needed in implicating these cysts with parasitic infection, as they could constitute inflammation responses to any of a variety of invasive entities. In the gills, the presence of *Trichodina* sp. in four fish was also found. *Trichodina* sp. is a common protozoan parasite in both freshwater and seawater fish (Lom and Dyková 1992). In the wild, *Trichodina* sp. are usually found at low intensity and their

presence is not threatening to fish health; when present in high densities they may cause mortality by damaging their hosts' gills (Bunkley-Williams and Williams 1994). Gill pathogens are a very common form of infection (Kearn 2005), and the costs of these pathogens are diverse and high (e.g., Omrani et al. 2010; Slavík et al. 2017).

By comparing the parasite composition of *L. dimidiatus* with the 14 other wrasse species (using parasite prevalence and visualised with a Component Analysis; Fig. 10), differences at the family level were found with Bucephalidae in *L. dimidiatus* driving the separation from other wrasses (Fig. 10B). While Muñoz et al. (2007) suggested that differences in the parasite composition between the 14 labrid species sampled was mainly due to distinctive host attributes such as fish body weight, fish abundance and fish swimming abilities, it is more likely that, here, the differences in parasite assemblage between *L. dimidiatus* and the other wrasses are mainly due to *L. dimidiatus'* behavioural attributes. Indeed, as mentioned above, bucephalid parasites use *L. dimidiatus* as a final host probably via the ingestion of metacercariae when cleaning other fishes. This indicates that cleaning behaviour ecologically separates cleaners from all other wrasse species. This is further supported by the fact that internal parasites that would otherwise be infecting predatory fishes exclusively infect labrids that clean other fishes.

No correlation was found between fish weight or location and parasite composition (number of parasites or presence/absence per parasitic group). However, the parasite abundance (adjusted for the weight of fish) in *L. dimidiatus* was found to be comparable (and higher for three groups out of eight) to other wrasse species such as *H. melapterus* (see Muñoz and Cribb 2005) and *C. batuensis* (see Muñoz and Cribb 2006). This indicates that *L. dimidiatus*, as other wrasses, are not exempt from parasite infection. Individual *L. dimidiatus* interact with a large number of fish (around 2,300 clients each day; Grutter 1996a) and have very close and intimate contact with their clients (e.g., tactile stimulation, Grutter 2004). Our findings suggest that from the nature of direct transmission, six ectoparasitic groups found here may use cleaners as disease transmitter to facilitate transmission of viable parasites (i.e., they can still live and reproduce) to new hosts (i.e., clients; **Chapter 2**; Narvaez et al. 2021a). The role of cleaner fish as a disease transmitter remains poorly understood and further investigation will be necessary to evaluate the balance between the cost and benefit of being cleaned by infected cleaner fish.

It may also be that some parasite groups exploit cleaning symbiosis for the purposes of transmission. Bronstein (2001b) states that exploiter organisms may benefit from a mutualistic relationship with other species: thus, parasites may act as exploiters of the interaction between client and cleaner fish. This paradigm has been observed in aquaculture systems such as on salmonid farms in the North Sea, where temperate cleaner wrasses (e.g., *Symphodus*, *Ctenolabrus* and *Centrolabrus* spp., Labridae) have been used as biocontrols to reduce the number of copepod ectoparasites infecting on the skin of farmed fish (Deady et al. 1995; Tully et al. 1996). However, the introduction of cleaner fish has been associated with the potential transmission of other pathogens from cleaner fish to salmonids (see Erkinharju et al. 2020 for review). Considering the relatively high abundance and diversity of parasites that occur on and in wild *L. dimidiatus*, I hypothesise that, while some parasites (e.g., gnathiid isopods) are prey for cleaner fishes, others exploit and benefit from the interaction between cleaners and their clients. Simultaneously evaluating the parasite community of cleaners and clients, as well as increasing taxonomic resolution through further study of the parasite community of *L. dimidiatus*, would help resolve which species of parasites hosted by *L. dimidiatus* may be transmitted between different species of fish during cleaning interactions. Furthermore, it is worth noting that comparisons between our data and that of Muñoz and Cribb (2005, 2006) and Muñoz et al. (2007) were made with caution because sampling biases such as seasonal and annual variations, methods of collections, sampling sensitivity and parasite species identification could vary between studies.

The concept of mutualism presents many challenges regarding several aspects of cooperation and the costs associated with it (Bronstein 2001a). While it is well known that selfish behaviour such as cheating is commonly performed by *L. dimidiatus* (Bshary and Grutter 2002), other costs associated with parasitic infection require further study. *L. dimidiatus* is clearly susceptible to parasitic infections and exhibits a comparable parasite composition to other wrasses from the same region. However, considering the role that *L. dimidiatus* has as a dedicated cleaner fish and its plausible role as a disease vector, the consequences of parasitic infection for *L. dimidiatus* may be higher than for other wrasses and the overall fish assemblage from the Indo-Pacific reefs.

Chapter 4: Cleaner fish are potential super-spreaders

This chapter has been prepared for submission to *Journal of Experimental Biology*
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Abstract

Cleaning symbiosis is critical for maintaining healthy biological communities in tropical marine ecosystems. However, potential negative impacts of mutualism, such as the transmission of pathogens and parasites during marine cleaning interactions, have rarely been evaluated. Here, I investigated whether the dedicated cleaner wrasse, *Labroides dimidiatus* is susceptible to, and can transmit generalist ectoparasites potentially associated with client fishes. In laboratory experiments, *L. dimidiatus* were exposed to infective stages of three generalist ectoparasite species with contrasting life-histories. *Labroides dimidiatus* was susceptible to the gnathiid isopod species tested compared to the control host species. However, *L. dimidiatus* was significantly less susceptible to the ciliate protozoan and the monogenean flatworm species compared to the control host species. Potential for parasite transmission from a client fish to the cleaner fish was simulated using experimentally transplanted viable (i.e., egg-producing) adult monogenean flatworms on *L. dimidiatus.* Parasites attached to cleaners for an average of two days during which parasite egg production was observed. Over such a timespan, a wild cleaner has an average 4,600 cleaning interactions, providing numerous opportunities for parasites to exploit cleaners as vectors. Our study provides the first experimental evidence that *L. dimidiatus* exhibits resistance to infection by some parasites, yet has the potential to temporarily carry viable parasites. Translocated parasites also produced embryonated eggs that hatched into viable larvae, which could subsequently infect other fishes, either through cleaning symbiosis or other dispersal mechanisms.

Introduction

Cleaning symbiosis between fishes is one of the most emblematic mutualistic relationships in marine environments. Cleaner fish are widespread in both tropical and temperate reef systems (Quimbayo et al. 2021) where they remove and ingest parasites from the body surface of other fish, called 'clients' (Côté 2000). Cleaner fish can clean throughout their entire lifespan and use cleaning as their primary way of gaining food (dedicated cleaner), while some cleaners engage in cleaning symbioses most frequently as juveniles and feed on other food sources (facultative cleaner; Vaughan et al. 2017). Research on cleaner fish often highlights the benefits of cleaners to their clients and associated communities. For example, by removing parasites (Grutter 1999a; Arnal and Morand 2001; Narvaez et al. 2015), aiding the healing process of injuries and wounds (Foster 1985; Vaughan et al. 2018c; Grutter et al. 2020a), and decreasing the stress level of fish (Soares et al. 2011), cleaner fish are critical for maintaining healthy biological communities (e.g., Clague et al. 2011a; Waldie et al. 2011; Sun et al. 2015; Binning et al. 2018; Ros et al. 2020).

However, costs are also associated with cleaning symbiosis. Cheating behaviour by cleaner fishes for example, is a cost that has been explored as a potentially negative impact that cleaner fish may have on their clients (e.g., Poulin and Vickery 1995; Soares et al. 2014; Truskanov et al. 2020). Cleaner fish, such as the wrasse *Labroides dimidiatus,* prefers to eat client fish mucus than parasites, because of the higher nutritional value of mucus (Arnal and Morand 2001). Nevertheless, the loss of mucus is detrimental to fish (Grutter and Bshary 2004) and can result in the client terminating the cleaning interaction by jolting and swimming off or chasing the cleaner fish (Bshary and Grutter 2002). Cleaner fish can also be eaten by their clients, making engagement in the interaction a potentially risky business (e.g., Francini-Filho et al. 2000; Messias and Soares 2015). Another cost that has been investigated is the potential for parasite transmission between cleaners and salmon species during cleaning interactions on fish farms (where cleaner wrasses are used as a biocontrol to remove sea lice in Northern Europe fish farms; **Chapter 2**; Narvaez et al. 2021a). Following the introduction of cleaner wrasses in salmon farming in the early 1990s, researchers have found that cleaners can also have parasites and be susceptible to salmon pathogens themselves (Treasurer 1997; Treasurer 2012). Thus, cleaners could

potentially act as a reservoir and vector of diseases for salmon (see Erkinharju et al. 2020 for review). Disease transmission by cleaners in the wild is poorly studied, with only one recent study that highlighted the potential for a wild cleaner fish (cleaner goby *Elacatinus prochilos*) in the Caribbean to share potentially harmful skin microbiome (i.e., bacteria) with their clients and *vice versa* (Xavier et al. 2019). Understanding the role of cleaner fish in the transmission of pathogens such as parasites have never been evaluated and presents a critical next step to identify if parasite transmission is a potential cost associated with cleaning symbiosis.

The aim of this study was to investigate whether the dedicated cleaner wrasse, *L. dimidiatus* is susceptible to, and can transmit, generalist ectoparasites potentially associated with their clients. To do so, individual *L. dimidiatus* and control fish species were exposed to infective stages of three generalist ectoparasites species with contrasting life-histories: the gnathiid isopod *Gnathia aureamaculosa*, the ciliate protozoan *Cryptocaryon irritans*, and the monogenean flatworm *Neobenedenia girellae*. These three ectoparasites are known for their low host-specificity (Ferreira et al. 2009; Li et al. 2021; Whittington and Horton 1996, respectively), cosmopolitan distribution (Ferreira et al. 2009; Diggles and Adlard 1997; Brazenor et al. 2018, respectively) and pathological effects (Smit and Davies 2004; Colorni and Burgess 1997; Trujillo-González et al. 2014). Potential for transmission from a client fish to the cleaner fish was then simulated using experimentally transplanted viable (i.e., egg-producing) adult *N. girellae* on *L. dimidiatus.* Based on the diverse parasite community of *L. dimidiatus* that has been explored and recorded in the wild (**Chapter 3**; Narvaez et al. 2021b) and the generalist nature of the experimental parasite species, I hypothesised that *L. dimidiatus* would be susceptible to the three generalist parasites tested here. Furthermore, due to repeated and close contact between cleaner fish and clients, I hypothesised that the mobile *N. girellae* could survive for produce viable eggs when experimentally transmitted from clients to cleaners.

Methods

Fish acquisition and husbandry

The dedicated cleaner wrasse, *L. dimidiatus*, was used as the study species given it is one of the most geographically widespread, conspicuous, and active marine cleaners reported to date (Côté and Cheney 2004; Cheney and Côté 2005; Froese and Pauly 2021). Susceptibility experiments were performed with the gnathiid isopod species *G. aureamaculosa* at Lizard Island Research Station (Great Barrier Reef, Australia). Twenty bluestreak cleaner wrasse, *L. dimidiatus* (73.6 ± 1.34 mm ± SD TL) and 20 Batu rainbow-wrasse *Coris batuensis* (control species 78.6 ± 1.61 mm ± SD TL) were collected using SCUBA at Lizard Island in January 2020 with barrier- and hand-nets. For this experiment, *C. batuensis* was used as a positive control because it has been previously found to be infected with gnathiid parasites at Lizard Island and is of similar size to *Lab. dimidiatus* (Muñoz and Cribb 2006; Muñoz et al. 2007). The fish were acclimatised for one week prior to experiments. Cleaner fish were held in 32 L tanks (dimensions: 39 x 28 x 30 cm; L x W x H) individually or in pairs. Individuals of *C. batuensis* were held in two 300 L round plastic tanks (dimensions: 1.0 m diameter x 0.4 m deep). These tanks were connected to a flow-through aquarium system, with water directly pumped from the adjacent reef via a holding tank. Varying sizes of Polyvinyl Chloride (PVC) plastic tubes were introduced in the tanks as shelters and fed the fish twice daily with frozen *Mysis* sp. shrimp. The seawater temperature was 30.4 ˚C ± 0.12 (± SD) during the experiment (i.e., the same water temperature as the adjacent reefs).

The susceptibility experiments were performed with the protozoan ciliate *C. irritans* and the flatworm monogenean *Neobenedenia girellae* at James Cook University (Townsville, Australia). Twenty adult bluestreak cleaner wrasses *Lab. dimidiatus* (65.55 ± 2.19 mm, TL) were purchased from a commercial ornamental fish supplier, Cairns Marine, in April 2019. Twenty barramundi, *Lates calcarifer* (169.35 ± 4.04 mm, TL) (Latidae) were also purchased from an aquaculture supplier, Spring Creek Barramundi farm, in February 2019. For these two experiments, *Lat. calcarifer* were used as a positive control, because this species is known to be susceptible to *N. girellae* and *C. irritans* under laboratory conditions (Skilton et al. 2020) and is routinely used as a host for *in vivo* parasite cultures in the Marine Parasitology Laboratory at James Cook University. On arrival, *Lab. dimidiatus* were quarantined for two weeks in separate tanks (dimensions: 22 x 14 x 13 cm; L x W x H; 4 L) and in two recirculating

systems and monitored for clinical signs of disease. Water parameters were monitored daily (salinity, pH, ammonia, nitrate, nitrite, and T ˚C). Each tank contained one PVC plastic tube as shelter. Fish were fed daily to satiation with *Mysis* sp. shrimp. Barramundi *Lat. calcarifer* were housed in a freshwater tank (dimensions: 63 x 37 x 45; L x W x H; 100 L) and the water was renewed once a day. They were fed with commercial pellets specifically formulated for *Lat. calcarifer* (Ridley Aquafeed - Marine float Range). Artificial lights were set on 12-hour day/night light regime. The water temperature was maintained at 26 ˚C and the salinity at 35 ppt (for cleaner wrasse system). The two recirculating systems used were built following Vaughan et al. (2018b), where the seawater was recirculated through an algae scrubber containing *Caulerpa taxifolia* for nitrate export. In the sump, bio balls were used for the growth of beneficial bacteria as well as a protein skimmer and a seawater UV treatment system.

Parasite cultures

The marine isopod gnathiid *G. aureamaculosa* were cultured continuously at Lizard Island Research Station from 2001 until 2020 (Nagel and Grutter 2007; Grutter et al. 2020b). Gnathiids are parasitic during their three larval stages before becoming adult where they aggregate on the benthos to reproduce (Tanaka 2007). In between each larval stage, gnathiids also return to the benthos to moult to the next stage (Tanaka 2007). Therefore, suitable benthic habitat such as turf and dead coral is necessary in the culture. A few fish are also needed for the gnathiids to feed on (see Grutter et al. 2020b).

The marine monogenean *N. girellae* was continuously cultured at James Cook University in the Marine Parasitology Laboratory between 2010 and 2019 (see Hutson et al. 2018) using barramundi, *Lates calcarifer*, as the principal host. In brief, freshwater *Lat. calcarifer* (maintained in 100 L aquarium) were gradually acclimatised to seawater over three days before being introduced to the seawater culture tank. Monogeneans *N. girellae* develop on the host until reaching sexual maturity (~ 7 days post-infection at 26 ˚C and 35 ppt salinity; Brazenor and Hutson 2015) and produce eggs for the next 17 consecutive days (Hoai and Hutson 2014). Eggs were incubated at the same temperature (26 ˚C) in a Petri dish containing fresh, filtered seawater (35 ppt salinity). Finally, free-swimming larvae (oncomiracidia), which hatch after 4 days of incubation (see Brazenor and Hutson 2015), were counted, and collected using a pipette and transferred to a beaker of fresh seawater for re-infection of naïve

acclimated *Lat. calcarifer*. Fish and parasites were maintained using artificial light with a 12 h day/night light regime.

The marine ciliate protozoan *C. irritans* has been cultured at James Cook University in the Marine Parasitology Laboratory for previous studies (Vaughan et al. 2018a; Skilton et al 2020). Similar to the methodology for *N. girellae* culture, freshwater *Lates calcarifer* were gradually acclimatised to seawater over three days and then introduced to the seawater culture tank (100 L tank). Twenty reproductive stages (tomonts) were counted and added into the culture tank (26 ˚C, 35 ppt). Fish presented clinical signs of infection (via the infective theront), as evidenced by white spots on the skin, after three days (trophonts). Trophonts leave the host and encyst into tomonts (reproductive stage; Diggles and Lester 1996) which were apparent on the bottom of the culture tank with the naked eye and collected from the substrate by scraping the bottom using a microscope slide and collecting them using a pipette. At the same time, fish were removed from the culture tank and acclimatised back to fresh water over three days to remove any remaining parasites. Trophonts were incubated at 26 ˚C in a Petri dish containing fresh seawater (35 ppt) for theronts' release. An artificial light was used and set on 12 h day/night light regime.

Experiment 1: Susceptibility of Labroides dimidiatus *to generalist parasites*

The susceptibility of *L. dimidiatus* to generalist parasites was determined in challenge trials following the World Organisation Animal Health (OIE) Aquatic Code recommendations for 'listing species as susceptible to infection with a specific pathogen' (available at http://www.oie.int/standard-setting/aquatic-code/access-online/). This includes criteria to: "1) determine whether the route of transmission is consistent with natural pathways for the infection", i.e., exposure to infective stages in the water column; "2) determine whether the pathogenic agent has been adequately identified", i.e., the cultures used have been identified using combined morphological and molecular approaches (*N. girellae*: Brazenor et al. 2018; *C. irritans*: Bastos Gomes pers. comm., *G.* aureamaculosa: Ferreira et al. 2009); and "3) determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection", which I defined as parasites surviving to adulthood for *N. girellae* and *C. irritans*, and *G. aureamaculosa* dropping off their hosts in an engorged state. Two different aquatic

anaesthetics were used for the following experiments according to the stage of anaesthesia needed. AQUI-S® was used for deeper anaesthesia, while 2-phenoxyethanol was used for light anaesthesia. 2-phenoxyethanol does not block the involuntary muscle reflexes and could have caused damage to the fish while using microscope during parasite transfer experiments (Ackerman et al. 2005).

(i) Gnathia aureamaculosa

Gnathiid isopods were cultured and used experimentally following Grutter et al. (2020b). After one week of acclimation, the cleaner fish *L. dimidiatus* collected at Lizard Island (n = 20) and the control host species *C. batuensis* (n = 20) were introduced to experimental tanks (36 x 21 x 20 cm; L x W x H) with fresh seawater. No water recirculation was used for the experiment and water was aerated using air stones. The fish were first acclimatised for 30 min to reduce the stress of handling. After the acclimation period, five unfed infectious juvenile third stage gnathiid isopod *G. aureamaculosa* were added to each tank (Fig. 11A). Only third stage infectious juvenile gnathiids were used because they are larger than the two first stages (~ 1.05 – 1.45 mm; Grutter 2003) and the most visible by naked eye (Nagel and Grutter 2007). The experiment started at 11:00 hrs and fish remained in the tank for two hours. This time is considered enough for a gnathiid parasite to attached and feed on labrid host (see Appendix B Fig B5 for an example of gnathiid attached to *L. dimidiatus*), before dropping off (Grutter 2003). During the two hours of experiments, the fish were visually checked every 15 min, looking for visible gnathiids on the skin of the fish. After two hours of exposure, the cleaner fish were removed from the tank and introduced them to dechlorinated freshwater bath for 5 min. Then, the fish was measured (total length, TL) and transferred the fish to a seawater bath for recovery. The water was filtered from all tanks (including experimental tank, fresh- and seawater recovery bath) with a 60 µm sieve. Engorged and unfed third stage gnathiid were recovered from the solutions, counted, and preserved in 70 % ethanol. To control for potential predation from the fish on gnathiids and the loss of gnathiids during fish transfers, control tanks (n = 20) with no fish were also exposed to *G. aureamaculosa.*

(ii) Cryptocaryon irritans

Twenty *Lab. dimidiatus* and 20 *Lat. calcarifer* were transferred to individual 30 L tanks (T°C: 26 °C and salinity: 35 ppt), exposed to ten *C. irritans* tomonts (reproductive stage) each (Fig. 11B). Numerous theronts (infective stage) hatch from tomonts and encyst in fish as trophonts (parasitic stage). The fish exposed to *C. irritans* tomonts were monitored for 10 days to enable the hatching of theronts as well as the development of trophonts on the fish which subsequently produce the second generation of tomonts. Forty-eight hours post introduction (to optimise theront infection), I performed a daily seawater exchange (70 to 80 % of the water). Water changes were done in a biosecure manner to prevent contamination of equipment, which was bleached daily using assigned equipment to different treatments. After the first appearance of trophonts infection on the fish (~ day 5; see Appendix B Fig. B6), I maintained the fish for a further five days with daily checks for remaining evidence of trophonts under the skin and tomonts (reproductive stage) on the bottom of the tank. After ten days, fish were removed from their tanks, anaesthetised with AQUI-S® (~ 85 µL/L) in seawater and transferred to a deep Petri dish (for *Lab. dimidiatus*) and a tray (*Lat. calcarifer*). I then assessed their skin under the dissection microscope (magnification x 20 and x 40; see Appendix 2 Fig. B6) for remaining evidence of *C. irritans* infection for up to two minutes before being released to recovery tanks. The bottom of each experimental tank was scraped using a new, synthetic sponge for each tank to suspend the tomonts in the water column which was then filtered using a 60 µm mesh sieve. Contents were collected in Petri dish and examined under the dissection microscope. The number of tomonts were counted and preserved them in 70 % ethanol. After the visual check, *Lab. dimidiatus* were placed into quarantine tanks (36 x 21 x 20 cm; L x W x H) (four per tank maximum, with similar size, or isolated if aggressive toward the others). Fish were moved to new, clean tanks every day over a period of one week to break the *C. irritans* life cycle in the unlikely event that 100 % of parasites were not recovered. I added the individual *Lat. calcarifer* to a half freshwater half seawater individual tanks before transferring them to freshwater again; *C. irritans*, being a marine organism, cannot persist in freshwater.

(iii) Neobenedenia girellae

In the interest of animal ethics and reduction, fish from the *Cryptocaryon* challenge were rested 30 days and then subsequently used for the *Neobenedenia* challenge experiments. Twenty *Lab. dimidiatus* and 20 *Lat. calcarifer* were transferred to individual 30 L tanks (dimensions: 38 x 28 x 29 cm; L x W x H) with PVC pipe for shelter and air stones for air supply. Experimental fish were exposed to 20 freshly hatched free-swimming oncomiracidia larvae (infectious stage) of *N. girellae,* previously collected from the laboratory culture using a pipette and transferred to a glass beaker of fresh seawater (Fig. 11C). Fish were maintained in their experimental tanks and monitored the water for up to 18 days for evidence of egg production (i.e., parasites typically infect fish within 48 h at ~ 25 ℃; Trujillo-González et al. 2015) and sexual maturity on approximately day 7 post-infection at 26 ℃ and salinity 35 ppt (Brazenor and Hutson 2015). To collect monogenean eggs, a piece of 3 cm^2 clean bridal tulle was immersed in each tank for the period of the experiment. At the end of the monitoring period, fish were individually given a dechlorinated fresh water bath for 5 min with an anaesthetic concentration of 2-phenoxyethanol at 0.10 to 0.15 ml/L for five minutes. This process anaesthetised the fish and removed the parasites present on the skin (Vaughan et al. 2018a). After the freshwater bath, adult *N. girellae* were removed from the water using a pipette or tweezers. Freshwater was filtered using a 60 μm mesh sieve and the contents examined in a Petri dish under a dissection microscope. The number of adult *N. girellae* was counted and preserved in individual vials per fish with 70 % ethanol.

Experiment 2: Egg production of adult Neobenedenia girellae *on* Labroides dimidiatus *post transfer*

I investigated whether adult *N. girellae* remain attached, survived, and produced viable eggs when attached to the skin of live *Lab. dimidiatus*. To do so, individual barramundi *Lates calcarifer* (n=15) were used as donors of adult parasites. Ten *Lab. dimidiatus* and ten *Lat. calcarifer* were used as receivers that were not previously infected by *N. girellae* (to avoid potential immunity following exposure during Experiment 1; Hutson et al. 2018). Survival and egg-laying ability of adult parasites were assessed concurrently in seawater as a control (n=10). Five adult *N. girellae* (between 16 and 20 days post-hatch) were transferred to each receiving fish following Hutson et al. (2018). In brief, I separately and simultaneously anaesthetised two fish (the donor and the receiver) using AQUI-S (85

µL/L). While anaesthetised, I placed *Lat. calcarifer* in trays and *Lab. dimidiatus* in a deep Petri dish filled with seawater. Two dissecting microscopes were used simultaneously, one for the donor and one for the receiver. Adult parasites were gently removed from the donor using a blunt-edged blade and a paintbrush placed underneath the attachment organ (the haptor) and transferred immediately to the body of receiver fish (flanks or tail; Fig. 11D). Successful transfer was considered when *N. girellae* was reattached to its new host as observed under the microscope and the parasite did not detach in the following few minutes post-transfer (see Appendix B Fig. B7 for *N. girellae* successfully attached to the new host). Following successful transfers (n = 5 per fish), I immediately placed the donors into a recovery tank and the receivers into individual experimental tanks (30 L tanks, dimensions: 38 x 28 x 29 cm; L x W x H) with PVC pipe for shelter and air stone for air supply. Receiver fish were monitored over the following seven consecutive days. To collect monogenean eggs, a piece of 3 cm^2 bridal tulle was immersed in each tank and replaced with a new piece one each day. The tulle previously immersed in the tank was observed under the dissecting microscope and the number of eggs produced was counted. Because *Lab. dimidiatus* produces nocturnal mucus envelopes (Lenke 1991), this mucus was removed daily and observed under the dissecting microscope for the presence of entangled eggs. Air stones were also examined for possible entangled eggs. A seawater exchange was performed daily (70 to 80 % of the water) using a siphon and a 60 µm filter. The remaining material filtered was examined under the dissecting microscope for detached and dead *N. girellae* as well as eggs (preserved in 70 % ethanol). After seven days, I removed and placed the fish into a seawater 2-phenoxyethanol bath (0.10 to 0.15 ml/L) and then a dechlorinated freshwater 2-phenoxyethanol bath (same concentration) to remove any remaining attached *N. girellae*. The water from each tank was filtered using a 60 µm filter and the tank was placed into a freshwater bath to determine whether any potential monogeneans that survived *in vitro* with no host (that can be detached from the host but still attached and surviving on the bottom of the tank: e.g., Tubbs et al. 2005; Ogawa et al. 2014; Reyes-Becerril et al. 2017). For the control experiment without a host, the same transfer procedure was done from a barramundi donor but the adult *N. girellae* were attached to the bottom of the tank. Similar daily procedures were applied to control tanks (i.e., changing tulle every day and siphoning). Each day, the control tanks were scrutinised

using a flashlight looking for dead *N. girellae* on the bottom (easily detected due to the fact they are opaque when dead), which were removed with tweezers and preserved in 70 % ethanol.

Experiment 3: Survival of adult Neobenedenia girellae *on* Labroides dimidiatus *post transfer*

In the third experiment I tested whether *Lab dimidiatus* remained infected with transposed adult *N. girellae* over 48 hours. The same procedure of transfer as per the previous experiment was performed. New *Lab. dimidiatus* individuals (n = 10) were used that were not subjected to the experiment above. After transferring adult *N. girellae* to each fish (n = 5), they were allocated to an experimental tank (dimensions: 22 x 14 x 13 cm; L x W x H) and checked for parasite presence by naked eye at 30 min, 1, 2, 4, 8, 24, and 48 h post-infection. The flatworm *N. girellae* is mostly transparent and hard to locate when attached to the host. However, after transfer, it was often possible to distinguish them on the body of the new host (see Appendix B Fig. B7 for *N. girellae* colouration). After each visual check, I gently moved the fish into their PVC pipe (with only one side open and the other side sealed) and transferred them, immersed in seawater, to another tank with new seawater. The water from the previous tank was filtered using a 60 µm filter and the contents analysed under a dissection microscope for detached and/or dead *N. girellae*. The previous tank was also immersed in freshwater bathed to remove any potential *in vitro* parasites. After 48 h, *Lab. dimidiatus* were removed and introduced to a seawater bath with 2-phenoxyethanol at 0.10 to 0.15 ml/L and then to a dechlorinated freshwater bath, with the same concentration of 2-phenoxyethanol to recover any remaining parasites attached to the host.

To check the egg viability of the parasites used in this experiment, a subsample of eggs produced by *N. girellae* from the susceptibility experiment with *Lat. calcarifer* and from the transfer experiment with *Lat. calcarifer* and *Lab. dimidiatus* were maintained in filtered seawater in Petri dishes until hatching. All eggs from the subsamples developed and hatched into live and mobile larvae (infective stage oncomiracidia). Infection success was not examined.

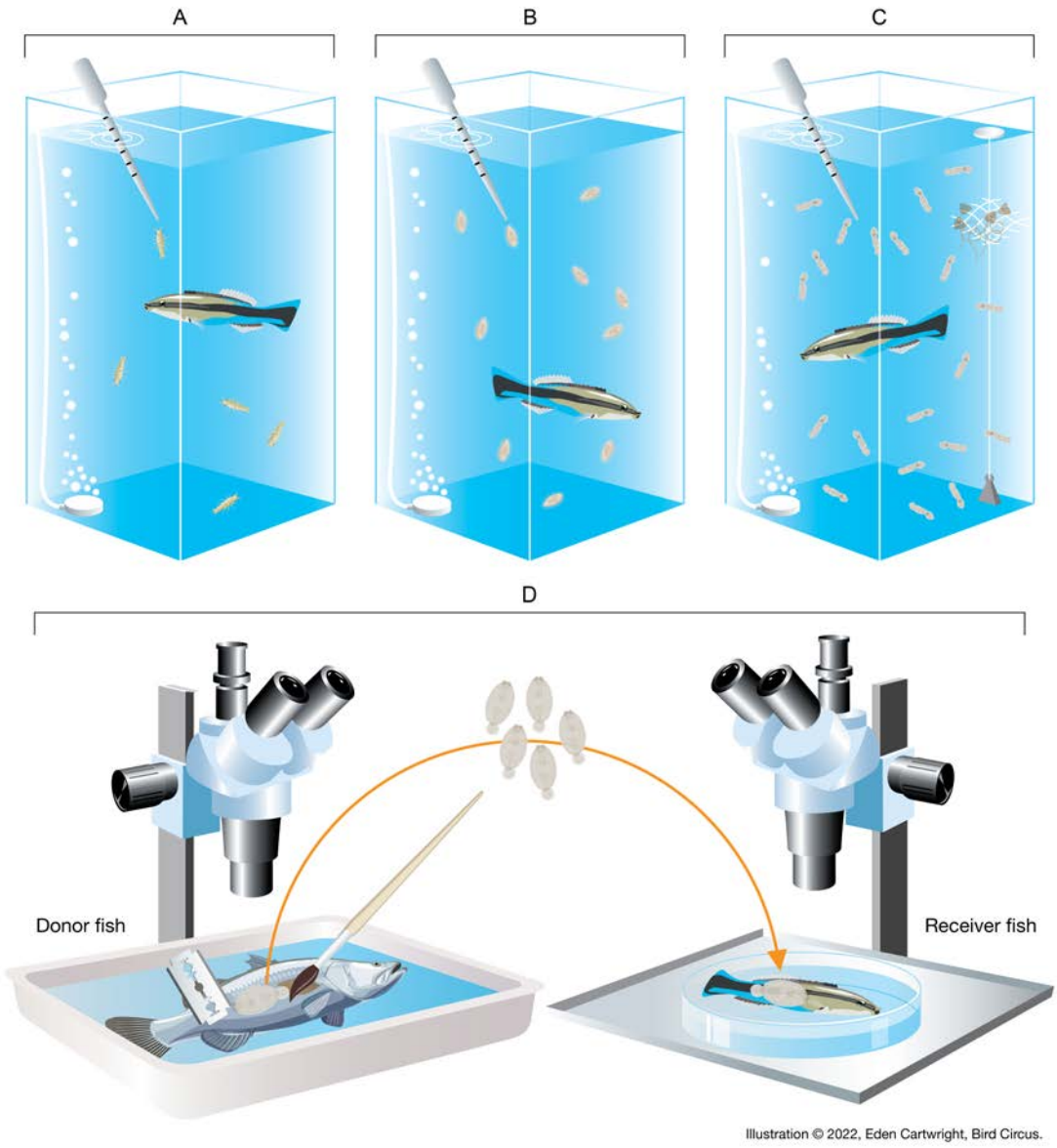

Figure 11 Schematic showing experimental treatments conducted in this study. First, susceptibility of the cleaner wrasse *Labroides dimidiatus* to (A) the gnathiid isopod *Gnathia aureamaculosa* third stage, (B) the ciliate protozoan *Cryptocaryon irritans* theronts and (C) the flatworm monogenean *Neobenedenia girellae* oncomiracidia were tested. Then, to test potential transmission of viable parasites from client to cleaner, adult *N. girellae* (D) were transferred from a susceptible fish to the cleaner and determine their survival on the cleaner

Quantification and statistical analysis

All data analyses were performed in R (R Core Team, 2020). A Bayesian analytical framework was used to structure Generalised Linear Models and Generalised Linear Mixed Models to test for 1)

differences in the recovery rates of gnathiids at the end of the susceptibility experiment (that could be due to potential predation on gnathiids by the fish); 2) differences in the susceptibility of *Lab. dimidiatus* and the control species to each parasite species; 3) differences in the egg production of *N. girellae* following the transfer experiment over 7 days between *Lab. dimidiatus*, the control fish, and the control with no fish; and 4) survival rates of adult *N. girellae* transferred from *Lat. calcarifer* to *Lab. dimidiatus*, to the control fish, and to the control with no fish over a 48 h time period. Markov Chain Monte Carlo (MCMC) chains were implemented using the No-U-Turn sampler algorithm in the Stan language with the rstanarm interface to R (Goodrich et al. 2018; Stan Development Team, 2018). For all models, 5,000 iterations, three chains, a 50 % warmup and a thinning of 1 every 3 iterations was used. Default priors were kept, which in most cases consisted in normally-distributed priors on both the intercept and coefficients (mean = 0, SD = 2.5), and an exponential (rate = 1) auxiliary prior. Each model was inspected using posterior predictive checking, trace plots of MCMC chains, effective sample sizes, Rhat values, and autocorrelation plots for each parameter. All indicators for all models were satisfactory.

For the models in 1, I tested for potential differences in the recovery rate of gnathiids between tanks with fish (both cleaner and control) and tanks without fish (control for the recovery). This model is meant to estimate potential predation rates from *L. dimidiatus* and *C. batuensis* on gnathiids. A Negative Binomial Bayesian generalised linear model was fitted, with the total number of gnathiids recovered from each tank as the response variable and the treatment (*L. dimidiatus*, *C. batuensis* or control) as the predictor variable. The recovery rate was lower for the fish treatments, and particularly for *C. batuensis*, compared to the no-fish treatment (*L. dimidiatus* = 2.6 [CI_{95}: 1.8-3.5]; *C. batuensis* = 1.39 [CI_{95}: 0.9-2] and no fish treatment= 4.4 [CI_{95}: 3.2-5.8] parasites recovered, mean and 95% credibility interval from the posterior distribution of parameters; Appendix B Fig. B1). Therefore, this indicated that comparisons of the susceptibility to gnathiids between species had to account for the differential detections of parasites. Consequently, two different tests were performed using the two different variables for gnathiid detection (visual count and fed recovered).

For the models in 2, interspecies comparisons required accounting for differences in body surface area exposed to experimental infections. Given logistical constraints that precluded surface area from being directly measured for the experimental fishes, I first devised a modelling procedure relating body length

with visible external surface area (i.e., not including gill area) from pictures of different individuals of each species. I first retrieved lateral pictures with associated length measurements for a minimum of eight individuals for each of the three fish species used. I used both my own photos and photos publicly available from FishBase (Froese and Pauly 2020). The visible external surface area was estimated from each photo by measuring the total lateral area of the fish using the software Fiji (Schindelin et al. 2012) and multiplying by two. Estimated visible external surface area as a function of body length was then modelled and the obtained relationship used to predict the visible external surface area for each of the fish individuals used across the susceptibility experiments. To test for the susceptibility to *Gnathia aureamaculosa*, two independent tests were done: 1) the number of gnathiids visually observed attached to each individual fish (*L. dimidiatus* or *C. batuensis*) during the experiment; and 2) the number of fed gnathiids recovered at the end of the experiment. For each of these two models, a Binomial Bayesian Generalised Linear mixed-effect model was fitted, with each parasite as an observation and the outcome (success or failure) as the response variable. For the first model, each outcome represented successfully detecting vs not detecting each individual gnathiid infecting the fish by visual means, with fish species as the predictor variable and fish individual as a random effect. For the second model, each outcome represented direct evidence of successful feeding vs unsuccessful feeding for each individual gnathiid. In this case, parasites either not recovered or recovered unfed were considered as representing an unsuccessful infection event. Similar to the previous model, fish species was used as the predictor variable and fish individual as a random effect, but I additionally incorporated the estimated visible external surface area of each fish individual (mm^2) as a log-link offset term. To test for the susceptibility to *C. irritans* and *N. girellae*, two Negative Binomial Bayesian generalised linear models were fitted. The number tomonts collected and the number of adults collected at the end of the experiment for *C. irritans* and *N. girellae*, respectively, were used as the response variable, and fish species as the predictor variable. In both cases the estimated surface area of each individual fish (mm^2) was used as a log-link offset term.

For the models in 3, I tested whether *N. girellae* egg production changed during the 7 day experiment involving experimental parasite transfer from a donor host to a receiver host. A Negative Binomial generalised linear mixed-effect model was fitted using the number of eggs produced per treatment (*Lab.*

dimidiatus, *Lat. calcarifer* or control) per experimental day as the response variable, and experimental day (from 1 to 7), including an interaction with species, and parasite age (as this is known to impact fecundity; Hoai and Hutson 2014, Brazenor et al. 2020) as the predictor variables. Parasite age had no effect on the model and was therefore removed (effect = -0.005; CI_{95} = -0.199 – 0.179). Experimental day was included as a categorical variable, rather than a numerical variable, to account for the possibility of non-linear responses in egg production. Finally, this model also included the fish individuals as a random effect.

For the models in 4, the survival rates of *N. girellae* were tested at different time points during the 48 h experiment involving experimental parasite transfer from the donor host to the receiver host. Two Binomial Bayesian generalised linear models were fitted. In the first model, the number of visible *N. girellae* still attached to *Lab. dimidiatus* (i.e., number of successes) relative to the number unattached (i.e., failures) during the experiment were considered the response variable and the different time points assessed (i.e., 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h) as the categorical predictor variable. For the second model, instead of the number of visible individuals of *N. girellae*, the number of dropped individuals (dead and *in vitro*) relative to the number of attached individuals was used as the number of successes/failures for the response variable, respectively, and time as the predictor variable.

Results

Susceptibility of the cleaner wrasse Labroides dimidiatus *to generalist ectoparasites*

The cleaner wrasse *Lab. dimidiatus* was more susceptible to *G. aureamaculosa* than *C. irritans* and *N. girellae*. During the exposure to *G. aureamaculosa*, the probability of visually detecting a gnathiid attached to the cleaner was 2.24 times higher (95 % Credibility Interval [CI_{95}]: 1.36 - 3.55; $Prob_{Ldim}$ = 0.46 [CI_{95}: 0.36 - 0.56]; $Prob_{Cbat}$ = 0.20 [CI_{95}: 0.12 - 0.29]) than for the control species, *C. batuensis* (Appendix B Fig. B1), and after two hours of exposure I recovered a median 2 (interquartile range [IQR] = 0.75 – 4) and 1 (IQR = 0 - 2) *G. aureamaculosa* individuals infecting the cleaners and the control, respectively (Fig. 12A). Differences in the likelihood of fish predation on *G. aureomaculosa* (Appendix Fig. B2) were accounted for by using the number of fed parasites recovered at the end of the experiment. The rate of recovery of fed gnathiids from tanks with cleaner fish was slightly higher than

from the control tanks, although their credibility intervals overlapped (Rec. $Rate_{Ldim}$ = 0.90 [CI_{95}: 0.77 - 0.999]; Rec. $Rate_{Cbat}$ = 0.73 [CI_{95}: 0.47 - 0.992]) (Fig. 12B).

All individuals of *Lab. dimidiatus* and all individuals of the control fish, *Lat. calcarifer*, were successfully infected by theronts (i.e., the infective stage) of the ciliate protozoan *C. irritans*. However, the median number of tomonts (i.e., the reproductive stage) produced by *C. irritans* (i.e., an indicator of life cycle progression) was considerably lower when infecting cleaners (median = 8; IQR = 5 – 19.2) compared to control fish (median = 438; IQR = 170 - 707; Fig. 12C). When accounting for differences in body surface area between the two host fish species, the cleaner was on average 73 % less susceptible to *C. irritans* infection than the control (CI_{95}: 54 - 86 %; Fig. 12D).

Finally, no *N. girellae* flatworm larvae successfully infected *Lab. dimidiatus*. This was in stark contrast with the control *Lat. calcarifer*, in which all individuals were infected with a median 5.5 (IQR = 5 – 11) parasites per fish, from an exposure of 20 (Fig. 12E). Accounting for differences in body surface area between species and conservatively assuming limitations on the capacity to detect zero infections, provided a predicted susceptibility to *N. girellae* infection for the cleaner fish that was on average 97 % lower than the control species (CI_{95}: 87 - 100 %; Fig. 12F).

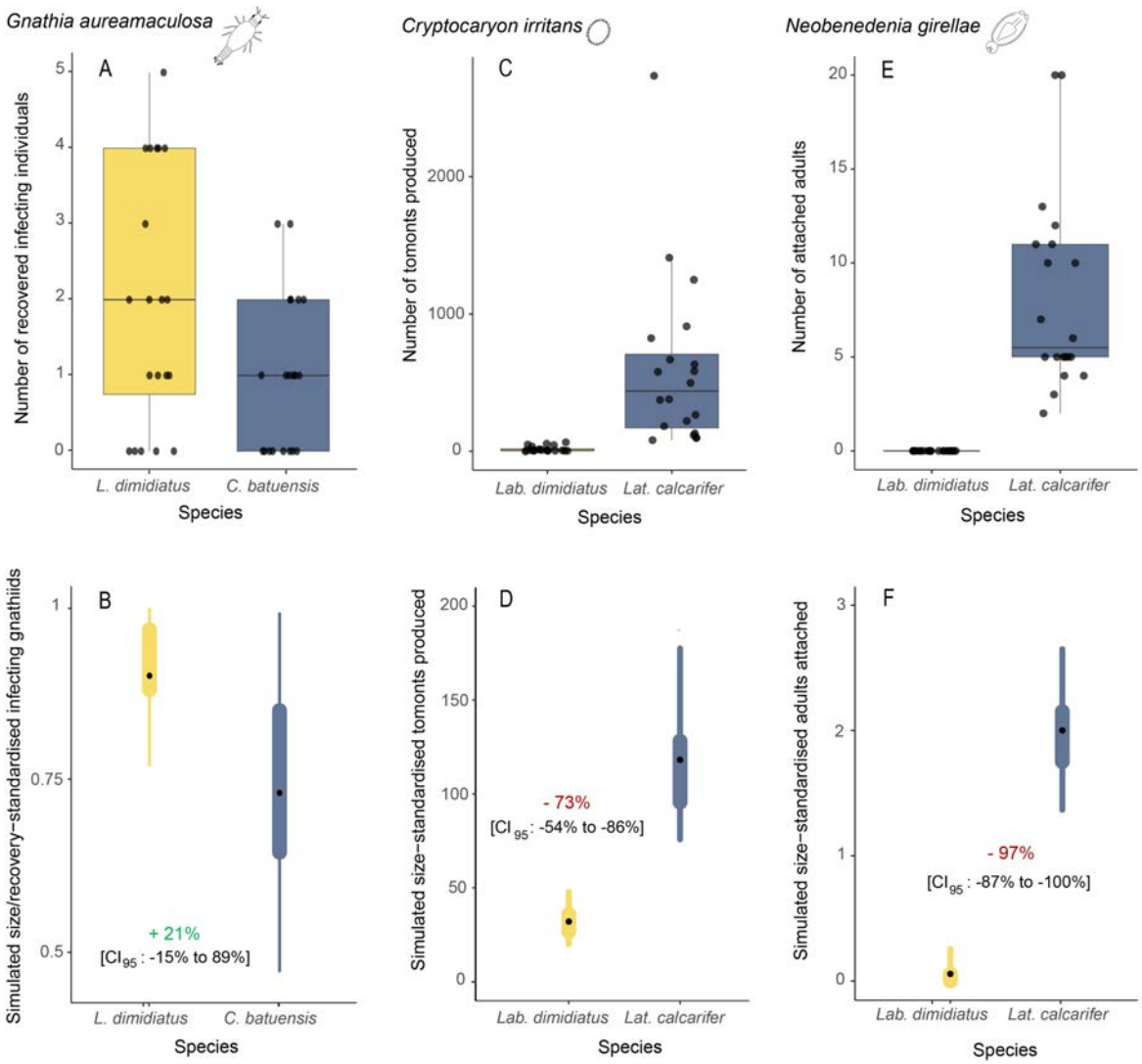

Figure 12 Raw data and predictive models describing rates of infection of the cleaner wrasse *Labroides dimidiatus* (yellow) by three types of ectoparasite compared to control species *Lates calcarifer* or *Coris batuensis* (both in blue). Panels in the top row (A, C and E) show the raw data from each susceptibility experiment. (A) Number of gnathiid isopods, *Gnathia aureamaculosa*, recovered fed at the end of the experiment, from a total of five originally added per trial. (C) Number of second generation tomonts produced by the protozoan ciliate *Cryptocaryon irritans* after initial exposure to 10 tomonts per trial; (E) Number of adult flatworms, *Neobenedenia girellae*, attached to the host following exposure to 20 infective stage larvae (oncomiracidia) per trial. Black dots (data points) were jittered on the x axis. Boxplots show the median (horizontal line), the 25 % and 75 % interquartile intervals (box), and maximum and minimum values (whiskers). Panels in the bottom row (B, D and F) show model coefficients and simulated data for each experiment while accounting for differences in surface area between individuals from the two fish species. (B) Simulated infection of *G. aureamaculosa* at the end of the experiment (fed gnathiids), with 0 and 1 representing unsuccessful and successful infection, respectively. (D) Simulated number of tomonts produced by *C. irritans* at the end of the experiment. (F) Simulated number of adult *N. girellae* attached to the fish at the end of the experiment. Legends in (B) to (F) show the effect size of the comparison between cleaner and control (percent ratio) in each case, with CI_{95} being the 95 % Credibility Interval. See Appendix B Fig. B3 for the Posterior Density curves associated with these analyses

Despite appearing to be resistant to infection by *N. girellae* larvae (Fig. 12E, F), it is conceivable that adult parasites, which are highly mobile and can crawl along the skin surface (see online supplementary video – Hutson and Narvaez 2022: https://doi.org/10.5281/zenodo.6207357), could transfer to *Lab. dimidiatus* from an infected client during close physical contact while cleaning. Whether adult parasites that may become attached to cleaner wrasses during cleaning interactions survive to enable transmission from *Lab. dimidiatus* to new clients is unknown.

Egg production and viability of adult Neobenedenia girellae *flatworms experimentally transferred to* Labroides dimidiatus

In the first 24 h after being transferred from the donor fish to the recipient fish, the number of eggs produced by flatworms on the cleaner fish was only 7 % (CI_{95}: 2 - 15 %) of the eggs produced on the *Lates calcarifer* treatment (Fig. 13A; Appendix B Fig. B4; Appendix B Table B1). Between 48 and 72 h post-transfer, flatworms attached to cleaner fish produced only 16.6 % (CI_{95}: 0.87 % - 25.8 %) of their initial egg production (i.e., first 24 h), and only 1.6 % (CI_{95}: 0.3 - 4 %) of the eggs produced by flatworms attached to *Lat. calcarifer* in the same period. Egg production on the cleaner treatment declined to only 6.7 % of initial egg production between 72 and 96 h (Appendix B Table B1). At the end of the experiment (i.e., 7 days), no adult flatworms were alive in the cleaner treatment, with 20 % found dead and detached from the fish, and 80 % not recovered, *i.e.,* degraded or possibly consumed by the cleaners (Fig. 13B). *In vitro* flatworms produced only 1.4 % (CI_{95}: 0.2 - 3.9 %) of their initial egg production between 48 and 72 h (Fig. 13A; Appendix B Fig. B4) and, after 4 days, all parasites had died (Fig. 13B), with no further egg production. Flatworms transferred to *Lat. calcarifer* maintained high egg production throughout the experiment, with 35 % of the initial egg production (CI_{95}: 2.3 - 111 %) after seven days. At the end of the experiment, 36 % of flatworms transferred to barramundi were alive on the fish, 30 % had died and detached from the fish, and 34 % were not recovered (Fig. 13B).

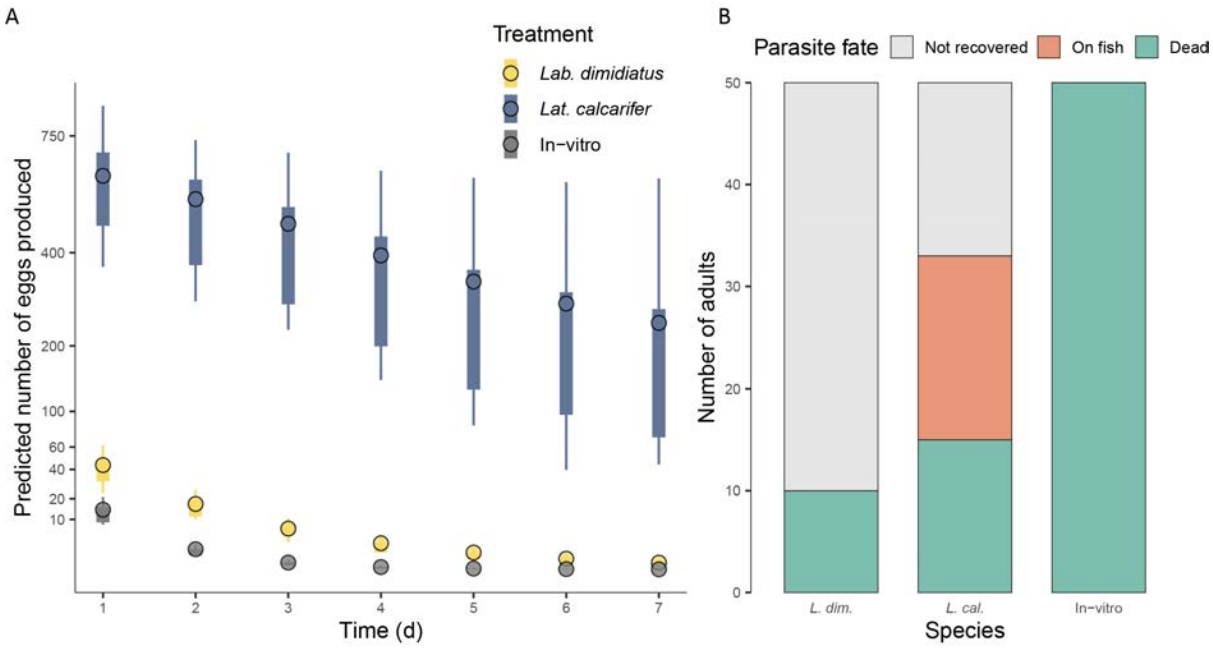

Figure 13 (A) Estimated number of eggs produced at daily time intervals by adult *Neobenedenia girellae* flatworms transferred from donor *Lates calcarifer* to the cleaner wrasse *Labroides dimidiatus* (yellow), uninfected *Lates calcarifer* (blue) and *in vitro* control (grey). (B) Fate of adult *N. girellae* after seven days post-transfer on *Lab. dimidiatus*, *Lat. calcarifer* and *in vitro* control treatments. Parasite fate "not recovered" (grey) includes *N. girellae* that dropped off during the experiment and either degraded in the water or were consumed by the fish. Parasite fate "on fish" (orange) represents *N. girellae* recovered after dropping off from the fish following a freshwater bath treatment on day 7. Parasite fate "dead" (green) represents parasites found dead at any point in time during the experiment

Egg production of adult flatworms transferred from *Lates calcarifer* to cleaner wrasse continued for at least 96 h. Egg production was substantially lower compared to parasites transferred to *Lat. calcarifer* and oviposition rate declined faster (Fig. 13).

Given that most adult flatworms (80 %) experimentally transferred to the cleaners were not found at the end of the experiment (i.e., 7 days), a third experiment was performed to determine the survival of these parasites on the cleaner *Lab. dimidiatus*. After transferring adult *N. girellae* flatworms (n = 5 parasites per trial; 16 to 20 days post-hatch) from donor barramundi *Lat. calcarifer* to cleaner *Lab. dimidiatus* (n = 10 trials), the probability of detecting parasites still visible while attached to *Lab. dimidiatus* was ~ 68 % at 30 min, 1 h and 2 h (CI_{95}: 55 - 81 %; Fig. 14). No parasites were found dead or living *in vitro* on the bottom of the tank for the first 8 h of the experiment. After this time, the number of detached parasites (dead or alive) increased, with a probability of 23.8 % (CI_{95}: 13.1 - 36 %) and 27.8

% (CI_{95}: 16.2 - 40.5 %; Fig. 14) of finding a detached parasite after 24 h and 48 h, respectively. After 24 h, approximately half of the transferred parasites were still visible on the bodies of *Lab. dimidiatus*. However, the probability of finding a visible flatworm on fish plummeted from over 48% (CI_{95}: 34.4 - 61.3 %) after 24 h to 17.7 % (CI_{95}: 8.2 - 28.6 %) after 48h. At the end of the experiment (48 h post transfer), 5 of 10 cleaners still had *N. girellae* attached with a median of 0.5 (IQR = 0 – 2.5) flatworms per fish.

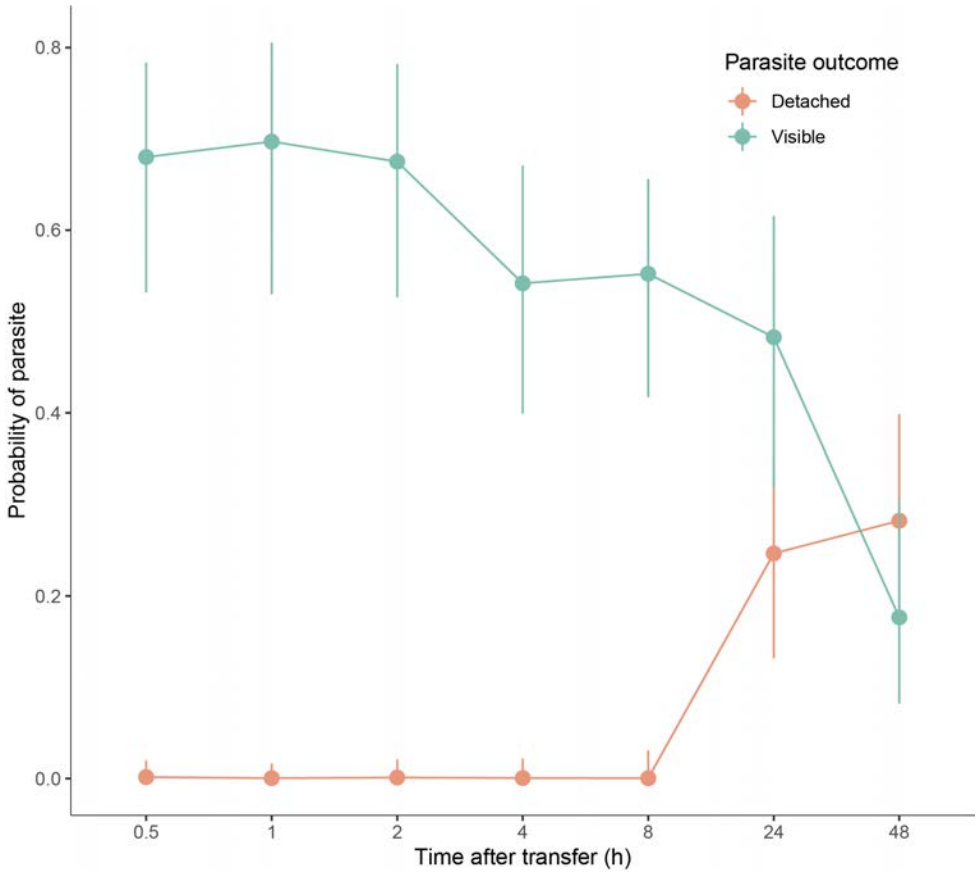

Figure 14 Estimated probability of different outcomes of *Neobenedenia girellae* transfer from donor fish *Lates calcarifer* to cleaner fish *Labroides dimidiatus* (n = 10) at different time intervals over a 48 h experiment. *N. girellae* were found either "detached" inside the tank (dead or alive) or "visible", attached to the body of *Lab. dimidiatus.* The dots represent the posterior probability for each outcome and the vertical lines the 95 % Credibility Interval

Discussion

I explored a poorly investigated, potentially negative aspect of cleaning symbiosis: that the widely distributed cleaner fish, *L. dimidiatus*, may be susceptible to, and act as a vector of, generalist ectoparasites (**Chapter 2**; Narvaez et al. 2021a). First, the susceptibility of *L. dimidiatus* to experimental infection by three parasite species: the gnathiid isopod *G. aureamaculosa*, the ciliate protozoan *C. irritans* and the monogenean flatworm *N. girellae* was tested. Second, parasite transmission from client to cleaner was simulated by manually transplanting adult *N. girellae* flatworms from a donor host to the cleaner fish, *L. dimidiatus*. Third, the success of experimentally transposed infections was quantified by monitoring viable egg production, and the survival of adult *N. girellae* on the cleaner fish host.

Susceptibility of the cleaner wrasse Labroides dimidiatus *to generalist ectoparasites*

The cleaner wrasse *L. dimidiatus* can host gnathiid isopods and this supports recent evidence that wild cleaners are subject to gnathiid infection abundance comparable, and often even higher, than that of other wild wrasse species from the same environments (including *C. batuensis*; **Chapter 3**; Narvaez et al. 2021b). Gnathiids are the main prey item of wild *L. dimidiatus*, constituting up to 95 % of their diet (Grutter 1997, 2000). However, gnathiids are not permanently attached to their hosts and can quickly drop off after successfully feeding, or as a response to increased host stress or risk of predation (Penfold et al. 2008; Grutter et al. 2020b). Given the fast attack speeds of gnathiids (up to 24.5 cm/sec for an average length of 1.5 mm; Grutter 2003; Grutter et al. 2020b), and their quick response to disturbance, the horizontal transmission from infected client fishes to cleaners and *vice versa* is plausible (**Chapter 2**; Narvaez et al. 2021a).

After exposure to the ciliate protozoan *C. irritans*, individuals *Lab. dimidiatus* were less susceptible to infection by *C. irritans* than control barramundi, *Lat. calcarifer.* Results also showed that *Lab dimidiatus* exhibits some level of resistance toward infection by *N. girellae*. These results are surprising because *C. irritans* and *N. girellae* are geographically widespread and exhibit remarkably low host-specificity among marine fishes (Colorni and Burgess 1997, Luo et al. 2008; Whittington and Horton 1996; Brazenor et al. 2018). Indeed, *C. irritans* can infect more than 30 fish species from 17 families in the wild (Burgess and Matthews 1995; Bunkley-Williams and Williams 1994; Diggles and Lester 1996)

and more than 120 species in captivity (Burgess 1992). Outbreaks of *C. irritans* in captivity can affect multiple species simultaneously and cause very high mortality (Burgess and Matthews 1995; Colorni and Burgess 1997; Montero et al. 2007). *N. girellae* has been reported to infect more than 135 marine fishes from 43 families, both in the wild and in captivity, including Labridae and Latidae, among others (Brazenor et al. 2018). Differences in host-susceptibility to *C. irritans* with a similar magnitude to the one observed here have been reported from laboratory experiments for the rabbitfish, *Siganus canaliculatus* (compared to six other marine fish species: Wang et al. 2010). This resistance is likely due to a blood protein which induces protozoan cilia detachment and the cell membrane rupture (Wang et al. 2010). Furthermore, components of fish innate immune system such as physical and physiological (e.g., antibacterial peptides, lysozyme, lectins) mucus parameters may be effective barriers against some pathogens (Magnadóttir 2006; Parida et al. 2018; Reverter et al. 2018). It is plausible that the cleaner wrasse *L. dimidiatus* may be resistant to *C. irritans* and *N. girellae* due to specific blood protein and/or mucus composition that protect them from some parasitic infection, however, future studies are needed to understand the mechanism/s behind this resistance.

The role of cleaner fish in ectoparasite transmission

Given that *C. irritans* penetrates and settle in the epithelium, within the first and secondary lamella layers of the epithelium, where it becomes encysted (Yambot et al. 2003), it may be unlikely that cleaners, interacting with infected clients, enter physical contact with this parasite. On the other hand, *N. girellae* most often attaches to the outer surface of the skin of its hosts and is highly mobile (Trujillo-González et al. 2015). As such, due to the intimate and frequent contact between cleaner fish and clients, it is reasonable to hypothesise that *N. girellae* or other monogenean ectoparasites may potentially come in physical contact with the cleaner wrasse and become attached to it during cleaning interactions (**Chapter 2**; Narvaez et al. 2021a). Therefore, while not observing successful experimental infections of *L. dimidiatus* by *N. girellae*, I showed that simulated inadvertent attachment of adult ectoparasites can be conducted successfully. Adult *N. girellae* experimentally transferred to *L. dimidiatus* largely remained attached to the fish and produced viable eggs for up to 4 days, demonstrating that it is plausible

for *N. girellae* to be transported by *L. dimidiatus* for at least 48 h (68 % parasites were still attached to fish body for up to at least 2 days). Parasites transferred to cleaner fish have the potential to continue to contribute to the population of parasites through the production of eggs. Monogenean eggs have filamentous structures that facilitate entanglement on uneven surfaces such as the gill rakers or opercula of fish host (Whittington and Deveney 2011; Hutson et al. 2018). Furthermore, these eggs display strong hatching responses to stimuli (physical and chemical) indicating potential host proximity (e.g., shading, water displacement, mucus) (Whittington and Kearn 2011; Skilton et al. 2020).

How ecologically realistic are the conditions simulated here? In the wild, *L. dimidiatus* individuals interact with an average of 2,300 fish per day, for an average 260 min per day (Grutter 1996a). In a high proportion of these interactions, *L. dimidiatus* executes detailed gill inspections (48 to 78 %; Grutter et al. 2002a), which often involve the cleaner inserting its whole body inside the client's gill chamber. Moreover, nearly 50 % of all these interactions also involve tactile stimulation (i.e., when cleaner fish touch the client's body with its pectoral and pelvic fins; Bshary and Würth 2001). It is well known that social interactions offer abundant opportunities for parasite transmission and spread (Godfrey et al. 2009; Johnson et al. 2011; MacIntosh et al. 2012; Craft 2015; Evans et al. 2020). Therefore, during cleaning interactions, ectoparasites such as adult *N. girellae*, which are mobile and can crawl across the body surface of fish (Trujillo-González et al. 2015; see online Supplementary video - Hutson and Narvaez 2022: https://doi.org/10.5281/zenodo.6207357), have ample opportunity of being horizontally transmitted via direct contact between fish (**Chapter 2**; Narvaez et al. 2021a). Some ectoparasites such as gyrodactylid monogeneans (Bakke et al. 2007) or caligid copepods (Ritchie 1997) are mobile and can switch hosts when conditions are not suitable (e.g., stress, host immunity, death) or by accidental dislodgement (Bakke et al. 2007). In cleaning symbiosis context, cleaning interactions present a potential stress for parasites because cleaner organisms feed on them and therefore detachment is likely when approached by a predator (**Chapter 2**; Narvaez et al. 2021a).

Finally, studies have found that parasites may evolve to exploit the original host's predator (Strona 2015; Antonovics et al. 2017) and develop host-finding and infection strategies based on the interactions between predators and prey (Strona 2015). When considering the close and intimate contact between

cleaner and client fish, cleaning interactions may be compared to prey/predator interactions and could therefore, facilitate parasite transmission in a similar way to prey/predator interactions (Jones et al. 2004).

Conclusion and future research

The benefits of cleaning symbiosis in marine environments appear obvious and are seemingly identifiable (e.g., parasite removal, enhanced abundance of client fish). However, there may be costs to this interaction associated with the possibility of parasite transmission from infected cleaners. Difficulties with detecting cryptic, small parasites in the field and even in captivity, as well as potential assumptions that cleaning symbiosis involve mostly positive outcomes may have contributed to the historical omission of considering this negative aspect of cleaning symbiosis in the past. Given the apparent resistance of cleaner fish to some generalist ectoparasites as observed here, experiments simulating direct transmission of parasites between cleaners and clients – an obvious next step – should focus on pathogens shared between cleaners and clients. This study presents the first experimental examination of the susceptibility of a common cleaner fish to generalist parasites and reveals their potential role in the transmission of parasites during cleaning interactions. Given the prolific nature of dedicated cleaners such as *L. dimidiatus*, which interacts with thousands of clients every day, client ectoparasite infection holds the potential for significant transmission rates and, as such, ecological impacts. Thus, understanding the frequency of direct parasite transmission and downstream outcomes for parasite survival and reproduction is key to accurately assess the potential for an underlying cost to a well-known mutualism.

Chapter 5: Investigating the diet of tropical cleaner organisms through DNA metabarcoding

This chapter has been prepared for submission to *Environmental DNA*
Expected authors: Pauline Narvaez, Roger Huerlimann, Jordan Casey, Alejandro Trujillo-González, David Vaughan and Cecilia Villacorta-Rath

Abstract

Marine cleaner organisms provide a beneficial service by removing ectoparasites from other 'client' fishes. While cleaners are presumed to eat mainly crustacean ectoparasites, morphological identification is challenging due to gut contents becoming rapidly unrecognisable. Here, we investigated the diet of three common cleaner organisms; the dedicated cleaner wrasse *Labroides dimidiatus*, and two cleaner shrimp species *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* using DNA metabarcoding techniques. Cleaner wrasses (n=30) and cleaner shrimps (n=15 for each species) were collected at Lizard Island, Great Barrier Reef. DNA extractions were conducted using the CTAB extraction protocol, and two universal primers targeting the *COI* and *16S* gene regions were chosen. Libraries were prepared and sequenced using MiSeq reagent kit v2 (500-cycle). For the *16S* primer set, sequencing data was filtered for genus or species-level taxonomy with > 98 % similarity. For the *COI* primer set, sequencing data was filtered for genus or species-level taxonomic assignment (97 % sequence similarity) and phylum (≥ 80 %). For *L. dimidiatus* using the *16S* primer set and after filtering for eukaryote, parasites were detected and assigned to the phylum Nematoda, the parasitic protist *Goussia* sp. and the flagellate protist from the Scytomonadidae family. For the two cleaner shrimp species, two parasite species from the subclass Digenea and Eucestoda were detected. Sequences obtained from the *COI* primer yielded 244 high-resolution OTUs for *L. dimidiatus*. From these, 191 OTUs (78.3 %) were assigned to 36 species of fish and 53 OTUs (21.7 %) to three phyla (Chordata, Arthropoda and Mollusca). For the cleaner shrimps, 719 OTUs were assigned to the phylum level Arthropoda. This study is the first to use molecular approach to investigate the diet of cleaner organisms. The presence of a high percentage of fish sequences encountered with the *COI* primer set for *L. dimidiatus* supports

the hypothesis that this cleaner removes fish tissues while cleaning. On the other hand, the universal primer targeting the *16S* region was useful to detect parasitic organisms for the three cleaner organisms and indicates cleaner fishes may eat a range of parasitic taxa from client fishes

Introduction

Marine cleaner organisms such as fish and shrimp have been widely studied in tropical environments because of the important role they play in the ecosystem (Bshary 2003; Grutter et al. 2003; Chapuis and Bshary 2009; Soares et al. 2011; Waldie et al. 2011; Vaughan et al. 2018a). Cleaners remove unwanted item such as parasites (e.g., Grutter 1999a; Arnal and Morand 2001) and dead tissue (Foster 1985) from other fish, called the 'clients' that in return, provide food for the cleaners. Therefore, cleaning interactions are characterised as mutualistic relationships because both cleaners and clients predominantly benefit from the interaction. While some cleaners carry out this service during their entire life (i.e., dedicated cleaners) other clean only during some life stages (i.e., facultative cleaners) (Vaughan et al. 2017).

In the marine environment, the diet of 36 cleaner fish species and nine cleaner shrimp species have been investigated using morphological approaches in wild-caught individuals and experimentally through food choice trials (see Vaughan et al. 2017 for review). The most predominant item found in the diet of both cleaner organisms (98.6 % of their diet) comprise crustacean ectoparasites such as gnathiid isopods (e.g., Arnal and Côté 2000; Arnal and Morand 2001; Narvaez et al. 2015), whereas the remaining items belong to other group of parasites such as monogenean flatworms (e.g., Gorlick et al. 1987; Grutter et al. 2002b; Grutter and Bshary 2003). While these findings possibly reflect a high food preference of cleaner organisms for crustacean parasites, these results need to be considered with caution because crustaceans possess exoskeletons that are not digested easily and therefore can be more readily identified (Vaughan et al. 2017). On the other hand, parasites such as worms are soft-bodied and become quickly unrecognisable in the gut (Becker and Grutter 2004). The removal of healthy tissue such as mucus have been also reported to be ingested by the cleaners, however, records are limited (e.g., Gorlick

1980; Grutter 1997), because they are often unidentifiable and unquantifiable in the gut of cleaners (Arnal and Côté 2000).

To overcome the challenges of morphological identification of gut contents, DNA metabarcoding has been recently used to maximise species-level identification of remnant tissue that were previously undetected by traditional methods (Takahashi et al. 2020). DNA metabarcoding combines DNA-based identification and high-throughput DNA sequencing (Siegenthaler et al. 2019a), allowing for a more comprehensive way of studying gut content. This technique has been shown to be efficient for many marine organisms such as fish (Su et al. 2018; Takahashi et al. 2020; Casey et al. 2019; Brandl et al. 2020) and crustaceans (O'Rorke et al. 2012; Siegenthaler et al. 2019a, b). Besides giving valuable information on the diet of many organisms, DNA metabarcoding can reveal ecological aspects that have been previously overlooked, such as reconstructing food webs with high resolution (Casey et al. 2019; Zamora-Terol et al. 2020), investigating the parasite community of fish (Scheifler et al. 2019) as well as quantifying biodiversity (Leray and Knowlton 2015; Tan and Liu 2018).

The main objective of this chapter was to use DNA metabarcoding to analyse the diet of three cleaner organisms in tropical reefs. I investigated the diet of a common tropical cleaner fish, the bluestreak cleaner wrasse *Labroides dimidiatus* and two species of cleaner shrimp, *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* that co-habit with *L. dimidiatus* (Froese and Pauly 2021; Horton et al. 2021). Because these three species of cleaners may inhabit the same or similar reefs in the Great Barrier Reef (pers. obs.), it is expected that their feeding habits will complement each other and targeting different prey. Moreover, *L. dimidiatus* is active only during the day (Grutter 2002) and *U. antonbruunii* and *U.* cf. *cyrtorhyncha* are active also at night (Bos and Fransen 2018; Vaughan et al. 2018a), therefore, difference in the diet between cleaner fish and shrimp could be expected.

Methods

Target species collection

Cleaner wrasse, *L. dimidiatus*, (n = 30) were collected in November 2018 at Lizard Island (14° 40' S, 145° 28' E), northern Great Barrier Reef, Australia (Fig. 15) using barrier nets and hand nets. Fish were

individually placed in sealed plastic bags and then transferred by boat to the research station, where they were euthanised using cold thermal shock. Cleaner fish were first scrutinised for parasites (see **Chapter 3**; Narvaez et al. 2021b) by removing the gut contents and placing them into a sterile petri dish to investigate for gut endoparasites. After parasite screening, the remains of the gut content were collected and preserved in RNA*later* and stored at 4 ˚C for 12h before freezing at -20 ˚C.

Cleaner shrimp, *Urocaridella antonbruunii*, (n = 15) and *U.* cf. *cyrtorhyncha* (n = 15) were collected using hand nets in January 2020 also at Lizard Island (Fig. 15). Shrimps were individually placed in sealed plastic bags and then transferred by boat to the research station, where they were euthanised using cold thermal shock and were directly kept in 80% ethanol in a -20 ˚C freezer. After a week, ethanol was flushed and replaced by new 80% ethanol solution.

The preservation methods differ between cleaner fish and shrimp to match the methods previously used for another project that shared the samples. As differently preserved fish and shrimp were not compared, this is not expected to affect any of the results in this study.

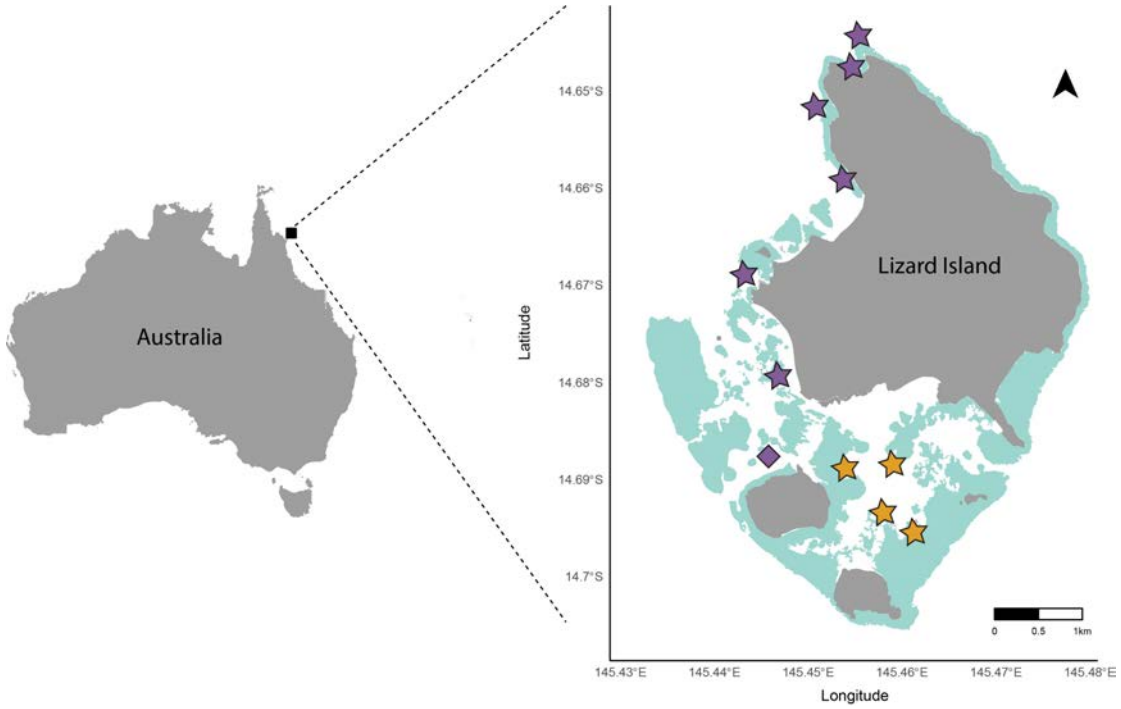

Figure 15 Sampling sites on Lizard Island (northern Great Barrier Reef, Australia) where cleaner specimens were collected. Cleaner fish were collected at different locations around the island and were subsequently qualified as 'lagoon' (orange stars) and 'non-lagoon' (purple stars). Cleaner shrimp individuals for both species were collected at the same 'non-lagoon' location (purple diamond)

DNA extraction and universal primer testing

DNA extraction and polymerase chain reaction (PCR) were done at the Molecular Ecology and Evolution Laboratory (MEEL), James Cook University, Australia. For each sample, approximately 100 mg of homogenised gut contents were used, and DNA extractions were conducted using the CTAB extraction protocol following Adamkewicz and Harasewych (1996). DNA quality was assessed on a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA) and the DNA quantity was determined using a Quantus™ Fluorometer (Appendix C Table C1 and C2). DNA extracts were diluted to a final concentration of 10 ng/µL for library preparation.

Two sets of universal primers targeting two different mitochondrial gene regions were used to amplify fish and shrimp gut contents. The first primer pair targeted a 313 base pair (bp) fragment of the *cytochrome c oxidase subunit I* (*COI*) (Table 4). This set of primers has been previously used in different studies due to their versatility amplifying metazoan prey from a wide range of phylum such as Platyhelminthes, Arthropoda, Mollusca, Nematoda and Chordata (Geller et al. 2013; Leray et al. 2013a; Casey et al. 2019). The second set of primers targeted a 450 bp fragment of the *16S* ribosomal DNA (rDNA) V4-V5 region (Table 4) and has been successfully used to characterise the parasite community of fishes in the Mediterranean Sea (Schleifler et al. 2019). *In-silico* tests were done using the software Geneious Prime (version 2020.2) to align sequences of the potential prey of the cleaner fish and cleaner shrimp to both sets of primers and determine their suitability for amplifying the suspected prey items.

PCR reactions consisted of a total volume of 20 µL containing: 1µL forward primer (10 µM), 1 µL reverse primer (10 µM); 10 µL of MyTaq™ Red Mix; 6 µL MilliQ water and 2 µL template DNA. Thermocycling conditions for the *16S* universal primers were as follow: 3 min of initial denaturation at 95 ˚C followed by 22 cycles of 95 ˚C for 45 s (denaturation), 50 ˚C for 45 s (annealing), and 68 ˚C for 90 s (extension), with a final extension at 68 ˚C for 5 min (Schleifler et al. 2019). For the *COI* universal primers, thermocycling conditions were as follow: 10 min of initial denaturation at 95 ℃ followed by two sets of cycles. The first set of 16 cycles includes: 95 ℃ for 10 s (denaturation), 62 ℃ (−1 ℃ per cycle) for 30 s (annealing) and 72 ℃ for 60 s (extension). The second set included 20 cycles with the same denaturation and extension steps, but with annealing at 46 ℃ for 30 s as well as a final extension at 72 ℃ for 7 min (Casey et al. 2019). Following amplification, 2 µL of amplicons were loaded in 0.8

% agarose gel at 70 V for 60 min and the gel image was visualised under UV light and with the imaging system VisionCapt v16.12 (Appendix C Fig. C1; C2; C3). The purified amplicons were shipped to the Australian Genome Research Facility (AGRF, North Melbourne, VIC, Australia).

Blocking primer design

A blocking primer was designed for the two species of cleaner shrimp to avoid amplification of the host DNA when using universal primers that target crustaceans (Vestheim and Jarman 2008). Genetic reference sequences were only available for *Urocaridella antonbruunii* and therefore, genomic DNA (gDNA) from *U.* cf. *cyrtorhyncha* was extracted using the CTAB method as described above. The same primer targeting the *16S* ribosomal DNA (rDNA) V4-V5 regions (and also matching eukaryotic 18S rRNA; Parada et al. 2016) described above was used to amplify the extracted *U.* cf. *cyrtorhyncha* gDNA and PCR amplicons were Sanger sequenced at the Australian Genome Research Facility (AGRF). Sequences from both species were aligned against those of known prey items, as well as the *16S* universal primers using the software Geneious Prime (version 2020.2). Genetic sequences of both cleaner shrimp species only matched the reverse *16S* primer at 100% identity and therefore, only one reverse blocking primer was designed. This blocking primer overlapped with the 3' end of 926R reverse primer (10 bp overlap) and extended into the two species of shrimp sequence (15 bp of shrimp sequence), and was modified at the 3' end with a C3 spacer to prevent elongation during annealing (Vestheim and Jarman 2008; Leray et al. 2013b; Takahashi et al. 2020) (see Table 4). *In-silico* and *in-vitro* tests were done to validate the blocking primer (Appendix C Fig. C4 and C5). *In-vitro* tests were done to test the efficiency of the blocking primer on 1) gDNA of both shrimp species and 2) the shrimp gut content. A PCR with gradient temperature from 50 ˚C to 58 ˚C was run and at different concentrations to determine the most appropriate annealing temperature (see Appendix C Fig. C4 and C5). No blocking primer was used for the cleaner wrasse species *L. dimidiatus* because no nucleotide matches were found between the primers selected and *L. dimidiatus* genomic sequences during the *in-silico* tests.

Table 4 Primer pairs used for this study. The blocking primer was used for both cleaner shrimp species, *Urocaridella antonbruunii* and *U. cyrtorhyncha*. Base pairs in bold in the blocking primer overlap with the 3' end of the reverse 926R primer. The 15 following base pairs represent the host sequences (same for both shrimp species). The C3 spacer at the end of the 3' end of the blocking primer that allows to inhibit annealing

Primer label	Primer type	Sequence 5'- 3'	Target gene region	Reference
mlCOIintF	Universal	GGW ACW GGW TGA ACW GTW TAY CCY CC	*COI*	Leray et al. (2013a)
jgHCO2198	Universal	TAI ACY TCI GGR TGI CCR AAR AAY CA	*COI*	Geller et al. (2013)
515F-Y	Universal	GTG YCA GCM GCC GCG GTA A	V4-V5 region:*16S* rRNA	Parada et al. (2016)
926R	Universal	CCG YCA ATT Y**MT TTR AGT TT**	V4-V5 region:*16S* rRNA	Parada et al. (2016)
926R- blkShrimp	Blocking primer	**MTT TRA GTT T**CA GTT TTG CAA CCA T /C3/	V4-V5 region:*16S* rRNA	This study

Library preparation

DNA metabarcoding libraries were prepared and sequenced at AGRF. PCR amplicons were generated using nextera-overhang primers (see Table 4). A first stage PCR was carried out in the Applied Biosystem 384 Veriti platform. PCR assays consisted of a total volume of 8 µL without the blocking primer and 10 µL with the blocking primer. For the samples with no blocking primer, the master mix composition was: 1 µL of 0.625 µM forward primer, 1 µL of 0.625 µM reverse primer; 4 µL of SuperFi II (Life Technologies, Australia); and 2 µL of template DNA. For the samples with blocking primer, the master mix composition was: 1 µL of 0.25 µM forward primer, 1 µL of 0.25 µM reverse primer; 2 µL of 2.5 µM blocking primer; 4 µL of SuperFi II (Life Technologies, Australia); and 2 µL of template DNA. PCR cycling program used was: 30 s of initial denaturation at 98 ˚C followed by 35 cycles of 98 ˚C for 10 s (denaturation), 60 ˚C for 10 s (annealing), and 72 ˚C for 30 s (extension), with a final extension at 72 ˚C for 5 min. Amplicons resulting from the first stage PCR were cleaned using magnetic beads (1.8 x ratio) to enrich for fragments > 100 bp and therefore remove primer dimers. Samples were then visualised on a 2 % Sybr Egel (Thermo-Fisher). A second stage PCR was used to index the amplicons and was achieved using the Platinum SuperFi II master mix (Thermo-Fisher). Resulting

amplicons were cleaned again using magnetic beads to remove primer dimers, measured by fluorometry (Promega Quantifluor) and normalised. Normalised amplicons were pooled and cleaned a last time utilising magnetic beads and the pooled libraries were measured using a High-Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pooled library was diluted to 5 nM and molarity was determined using a Qubit High Sensitivity dsDNA assay (ThermoFisher). Finally, libraries were sequenced on an Illumina MiSeq (San Diego, CA, USA) using a V2, 500 cycle kit (2 x 250 base pairs paired-end) and a 20 % PhiX spike-in to improve nucleotide diversity.

Analysis of sequencing data

For the *16S* primer set, diversity profiling analysis were performed using the Quantitative Insights into Microbial Ecology QIIME 2 software (version 2019.7; Bolyen et al. 2019). Raw reads were demultiplexed using the Illumina bcl2fastq2 (version 2.20). Demultiplexed raw reads were primer trimmed and quality filtered using the cutadapt plugin followed by denoising with DADA2 (via q2-dada2; Appendix C Table C3, C4) (Callahan et al. 2016). Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich et al. 2018), a naïve Bayes taxonomy classifier, Silva (version 132) as references. Genus or species-level taxonomy was assigned for sequences with > 98% similarity with the reference database. If a sequence had < 98 % similarity, it was discarded from downstream analyses. If a sequence matched two or more genera or species with the same percentage of similarity (≥ 98 %), the sequence was not assigned and discarded from downstream analyses (Scheifler et al. 2019). Since our CTAB DNA extraction protocol did not include a bead beating step (essential step to avoid bias toward Gram-positive or Gram-negative bacteria when analysing bacterial community; Infante-Villamil et al. 2021), the number of bacterial sequences were not included in our results (reported under Appendix C Fig. C6 to C11).

For the *COI* primer set, paired-ends reads were assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5; Zhang et al. 2014). Primers were identified and trimmed using custom python scripts. Trimmed sequences were processed using QUIIME 1.8 software (Caporaso et al. 2010) USEARCH (version 8.0.1623, Edgar 2010; Edgar et al. 2011) and UPARSE software. Demultiplexed, trimmed sequences were quality-filters in USEARCH tools, full length duplicate sequences were

removed and sorted by abundance (Appendix C Table C5, C6). Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using the SILVA database (silva32) as reference. To obtain number of reads in each Operational Taxonomic Unit (OTU), reads were mapped back to OTUs with a minimum identity of 97 % (genus-level taxonomy). Phylum-level taxonomy was assigned when sequence similarity was ≥ 80 % (Casey et al. 2019) and host 'self-hits' were removed. Representative sequences of each OTU were taxonomically assigned using the BOLD and NCBI databases. If an OTU had several hits to different taxonomic assignments. Finally, sequences assigned to terrestrial organisms (e.g., human, plants, etc.) were discarded as the result of potential environmental contamination.

Statistical analyses

Diet diversity of the cleaner fish obtained through the *COI* universal primer was analysed according to fish size and fish habitat (lagoon *vs* non-lagoon). I used two different modelling procedures, one in which I tested differences between individual cleaner fish in the composition and number of hits of the three detected phyla (i.e., molluscs, arthropods and chordates), and another one using all detected species-level fish OTUs. A model-based multivariate generalised linear model analysis using the 'manyglm' function of the R package "mvabund" (Wang et al. 2012; Warton et al. 2012) with a negative binomial was performed to test for differences between fish individuals of different sizes and collected in the distinct habitats. To illustrate patterns, I also ran a Principal Coordinates Analysis (PCoA) for each taxonomic level (i.e., phylum or species) of dietary groups, which is represented by biplots with the first two PCoA axes. To visualise the association between cleaner wrasse *L. dimidiatus* individuals and targeted taxa, a Sankey diagram was generated in the "networkD3" (Allaire et al. 2017) R package. Here, the network nodes represent the number of hits for each target taxa and the network edges represent each *L. dimidiatus* individual and the target taxa. To visualise the targeted taxa to at the phylum level, a tile plot was generated using the R package "ggplot2" (Wickham 2016). Statistical analyses were performed using the software R version 4.1 (R Core Team 2020).

Results

Sequences obtained from the 16S universal primer

A total of 3,174,921 reads were obtained for the cleaner wrasse *L. dimidiatus* (n=30). From these, 2,418,428 belonged to eukaryote (76.2 %) but were unassigned and discarded because they matched to more than one genus with the same percentage of similarity. Four sequences were host self-hits and 756,452 sequences (23.8 %) corresponded to bacteria (Appendix C Fig. C6 and C7). After filtering for eukaryote at 98 % similarity, three OTUs represented by 12 sequences and were assigned to the phylum Nematoda, the parasitic protist *Goussia* sp. and the flagellate protist from the Scytomonadidae family (Table 5). For Archaea, two OTUs were obtained and were assigned to the phylum Thaumarchaeota (Table 5).

On the other hand, a total 3,116,582 raw reads were obtained for both cleaner shrimp, *Urocaridella antonbruunii* and *U. cyrtorhyncha,* using the blocking primer and 4,412,203 raw reads were obtained without blocking primer. For *U. cyrtorhyncha*, a lower percentage of self-hits were obtained when using blocking primer (89.4 %) than without it (95.9 %). For *U. antonbruunii*, there was no apparent difference in the number of self-hits with and without blocking primer (99 % and 99.5 % respectively). The number of hits representing bacteria were higher when using the blocking primer (179,616 and 12,144 for *U.* cf. *cyrtorhyncha* and *U. antonbruunii* respectively) compared with no blocking primer (93,000 and 2,715 for *U.* cf. *cyrtorhyncha* and *U. antonbruunii* respectively; see Appendix C Table C7 and Fig. C8 and Fig. C9). When using the blocking primer, the number of eukaryote sequences at 98 % similarity was 52 and 53 for *U.* cf. *cyrtorhyncha* and *U. antonbruunii* respectively and without blocking primer, 292 and 17 respectively (Table 5).

Table 5 Diet taxa (organisms amplified) summary using the *16S* universal primer for the three study species: the cleaner wrasse *Labroides dimidiatus* and the two cleaner shrimp *Urocaridella* cf. *cyrtorhyncha* and *U. antonbruunii*. For the cleaner shrimp, the number of total number of hits was reported either using or not using the blocking primer designed in this study

Diet taxa	Cleaner species	Number of hits without blocking primer	Number of hits with blocking primer
Eukaryota (Domain) Nematoda (Phylum) Raphidascarididae (Family)	*Labroides dimidiatus*	7	NA
Eukaryota (Domain) Apicomplexa (Phylum) Eimeriidae (Family) Goussia sp. (Genus)	*Labroides dimidiatus*	3	NA
Prokaryota (Domain) Archaea (Kingdom) Thaumarchaeota (Phylum)	*Labroides dimidiatus*	11	NA
Eukaryota (Domain) Euglenozoa (Phylum) Scytomonadidae (Family)	*Labroides dimidiatus*	2	NA
Eukaryota (Domain) Platyhelminthes (Phylum) Digenea (Subclass) *Helicometra fasciata*	*Urocaridella* cf. *cyrtorhyncha*	289	52
Eukaryota (Domain) Platyhelminthes (Phylum) Eucestoda (Subclass) *Echinobothrium* cf. *heroniense*	*Urocaridella antonbruunii*	17	53
Eukaryota (Domain) Fungi (Kingdom) Basidiomycota (Phylum) *Cystobasidium lysinophilum*	*Urocaridella* cf. *cyrtorhyncha*	3	0

Sequences obtained from the COI universal primer

A total of 5,302,435 reads were obtained for the cleaner wrasse *L. dimidiatus*, out of which 5,286,506 reads were self-hits. After filtering sequences for genus or species-level taxonomic assignment (97 % sequence similarity) and phylum assignment (≥ 80 %), a final 244 OTUs were obtained. From these, 191 OTUs (78.3 %) were assigned to the species level and 53 OTUs (21.7 %) to the phylum level. All 191 OTUs were assigned to 36 species of teleost fish from 13 different families. The most abundant OTUs were assigned to the floral wrasse *Cheilinus chlorourus* (Labridae; 40 OTUs and 2810 sequences), the needlefish *Tylosurus crocodilus* (Belonidae; 21 OTUs and 2645 sequences), the dash-and-dot goatfish *Parupeneus barberinus* (Mullidae; 32 OTUs and 2162 sequences) and the monocle

bream *Scolopsis monogramma* (Nemipteridae; 28 OTUs and 1856 sequences; Fig. 16). The Sankey network plot shows the abundance as well as the diversity of fish targeted by *L. dimidiatus* (Fig. 16).

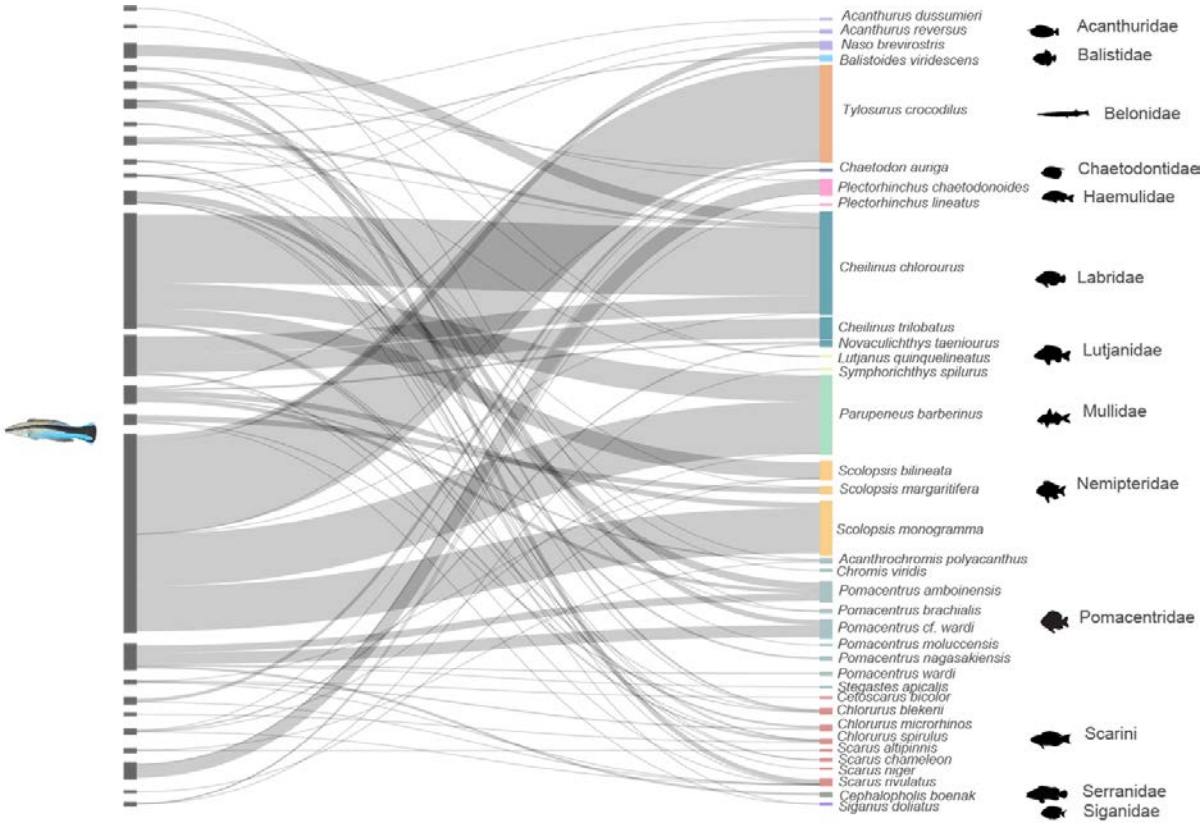

Figure 16 Cleaner wrasse *Labroides dimidiatus* individuals (left) and their associated targeted taxa (right), including 191 OTUs obtained through sequencing a fragment of the *COI* gene. The 13 different colours and fish silhouettes on the right represent the fish diversity targeted by cleaner fish and within each colour, the name of each fish species is included. The width of the lines represents the number of total sequences assigned to each target species

The OTU assigned to the phylum level (n = 53 OTUs) were assigned to Chordata (2 OTUs), Arthropoda (39 OTUs) and Mollusca (12 OTUs). When combining the 191 OTUs assigned to fish sequences obtained at the species level to their respective phylum (Chordata; n total = 193 OTUs), Chordata were the most represented phylum (out of three phylum) and were found in the diet of 25 *L. dimidiatus* (83.3 %; Fig. 17). Arthropoda were targeted by 14 *L. dimidiatus* (46.7 %) and Mollusca by 11 *L. dimidiatus* (36.7 %; Fig. 17). Three *L. dimidiatus* were found with none of these targeted phyla.

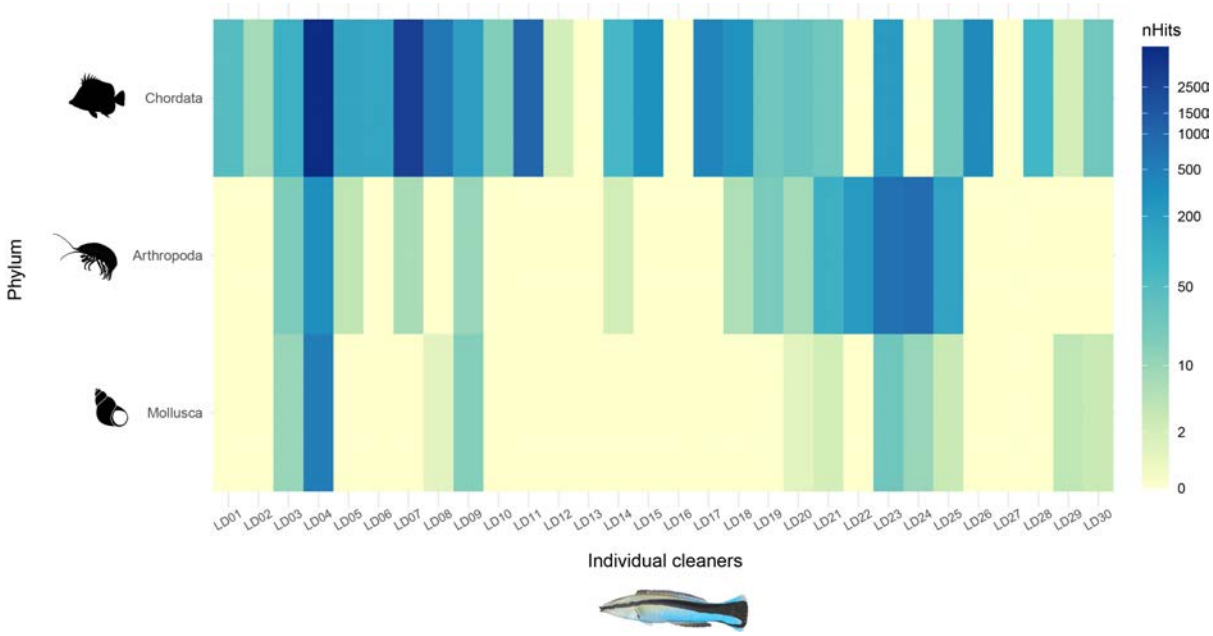

Figure 17 Phylum abundance in the diet for each cleaner wrasse *Labroides dimidiatus* individuals (n=30) collected at Lizard Island obtained through sequencing a fragment of the *COI* gene

There was no evidence of either habitat or size effects on dietary prey composition and number of hits between cleaner fish individuals at the species level, with no clear clustering by size or habitat (Appendix C Table C8; Fig. C12A). At the phylum level, prey composition showed a slightly clearer separation between individuals of different sizes and habitats, although with considerable overlap (Appendix C Table C8, Fig. C12B).

For the cleaner shrimp, a total of 2,255,567 raw reads for both species were obtained. From these, 2,205,437 were self-hits. After filtering sequences for genus or species-level taxonomic assignment (97 % sequence similarity) and phylum assignment (≥ 80 %), 729 OTUs remained. From these, 10 OTUs were assigned to the species level the teleost fish *Spratelloides delicatulus* (Clupeidae) for one individual *Urocaridella* cf. *cyrtorhyncha* and 719 OTUs were assigned to the phylum level (≥ 80 %) Arthropoda (Fig. 18).

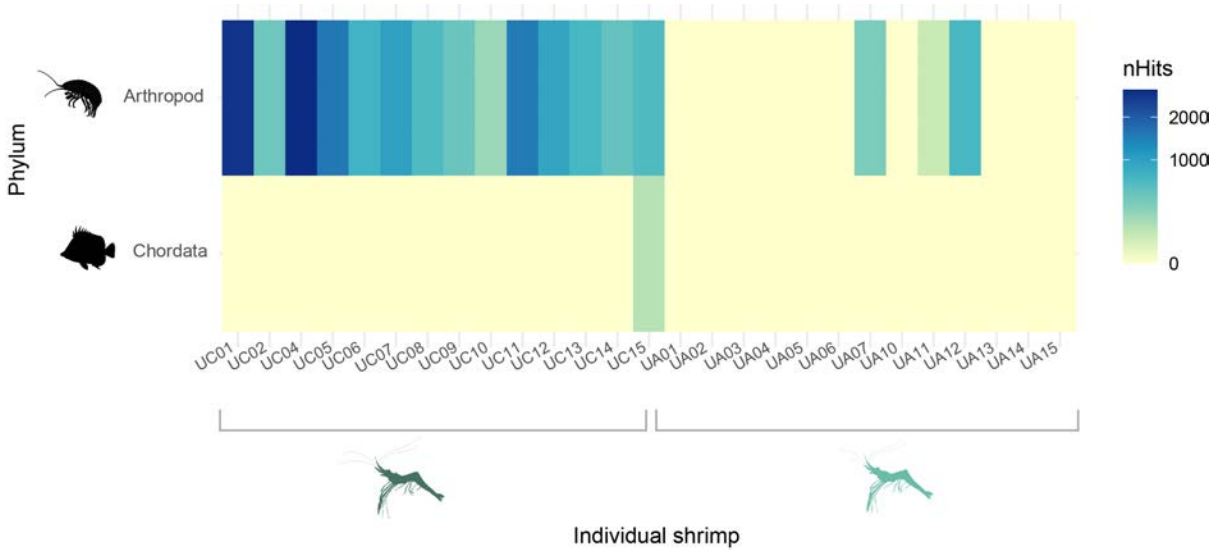

Figure 18 Phylum abundance in the diet for each cleaner shrimp individual from the two species (*Urocaridella* cf. *cyrtorhyncha* in dark green and *U. antonbruunii* in light green) collected at Lizard Island obtained through sequencing a fragment of the *COI* gene

Discussion

In the present chapter, I used a DNA metabarcoding approach to assess the gut content of three cleaner organisms inhabiting coral reefs, the cleaner wrasse *L. dimidiatus* and the cleaner shrimp *Urocaridella antonbruunii* and *U. cyrtorhyncha*. This molecular approach was used to overcome the challenges of under-detecting soft-bodied prey items and therefore to determine with more accuracy gut content of the target organisms. This constitutes the first study to use molecular tools to investigate the diet of cleaner organisms. The simultaneous use of two different universal primers to analyse the diet of cleaners allowed amplification of a wide range of organisms, from Chordata to Mollusca, Arthropoda, Nematoda, Platyhelminthes and Protozoa. The universal primer targeting the *COI* gene was useful to amplify fish to the species level targeted by the cleaner wrasse *L. dimidiatus*. On the other hand, the universal primer targeting the *16S* rDNA V4-V5 region was useful to detect parasitic organisms for the cleaner wrasse *L. dimidiatus* and the cleaner shrimp *U. antonbruunii* and *U. cyrtorhyncha*.

Cleaner fish gut content

The presence of fish DNA in the gut of the cleaner wrasse *L. dimidiatus* may reflect both an opportunistic and mutualistic behaviour of this organism. First, *L. dimidiatus* could have opportunistically removed fish client mucus while interacting with them. In the wild, taxonomic identification of *L. dimidiatus* gut content suggests that this species ingests fish mucus (Grutter 1997). In captivity, *L. phthirophagus* was found to remove and ingest considerable amount of mucus from clients while cleaning (Gorlick 1980). Interestingly, different species of cleaner wrasses in both tropical and temperate environments have been found to spend more time and more frequently with clients that have better quality of mucus (i.e., caloric value and amount of mucus produced; Gorlick 1980; Arnal and Morand 2001) and mucus load (Grutter and Bshary 2004). For example, food choice experiments suggested that *L. dimidiatus* prefer to eat parrotfish mucus instead of gnathiid isopod parasites (Grutter and Bshary 2003). However, it has been reported that gnathiid parasites constitute *L. dimidiatus'* main food source in the wild (Grutter 1997; Grutter 2000). Therefore, it is plausible that depending on the availability of parasites on the fish or their hunger level, *L. dimidiatus* may choose between various food items from its client fish (Cheney and Côté 2005).

On the other hand, the presence of fish sequences in the gut content of *L. dimidiatus* could also indicate necrotic tissue removal of wounded fish clients, being considered a mutualistic behaviour. In the wild, behavioural observations over an 18-month period found that severely wounded individual client fishes visited cleaner fishes more regularly and over longer periods of time than after their wounds were healed (Foster 1985). Moreover, these observed cleaners seemed to specifically target wounded areas (Foster 1985). Similar behaviour was observed with cleaner shrimp *Ancyclomenes* sp. in the wild, picking at clownfish wounds and reducing the lesions considerably (Grutter et al. 2020a). In captivity, injured clients with access to the cleaner shrimp *Lysmata amboinensis* exhibited a significant reduction in injury redness (i.e., inflammatory response as consequence of blood flow boost; Vaughan et al. 2018c) when compared to clients with no access to cleaner shrimp (Vaughan et al. 2018c). This suggests that cleaner shrimp help the healing process of their clients, by removing dead or infected tissues reducing therefore secondary bacterial or viral infections (Vaughan et al. 2018c). Interestingly, contrary to what was found by Foster (1985) and Grutter et al. (2020a) in the wild, experimental trials showed that *L. amboinensis*

was not specifically targeting the wounded area on the injured client (Vaughan et al. 2018c). In this study, cleaning observations of *L. dimidiatus* were not performed before collection, therefore, there is no evidence suggesting that *L. dimidiatus* was targeting wounds from their clients in the wild.

The fish DNA sequences present in *L. dimidiatus* gut content could also derive from ingestion of parasites attached to the body of *L. dimidiatus* clients, which ingest fish tissues (e.g., skin, gills) or fluids (e.g., blood, plasma). In fact, the application of invertebrate-derived DNA (iDNA) is a recently new method to understand the biodiversity of organisms through parasitic invertebrates such as terrestrial leeches (Drinkwater et al. 2021; Nguyen et al. 2021; Wilting et al. 2021). In the marine environment, fish DNA has been found in gnathiid isopod parasites' gut content (Nagel and Lougheed 2006; Jones et al. 2007; Hendrick et al. 2019). In this study, *L. dimidiatus* guts were examined to investigate the endoparasite gut community prior to preservation (**Chapter 3**; Narvaez et al. 2021b). During this process, gnathiid isopods (n = 20) were visually found in the gut of only one specimen. Furthermore, knowing that crustaceans such as gnathiids have a hard exoskeleton (Kearn 1978) and that food ingested by *L. dimidiatus* requires approximately 4 hours to pass through the digestive tract (Grutter 1996a), there is a high likelihood that the remaining 29 *L. dimidiatus* individuals did not in fact feed on gnathiids this same morning of collection. This may be unexpected knowing that the peak time of feeding on gnathiid by *L. dimidiatus* was found to be in between 8:00 h and 10:30 h in the morning (Grutter 1996a), which correspond to the time of *L. dimidiatus* collection. A potential explanation for this result may be that the collection period (i.e., October 2018) corresponds to a decline of number of gnathiids collected from the benthos across several reefs at Lizard Island (Sikkel et al. 2019). Consequently, it can be hypothesised that the decrease in gnathiid abundance may have affected *L. dimidiatus* diet, which would have fed on other food sources from their clients such as mucus, to compensate the lack of gnathiids (Cheney and Côté 2005).

In all cases, the 36 fish species found to be targeted by *L. dimidiatus* are common fish species at Lizard Island (Muñoz et al. 2006; Jones et al. 2007; Hoey and Bellwood 2008; Fulton and Bellwood 2002; Kerry and Bellwood 2016; Morais and Bellwood 2020) and therefore, reflect with accuracy our results. The presence of arthropods and molluscs in the diet of the *L. dimidiatus* may represent the removal of parasites from the body of fish clients. However, the taxonomical classification to the phylum level for

both arthropods and molluscs does not allow concluding this. This limitation may be due to the lack of sequence of organisms such as parasites (Wylezich et al. 2019) but also could be due to level of identification not low enough or incorrectly identified on GenBank (Valkiūnas et al. 2008).

Finally, nematodes from the Raphidascarididae family and the protozoan *Goussia* sp. were amplified with the *16S* universal primer from the gut contents of *L. dimidiatus*. The life cycle from a few species of nematodes from the Raphidascarididae family have been investigated and crustaceans such as copepods and isopods are commonly the first intermediate host before infecting fish as second intermediate host (González 1998; Klimpel and Rückert 2005; Ghadam et al. 2018). Therefore, it is possible that *L. dimidiatus* became infected by Raphidascarididae nematodes by removing infected crustaceans from the body of other fish. On the other hand, coccidian parasites, such as *Goussia* sp., have predominantly direct life cycle, developing in several organs of fish such as swim bladder or intestine, without intermediate hosts (Rohde 2005). Hence, it is likely that *L. dimidiatus* have been infected directly from the environment. The presence of Thaumarchaeota (Archae) and Scytomonadidae (Euglenozoa) could have been associated to ingestion of surrounding water or contamination from the environment. In fact, both microorganisms are mostly planktonic and abundant through the water column in marine environment (Liu et al. 2017; Yubuki and Leander 2018).

Cleaner shrimp gut content

Contrary to *L. dimidiatus*, only one fish species was found in the gut contents of the cleaner shrimp *Urocaridella cyrtorhyncha*, the blueback sprat *Spratelloides delicatulus* and at the phylum level, arthropods were found in the gut contents of individuals of both cleaner shrimp species. Although *S. delicatulus* is common in the GBR, it swims in large schools and is often pelagic (Froese and Pauly 2021). Also, other fish species such as *Cephalopholis cyanostigma* and *Plectropomus leopardus* (Serranidae) are more common at cleaner shrimp cleaning stations (Becker et al. 2005 and Grutter pers. obs.) but were not amplified here. Therefore, this result should be interpreted with caution since this could be the result of potential contamination.

The lack of diversity found in the gut content of the cleaner shrimp may be due to two different factors. First, due to the small size of the cleaner shrimp (1–3 cm: Bruce 1967; Fujino and Miyake 1969), whole

shrimp individuals were preserved, therefore, the concentration of shrimp DNA was substantially high in the extracted DNA (see Appendix C Table C2). This would have yielded DNA sequences in orders of magnitude higher than that of prey items. To overcome this challenge, a blocking primer targeting host shrimp sequences and the *COI* primer could have been used, however, *in-vitro* tests failed to identify any matches between the two species of cleaner shrimp and the *COI* primer set. Therefore, no blocking primers were used in combination with the *COI* universal primers. Second, cleaner shrimp species are active both diurnally and nocturnally. While the cleaner shrimp *Urocaridella antonbruunii* have been reported to clean at night in the wild (Yokes and Galil 2006; Bos and Fransen 2018) and in captivity (Vaughan et al. 2018a) cleaner shrimp from the genus *Urocaridella* were also recorded to clean during day light (Becker et al. 2005). It is possible that *U. antonbruunii* and *U.* cf. *cyrtorhyncha* have different peaks of cleaning activity in the wild that could reflect the gut content item diversity and their proportion. Therefore, more studies are necessary to determine with more accuracy the cleaning patterns of these two species of cleaner shrimp, both during the day and at night.

From the sequences yielded by the *16S* primer, the digenean *Helicometra fasciata* and the eucestode *Echinobothrium* cf. *heroniense* were present in *Urocaridella* cf. *cyrtorhyncha* and *U. antonbruunii* specimens, respectively. The digenean *H. fasciata* is cosmopolitan and has a complex life cycle where crustacean decapods are second intermediate hosts, and the final host is often the intestine of teleost fish (Bray and Cribb 1989). The life cycle of diphyllidean eucestodes, such as *E. heroniense* also include invertebrate such as amphipods, crabs and shrimps as intermediate hosts as well as teleost fish before reaching elasmobranchs as final hosts (Haseli et al. 2012). Therefore, it is highly possible that these two organisms represent parasites from the cleaner shrimp themselves, not necessarily present in the gut content.

When investigating the differences between using a blocking primer or not, very distinct results were found for each cleaner shrimp species. While the use of blocking primer reduced the number of self-hits (from 95.9% to 89.4%) in *Urocaridella cyrtorhyncha*, this was not the case with *U. antonbruunii* (99.9% to 99%). Interestingly, results with blocking primer resulted in higher bacterial sequences for both species of shrimps, with almost twice as many sequences for *U.* cf. *cyrtorhyncha* and almost five times more sequences with *U. antonbruunii* (Appendix C Table C7). The use of blocking primer has

been found to be useful in other studies to remove predator DNA (e.g., Leray et al. 2013b; Su et al. 2018; Liu et al. 2019; Takahashi et al. 2020) suggesting their importance. However, blocking primers need to be used with caution since it may also block sequences from other potential target (and unknown) species (Vestheim and Jarman 2008; Leray et al. 2013b; Piñol et al. 2015), reducing or excluding their amplification. This could have been the reason behind the small amount of eukaryote reads yielded in the sequencing run (83 and 70 eukaryote sequences compared to 179,616 and 12,144 bacterial sequences with *U.* cf. *cyrtorhyncha* and *U. antonbruunii* respectively, see Appendix C Table C7) in the presence of the blocking primer.

Finally, it is important to note that methodological factors such as the number of template-primer mismatches between universal primer and target species, concentration of blocking primer, annealing temperature and the number of PCR cycles influence the efficiency and outcome of reads (Piñol et al. 2015). In this study, different annealing temperatures as well as the concentration of blocking primer were tested beforehand to increase amplification efficiency. The number of mismatches between potential targeted prey and the blocking primer were also analysed and the number of base pairs mismatches ranged between 1 and 3 only (see Appendix C Table C9). However, during PCR reactions, blocking primer could co-block potential preys with 4 mismatches (Piñol et al. 2015; Takahashi et al. 2020). Therefore, this region targeted by the universal primer was extremely conserved across a wide range of organisms and this could have been one of the reasons why some eukaryote sequences may not have been amplified when using the blocking primer. Further studies may be needed to test other specific primers that target more specific prey items.

Conclusions

The use of DNA metabarcoding enhanced the detectability of soft tissue from the gut of the cleaner fish *L. dimidiatus*, emphasising the suitability of molecular approaches to add another perspective to the ecological role of cleaner organisms. More specifically, the *COI* primer allowed identifying client fish to the species level, highlighting a high diversity of clients (i.e., 36 species) and that *L. dimidiatus* could preferentially remove tissues from their own clients. However, lower taxonomical assignment for molluscs and arthropods was not achievable, possibly due to lack of a reference database, resulting in a

lack of information on those specific diet items during universal primer design. The fact that gnathiid isopods were not found in the diet of *L. dimidiatus*, despite being indicated as a preferred food item, could be due to the low abundance of gnathiids on the reefs at this time (Sikkel et al. 2019) or simply the choice of non-removal from *L. dimidiatus*. However, gnathiids were visually found in the gut of one *L. dimidiatus* individual but were not amplified. This suggests that gnathiid hard exoskeleton could have inhibited PCR amplification (Nagel and Lougheed 2006) and led to false-negative results. The use of blocking primer was efficient at amplifying more bacteria than in the absence of blocking primer, but was not as effective at amplifying eukaryote sequences. This may be probably because the region of the universal primer was highly conserved across organisms. Finally, further studies investigating the diet of cleaner shrimps could integrate cleaning activity of cleaner shrimp during the day and during the night to understand if their diet may fluctuate across a 24-hour period.

Chapter 6: Habitat degradation drives increased gnathiid isopod ectoparasite infection rate on juvenile but not adult fish

Published as: Pauline Narvaez, Renato A. Morais, Kate S. Hutson, Mark I. McCormick, Alexandra S. Grutter (2021) Habitat degradation drives increased gnathiid isopod ectoparasite infection rate on juvenile but not adult fish. *Coral Reefs* 40:1867–1877. https://doi.org/10.1007/s00338-021-02166-y

Abstract

Widespread coral mortality is leading to coral reef degradation worldwide. Many juvenile reef fishes settle on live coral, and their predator-avoidance behaviour is disrupted in seawater exposed to dead corals, ultimately increasing predation risk. Gnathiid isopods are micropredatory fish ectoparasites that occur in higher abundances in dead coral. However, the effect of seawater associated with dead coral on the susceptibility of fish to micropredators has never been investigated. We tested whether the infection rate of cultured gnathiid ectoparasites on individual damselfish, *Pomacentrus amboinensis* from two different ontogenetic stages (juveniles and adults) was influenced by seawater exposed to three different treatments: dead coral, live coral, or no coral. Seawater treatments were presumed to contain different chemical properties and are meant to represent environmental changes associated with habitat degradation on coral reefs. Gnathiid infection of juvenile fish in seawater exposed to dead coral was twice as high as that of fish in live coral or no coral. Infection rates did not significantly differ between live coral and no coral treatments. In contrast to juveniles, the susceptibility of adults to gnathiids was not affected by seawater treatment. During experiments, juvenile fish mortality was relatively low, but was higher for infected fish (9.7 %), compared to fish held without exposure to gnathiids (1.7 %). No mortality occurred in adult fish that became infected with gnathiids. Our results suggest that chemical cues released from dead corals and/or dead coral colonisers affect the ability of juvenile, but not adult fish to avoid parasite infection. Considering increased habitat degradation on coral reefs and that gnathiids are more abundant in dead coral substrate, it is possible that wild juvenile fish may experience increased susceptibility to parasitic infection and reduced survival rate. This highlights the importance of including parasitism in ecological studies of global environmental change.

Introduction

Coral reefs are one of the richest and most biologically diverse ecosystems in the world (Burt et al. 2020). However, coral reefs are particularly vulnerable to habitat degradation from stressors including pollution, overfishing and climate change (McCauley et al. 2015). Following long periods of thermal stress, corals may lose their algal symbionts and bleach. These bleaching events may be reversible but, if the thermal stress is too extensive and prolonged, damage can be permanent and lead to coral mortality (Hughes et al. 2017). In the aftermath, a series of organisms including algae, bacteria, and other invertebrates begin to settle on the coral skeleton (McCormick et al. 2017a). In recent years, coral bleaching, storms, and crown-of-thorns starfish outbreaks have led to high coral cover losses on the Great Barrier Reef (GBR) (see MacNeil et al. 2019). Because corals are ecosystem engineers on reefs, any loss of coral can disrupt other closely associated organisms.

Many coral reef fishes rely on coral substrate and structure to feed (Graham et al. 2009; Huertas and Bellwood 2018), to use as habitat and shelter (Khan et al. 2017; Morais et al. 2020), and as nursery habitat (Jones et al. 2004; Levin 2006; Almany et al. 2017). Most coral reef fishes have a larval pelagic phase (Leis and McCormick 2002), and this phase is thought to be a strategy to enhance dispersal, access food resources in the water column and avoid reef predators and parasites (Strathmann et al. 2002; Duong et al. 2019). While the pelagic phase is vital for most fishes, settling back on the reef can also present many challenges, such as competition, predation, and parasitism (McCormick and Holmes 2006; Bonin et al. 2009; Grutter et al. 2017). Juvenile damselfish rely extensively on sensory cues, particularly olfactory cues to settle near conspecifics (Lecchini et al. 2005). Habitat degradation may add further challenges during this phase. For example, chemical alarm cues released from dead corals can affect risk assessment in juvenile damselfish species by reducing neophobic behaviour, essential to avoid predation (McCormick et al. 2017b). In the wild, this translates to lower survival rates of fish previously exposed to chemical cues from dead coral colonisers (McCormick et al. 2017b). These chemical cues associated with dead coral also reduce the escape response of juvenile damselfish species (McCormick and Allan 2017) and hinder the ability of fish to react to odour alarm cues (McCormick et al. 2017a).

While the pelagic habitat may allow embryos and larvae of fish to avoid reef parasites ('parasite avoidance hypothesis', see Duong et al. 2019), upon settlement juvenile fish remain vulnerable to ectoparasite infection, particularly from temporary blood-feeding parasites (i.e., micropredation, Lafferty and Kuris 2002). Gnathiid isopods, common ectoparasites in tropical environments (Grutter et al. 2011a), are temporary ectoparasites and are often characterised as 'mosquitoes' or 'ticks of the sea' (Grutter et al. 2011b; Santos and Sikkel 2019) and hence also micropredators. While gnathiids have three parasitic stages as larvae, they are not parasitic as adults and stay in the benthos to reproduce. After hatching, they emerge from the substrate, find and attach to a host for a few minutes to several hours until engorged with host fluids (e.g., blood and plasma) before returning to the benthos to moult (Smit and Davies 2004). Newly settled fish can succumb to gnathiid infection, even when infected with only one or a few gnathiid individuals (Grutter et al. 2008; Artim et al. 2015; Grutter et al. 2017). Additionally, gnathiid infections can also drastically decrease the swimming performance (Grutter et al. 2011a), escape response of juvenile fish (Allan et al. 2020) and reduce fish growth within the first days of settlement (Jones and Grutter 2008). Gnathiids prefer or occur more often in dead and degraded coral microhabitat compared to live coral (Artim and Sikkel 2013; Santos and Sikkel 2019; Paula et al. 2021) likely due to risk of predation from live corals (Artim and Sikkel 2013; Paula et al. 2021). Paradoxically, decreases in gnathiid abundance in the benthos have been associated with bleaching events in the GBR (Sikkel et al. 2019), possibly to due in part to an acute effect of increased temperature on gnathiid survival rate (Shodipo et al. 2020). However, in the subsequent cooler months post-bleaching, the abundance of gnathiids was higher and comparable to non-bleaching months, suggesting that, in the long term, the loss of coral cover was favourable to gnathiid recovery (Sikkel et al. 2019).

Despite the body of work associating habitat degradation to disruption of chemical alarm cues for juvenile fish and to changes in gnathiid abundance, potential effects on infection rates and survival of infected fishes have not been evaluated. The aim of this study was to compare the infection rate of gnathiid isopods on juvenile and adult common coral reef damselfish *Pomacentrus amboinensis* exposed to three different seawater treatments mimicking degraded (i.e., dead coral substrate covered by a variety of colonisers; McCormick et al. 2017a), non-degraded (i.e., high live coral cover) reefs and

seawater not recently exposed to corals (control). Our prediction was that seawater mimicking degraded conditions would hamper the ability of fish to evade gnathiid attachment and feeding.

Methods

Fish and coral collection and location

This study was conducted at Lizard Island Research Station (14° 40' S, 145° 28' E) during two austral summers in 2018 (from 18 November to 17 December) and 2020 (from 11 to 20 February). Experiments with both juvenile and adult *P. amboinensis* were carried out during different years due to space and time constraints. The reefs around Lizard Island group, northern Great Barrier Reef (GBR), where this study was conducted, have been severely affected by two severe tropical cyclones (categories 4 and 5) and two major bleaching events between 2014 and 2017. Consequently, the coral cover around Lizard Island declined abruptly over this period (up to 80 %, Madin et al. 2018).

Juveniles of *P. amboinensis* (n = 534) were collected in 2018 using light traps deployed at night and returned to the laboratory by early morning (07:00) and transferred to a 32 L tank (dimensions: 38.7 x 28.6 x 29.8 cm; L x W x H) with air stones and constant water flow, including Polyvinyl Chloride (PVC) pipes of different sizes for shelter. These fish were used for the experiment within two to four days of capture.

Adult *P. amboinensis* (n=60) were collected in 2020 using a dilute solution of the anaesthetic clove oil and hand nets on fringing reefs around Lizard Island. Fish were brought back to the laboratory and transferred to individual mesh cages (dimensions: 20 x 20 x 10 cm; L x W x H, mesh size: 2 mm) to minimise aggressive behaviour inside six 300 L holding tanks, where they were maintained for at least 48 h prior to their use in experiments. Juveniles and adult *P. amboinensis* were fed twice daily with live *Artemia* and frozen mysid shrimp, respectively.

To test if seawater that originated from different sources influenced the infection rate of gnathiids on fish, live and dead coral (covered with a variety of algae and other associated organisms, which occur naturally and quickly colonise corals after corals die, McCormick et al. 2017b) *Pocillopora damicornis* colonies (3 to 5) of approximatively 30 cm diameter were collected from the reef. Live and dead coral

colonies were separately transferred to a header tank (32 L) with a seawater flow system and, to avoid loss of live coral due to thermal and oxygen stress, fresh seawater flowed continuously at high rate (~1 L/min) with air stones for aeration (following McCormick et al. 2017b). The same conditions were applied for the dead coral colonies. Fresh seawater was provided by the piping system from the research station and sourced directly from the back reef lagoon, from a sandy area with no reef growth and at a depth of ~ 0.5 to 2 m depending on the tide. Coral colonies that presented signs of thermal stress were replaced before the next trial.

Laboratory studies

Gnathiid infection rates on juvenile and adult fish were recorded in three seawater treatments: a) seawater that flowed through a header tank with live coral *Poc. damicornis*; b) seawater that flowed through a header tank with dead *Poc. damicornis*; and c) seawater with no coral that flowed from the research station seawater system. Ectoparasite gnathiid isopods *Gnathia aureamaculosa* were sourced from a culture maintained at Lizard Island (see Grutter et al. 2020b). For the experiment with juvenile *P. amboinensis*, gnathiid larval stage two and three (the infective unfed stage) were used. For the experiment with adult *P. amboinensis*, stage three gnathiid larvae were used. Stage three gnathiids are often used in experiments because they are larger (~1.5 mm), which makes them easier to find and collect (Grutter et al. 2011a). However, because of the lower availability of third stages in the gnathiid culture, stage two gnathiids were also used for two of the juvenile trials.

Seawater temperature was measured at the beginning and the end of the trial (mean temperature ± SE was 25.7 ˚C ± 0.23 ˚C for Experiment 1 and 30.4 ˚C ± 0.12 ˚C for Experiment 2). For the experiment with juvenile *P. amboinensis*, dissolved oxygen (DO) levels were also measured on the first day of trial (mean DO ± SE at the beginning of the trial: 100.3 % ± 0.4%) and at the end of the trial (97.85 % ± 0.1%). No additional air supply was considered necessary. While Experiment 1 with juvenile fish was conducted outdoors and thus exposed to natural light, Experiment 2 with adults was conducted indoors due to laboratory space limitations. To examine potential variation in infection rates due to changing moonlight levels (Grutter et al. 2000; Welicky et al. 2013), moon luminosity (percentage of maximum

luminosity) was estimated using the date of every trial. Luminosity was obtained using a specialised astronomical website (©Astronomy Know How 2019) and was used as a proxy of lunar cycle (moon luminosity peaks during full moon and is lowest at new moon). Before the start of the experiments, each experimental tank was scrutinised for unintended organisms (e.g., fragments of algae or mobile invertebrates) that could have flowed from the header tanks to the experimental tanks.

Experiment 1: Gnathiid infection rate on juvenile fish

The protocols for this experiment followed Allan et al. (2020). Each day, and an hour before the experiment, *P. amboinensis* (mean size ± SD, 12.1± 0.6 mm, standard length [SL]) were fed to satiation with live *Artemia* to avoid predation on parasites. Afterwards, 60 fish were individually allocated to 900 mL non-recirculating black containers (dimensions: 15.5 x 10.5 x 5.5 cm; L x W x H). Black containers maximise contrast with the white-coloured parasite and facilitate monitoring. Trials were conducted each night, with the three seawater treatments randomly assigned to the 60 containers, with 20 containers per treatment. In each of half of the containers per treatment (n = 10), a single gnathiid individual was carefully added using a pipette (total gnathiids used each day = 30). The other ten replicates were used as procedural controls (i.e., no gnathiid, to control for effect of procedures on fish). The trial started at 1800 hrs and fish remained in the container for 12 h (end of the trial). During the trial, fish and gnathiids (gnathiid treatment only) were visually checked every two hours with a red light to record possible gnathiid infection on the fish (i.e., gnathiid attachment on fish or feeding status, based on the presence or absence of an engorged gut) or previous infections (gnathiid had dropped off fish and was categorised as “fed”). Fish mortality and absence of the gnathiid in replicate aquaria was also recorded to account for possible micropredation by the gnathiid on the fish resulting in fish mortality, and predation on the gnathiid by the fish, respectively. The experiment was repeated for ten nights over a period of one month, using new *P. amboinensis* and gnathiids for trials, with fish and gnathiids only being used once, totalling 99 replicates per treatment with gnathiid exposure (during one of the trials, 27 parasites were used instead of 30 because of the low availability of gnathiids, i.e., one replicate reduced for each treatment). Whether the gnathiid was fed or unfed during the experiment and/or at the end of the trial was used as the response variable for fish susceptibility.

Experiment 2: Gnathiid infection rate on adult fish

Each day, six adult *P. amboinensis* (mean size ± SD, 56.8 mm ± 8.3 mm, SL) were individually allocated to 32 L tanks (dimensions: 38.7 x 28.6 x 29.8 cm; L x W x H). Before the experiments, fish were fed to satiation with mysid shrimps. The same three water source treatments as above were randomly assigned to the tanks and air stones were added to each tank to maintain oxygen levels. Each night, 12 tanks were used as follows: four replicate tanks randomly assigned per seawater treatment (dead coral, live coral, no coral). Out of the 12 tanks, half included only gnathiids but no fish (to compare the recovery rate of gnathiids after the experiments and to account for potential predation from the fish on gnathiids) and in the other half, fish and gnathiids were introduced. Five gnathiids that had been previously transferred from the culture tank to a 5 mL vial (half seawater, half air) were added to each tank (n = 60 gnathiids used per day). The trial started at 1800 hrs and continued for 12 h. Fish (fish treatment only) and gnathiids were checked as above but at 4 h intervals (2200, 0200, 0600 hrs). At the end of the trial, fish were removed from the experimental tanks and placed in a dechlorinated freshwater bath for 5 min to remove any attached gnathiids (Grutter et al. 2020b). Fish were then measured for length [total length (TL), SL, cm] and returned to a seawater tank to recover, prior to being released to the collection sites. The seawater from experimental tanks and from the fresh- and seawater bath was filtered with a 60 µm sieve and examined for gnathiids under a stereomicroscope. The experiment was repeated over ten consecutive nights, using new *P. amboinensis* and gnathiids for trials, with fish and gnathiids only being used once, with a total of 20 replicates per treatment. Fed and unfed gnathiids were counted to indicate the number of gnathiids that successfully infected (fed upon) fish from the original number introduced (n = 5 per tank).

Statistical analyses

Given the potential relationship between gnathiid infection rates and the lunar cycle (Grutter et al. 2000; Welicky et al. 2013), the differential availability of experimental facilities during the two experiments (outdoors for juvenile and indoors for adult *P. amboinensis*), and that the two experiments were done during different lunar cycles, I tested for a potential correlation between the proportion of maximum

moon luminosity and gnathiid infection rates for both experiments. For juvenile fish, a binomial Generalised Linear Model (GLM) was performed, using the proportion of maximum luminosity as the predictor variable and the presence *vs* absence of infection by the gnathiid for each individual fish exposed to a gnathiid (i.e., only one gnathiid added per individual fish tank) as the response variable. For adult *P. amboinensis*, a binomial GLM was also performed with the proportion of maximum luminosity as the predictor variable. However, because I used five gnathiids per individual fish tank instead of one, the response variable comprised the number of successful gnathiid infections (fed gnathiids) per fish relative to the number of unsuccessful infections (unfed gnathiids) (see Appendix D Fig. D1).

A clear effect of the proportion of maximum luminosity was detected on the infection rates for juvenile *P. amboinen*sis in the first experiment (GLM: $p = 0.017$; Fig. D1B), but not for adults in the second experiment (GLM: $p = 0.16$; Fig. D1A). Following these results, and also to account for other potential confounding effects associated with the consecutive dates of the experiments, but not with moon luminosity, for the subsequent analyses, model selection was performed involving: 1) a model including the proportion of maximum moon luminosity (numerical) as a fixed effect; 2) a model including the date of the experiment (categorical) as a random effect; and 3) a model without any of these variables. To keep consistency, model selection was performed for both experiments, *i.e.,* juveniles and adults. Models were selected by comparing their Akaike Information Criterion (AIC) and derived Δ AIC.

The effect of the three different seawater treatments (dead coral, live coral, no coral) on the infection rate of gnathiids on juvenile *P. amboinensis*, was tested by binomial Generalised Linear and binomial Generalised Linear Mixed-Effects Models (GLM and GLMM, as per the model selection above). Infection rate (presence or absence of a fed gnathiid) was used as the response variable and the seawater treatment and the stage of the parasites (second or third larval stage) were used as core fixed factors present in all three models compared (*i.e.,* with the proportion of maximum luminosity, with date, or without any of these). Here, there was clear evidence that including date as a random effect, but not proportion of maximum moon luminosity, improved the model (AIC GLMM with date = 278.3; Δ AIC GLM with moon = 4.00; Δ AIC GLM none of these = 10.78), and therefore this model was chosen. Body size varied very little among juvenile fish (CV = 5.1%), therefore was not included in the model.

For adult *P. amboinensis*, a binomial GLM and GLMM (as per the model selection above) were also used to determine if the number of successfully fed gnathiids per fish relative to the number of unsuccessful infections (from a total of five gnathiids added per individual fish tank) was influenced by the seawater treatment (dead coral, live coral, no coral). The body size (SL, cm) was included as an offset (i.e., a variable with fixed coefficient of 1 rather than an estimated coefficient) in the model to account for size heterogeneity in adults (CV = 14.7%). Contrary to juveniles, no evidence of improvement in the model was found by adding date as a random effect or proportion of maximum moon luminosity as a fixed effect (AIC GLM none of these = 167.0; Δ AIC GLMM with date = 0.82; Δ AIC GLM with moon = 1.86), and therefore the simpler model without these variables was kept.

Since not all juvenile fish exposed to a gnathiid became infected, the exposed fish were categorised into two post-trial groups, "fish exposed to a gnathiid and infected" and "fish exposed and not infected", in addition to the group of "fish not exposed to gnathiids". This is because being infected is expected to increase the likelihood of mortality for an individual. Therefore, to determine if the mortality rate of juvenile *P. amboinensis* was influenced by these three post-trial categories and/or the seawater treatments, a binomial GLMM was performed using fish mortality (yes or no) as the response, and seawater treatment and post-trial category as fixed factors and the date of the experiment as a random factor (following the detection of an effect of date on infection above). To calculate the probability of a fish dying in each post-infection category, the Estimated Marginal Means (EMMs) was calculated based on the GLMM model above. No adult fish died in Experiment 2, therefore, this analysis was not performed for adult *P. amboinensis*.

Finally, to test for potential predation by adult *P. amboinensis* on gnathiids, the rate of recovery of gnathiids was compared between tanks with or without fish at the end of the experiment (from the 5 gnathiids originally added to each tank). A Poisson GLM was used with the number of recovered parasites as the response variable and the treatment (with *vs* without fish) as a predictor variable.

For all analyses, the treatment "live coral" was used as the reference factor for comparison with the treatments "dead coral" and "no coral". This was done by reordering the levels of these three factors.

All statistical analyses were undertaken using the software R version 4.0.2 (R Core Team 2020), including the packages glmmTMB (Brooks et al. 2017) and emmeans (Lenth 2020).

Results

Gnathiid infection rate on juvenile fish

The infection rate of gnathiids on juvenile *P. amboinensis* varied from 7 % to 37 % among trials (10 trials in total), representing a mean (± SE) of 5.6 ± 1.31 gnathiids infecting juvenile fish per trial out of the 30 exposed to gnathiids (except for one trial with 27 gnathiids instead of 30). Of the 297 gnathiids exposed to fish, 101 gnathiids (34%) were lost and were presumed consumed, representing a mean (± SE) of 10.1 ± 1.7 gnathiids per trial. The infection rate of gnathiids differed among the three seawater treatments, but was not different between the two gnathiid larval stages (GLM test: p = 0.88; Appendix D Table D1; Fig. 19). When averaged across parasite life-stage, the probability of a fish in the dead coral treatment being infected by gnathiids (24.9 ± 8% SE; Table 6) was almost twice as high as the no coral (11.8 ± 5% SE; Table 6) and live coral (12.8 ± 5% SE; Table 6) treatments (GLM test: p = 0.03, Fig. 19; Appendix D Table D1).

Table 6 Marginal mean probability of gnathiid infection for juvenile *Pomacentrus amboinensis* in the three water treatments tested (no coral, live coral and dead coral) including lower and upper 95 % confidence levels

Water treatments	Probability	Std. Error	Df	Lower CL	Upper CL
No coral	0.118	0.05	292	0.05	0.25
Live coral	0.128	0.05	292	0.05	0.27
Dead coral	0.249	0.08	292	0.12	0.43

There was no difference in gnathiid infection success between the live coral seawater treatment and the no coral control (GLM test p = 0.84, Fig. 19; Appendix D Table D1).

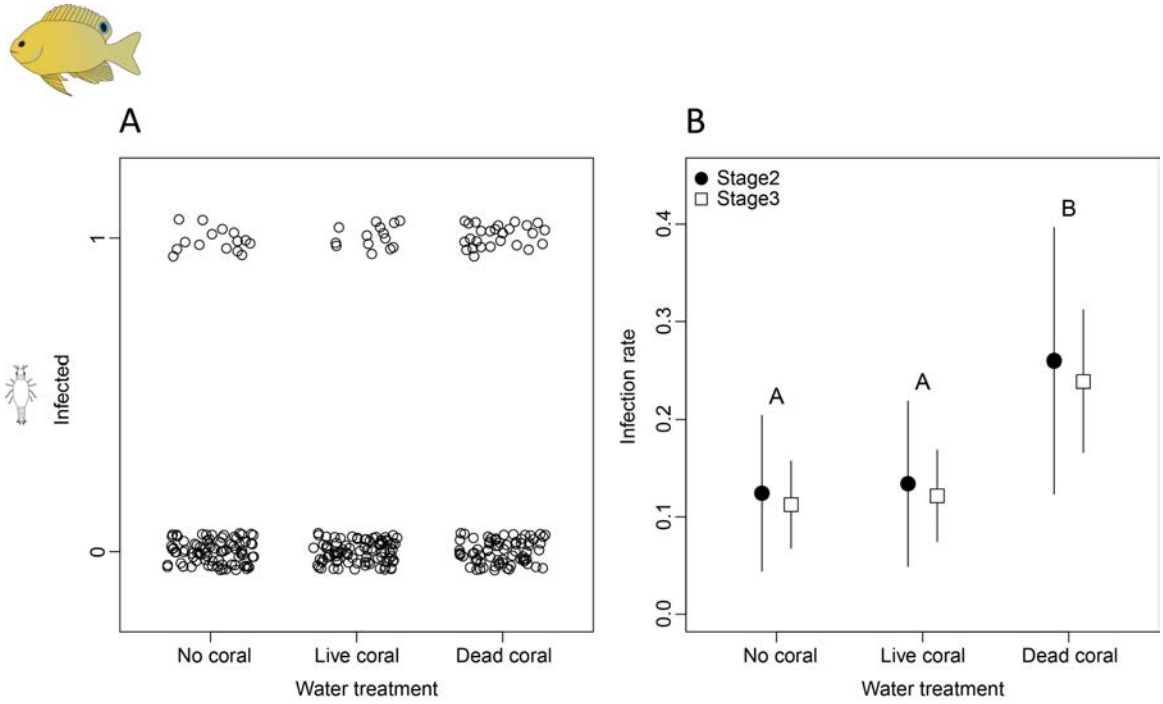

Figure 19 (A) Infection status for each juvenile fish *Pomacentrus amboinensis* tested (n=297) in Experiment 1 in the three seawater treatments with 0 representing "non-infected" and 1 "infected". (B) Infection rate of gnathiid on juvenile fish *P. amboinensis* in the three seawater treatments with stage 2 and 3 representing the larval stages of gnathiid used for the experiments. The letters A and B indicate significantly different groups

The overall mortality of juvenile fish was low (4.7 %) and did not differ between seawater treatments (Appendix D Table D2) but did differ between the infection categories (Appendix D Table D2). Fish that were exposed to a gnathiid and were infected had a higher probability of mortality (9.7 ± 7% SE, Table 7) than both fish that were not exposed to a gnathiid (1.7 ± 1% SE, GLM test: p = 0.003; Appendix D Table D2), and fish that were exposed to a gnathiid and were not infected (1.6 ± 1%, GLM test; p = 0.004; Appendix D Table D2).

Table 7 Marginal mean probability of mortality for the infection categories of juvenile *Pomacentrus amboinensis* including lower and upper 95 % confidence levels

Infection categories	Probability	Std. Error	Df	Lower CL	Upper CL
Fish exposed and infected	0.098	0.07	468	0.02	0.33
Fish exposed and not infected	0.016	0.01	468	0.004	0.07
Fish non exposed	0.017	0.01	468	0.004	0.07

Gnathiid infection rate on adult fish

From the five gnathiids exposed to each adult *P. amboinensis*, a mean (± SE) of 2.17 (± 0.31) gnathiids infected the fish was found in the no coral treatment, a mean of 2.22 (± 0.31) gnathiids infected the fish in the live coral treatment, and 2.12 (± 0.31) gnathiids infected fish in the dead coral treatment. No differences were found between gnathiid infection on fish between live coral and dead coral treatments (GLMM: p = 0.71; Fig. 20; Appendix D Table D3) and from no coral and live coral (GLMM: p = 0.76; Fig. 20; Appendix D Table D3).

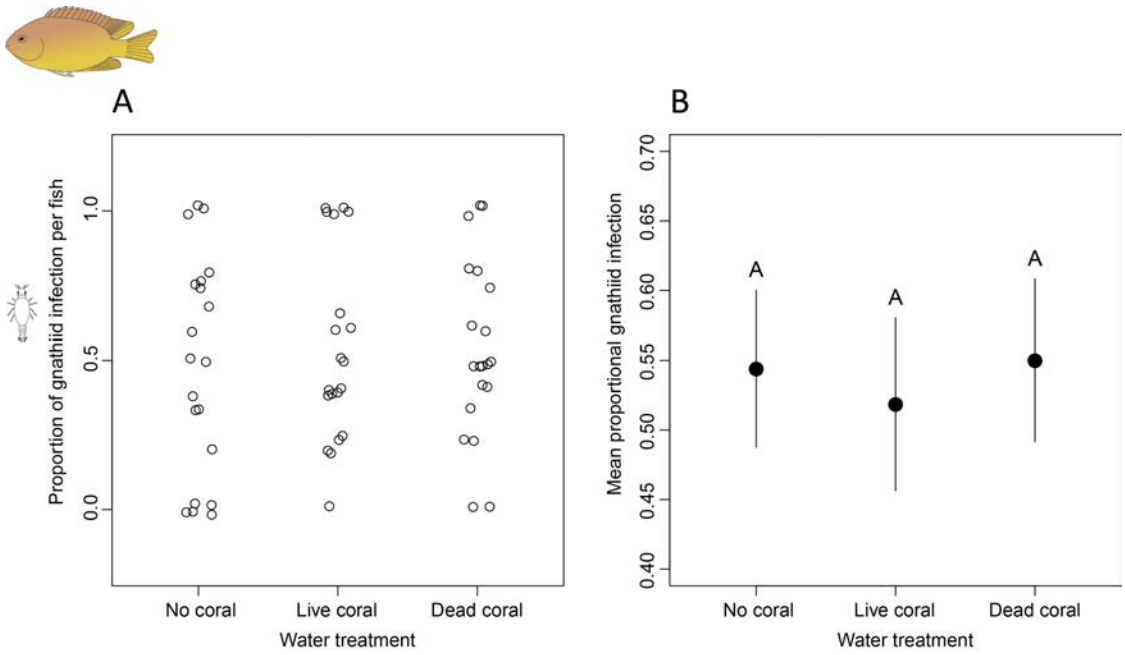

Figure 20 (A) Proportion of gnathiids (number of successfully fed gnathiids per fish relative to the number of unsuccessful infections, from a total of five gnathiids added per individual fish tank) infecting all adult fish tested (n=60) in Experiment 2 in three different seawater treatments. (B) Mean (± SE) proportion of the infection of gnathiids on adult fish *Pomacentrus amboinensis* in three different seawater treatments. The letter A indicates no statistically significant differences between categories

To account for possible predation from *P. amboinensis* on gnathiids during the experiment, the recovery rate of gnathiids in each tank (fish *vs* no fish) was compared and found no difference between tanks with fish and tanks with no fish (Poisson GLM test: p = 0.56) with a mean (± SE) of 4.13 (± 0.1) gnathiids recovered in tanks with fish (out of the 5 initially added) and a mean of 4.36 (± 0.1) gnathiids recovered in tanks with no fish (out of the 5 initially added). No adult fish mortality was observed.

Discussion

Coral reefs are increasingly exposed to stressors with associated changes in seawater chemistry, which may also affect the survival of coral reef organisms (e.g., Lecchini et al. 2014; McCormick et al. 2017a, b). Here, I tested whether parasite infection rate might be impacted by different seawater treatments associated with healthy and degraded coral reef habitats. I found that juvenile and adult damselfish are impacted differently when subjected to seawater flowing through dead compared to live coral, or to water that has not flowed through coral. Juvenile fish were twice as likely to be infected by gnathiids when exposed to dead coral seawater compared to live coral or no coral, while adult infection rate was not affected by seawater treatment.

For juvenile fishes, selecting suitable settlement habitats relies on sensorial cues including visual, vibratory and, particularly olfactory cues which provide mechanisms to detect conspecifics (Lecchini et al. 2005). Suitable settlement habitats are crucial for the survival and growth of these fishes (Lecchini et al. 2005). Previous studies have demonstrated that juvenile damselfishes are impacted by the cocktail of odours emanating from dead corals (McCormick et al. 2019a). For example, neophobia behaviour and predator-escape response of juvenile damselfish are negatively impacted by seawater flowing through dead coral (e.g., McCormick et al. 2017a; McCormick and Allan 2017). From the most common organisms found colonising dead corals in our study area, it appears that filamentous cyanobacteria *Okeania* sp., benthic diatoms *Pseudo-nitzschia* sp., and the red algae *Galaxaura rugosa* are the organisms responsible for hindering damselfish responses to alarm odour cues. (McCormick et al. 2017b). While it has been shown that anthropogenic contaminants affect signal receivers in fish (Ward et al. 20008; Van der Sluijs et al. 2011), to the best of our knowledge, which compounds released by dead coral and/or their colonisers impair behavioural responses of juvenile fish, and the physiological mechanisms involved are still largely unknown. Nevertheless, taking into consideration previous studies investigating the effect of chemical cues released by dead coral on juvenile fish (e.g., Lecchini et al. 2014; McCormick et al. 2017a, b; McCormick and Allan 2017; McCormick et al. 2019a, b), it is highly plausible that emanating compounds affect directly the sensorial cues used by juvenile fish in this critical phase. Consequently, physiological mechanisms such as stress responses and parasite

avoidance behaviour may be altered and warrant further investigation. Our results suggest novel and negative impacts that degraded environments containing dead coral may have for *P. amboinensis* settlement success (see Fig. 21) via enhanced micropredation from gnathiid isopod parasites.

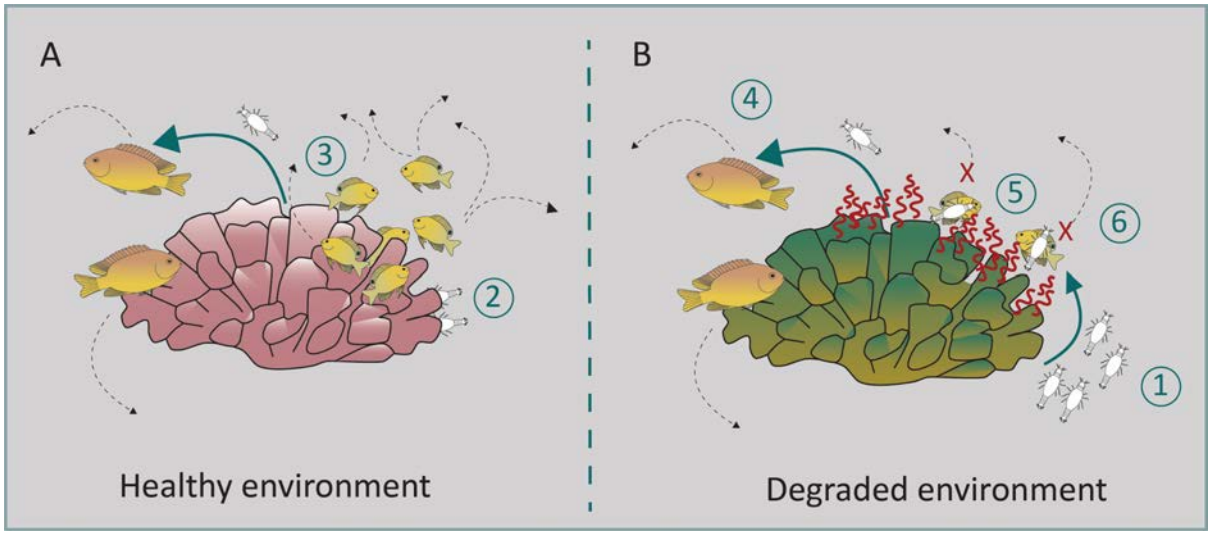

Figure 21 Schematic illustration of (A) a healthy and (B) a degraded environment proposed based on results from previous studies and this study. (1) Gnathiids have been found to be more abundant in dead coral compared with live coral microhabitats, habitats representing degraded compared to healthy environments (Santos and Sikkel 2019) and (2) this pattern could in part be due to predation from corals upon gnathiids (Artim and Sikkel 2013; Paula et al. 2021). (3) While juvenile *Pomacentrus amboinensis* damselfish preferentially choose to live in a healthy over a degraded environment, (4) adult *P. amboinensis* do not show any preference (McCormick and Weaver 2012). (5) This may be due to chemical cues released by dead coral (red wavy lines) that affect juvenile fish behaviour (e.g., McCormick and Allan 2017; McCormick et al. 2017a). The green arrows represent gnathiids emerging from the benthos and searching for a suitable host. The dotted arrows represent fish swimming away, avoiding gnathiids. (6) I propose that in a degraded habitat, juvenile fish may be more susceptible to gnathiid infection (red crosses) than in healthy habitats, and this may be due to chemical cues released by dead coral (this study). Gnathiids are not represented to scale

In contrast to juveniles, the susceptibility of adult *P. amboinensis* to gnathiids was not affected by seawater treatment. Interestingly, while the impact of chemical cues associated with habitat degradation on juvenile fish (all involving damselfish) have been extensively investigated (e.g., McCormick 2012; Chivers et al 2016; McCormick et al. 2017a, b; McCormick and Allan 2017), until the present study, no other study had performed similar experiments with adult damselfish. Nevertheless, behavioural responses in a species of predator fish, *Pseudochromis fuscus* (of similar size to the adult *P. amboinensis* tested here) exposed to the same seawater treatments as here were also not affected by seawater

treatment (Natt et al. 2017). This suggests that adult fish are not physiologically affected by changes in seawater chemistry taking place after coral mortality and may thus be able to avoid chemical cue interference (Natt et al. 2017). Thus, it is not impossible that juvenile reef fish, which rely extensively on olfactory cues, are particularly vulnerable to chemical cues emanating from dead coral compared to adults, resulting in higher stress and disrupted parasite avoidance capacity. Supporting this idea, although newly settled *P. amboinensis* settle preferentially on live coral (70%) compared to dead coral (20%) and rubble (10%) substrates (McCormick and Weaver 2012), older individuals (more than a month old) have comparable abundance across these three habitats (McCormick and Weaver 2012). This pattern suggests that habitat preferences may switch with ontogeny for *P. amboinensis*, possibly related to enhanced tolerance to chemicals released by colonisers of dead coral substrate (see Fig. 21). Finally, there are a number of other factors that may have masked the impacts of habitat degradation on gnathiid infection rates on adults including: 1) possible prior infections by wild gnathiids on adult fish, which could induce immune responses (Jenkins et al. 2018); 2) the development of behavioural avoidance strategies (Sarabian et al. 2018) and 3) non-quantified inter-annual variability between experiments.

Since gnathiid infections were either higher (juveniles) or similar (adults) in seawater from dead coral compared to live coral, it appears that gnathiid infection behaviour was not adversely affected by the chemicals released in the treatment mimicking degraded habitat. Ectoparasites rely heavily on sensory information to detect and attach to suitable hosts, mostly from chemical (e.g., Kearn 1986; Mikheev et al. 2004; Skilton et al. 2020) and visual (Genna et al. 2005) cues. Gnathiids have also been found to use cues to locate and attach to their hosts. For example, under laboratory conditions, nocturnal species of gnathiids use olfactory cues more efficiently than diurnal species (Nagel et al. 2008). In contrast, diurnal gnathiids use vision more efficiently in response to ambient light than nocturnal species (Nagel et al. 2008). Interestingly, gnathiids show habitat selection and preference for degraded reef substrate (Santos and Sikkel 2019; Artim and Sikkel 2013) compared to live corals. Furthermore, it has been also found that live corals prey on gnathiids (Artim and Sikkel 2013; Paula et al. 2021; see Fig. 21). Based on the information available on live coral preying on gnathiids and that gnathiids rely on olfactory cues, it is possible that gnathiids may respond to chemical cues released by live coral and adopt a predator

avoidance behaviour. However, no difference was found in the infection rates of adult fish between live and dead coral, therefore, we currently have no support for this hypothesis.

Few studies have experimentally evaluated the effects of environmental changes that lead to habitat degradation on marine fish ectoparasites. These have included the effects of thermal stress (Shodipo et al. 2020) and ocean acidification (Paula et al. 2020) on gnathiid survival rates. Our study, however, is the first to integrate the effect of the chemistry dynamics of corals on gnathiid infection rates. It is important to highlight that habitat degradation encompasses a multitude of processes and community states. While some stressors associated with environmental changes (e.g., high water temperature, eutrophication, fishing pressure, ocean acidification, coral cover loss) will drive positive, others will lead to negative impacts on parasite abundance and prevalence in marine systems (e.g., Lafferty 1997; Lafferty and Holt 2003; Wood et al. 2014; MacLeod and Poulin 2015; Marcogliese 2016; Artim et al. 2020). The dynamic association between habitat degradation and parasitic infection is complex and depends on factors such as parasite life cycle, degree of host dependence and change in host susceptibility due to habitat degradation (Lafferty and Holt 2003). Some types of habitat degradation such as eutrophication are favourable for specific groups of parasites (e.g., nematodes, monogeneans, cestodes, acanthocephalans and digeneans; Lafferty 1997) with a propensity for increased parasite virulence and density (Brunner and Eizaguirre 2016). On the other hand, higher levels of chemical and biological pollution (such as ammonium, nitrate, phosphate, turbidity) have been shown to reduce the abundance of ergasilid crustacean ectoparasite on cardinal fish in New Caledonia, suggesting a possible susceptibility of the ectoparasites to polluted conditions (Sasal et al. 2007). Additionally, parasites that rely on host-density for transmission, could be negatively affected by host depletion due to habitat degradation (Lafferty 2013). At the reef scale, further studies are needed to understand how other groups of common parasites are affected by coral mortality and how this may impact infection rates and survival of the fish host.

Combining our results with current knowledge on 1) the impact of chemical cues released by dead coral and their colonisers on juvenile fishes (e.g., McCormick and Allan 2017, McCormick et al. 2017 a, b); and 2) the impacts of gnathiid infection on juvenile fish (Grutter 2008; Grutter et al. 2011a; Allan et al. 2020), negative parasite-driven impacts on juvenile fish due to coral death are likely. This could have

broad implications for population and community dynamics in degraded environments. For adult fish, however, no evidence was found of an impact of coral death on gnathiid infection rates. Moreover, there were no detectible effects of dead coral water chemistry on gnathiid isopods directly, and this is perhaps unsurprising due to their preference to reside in dead coral rubble habitats. This study thus provides a better understanding of the small-scale interactions between fish and a single parasite species in a degraded environment. Coral reefs contain a myriad of other parasite groups (Rohde 2010, Cribb et al. 2014) for which the dynamics between parasites and their hosts is very complex. Further research on whether or how host-parasite relationships respond to coral reef degradation hold the potential for novel insights on the ecological limits of parasitism as a whole.

Chapter 7: General Discussion

A novel perspective of cleaning symbiosis

Cleaning symbiosis comprises in its essence a complex and, above all, dynamic relationship between organisms. The ecological role of cleaner organisms and, particularly, of cleaner fishes, has intrigued scientists, and therefore featured in aquatic research endeavours for many decades. These years of research have brought a vast amount of knowledge on the role of cleaners in their environment. Clearly, cleaners exert positive ecosystem impacts, both directly, by removing unwanted items from the body of other fishes (Grutter 1999a; Cheney and Côté 2005), and indirectly, by attracting and stimulating fish recruitment, and therefore boost the abundance and biodiversity on reefs where they inhabit (Waldie et al. 2011; Sun et al. 2015; see **Chapter 1**). From an evolutionary perspective, the emergence of specialised physical traits facilitating cleaning throughout the phylogenetic tree, including morphological features such as body shape, feeding mechanics (Baliga and Metha 2016; Baliga et al. 2017; Baliga and Metha 2019) and colour patterns (Arnal et al. 2006) have also been extensively investigated. Physiological studies have focused on dedicated cleaner fishes, aiming to understand the effects of certain chemical compounds, such as cortisol, arginine vasotocin and dopamine on the brain activity and subsequently, social interactions among cleaners and with other fishes (e.g., Soares et al. 2012; Soares et al. 2014; Messias et al. 2016).

Despite this vast and detailed body of work on cleaning symbiosis, and despite cleaners often engaging in intimate interactions with their clients, studies exploring potential mechanisms of parasite transmission during cleaning interactions are lacking. This is where my thesis is situated. I endeavoured to ground this thesis in the current knowledge of tropical marine cleaning symbiosis, and push the boundaries by exploring a previously neglected research angle. Social interactions between hosts are used by parasites for transmission and spread, a process commonly known as social transmission (Romano et al. 2021). Although social transmission has been explored in several groups of organisms, including lizards, fishes, and primates (Godfrey et al. 2009; Johnson et al. 2011; MacIntosh et al. 2012), social transmission has been rarely studied in the context of cleaning symbiosis. In **Chapter 2**, as a conceptual exercise, I included, for the first time, cleaner organisms as an extra element in the disease

triangle (Francl 2001; Scholthof 2007). This additional element revealed a substantial gap in the literature concerning data on pathogen transmission between fishes. This knowledge gap is surprising when considering 1) how fish interact with each other during cleaning interactions, that is, physically close and intimate contact (Grutter 2004); and 2) parasite evolution, which not only involves developing strategies to breach host defences but also to benefit from host behaviour and interactions (inter or/and intraspecific interactions) to expand their potential host basis (Lafferty 1999; Jones et al. 2004; Poulin 2010). Considering a scenario where parasite transmissibility is greater than zero, I proposed that cleaner fish may act as *parasite transmitters* and that therefore parasites may take advantage of cleaning interactions to reach a wider diversity and number of fish hosts. Furthermore, by analysing the literature on cleaning symbiosis, parasite behaviour and the strategies they employ to infect potential hosts, I proposed that cleaning stations may act as *parasite hotspots.* Near these hotspots, parasite pressure can be expected to be higher than further away from them, a hypothesis based on the abundance and diversity of infected fishes that aggregate and wait to be cleaned.

Cleaning symbiosis and parasite transmission

Chapter 2 provided a measure of the main knowledge gaps and challenges remaining to understand the potential role of cleaners on parasite transmission, which led me to two critically unanswered questions: 1) Are cleaner fish susceptible to parasites in the wild and in captivity and, 2) can cleaner fish potentially transmit parasites? In **Chapter 3**, I discovered that the cleaner fish *Labroides dimidiatus* is susceptible to parasite infection in the wild. I found that the parasite community of *L. dimidiatus* on eastern Australia's coral reefs is diverse, comprising representatives of eight taxonomic groups, including five ectoparasitic and three endoparasitic groups. Additionally, parasite composition and abundance were comparable to other wrasse species from the same environment. Contrary to other wrasses, *L. dimidiatus* individuals interact regularly with several fish species and thousands of individuals, which allows parasites to potentially exploit cleaning symbiosis as a means of facilitating transmission to new hosts.

A key aspect of vector transmission is that parasites can survive on the intermediary host for long enough to be transmitted. In the laboratory, I discovered that *L. dimidiatus* was not similarly susceptible

to three generalist parasites tested (**Chapter 4**). *L. dimidiatus* was highly susceptible to its main wild food source, gnathiid isopods, as represented by *Gnathia aureamaculosa*. Gnathiid isopods have been likened to 'mosquitos of the sea' (Grutter et al. 2011b), because of their brief period of attachment to the fish host to feed on blood and plasma before dropping off to the substrate to moult and reproduce (Tanaka et al. 2007). The infection mechanisms of gnathiids allow them to quickly detach from the host after feeding or when disturbed (i.e., from a predator threat such as cleaner fish), which can potentially allow them to re-infect a different fish in the vicinity (**Chapter 2**; Narvaez et al. 2021a). In the context of cleaning symbiosis, *L. dimidiatus* may engage in more than 2,000 interactions with individual fishes per day, which means that mobile gnathiids that avoid predation and attach to *L. dimidiatus* have ample opportunity to transmit to the next client fish.

L. dimidiatus had very low susceptibility to the cosmopolitan protozoan *Cryptocaryon irritans*, agent of white spot disease, and was not susceptible to the flatworm monogenean *Neobenedenia girellae* (**Chapter 4**). Lack of susceptibility suggests overall resistance from *L. dimidiatus* to harmful parasitic infections, particularly of *N. girellae*, which is surprising given the nature of these parasites (i.e., widely distributed geographically and across habitats and low host specificity). Further studies are needed to increase understanding of the mechanisms behind this resistance. Interestingly, while *L. dimidiatus* showed resistance to infection by the monogenean *N. girellae*, subsequent experiments showed that adult parasites that may become inadvertently attached to *L. dimidiatus* could survive and produce viable eggs for between 2 and 4 days (**Chapter 4**). This length of time could still provide numerous opportunities (~4,000 - 8,000 interactions) for these mobile parasites to infect a new host through inadvertent parasite attachment from close physical inspection during cleaning.

Exploring the diet of cleaners using molecular tools

DNA metabarcoding has been recently used to characterise the diet of many vertebrates, including mammals (Bohmann et al. 2018), birds (da Silva et al. 2020) and fishes (Takahashi et al. 2020). In **Chapter 5**, I accomplished the first exploration of the diet of cleaners using DNA metabarcoding techniques. To do so, I sampled three species of cleaners, that are common on reefs at Lizard Island on the Great Barrier Reef where this study was developed: the cleaner wrasse *L. dimidiatus*, and two

species of reef-inhabiting cleaner shrimp, *Urocaridella antonbruunii* and *Urocaridella cyrtorhyncha*. I discovered that the dietary contents of the cleaner fish *L. dimidiatus* mostly comprised DNA belonging to more than 30 fish species. This is likely a result of direct ingestion of fish tissues and/or mucus, or of parasites attached to their clients that themselves rely on blood or skin that contain host DNA. Although the source of the DNA is not entirely clear, these findings show with precision which fish species interacted with the cleaners prior to collection and, more importantly, provides support for the hypothesis that wild cleaners consume a large amount of items other than parasites, in particular fish tissues. Conversely, I discovered that the use of DNA metabarcoding on the dietary contents of the two species of cleaner shrimp presented substantial challenges for interpretation because the universal primers chosen for this study showed high matching to both parasite and cleaner shrimp DNA. Overall, with this study, I provided preliminary information on non-visually identifiable items of the diet of small cleaner fish and cryptic cleaner shrimp. In addition, it highlights challenges that need to be overcome to gain further understanding of cleaner organism diets, particularly of cleaner shrimp. This will require a larger library of parasite DNA and testing of other specific primers that target more specific prey items.

The disease triangle in a globally changing environment

Chapter 2 highlighted a gap in the literature on how climate change may impact the dynamics of interactions in the disease triangle, such as the impacts of reef habitat degradation on the parasite/host relationship. Along these lines, in **Chapter 6**, I discovered that after exposing juvenile damselfish *Pomacentrus amboinensis* to water conditioned with dead corals and their colonisers (i.e., mostly cyanobacteria and algae that grow on top of dead coral skeletons), the infection rate of the gnathiid *G. aureamaculosa* on *P. amboinensis* was higher than in water conditioned with live corals and unconditioned control water. This trend was not found for adult *P. amboinensis*, with no differences in parasite infection rates between treatments. These results provide insights on 1) how gnathiids respond to water directly surrounding different reef substrates, particularly increasingly common colonised dead corals and 2) how ontogeny may change the behavioural responses of *P. amboinensis* immersed in water surrounding dead corals. First, this experiment showed that the capacity to infect of gnathiids appears

not to be affected by presumed chemical distinctions in water surrounding dead or live corals. This is surprising given that it has been previously shown that gnathiid isopods prefer to moult, live, and reproduce in dead coral substrate (Artim and Sikkel 2013; Santos and Sikkel 2019) because live corals often prey on gnathiids (Artim and Sikkel 2013; Paula et al. 2021). Second, I found that juvenile damselfish are more susceptible to gnathiid infection in water surrounding dead corals, potentially due to the disturbance of key olfactory cues as has been found to be the case previously (e.g., McCormick and Allan 2017). Consequently, this chapter suggests that habitat degradation may disturb the interaction between parasites and hosts. I found this to be the case for juvenile fishes that are presumably more vulnerable to chemical components released by dead corals and/or coral colonisers, a substrate where gnathiid isopods are also more abundant, and therefore providing gnathiids with more opportunities to infect juvenile fish.

Advancements to understanding cleaning symbiosis and the disease triangle

My thesis provided the first comprehensive effort to integrate a novel perspective into a well-established concept, the disease triangle (Fig. 22). As a result, we have now a synthetic framework that permits including symbiotic mutualisms, like cleaning symbiosis, into the disease triangle, which advances our understanding in the interrelationship between host, pathogen, environment and cleaner organisms. This fourth dimension of the disease triangle emerges from the efforts in this thesis to characterise the links between cleaners and parasites, cleaners and clients, parasites and hosts, and parasites, host and environment. Among these links, we now know that there is a clear negative relationship between parasite and cleaners (number 1; negative interaction). Furthermore, *L. dimidiatus* is also negatively (number 2; negative interaction) or not affected (number 2; neutral interaction) by generalist parasites but it could participate on parasite transmission during cleaning interaction (number 3; positive interaction). It is now clear that *L. dimidiatus* removes clients' tissues which can be both positive (removal of dead tissues; number 4; positive interaction from cleaner to client) or negative interaction (removal of mucus; number 4; negative interaction). Furthermore, the clients are source of food for cleaners (number 4; positive interaction from client to cleaner). Finally, habitat degradation affected the

relationship between parasite and host both negatively (for juvenile fish; number 5; negative interaction) and positively (for parasites; number 5, positive interaction).

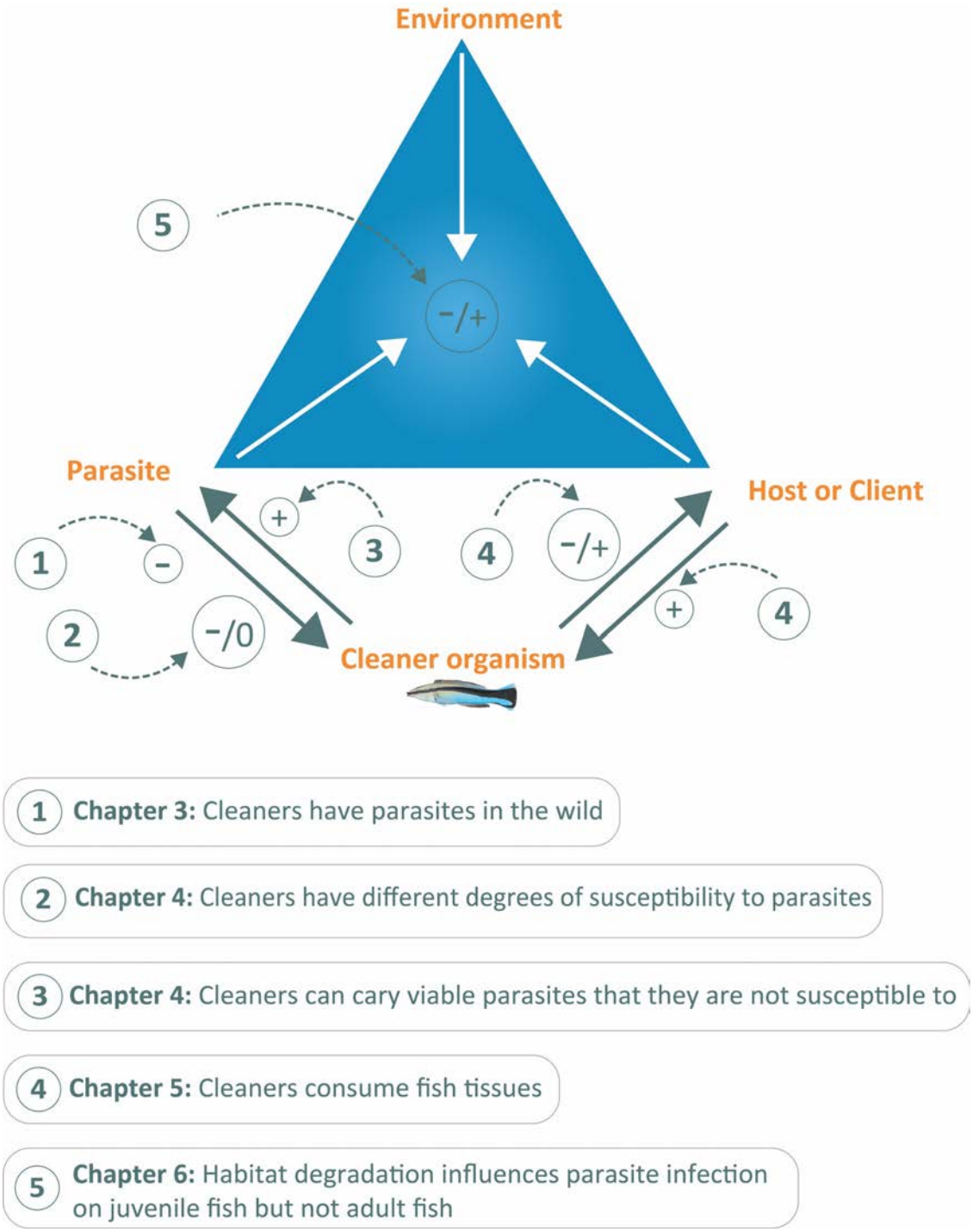

Figure 22 Final representation of the disease triangle including cleaner organisms as the fourth element. The solid green arrows represent the known relationships between cleaners, parasites and host/clients. The dashed green arrows represent the various findings of this thesis according to each chapter. – represents negative effect, 0 represents no effect; and + represents positive effect

However, this thesis has not only allowed me to explore the connections created by the updated disease triangle, but to further develop my conceptual thoughts on the inter-relationships between elements. When including ecologically relevant cleaning symbiosis (such as the one with *L. dimidiatus*), it may be that the representation of the disease triangle would be best represented by a three-dimensional shape, such as a tetrahedron (**Chapter 2**; Fig. 23). Thus, I suggest that this concept be termed, the disease tetrahedron.

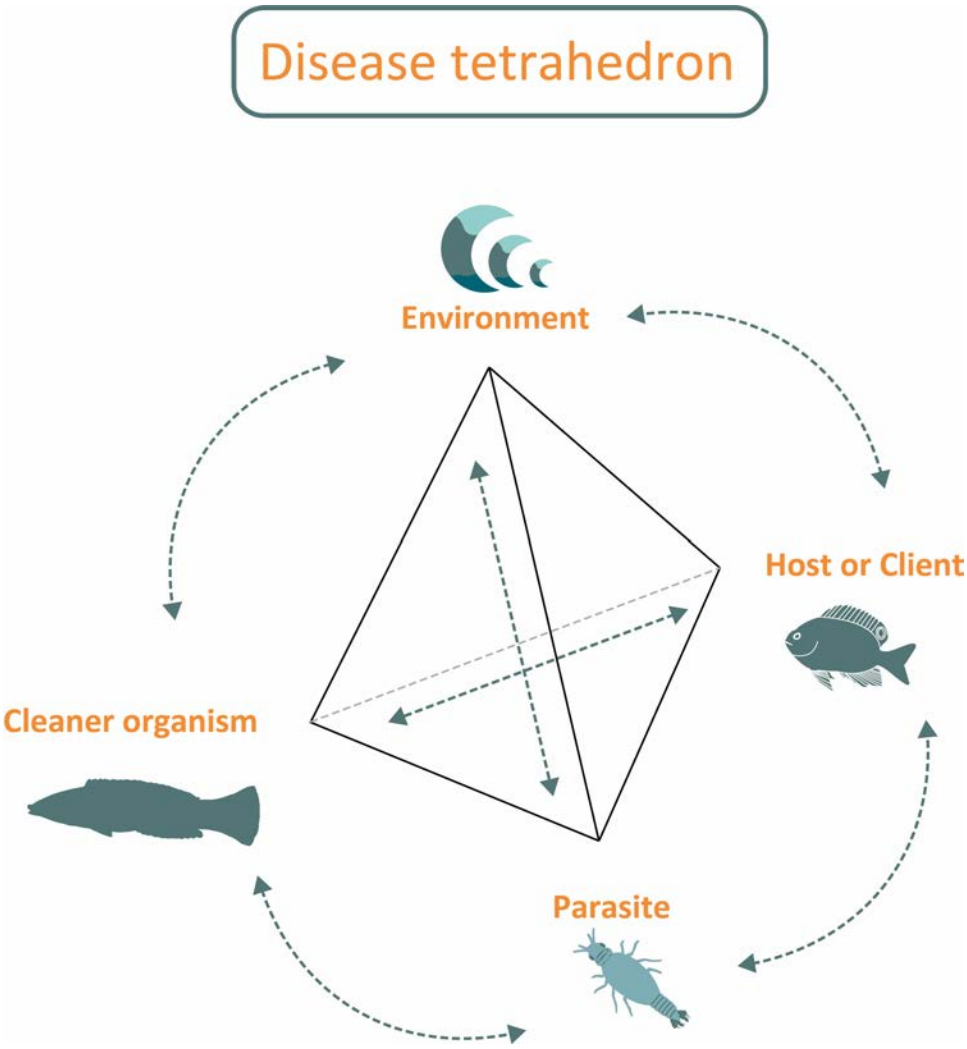

Figure 23 The disease tetrahedron in cleaning symbiosis context is a complex relationship between four elements: the environment, the cleaner, the host/client and the parasite corner

Future research directions

In this thesis, while I believe that many questions have been answered, this thesis also raised several further questions that could be addressed in future research. Below I explore some of these questions, highlighting the ones that I think are the most interesting:

1- **Chapter 2** provided several novel hypotheses relating cleaning symbiosis and parasitism. An exciting new avenue would be to examine whether cleaning stations are 'parasite hotspots' in the wild. Testing this hypothesis would require extensive fieldwork and specialised underwater equipment such as temporary sentinel fish and/or parasites traps at different distances to previously located cleaning stations and sediment vacuum samplers to collect parasites and eggs potentially shed to the bottom by host fishes at the cleaning station. Both approaches would need to pay particular attention to abiotic factors such as tides, currents and waves that could result in transport of bottom materials. Resolving whether cleaning stations may have the potential to aggregate parasites will be a useful next step towards an understanding the role of cleaning symbiosis in the transmission of parasites.

2- In **Chapter 3** I unveiled the diverse and abundant community of parasites harboured by the dedicated cleaner wrasse *L. dimidiatus*. Similar complementary studies could target other tropical dedicated cleaner fish species such as the Indo-Pacific wrasses *Labroides bicolor*, *L. phthirophagus* and *L. pectoralis*, or Atlantic cleaner gobies such as *Elacatinus prochilos* or *E. evelynae.* Detailed description of their parasite communities in the wild will provide a valuable baseline to further explore which species of parasites may exploit cleaning symbiosis as an evolutionary and/or ecological mechanism of spread.

3- In **Chapter 4**, I experimentally tested whether *L. dimidiatus* is susceptible to infection from three generalist parasites that had been used extensively in previous research and that are known to infect a wide range of fish host species. It would be interesting to expand the scope to other groups of common parasites, such as leeches, copepods, and other monogeneans or other taxonomic groups such as bacteria and viruses. Furthermore, it would be valuable to explore parasite transmission via a cleaner vector in mesocosms. Such a setup may enable access of the

cleaner to infected and non-infected clients (e.g., two aquaria separated by a tube that only the cleaner could pass through).

4- Further, in **Chapter 4** I also showed that cleaner fish is capable of transporting parasites to which it is not susceptible for a relatively extended period if the parasite is able inadvertently attach to the cleaner during cleaning interactions. In this case, clients with higher loads of parasites may pose the greatest risk of parasite infection or spread of parasites. From the cleaner fish perspective, it is conceivable that there may be a 'parasite infection threshold' past which the cleaner fish may choose not to interact with a client due to increased reduce the risk of infection or inadvertent attachment. It has been shown for other organisms, such as for mandrills *Mandrillus sphinx,* that individuals may recognise infected congeners using olfactory cues and avoid grooming (Poirotte et al. 2017). We know that cleaner fishes mainly use visual cues to approach and clean their clients (Vaughan et al. 2017), but more studies would be needed to understand if these or other sensory cues involved in cleaning symbiosis could also be used by cleaners to judge infection loads and avoid engaging in cleaning interactions with fish that present a high risk of infection spread.

5- DNA metabarcoding techniques can bring new ecological insights to the understanding of feeding ecology. In **Chapter 5**, among the targeted organisms, it was apparent that the DNA sequences of parasites accessible on databases such as GenBank are largely incomplete. An extensive collection effort targeting common and widespread parasites encompassing a broad range of taxonomic groups and using different DNA regions will thus be an important next step in assessing rates of parasite consumption. After obtaining these sequences, non-universal primers, targeting one or several parasitic groups could be designed accordingly, greatly expanding the taxonomic scope and resolution of parasite prospecting in cleaner organisms' diets. This would, in turn, provide the basis for broader ecological research aiming to understand variations in the diet of cleaner fish and shrimp at different temporal scales (day *vs* night or among seasons).

6- In **Chapter 6**, I investigated how environmental changes stemming from severe mortality of corals may affect parasite/host relationships on coral reefs. I specifically used the relationship

between parasite and host as a model, but further research could be done to include cleaner fish as well. This would cast further light on the purported population success of parasites after coral mortality, showing whether cleaner interactions are able to mitigate the increased parasite infection rates on juvenile fishes. Furthermore, other aspects of habitat degradation could be explored, including increases in water turbidity or acidification, particularly as these may in theory interfere with sensory cues on which both fish and parasites rely extensively during cleaning interactions (Vaughan et al. 2017; Sikkel et al. 2011).

Conclusion

Overall, this thesis provides a fresh perspective on cleaning interactions using the combination of ecological, experimental, and molecular techniques. The inclusion of cleaner organisms in the disease triangle was crucial and permitted me to explore new aspects of cleaning symbiosis, adding substantial findings on the highly complex dynamic between each element of the triangle. Cleaning interactions are not only limited to tropical coral reef environments but are found in multiple ecosystems such as temperate marine ecosystems, estuarine, freshwater (Vaughan et al. 2017; Sazima 2021) and terrestrial environments (**Chapter 1**). Therefore, this thesis offers grounds for further studies to explore the role of cleaners in parasite transmission in diverse ecosystems.

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Appendix A: Supporting Information for Chapter 2

1. Data gathering and methods to generate Figure 4

Publications from the peer-reviewed international literature about cleaner fish were gathered using the snowball methods (i.e., sampling until data saturation; Naderifar et al. 2017), searching for studies from 1950 to 2020 using Google Scholar database and references from each publication. A data base was then created using an excel spreadsheet with columns populated corresponding to author, paper title, year of publication and classification (temperate *vs* tropical). To generate Fig. 4A, the number of new studies per decade were summed. For the cumulative data, the number of studies for each decade were summed. To identify publications specifically related to "*Disease*" and "*Disturbance*", the titles, abstracts, key words, and methods of each publication gathered and checked for specific key words associated with cleaner fish. For the theme *Disease*, the words "virus", "pathology", "pathogens", "transmission", "susceptibility", "infection", "bacteria", "parasites", "diseases" were used. For the theme *Disturbance* the words "perturbations", "effects", "fishing", "ocean warming", "acidification", "climate change", "future", "CO2", "disruptions", "noise", "disturbances" were used. To generate Fig. 4B, each study was classified as either tropical or temperate study, or both, according to the geographic location of the study and/or the species of fish studied (tropics was considered above the Tropic of Capricorn and below the Tropic of Cancer).

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Appendix B: Supporting Information for Chapter 4

Figures

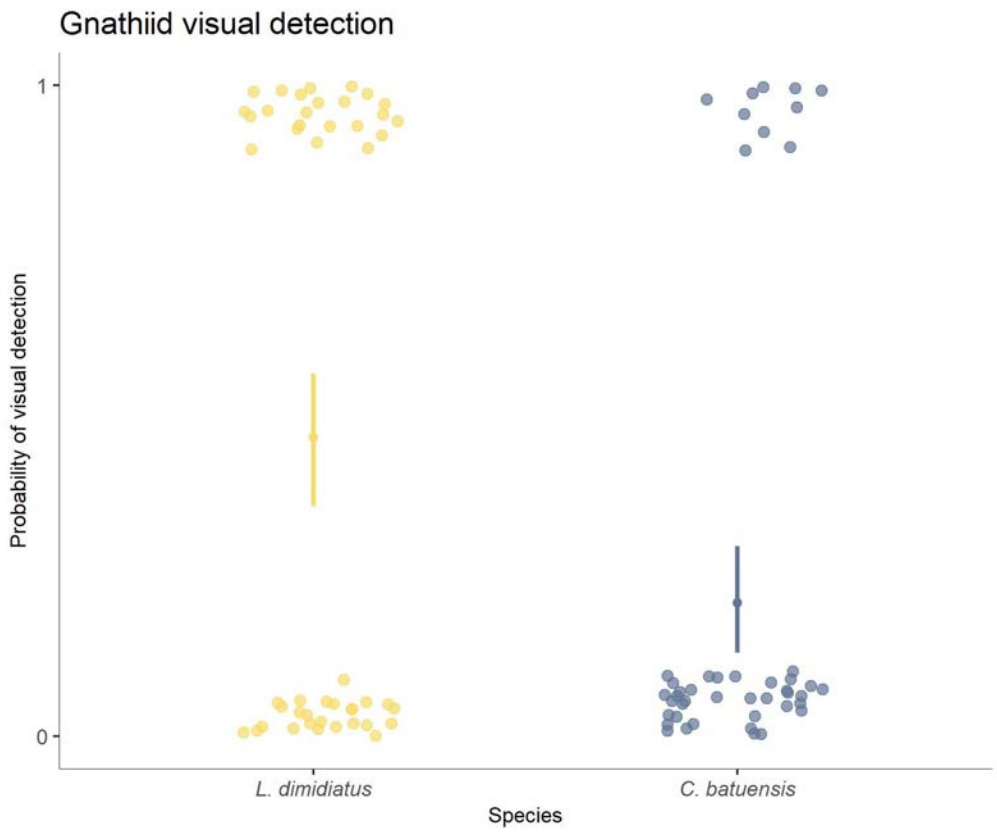

Figure B1 Probability of visually detect *Gnathia aureamaculosa* during the experiment with *Labroides dimidiatus* and *Coris batuensis* with 1 being the probability of detection and 0 no detection. The middle dot represents the median and the line the lower and upper 95 % Credibility Interval. Simulated data points were jittered in the x- and y- axis

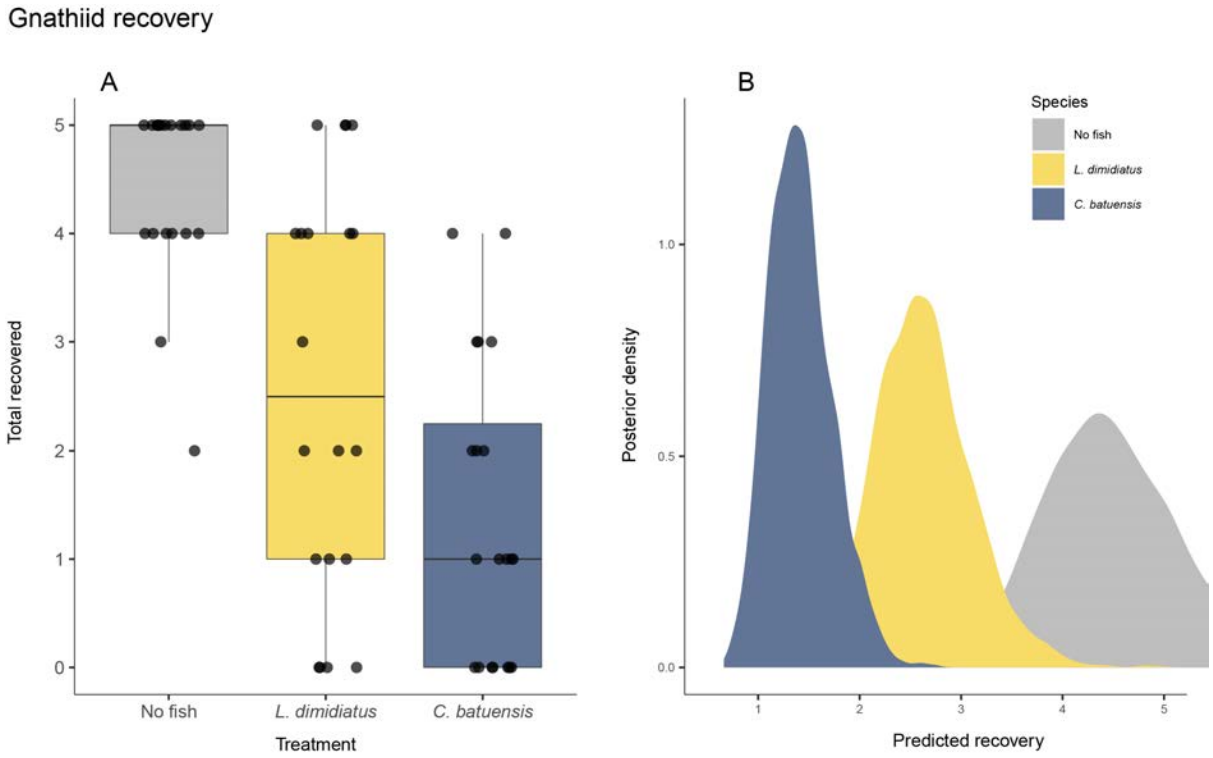

Figure B2 (A) Gnathiid recovered at the end of the experiment in control tank with no fish (grey), *Labroides dimidiatus* (yellow) and control fish *Coris batuensis* (blue). Black dots show jittered data points representing each individual or tank (for control with no fish). Boxplots show the median (horizontal bar), the interquartile (rectangle) and maximum and minimum values (error bars). (B) The predicted recovery was higher for the control with no fish than the two fish treatments. However, the prediction of recovering gnathiid from the *L. dimidiatus* tanks was higher than from *C. batuensis*

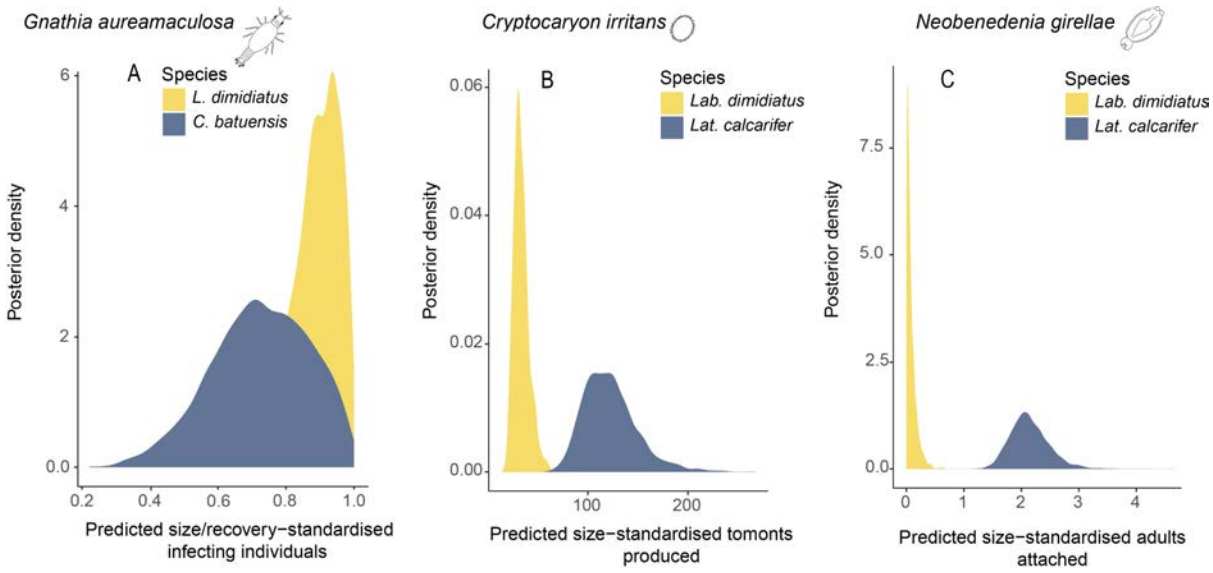

Figure B3 Posterior distributions of parameters from the susceptibility experiment for (A) the gnathiid isopod *Gnathia aureamaculosa*, (B) the ciliate protozoan *Cryptocaryon irritans* and (C) the flatworm monogenean *Neobenedenia girellae*

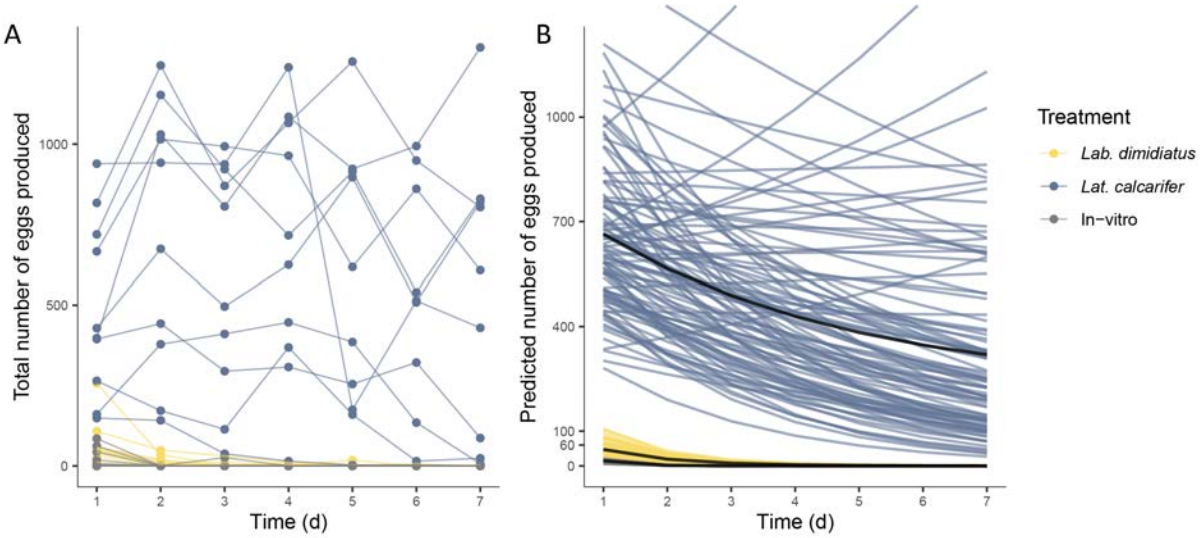

Figure B4 (A) Number of eggs produced by the monogenean *Neobenedenia girellae* during seven days post-transfer experiment (n=5 adult *N. girellae*) on *Labroides dimidiatus* (yellow), control fish *Lates calcarifer* (blue) and *in vitro* (grey). (B) Predicted number of eggs produced based on the Bayesian model for the seven days post-transfer experiment

Figure B5 A fed gnathiid *Gnathia aureamaculosa* (inside yellow circle) on an anaesthetised individual cleaner wrasse *Labroides dimidiatus*, slightly obscured by the gill operculum

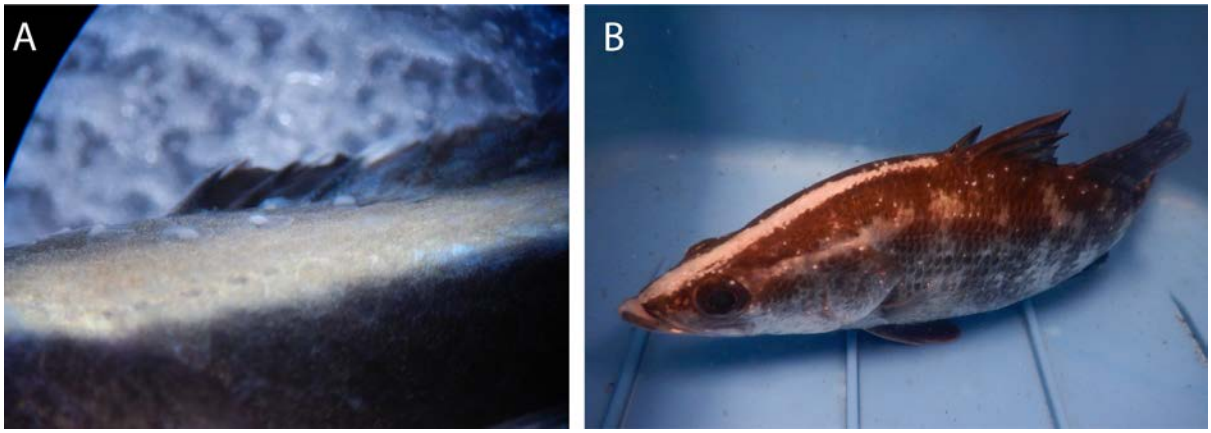

Figure B6 Trophonts (parasitic stage) of *Cryptocaryon irritans* infecting the cleaner wrasse *Labroides dimidiatus* were occasional and visible only with the binocular microscope (magnification x 40) (A). However, trophonts of *C. irritans* infecting barramundi *Lates calcarifer* were visible with the naked eye and in greater number (B)

Figure B7 Adult (parasitic stage) flatworm *Neobenedenia girellae* (inside the orange circle) on the body of an anesthetised individual cleaner wrasse *Labroides dimidiatus* after transfer from a donor fish

Table B1 (A) Egg production estimation based on the ratio of *Labroides dimidiatus* / *Lates calcarifer* and *L. dimidiatus* / *in vitro* using a Bayesian generalised linear models via Stan. Lower.HPD represents the lower endpoint of the highest posterior density interval and Upper.HPD represents the upper endpoint of the highest posterior density interval. (B) Total number of eggs produced per day of experiment for *Labroides dimidiatus* (n=10), *Lates calcarifer* (n=10) and *in vitro* (n=10) treatments

A Contrast	Day	Estimate	lower.HPD	upper.HPD
Lab.dim / *Lat.cal*	1	0.07	0.021	0.15
Lab.dim / *Lat.cal*	2	0.034	0.009	0.08
Lab.dim / *Lat.cal*	3	0.016	0.003	0.04
Lab.dim / *Lat.cal*	4	0.008	0.001	0.03
Lab.dim / *Lat.cal*	5	0.004	0	0.02
Lab.dim / *Lat.cal*	6	0.002	0	0.01
Lab.dim / *Lat.cal*	7	0.001	0	0.006
Lab.dim / *in vitro*	1	3.2	0.75	7.9
Lab.dim / *in vitro*	2	10.9	2.35	29.5
Lab.dim / *in vitro*	3	36.6	4.73	156
Lab.dim / *in vitro*	4	125	7.58	870
Lab.dim / *in vitro*	5	427	8.01	5175
Lab.dim / *in vitro*	6	1461	8.47	33088
Lab.dim / *in vitro*	7	5055	8.95	195309

B Total number of eggs produced			Day
Lab. dim	*Lat. cal*	*In vitro*	
710	4,938	223	1
146	7,195	1	2
48	5,882	29	3
41	6,834	0	4
30	5,588	0	5
7	4,842	0	6
3	4,911	0	7

Appendix C: Supporting Information for Chapter 5

Figures

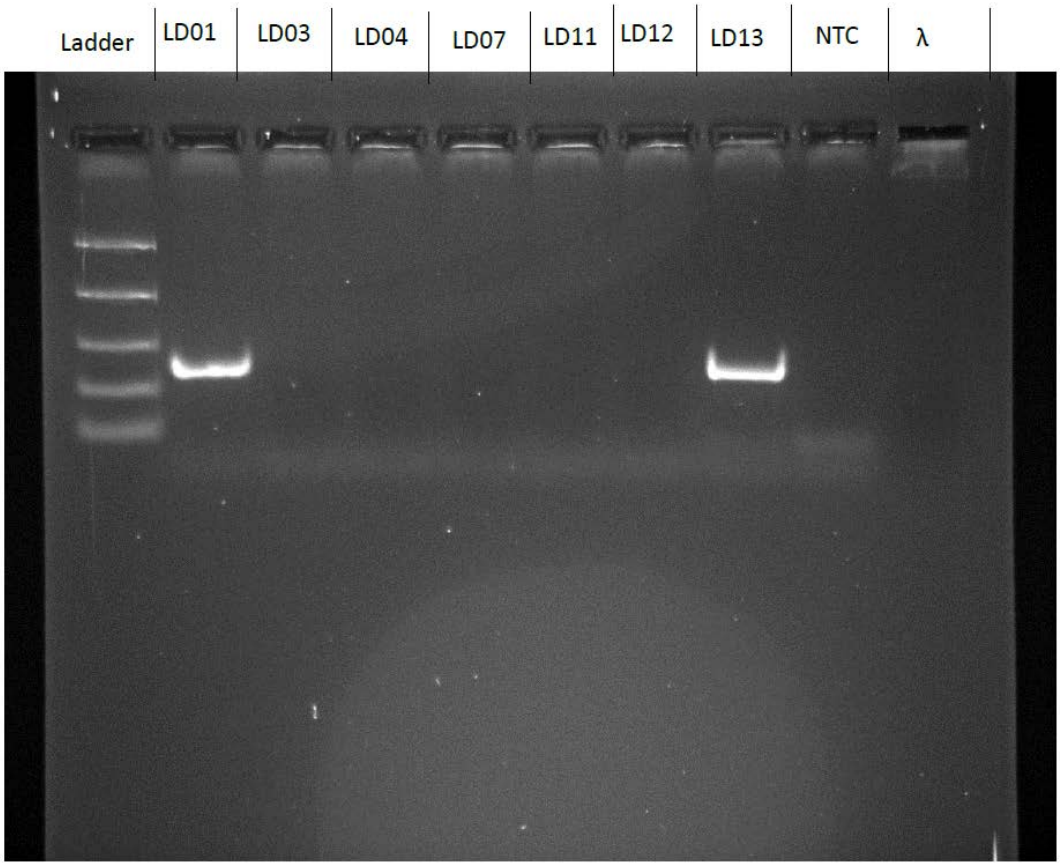

Figure C1 Test of the *COI* universal primer pair specificity on DNA of the gut content of cleaner fish *Labroides dimidiatus*, after diluting it to 10ng/µL. Bands show PCR product of approximately 350 bp length. Well labeled "λ" represents the lambda standard at a concentration of 10 ng

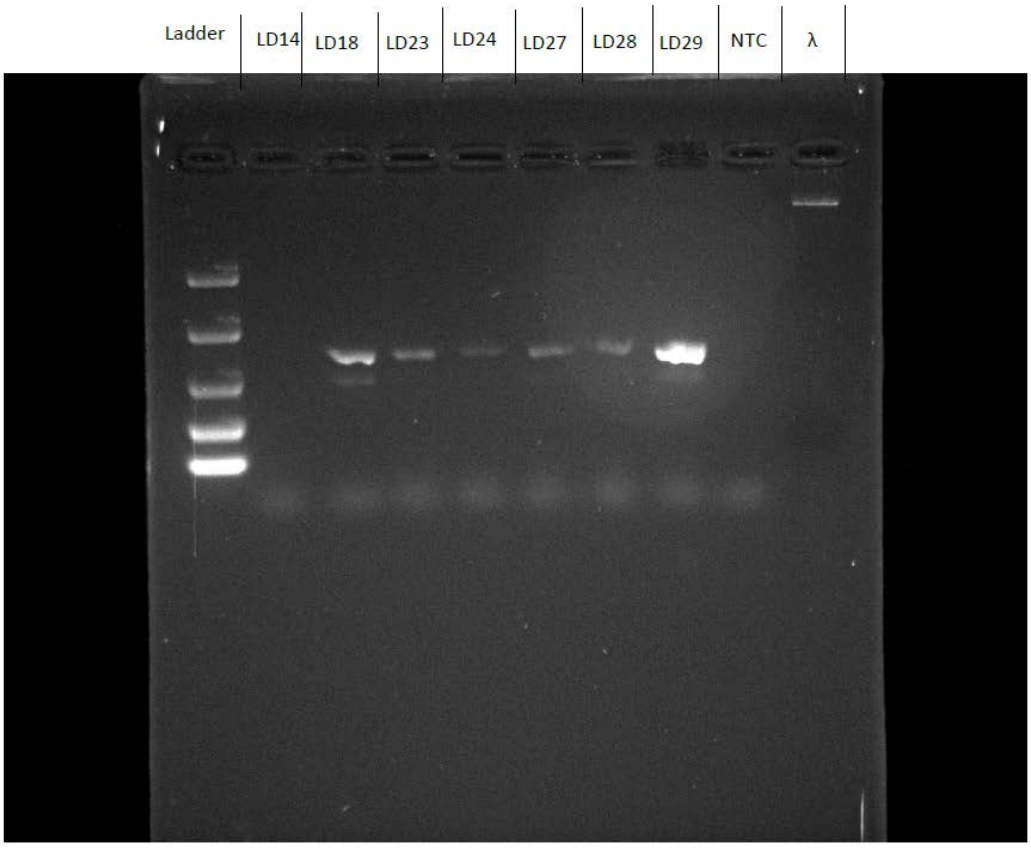

Figure C2 Test of the *16S* universal primer pair specificity on DNA of the gut content of cleaner fish *Labroides dimidiatus*, after diluting it to 10ng/µL. Bands show PCR product of approximately 750 bp length. Well labeled "λ" represents the lambda standard at a concentration of 10 ng

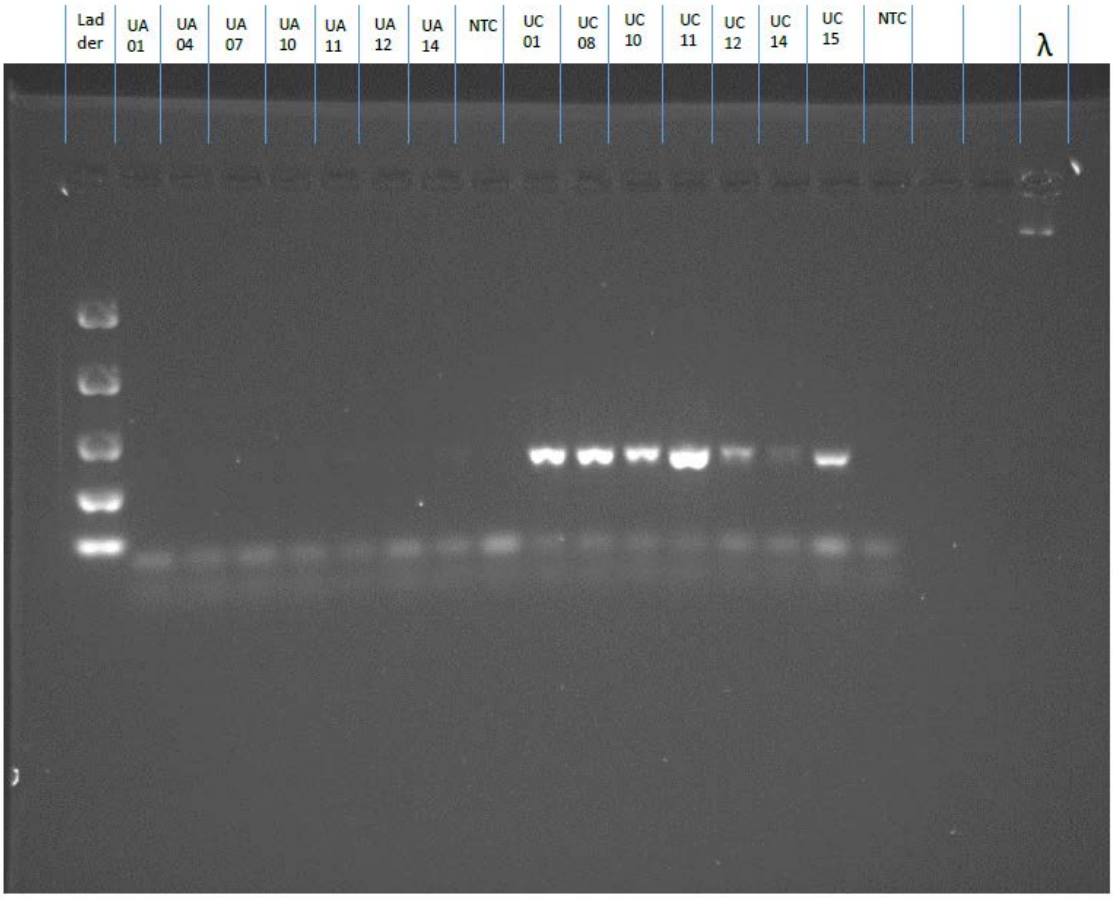

Figure C3 Test of the *COI* universal primer pair specificity on DNA of the gut content of the cleaner shrimp *Urocaridella antonbruunii and U. cyrtorhyncha*, after diluting it to 10ng/µL. Bands show PCR product of approximately 500 bp length. Well labeled "λ" represents the lambda standard at a concentration of 10 ng

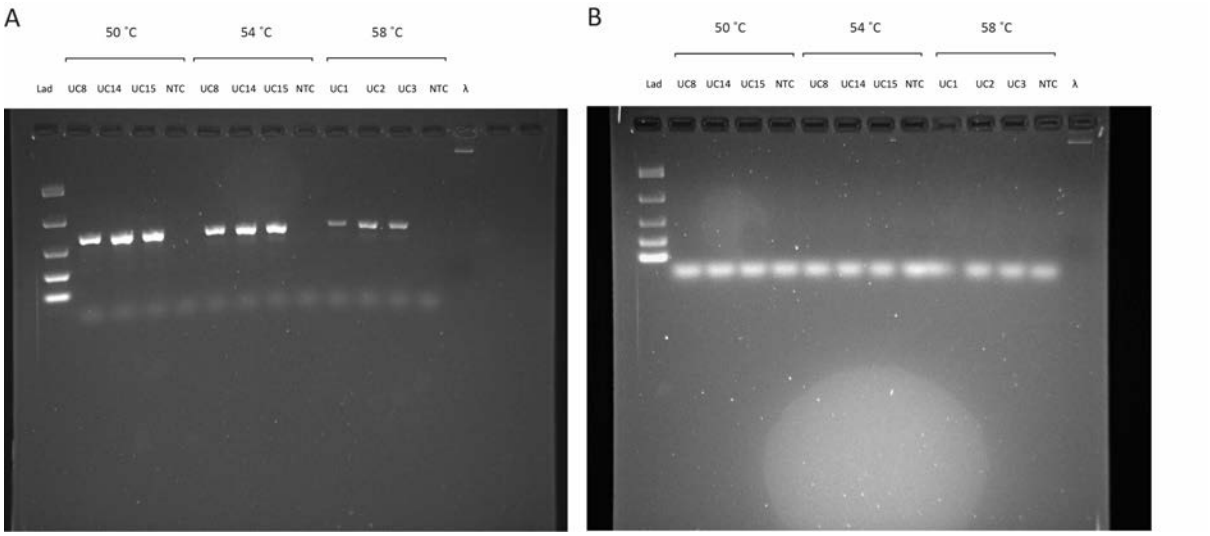

Figure C4 Test of the *16S* universal primer pair specificity on DNA of the gut content of the cleaner shrimp *Urocaridella cyrtorhyncha*, after diluting it to 10ng/µL and at different annealing temperature (A) without blocking primer and (B) with blocking primer. Bands show PCR product of approximately 750 bp length. Well labeled "λ" represents the lambda standard at a concentration of 10 ng

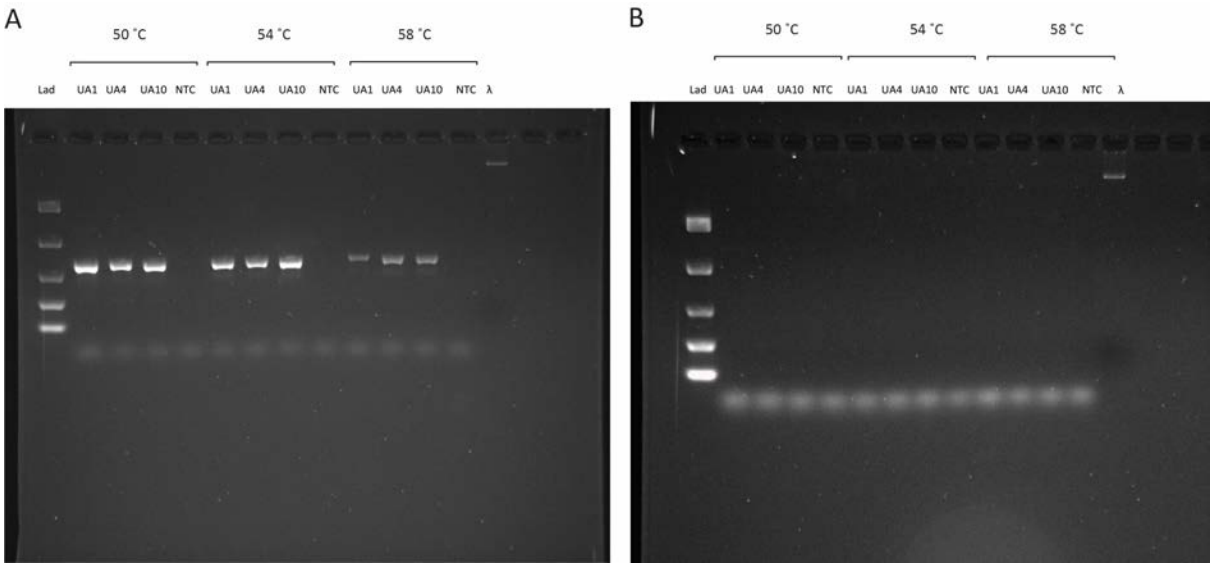

Figure C5 Test of the *16S* universal primer pair specificity on DNA of the gut content of the cleaner shrimp *Urocaridella antonbruunii*, after diluting it to 10ng/µL and at different annealing temperature (A) without blocking primer and (B) with blocking primer. Bands show PCR product of approximately 750 bp length. Well labeled "λ" represents the lambda standard at a concentration of 10 ng

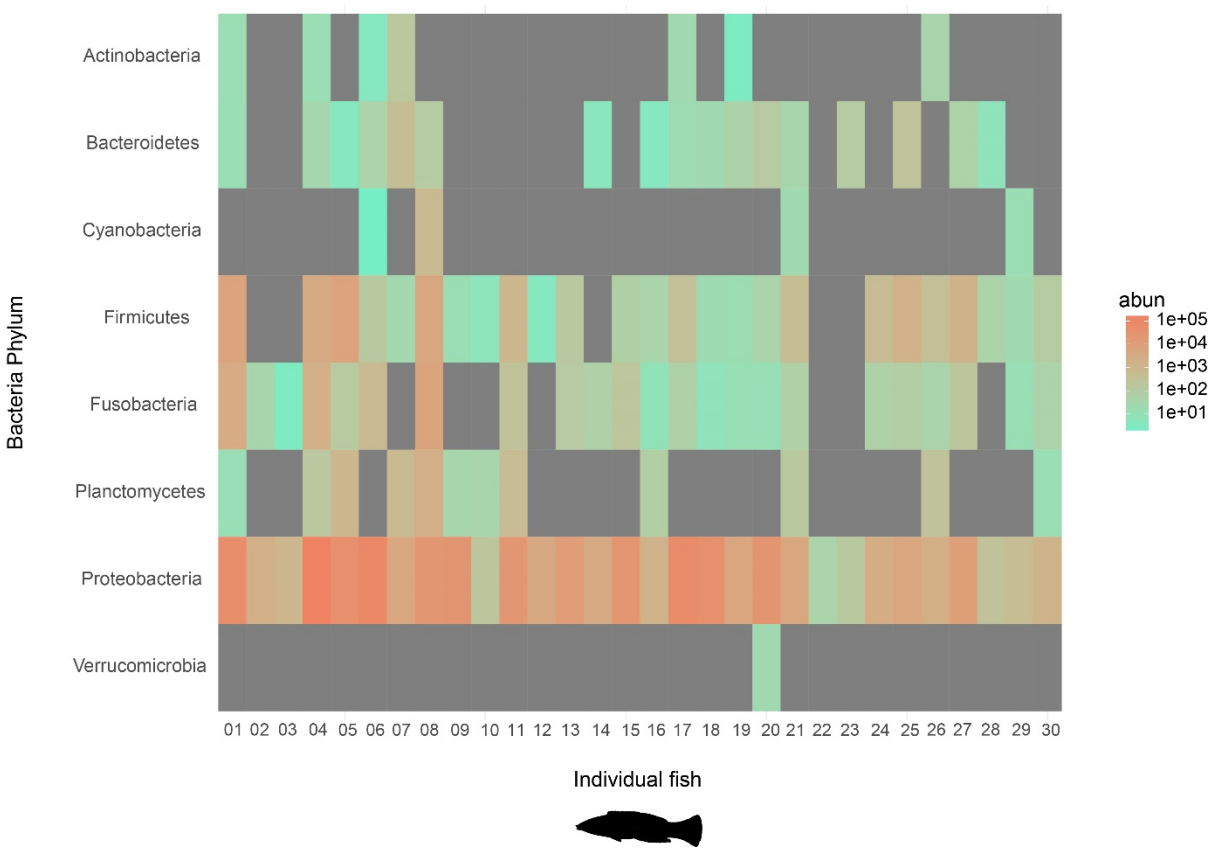

Figure C6 Bacterial community found in cleaner fish, *Labroides dimidiatus*, gut content at the phylum level. Scale is log-10 transformed (dark grey represents 0)

Figure C7 Bacterial community found in cleaner fish, *Labroides dimidiatus*, gut content at the family level. Scale is log-10 transformed (dark grey represents 0)

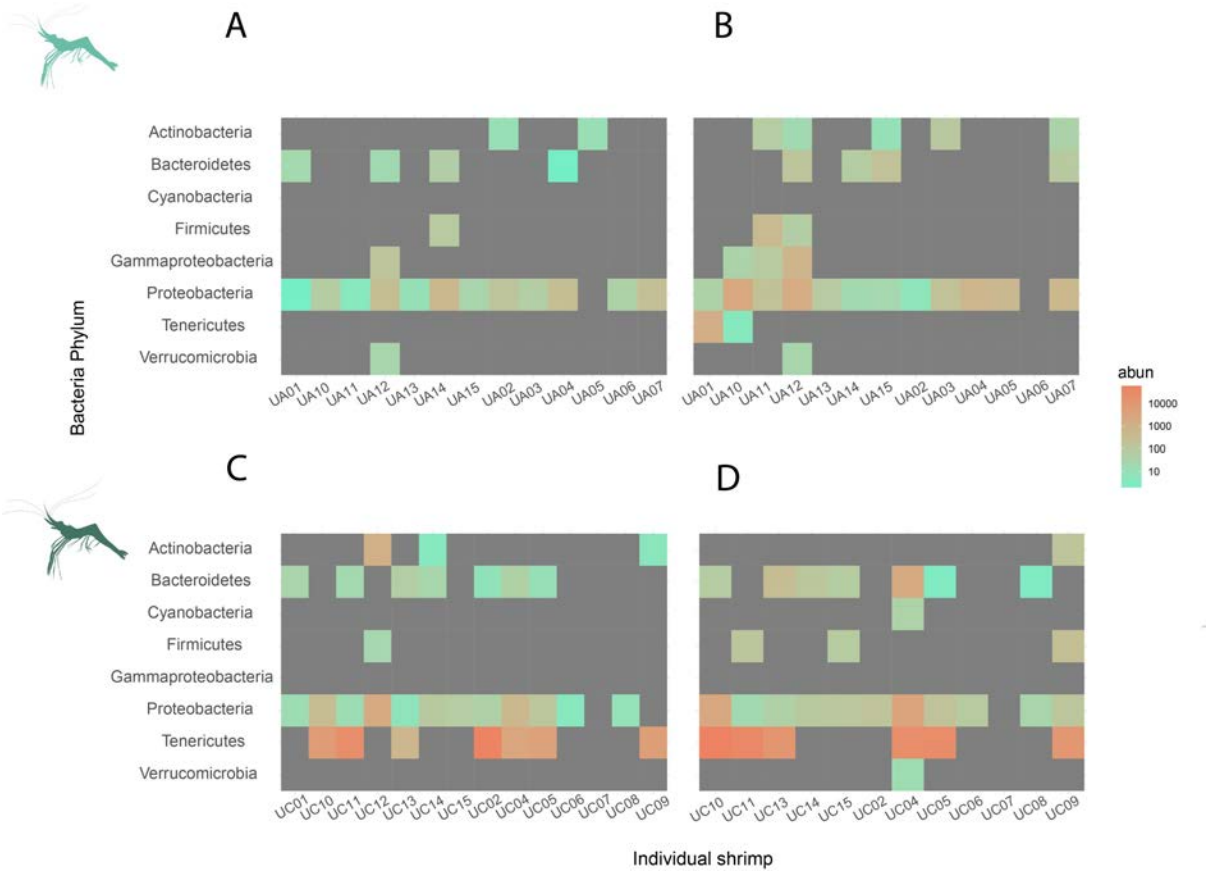

Figure C8 Bacterial community found in the two cleaner shrimp species at the phylum level with and without blocking primer: (A) *Urocaridella antonbruunii* without blocking primer, (B) *U. antonbruunii* with blocking primer, (C) *U.* cf. *cyrtorhyncha* without blocking primer, (D) *U.* cf. *cyrtorhyncha* with blocking primer. Scale is log-10 transformed (dark grey represents 0)

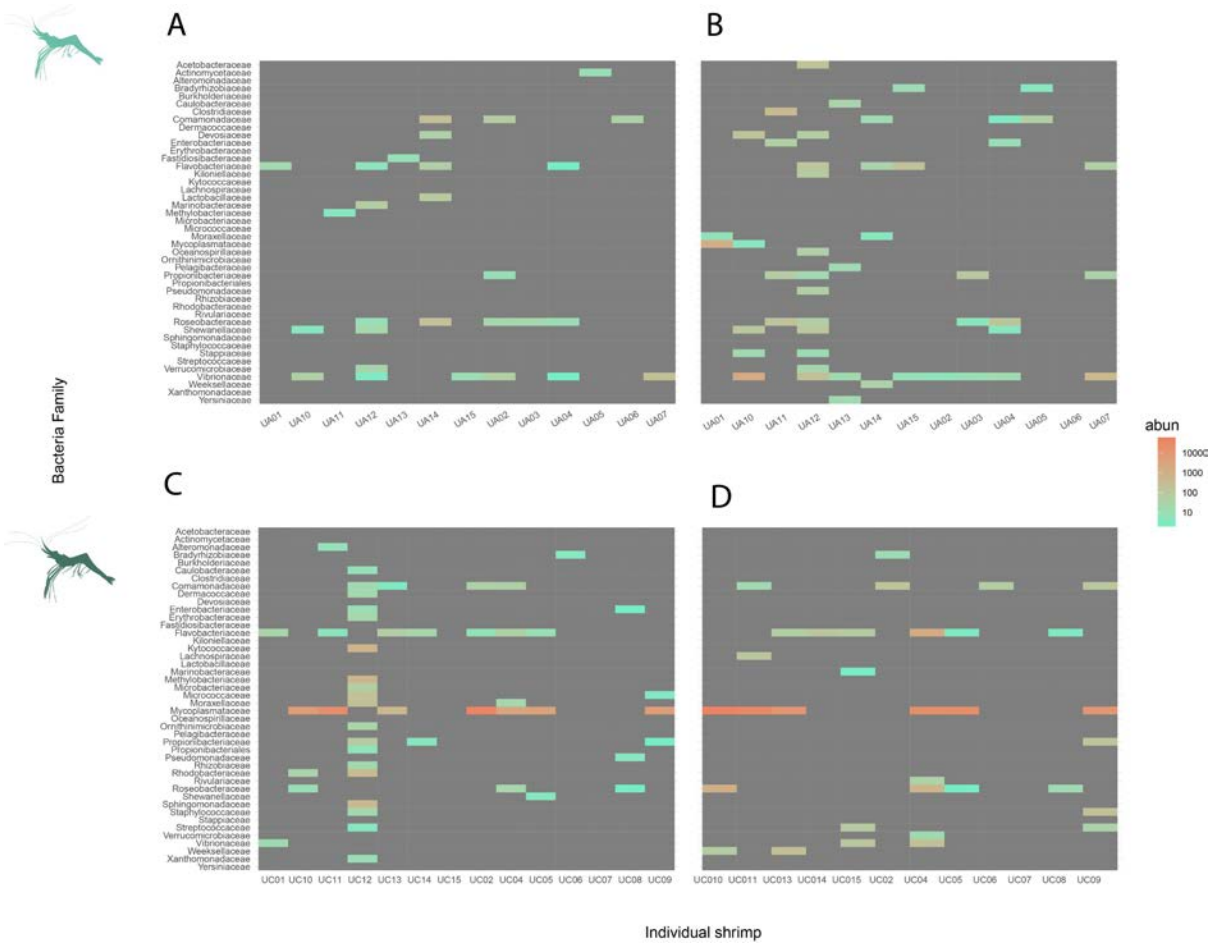

Figure C9 Bacterial community found in the two cleaner shrimp species at the family level with and without blocking primer: (A) *Urocaridella antonbruunii* without blocking primer, (B) *U. antonbruunii* with blocking primer, (C) *U.* cf. *cyrtorhyncha* without blocking primer, (D) *U.* cf. *cyrtorhyncha* with blocking primer. Scale is log-10 transformed (dark grey represents 0)

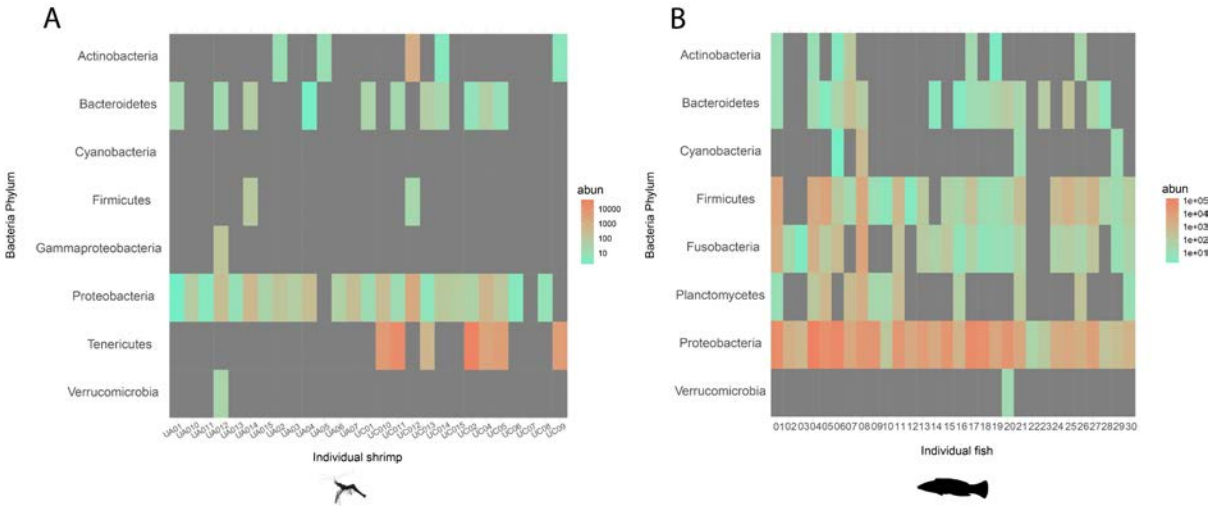

Figure C10 Bacterial community at the phylum level found in (A) the two cleaner shrimp species: *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* and (B) the cleaner fish *Labroides dimidiatus.* For this analysis, the shrimps with no blocking primer are displayed. Scale is log-10 transformed (dark grey represents 0)

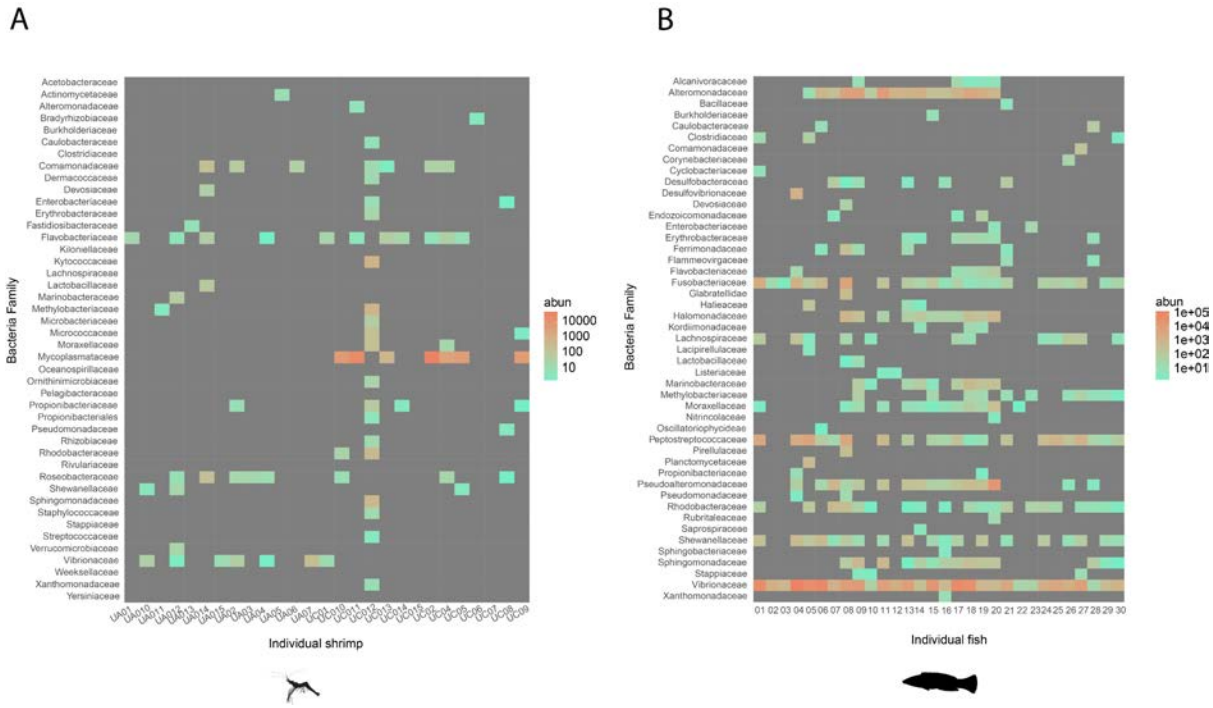

Figure C11 Bacterial community at the family level found in (A) the two cleaner shrimp *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* and (B) the cleaner fish *Labroides dimidiatus*. For this analysis, the shrimps with no blocking primer are displayed. Scale is log-10 transformed (dark grey represents 0)

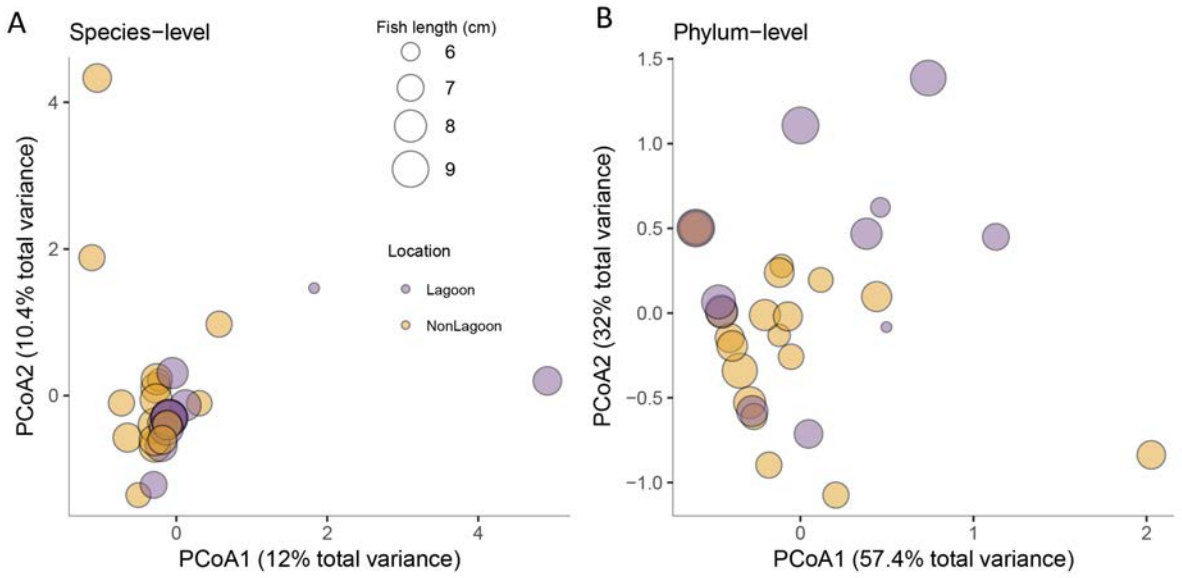

Figure C12 Prey composition of the cleaner fish *Labroides dimidiatus* at (A) the species level and (B) the phylum level according to *L. dimidiatus* individual size (cm) and location of collection (lagoon: purple circles and non-lagoon: yellow circles) represented with PCoA analysis (PCoA1 and PCoA2)

Table

Table C1 DNA concentration from the gut of cleaner fish *Labroides dimidiatus*

Organism	Organism ID	DNA concentration (ng/µL)
Labroides dimidiatus	LD01	34
Labroides dimidiatus	LD02	27
Labroides dimidiatus	LD03	21
Labroides dimidiatus	LD04	12
Labroides dimidiatus	LD05	15
Labroides dimidiatus	LD06	26
Labroides dimidiatus	LD07	14
Labroides dimidiatus	LD08	13
Labroides dimidiatus	LD09	22
Labroides dimidiatus	LD10	53
Labroides dimidiatus	LD11	16
Labroides dimidiatus	LD12	3.76
Labroides dimidiatus	LD13	73
Labroides dimidiatus	LD14	27
Labroides dimidiatus	LD15	71
Labroides dimidiatus	LD16	24
Labroides dimidiatus	LD17	48
Labroides dimidiatus	LD18	86
Labroides dimidiatus	LD19	33
Labroides dimidiatus	LD20	24
Labroides dimidiatus	LD21	23
Labroides dimidiatus	LD22	53
Labroides dimidiatus	LD23	34
Labroides dimidiatus	LD24	97
Labroides dimidiatus	LD25	20
Labroides dimidiatus	LD26	25
Labroides dimidiatus	LD27	29
Labroides dimidiatus	LD28	37
Labroides dimidiatus	LD29	88
Labroides dimidiatus	LD30	127

Table C2 DNA concentration from the cleaner shrimp *Urocaridella antonbruunii* and *U. cyrtorhyncha*

Organism	Organism ID	DNA concentration (ng/µL)
Urocaridella antonbruunii	UA01	184
Urocaridella antonbruunii	UA02	174
Urocaridella antonbruunii	UA03	160
Urocaridella antonbruunii	UA04	160
Urocaridella antonbruunii	UA05	123
Urocaridella antonbruunii	UA06	161
Urocaridella antonbruunii	UA07	143
Urocaridella antonbruunii	UA10	173
Urocaridella antonbruunii	UA11	183
Urocaridella antonbruunii	UA12	50
Urocaridella antonbruunii	UA13	118
Urocaridella antonbruunii	UA14	84
Urocaridella antonbruunii	UA15	125
Urocaridella cyrtorhyncha	UC01	123
Urocaridella cyrtorhyncha	UC02	34
Urocaridella cyrtorhyncha	UC04	154
Urocaridella cyrtorhyncha	UC05	177
Urocaridella cyrtorhyncha	UC06	162
Urocaridella cyrtorhyncha	UC07	163
Urocaridella cyrtorhyncha	UC08	138
Urocaridella cyrtorhyncha	UC09	153
Urocaridella cyrtorhyncha	UC10	108
Urocaridella cyrtorhyncha	UC11	159
Urocaridella cyrtorhyncha	UC12	124
Urocaridella cyrtorhyncha	UC13	116
Urocaridella cyrtorhyncha	UC14	165
Urocaridella cyrtorhyncha	UC15	160

Table C3 Quality control metric for each analysed sample of the diet of *L. dimidiatus* with the *16S* primer

Sample	Input	Filtered	Denoised	Non-chimeric
LD01-16S	131113	120163	119845	106120
LD02-16S	131105	111661	111544	104044
LD03-16S	130223	114586	114503	108244
LD04-16S	223841	209439	209123	193817
LD05-16S	104432	92219	92110	90727
LD06-16S	198704	185961	185629	166114
LD07-16S	10384	9364	9339	9339
LD08-16S	177628	165955	165856	165020
LD09-16S	145674	135396	135246	132957
LD10-16S	123108	107682	107655	101876
LD11-16S	140925	118546	118430	115199
LD12-16S	82336	77765	77739	77739
LD13-16S	142842	131042	130890	123381
LD14-16S	112668	96106	96046	90683
LD15-16S	163027	150912	150803	143753
LD16-16S	108844	86689	86563	79769
LD17-16S	148478	131124	130725	122566
LD18-16S	140590	128511	128290	119572
LD19-16S	108594	87023	86878	80014
LD20-16S	123151	101130	100935	94512
LD21-16S	98687	75850	75781	71707
LD22-16S	86599	72642	72604	69597
LD23-16S	125429	107891	107440	102400
LD24-16S	108685	93492	93411	90400
LD25-16S	114418	87566	87503	81626
LD26-16S	159551	148339	148289	144953
LD27-16S	119509	107093	107028	103959
LD28-16S	119718	82990	82972	74797
LD29-16S	112565	101374	101193	96960
LD30-16S	136287	119777	119580	113076
Mean	**127637**	**111943**	**111943**	**111871**
SD	**37082**	**36958**	**36958**	**36930**

Table C4 Quality control metric for each analysed sample of the diet of *U. antonbruunii* (UA) and *U.* cf. *cyrtorhyncha* (UC) with the *16S* primer without and with blocking primer (BLK)

Sample	Input	Filtered	Denoised	Non-chimeric
UA01_16S	164301	162522	162497	162497
UA01_16SBLK	170126	168216	168077	168077
UA010_16S	117364	115972	115933	115933
UA010_16SBLK	154163	151847	151808	151808
UA011_16S	180867	178704	178684	178684
UA011_16SBLK	121924	120430	120333	120333
UA012_16S	188975	186604	186482	186386
UA012_16SBLK	120158	118210	118183	118183
UA013_16S	164094	162202	162173	162173
UA013_16SBLK	22927	22660	22633	22633
UA014_16S	169647	167573	167534	167534
UA014_16SBLK	2522	2476	2467	2467
UA015_16S	163280	161412	161265	161187
UA015_16SBLK	35799	35336	35277	35277
UA02_16S	178525	176318	176205	176158
UA02_16SBLK	161296	159622	159441	159441
UA03_16S	168786	166698	166664	166664
UA03_16SBLK	134955	133371	133342	133342
UA04_16S	176515	174465	174307	174292
UA04_16SBLK	151465	149614	149594	149594
UA05_16S	108150	106872	106852	106852
UA05_16SBLK	95007	93630	93618	93618
UA06_16S	166543	164472	164432	164416
UA06_16SBLK	127222	125772	125486	125433
UA07_16S	182875	180595	180564	180564
UA07_16SBLK	138633	136685	136567	136567
UC01_16S	159964	157990	157908	157891
UC010_16S	142195	140091	140073	139915
UC010_16SBLK	179413	174683	174569	174473
UC011_16S	163827	160917	160684	160684
UC011_16SBLK	154958	151848	151785	151785
UC012_16S	210873	208136	207986	207818
UC012_16SBLK	156795	154654	154573	154320
UC013_16S	157471	155544	155511	155486
UC013_16SBLK	135966	133779	133715	133715
UC014_16S	182827	180734	180706	180700
UC014_16SBLK	225960	223063	223028	222999

UC015_16S	145831	144061	144034	143945
UC015_16SBLK	89579	88295	88213	88213
UC02_16S	176516	172554	172340	172189
UC02_16SBLK	247	160	155	155
UC04_16S	151231	149400	149358	149241
UC04_16SBLK	135039	131997	131647	131647
UC05_16S	170412	168293	168272	168255
UC05_16SBLK	181976	178812	178704	178632
UC06_16S	155780	153921	153887	153887
UC06_16SBLK	161219	159495	159470	159449
UC07_16S	176493	174524	174402	174336
UC07_16SBLK	155661	153870	153765	153690
UC08_16S	180916	178576	178432	178268
UC08_16SBLK	143599	141617	141586	141586
UC09_16S	168576	166507	166256	166248
UC09_16SBLK	166382	163531	163467	163467
Mean	**147091**	**145082**	**144999**	**144964**
SD	**45382**	**44773**	**44756**	**44739**

Table C5 Read counts for each analysed sample of the diet of *L. dimidiatus* before and after quality control (QC) with the *COI* primer

Sample	Raw Reads	After QC
LD01-COI	187279	176435
LD02-COI	206427	193763
LD03-COI	198856	188909
LD04-COI	294101	277728
LD05-COI	17632	16642
LD06-COI	255890	241031
LD07-COI	311146	288295
LD08-COI	361651	339903
LD09-COI	192690	181501
LD10-COI	173542	163700
LD11-COI	261603	246054
LD12-COI	68689	64380
LD13-COI	148629	140346
LD14-COI	229471	216333
LD15-COI	220250	207748
LD16-COI	146857	138637
LD17-COI	193251	182762
LD18-COI	211331	199137
LD19-COI	232898	219900
LD20-COI	197817	185757
LD21-COI	160909	152516
LD22-COI	167858	159069
LD23-COI	149411	141311
LD24-COI	240908	226805
LD25-COI	178037	168222
LD26-COI	163803	154613
LD27-COI	132814	125699
LD28-COI	158430	149369
LD29-COI	191199	180133
LD30-COI	152326	144391
Mean	**193523.5**	**182369.6**
SD	**65737**	**61525**

Table C6 Read counts for each analysed sample of the diet of *U. antonbruunii* (UA) and *U.* cf. *cyrtorhyncha* (UC) before and after quality control (QC) with the *COI* primer

Sample	**Raw Reads**	**After QC**
UC02-COI	210119	198538
UA06-COI	33598	30064
UA01-COI	22104	19769
UC05-COI	160046	151347
UA15-COI	18193	16767
UA13-COI	9790	8459
UC01-COI	205928	194725
UA14-COI	22009	19803
UC04-COI	164117	155093
UC07-COI	175540	166609
UA05-COI	4133	3831
UA02-COI	24425	22127
UC11-COI	120778	114571
UC08-COI	174327	164403
UC12-COI	194565	185019
UA11-COI	4169	3758
UC06-COI	166340	158384
UC15-COI	148390	140050
UC10-COI	127845	121671
UA12-COI	24716	22792
UA10-COI	18911	16502
UC13-COI	62378	59167
UA03-COI	18086	16159
UC09-COI	147517	139366
UA04-COI	11778	10875
UA07-COI	18611	16216
UC14-COI	152821	144927
Mean	**90416**	**85222**
SD	**75032**	**71432**

Table C7 Number of raw reads obtained after sequencing *Urocaridella antonbruunii* and *U.* cf. *cyrtorhyncha* gut content with and without the use of blocking primer

	Urocaridella cyrtorhyncha		*Urocaridella antonbruunii*	
Number of reads	Blocking (n=12)	No blocking (n=14)	Blocking (n=13)	No blocking (n=13)
Host (self-hits)	1,520,015 (89.4%)	2,215,561 (95.9%)	1,402,906 (99%)	2,100,416 (99.9%)
Bacteria	179,616	93,000	12,144	2,715
Eukaryote	159	299	1,585	203
Eukaryote with 98% similarities	83	292	70	70
Eukaryote with 90% similarities	17	5	478	69
Total	**1,699,809**	**2,308,863**	**1,416,773**	**2,103,340**

Table C8 Summary of model-based analyses ('mvabund') testing for potential multivariate relationships between the number of sequence of dietary items targeted by the cleaner wrasse *Labroides dimidiatus* and size and habitat at Lizard Island, Great Barrier Reef, Australia. Both (A) species and (B) phylum of targeted dietary items by *L. dimidiatus* are presented here

	Wald value	Pr(>wald)
(A) (Intercept)	8.375	0.346
Habitat	4.602	0.177
Size	10.894	0.328
(B) (Intercept)	6.204	0.0099
Habitat	2.936	0.2617
Size	4.715	0.0359*

Table C9 Number of matching base pairs between the potential organisms tested and the shrimp sequences following the 3' end of the 926R reverse primer. Species (n = 2 - 4) from each group reported here were tested *in-silico*. Red letters represent base pair mismatch

	3' end of 926R reverse primer (10 bp overlap)	Organism's sequence (15 bp) following the 10 bp of the primer
Shrimp Blocking primer	**MTTTRAGTTT**	**CAGTTTTGCAACCAT**
Organisms tested from digenean group	MTTTRAGTTT	CAGCTTTGCAACCAT
Organisms tested from capsalid group	MTTTRAGTTT	CAGCTTTGCAACCAT
Organisms tested from cestode group	MTTTRAGTTT	CAGCTTTGCAACCAT
Organisms tested from hirudinean group	MTTTRAGTTT	CGGCTTTGCAACCAT
Organisms tested from myxosporean group	MTTTRAGTTT	CAGCCTTGCGACCAT

Appendix D: Supporting Information for Chapter 6

Figures

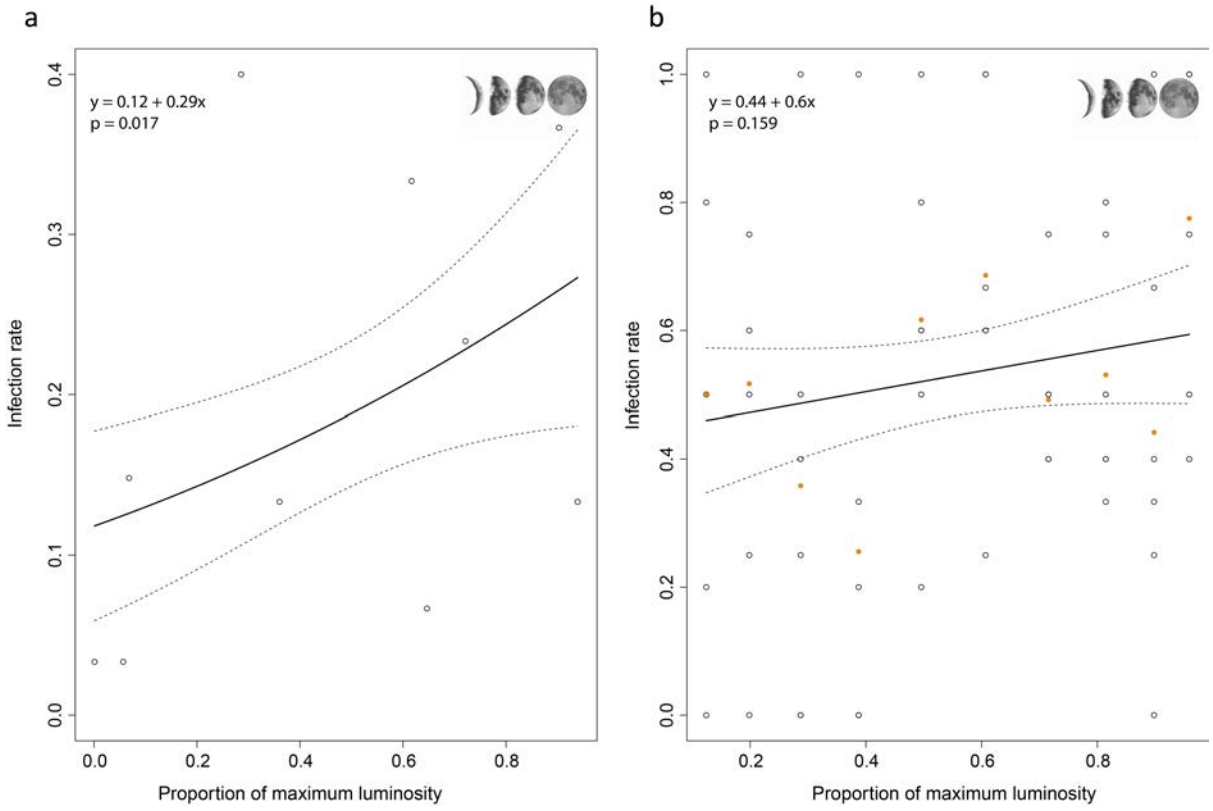

Figure D1 Gnathiid infection rates on (A) juvenile and (B) adult *Pomacentrus amboinensis* relative to the proportion of the maximum moon luminosity. (A) The dots represent the infection rates of gnathiids across all replicates (n=30) per night (n=10). (B) The unfilled dots represent the proportion of fed gnathiids (successful infection) relative to the total exposure of gnathiids (fed + unfed) for each fish tested (n=60) per consecutive night (n =10). The filled orange dots represent the mean proportion of fed relative to unfed gnathiid for each night

Table

Table D1 Gnathiid infection rates according to larval stage (stage 2 and 3) on juvenile *Pomacentrus amboinensis* for three seawater treatments (no coral, dead coral and live coral) performed with a Binomial Generalised Linear Mixed-Effects Model. Intercept represents live coral treatment and stage 2 gnathiid

Fixed effects	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	-1.86	0.73	-2.55	0.01 *
No coral	-0.09	0.42	-0.21	0.84
Dead coral	0.82	0.38	2.16	0.03 *
Gnathiid stage 3	-0.11	0.78	-0.14	0.88

Table D2 Mortality rates on juvenile *Pomacentrus amboinensis* in three seawater treatments (no coral, dead coral, live coral), with the different infection categories (fish exposed and infected, fish exposed and not infected, fish non exposed) performed with a Binomial Generalised Linear Mixed-Effects Model. Intercept represents live coral treatment and exposed and infected fish

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	-1.72	0.83	-2.09	0.037*
No coral	-0.81	0.56	-1.44	0.15
Dead coral	-0.69	0.54	-1.28	0.2
Fish exposed and not infected	-1.88	0.65	-2.89	0.004**
Fish non exposed	-1.83	0.62	-2.97	0.003**

Table D3 Proportion of successfully fed gnathiids (relative to the total number of parasites trialled) on adult *Pomacentrus amboinensis* in different seawater treatments (no coral, dead coral, live coral) performed with a Binomial Generalised Linear Model. Intercept represents live coral treatment

Fixed effects	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	-5.6	0.25	-22.57	<2e-16 ***
No coral	0.1	0.34	0.3	0.76
Dead coral	0.13	0.34	0.37	0.71

Appendix E: Publications arising from this thesis

Narvaez P, Morais RA, Hutson KS, McCormick Mark, Grutter AS (2021) Habitat degradation drives increased gnathiid isopod ectoparasite infection rate on juvenile but not adult fish. **Coral Reefs** 40:1867–1877. https://doi.org/10.1007/s00338-021-02166-y

Narvaez P, Yong RQ-Y, Grutter AS, Hutson KS (2021) Are cleaner fish clean? **Marine Biology** 168 (59). https://doi.org/10.1007/s00227-021-03858-3

Narvaez P, Vaughan DB, Grutter AS, Hutson KS (2021) New perspectives on the role of cleaning symbiosis in the possible spread of fish diseases. **Reviews in Fish Biology and Fisheries** 31:233–251. https://doi.org/10.1007/s11160-021-09642-2

Appendix F: Other publications during this thesis

Papers published in peer-reviewed journals

Quimbayo JP, Mendes TC, Barneche DR, Dias MS, Grutter AS, Furtado M, Leprieur F, Pellissier L, Mazzei R, **Narvaez P**, Sasal P, Soares MC, Parravicini V, Sazima I, Kulbicki M (2021) Patterns of taxonomic and functional diversity in the global cleaner reef fish fauna. **Journal of Biogeography** 48:2469–2485. https://doi.org/10.1111/jbi.14214

Paula JR, Sun D, Pissarra V, **Narvaez P**, Rosa R, Grutter AS, Sikkel PC (2021) The role of corals on the abundance of a fish ectoparasite in the Great Barrier Reef. **Coral Reefs** 40:535–542. https://doi.org/10.1007/s00338-021-02051-8

Narvaez P, Morais RA (2020) Filling an empty role: first report of cleaning by pygmy angelfishes (Centropyge, Pomacanthidae). **Galaxea** 22:31–36. https://doi.org/10.3755/galaxea.22.1_31

Allan BJM, Illing B, Fakan EP, **Narvaez P**, Grutter AS, Sikkel PC, McClure EC, Rummer JL, McCormick MI (2020) Parasite infection directly impacts escape response and stress levels in fish. **Journal of Experimental Biology** 223(16): jeb230904. https://doi:10.1242/jeb.230904

Grutter AS, Feeney WE, Hutson KS, McClure EC, **Narvaez P**, Smit NJ, Sun D, Sikkel PC (2020) Practical methods for culturing parasitic gnathiid isopods. **International Journal for Parasitology** 50:825–837. https://doi.org/10.1016/j.ijpara.2020.03.014

Morais R, Depczynski M, Fulton C, Marnane M, **Narvaez P**, Huertas V, Brandl S, Bellwood D (2020) Severe coral loss shifts energetic dynamics on a coral reef. **Functional Ecology** 34:1507–1518. https://doi.org/10.1111/1365-2435.13568

Sikkel PC, Richardson MA, Sun D, **Narvaez P**, Feeney WE, Grutter AS (2019) Changes in abundance of fish-parasitic gnathiid isopods associated with warm-water bleaching events on the northern Great Barrier Reef. **Coral Reefs** 38:721–730. https://doi.org/10.1007/s00338-019-01835-3

Book chapter

Grutter AS, Feeney WE, Hutson KS, McClure EC, **Narvaez P**, Smit NJ, Sun D, Sikkel PC (2021) *Gnathia aureamaculosa* and *G. marleyi*. In: Bobadilla SA, Bron JE, Wiegertjes G, Piazzon MC (eds): **Fish Parasites: A handbook of protocols for their isolation, culture and transmission**. 5m Publishing. Sheffield, United Kingdom

Appendix G: Grants, Awards and Honours

Grants and Awards

2018 – James Cook University Postgraduate Research Scholarship - February 2018 to February 2022 ($AUD 27,082 per annum)

2018 – Michael Hall Student Innovation Award – Australian Society for Fish Biology ($AUD 1,000): "Cleaner fish as vectors of parasitic disease"

2019 – Holsworth Wildlife Research Endowment Grant ($AUD 5,795): "DNA metabarcoding analysis to investigate parasite consumption by cleaner fish and shrimp"

2019 – Competitive Research Training Grant – James Cook University ($AUD 3,000)

2019 – John Glover travel Award – Australian Society for Fish Biology ($AUD 400): "Cleaner fish as vectors of parasitic disease"

2021 – David Yellowlees Excellence in Research Award – ARC Centre of Excellence for Coral Reef Studies ($AUD 3,000): "Understanding the ecological role of cleaner fish and cleaner shrimp using DNA metabarcoding techniques"

Honours

2021 – A new species of shark parasite was named on my behalf: "*Scyliorhinocotyle narvaezae*" in Vaughan D B, Christison KW, Hansen H (2021) New monogeneans from the bathydemersal southern African endemic catshark, *Holohalaelurus regani* (Gilchrist, 1922). **Systematic Parasitology** https://doi.org/10.1007/s11230-021-09982-4