Mitigating the impact of the *Acropora*-eating flatworm, *Prosthiostomum acroporae* on captive *Acropora* coral colonies
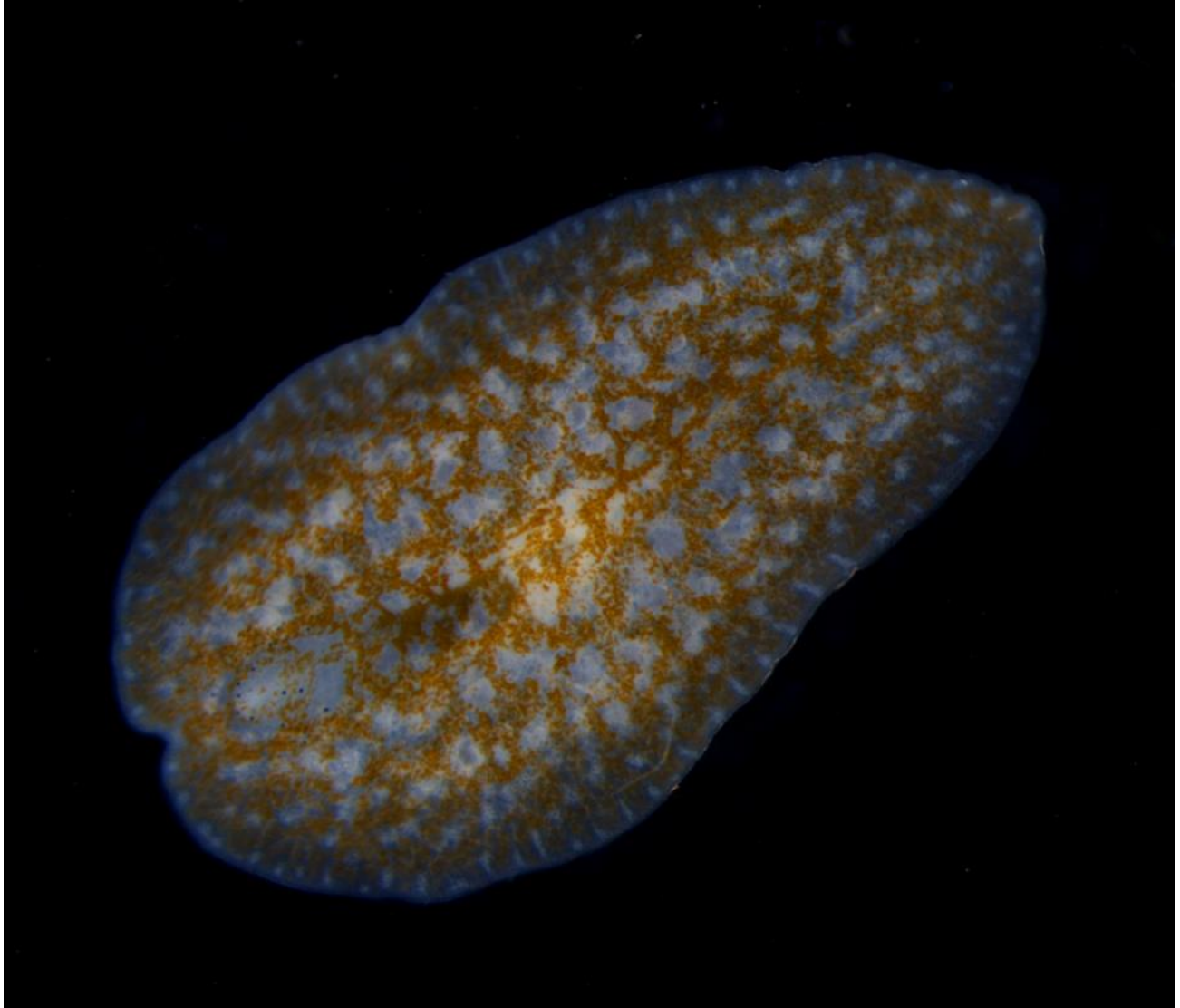
Thesis submitted by Jonathan Barton in May 2020

for the degree of Doctor of Philosophy

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Front piece: Whole, live *Prosthiostomum acroporae* from the captive culture we established and maintained at SeaSim, Australian Institute of Marine Science. Approximate length 7 mm. Photo: JB and Matt Salmon

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Statement of Contribution of Co-Authors

The following chapters have been accepted, are in review or in preparation for publication. Below is an overview of each co-author's contributions to the publications.

Chapter	Publication details	Contributions of each author
2	Barton JA, Bourne DG, Humphrey C, Hutson KS (2020) Parasites and coral-associated invertebrates that impact coral health. *Reviews in Aquaculture* Early View (awaiting issue placement) doi: 10.1111/raq.12434	All authors conceptualized the scope of the review. Barton sourced literature and wrote the manuscript with editing input from co-authors.
3	Barton JA, Hutson KS, Bourne DG, Humphrey C, Dybala C, Rawlinson KA (2019) The Life Cycle of the *Acropora* Coral-Eating Flatworm (AEFW), *Prosthiostomum acroporae*; The Influence of Temperature and Management Guidelines. *Frontiers in Marine Science*, 6: 524. doi: 10.3389/fmars.2019.00524	Co-authors assisted in conceptualization of experiments. Barton ran three experiments, Rawlinson assisted with two experiments, Dybala assisted with one. Barton conducted data analysis and managed draft preparation with editing input from co-authors.
4	Barton JA, Smith HA, Hung JYH, Humphrey C, Bourne DG, Hutson KS, Rawlinson KA (in prep for *Coral Reefs*) Polyclad flatworms associated with acroporid corals of the Great Barrier Reef: distribution and phylogenetics.	Barton screened all corals and collected flatworm samples. Smith and Huan-Soung conducted molecular work. Barton and Rawlinson did the morphological analysis. Barton managed draft preparation with editing input from co-authors.
5	Barton JA, Humphrey C, Bourne DG, Hutson KS (2020). Biological controls to manage *Acropora*-eating flatworms in coral aquaculture. *Aquaculture Environment Interactions* 12: 61-66. doi: 10.3354/aei00347	Barton conducted all experiments. Co-authors assisted in conceptualization of research. Barton managed draft preparation with editing input from co-authors.
6	Barton JA, Neil RC, Bourne DG, Humphrey C, Hutson KS (2020) Efficacy of chemical treatments of captive infestations of the *Acropora*-eating flatworm infestations. *Aquaculture* 532. doi: 10.1016/j.aquaculture.2020.735978	Co-authors assisted in conceptualization of experiments. Barton and Neil conducted experiments and conducted data analysis. Barton managed draft preparation with editing input from co-authors.

Declaration of Ethics

The research presented and reported in this thesis was conducted in compliance with the Great Barrier Reef Marine Park Regulations 1983 (Commonwealth) and the Marine Parks Regulation 2006 (Queensland), permitted by the Great Barrier Reef Marine Park Authority (GBRMPA) and the Queensland Parks and Wildlife Service under GBRMPA Permit No. G12/3236.1. The work was undertaken according to James Cook University Animal Ethics Committee approval, permit number A2466.

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Statement of Thesis Access

I, Jonathan Barton, the author of this thesis, understand that James Cook University will make it available to use within the University Library and via the Australian Digital Thesis Network for use elsewhere.

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I, Jonathan Barton, declare that the electronic copy of this thesis provided to James Cook University is an accurate copy of the submitted thesis, within the limits of the available technology.

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General Abstract

Maintaining the health of captive corals is vital to supply the industries and related activities associated with the ornamental trade, research, and reef restoration. However, our limited understanding of the biology of captive pests, their management and the stressors coral-associated invertebrates exert on their hosts, represents a potential bottleneck in captive maintenance and aquaculture production. The relationship between corals and their diverse associated invertebrates may change as corals enter captivity, and therefore must be understood to inform best practice captive pest management procedures. The present thesis aimed to provide an understanding of which coral-associated organisms impact the health of corals in captivity. Coral-associated invertebrates were examined in the context of their potential to harm corals in captivity (Chapter 2). Organisms that exhibit high fecundity, direct consumption of coral tissue and exhibit a propensity to reach unnatural densities in captivity are usually associated with a reduction in coral health, ultimately leading to colonial mortality or disease. The primary focus of this thesis, the *Acropora*-eating flatworm *Prosthiostomum acroporae,* exhibits these characteristics and is a particularly destructive coral-associated invertebrate in captivity.

Prosthiostomum acroporae feeds on *Acropora* coral tissue and its high fecundity and cryptic disposition facilitate spread and proliferation to high densities in captivity. Given the paucity of information available on *P. acroporae,* the present thesis sought to develop an integrated pest management framework based on investigation of the *P. acroporae* life cycle (Chapter 3), the distribution of *P. acroporae* on the Great Barrier Reef (Chapter 4), identification of potential biological control organisms (Chapter 5) and chemical treatment options (Chapter 6). Information and evidence gathered was used to develop a decision support tool to assist a variety of stakeholders to aid the diagnosis, treatment and management of these pests in captivity (Chapter 7; General Discussion).

We demonstrated that temperature alters the life cycle of *Prosthiostomum acroporae*, with higher temperatures reducing embryonation duration and the time required to reach sexual maturity. We

examined temperatures relevant to captive coral husbandry on the life cycle parameters of *P. acroporae* (Chapter 3). To do this, we established and maintained an *in vivo* culture for this and all subsequent experimentation in the thesis. Using temperatures relevant to captive coral husbandry (24°C, 27°C and 30°C) we studied the embryonation period (time to hatch) of egg capsules (and metamorphosing embryos), the time to starvation of metamorphosed hatchlings, and the time to sexual maturity of these hatchlings after successful settlement on host coral, *Acropora millepora* (Ehrenberg, 1834). Temperature had a significant influence on the embryonation period and time to sexual maturity, but not hatchling longevity. Linear mixed effects models were used to predict the embryonation period and time to sexual maturity between 21°C - 30°C. This model informs stakeholders about treatment intervals (time between initial and successive treatments) designed to disrupt the life cycle of *P. acroporae* infesting captive coral aquaria. Because eggs appear impervious to chemical immersions, treatment intervals are of critical importance to remove hatched *P. acroporae* before they can reach sexual maturity and contaminate the system through egg deposition.

Sampling of *Acropora* from several locations on the Great Barrier Reef (GBR) demonstrated that *P. acroporae* are living in association with *Acropora* hosts in all sampled wild locations, with distribution extending from Lizard island (14.6645° S) in the north to the Keppel Islands (23.1894° S) in the southern GBR (Chapter 4). Of 154 sampled coral colonies, 56% ± 4% (mean ± SE) were infested with flatworms, with approximately 5.89 ± 0.51 (mean ± SE) flatworms found per coral fragment. Coral species had a significant effect on whether coral fragments were infested, and on the number of flatworms found on coral fragments with *Acropora loripes* (Brook, 1892) the most frequently infested species (82.4 ± 7.9 %; mean ± SE) with approximately 5.5 ± 1.20 *P. acroporae* (mean ± SE) flatworms found per coral. Post hoc pairwise comparison revealed significant differences in the percentage of infested colonies between *A. loripes* and *Acropora* sp. (Dunn Test with Bonferroni adjustment; $P < 0.008$). We also observed captive *Montipora* colonies with 2-3 mm blotchy feeding scars and organized egg clusters deposited on exposed

coral skeleton. Sampling revealed a polyclad flatworm similar to *Prosthiostomum montiporae* Poulter, 1975 from Hawaii in association with *M. digitata,* and *Montipora tuberculosa* (Lamarck, 1816) . Morphological comparison between Great Barrier Reef specimens and the original description of *P. montiporae* by Poulter (1975) showed that the specimens obtained in this study conformed to *Prosthiostomum montiporae* based on eye arrangement, a distinctive cleft pharynx, and reproductive anatomy. 28s RNA sequencing and subsequent comparison demonstrated three clear subclades within *Prosthiostomum*; one composed of *P. acroporae*, another containing GBR *P. montiporae* and one other large *Prosthiostomum* sp. from *Montipora digitata* (Dana, 1846), and *Prosthiostomum* sp. Z which grouped with the free living flatworms *Prosthiostomum torquatum* Tsuyuki, Oya & Kajihara, 2019 and *Prosthiostomum trilineatum* Yeri & Kaburaki, 1920*.*

To assist in captive management of *P. acroporae,* we identified two potential biological controls. Chapter 5 demonstrated that the sixline wrasse, *Pseudocheilinus hexataenia* (Bleeker, 1857) *,* and the peppermint shrimp, *Lysmata vittata* (Stimpson, 1860), reduced populations of *P. acroporae* on infested *Acropora* in captive environments. Experiments introduced *A. millepora* infested with *P. acroporae* and skeletal fragments with egg capsules laid on coral skeleton to aquaria over 24 hours in the presence or absence of *P. hexataenia* or *L. vittata. P. hexataenia* consumed 100% of adult flatworms from *A. millepora* fragments (n = 9; 5 flatworms fragment^{-1}), while *L. vittata* consumed 82.0 ± 26.76% of adult flatworms (mean ± SD; n = 20). *Pseudocheilinus hexataenia* did not consume any *Prosthiostomum acroporae* egg capsules, while *L. vittata* consumed 63.67 ± 43.48% (n = 20) of egg capsules on the *Acropora* skeletons. Mean handling losses in controls were 5.83% (shrimp system) and 7.50% (fish system) of adult flatworms and 2.39% (fish system) and 7.50% (shrimp system) of egg capsules. Both exposure to *L. vittata* and *P. hexataenia* resulted in the predation of *P. acroporae* life stages from the *Acropora* coral host and represent viable biological control candidates to reduce infestations of *P. acroporae* in aquaculture systems.

To add to the tools for *P. acroporae* management, we evaluated the use of therapeutic immersions in praziquantel and levamisole (Chapter 6). Experiments measured the removal efficacy of these anthelmintics and the treated *A. millepora* fragments were monitored for subsequent evidence of bleaching and basal growth for four weeks following treatment. Levamisole and praziquantel immersions removed significantly more flatworms from *A. millepora* fragments (93% ± 3.8 and 95.0% ± 2.6 respectively; mean ± SE; p < 0.05) compared to the seawater handling control (26% ± 7.5%). Chemical treatments had no significant effect on basal growth, with fragments across all treatments (including controls) increasing basal area by 73.31 ± 3.82% (mean ± SE). Furthermore, bleaching was not observed for any *A. millepora* fragments across the treatments and controls. Results from this study demonstrate that levamisole and praziquantel used in conjunction with water movement are effective at removing> 90% of *Acropora* eating-flatworms with no observable negative impacts on coral growth or health.

Based on the finding that potentially more than half of wild *Acropora* on inshore areas of the Great Barrier Reef host *P. acroporae* (Chapter 4), there is a considerable risk of introducing corals infested with *P. acroporae* into captive systems from wild collections. This is especially true without established biosecurity, quarantine, or management strategies. A management plan for appropriate prevention, diagnosis and treatment will help to support research activities, reef restoration and the marine ornamental trade that maintain *Acropora* in captivity. We synthesized an Integrated Pest Management (IPM) framework for control of *P. acroporae* on captive *Acropora* corals (Chapter 7; General Discussion). We identify appropriate prevention methods including processing incoming corals, quarantine procedures, rapid real-time diagnostic methods for visual identification of adult worms and eggs, transmission mitigation behaviors, biological controls, chemical treatments, and fallowing (aquaria empty of devoid of *Acropora* for a given duration). For each of these key steps we consider site-specific challenges of different stakeholders for captive coral husbandry. The stakeholders discussed are coral

researchers, reef restoration practitioners, coral collectors, coral aquaculture facilities, and distributers/suppliers. A web-based decision support tool was developed to make this information accessible to stakeholders in the husbandry of *Acropora* corals (https://cawthron.shinyapps.io/acropora_eating_flatworm/). The app provides information on detection, diagnosis and real time life cycle data based on aquarium system temperatures from 21-30ºC (also ºF conversion) to effectively observe intervals between initial and successive chemical treatments.

The present study provides information for the management of *P. acroporae*, where previously no such information existed. This new research includes a large extension of the previously known distribution of *P. acroporae* and explores the abundance of these flatworms present on *Acropora* spp. collected from the wild, which informs collectors for research or the ornamental trade of the considerable risk of infestation when wild corals are brought in to captivity from the field. The extensive examination of the life cycle of *P. acroporae* as influenced by temperature provides critical data allowing for the disruption of the flatworm's life cycle, significantly improving treatment options for infestations in captive systems. The discovery of two organisms, *L. vittata* and *P. hexataenia*, that can be used as biological controls, provides the first evidence of the efficacy of such organisms to treat parasites in coral aquaculture. Furthermore, the thesis provides the first empirical evidence of the efficacy of anthelmintics (levamisole HCl and praziquantel) for the removal of flatworms from infested colonies, coupled with no observable evidence of negative consequences to treated *Acropora* hosts. The novel contribution this thesis provides to the field of pest management in coral aquaculture provides a foundation to optimize management of *P. acroporae* and similarly destructive pests in the future. The use of the *P. acroporae* Integrated Pest Management web application provides important management information that is distilled to reach both academic and non-academic stakeholders and maximize the contribution of this thesis to coral husbandry practices.

Table of Contents

List of Tables

List of Figures

(arrowheads) (Bi) or lost entirely (Bii) (live hatchlings). (C) Hatchlings that have lost their lobes retain the ciliary

tufts (arrow; fixed specimen). Scale = 100 mm. (D) Hatchling longevity (the number of days a hatchling survives in

the absence of coral) at 24°C (n = 64, 4 egg clusters), 27°C (n = 81 individuals, 4 egg clusters), and 30°C (n = 62, 5

egg clusters) (error bars = min. and max. values, boxes = lower and upper quartiles, line = median, and dot =

mean).

Figure 3-4: A) The time for hatchlings to reach sexual maturity (error bars = min. and max. values, boxes = lower

and upper quartiles, line = median, and dot = mean). (B) Mean body length (± SE) of individuals at sexual maturity.

Treatment boxes and bars with the same letter are not significantly different from each other (p > 0.05).

Figure 3-5: The life cycle of *P. acroporae* measured at 3°C increments from 24 to 30 °C and the recommended

timings of treatments (see section "Discussion").

Figure 4-1: Map showing the distribution of *P. acroporae* on the Great Barrier Reef, color coded corresponding to

samples collected for this study (blue), areas known from collector input (green), and from the first report *of P.*

acroporae on the GBR (orange; Rawlinson and Stella 2012). Collector input (green) highlight regions of reef where

licensed coral collectors encounter *P. acroporae*.

Figure 4-2: Evidence of Montipora-eating flatworms (MEFW) infestation of Montipora digitata from the Great

Barrier Reef. A. Infested M. digitata colony covered in feeding scars (scale = 3 cm), B. *Indicates egg capsules laid

on freshly exposed skeleton on the margin of tissue loss (arrows) on M. digitata (scale = 1 mm), C. Two MEFW

specimens removed from M. digitata colony (scale = 2 mm), D. Cross section of MEFW with Symbiodiniaceae in

branching gut (scale = 1 µm).

Figure 4-3: The morphology of the MEFW. A. Ventral view of a live specimen showing the mouth (m), pharynx (p),

female gonopore (fg), cement glands (cg), sucker (s) uteri (u), and ovaries (o), Scale = 1 mm; B. Dorsal anterior view

of wholemount showing coniform eye arrangement of cerebral eyes (ce) and positioning of marginal eyes (me,

Scale = 1 mm; C. Sagittal section showing anterior third of worm; mouth (m), pharynx (p), brain (b), cerebral (ce),

and the gut (g), Scale = 1 mm; D. Cross section showing the sucker (s), paired uteri (U), eggs (e), sperm (sp), cement

glands (cg) and main tract (mt) of branching gut ; E. Sagittal section showing the main tract of branching gut (bg),

seminal vesicle (sv), prostatic vesicles (pv), also known as accessory vesicles), spermiducal vesicles (spv), ejaculatory duct (ed), male atrium (ma), penis sheath (ps) and male gonopore (mg); F. Cross section showing the gut (g), uterus (u), egg/oocyte (e), vagina (v), cement glands (cg), egg pouch (ep) and female atrium (FA), Scale = 0.2 mm; G. Cross section showing the retracted scroll-like cleft pharynx (p).

Figure 4-4: A maximum likelihood tree of Prosthiostomidae 28s rRNA sequences. Numbers next to nodes are SH-aLRT support (%) / ultrafast bootstrap support (%). Highlighted specimens in red and blue indicate two inferred sub-clade within Prosthiostomidae. Figure 4-5: Dorsal photograph of Prosthiostomum sp. Z specimen; scale 2 mm.

Figure 5-1 Proportion of *Acropora*-eating flatworm individuals and egg capsules removed (error bars: ±SD) in the presence and absence of biocontrols. (A) *Lysmata vittata* and flatworm individuals (n = 24), (B) *L. vittata* and flatworm eggs (n = 20 egg clusters), (C) *Pseudocheilinus hexataenia* and flatworms (n = 9), and (D) *P. hexataenia* and flatworm eggs (n = 9 egg clusters). *: statistical significance between treatments and controls. Photos: = *L. vittata* and *P. hexataenia*. (*P. hexataenia* photo credit: creative commons license istockphoto.com user: marrio31 id#471448553)

Figure 6-1: Schematic showing immersion procedure with all treatments (levamisole, praziquantel, handling control, EtOH control, and no handling).

Figure 6-2: Photographs of experimental design: A. *Acropora millepora* fragments in their respective 2 L beakers during a one-hour chemical immersion, B. Containers with filtered seawater use for the 'shake step' after chemical immersion, C. Camera cart used for taking photos of *A. millepora* fragments, D. Initial photo (before chemical immersion) taken of *A. millepora* fragments, E. Day 28 photo taken of the same tray of *A. millepora* fragments.

Figure 6-3: Stacked bar plot showing the mean percentage of *P. acroporae* recovered from *A. millepora* fragments from each associated chemical treatment (Handling control, Levamisole, and Praziquantel) from each immersion, shake step, and the mechanical screening step to recover remaining flatworms. The letters (a) and (b) indicate treatments with statistical differences from each other.

Chapter 1: General Introduction

Keeping corals in captivity is a required component of research, reef restoration, and the ornamental trade. The overarching objectives of the present thesis are to expand our knowledge of *Prosthiostomum acroporae* in a way which supports captive coral husbandry and the management of this destructive pest associated with captive *Acropora* populations. At the time of commencing my candidature, there was a distinct knowledge gap in our understanding of *P. acroporae*, with investigations limited to an account of a lethal infestation in captivity (Nosratpour 2008), the species description from captive specimens (Rawlinson et al. 2011), the first account of *P. acroporae* in the wild (Rawlinson and Stella 2012), and an examination of the consequences of infestation on the photoacclimation ability of *Acropora* fragments (Hume et al. 2014). The chapters in this thesis aim to address the paucity of information available on *P. acroporae* by investigating key components which advance or support the further development of effective management strategies for *P. acroporae* in captivity.

To further inform management decisions, Chapter 2 aims to understand the common characteristics which make coral pests most destructive to captive coral populations. The traditional model of captive pest management aims to disrupt the life cycle of pest species, removing individual pests before they become sexually reproductive, rather than continuously removing already reproductive individuals. The life cycle of poikilothermic organisms are often influenced by temperature (Howe, 1967; Hoegh-Guldberg and Pearse, 1995; Golizadeh et al. 2007; Wudarski et al. 2019), where the general trend is an increase in developmental rate associated with an increase in temperature to a physiological threshold, and subsequent completion of the life cycle in shorter periods of time. The influence of temperature on parasite development is studied within the context of finfish aquaculture (Brazenor and Hutson, 2015), where this approach effectively informs the timing of treatment regimens at different temperatures. Given the variability of temperatures in coral aquaria (24 - 30° C), it is imperative to understand how the

development of *P. acroporae* similarly changes at different temperatures. While Rawlinson et al. (2011)

examined the life cycle of *P. acroporae*, there was a lack of understanding of the influence of

temperature on the life cycle of this pest; specifically the influence of temperature on the embryonation

period (time to hatching of eggs), longevity of hatchlings, and the time to reach sexual maturity.

Accordingly, in Chapter 3 we investigated the influence of temperature on the embryonation period,

hatchling longevity, and time to sexual maturity of *P. acroporae*. Using the information derived from this

investigation, we were able to later identify the interval between initial and subsequent treatments

which most effectively removes flatworms from infested coral colonies before reaching sexual maturity,

but not after egg capsules associated with these colonies are able to hatch.

With only one report of *P. acroporae* infesting wild *Acropora* (*Acropora valida* on Lizard Island, GBR*;*

Rawlinson and Stella 2012), Chapter 4 aims to understand where else *P. acroporae* are prevalent. This is

to inform where harvest of *Acropora* from the GBR presents the risk of introduction of *P. acroporae* to

captive environments, and the subsequent risk of proliferation in these aquaria. While biosecurity best

practices are largely established for the aquaculture of finish and some invertebrates (Bondad-Reantaso

et al. 2005; Lightner 2007), biosecurity practices in coral aquaculture remain in their infancy.

Accordingly, we developed a screening method for *Acropora* colonies used throughout my candidature

to detect infested colonies post-harvest upon entering the National Sea Simulator (SeaSim) at the

Australian Institute of Marine Science (AIMS). Using data recorded from screening 154 *Acropora*

colonies, we can gain a better understanding of which coral reefs harbor infested *Acropora* colonies*.*

Furthermore, communication with coral collectors from the ornamental trade on the GBR provided

further information on the distribution of *P. acroporae* on the GBR.

Following the investigation of *P. acroporae* introduction to captive aquaria, and how temperature

influences their subsequent development, we aimed to provide captive management tools in the form

of biological controls. Biological control is the use of predators or parasites of a given pest organism to

suppress or eliminate the pest populations in an agricultural or aquaculture environment (Smith and

Basinger 1947; Simmonds et al. 1976; Greathead 1994; Tully et al. 1996; Eilenberg et al. 2001). Perhaps

one of the most successful modern models of biological controls is the use of the ballan wrasse *Labrus*

bergylta Ascanius, 1767 and lumpfish *Cyclopterus lumpus* Linnaeus, 1758 to control sea lice in salmonid

(*Salmo salar* Linnaeus, 1758) aquaculture (Tully et al. 1996). Accordingly, in Chapter 5 we found

biological control organisms suitable for coral aquaria. We investigated the potential of two different

organisms to consume *P. acroporae* and their egg capsules *in situ* (on the coral host). Any potential

biological control would ideally have an established fishery or captive breeding program (preferable)

and be suitable for most captive aquaria (Powell et al. 2017; Brooker et al. 2018). While no empirical

evidence existed, some hobbyists have historically asserted that the sixline wrasse *Pseudocheilinus*

hexataenia consumed *P. acroporae* from captive *Acropora* populations. The popularity of this organism

in the marine ornamental trade made *P. hexataenia* and ideal candidate for testing. The second

organism we examined was inspired by the previous work of Rhyne et al. (2004) that demonstrated

peppermint shrimps *Lysmata* spp. consume *Aiptasia* sp. in captivity. Furthermore, Vaughan et al.

(2018a, b) demonstrated the ability of *L. vittata* to consume a variety of finfish parasites at different life

stages in captivity. The previously demonstrated efficacy of peppermint shrimps, combined with their

popularity in the marine ornamental trade prompted my investigation of *L. vittata* as a potential

biological control organism for captive infestations of *P. acroporae*. Based on the results of exposing

infested coral fragments to a potential predator or control treatment, we evaluate their efficacy at

consuming a known number of flatworms or egg capsules.

Similarly, to the treatment of any pest in aquaculture, managers generally rely on having more than one

treatment approach. To support management of *P. acroporae*, additional tools are required to support

prevention, mitigation, and treatment methodology to ultimately advance biosecurity practices. With no

current empirical evidence for effective chemical treatments to remove *P. acroporae* from infested

Acropora colonies, Chapter 6 aims to identify suitable chemicals for *P. acroporae* treatment. Given the specificity of levamisole HCl and praziquantel (anthelmintics) at targeting platyhelminths (Ribeiro et al. 2005; Doenhoff et al. 2008), we evaluate their use in therapeutic chemical immersions. As with any medication, high efficacy is irrelevant if the host is harmed by the treatment. *Acropora millepora* fragments were photographed before and four weeks after treatment, then analyzed to discern if any bleaching occurred, or if basal growth was at all compromised by the treatment. From the results of the study, we make recommendations for or against their use in for treating *P. acroporae* infestations.

The general discussion (Chapter 7) aims to combine the knowledge gained from previous chapters of this thesis into the framework of integrated pest management (IPM) that can be used by various stakeholders in captive coral husbandry. These components include biosecurity practices such as processing incoming coral colonies, quarantine procedures, transmission mitigation behaviors, and reduction through biological controls and/or treatment using chemicals. We consider and discuss the challenges of applying these resources in the context of different stakeholders including coral husbandry for reef restoration, research, coral collectors, wholesalers, aquarium shops, the hobbyist aquarium, and how socioeconomic status may influence the application of these components. Finally, we describe the web application developed as a free educational tool for stakeholders, which can also be used to recommend treatment intervals depending on the temperature (in degrees centigrade or fahrenheit) of a given system based on the models from Chapter 3.

Chapter 2: Parasites and coral-associated invertebrates that impact coral health

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Abstract

Scleractinian corals both directly and indirectly provide both habitat and a source of food to a variety of associated invertebrates. While this associated biodiversity can be by in large positive to the health of coral reefs, sometimes coral-associated invertebrates can have deleterious effects on coral health. This is especially relevant in the context of coral aquaculture, where the study of potential coral pests can inform managers if intervention is necessary to achieve maximum growth and survivorship of their captive corals. Here we review the complex relationships between corals and their associated symbiotic organisms, identify invertebrates that may harm the corals, and suggest known management techniques in captivity. Groups considered included acoels (Xenacoelomorpha: Acoela), digeneans (Trematoda: Digenea), polyclads (Rhabditophora: Polycladida), gastropods (Mollusca: Gastropoda), decapods (Malacostraca: Decapoda), copepods (Hexanauplia: Copepoda), and pyrgomatids (Cirripedia: Pyrgomatidae). There are few empirically validated management techniques for coral pests, particularly in terms of largescale aquaculture, emphasizing the need for further directed research in this area. Information generated through the ornamental trade and hobbyists is valuable to inform future research direction targeted towards captive coral husbandry, reef ecosystem management and restoration strategies.

Keywords: symbiosis, pest management, reef restoration, coral aquaculture, ornamental trade

Introduction

Coral diseases can contribute to decline in coral ecosystem health (Aronson and Precht 2001; Willis *et al.* 2004; Bourne et al. 2009; Sweet et al. 2012); however, the etiology of coral diseases can be difficult to derive and are often complex (Mera and Bourne 2018). Several reviews have provided comprehensive examinations of coral diseases associated with bacteria and ciliate infections (e.g., Willis *et al.* 2004;

Rosenberg and Kushmaro, 2011; Sweet et al. 2012; Sheridan et al. 2013; Sweet and Bulling 2017; Mera and Bourne 2018), however to date there has been limited consideration of other potential pathogenic agents such as parasites and symbiotic invertebrates on coral health (Sweet et al. 2012). Currently there are more than 165,000 described species of coral-associated invertebrates along a spectrum of symbiosis from mutualism to commensalism and parasitism (Ray and Grassle 1991; Ruppert et al. 2004; Rotjan and Lewis 2008; Stella et al. 2011), yet the scientific community lack understanding of the influence these invertebrate symbionts can have on their coral host.

The confronting predictions for coral reefs over the coming century (Hoegh-Guldberg et al. 2007; Veron et al. 2009; Hughes et al. 2014; Hughes et al. 2017, 2018) have led to an increase into research on active intervention strategies in an attempt to restore reefs and build ecosystem resilience (van Oppen et al. 2015, 2017; Barton et al. 2017). Research is currently underway to demonstrate the feasibility of large-scale coral aquaculture to provide a source of corals to transplant onto degraded coral reefs (Baria et al. 2012; Villanueva and de la Cruz 2016). Pests and disease have a history of negatively impacting aquaculture efforts; the cost of sea lice (ectoparasitic copepod) control in the salmonid aquaculture costs the industry approximately 6% of production value of a US$8.4 billion industry (in 2006) (Costello 2009). Assessment of costs associated with pests in coral aquaculture require the consideration of which of these pests exist and how they can be treated effectively. While limited practical means of pest control exist after corals are transplanted, it is pertinent to understand which organisms pose a threat to corals during the 'nursery' phase or coral grow-out period to implement countermeasures preceding transplantation for biosecurity and optimal growth (Epstein and Rinkevich 2001; Rinkevich 2006). This is a critical phase in both sexual and asexual coral propagation techniques, whereby corals must reach a size refuge to increase survivorship before transplantation (Epstein et al. 2001; Lirman et al. 2010; Barton et al. 2017). Survivorship is also important for scleractinian corals that are wild-harvested or cultured in aquaculture facilities. The natural balance between potentially harmful coral-associated

invertebrates and their predators may be compromised in captive environments, leaving corals particularly vulnerable to pest proliferation. Given the considerable costs associated with coral aquaculture, optimization of coral pest control could reduce costs per coral in the 'nursery phase'.

This review examines parasites and other coral-associated invertebrates that potentially impact scleractinian coral health in wild and captive coral colonies. We considered acoels (Xenacoelomorpha: Acoela), digeneans (Trematoda: Digenea), polyclads (Rhabditophora: Polycladida), gastropods (Mollusca: Gastropoda), decapods (Malacostraca: Decapoda), copepods (Hexanauplia: Copepoda), and pyrgomatids (Cirripedia: Pyrgomatidae) for this review. The crown-of-thorns starfish, *Acanthaster planci* (Linneaus, 1758) and microorganisms implicated in coral disease (i.e., bacteria, viruses, and ciliates), were excluded because of the existing body of work available which examines their impacts on coral reefs (e.g., Brodie et al. 2005; Kayal et al. 2012; Sweet and Séré 2016; Pratchett et al. 2017; Mera and Bourne 2018; Buerger and van Oppen 2018). Current management practices for parasites and pests of captive coral are assessed and for cases where no management is known, potential approaches are suggested.

Aceola

Acoels (Acoela: Acoelomorpha) are generally free-living, soft-bodied animals commonly found in marine environments (Ogunlana *et al.* 2005). Many acoels live in association with sediments, but some are epizoic, considered by many to live non-parasitically on the surface of corals (Winsor 1990; Barneah et al*.* 2004, 2007, 2012). Acoels occur on corals globally, including reefs in the Red Sea (Ogunlana et al. 2005), Coral Sea (Winsor 1990), Indonesia (Haapkyla *et al.* 2009), Micronesia (Trench and Winsor 1987), Western Australia (Cooper et al. 2015), and on the GBR (Winsor 1990). *Waminoa* spp. (Winsor 1990; Figure 2-1B) are coral-associated acoels of the family Convolutidae that infest more than twenty genera

of soft and stony corals (Barneah et al. 2004, 2007; Haapkyla et al. 2009; Hoeksema and Farenzena 2012; Table 2-1).

Waminoa spp. may have negative consequences for their coral hosts through the consumption of coral mucus (Barneah et al. 2007; Naumann et al. 2010), inhibiting host biochemical processes (e.g. Symbiodinacea photosynthesis; Barneah et al. 2007; Hoeksema and Farenzena 2012). They also have a propensity to remove and consume zooplankton caught in coral polyps (Wijgerde *et al.* 2013). The consumption of coral mucus by *Waminoa* spp. may weaken coral immunity and compromise the ability of the coral to resist unfavorable environmental conditions such as increases in UV light and sedimentation (Naumann et al. 2010). Barneah et al. (2007) observed soft coral *Stereonepthya cundabiluensis* Verseveldt, 1965 infested with *Waminoa* sp. lacked a mucus layer and apparently modified the phenotype of infested corals which subsequently developed distinct microvilli. The authors also observed the translocation of carbon and nitrogen isotopes (^{13}C and ^{15}N respectively) from coral mucus into *Waminoa* sp. tissue. Infestation with *Waminoa* sp. has been observed to inhibit photosynthetic potential of Symbiodiniaceae, which may cause indirect negative effects on the host coral through a reduction in net energy production (Barneah et al. 2007). Hoeksema and Farenzena (2012) observed the fungid coral *Danafungia scruposa* Klunzinger, 1879 fully covered by *Waminoa* sp., and suggested impairment of coral respiration and feeding, though further empirical studies are required. Wijgerde et al. (2013) demonstrated that *Waminoa* sp. impaired zooplankton feeding by the octopus coral *Galaxea fascicularis* Linnaeus, 1767, with single polyps infested with worms (density of 3.6 ± 0.4 individuals per polyp) having significantly reduced prey ingestion rates relative to polyps without worms; between 5 to 50% of total prey captured by the polyps was stolen by *Waminoa* individuals. These results prompted the classification of *Waminoa* sp. as parasites exhibiting kleptoparasitism, or the removal of acquired prey items from the coral polyps (Wijgerde et al. 2013). Interference with heterotrophic feeding may result in organic nutrient deficiencies in the form of fatty acids and amino

acids, though studies measuring coral growth with and without *Waminoa* sp. are needed to evaluate whether these animals present a considerable burden to coral health.

Sometimes mistaken for *Acropora*-eating flatworms or 'red planarians' by aquarists, *Waminoa* spp. and other convolutids are viewed as unwanted pests by aquarists and appear to multiply rapidly within aquaria (Ogunlana et al. 2005; Table 2-1). While they all bear similar coloration, morphological differences make differentiation between Waminoa spp., *Convolutriloba retrogemma* Hendelberg and Akesson, 1988 and *Heterochaerus australis* Haswell, 1905 possible. *Waminoa* are typically ovoid in shape, often with a posterior cleft (Winsor 1990). *H. australis* has an oblong body shape with a pair of caudal appendages or lappets (Figure 2-1C), while *C. retrogremma* has a median lobe in addition to caudal appendages (Hendelberg and Akesson 1988; Winsor 1990)*. Both *Convolutriloba* and *Heterochaerus* appear able to populate all areas of an aquarium, unlike *Waminoa* which is found preferentially on coral hosts. There are several potential treatments for these pests. Salifert's product 'Flatworm Exit' (in tank treatment) and essential oil-based coral dips (i.e., Coral Rx, Revive Coral Cleaner) are commonly used to treat aquariums for acoel infestation (see Lynford 2009; Sweet et al. 2012; Table 1). Freshwater immersion coupled with vigorous shaking can be highly effective for treating infestations on corals which will briefly tolerate freshwater (e.g. *Echinophyllia,* Coraliomorpha, *Palythoa*; author pers. obs.). The blue velvet nudibranch, *Chelidonura varians* Eliot, 1903*, is also considered by the hobbyist community to be a successful biological control for these pests in aquaria, but these nudibranchs starve once the population of their prey has been reduced and are not widely available.

Table 2-1: A summarization of coral-associated invertebrates, the known consequence of their presence on coral health, and known treatment methodologies.

Classification	Genus	Prone to high density	Consumes coral tissue	Demonst rated impact on coral	Affiliation with disease	Associated with coral mortality	Signs of infestation	Treatment	References
Acoela	*Convolutriloba*	Y	N	N	N	N	Oblong body shape with median lobe between two caudal appendages	Essential oil-based dips, freshwater dips, or manual removal	Hendelberg and Akesson 1988; Winsor, 1990; Lynford 2009
	Waminoa	Y	N	Y	N	N	Fleshy ovoid shapes on coral tissue; heavy infestations can cover 100% of coral surface area	Essential oil-based dips, freshwater dips, or manual removal	Winsor 1990; Ogunlana et al. 2005; Barneah et al. 2007; Lynford 2009; Naumann et al. 2010; Hoeksema and Farenzena 2012; Wijgerde et al. 2013
	Heterochaerus	N	N	N	N	N	Oblong body shapes with two caudal appendages; can often be found on unfouled substrates	Essential oil-based dips, freshwater dips, or manual removal	Hendelberg and Akesson 1988; Winsor, 1990
Digenea	*Polypipapiliotrema stenometra*	N	N	Y	Y	N	Pink nodules or irregular growths on *Porites* coral tissue	Removal of intermediate or chaetodontid host	Aeby 1991, 1998, 2003, 2007
Polycladida	*Prosthiostomum acroporae*	Y	Y	Y	Y	Y	Characteristic circular feeding scars and egg clusters typically found around the base of coral colony, underside of and in between branches. *Acropora* host	Isolation of infested colony, removal of individuals and all egg clusters; Levamisole HCl, biological control with *Lysmata vittata* and *Pseudocheilinus hexataenia*.	Carl et al. 2008, Nosratpour 2008; Barton et al. 2019a, b
	Prosthiostomum montiporae	Y	Y	Y	Y	Y	Characteristic circular feeding scars and egg clusters typically found around the	Similar approach to *P. acroporae* control	Jokiel and Townsley 1974; Poulter 1975; Barton et al. 2019

base of coral colony, underside of and in between branches. *Montipora* host

							Signs of infestation	Treatment	References
Muricidae	*Coralliophila*	N	Y	Y	Y	Y	Usually conspicuous around the site of tissue loss on coral colonies. Shells are often purple form CCA	Mechanical removal using forceps	Johnson and Cumming 1995; Baums et al. 2003a, 2003b; Potkamp et al. 2017; Shaver et al. 2008;
	Drupella	N	Y	Y	Y	Y	Usually conspicuous around the site of tissue loss on coral colonies. Shells are often purple form CCA	Mechanical removal using forceps	Johnson and Cumming 1995; Baums et al. 2003a, 2003b; Potkamp et al. 2017; Shaver et al. 2008;
Littorinimorpha	Vermetidae	Y	N	Y	N	N	Distinct growth anomalies on coral surface; size varies between genera	Mechanical removal or sealing of shell to prevent reproduction	Hughes and Lewis 1974; Zvuloni et al. 2008; Bergsma 2009; Shima et al. 2010, 2013; Phillips 2011; Zill et al. 2017; Brown and Osenberg 2018
Nudibranchia	Opisthobranchs (*Phestilla, Pinufius, Tenellia*)	Y	Y	Y	N	Y	Egg capsules generally present on the underside of coral surface. Although generally camouflaged, the cerata of individuals can indicate their presence	Isolation of infested colony, chemical dips, mechanical removal of adults/eggs, potential biological controls	Gochfield and Aeby 1997; Wong et al. 2017
Copepoda	Xarifiidae (*Xarifia*)	N	N	N	N	N	N/A	N/A	Humes 1985b, 1985a; Cheng and Dai 2009; Cheng et al. 2010; Ho et al. 2010
	Tegastidae (*Tegastes acroporanus, Parategastes*)	Y	Y	Potential	N	N	Black or red copepods on coral tissue	Chemical dips (otic melbemycin oxime)	Carl 2008; Riddle 2010; George 2011; Christie and Raines 2016
	Rhynchomolgidae (gall)	N	N	N	N	N	Presence of galls (size mm)	N/A	Dojiri 1988; Patton 1994; Kim and Yamashiro 2007

Family	Genus						Description	Removal	References
Cryptochiridae	*Cryptochirus*	N	Potential	N	N	N	Presence of gall or pits	N/A	Verrill 1867; Patton 1967; Kropp 1986, 1990; Simon-Blecher and Achituv 1997; Simon-Blecher et al. 1999; Carricart-Ganivet 2004 Wei et al. 2013
	Haplocarcinus	N	Potential	N	N	N	Presence of gall or pits	N/A	Verrill 1867; Patton 1967; Kropp 1986; Simon-Blecher and Achituv 1997; Wei et al. 2013; van der Meij 2014
Trapezioidea	*Tetralia*	N	N	N	N	N	Reside between branches of *Acropora* host; tissue loss can occur if coral if unhealthy	N/A	Abele and Patton 1976; Patton 1994; Stella et al. 2011
	Trapezia	N	N	N	N	N	Reside between branches of *Pocillopora* host; tissue loss can occur if coral if unhealthy	N/A	Abele and Patton 1976; Patton 1994; Stella et al. 2011
Xanthidae	*Cymo melanodactylus* and likely many undescribed	N	Potential	Y	N	Potential	Dark-colored with hairy legs, often tissue discoloration or mortality around crab	Mechanical removal (forceps or barbed skewer) or trapping	Patton 1994; Pratchett et al. 2010, 2013; Pollock et al. 2012
Balanoidea (Pyrgomatidae)	*Hoekia*	N	Y	Y	N	N	Growth abnormalities with smooth tissue surrounding orifice. Lack the presence of cirral nets (filter-feeding apparatus)	Destruction of barnacle using (skewer or ice pick) or sealing of opercular plate	Anderson 1992; Ross 2000; Ross and Newman 1969, 1995, 2000
	~24 or more filter feeding genera (*Darwiniella, Galkinius, Savignium*)	N	N	N	N	N	Growth abnormalities (raised smooth tissue sometimes with pink appearance) between corallites with central orifice. Cirral net should be exposed when left undisturbed	Destruction of barnacle using (skewer or ice pick) or sealing of opercular plate	Cook et al. 1991; Anderson 1992; Achituv and Mizrahi 1996; Tsang et al. 2014; Simon-Belcher et al. 2016
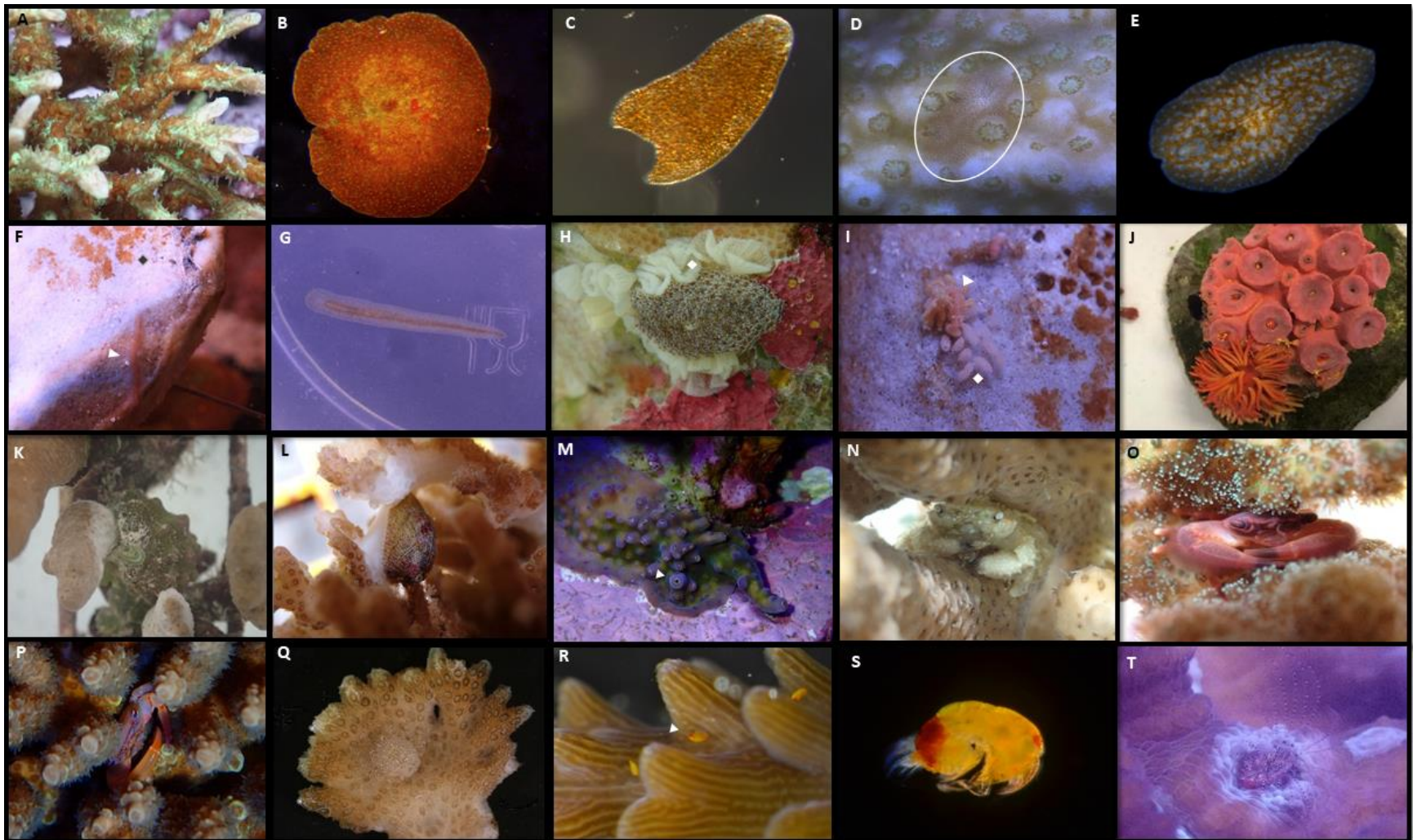
Figure 2-1: Photographs of coral-associated invertebrates *in situ* or *ex situ* to their respective coral hosts. **A** *Waminoa* sp. on *Hydnophora exesa*;

B *Waminoa* sp.; **C** *Heterochaerus australis*; **D** *Prosthiostomum acroporae* camouflaged on *Acropora millepora* host; **E** *Prosthiostomum acroporae*;

F *Montipora*-eating flatworm *Prosthiostomum* sp. (triangle) and egg clusters (diamond) on underside of *Montipora* host; **G** *Montipora*-eating

flatworm *Prosthiostomum* sp.; **H** *Phestilla sibogae* and egg masses (diamond) deposited on the skeleton of *Porites lutea*; **I** *Phestilla subodiosus*

(triangle) feeding adjacent to eggs (diamond) on the underside of *Montipora* host ; .**J** *Phestilla melanobrachia* (Dylan Hoemberg) feeding on

Tubastrea sp. host; **K** *Prosthiostomum* sp. ; **L** *Coralliophila* sp. feeding on *Pocillopora acuta* host; **M** Unidentified vermetid; **N** Unidentified xanthid

crab on *A. millepora* host; **O** *Trapezia* sp. on *Pocillopora damicornis* host; **P** *Tetralia* sp. in *Acropora tenuis* host; **Q** *Hapalocarcinus marsupialis* on

Seriatopora sp. host (Sancia van der Meij) **R, S;** *Tegastes acroporanus* on *Acropora formosa* (triangle) (Christie and Raines 2016; Figure 1); **T**

Unidentified pyrogomatid barnacle in *Mycedium elephantotus* host.

Platyhelminthes

Trematoda

Trematodes (Platyhelminthes: Trematoda) are a group of parasitic flatworms or flukes, composed of

digenea (Olson et al. 2003) and aspidogastrea (Rohde et al. 2001). Trematodes exhibit complex life

cycles with both sexual and asexual phases involving two or more hosts (Rohde et al. 2001; Olson et al.

2003; Gryseels et al. 2006). Trematode metacercariae are known to encyst in coral polyps, with

Polypipapiliotrema stenometra (Pritchard, 1966) observed to cause irregular pink growth nodules on

Porites spp. (Link, 1807) (Aeby 1998, 2003, 2007; Cheng and Wong 1974; Martin et al. 2018; Table 2-1).

This phenomenon, first described by Cheng and Wong (1974) on *Porites compressa* (Dana, 1846) and

Porites lobata (Dana, 1846) in Kaneohe Bay, Oahu, Hawaii, is referred to as *Porites* trematodiasis (Aeby

2003, 2007). Widespread in the Hawaiian Archipelago (Aeby 2007), *Porites* trematodiasis has also been

observed in Guam, Papua New Guinea, the Great Barrier Reef (Bray and Cribb 1989), and French

Polynesia (Aeby 2003). *P. stenometra* infests a molluscan intermediate host, then utilizes *Porites* spp. as

a second intermediate host prior to maturation in the gastrointestinal tract of corallivorous

chaetodontids (butterfly fishes; Aeby 1998; 2003).

Aeby (1998) found that the pebbled butterflyfish *Chaetodon multicinctus* Garrett, 1863 preferentially

feed on the pink-colored growths on *Porites* sp. over healthy coral tissue. She observed a 100%

prevalence of infestation of *P. stenometra* in *C. multicinctus* when they fed on these nodules. It is

possible that this pigmentation attracts this fish to feed on infested epidermal tissue, subsequently

transferring infestation. The complexity of the *P. stenometra* life cycle appears to exploit the red

fluorescent protein (RFP) responsible for the pigmentation in infested *Porites* spp. nodules. The

presence of RFP is thought to be directly related to an inflammatory response (Palmer et al. 2009),

which compliments an up-regulation of the melanin pathway (produce melanin-containing granular cells

that protect coral cells via cytotoxic effects from invading organisms; Palmer et al. 2008), and

phenoloxidase (PO) activity (Mydlarz et al. 2009) as part of a general immune response (Palmer et al. 2009, 2010). This is supported further by observations of the same pigmentation in pink spots on *Porites lutea* (Milne Edwards, 1860) colonies in South Yemen, apparently caused by barnacles settling on the coral tissue (Benzoni et al. 2010). These pink nodules persist until they are removed either by corallivorous fish or by senescence (Aeby 2003). Aeby (1991) observed the presence of *P. stenometra* limiting the colonial growth of *Porites* spp. (measured by weight) by up to 50%, suggesting that infestation represents a metabolic cost to the coral host.

Despite being implicated in reduced coral growth (Aeby 1991), there is no evidence of mortality associated with *Porites* pink spot syndrome. However, the clear elicitation of an immune response requires energy investment, which could otherwise be spent on growth or reproduction. Infestation is uncommon in captive environments given the complexity of the *P. stenometra* life cycle. If such an infestation were to occur, the removal of the primary chaetodon hosts would prevent further spread (Table 2-1). The limited consequences of *Porites* pink spot syndrome combined with low prevalence and apparent treatability of infestation, render infestation to be of low concern to captive coral colonies.

Polycladida

Polyclad flatworms (Family: Polycladida) are traditionally free-living animals found in a variety of marine habitats (Rawlinson 2014), and are generally predatory, feeding on a variety of marine invertebrates (Poulter 1975; Littlewood and Marsbe 1990; Newman 1994; Murina et al. 1995; Pérez-Portela and Turon 2007; Rawlinson et al. 2011). While the prey specificity of polyclads is poorly understood, some species such as the tiger flatworm *Maritigrella crozieri* Crozier, 1917, exhibit high specificity, feeding only on the mangrove ascidian *Ecteinascidia turbinata* Herdman, 1880 (Newman 2000).

The *Acropora*-eating flatworm *Prosthiostomum acroporae* (Rawlinson, Gillis, Billings, and Borneman, 2011) feeds exclusively on members of *Acropora* Oken, 1815 (Nosratpour 2008; Rawlinson et al. 2011;

Rawlinson and Stella 2012; Hume et al. 2014; Figure 2-1E). These cryptic animals were first observed by hobbyists in reef-keeping communities, who have struggled with infestations of *P. acroporae* on captive *Acropora* colonies for many years (Sprung 2001; Delbeek and Sprung 2005; Figure 2-1D).

Acropora spp. colonies infested with *Acropora*-eating flatworms can exhibit rapid decline of health and subsequent colonial mortality (Nosratpour 2008). The impact of *Acropora*-eating flatworms on public and private aquaria led to the description of '*Acropora*--eating flatworm' as *P. acroporae,* with hobbyists providing material to the authors (Rawlinson et al. 2011). The first report of *P. acroporae* on coral in the wild was off Lizard Island, Great Barrier Reef, Australia (Rawlinson and Stella 2012) and subsequently, the species has been found to infest *Acropora* colonies observed to have a cosmopolitan distribution along the Great Barrier Reef (author pers. obs). This species is common throughout the ornamental trade with frequent mention among popular reef-keeping forums. *Prosthiostomum acroporae* consume tissue of *Acropora* spp., leaving circular pale feeding scars of approximately ~1mm (Nosratpour 2008; Rawlinson et al. 2011; Hume et al. 2014; Table 2-1). Tissue consumed in this manner is digested in a branching gut spanning the almost complete lateral surface area of the animal, providing exceptional camouflage to the otherwise opaque white appearance of starved specimens (Rawlinson et al. 2011). The Symbiodiniaceae present within the branching gut and parenchyma of feeding *P. acroporae* remain photosynthetically active for an unknown duration (Rawlinson et al. 2011; Hume et al. 2014). Molecular analysis of *P. acroporae* gut contents reveal Symbiodiniaceae acquired in this manner match those of their coral hosts (Rawlinson et al. 2011; Hume et al. 2014) providing direct evidence of consumption of coral tissue. Hume et al. (2014) described the progressive loss of the coral's ability to acclimate to higher light levels (photoacclimation) with intense *P. acroporae* feeding. Additionally, host coral fluorescence declined with the duration and intensity of infestation (Hume et al. 2014). Hume et al. (2014) noted an absence of fluorescent pigments in the feeding scars, suggesting that *P. acroporae* extract pigments from the host during the feeding process. These pigments were observed to be evenly distributed in *P.*

acroporae parenchyma, subsequently enhancing their camouflage (Hume et al. 2014). This strategy of apparent predation avoidance is also observed in related taxa, with monogeneans exhibiting similar camouflage on their finfish hosts (Whittington 2009; Trujillo-González et al. 2015).

The presence of unfired nematocysts in the gut contents of *P. acroporae* suggest that these animals can circumvent or inhibit the innate threat recognition pathways of infested host corals (Rawlinson et al. 2011; Rawlinson and Stella 2012). Unhindered continuous feeding by *P. acroporae* commonly leads to either slow tissue necrosis (STN) or rapid tissue necrosis (RTN) of the host *Acropora* colony (author pers. obs), with colonial mortality often occurring as a result (Nosratpour 2008). These polyclad flatworms can capitalize on the resulting exposed coral skeleton as a substrate for egg deposition as the eggs are only laid on coral skeleton and not on live coral tissue. A biological cement produced by the flat worms surrounds the egg cluster anchoring them in place on the skeletal matrix of the host colony (Barton et al. 2019a). Following hatching, young *P. acroporae* either recruit onto the same host or disperse to another nearby *Acropora* colony.

While *P. acroporae* acquire resources from their coral host, and are associated with colonial mortality, it is also probable a combination of factors contributes to host colonial mortality. Feeding scars could contribute to colonization of opportunistic microorganisms and potential pathogens such as *Vibrio* spp. (Luna et al. 2007), facilitating the spread of disease (Katz et al. 2014). Overall, there is a paucity of information on the ecology, population dynamics and impact of *P. acroporae* on wild coral populations. Another flatworm known to infest scleractinian corals is the *Montipora*-eating flatworm, *Prosthiostomum montiporae* (Poulter, 1975), which also demonstrates high host-specificity, known only to feed on *Montipora* species (Jokiel and Townsley 1974; Poulter 1975; Figure 2-1F,G). This polyclad flatworm demonstrates a remarkably similar ecology to *P. acroporae*; feeding on coral tissue, leaving characteristic circular feeding scars, acquires camouflage from consuming the tissue of the host and depositing its egg clusters on freshly exposed coral skeleton. The egg capsules of *P. montiporae* are

much smaller (still contain multiple embryos) and can be more difficult to detect compared to those deposited by *P. acroporae*, which are usually easily identifiable with the naked eye. The small egg capsule size only influences detection and not removal, with entire egg clusters easily removed with tweezers or a scalpel similarly to *P. acroporae* egg removal recommendations (Barton et al. 2019a; Table 2-1).

Both of these prosthiostomids appear to present a considerable risk to captive coral populations because they actively consume host tissue, are associated with colonial mortality, and their cryptic nature makes early detection of infestations difficult. While there are yet to be empirical validation of various chemical emersion treatments or 'dips' for *P. acroporae*, some suggestions can be gleaned from the grey literature. Carl (2008) suggests that the use of immersion in 40 mg L^{-1} Levamisole HCl can be an effective way to remove flatworms from *Acropora* hosts. Within aquarist forums, such as Reef Central (http://www.reefcentral.com/), there are many purported treatments claiming to be effective to treat infestations of polyclad worms including Levamisole HCl, pesticides (e.g. Bayer Advanced Insecticide), and essential oil-based dips (Coral Rx, Revive Coral Cleaner, Dettol, Melafix, etc.; see Sweet et al. 2012; Table 2-1). In many cases, coral colonies can die following the application of treatments (Nosratpour, 2008). Whether this is because of compromised coral health, handling, differences in treatment application or in response to chemical immersion is unclear. Further investigation to optimize the effectiveness of such dips, minimize the impact on host coral health, and the use of novel treatments would likely provide better health outcomes for the afflicted coral colonies.

Although there is no data to support which chemical treatments should be used, recent advances in our understanding of the life cycle of *P. acroporae* provides crucial information regarding ideal treatment intervals to remove this pest. Barton et al. (2019a) demonstrated that the developmental rate of *P. acroporae* is closely related to temperature. Since eggs are resistant to most treatments, the authors recommend treating *Acropora* juveniles and adults at timed intervals which prevent newly hatched *P.*

acroporae from reaching sexual maturity between treatments (see Barton et al. 2019a). Despite developmental variation with temperature, spacing treatments between two or three weeks apart should break their life cycle. Similar studies to understand the influence of temperature on the *P. montiporae* life cycle would greatly benefit treatment regimens of this pest as well. Manual removal of egg clusters is recommended in conjunction with chemical immersion of the coral colony (Table 2-1). There also exists the opportunity for the use of biological controls in captive systems to compliment strict quarantine practices and chemical treatments. The peppermint shrimp *Lysmata vittata* (Stimpson, 1860) and sixline wrasse *Pseudocheilinus hexataenia* (Bleeker, 1862) were suggested by Barton et al. (2019b) as suitable biological control candidates in captive systems. The authors found *L. vittata* removed 82.0 ± 26.76% (mean ± SD) egg capsules laid on *Acropora* skeleton and 63.67 ± 43.48% of flatworm individuals from *Acropora millepora* fragments. *P. hexataenia* consumed 100% of flatworms from each *A. millepora* fragment (n=9) but had no interactions with *P. acroporae* eggs. These experiments were conducted without the presence of alternative food sources. Therefore, while the addition of *L. vittata* or *P. hexataenia* is unlikely to eradicate a flatworm infestation, applied application of these organisms could help reduce flatworm infestations in captivity (Table 2-1).

Gastropods

Muricidae

Muricidean snails (Gastropoda: Muricidae) are well-documented to have negative effects on the coral hosts they feed on (Robertson 1970; Cumming, 1999, 2009; Miller 2001; Baums et al. 2003a, 2003b; Kruzic *et al*. 2013). The two principal corallivorous genera include *Drupella* (Thiele, 1925) and *Coralliophila* (H. Adams and A. Adams, 1853), which have been reported on reef systems globally (Cumming and McCorry 1998; Miller 2001; Baums et al. 2003a, 2003b; Schoepf et al. 2010; Kruzic et al. 2013). They graze on live coral tissue with a specialized radula (Cernohorsky 1969; Fujioka 1982) striping

coral tissue from the calcium carbonate skeleton (Cumming 1996, 1999). *Drupella* (Figure 2-1K) and

Coralliophila (Figure 2-1L) preferentially consume *Acropora* tissue, with their affinity for *Acropora*

thought to have positive implications on the fitness (e.g., faster growth, longer life span, more predicted

offspring) of these muricids (Baums et al. 2003a, 2003b; Johnston and Miller 2007; Schoepf et al. 2010).

They can however be somewhat generalist in their prey specificity, demonstrating plasticity when

preferred species are absent (Baums et al. 2003a, 2003b; Johnston and Miller 2007; Schoepf et al. 2010).

Muricid gastropods exert both direct and indirect consequences on the coral colonies they feed on. At

high densities these animals can directly cause colonial mortality (Brawley and Adey 1982; Miller 2001;

Baums et al. 2003b) and even reduce coral cover (Turner 1994; Cumming 1999). Indirect consequences

to coral colonies include growth reduction (Meesters et al. 1994), susceptibility to disease (Nicolet et al.

2013; Clemens and Brandt 2015), and post-bleaching survivorship (Shaver et al. 2018). For example,

Meesters et al. (1994) inflicted minor artificial injuries of 1cm^2 on *Orbicella annularis* (Ellis and Solander

1786), mimicking the damage caused by gastropod feeding, and observed a 32% reduction in growth

over 56 days (Meesters et al. 1994). *Drupella* and *Coralliophila* may act as disease vectors, with Clemens

and Brandt (2015) finding that *Coralliophila erosa* (Röding, 1798) transmitted the Caribbean coral

disease, white plague, between *Orbicella annularis* specimens. Nicolet et al. (2013) also observed

Drupella individuals to facilitate brown band disease (BrB) transmission on the GBR. In degraded coral

reef environments, or those which recently experienced disturbance, recovery can be seriously impeded

by the predation pressure of *Drupella* and *Coralliophila* (see Baum et al. 2003a; Shaver et al. 2018).

Shaver et al. (2018) found the severity of bleaching observed in the grooved brain coral *Diploria* Milne

Edwards and Haime, 1848 and symmetrical brain coral *Pseudodiploria* Fukami, Budd and Knowlton, 2012

to increase with more *C. erosa* present on these corals.

Reducing muricid snails on wild populations is labor intensive but captive control is a more

straightforward process. Their non-cryptic habit renders them relatively obvious to detection on coral

compared to nudibranchs or polyclad flatworms. Muricid snails can often be found feeding in the center

of branching colonies and conspicuous patches of exposed skeleton can indicate recent feeding activity

(Table 2-1). Herbivorous snails such as *Trochus* spp. or detritivores like *Strombus* spp. rarely spend any

extended periods of time on living coral tissue. Any snail detected on the tissue margins of a coral colony

in captivity (apart from *Stomatella* spp.) could be considered as a potential coral predator. Positive

identification of muricid snails can be made with the distinctive features of their shells (Johnson and

Cumming 1995; Baums et al. 2003a, 2003b; Potkamp et al. 2017; Figure 2-1K). Routine inspection of all

corals entering a facility will enable these animals to be easily removed by hand or with tweezers. Early

detection and removal will prevent horizontal transmission between coral colonies (Table 2-1).

Nudibranchia

Nudibranchs are soft-bodied molluscs found in marine environments worldwide, with many residing on

coral reefs (Debelius and Kuiter 2007; Gosliner et al. 2008). Nudibranchs are a diverse group of

organisms which are known to demonstrate dietary specificity (Todd 1991). Corallivorous nudibranchs

of the genera *Phestilla* (Bergh, 1874) and *Pinufius* Er. Marcus and Ev. Marcus, 1960 feed exclusively on a

narrow range of scleractinian coral host species (Harris 1975; Gochfeld and Aeby 1997; Rudman 1981,

1982; Ritson-Williams et al. 2003, 2009; Dalton and Godwin 2006). For example, *Phestilla melanobrachia*

Bergh, 1874 (Figure 2-1J) typically only feeds on coral of the family Dendrophylliidae (Harris, 1975),

while *Phestilla sibogae* Bergh, 1905 (Figure 2-1H) feeds exclusively on the corals of the genus *Porites*.

Feeding activity of nudibranchs can have serious consequences on coral. For instance, Dalton and

Godwin (2006) observed host tissue sloughing after *Phestilla* sp. fed on *Turbinaria mesenterina*

(Lamarck, 1816), noting various bacteria and ciliates adjacent to the margin of sloughing tissue. There is

insufficient evidence to draw a causal relationship between feeding and tissue sloughing, but it is

possible that *Phestilla* spp. may serve as a disease vector similarly to the interaction observed with *C.*

erosa and white plague (Dalton and Godwin 2006; Clemens and Brandt 2015). *Phestilla* spp. are highly fecund, but we have a poor understanding of their life cycles (Carl 2008). The size of egg masses appears to be largely variable, with those found on zoanthid polyps to be 2-3mm in diameter, while those of *P. sibogae* Bergh, 1905 exceed 10 mm in diameter. Given their high fecundity and cryptic nature, this pest can be particularly problematic in captivity.

Many corallivorous nudibranchs remain undescribed, likely in part because of their cryptic habit; however, they may be readily observed by aquarists because they can be destructive in captivity. *Montipora*-eating nudibranch is yet to be observed in the wild but has recently been formally described as *Phestilla subodiosus,* Wang, Conti-Jerpe, Richards, Baker 2020 from captively sourced specimens because of its frequency as a pest in coral aquaria (Carl 2008; Wang et al. 2020) Figure 2-1F,G). Like other *Phestilla* nudibranchs, it feeds exclusively on *Montipora* species and is found predominantly on shaded areas of coral (Figure 2-1F). *Zoanthus*-eating nudibranchs are also frequently encountered in coral aquaria, where they feed exclusively on the zoanthid coral *Zoanthus* spp*.* Lamarck, 1801 Hobbyists note that these specimens incorporate fluorescent pigments from their zoanthid hosts into their cerata, resulting in host-specific coloration of these animals (Figure 2-1). Given the typical camouflage of these organisms, evidence of infestation is most likely to be observed from inspection of an unhealthy coral colony (i.e., color contrast between the nudibranch and dead coral tissue), observing their characteristic cerata, or by the observation of white egg masses typically deposited in sheltered areas of the coral host (Figure 2-1H,I,J).

Current reported effective chemical treatments for infestations of nudibranchs in captivity are largely limited to grey literature. The cryptic nature of these invertebrates usually results in management when coral health is in serious decline (Carl 2008). Mechanical removal of adults and egg masses appears to be an effective management method but can be difficult in large systems (Carl 2008). *Phestilla* spp. appear to have natural predators in the wild, so suitable predators (i.e., biological controls) could be used in

captivity (Table 2-1). Indeed, Bochfield and Aeby (1997) observed the saddle wrasse *Thalassoma duperrey* (Quoy and Gaimard, 1824) and threadfin butterflyfish *Chaetodon auriga* (Forsskål, 1775) feeding on *P. sibogae* in the wild. They also observed that the xanthid crab, *Phymodius monticulosus* (Dana, 1852) consumed nudibranchs under laboratory conditions. It is also possible that wrasses (e.g. *Coris, Pseudocheilinus* and *Thallasoma* spp.) may also reduce infestation intensity and these organisms (Carl 2008). Some aquarists report success with treatments such as levamisole HCl and essential oil-based dips. Short 30 s to 1 min dechlorinated freshwater dips are suitable for zoanthids but are likely to result in mortality of scleractinian corals such as *Montipora* (author pers. obs.). Further research is required to determine the effectiveness of these dips, and to observe subsequent consequences for host coral health.

Vermetidae

Vermetid snails are marine gastropods that hatch as planktonic larvae and then settle on a variety of surfaces, where they metamorphose and begin to secrete a calcareous tube that individuals inhabit (Hughes and Lewis 1974; Bergsma 2009; Phillips 2011). Many vermetid gastropods, including *Ceraesignum maximum* (G.B. Sowerby I, 1825), use live coral tissue as a settlement substrate (Bergsma 2009; Phillips 2011). This can result in characteristic finger-like growth anomalies (Bergsma 2009; Figure 2-1M) or irregularly flattened coral branches (Zvuloni et al. 2008). While vermetid gastropods do not feed on their coral hosts, they elicit a physiological response in the form of these growth anomalies (Shima et al. 2010, 2013; Zill et al. 2017). Coral biomineralization is an energetically costly biochemical process (Tambutté et al. 2011), rendering energy spent in this manner to encapsulate vermetid shells essentially forfeited. *Ceraesignum maximum* is a filter feeder that uses a mucus net to trap food from the water column. In doing so, sediment is retained in the mucus net where it can negatively affect the coral host (Hughes and Lewis 1974; Kappner et al. 2000; Zill et al. 2017). Brown and Osenberg (2018)

also observed mucus net feeding by *C. maximum* to increase the thickness of the boundary layer around

the host, the retention time of water within the mucus net, and a reduction of water flow around the

host. A thickening of the boundary layer around host corals results in a lower oxygen concentration at

the surface of and within associated coral tissue, which could considerably influence host coral

metabolism (Kuhl et al. 1995). Accordingly, the abundance of *C. maximum* has a negative correlation

with coral cover, with their presence in high density observed to decrease survivorship and colonial

skeletal growth (Shima et al. 2010, 2013).

Despite evidence of negative consequences of vermetid snails to coral, information on treatment

options remain limited, with little known about treatment in captive environments. In addition to a

protective shell, some vermetids possess an operculum which renders chemical treatment applications

difficult. Many aquarists use a small amount of cyanoacrylate gel (super glue) to seal the opening of the

shell, resulting in the death of the animal (Table 2-1). This method is effective to limit the reproductive

capacity of vermetid snails if carefully applied. Manual removal is possible using sharp tweezers, but this

process is quite invasive and could cause further coral damage. Quarantine and exclusion appear the

best preventative measures.

Crustacea

Crustaceans are a diverse group of arthropods found in terrestrial and aquatic environments. A wide

variety of these invertebrates are found in coral reefs around the world, where they have a range of

relationships with scleractinian corals. Many of these crustaceans likely use the structure of coral hosts

as their home, while other may utilize corals as a food source. Here we examine decapods, copepods,

and cirripeds; three groups of crustaceans found living in close association with host corals (Stella et al.

2011).

Cryptochiridae

While decapods can be free-living species, many organisms live in close association with their hosts
(Castro 1976; Stella et al*. 2011*). Cryptochirids (Decapoda: Cryptochiridae) for instance have evolved a
life history strategy where female adults reside within the confines of coral dwellings often referred to
as 'galls' (Figure 2-1Q). There are over 55 described species of cryptochirids representing 20 genera
(WoRMS Editorial Board, 2019) that live in symbiosis with over 42 genera of coral hosts (Kropp 1990;
van der Meij 2014). Gall crabs are thought to settle on coral tissue as larvae, where they metamorphose
and eventually induce encasement by the coral host via abrasive action typically from spiny projections
on their legs (Verrill 1867; Patton 1967; Kropp 1986; Simon-Blecher and Achituv 1997; Wei *et al.* 2013).
The resulting skeletal modifications in host corals result in changes in water flow patterns, creating
eddies that trap particulates which supply the gall crab with food (Abelson et al. 1991).

Some consider gall crabs to be parasitic (Verrill 1867), because they have been observed to consume
coral mucus and coral tissue (Stimpson 1859; Kropp 1986). Corals naturally exude mucus to remove
sediment from their tissues for instance, and therefore the consumption of said mucus may or may not
represent a metabolic drain; however, because the scraping action of gall crabs produces extra mucus, it
has been argued that the mucus was not produced to remove sediment (Simon-Blecher et al. 1999).
Furthermore, Simon-Blecher et al. (1999) suggested that the gall crab, *Cryptochirus coralliodytes* (likely a
complex comprised of several species, Sancia van der Meij pers. comm.) inhibits the growth rate of
corals and fosters the settlement of algae and fungi within the coral dwelling. Carricart-Ganivet (2004)
suggest that algae around the opening of host corals are pruned similarly to a garden, and act to
supplement food that enters the pit or gall. Recent research does not provide consensus on whether gall
crabs are commensal or parasitic (Kropp 1986; Simon-Blecher and Achituv 1997; Simon-Blecher et al.
1999; Terrana et al. 2016; Vehof et al. 2016). Laboratory based, long-term examination of hosts with
varied densities of cryptochirids are required to understand the energetic cost to corals that host gall

crabs. With no consensus regarding the impact of gall crabs on coral health and no evidence to suggest that these organisms proliferate considerably in captivity, the risk to captive coral colonies appears negligible and is not likely to hinder coral propagation efforts (Table 2-1).

Misc. Decapods

There is little information regarding the consequences of many coral-associated decapods to their coral hosts. The crab families, Trapezioidea and Xanthidae each possess a number of species considered to be obligate associates on corals, consuming coral tissue (Stella et al. 2011). Commonly observed representatives of Trapezioidea include *Trapezia* (Figure 2-1O) which are found among the branches of Pocillorporid hosts and *Tetralia* (Figure 2-1P) which inhabit *Acropora* host colonies (Abele and Patton 1976; Patton 1994; Stella et al. 2011). These two genera are generally considered to be harmless coral-associated invertebrates by hobbyists, but no study has examined the metabolic impacts of *Trapezia* and *Tetralia* species.

In contrast, xanthid crabs are well known among aquarists for corallivory and are removed from captive aquaria (as identified by hairy legs and sharp black claws; Figure 2-1N; Table 2-1). While this over-simplification may be effective, the interaction between xanthid crabs and their coral hosts remain poorly understood. *Cymo melanodactylus* Dana, 1852 for instance is obligate to *Acropora* spp. (Patton, 1994) and was observed in association with lesions on the tissue of *Acropora cytherea* Dana, 1846 (Pratchett et al. 2010), but further examination found that *C. melanodactylus* was also associated with a reduced rate of lesion progression (Pollock et al. 2012), presumably via cleaning of these lesions. While *C. melanodactylus* may be helpful in the presence of disease lesions, Pratchett et al. (2013) found over 75% of recent wounds on *A. cytherea* associated with infestation, and that no crabs were observed on apparently healthy colonies. The consequences of symbiosis exerted on host corals (positive or negative) and how symbiosis is initiated by xanthid crabs (attracted to lesions or causal in lesion development)

remain debatable and could vary interspecifically. Further investigation of these relationships is required to identify what threat different species or genera may pose to wild and captive corals.

Copepoda

Copepods are a diverse group of crustaceans that have evolved intimate, often obligate, associations with scleractinian corals over the course of evolutionary history (Humes 1985b, 1960, Cheng and Dai 2009; Stella et al. 2010, 2011) with 363 copepod species from 99 genera, 19 families and three orders currently recorded to associate with scleractinian corals (Cheng et al. 2016). Given this considerable diversity, research effort has primarily focused on the taxonomy of symbiotic copepods (Cheng et al. 2016; Humes 1960). Our understanding of the ecology of these organisms and specifically their interaction with their respective coral host is limited, preventing our ability to label them as genuinely parasitic, negatively affecting the fitness of their hosts, or commensal symbionts benign to coral health (Cheng et al. 2016). Determination of these interactions will be difficult without a shift in research focus towards ecological interactions and emphasis on the impact coral-associated copepods have on their respective hosts. The three groups of coral-associated copepods particularly warranting ecological consideration are the endosymbiotic (Butter 1979; Humes 1985b, Cheng and Dai 2010; Ho et al. 2010), gall-inducing (Kim and Yamashiro 2007) and ectoparasitic copepods (Riddle 2010; Cheng et al. 2016; Christie and Raines 2016). These copepods are distinguished by their relative location on host corals, and morphological differentiation to suit these niches.

Endosymbiotic copepods

Endosymbiotic copepods are found living inside the gastrovascular cavities of coral polyps. They typically possess elongated slender bodies, well-suited for entry and exit from coral polyps (Humes 1985b, 1985a; Cheng et al. 2010; Ho et al. 2010). It is unknown what mechanism these copepods use to enter the coral

polyp; however, Cheng and Dai (2009) observed coral polyps to relax when approached by *Xarifia obesa* Humes and Ho, 1968; a behavior typically associated with coral feeding (Cheng and Dai 2009). Cheng and Dai (2009) suggest that these copepods use a chemical cue to facilitate this relaxation, but there is currently no evidence to support this hypothesis. Other parasitic crustaceans have been shown to 'tickle' bivalve hosts to gain entry (Trottier and Jeffs 2015). Cheng and Dai (2010) observed that *Xarifia fissilis* (Humes, 1985) consumed Symbiodiniaceae within their cauliflower coral *Pocillopora damicornis* (Linnaeus, 1758) hosts, which remained photosynthetically viable in the gut of these copepods after two weeks. Although endosymbiotic copepods are typically referred to as 'endoparasitic' copepods, it remains unclear whether the consumption of Symbiodiniaceae adversely affects coral fitness. Because the coral holobiont is generally able to regulate Symbiodiniaceae densities under stable environmental conditions (Falkowski et al. 1993), endosymbiotic copepods may be commensal organisms, but could have negative effects on bleached host corals depleted of their Symbiodiniaceae (Cheng and Dai 2010).

Gall-inducing copepods

Like gall-crabs, some copepods settle on a coral host and induce the formation of galls (Dojiri 1988; Patton 1994; Kim and Yamashiro 2007). Dojiri (1988) described the first gall-inducing cyclopoid copepod *Isomolgus desmotes* Dojiri, 1988 from the birdsnest coral *Seriatopora hystrix* Dana, 1846. The females are believed to be unable to leave galls after formation because of their characteristically swollen prosome, while the smaller males can presumably come and go for mating purposes. The settlement and subsequent irritation caused by gall-inducing copepods supports the assertion that these copepods cause a form of physiological distress and energy expenditure from their host, albeit minor (Dojiri 1988). There is currently no data examining the effects that any metabolic drain or growth inhibition associated with endoparasitic copepods and their coral hosts.

Ectoparasitic copepods

Ectoparasitic copepods are typically harpacticoids (Copepoda: Harpacticoida) which live on the coral epidermis, where they likely consume coral tissue and mucus (Carl 2008; Riddle 2010; Cheng et al. 2016; Christie and Raines 2016; Figure 2-1R,S). While these epifaunal copepods are observed in natural coral reef environments (Humes 1960, 1984, 1985a, 1985b; Kim 2003; Stella et al. 2011; Cheng et al. 2016), the consequences of the association between copepods and their coral hosts is predominantly documented under captive conditions (Carl 2008; Riddle 2010; George 2011; Christie and Raines 2016). Riddle (2010) suggested that *Tegastes* Norman, 1903 and *Parategastes* Sars GO, 1904 are the predominant genera of harpacticoid copepods encountered in coral reef aquaria. *Tegastes acroporanus* Humes, 1981 are referred to as 'red bugs' because of the red coloration in the urosome contrasted by a yellow prosome (Riddle 2010; Christie and Raines 2016; Figure 2-1S). Acutely affected (severe infestation) *Acropora* colonies generally display two or more of the following states: tissue loss radiating upward from the coral base, atypical polyp extension, generalized loss of pigmentation, elevated mucus production, and/or loss of distal coloration in axial corallites, suggesting a discontinuation of colonial growth (Carl 2008; Sweet et al. 2012; Christie and Raines 2016). The negative consequences of infestation with high density of *Tegastes acroporanus* on *Acropora* spp. prompted experimental treatment with Otic Milbemycin Oxime, which appears to be a viable option (Christie and Raines 2016; Table 2-1).

Pyrgomatidae

Pyrgomatids (Cirripedia: Pyrgomatidae) are obligate endosymbiont barnacles on a diverse range of coral families (see Tsang et al. 2014; Simon-Belcher et al. 2016; Figure 2-1T). They typically settle between coral polyps as cyrpid larvae, then metamorphose and become encapsulated by the coral host (Liu et al. 2016). The nature of their symbiotic association with host corals is unclear and often contested (Ross

and Newman 1969, 1995; Cook et al. 1991; Achituv and Mizrahi 1996; Ross 2000; Liu et al. 2016).

Pyrgomatids feed in different ways; the majority are suspension feeders, but some directly feed from their host corals (Ross and Newman 1969, 1995, 2000; Ross 2000). For instance, Ross (2000) found that *Hoekia monticulariae* (Gray, 1831) feed on adjacent tissue within the horn coral *Hydnophora exesa* (Pallas, 1766) host's coelenteron. *Hoekia* barnacles represent 'energy sinks' to their host corals (Pearse and Muscatine 1971; Oren et al. 1997, 1998; Ross and Newman 1969, 1995; Ross 2000) by slowly consuming tissue and forcing their hosts to constantly expend energy for tissue regeneration (Ross 2000). Although to a lesser degree than *Hoekia* spp., suspension feeding pyrgomatids also impose physiological consequences for their coral hosts (Benzoni et al. 2010; Liu et al. 2016). Liu et al. (2016) investigated the settlement process of the barnacle *Darwiniella angularis* Chan, Chen and Lin, 2012 on *Cyphastrea chalcidicum* (Forskål, 1775) coral hosts, describing a six-step process between first interaction with the host and the commencement of suspension feeding as metamorphosed adults. Most notably, in phases one (probing stage) and two (battling stage), the cyprid juveniles penetrate the host coral tissue with their antennules and attempt to move deeper into the host, eliciting a physiological response from the host, including the protrusion of mesenteries as a defensive mechanism (Liu et al. 2016).

Despite the evidence of negative consequences to the coral host (Ross and Newman 1969, 1995, 2000; Ross 2000; Benzoni et al. 2010; Liu et al. 2016), some studies suggest that pyrgomatid barnacles may benefit the coral holobiont via nutrient provision (Cook et al. 1991; Achituv and Mizrahi 1996). Cook *et al*. (1991) suggested that carbon and phosphorus ingested by *Neotrevathana elongata* Hiro, 1931 was terminally excreted and taken up by Symbiodiniaceae within the fire coral *Millepora dichotoma* Forskål, 1775 host, while nitrogen in the form of ammonium (NH_{4+}) was also provided to the coral holobiont by *N. elongatum* (see Achuiv and Mizrahi 1996). Therefore, a trade-off between the carbon, nitrogen and phosphorus provided to the coral holobiont by pyrgomatid metabolic excretion, and energy expenditure

associated with biomineralization (Tambutté et al. 2011) to isolate the barnacle (Santos et al. 2012) and physiological defense response (Benzoni et al. 2010; Liu et al. 2016). Another cost may also include the host surface area not contributing to heterotrophic feeding. The total energy budget of this association is complicated further by the potential variable influence of different pyrgomatid species, and the infestation density on the coral host. While the density of infestation could be high in some circumstances, such phenomena are not documented in captivity, potentially because the captive environment renders reproduction difficult. However, *in situ* nursery applications may be threatened by high infestations and should be monitored accordingly.

With no current knowledge of biological controls or suitable chemical treatments, mechanical removal of pyrgomatids remains the only current management option. Rather than excavating a given pyrgomatid from the coral tissue and skeleton which is likely to cause more harm to coral than the animal itself, a sharp length of metal resembling an ice pick can be used to destroy the shell with minimal damage to the surrounding coral (Table 2-1). This practice can limit any further energetic loss from biomineralization to accommodate the pyrgomatids and prevent them from reproducing.

Discussion

This examination of a diverse range of coral-associated invertebrates within the context of coral aquaculture highlights the knowledge gaps in our understanding of the consequences of symbiosis on the host coral. Organisms which threaten the health of corals in aquaculture, the ornamental trade, or reef restoration should be controlled in these systems to maintain healthy and growing coral colonies or propagules and limit biosecurity risks associated with the translocation of infested coral propagules. When considering the perceived risk coral pests present to host corals, it is helpful to consider which traits of these organisms are most likely to contribute to negative health outcomes (e.g., tissue loss, mortality) to host colonies. Any coral-associated organism which 1) actively consumes coral tissue, 2)

possesses high reproductive capacity, 3) is prone to infestation of coral populations in high densities, and 4) is associated with coral mortality (especially in captivity) should be of primary concern to coral husbandry efforts. Of those organisms examined in this review, those which fit the above criteria include muricid snails, corallivorous nudibranchs (Trinchesiidae), and the polyclad flatworms *P. acroporae* and *Prosthiostomum montiporae*. While these organisms represent potentially high risk to coral health, corals can still be blighted by opportunistic infestations of other coral-associated invertebrates.

While pest infestations can occur in both *in situ* and *ex situ* aquaculture environments, the application of pest control protocols in coral aquaculture presents different challenges in different culture environments. For instance, *in situ* coral propagation facilities have very limited means of preventing the recruitment of unwanted coral-associated invertebrates. While 'caging' of transplanted corals may be practical for preventing corallivory by larger organisms (e.g. chaetodontids), a net or cage is unlikely to hinder the introduction of pathogens, parasites or symbionts. In addition, the exclusion of herbivores like the straited surgeonfish *Ctenochaetus striatus* (Quoy & Gaimard, 1825) and further biofouling is likely more consequential to coral health than corallivory (Knoester et al. 2019).

In contrast, *ex situ* aquaculture permits quarantine protocols and standardized biosecurity practices. Effective quarantine procedures can prevent the introduction of unwanted organisms through initial isolation and rigorous observation of newly introduced aquaculture broodstock (sexual or asexual propagation). Isolation and observation allow for the detection and subsequent treatment of any potentially harmful coral-associated invertebrate. While *ex situ* aquaculture may have the advantage with quarantine and treatment methods, organisms such as *P. acroporae* can persist in recirculating aquaculture systems through horizontal transmission (Barton et al. 2019a). Pest larvae with the ability to swim to find a new host can also take advantage of high stocking densities (subsequent close proximity) and monospecific culture typical of *ex situ* aquaculture systems, highlighting the risk of lateral transmission of infestation. To help mitigate this risk, the use of maintenance equipment restricted to

34

specific aquaria (e.g., siphon hoses, algae scrapers) can prevent instances of cross contamination and subsequent transmission. Additionally, UV sterilization in *ex situ* environments could reduce the density of ciliates and planktonic juvenile coral pests in the water column.

In *ex situ* applications, the visual identification and physical removal of muricid snails should effectively reduce their impact on coral colonies. However, the management of infestations when conducting *in situ* aquaculture is more complex, with less control of the natural environment. Active removal would appear the most suitable practice, but without the presence of natural predators of one or more life history stages for a given organism, it would be difficult to inhibit the recruitment of *Drupella cornus* onto a dense thicket of growing *Acropora muricata* for instance. In contrast to muricids, both corallivorous nudibranchs and the polyclad species have cryptic habits which render visual identification and subsequent active removal more difficult both *in situ* and *ex situ.* Delayed identification coupled with high fecundity can present circumstances where coral colonies are in irreversible decline in health before intervention is possible. This regular occurrence presents the need for active management tools such as biological controls. While it is possible for natural predators to mitigate population growth of these organisms in the wild, this interaction must be fostered in captivity. The saddle wrasse *Thalassoma duperrey* Bochfield and Aeby (1997) observed to consume *Phestilla sibogae* for instance, could perhaps be utilized to treat or mitigate outbreaks of this pest in *ex situ* environments if a sustainable fishery exists.

Barring the facilitation of continuous predation on these organisms by a natural predator, chemical treatments may be suitable for particular applications, where such treatments present limited impact to the coral host or surrounding environment. Again, the suitability of this approach remains limited to *ex situ* environments, compared to *in situ* aquaculture where it is difficult to treat open systems and neighboring fauna could be impacted. It could however be possible to temporarily move corals into *ex situ* holding for treatment, allowing them to heal and be free of any chemicals before returning them to

an *in situ* nursery. Regardless of the treatment environment, a thorough understanding of the life cycle

of any treated pest is critical to the successful removal and management of such organisms. For

example, our understanding of the life cycle of *P. acroporae* allows for treatment methodology which

considers the embryonation period (time to hatching) and time required for these polyclads to reach

sexual maturity to break the life cycle of this pest (Barton et al. 2019a). Similar studies are required

across the life cycle of the wider range of coral symbionts, not only for effective management strategies

but also understand how these organisms influence the host at different stages of their complex life

histories.

The development of protocols which suit the biology and ecology of a coral pest highlights the risk of

assigning coral-associated invertebrates into distinct states of symbiosis (mutualist, commensal and

parasitic). This practice undermines the fluidity and potential variability of symbiotic interactions along

the mutualistic-parasitic continuum of symbiosis (Skelton et al. 2016). Aquarists and the scientific

community should avoid making inferences about the ecological roles of coral-associated organisms

solely based on morphological evidence, without assessing the dynamics of the symbiosis

experimentally. Even a traditional example of mutualistic symbiosis between Symbiodiniaceae and

scleractinian corals can shift into a parasitic relationship under particular conditions (Lesser et al. 2013;

Baker et al. 2018). How environmental pressures and the density of coral-associated invertebrates can

change the consequences of symbiosis on the coral host warrants further investigation, as does the

interaction between these variables and coral immunity.

Chapter 3: The Life Cycle of the *Acropora* Coral-Eating Flatworm (AEFW), *Prosthiostomum acroporae*; The Influence of Temperature and Management Guidelines

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Abstract

The *Acropora* coral-eating flatworm (AEFW), *Prosthiostomum acroporae* (Platyhelminthes: Polycladida: Prosthiostomidae) feeds on wild and cultivated *Acropora* species and its inadvertent introduction into reef aquaria can lead to the rapid death of coral colonies. To improve the treatment of infested corals we investigated the flatworm's life cycle parameters at a range of temperatures that represent those found in reef tanks, coral aquaculture facilities and seasonal fluctuations in the wild. We utilized *P. acroporae* from a long-term in vivo culture on *Acropora* species to examine the effects of temperature (3°C increments from 21 to 30°C) on flatworm embryonation period, hatching success, hatchling longevity, and time to sexual maturity. Our findings show that warmer seawater shortened generation times; at 27°C it took, on average, 11 days for eggs to hatch, and 35 days for flatworms to reach sexual maturity, giving a minimum generation time of 38 days, whereas at 24°C the generation time was 64 days. Warmer seawater (24–30°C) also increased egg hatching success compared to cooler conditions (21°C). These results indicate that warmer temperatures lead to higher population densities of *P. acroporae*. Temperature significantly increased the growth rate of *P. acroporae*, with individuals reaching a larger size at sexual maturity in warmer temperatures, but it did not influence hatchling longevity. Hatchlings, which can swim as well as crawl, can survive between 0.25 and 9 days in the absence of Acropora, and could therefore disperse between coral colonies and inter-connected aquaria. We used our data to predict embryonation duration and time to sexual maturity at 21–30°C and discuss how to optimize current treatments to disrupt the flatworm's life cycle in captivity.

Introduction

Trade in live coral has increased by 10-50% annually since 1987 (Rhyne et al. 2009) and is valued at between $US 200-330 million each year (Wabnitz 2003). Stony corals (Order: Scleractinia) in the genus *Acropora* are one of the most popular corals collected for the global marine aquarium trade because of

their vibrant colors and diversity of growth forms (Rhyne et al. 2014; Barton et al. 2017). *Acropora* sp*.*
are suitable candidates for aquaculture in situ or ex situ in land-based facilities and are propagated in a
variety of geographic and socioeconomic regions including the Indo-Pacific, Caribbean, and the Great
Barrier Reef. Rearing acroporid corals for a sustainable marine aquarium trade could relieve the
pressure that conventional collection strategies have placed on wild stocks (Tlusty et al. 2013; Rhyne et
al. 2014). For example, recent studies show that the majority of acroporids exported by Indonesia are
now cultured (Rhyne et al. 2012, 2014). Coral aquaculture endeavors also form the basis for active
restoration programs to restore denuded reefs and mitigate the cumulative pressures on reef
ecosystems (e.g., sedimentation, climate change; Fabricius 2005; Hoegh-Guldberg et al. 2007; Doney et
al. 2009; De'ath et al. 2012; Hughes et al. 2017). *Acropora* sp*.* are common target species for reef
restoration because of their fast growth-rates relative to other scleractinian corals and their
contribution to structural complexity (Craggs et al. 2017; Pollock et al. 2017). Coral propagation efforts
can be threatened by the introduction or natural occurrence of coral predators, pathogens, and
parasites. The *Acropora*-eating flatworm, *Amakusaplana acroporae* (Rawlinson et al. 2011) [now known
as *Prosthiostomum acroporae*] (Litvaitis et al. 2019)] has been a problematic pest for the coral hobbyist
community globally for over a decade (Nosratpour 2008). Their inadvertent introduction into coral
aquaria can lead to irreversible tissue damage and ultimately to the death of entire *Acropora* colonies
(Delbeek and Sprung 2005; Carl 2008; Nosratpour 2008; Rawlinson et al. 2011; Hume et al. 2014).
Prosthiostomum acroporae is a polyclad flatworm (Platyhelminthes: Polycladida) belonging to the sub-
order Cotylea and family Prosthiostomidae. It lays its egg clusters on bare coral skeleton, and each
cluster contains multiple egg capsules within which multiple embryos develop (Rawlinson et al. 2011).
Like other cotylean polyclads, *P. acroporae* development proceeds via a larval form with lobes and ciliary
bands for swimming and feeding in the water column (Rawlinson 2014), but unlike other cotyleans,
these larval features develop and are then reduced and lost while still inside the egg capsule, i.e., it is an

intracapsular larva that undergoes metamorphosis before hatching as a juvenile (Rawlinson et al. 2011).

A consequence of this life history strategy could be increased retention of hatchlings on the natal coral

and limited dispersal potential, both of which contribute to its rapid proliferation in captive systems. A

further factor aiding its success in captive systems is its camouflage and cryptic habit (Rawlinson et al.

2011; Hume et al. 2014). The long supply chain within the ornamental trade (Wabnitz 2003; Rubec and

Cruz 2005; Cohen et al. 2013; Fujita et al. 2014) not only presents the opportunity for flatworms to

spread between corals at each holding location, but the stress from transportation may increase the

susceptibility of *Acropora* colonies to infestation. Despite a long-standing infamy among coral hobbyists,

P. acroporae was only recently reported in the wild from Lizard Island on the Great Barrier Reef,

Australia, and the biogeographic range and impact on wild acroporids is not known (Rawlinson and

Stella 2012). There have been no empirical studies on the management of *P. acroporae* in captivity or in

the wild. Effective management of this species requires a comprehensive understanding of its life cycle

so that vulnerable life stages can be identified and eradicated through appropriate treatments. The

developmental rate of poikilothermic animals, including polyclad flatworms, is greatly influenced by

temperature (Gammoudi et al. 2012), therefore it is important to consider *P. acroporae* development at

a range of temperatures relevant to seasonal fluctuations in the wild and within the temperature ranges

of corals maintained in aquaria. The aim of this study was to provide the knowledge that will help

formulate approaches that disrupt the life cycle of *P. acroporae* by investigating the effects of

temperature on the embryonation period and hatching success, the longevity of hatchlings and the time

to, and size at, sexual maturity (Figure 3-1). Additionally, there was morphological variation observed for

hatchlings, with some emerging with lobes and others without, which may affect their dispersal

potential (i.e., those with lobes may have greater dispersal ability). Although difficult to accurately

quantify the variation in hatchling morphology, the survivorship was determined across these

morphologies as a measure of their dispersal potential.

Materials and Methods

Prosthiostomum acroporae Culture

Prosthiostomum acroporae were collected from *Acropora* spp. colonies (*A. millepora, A. spathulata, A. loripes, A. tenuis, A. microclados, A. nasuta, A. microphthalma, A. rosaria*) harvested between May 2016 and January 2018 from various inshore (Esk Reef 18° 46.4200 S 146° 31.3720 E) and midshelf reefs (Trunk Reef 18°23020.40 0 S 146°48025.80 0 E, Davies Reef 18°49021.60 0 S 147°39012.50 0 E and Rib Reef 18°28047.10 0 S 146°52000.90 0 E) that form part of the central Great Barrier Reef of Australia (GBRMPA Permit No. G12/3236.1). Flatworms were removed from the corals using a jet of filtered seawater (see Supplementary Methods for details on screening corals for flatworms). A continuous culture of *P. acroporae* was established on a mixture of captive *Acropora* species (primarily *A. millepora, A. spathulata, A. tenuis, A. loripes, and A. nasuta*) housed at the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS), Queensland. The culture was maintained at 27°C in two 250 L flow-through aquaria supplied with fresh filtered seawater at approximately 2 L min^{-1}. Colony fragments were added to replace dead corals as needed and density adjustments of flatworm infestation were conducted regularly (see Supplementary Material for details on coculturing flatworms and corals). *P. acroporae* lay their eggs on bare coral skeleton (Figure 3-1) which renders microscopic observations of embryonic development difficult. Subsequently, in vitro laid egg clusters used in experiments were collected using two methods. The first approach involved removing *P. acroporae* adults from host *Acropora* colonies using a jet of water and individual worms being placed in plastic bags (SandvikR plastic bags [127 mm * 200 mm]) containing filtered seawater (1 mm). Visual inspection for egg clusters and 75% water changes were performed daily, and egg clusters were collected within 24 h of oviposition. This method was used for experiment 1 to determine the effect of temperature on embryonation period and hatching success. A second approach was later developed to bolster *P. acroporae* cultures and provide more egg clusters for experimentation and was subsequently used for

experiments 2 (hatchling longevity and morphology) and 3 (time to sexual maturity and size at sexual maturity). This method used rectangles of clear plastic (2 cm * 5 cm) cut from clean plastic bags, pegged with metal-free clothes pegs onto infested *Acropora* colonies proximal to feeding scars (see Supplementary Material). The plastic substrates were monitored daily so that egg clusters could be collected within 24 h of oviposition. Egg clusters collected using the second method were only used if they were encased in a continuous layer of 'cement' (Figure 2A), indicating that all eggs in the cluster were the product of one laying event from a single parent. Extraneous egg capsules (not covered by a continuous cement layer) were removed.
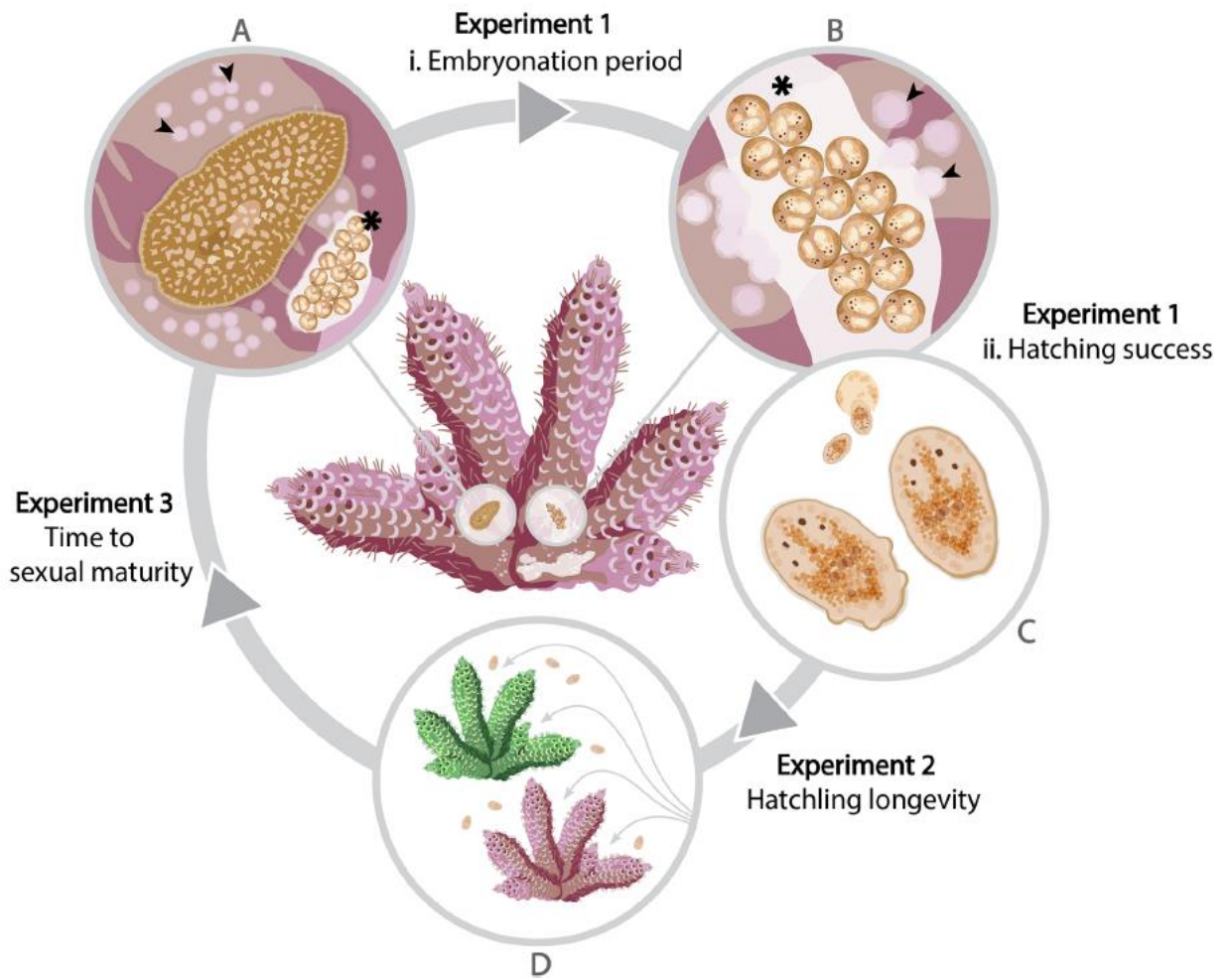
Figure 3-1: A schematic of the life cycle of *Prosthiostomum acroporae* showing three experiments to assess the effect of temperature on development. Adult flatworms (A) leave circular feeding scars (arrowheads) on the coral tissue and deposit egg clusters (asterisk) on the coral skeleton. The time from (A) oviposition of the egg cluster to (B, C) hatching is referred to as the embryonation period. Hatchlings (D) may settle on their natal coral (pink), or find a new *Acropora* host (green), where they feed and develop to sexual maturity, as evidenced by the appearance of the next generation of eggs.

Experiment 1 – Embryonation Period and Hatching Success

To examine the influence of temperature on embryonation period and hatching success, *P. acroporae* egg clusters were placed in four separate temperature treatments [21, 24, 27, or 30°C (±°0.2°C; precision of temperature control)]. These temperatures represent the range at which *Acropora* spp*. grow along the Great Barrier Reef, Australia, according to the average monthly water temperature in the northern (Lizard Island), middle (Davies Reef), and southern (Heron Island) portion of the reef. The chosen temperature range also includes the range of temperatures at which *Acropora* spp*. are commonly kept in captive aquaria. Thirty-six *P. acroporae* egg clusters were placed in individual 500 mL flow-through hatching chambers and distributed equally among the four temperature treatments (during September to November 2016). For each temperature treatment, nine hatching chambers were split between three incubation tanks (i.e., three replicate hatching chambers per tank; three tanks total) that were randomly positioned within the experimental room. Hatching chambers were provided with individual water supply and housed in triplicate to ensure temperature stability within each chamber. A 12:12 (light:dark) cycle was used with the first and last hour ramping to the desired intensity of 24 °C mmole m^{-2} s^{-1} provided by AquaIllumination Hydra 52 LED modules. This light intensity was selected based on measurement of the light intensity (LI-COR LI-250A light meter and LI-190R Quantum Sensor) reaching egg capsules that were laid on the underside of *A. millepora* colonies. Egg clusters were

examined daily under a dissection microscope (Leica MZ16; 10-40x) to monitor the development of the embryos in each egg capsule. Observation of each egg cluster continued until the last individual was observed to hatch, or when it was determined that no further embryos were viable. The embryonation period of each egg capsule within a given cluster (Figures 2A,B) was defined as the number of days postoviposition until the emergence of all viable individuals from the egg capsule through the operculum. Hatching success was expressed as the proportion of capsules within a cluster that gave rise to hatchlings from the total number of capsules in a cluster.

Experiment 2 – Hatchling Longevity and Morphological Variation

We observed variation in the morphology of *P. acroporae* hatchlings, so to investigate if there was variation within and between egg clusters, we collected all the hatchlings from three clusters reared at 27°C, made live observations of their swimming/crawling movements and subsequently fixed them in 4% paraformaldehyde (PFA) for sorting by morphological variation. To assess dispersal potential, we measured how long *P. acroporae* hatchlings could survive in the absence of coral (hatchling longevity) and whether temperature impacted this. Freshly laid egg clusters (using the egg collection method 2 described above) were reared in individual Petri dishes of filtered seawater (1 mm) incubated in tanks at 24, 27, or 30°C (0.2°C), with five replicates per treatment. The 21°C treatment was excluded because of the poor hatching success rates in experiment 1. Hatchlings were collected as they emerged from their egg capsules and incubated in Petri dishes of filtered seawater at the temperature in which they were reared. Each Petri dish contained 10 hatchlings, which were collected at the same time and then monitored every 6 h to assess survival. Hatchlings were considered dead once they showed no signs of motion and failed to respond to a gentle stream of water from a plastic pipette, or when they failed to regulate their position in the water column following gentle centrifugal motion of their Petri dish. Once pronounced dead, they were examined in the subsequent monitoring period for confirmation. Longevity of each hatchling was expressed as the time elapsed from emergence from the egg capsules to death.

44

Experiment 3 – Time to Sexual Maturity and Size at Sexual Maturity

The time for *P. acroporae* to reach sexual maturity was assessed by determining the time between coral infestation with hatchlings to the first appearance of eggs on the coral skeleton (Figure 3-1). Uninfested coral fragments for this experiment were prepared from an *A. millepora* colony collected from Davies Reef (18°49021.60 0 S 147°39012.50 0 E; GBRMPA Permit No. G12/3236.1) in November 2017 and the experiment was conducted between February and April 2018. Egg clusters (27 clusters; nine per treatment) were collected from the culture on plastic strips and incubated and monitored using three treatment temperatures 24 ± 0.2°C, 27 ± 0.2°C, or 30 ± 0.2°C. A constant supply (0.2 L/min) of filtered seawater provided stable temperature and water quality within the 27, 1.5 L PVC infestation chambers. Each chamber housed one *A. millepora* fragment and one *P. acroporae* egg cluster. Aeration was provided to each infestation chamber to maintain water flow and was only reduced during hatching to facilitate *P. acroporae* recruitment. Each infestation chamber was placed in a group of three within temperature-controlled water baths, with replication to account for potential tank effects (i.e., three incubation aquaria per temperature; three infestation chambers per water baths). Before the addition of egg clusters, *A. millepora* fragments were acclimated to their assigned treatment within the experimental system with temperature change no greater than 0.8°C per week. Daily monitoring of egg clusters informed when hatching would occur; in the embryo pigmentation of the gut and development of five or more eye spots indicated imminent hatching. A section of coral tissue was removed with pressurized air (~4 mm) to expose the coral skeleton and provide substrate for *P. acroporae* to deposit eggs.

Each infestation chamber was fitted with 60 mm mesh 'banjo filters' on each chamber outlet to prevent loss of hatchlings when hatching was imminent. These filters were cleaned three to four times per day to remove biofouling. Egg capsules were checked twice daily (morning and evening) for hatching. The

first day of hatching was considered day zero of time to reach sexual maturity. The exact numbers of

fresh hatchlings were not examined, because this process would disrupt recruitment of *P. acroporae* to

the host *Acropora millepora* fragment. Daily checks of the coral using a magnifying lens (SubSee C10

Diopter) were made to assess the progression of *P. acroporae* infestation (e.g., feeding scars) and look

for the next generation of egg capsules. Egg deposition on the host coral was used as a proxy for the first

attainment of sexual maturity in each cohort. Once eggs were observed, adult worms were collected by

holding the infested coral over a 2 L Pyrex® bowl and removing them with streams of water from a

'turkey baster.' Each flatworm was measured using a ruler (to the nearest mm) and Olympus® Tough

camera to determine the mean size at sexual maturity at each temperature.

Statistical Analysis

Data were analyzed using RStudio (Version 1.0.143) for the influence of temperature on the

embryonation period, hatching success, time to hatchling death, time to sexual maturity, and size at

sexual maturity of *P. acroporae*. Normality was assessed using QQplot and Shapiro-Wilk tests. A linear

mixed effects model [LME; R package "nmle" (Pinheiro et al. 2019)] was used to examine the influence

of temperature on the time to sexual maturity (Shapiro–Wilk; $p < 0.05$). Because data from

embryonation period and hatchling longevity experiments did not meet the assumption of normality

and are time-to-event experiments, a time-to-event semi-parametric mixed effects Cox proportional

hazards model [COXME; R package "survival" (Therneau 2015)] was performed instead. Each model

considered temperature fixed effect, and the cluster each egg capsule belonged to as a random effect,

with significance level defined at $p < 0.05$. Adult length was also considered a fixed effect for

embryonation period and hatching success data. Post hoc pairwise comparisons with Bonferroni

correction were also performed for both LME [R package "emmeans" (Lenth 2019)] and COXME (R

package "survival") analyses, to examine differences between temperature treatments. Because this

study aimed to provide predictions of how long flatworms take to hatch (embryonate) and reach sexual

maturity at different temperatures, models were used to estimate these parameters from temperatures 21–30°C. Kaplan-Meier survival estimates of the Cox model (R package "survival" survfit function) were used to estimate the duration of the embryonation period (95% CI) at temperatures 21–30°C. Similarly, the linear mixed effects model was used to estimate time to sexual maturity at temperatures 21–30°C based on the relationship between temperature and rate of attaining sexual maturity. A Chi-squared test was performed to investigate the influence of temperature on hatching success (temperature treatment vs. number of eggs hatched and unhatched), followed by independent pairwise comparisons between each treatment with subsequent Bonferroni adjustment to assess significant differences ($p < 0.008$) in hatching success between temperature treatments. Because of the non-normal distribution

of size at sexual maturity data (Shapiro-Wilk; $p < 0.05$), a Kruskal-Wallis test was used to examine the influence of temperature on size at sexual maturity, and a Dunn test with Bonferroni adjustment examined differences between temperature treatment. The life cycle generation time, or minimum time to reinfestation by sexually mature worms, was calculated as the sum of time taken for eggs to begin hatching and minimum time to sexual maturity. Hatchlings were considered to be able to infest coral immediately following hatching.

Results

Experiment 1 – Embryonation Period and Hatching Success

Temperature had a significant effect on the duration of the *P. acroporae* embryonation period [$p < 0.001$; 1.76 ± 0.16 (coefficient ± SE); COXME; Figure 3-2C]. Pairwise comparison revealed significant differences between all temperature treatments ($p < 0.001$; Tukey post hoc). At 21, 24, 27, and 30°C mean embryonation period for *P. acroporae* egg capsules was 26, 15, 11, and 9 days, respectively (Figure 3-2C and Table 3-1). The first and last day of capsule hatching in all egg clusters at a given temperature is shown in Figure 3-2C and Table 3-1. Predicted hatching probability curves using

experimental embryonation data (Kaplan-Meier survival estimates) suggest that the embryonation

period should range from 26 days at 21°C to only 9 days at 30°C (Table 3-2).

Table 3-1: Life cycle parameter table for *Prosthiostomum acroporae*. FH/LH – days to first and last egg capsule

hatching in egg clusters (number of egg capsules counted over 6–9 egg clusters at each temperature); HL –

average hatchling longevity ± SE, Smin – minimum time to sexual maturity; Smean – mean minimum time to sexual

maturity ± SE; LC – minimum time to completion of life cycle (FH C Smin).

Temperature (°C)	FH/LH (days) (mean ± SE)	HL (days)	S_{min} (days)	S_{mean} (days)	LC (days)
21	22/28 (25.9 ± 0.22) Capsules n = 125, Clusters n = 6	-	-	-	-
24	10/16 (14.83 ± 0.11) Capsules n = 124, Clusters n = 7	2.27 ± 0.26	54	56 ± 1.15	64
27	6/14 (11.3 ± 0.10) Capsules n = 144, Clusters n = 9	0.93 ± 0.06	32	34.5 ± 0.9	38
30	6/12 (9.4 ± 0.12) Capsules n = 129, Clusters n = 9	2.23 ± 0.29	30	32.5 ± 2.50	36

Table 3-2: Predicted time of embryonation from hatching probability curves using experimental embryonation

data (Kaplan–Meier survival estimates), and time to sexual maturity (linear mixed effects model) in days.

Temperature (°C)	Embryonation period (median days)	Time to sexual maturity (days)
21	26	141
22	22	94
23	16	70
24	15	56
25	14	47
26	13	40
27	12	35
28	11	31
29	10	28
30	10	25

Temperature also had a significant influence on the hatching success of *P. acroporae* from their egg capsules (p < 0.05, Chisquared test; Figure 3-2C). Pairwise comparison demonstrated that the hatching success of worms from egg capsules in the 21°C treatment [43 ± 4% (mean ± SE)] was significantly lower (p < 0.008, Chi-squared test) than the other treatments (24, 27, 30°C). There were however no significant differences (p > 0.008) between the hatching success of temperature treatments of 24°C (80 ± 4%), 27°C (91 ± 2%), and 30°C (81 ± 3%) in any combination. Across the total of 36 egg clusters examined in this experiment, the number of egg capsules that made up a cluster was variable with a mean of 14 ± 1.3 capsules (mean ± SE) and a range of 3 to 36. The number of embryos per capsule ranged from 1 to 5. Not all embryos within each egg cluster successfully hatched at all measured temperatures. We observed cases of incomplete embryonic development, oversized embryos (potentially too large to exit through the capsule operculum), or a general inability of the embryos to exit a given capsule. We considered an egg capsule to be successfully hatched when all viable individuals exited the egg capsule through the operculum.

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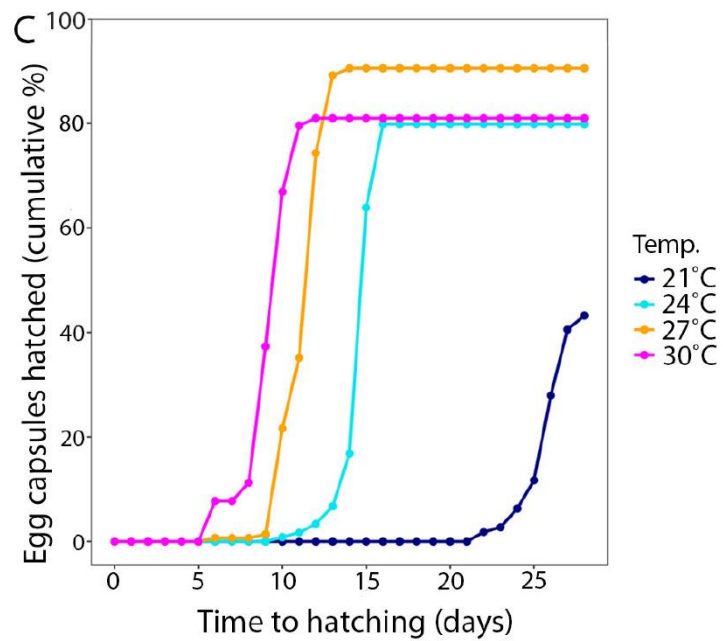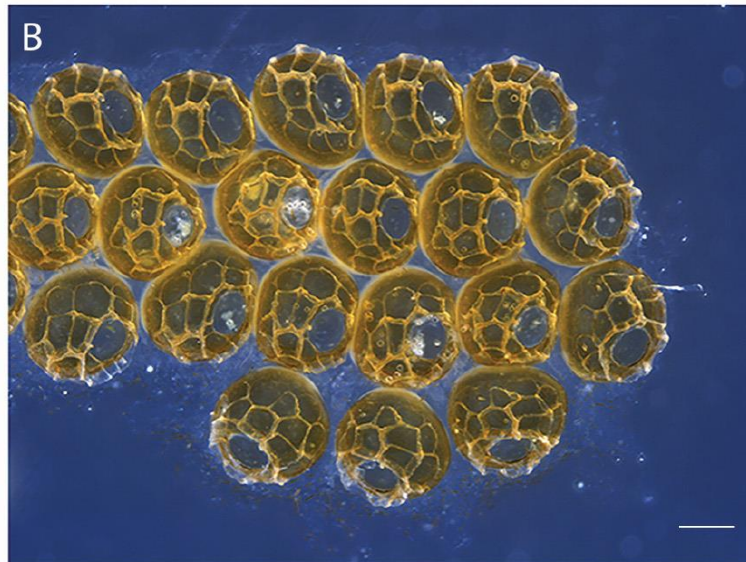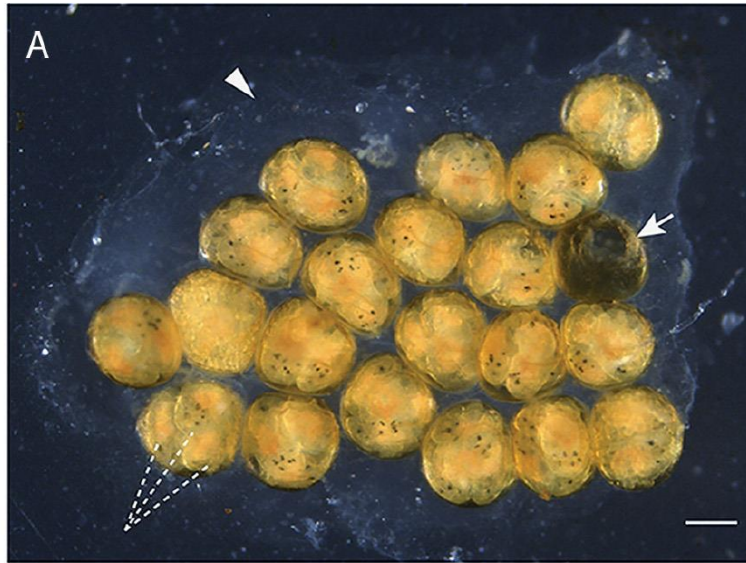
Figure 3-2: (A) A *P. acroporae* egg cluster showing multiple embryos (dashed lines) inside each egg capsule (arrow points to a hatched capsule), cemented to the substrate (arrowhead) (scale = 200 mm). (B) An egg cluster showing capsules after the hatchlings have emerged. (C) A cumulative hatching plot showing the effect of temperature on embryonation period and hatching success (n = 125 egg capsules at 21°C, n = 124 egg capsules at 24°C, n = 144 egg capsules at 27°C, n = 129 egg capsules at 30°C; nine replicate egg clusters per temperature).

Experiment 2 – Hatchling Longevity and Morphology

During embryogenesis, *P. acroporae* developed larval characters typical of other cotylean polyclads (i.e., lobes with ciliary tufts for swimming during a pelagic phase; Figure 3A). There was variation in hatchling morphology, which ranged from having reduced lobes to no lobes (Figure 3B), but all individuals were able to swim and crawl. This morphological variation was subtle and difficult to observe in live specimens due to the plasticity of their body shape. Fixation of the hatchlings enabled examination of the morphological variation within and between clusters and revealed that specimens that had reabsorbed their lobes still retained the ciliary tufts (Figure 3C). It also appeared that there may be variation between egg clusters laid by different parents as all hatchlings from one cluster had reduced lobes, whereas hatchlings from another had completely reabsorbed their lobes (Supplementary Figure3-1). Even in fixed hatchlings, the degree of lobe reabsorption was a subtle and a continuous character that was difficult to score. Therefore, due to the lack of clear dimorphism, there was no attempt to quantify the variation in hatchling morphology. Instead, because all hatchlings could swim, we determined the longevity (or survivorship) of hatchlings as a measure of their dispersal potential. Hatchling survival ranged from 0.25 to 9 days in the absence of coral; the mean number of days (± SD) to death was 2 (± 2.12), 1 (± 0.52), and 2 (± 2.27) at 24°C, 27°C, and 30°C respectively (Table 3-1). Temperature did not have a significant influence on hatchling longevity (p > 0.05; COXME; Figure 3A). Pairwise comparison demonstrated significant differences between temperature treatments 24 and

27°C (p < 0.001), 27 and 30°C (p < 0.001), but none between 24 and 30°C (p < 0.001). Hatchling longevity was variable between individuals sourced from within a cluster and also between clusters (Figure 3D).
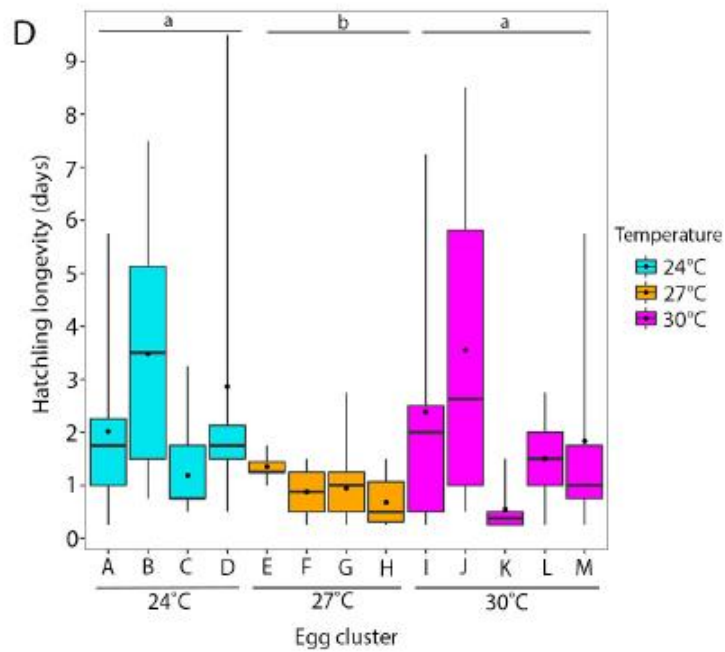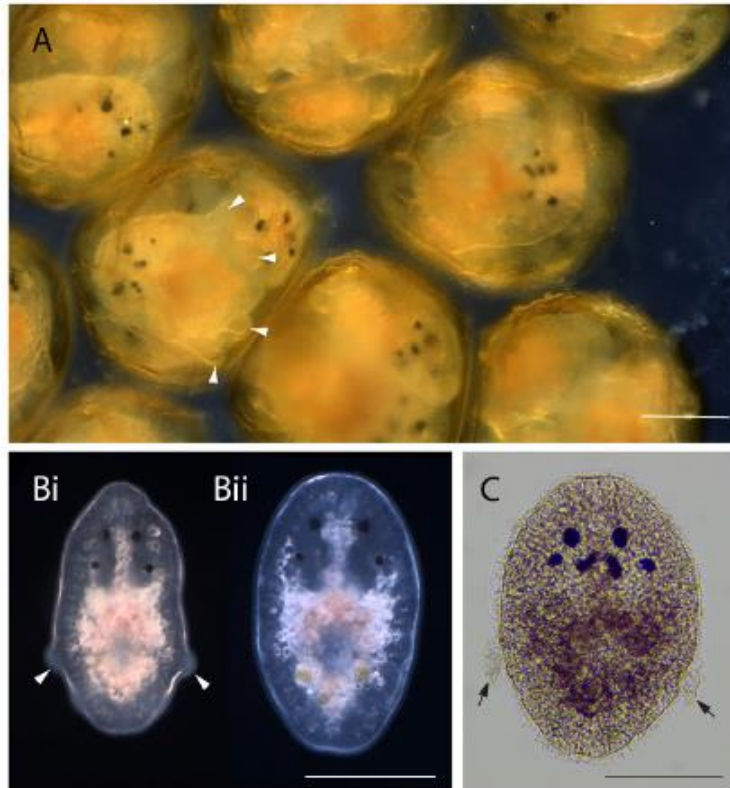
Figure3-3: Hatchling morphology and longevity of *P. acroporae*. (A) Live embryos developing inside the egg capsule showing pronounced larval lobes (arrowheads). (B) At hatching these lobes are either much reduced (arrowheads) (Bi) or lost entirely (Bii) (live hatchlings). (C) Hatchlings that have lost their lobes retain the ciliary tufts (arrow; fixed specimen). Scale = 100 mm. (D) Hatchling longevity (the number of days a hatchling survives in the absence of coral) at 24°C (n = 64, 4 egg clusters), 27°C (n = 81 individuals, 4 egg clusters), and 30°C (n = 62, 5 egg clusters) (error bars = min. and max. values, boxes = lower and upper quartiles, line = median, and dot = mean).

Experiment 3 – Time to Sexual Maturity and Size at Sexual Maturity

Temperature had a significant influence on the time for newly hatched *P. acroporae* to reach sexual maturity ($p < 0.005$, $R^2 = 0.7754$, SE = 0.0126, LME; Figure 3-4A and Table 3-1). Pairwise comparison (emmeans) of rate of sexual maturity between temperature treatments revealed significant differences between 24 and 27°C ($p < 0.005$), 24 and 30°C ($p < 0.005$), but no significant difference between 27 and 30 °C ($p > 0.005$). Predicted time to sexual maturity is up to 141 days at 21°C and as short as 26 days at 30 °C (Table 3-2).

One 27 °C replicate flatworm resided on an unhealthy coral (bleached) and was considered an outlier in the data set, reaching sexual maturity at 58 days compared to 39.4 ± 4.97 days (mean SE) and was removed from the analysis. Results were limited by the number of replicates that housed sexually mature flatworms, specifically in the 30 °C treatment where only two replicates had individuals that reached sexual maturity. One of these 30 °C replicates had five flatworms at the time of oviposition of the first egg cluster (30 egg capsules), while the other fragment had only one flatworm when the first egg cluster was recorded (nine egg capsules).

Although successful initial settlement of hatchlings is not verifiable within our experimental design, it appears that low settlement occurred in the 30 °C treatment, at least that which yielded symptoms of infestation. Eight of the nine replicate clusters (one cluster failed to hatch after appearing to complete development) successfully hatched at 30 °C, but of these, six failed to infest their associated *Acropora*

millepora fragments. At 25 days post-oviposition, no flatworms were associated with the remaining six replicates.

Temperature had a significant effect on total body length of *P. acroporae*, where individuals were larger at sexual maturity in warmer temperatures (Kruskal-Wallis, p < 0.001, F = 20.29; Figure 3-4B). Pairwise comparison revealed significant differences between temperature treatments 24 and 27 C (Dunn's Test, p < 0.001), 24 and 30 C (p < 0.001), but not between 27 and 30 C (p > 0.001). The consequence was that flatworms reaching sexual maturity at warmer temperatures had total body lengths of 5.81 ±0.82 and 6.12 ± 0.37 mm (mean ± SE) (27 and 30 °C respectively) and were larger than flatworms in cooler conditions (24 C) with a body length of 4.00 ± 0.27 mm.
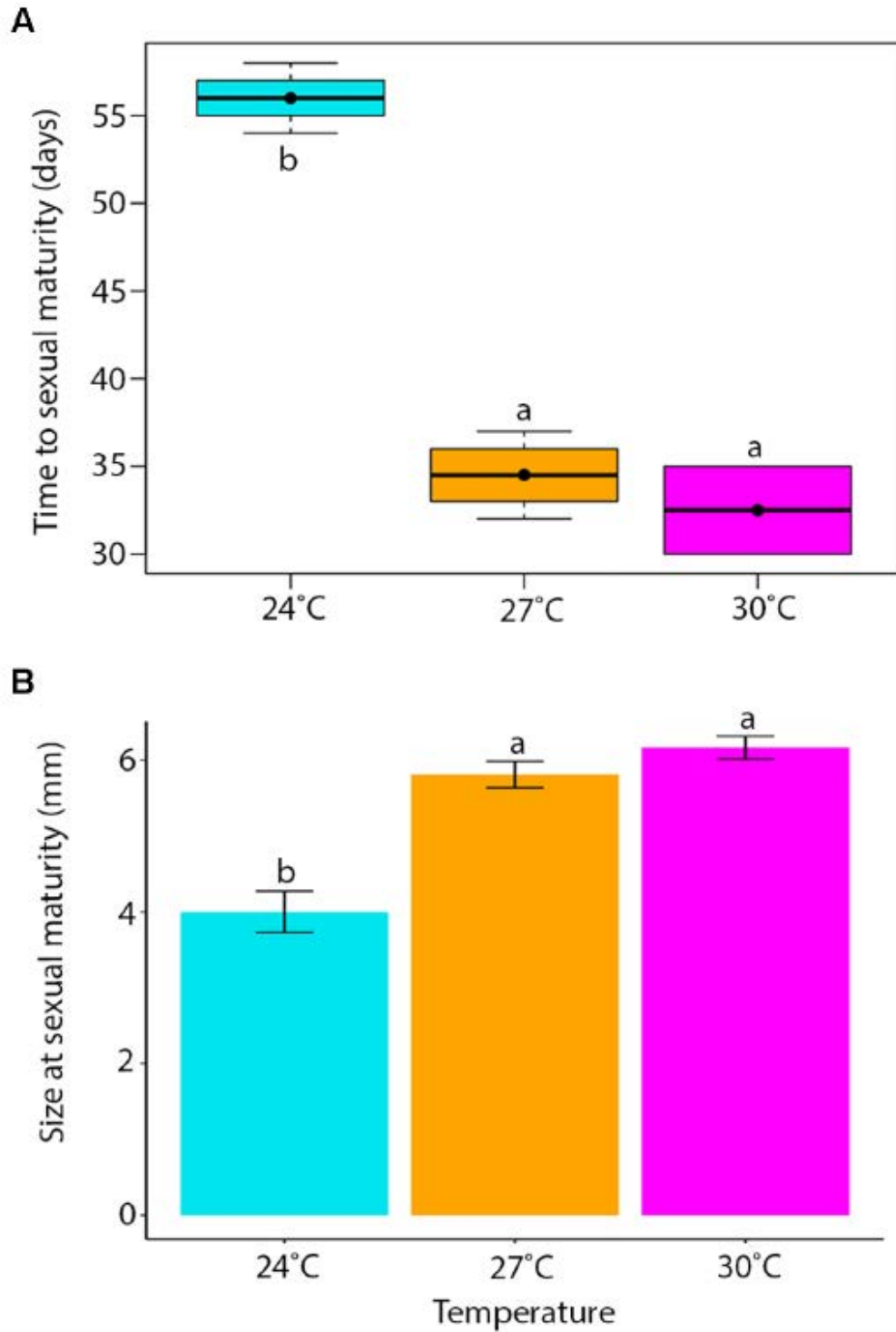
Figure 3-4: A) The time for hatchlings to reach sexual maturity (error bars = min. and max. values, boxes = lower and upper quartiles, line = median, and dot = mean). (B) Mean body length (± SE) of individuals at sexual maturity. Treatment boxes and bars with the same letter are not significantly different from each other (p > 0.05).

Discussion

Advances in Our Understanding of the Life Cycle of Prosthiostomum acroporae

The polyclad flatworm *P. acroporae* presents a serious problem to captive held Acropora corals, impacting their health in both hobbyist aquariums and large-scale coral aquaculture facilities. Effective management of this worm requires detailed understanding of its life cycle, and in this study we determined its embryonation period, hatching success, hatchling longevity, and time to sexual maturity across a range of biologically relevant temperatures (Berkelmans and Willis 1999; Berkelmans and Van Oppen 2006; Howells et al. 2013). To our knowledge, this is the first study to determine the timeline of a complete life cycle in a polyclad flatworm. The empirical data on life cycle parameters of *Prosthiostomum montiporae* allows us to calculate the generation time from oviposition of the parental generation to oviposition of the first generation. In comparison to other flatworms, a life cycle of 38 days for *P. acroporae* (at 27 °C) is long compared to 10-13 days for the skin fluke *Neobenedenia girellae* (Hargis, 1955) (at 26 and 28 C) (Brazenor and Hutson 2015), and 2-3 weeks for the free-living flatworm *Macrostomum lignano* Ladurner, Schärer, Salvenmoser & Rieger, 2005 at 20 °C (Morris et al. 2004; Wudarski et al. 2019), but short compared to 80 days for the trematode flatworm *Schistosoma mansoni* Sambon, 1907 at 28 °C (in snail host and freshwater) and 37 °C (in mammalian host) (Rawlinson personal observation). This information has important implications for population numbers of *P. acroporae* and these findings can be used to identify the timing of treatments to disrupt the life cycle of this coralivorous flatworm.

Interestingly, variation in the morphology of the hatchlings was observed with some hatching with lobes and ciliary tufts, and others hatching without lobes but with ciliary tufts. As this variation was subtle and continuous it may be due to variation in the timing of hatching, with hatchlings emerging at different

timepoints during metamorphosis, during which lobes are reabsorbed and ciliary tufts are eventually

shed (Kato 1940; Ruppert 1978). It does not appear to be a case of clear developmental dimorphism (or

poecilogony) with distinct types of embryos within a cluster developing into either long-lived larvae with

an obligate feeding period or short-lived larvae that can settle without feeding in the plankton (Krug

2009). Our observations on fixed hatchlings also suggest that there may be variation between egg

clusters, indicating either a parental effect or, more remotely, a case of cryptic species. However, as we

only assessed variation in hatchling morphology in three clusters and at one temperature (27 °C), a

more extensive examination of variation within and between clusters is necessary to draw conclusions

on the significance of this finding. Variation in hatchling morphology has interesting ecological,

developmental, and evolutionary consequences, with those hatchlings retaining lobes potentially able to

swim further and for longer durations than those that have already reabsorbed their lobes. Many

benthic marine invertebrates, that have a dispersive larval stage, develop tufts of long cilia for swimming

during the pelagic phase; and their placement on lobes increases the volume of water moved per ciliary

stroke relative to placement of cilia on a flat surface (Emlet 1991). *P. acroporae* has retained the

dominant life history strategy found in other cotylean polycads, i.e., indirect development via a larval

form, but delays hatching until metamorphosis is almost complete. Our results suggest that as

hatchlings can swim and have ciliary tufts (and some have lobes), there is potential to disperse between

coral colonies. However since they are also able to crawl and can survive for up to 9 days (a relatively

short time compared to hatchlings of other cotylean species, reviewed in Rawlinson 2014) in the

absence of coral, they could also be competent to settle given the right cues. Our development of life

cycle rearing techniques and measurements of life cycle parameters provide a foundation for

investigating if any genetic and/or epigenetic factors may influence time of hatching, and hatchling

survivorship and dispersal.

Temperature Effects on the Life Cycle and the Timing of Treatments

It is common for poikilothermic animals to exhibit elevated developmental rates with increased temperatures (Howe 1967; Hoegh-Guldberg and Pearse 1995; Golizadeh et al. 2007; Wudarski et al. 2019). This phenomenon has historically been studied by entomologists to inform pest control methodology in agriculture and more recently for parasitic disease management in aquaculture (Tubbs et al. 2005; Brazenor and Hutson 2015). Knowledge of timing of key life cycle stages at different temperatures for *P. acroporae* will increase the efficacy of treatment regimens to disrupt its life cycle and help advance coral husbandry practices. Currently, a variety of 'dips' (such as Levamisole HCl solutions (see Carl 2008) and other commercial products) are used to treat infestations of *P. acroporae* in captivity. Here we suggest, that after an initial treatment for *P. acroporae*, the host colony must be treated again, to target new hatchlings after they emerge from their protective egg capsules. The second treatment should take place before offspring reach sexual maturity (Figure 3-5 and Table 3-2). For example, *P. acroporae* eggs in an aquarium operating at approximately 28 °C would complete embryonation in approximately 11 days (Table 3-2). Assuming these hatchlings find a susceptible Acropora host, they would reach sexual maturity in approximately 31 days at 28 °C (Table 3-2). Therefore, this *Acropora* colony should receive a second treatment between 13 (11 days embryonation period plus 1 or 2 days for settlement) and 31 days (approximate time to sexual maturity) following the first treatment (Figure 3-5). In this case (28 °C) and at all temperatures, we recommend applying the second treatment at a time interval greater than the duration of embryonation, but less than the estimated time to sexual maturity. Importantly however, the present study did not evaluate the efficiency of any given chemical treatment to remove *P. acroporae* individuals, and therefore the suggested treatment strategy needs to be rigorously evaluated (see Carl 2008). The effectiveness of these treatments can be enhanced by the mechanical removal of egg capsules using a scalpel or razor blade. If more than one coral colony in the aquarium system is infested, reinfestation is likely if all

infested corals are not treated simultaneously. Although most *P. acroporae* hatchlings will starve within

2 days without any host material, the most resilient survive up to 9 days. Therefore, it is recommended

that treated corals be housed in an isolated quarantine tank between treatments, if possible, as this

practice will give sufficient time for any *P. acroporae* hatchlings left in the infested system to starve. *P.*

acroporae can lay their eggs on most hard substrates, which could foster reinfestation if *Acropora* tissue

is still present.
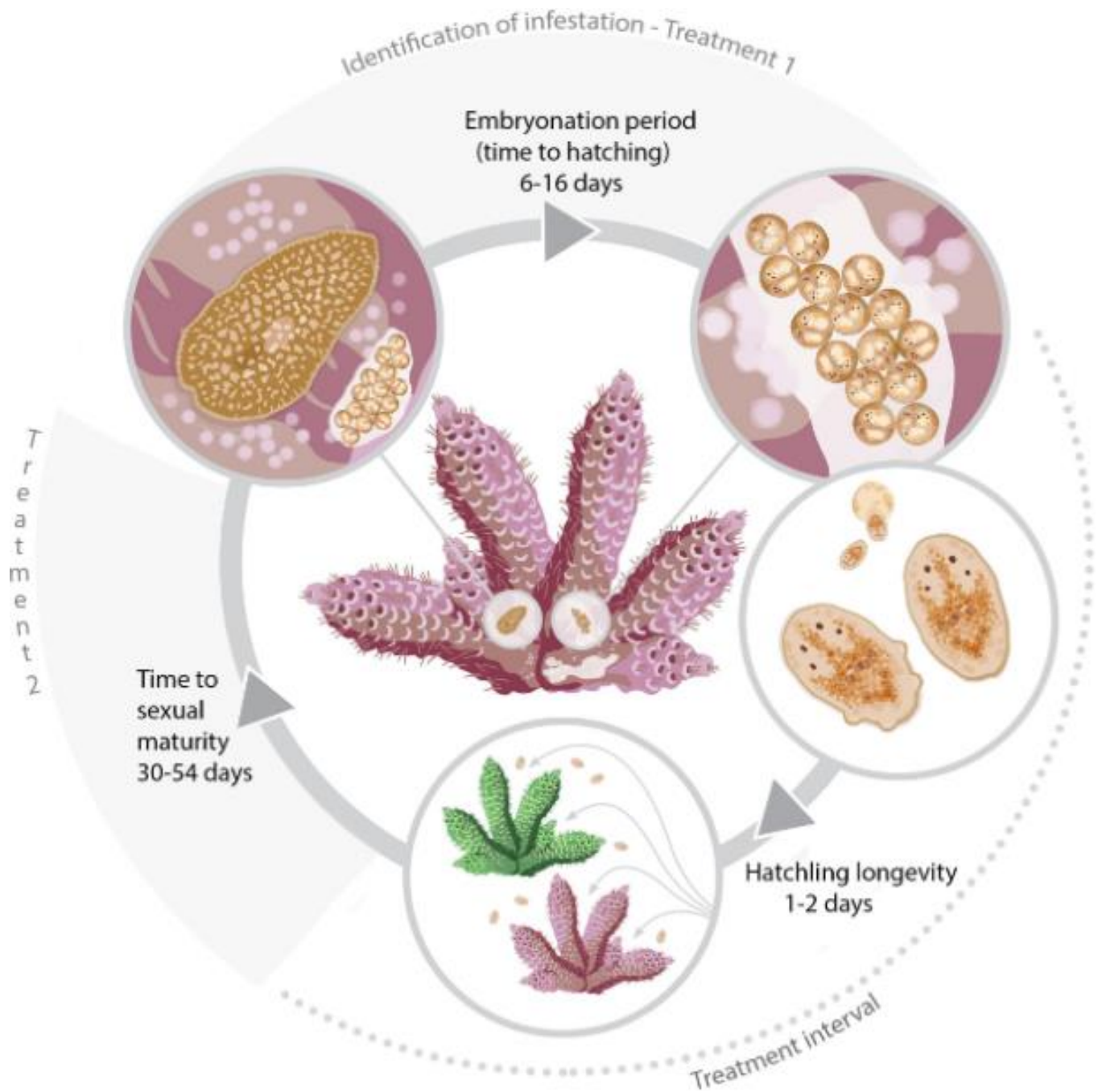
Figure 3-5: The life cycle of *P. acroporae* measured at 3°C increments from 24 to 30 °C and the recommended timings of treatments (see section "Discussion").

In addition to optimizing coral dipping treatments to disrupt the flatworm's life cycle, our data could also be used to minimize *P. acroporae* numbers along the supply chain and to inform the duration of fallow "*Acropora*-free' periods within a system. The relatively slow development and poor hatching

success of *P. acroporae* in the 21 °C treatment suggests that captive population numbers will be lower in cooler waters. However, hatching success and/or settlement success may have been higher using flatworms from reefs of the southern GBR (e.g. Heron Island) where waters are typically cooler. Although lower temperatures could potentially be used to limit *P. acroporae* numbers (e.g., during transport of coral), temperature adjustments should primarily be based on the thermal tolerance of the *Acropora* colonies. Combining knowledge on the lower thermal tolerance of the *Acropora* species with our generation time predictions at different temperatures (Table 3-2) could allow a compromise temperature to be found that would minimize flatworm-related, and thermal, stress to the coral. Our data can also guide 'fallow' periods in a captive system; for example, by increasing the temperature of a system to 30 °C and providing no live *Acropora* tissue, we would predict that any remaining egg capsules would hatch within 12 days and all subsequent hatchlings would be starved if held a further 9 days, equating to a 21 day fallow period. By contrast, at 24 degrees the same process would take 25 days (i.e., egg capsules hatch within 16 days and a 9-day hatchling starvation period).

Implications for Wild Population Numbers of *Prosthiostomum acroporae*

Investigating the life cycle of *P. acroporae* in captivity provides a foundation to understand how populations may fluctuate seasonally in the wild. Based on our findings, we would predict that shorter life cycles during the warmer months could lead to higher population densities of *P. acroporae*, assuming that no deleterious effects associated with increased development occur (not observed in this study. In contrast, it appears that *P. acroporae* development slows considerably in cooler temperatures with reduced hatching success, indicating that wild population numbers may fluctuate considerably throughout the year. On Davies Reef, for example, the yearly average water temperature ranges from 29 °C in December to 23 °C in April.

Increased sea surface temperatures (SSTs), attributed to climate change, already lead to thermal stress on Acropora populations (De'ath et al. 2012; Hughes et al. 2017). Thermal stress can predispose corals to higher rates of mortality from the feeding activity of corallivores [e.g., *Drupella* gastropod (Shaver et al. 2018)]. An increase in *P. acroporae* population density during prolonged thermal stress could exacerbate consequences to host *Acropora* colonies in a similar fashion if flatworm populations are not controlled by their natural predators. Identifying (and conserving) the natural predators of *P. acroporae* could help limit numbers in captivity and the wild. In addition to any potential climate change related threats *P. acroporae* could pose, our data highlights a trade-off between growth and reproduction at different temperatures. In cooler temperatures, *P. acroporae* appears to sacrifice size to reach sexual maturity, possibly investing energy into reproduction over body size. While the ecological ramifications of reduced size are unknown, this phenotypic plasticity could be advantageous to survival in the temperature gradient along the GBR (21-30 °C; Berkelmans and Willis 1999; Berkelmans and Van Oppen 2006; Howells et al. 2013).

Conclusion

Warmer water temperatures lead to faster rates of development (pre- and post-hatching), shorter generation times and increased hatching success of the corallivorous flatworm *P. acroporae*. The data and models provided in this study detail the timelines for life cycle parameters at a range of biologically relevant temperatures, information critical to aquarists looking to disrupt the coralivorous flatworms' life cycle. As the coral aquaculture trade grows, more effective management tools are required to control *P. acroporae* numbers in captive settings and this not only includes better targeted chemical treatments but also identifying natural predators of *P. acroporae* that are also suitable to captive conditions.

Chapter 4: Polyclad flatworms associated with acroporid corals of the Great Barrier Reef: distribution and phylogenetics

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Abstract

The collection of corals and subsequent introduction of coral-associated pest species into captivity can have negative consequences on these corals if infestations intensify and spread. Understanding the distribution of these organisms along the Great Barrier Reef, and how prevalently they are found in association with their respective coral hosts informs stakeholders of which coral reefs and coral hosts these pests can occur in association with. Two corallivorous polyclad flatworms, *Prosthiostomum acroporae*, the *Acropora*-eating flatworm (AEFW) and *Prosthiostomum montiporae*, the *Montipora*-eating flatworm (MEFW) consume coral tissue (along with the symbiotic Symbiodiniaceae) and in captive conditions can proliferate to densities which cause colonial mortality. This study examined the prevalence of *P. acroporae* on coral colonies collected from the Great Barrier Reef (GBR). Among 154 screened captive and wild harvested *Acropora* coral fragments (representing *Acropora millepora*, *A. spathulata*, *A. tenuis*, *A. loripes* and *Acropora* sp.), 56% ± 4% (mean ± SE) were infested with flatworms, with approximately 5.89 ± 0.51 (mean ± SE) flatworms found per coral. The distribution of *P. acroporae* ranged from Lizard Island in the northern GBR to over 1000 km south to the Keppel Islands in the southern GBR. We encountered the first evidence of MEFW on corals of the GBR, and we used morphological and molecular evidence to determine if MEFW specimens found infesting captive corals from the GBR (*Montipora digitata* and *Montipora tuberculosa*) are the same species as *P. montiporae* found infesting *Montipora capitata* in Hawaii. Based on histological evidence of shared morphological traits, we propose that GBR MEFW specimens are *Prosthiostomum montiporae.* While we were unable to include *P. montiporae* sequence data from Hawaiian MEFW, our molecular phylogeny of the family Prosthiostomidae using 28s rDNA sequences of *Prosthiostomum* spp. specimens (n = 30) collected from the GBR suggested the family Prosthiostomidae is a monophyletic clade composed of three distinct subclades: 1) the AEFW *P. acroporae*; 2) the MEFW *P. montiporae* with two other large specimens (~13 mm and 17 mm respectively) from *Acropora* sp. and *Euphyllia glabrescens*; 3) *Prosthiostomum*

torquatum Tsuyuki, Oya & Kajihara, 2019 and *Prosthiostomum* sp. Z. No other polyclad flatworms from other families were found in this study or any other. Future screening of various coral families is required to broaden our understanding of the diversity of symbiotic flatworms.

Introduction

Coral reefs are facing increased pressures including anthropogenically driven climate phenomena (e.g. coral bleaching/cyclones), declining coastal water quality, and outbreaks of coralivorous invertebrates such as the crown-of-thorns starfish *Acanthaster planci* (see Kayal et al., 2012; Pratchett et al., 2014, 2017) and corallivorous snails *Drupella* (see Cumming, 1996; 1999; Baums et al., 2003; Raymundo et al., 2016). The global decline in the health of reef ecosystems, including the Great Barrier Reef (GBR) which has continued to lose coral cover over the past three decades (De'ath et al., 2012; LTRMP 2018/2019), highlights the importance of understanding and mitigating the pressures faced by coral reefs. Corals are held in captivity to support research to understand these pressures, and increasingly to support active replenishment of degraded coral reef habitats (e.g. reef restoration Epstein, 2001; Rinkevich 2014; van Oppen et al., 2017; Omori, 2019; Randall et al., 2020).

Captive corals held for extended periods of time can become infested with coral-associated invertebrates (e.g. obligate corallivores) which can be harmful at high densities (Stella et al., 2010; Stella et al., 2011; Sweet et al., 2012). Polyclads for instance are typically free-living, marine, predatory flatworms with only two species, *Prosthiostomum acroporae* (Rawlinson, Gillis, Billings & Borneman, 2011) and *Prosthiostomum montiporae* (Poulter, 1975) known to be obligate corallivores of acroporid corals (belonging to *Acropora* and *Montipora*). *P. acroporae*, commonly known as the *Acropora*-eating flatworm, were discovered in the ornamental trade on captive *Acropora* coral species (Rawlinson, Gillis, Billings & Borneman, 2011) and subsequently found in wild coral populations around Lizard Island, in the

northern GBR (Rawlinson & Stella 2012). *Prosthiostomum montiporae* was described from wild sourced

Montipora capitata corals in Kaneohe Bay, Hawaii, and like *P. acroporae*, it is well camouflaged because

of its dorso-ventrally compressed body and retention of host coral tissue and Symbiodiniaceae (Poulter

1975; Jokiel and Townsley 1974). As such, infestations in wild and captive coral colonies can be difficult

to detect until colonies display feeding scars, tissue necrosis, and/or the presence of egg clusters.

Despite the observation of *P. acroporae* and *P. montiporae* in wild and captive environments, our

understanding of the prevalence, distribution, and diversity of these organisms is in its infancy.

Morphological characters used to identify or distinguish between members of Prosthiostomidae include

eye arrangement, male and female reproductive system features, the presence or absence of an

attachment organ, and the morphology of the pharynx (Poulter 1975; Prudhoe 1985; Cannon 1986;

Rawlinson et al. 2011). Perhaps the most distinctive character shared among known corallivorous

Prosthiostomum speci*es* is the coiled muscular pharynx. Our understanding of Prosthiostomidae can

benefit from recent advances in molecular taxonomy of polyclad flatworms, which provide further

resolution of family structure using past and novel 28s rDNA sequences (Litvaitis et al. 2019; Tsuyuki and

Kajihara 2020). What remains less understood however is the adaptation of corallivory by members of

Prosthiostomum (e.g. *Prosthiostomum acroporae*), how this is reflected by their positioning within

Prosthiostomidae, and the diversity of host species they can infest.

The aim of this study was to investigate the prevalence and distribution of *P. acroporae* associated with

acroporids of the GBR. In the process of our assessment of *P.* acroporae we encountered specimens

infesting captive *Montipora* spp., which we assessed morphologically to compare to *P. montiporae* from

Hawaii. Using 28s rDNA sequences of *Prosthiostomum* spp. specimens (n = 30) collected from the GBR

and from GenBank, we created a molecular phylogeny of the family Prosthiostomidae to provide

resolution among corallivorous prosthiostomids using a molecular phylogeny.

Materials and Methods

Coral collection

Acroporid corals (*Acropora millepora* (Ehrenberg, 1834), *Acropora spathulata* (Brook, 1891), *Acropora tenuis* (Dana, 1846), *Acropora loripes* (Brook, 1892) and *Acropora* sp.) were collected between 2016 and 2018 (ten separate trips; GBRMPA Permit G12/3236.1) from depths ranging from 3 to 9 m, from inshore reefs (Esk Reef (18°45'49.6"S 146°110'31.372"E); Orpheus Island (18°36'18.7128"S 146°28'59.7792"E); Magnetic Island (19°9'7.2648"S 146°52' 32.466"E); Falcon Reef (18°46'43.8636"N 146°32'27.402"E) and mid-shelf reefs (Trunk Reef 18°23'20.4"S 146°48'25.8"E); Davies Reef (18°49'21.6"S 111 147°39'12.5"E); Backnumbers Reef (18°30' 06.2"S 147°09' 14.8"E) and Rib Reef (18°28'47.1"S 146°52'00.9"E)) of the GBR. A hammer and chisel were used by divers to collect corals which were transported in flow through aquaria to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS). Each fragment was screened for flatworms before being transferred into flow-through, filtered seawater (1 µm) aquaria, with temperature controlled to match that of collection sites. Although the behavior was not observed, there is a possibility that individuals moved between coral specimens collected together (ten separate collections), because corals were not held separately in transit.

Flatworm screening and collection

Field collected and captive acroporid (*Acropora*) colonies were screened (n = 154) to mechanically remove flatworms present using the methods described in Barton et al. (2019). Briefly, a jet of filtered seawater (of the appropriate temperature) was sprayed over all surfaces of the collected coral fragment which were held inside or just above a 100L aquarium, modified to maintain a constant water level and catch any dislodged flatworms in a 300 µm strainer. The size of coral fragments sampled ranged from approximately 6 x 6 cm fragments to larger 20 x 12 cm (height x width) fragments, with each sample collected from different donor colonies (i.e., one sample per colony). Fragments were screened

immediately when they entered the facility before *P. acroporae* had the opportunity to spread within aquaria. The number of flatworms removed from each fragment was recorded to determine the density of infestation before the corals entered the captive systems. While the majority of screened flatworms were retained live for culture of *P. acroporae* for later experimentation (see Chapter x, x and x), 22 specimens collected from *Acropora* spp. were preserved in 95% ethanol for later identification and analysis (Table A-1).

Opportunistically, captive colonies of the finger coral *Montipora digitata* (Dana, 1846) infested with a corallivorous polyclad flatworm (*Montipora*-eating flatworm, MEFW) were discovered after mechanical screening of fragments with feeding scars. MEFW were found on *Montipora digitata* colonies living in a 2000L (only *M. digitata* corals) flow through aquarium system at the SeaSim, AIMS, where they had been held for seven months. A second grossly distinct prosthiostomid flatworm (designated *Prosthiostomum* sp. Z) was also recovered during mechanical screening from among the *M. digitata* and their associated mesocosm during screening/removal of biofouling organisms. Three MEFW specimens and three *Prosthiostomum* sp. Z were preserved in 95% ethanol for molecular analysis (Table A-1). Ten MEFW specimens were fixed for histology (see protocol below). A later infestation of MEFW was observed on *Montipora tuberculosa* (Lamarck, 1816) at a licensed commercial coral collection facility in Mackay, Queensland, and screening of a specimen of the gold torch coral *Euphyllia glabrescens* (Chamisso and Eysenhardt, 1821) provided two additional specimens which were preserved in 95% ethanol for analysis (Table A-1). In total, 30 prosthiostomatid flatworms were collected, representing three distinct species (AEFW, MEFW and *Prosthiostomum* sp. Z). Communication with licensed coral collectors along the coast of Queensland provided additional anecdotal distribution information based on their experiences with *P. acroporae* pest control (no physical samples) when collecting *Acropora* from non-specific locations around Cairns, Bowen, Swain Reefs, and the Keppel Islands (JBarton per comm; Figure 4-1).

DNA extraction and 28S rDNA gene phylogenetic analysis

Genomic DNA was extracted using the salting-out method (See Ferrara et al. 2006) from 30 prosthiostomid specimens (whole specimens), including 22 *Prosthiostomum acroporae* and 8 *Prosthiostomum* spp., following the manufacturer's protocol. The D1–D2 region of the 28S rDNA gene was amplified using a forward (5' – 3'; AGCACCGAATCCTTCACC) and reverse (5' – 3; TAGTTCACCATCTTTCGGGT) primer pair specific for *Prosthiostomum acroporae* (Rawlinson et al 2011). PCR was carried out in 10 µl reactions including 1 µl DNA template, 5 µl AmpliTaq Gold 360 Mastermix (Applied Biosystems, CA, USA), and 2 µl of each 2 uM forward and reverse primers. PCR was carried out using the following cycle temperatures/times: 10 min at 95°C; 30 cycles of 30 s at 95°C, 60s at 57°C and 60s at 72°C; 7 min at 72°C for a final extension. PCR products were visualized using electrophoresis in a 1.5% agarose gel with an estimated 950 bp products. Samples of *P. montiporae* (n = 4) from the rice coral *Montipora capitata* (Dana, 1846) collected in Hawaii and provided by Hawaii Institute of Marine Biology (at Coconut Island) for this study, failed to amplify with the primers used. PCR products of the successful samples (n = 30) were sequenced at Macrogen Inc (South Korea) for purification and sequencing (Sanger) in both directions.

All 28s rDNA sequences were quality checked and assembled using Geneious v10.2.4 (https://www.geneious.com). The generated sequences (see Table A-2) were aligned with previous deposited 28s rDNA isolate sequences in GenBank from 19 polyclads (predominantly prosthiostomid species; see Table A-2), and three other cotylean species (outgroups; See Table A-2). All sequences were aligned using the Multiple Alignment Fast Fourier Transform (MAFFT) v7.450 plugin in Geneious, using default values and trimmed to 922 base pairs (bp). A maximum likelihood (ML) tree was generated using the IQ-TREE web server v1.6.11 (Trifinopoulos et al. 2016) with 1000 ultrafast bootstraps (UFBS) (Hoang et al. 2018), 1000 iterations and performed SH-aLRT branch test at 1000 replicates.

Histological preparation of *Prosthiostomum* sp.

For histological species identification, *Prosthiostomum* sp. specimens (MEFW) from *Montipora digitata* were processed for histology and whole mount investigations following protocols of Rawlinson et al., (2011). In brief, adults (*Prosthiostomum* sp. n = 10) were fixed on 4% frozen paraformaldehyde in sea water and stored overnight at room temperature, then rinsed in sea water multiple times before either being dehydrated into 75% ethanol for storage or processed directly for histology. For histology, whole specimens of *Prosthiostomum* sp. were graded into 100% ethanol. Specimens were then cleared in Histoclear (National Diagnostics) for 24 h, infiltrated with 1:1 histoclear/paraffin for 24 h and equilibrated in molten paraffin for 24 h (all steps performed in a 60°C paraffin oven, with several changes at each step). Specimens were then embedded in fresh paraffin and left to harden at room temperature for 24 h prior to sectioning. Five MEFW individuals were sectioned in the transverse plane (i.e., in cross section), three individuals were sectioned in the sagittal plane, and two individuals were mounted for whole mounts. Paraffin-embedded sections (6 µM) were stained following a Masson's trichrome protocol. For whole mounts, specimens were graded from 70% ethanol into 100% ethanol and then cleared for 1 h in histosol at room temperature (with three changes). Specimens were then equilibrated in DPX and mounted. Specimens were imaged on a Lieca DM750 compound microscope at 100-400x magnification.

Statistical analysis

Mechanical screening data detailing which corals were infested and how many flatworms were present on infested colonies was assessed for normality using Shapiro-Wilks test and QQnorm plots. Given the non-normal distribution of the data and the uneven sampling of *Acropora* species (unidentified species considered *Acropora* sp.), a Kruskal-Wallis test was used to assess the statistical significance ($P < 0.05$

significance threshold) of coral species and their respective harvest sources (inshore or midshelf reef) on whether sampled corals were infested with *P. acroporae*, and separately on the mean number of flatworms found on each coral fragment to assess infestation severity. A Dunn Test with Bonferroni adjustment; ($P > 0.008$) was performed as a post-hoc pairwise comparison.

Results

Prevalence and Distribution of *Prosthiostomum acroporae*

Mechanical screening of wild and captive *Acropora* coral species (n = 154; *A. millepora, A. spathulata, A. loripes, A. tenuis, Acropora* sp.) revealed 56 ± 4% (mean ± SE) had at least one *P. acroporae* individual (Table 4-1). Infested *Acropora* fragments had an average of 5.89 ± 0.51 flatworms (mean ± SE; n = 154), with the number of flatworms present ranging from 1 to 100 individuals. The harvest location of *Acropora* hosts (inshore or midshelf) had no significant effect on whether coral fragments were infested with *P. acroporae* or how many flatworms were found on each coral fragment (Kruskal-Wallis; $P > 0.05$). Coral species sampled had a significant influence on whether coral fragments were infested or not, and on the number of flatworms found on each infested coral fragment ($P < 0.05$). Post hoc pairwise comparison revealed significant differences between *A. loripes* and *Acropora* sp. (Dunn Test with Bonferroni adjustment; $P > 0.008$) with *A. loripes* more likely to be infested. Of the 23 *A. loripes* fragments screened, 82.4 ± 7.9 percent of screened colonies were found to be infested with an average of 5.5 ± 1.2 flatworms per fragment screened. No other significant differences were observed between other *Acropora* species.

Table 4-1: The contribution of wild *Acropora* sampling (n = 154) *P. acroporae*, with the mean ± SE percentage of corals found infested upon screening, the mean ± SE number of flatworms found per coral species.

	A. millepora	*A. spathulata*	*A. tenuis*	*A. loripes*	*Acropora* sp.
No. screened	82	9	9	23	31
Percent	49.9 ± 5.5 %	55.6 ± 16.6 %	40.0 ± 16.3 %	82.4 ± 7.9 %	30.3 ± 8.3 %
P. acroporae	3.99 ± 1.07	1.56 ± 0.72	0.5 ± 0.22	5.5 ± 1.20	0.64 ± 0.44

The present study found *P. acroporae* infesting *Acropora* spp. from Britomart Reef, Trunk Reef, Backnumbers Reef, the Palm Islands (Orpheus and Pelorus), Magnetic Island and the Mackay region. Collector input indicates that *P. acroporae* likely infest corals in the Cairns region, Bowen region, Swain Reefs, and the Keppel Islands. This combines for a known species distribution of *P. acroporae* that ranges from Lizard Island (14°41'913.04"S, 145°27'920.06"E) in the northern GBR (Rawlinson and Stella 2012), to over 1000 km south to the Keppel Islands (23°10'15.6"S 150°55'52.2"E in the southern GBR (Figure 4-1).
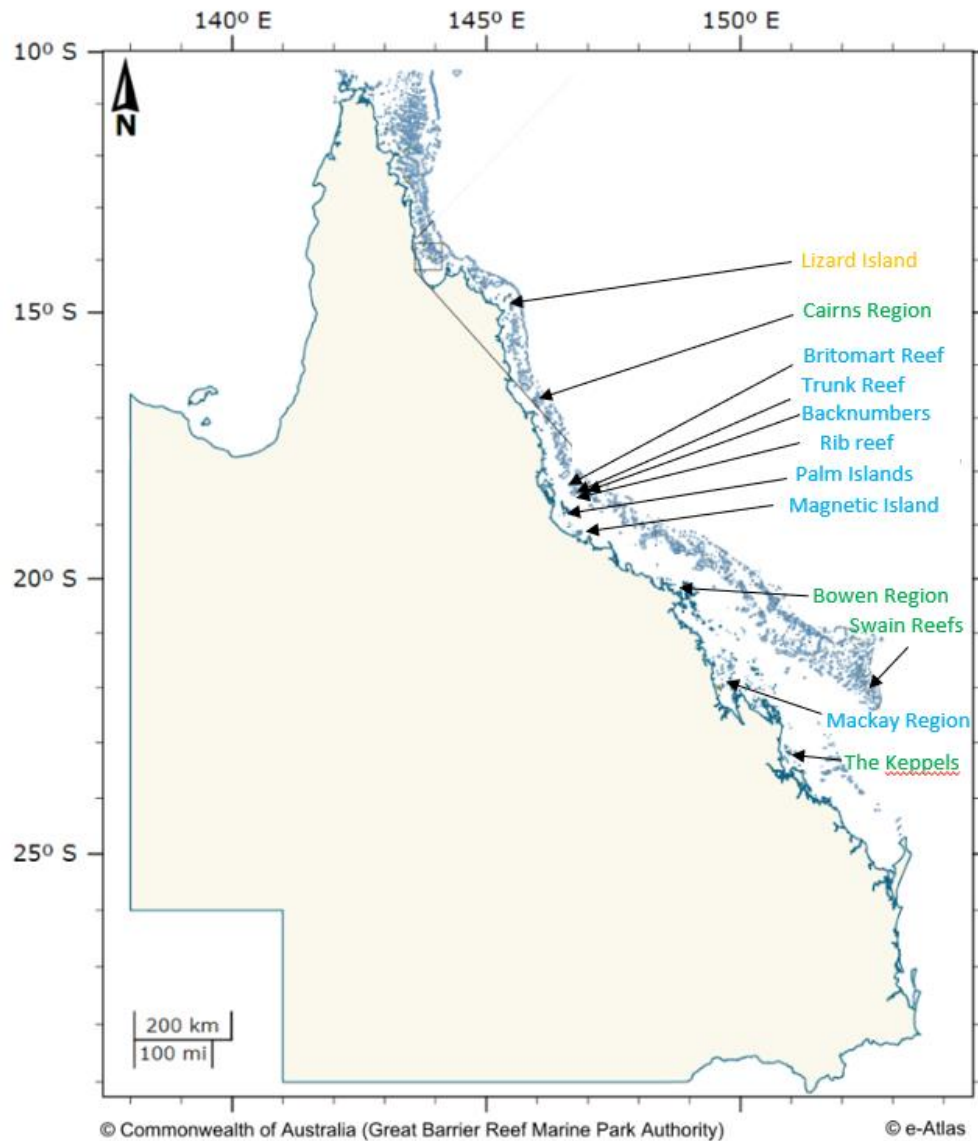
Figure 4-1: Map showing the distribution of *P. acroporae* on the Great Barrier Reef, color coded

corresponding to samples collected for this study (blue), areas known from collector input (green), and

from the first report of *P. acroporae* on the GBR (orange; Rawlinson and Stella 2012). Collector input

(green) highlight regions of reef where licensed coral collectors encounter *P. acroporae*.

Discovery and morphological analysis of *Montipora*-eating flatworms (MEFW) on captive *Montipora* hosts

Observations of captive *Montipora digitata* colonies showed signs of polyclad flatworm corallivory; 2-3 mm blotchy feeding scars (Figure 4-2A, B) and egg clusters deposited on exposed coral skeleton (Figure 4-2B). Screening revealed polyclads (Table A-1; *Prosthiostomum* sp. 1, 2, 3) resembling *Prosthiostomum montiporae* Poulter, 1975 (Figure 4-2C). Symbiodiniaceae were found in the gut contents of this polyclad (Figure 4-2D). Further specimens were collected from a commercial coral collector's facility in Mackay, Queensland, where *Montipora tuberculosa* (Lamarck, 1816) were found exhibiting similar blotchy feeding scars.
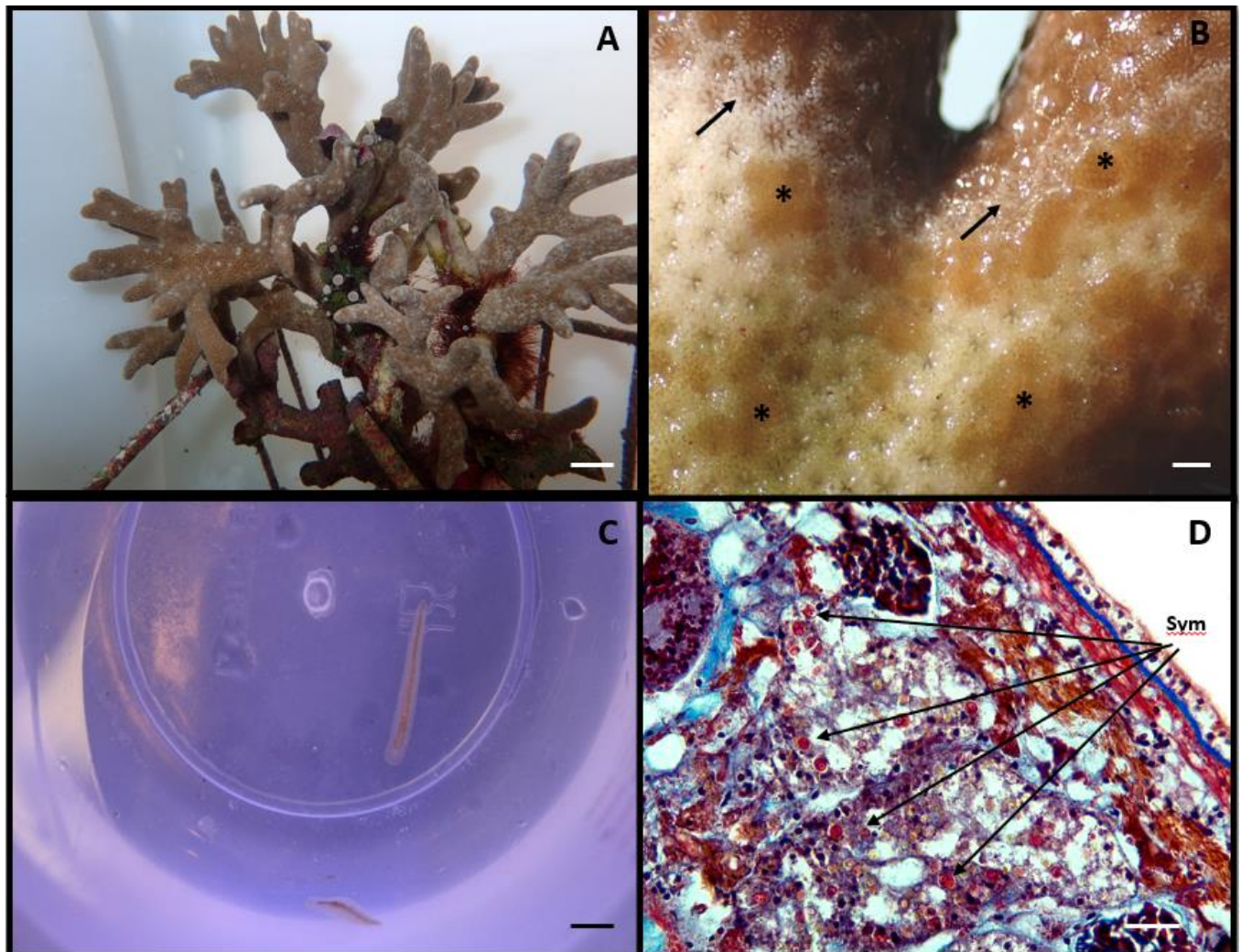
Figure 4-2: Evidence of *Montipora*-eating flatworms (MEFW) infestation of *Montipora digitata* from the Great Barrier Reef. **A.** Infested *M. digitata* colony covered in feeding scars (scale = 3 cm), **B.** *Indicates egg capsules laid on freshly exposed skeleton on the margin of tissue loss (arrows) on *M. digitata* (scale = 1 mm), **C.** Two MEFW specimens removed from *M. digitata* colony (scale = 2 mm), **D.** Cross section of MEFW with Symbiodiniaceae in branching gut (scale = 1 μm).

Morphological analysis of MEFW

Histological specimens of MEFW from captive *Montipora digitata* from the GBR (Figure 4-3) were collected and examined to determine whether these specimens are the same species as *Prosthiostomum montiporae* obtained from *M. capitata* hosts in Hawaii (Jokiel and Townsley 1974; Poulter 1975; Chapter 2). GBR specimens possess family and genus level characters which lead us to consider them *P. montiporae*. Morphological characters relevant to family level taxonomy of Prosthiostomidae are an ovoid to elongate body shape, the absence of marginal tentacles (Figure 4-3A), paired muscular prostatic vesicles (Figure 4-3E), and a cylindrical pharynx (Figure 4-3C). Genus level characters of *Prosthiostomum* include the possession of a ventral eye as part of each eye cluster, median intestinal branch (Figure 4-3C, D, E) which extends anteriorly, and a cylindrical cleft pharynx (Figure 4-3C, G) adapted for corallivory within the genus (particularly for *P. acroporae* and *P. montiporae;* Poulter, 1975; Prudhoe 1985; Rawlinson et al. 2011; Litvaitis et al. 2019). *P. montiporae* from the GBR exhibited paired clusters of cerebral eye spots which form coniform groups with 15-18 eye spots per cluster (Figure 4-3B), and marginal eyes forming a band proximal to the anterior margin that extends posteriorly until the cerebral eyes (Figure 4-3B). Posterior to the eyes is the ventral mouth and the coiled tubular pharynx with a longitudinal cleft (Figure 4-3C, G). Posterior to the pharynx lies the anterior portion of the male reproductive system.

Figure 4-3: The mmorphology of the MEFW. **A**. Ventral view of a live specimen showing the mouth (m), pharynx (p), female gonopore (fg),

cement glands (cg), sucker (s) uteri (u), and ovaries (o), Scale = 1 mm; **B.** Dorsal anterior view of wholemount showing coniform eye arrangement

of cerebral eyes (ce) and positioning of marginal eyes (me, Scale = 1 mm; **C**. Sagittal section showing anterior third of worm; mouth (m), pharynx

(p), brain (b), cerebral (ce), and the gut (g), Scale = 1 mm; **D**. Cross section showing the sucker (s), paired uteri (U), eggs (e), sperm (sp), cement

glands (cg) and main tract (mt) of branching gut ; **E**. Sagittal section showing the main tract of branching gut (bg), seminal vesicle (sv), prostatic vesicles (pv), also known as accessory vesicles), spermiducal vesicles (spv), ejaculatory duct (ed), male atrium (ma), penis sheath (ps) and male gonopore (mg); **F**. Cross section showing the gut (g), uterus (u), egg/oocyte (e), vagina (v), cement glands (cg), egg pouch (ep) and female atrium (FA), Scale = 0.2 mm; G. Cross section showing the retracted scroll-like cleft pharynx (p).

The male reproductive system consists of a seminal vesicle , two associated prostatic vesicles and

spermiducal vesicles filled with sperm (Figure 4-3D, E). The prostatic vesicles are adjacent to each other

but not bound together with the ejaculatory ducts, the only tissue separating them. Spermiducal

vesicles are ventral relative to seminal vesicles and are adjacent each side of the branching gut (Figure 4-

3D, E). The prostatic vesicles feed into the ejaculatory duct which originates from the seminal vesicle.

The ejaculatory duct narrows but our view of the penis papilla and actual penis were obstructed,

however the sectioned remnants of a penis sheath protrude into the male atrium that subsequently

narrows to the male gonopore (Figure 4-3E).

The female reproductive system of *Prosthiostomum* sp. (Figure 4-3A) shows the female copulatory

apparatus and abundant maturing ova between the musculature and the body wall. A pair of oviducts

posterior to the female copulatory apparatus feed developing ova into paired uteri medially relative to

the ventrally situated cement glands (Figure 4-3A, D, F). The egg pouch visible in Figure 3F is surrounded

by cement glands, which receive and align eggs for deposition, before entering the female atrium and

subsequently exiting the female gonopore (Figure 4-3A). The female gonopore is approximately

equidistant between the male gonopore (mg; Figure 4-3A, E) and the sucker (S; Figure 4-3A, D). Between

the male gonopore and the female gonopore, the sucker is an attachment organ with well-developed

and muscular tissue, in contrast to the adjacent tissue of the body wall (Figure 4-3D).

Molecular analysis

There were four aims of our molecular phylogeny; 1) to assess the placement of corallivorous

Prosthiostomidae and identify sister groups; 2) determine if MEFW from the GBR are closely related to

Prosthiostomum acroporae; 3) to assess if molecular evidence supports the morphological assessment

of GBR MEFW as *P. montiporae*, and ; 4) determine if the second polyclad (*Prosthiostomum s*p. Z) found

associated with *M. digitata* belongs in Prosthiostomidae. The maximum likelihood (ML) tree suggested

that all newly sequenced GBR specimens are grouped within Prosthiostomidae (Figure 4-4; Table 4-2).

The sequenced samples (Table A-1) and GenBank *Prosthiostomum* sequences (Table A-2) suggest a

monophyletic clade with three distinct subclades of *Prosthiostomum* strongly supported by BS > 95

(Figure 4-4). These subclades are: 1) *Prosthiostomum acroporae* sequences (new and Genbank) from

Acropora sp. hosts; 2) *Prosthiostomum* sp. #1 (MEFW), 4, and 5 collected from three host genera

(*Montipora, Acropora,* and *Euphyllia*, respectively) and; 3) *Prosthiostomum s*p. Z (Figure 5) and

Prosthiostomum torquatum grouped closely. Subclade one had the largest proportion of sampling, with

20 of the 30 28s rDNA isolate samples from *P. acroporae*. The sequence similarity among specimens in

subclade one is >99% to *P. acroporae* specimens (Blastn)*.* While the *Prosthiostomum* sp. specimens

grouped in subclade two demonstrated >97% similarity to *P. acroporae*, they are more closely related to

each other. Despite their presence on three host genera they form a corallivorous subclade distinct

from the *P. acroporae* in subclade one. Unfortunately, the 28s gene from *P. montiporae* specimens from

Hawaii could not be amplified, so we were unable to verify whether MEFW from the GBR is *P.*

montiporae through molecular analysis. While subclade three which contains *Prosthiostomum* sp. Z, still

showed 94% sequence similarity with *P. acroporae*, there was more sequence similarity between

Prosthiostomum sp. Z and free-living relatives *P. torquatum* from Japan and *P. trilineatum* from Guam

(96%).

Pericelis cata (EU679114)

Cestoplana rubrocincta (Phillip Island, Australia) (HQ659009)

Hoploplana californica (California, USA) (KC869850)

Imogine zebra (Woods Hole, Massachusetts, USA) (MH700313)

Notoplana atomata (New Hampshire, USA) (MH700330)

Prosthiostomum acroporae (New York, USA) (HQ659010)

Prosthiostomum acroporae #24 (Pioneer Bay, Oprheus Acropora sp.)

Prosthiostomum acroporae #1 (SeaSim A. millepora)

Prosthiostomum acroporae (Lizard Island, Australia) (JQ791553)

Prosthiostomum acroporae #17 (Magnetic Island A. tenuis)

Prosthiostomum acroporae #4 (SeaSim A. loripes)

Prosthiostomum acroporae #6 (SeaSim A. millepora)

Prosthiostomum acroporae #11 (SeaSim A. millepora)

Prosthiostomum acroporae #12 (SeaSim A. millepora)

Prosthiostomum acroporae #14 (Backnumber reef, A. millepora)

Prosthiostomum acroporae #15 (Magnetic Island A. tenuis)

Prosthiostomum acroporae #13 (SeaSim A. millepora)

Prosthiostomum acroporae #16 (Magnetic Island A. tenuis)

Prosthiostomum acroporae #23 (Little Pioneer Bay, Orpheus A. spathulata)

Prosthiostomum acroporae #18 (Trunk Reef A. millepora)

Prosthiostomum acroporae #2 (SeaSim A. millepora)

Prosthiostomum acroporae #7 (SeaSim A. millepora)

Prosthiostomum acroporae #3 (SeaSim A. millepora)

Prosthiostomum acroporae #5 (West Pelorus Acropora sp.)

Prosthiostomum acroporae #8 (SeaSim A. millepora)

Prosthiostomum acroporae #9 (SeaSim A. millepora)

Prosthiostomum acroporae #10 (SeaSim A. millepora)

Prosthiostomum sp.1 (SeaSim M. digitata; n=3)

Prosthiostomum sp. 4 (Pioneer Bay, Orpheus Island Acropora sp.)

Prosthiostomum sp. 5 (Mackay, Australia E. glabrescens)

Prosthiostomum vulgaris (Japan) (LC100091)

Prosthiostomum purum (Gulf of Aqaba, northern Red Sea) (MH700374)

Prosthiostomum torquatum (Tanabe Bay, Japan) (LC504234)

Prosthiostomid z (SeaSim M. digitata; n=2)

Prosthiostomum trilineatum (Ritidian Point, Guam) (MH700376)

Prosthiostomum cynarium (Reef Bay, St. John, US Virgin Islands) (MH700371)

Prosthiostomum utarum (Carmabi Beach, Piscadera Baai, Curacao) (MH700377)

Prosthiostomum milcum (Old Dan Bank, Long Key, Florida, USA) (MH700373)

Prosthiostomum siphunculus (Mataró, Spain) (HQ659012)

Prosthiostomum grande (Ebisu Island, Japan) (LC100090)

Prosthiostomum sp. (Eilat, Israel) (MH700375)

Prosthiostomum lobatum (Florida, USA) (MH700372)

Euprosthiostomum mortenseni (Parish, Jamaica) (MH700304)

Enchiridium evelinae (Brazil) (KY263683)

Enchiridium periommatum (Panama) (MH700300)

Enchiridium sp. (Heron Island, Great Barrier Reef, Australia) (MH700303)

Echiridium japonicum (Eilat, Israel) (MH700298)

Prosthecereaus crozieri (Long Key, Florida, USA) (HQ659013)

0.05

Figure 4-4: A maximum likelihood tree of Prosthiostomidae 28s rRNA sequences. Numbers next to nodes are SH-aLRT support (%) / ultrafast bootstrap support (%). Highlighted specimens in red and blue indicate two inferred sub-clade within Prosthiostomidae.

Figure 4-5: Dorsal photograph of Prosthiostomum sp. Z specimen; scale 2 mm.

Table 4-2: Summary table of specimens provided in this study including their coral host, form, location, and subclade.

Specimen	Coral host	Form	Location	Subclade
Prosthiostomum acroporae	*Acropora* spp.	Parasitic	GBR and captivity	1
Prosthiostomum sp. #1 (*Prosthiostomum montiporae*)	*Montipora* spp.	Parasitic	GBR, Hawaii, and captivity	2
Prosthiostomum sp. #4	*Acropora* sp.	Parasitic	GBR and captivity	2
Prosthiostomum sp. #5	*Euphyllia glabrescens*	Undetermined	GBR and captivity	2
Prosthiostomum sp. z	*Montipora digitata*	Free-living	GBR and captivity	3

Discussion

Distribution and prevalence of *P. acroporae*

By combining collector input with samples from the present study and the previous work of Rawlinson and Stella (2012), we are able to demonstrate the distribution of *P. acroporae* across at least 1,000 km of the Great Barrier Reef. This demonstrates a broad distribution of *P. acroporae* and suggests that their presence is likely on coral reefs where *Acropora* are prevalent in general. While we found *P. acroporae* infesting a number of *Acropora* coral species, *A. loripes* had a particularly high percentage of fragments infested with *P. acroporae* (82.4 ± 7.9 % of colonies infested) relative to other species screened. It is difficult to discern which species other than *A. loripes* are susceptible to infestation or preferred by *P.*

acroporae; or alternatively, which particular species may be resistant to *P. acroporae* settlement*.*

Infestations observed in *A. loripes* from the present study could have been influenced by seasonal

factors (temperature) and/or the uneven sampling of coral species. More data is required for more

species of *Acropora* to discern if *P. acroporae* have particular host preferences, and if those preferences

are reflected by the prevalence of infestation in potentially favored host corals.

Despite finding over 56% of screened *Acropora* fragments to be infested with ~5.8 *P. acroporae* per

coral fragment, screened corals appeared otherwise healthy. *Acropora* appear able to tolerate

infestations of *P. acroporae* in healthy coral reef environments where an unknown compliment of

trophic interactions (e.g. natural predators; see Gochfield and Aeby 1997; Stella et al. 2010; Chapter 5)

and robust coral health maintain a balance, allowing corals to remain healthy. However, when corals are

brought into captivity the difficulty of maintaining this balance is evident by the occurrence high

flatworm densities and subsequent mortality of captive *Acropora* (Nosratpour 2008; Chapter 2, 3).

Understanding these trophic relationships and how they are mediated in the context of captive coral

systems could benefit *P. acroporae* management to support *Acropora* coral health in captivity.

Prosthiostomum montiporae of the GBR

Morphological analysis of MEFW infesting *Montipora digitata* demonstrated morphological similarity to

Hawaiian *Prosthiostomum montiporae* as evident by the female reproductive system (Figure 4-3D, F),

male reproductive system (Figure 4-3D, E), eye arrangement (possession of a ventral eye as part of each

eye cluster) (Figure 4-3B), presence of a sucker (Figure 4-3D,) median intestinal branch which extends

anteriorly, and the cylindrical cleft pharynx adapted for corallivory within the genus (particularly for *P.*

acroporae and *P. montiporae;* Poulter, 1975; Prudhoe 1985; Rawlinson et al. 2011; Litvaitis et al. 2019;

Table 4-2). The sclerotized penis stylet typically associated with the male copulatory apparatus (Poulter

195; Litvaitis 2019) is the only feature not able to be verified because of histological artifacts. Poulter

(1975) outlined that *P. montiporae* can be distinguished from *P.* (*Lurymare*)*, P. purum* and other

members of *Prosthiostomum* by a combination of eye arrangement, extremely short pharynx length,

distinctive cleft-like morphology of the pharynx, and some details of the copulatory apparatus. *P.*

montiporae can be distinguished from *Amakusaplana ohshimai* by the morphology of the pharynx,

differing eye arrangement, general shape, and the absence of a sucker in *A. ohshimai*. Despite *P.*

acroporae having similar pharynx morphology to *P. montiporae*, *P. montiporae* are more than double

the size of *P. acroporae* which only reach approximately 7 mm in length compared to the ~18 mm

reached by *P. montiporae* (Jokiel and Townsley 1974; Poulter 1975)*.* While the sucker is retained in GBR

P. montiporae specimens (Figure 4-3A, D), this attachment organ is distinctly absent in *P. acroporae*

(Rawlinson et al. 2011). While both *P. montiporae* and *P. acroporae* have multiple developing embryos

per egg capsule (Jokiel and Townsley 1974; Rawlinson et al. 2011), *P. montiporae* are suggested to

hatch as Muller's larvae (typically dispersive), while *P. acroporae* hatchlings emerge with a gradient of

development between Muller's larvae and fully metamorphosed individuals. It remains unknown if this

difference is functional (e.g. dispersal or fecundity), or merely a divergent character from a shared

common ancestor.

Monophyletic *Prosthiostomum* composed of three subclades

The comparison of 28s rDNA from polyclads collected from captive and wild acroporid corals from the

GBR and 25 GenBank sequences demonstrated strong support (BS > 95) for all specimens sequenced for

this study belonging to the family Prosthiostomidae and genus *Prosthiostomum*. The first and largest

(sample representation) of the three distinct subclades (BS > 95) discussed here include the 22 *P.*

acroporae sampled in this study and previous 28s rDNA from a captive specimen collected from Atlantis

Marine World in New York and from the Lizard Island specimen from Rawlinson and Stella (2012). This

remarkable conservation in the 28s rDNA is reflected by the >99% sequence similarity among these

specimens. The origin of *P. acroporae* infested *Acropora* brought into Atlantis Marine World is unknown,

and it is plausible that the specimen came from the ornamental trade (e.g. Indonesia, Australia, or another exporter).

The second well supported subclade (BS > 95) interestingly included *Prosthiostomum* sp. specimens #1 from *Montipora '*MEFW ' (n=3), #4 from *Acropora* sp., and #5 from *Euphyllia glabrescens*. We demonstrate morphologically by the structure of the pharynx and presence of the sucker, which is absent from *P. acroporae,* that *Prosthiostomum* sp. #1 individuals are distinct from *P. acroporae*. The presence of another corallivorous *Prosthiostomum* species distinct from *P. acroporae* suggests that these *Acropora*-eating flatworms (AEFW) include more than a single species. While it is possible that *Prosthiostomum* sp. #1, #4 and #5 are generalists of the same species, the apparent absence of feeding scars or egg clusters on *E. glabrescens* from *Prosthiostomum* sp. #5 is in contrast to obvious presence on all *Montipora* infested with MEFW (including Mackay specimens) and *Acropora* sp. infested by *Prosthiostomum* sp. #4. The absence of feeding scars or eggs could be associated with the timing or intensity of infestation, or the manner in which *E. glabrescens* heals. We speculate that the Hawaiian *P. montiporae* specimens would be grouped within this corallivorous subclade. Furthermore, it is possible that the *P. acroporae* subclade (first) and *Prosthiostomum* sp. #1, #4 and #5 (second) subclade may share a common corallivorous ancestor.

The third well supported clade contained *Prosthiostomum s*p. Z (Figure 4-5, *Prosthiostomum torquatum*, and *Prosthiostomum trilineatum* Yeri & Kaburaki, 1920. While *P. torquatum* is considered a free-living prosthiostomid collected from intertidal waters of Japan, *Prosthiostomum trilineatum* was originally described from a *Porites* host in Mexico. We suspect that *Prosthiostomum* sp. Z is not a corallivore but rather associated with the biofouling community. Similar to *P. torquatum, Prosthiostomum* sp. Z has a pigmented dorsal epidermis (Figure 4-5; Tsuyuki et al. 2019) for apparent camouflage, in contrast to *P. montipora and P. acroporae* which are pigmented from the coral tissue and Symbiodiniaceae they

ingest. Future histological sampling to verify the absence of Symbiodiniaceae in the gut could assist in the description of *Prosthiostomum* sp. Z.

Conclusions

The present study identified polyclads associated with captive and wild acroporid corals of the GBR, including the first morphological and molecular assessment of MEFW (*P. montiporae*). We provide the most comprehensive understanding of *P. acroporae* distribution infesting *Acropora* of the GBR, and presented a molecular phylogeny of the family Prosthiostomidae using 28s rDNA sequences of *Prosthiostomum* spp. specimens (n = 30) collected from the GBR and from GenBank to provide resolution among corallivorous Prosthiostomids. Morphological evidence suggests consistent morphology of these specimens to *P. montiporae* of Kaneohe Bay, Hawaii. These MEFW are grouped within a new subclade with high sequence similarity to two distinct specimens from *Acropora* sp. and *Euphyllia glabrescens* (also this study). Without direct evidence of corallivory by the *Prosthiostomum* sp. on *E. glabrescens,* we cannot draw conclusions about any threat they pose to captive *Euphyllia* spp. However, the collection of wild or captive *Acropora* and *Montipora* corals for any purpose should consider biosecurity and the threat of introduction into captive systems if populations are not managed. While the cryptic nature of corallivorous *Prosthiostomum* spp. is likely to have hindered our understanding of the diversity of these camouflaged corallivores and that of their associated coral hosts in the past, the implementation of screening protocols provides a way to prevent introduction of potential pests into captive aquaria and explore flatworm diversity further in the future.

Chapter 5: Biological controls to manage Acropora-eating flatworms in coral aquaculture

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Abstract

Pest management in aquaculture has historically used of a variety of methods to both prevent and treat parasite outbreaks. One non-chemical method of intervention is the use of biological controls, in which natural predators or pathogens are intentionally introduced to suppress or remove the unwanted pest organism. Given the importance of *Acropora* to coral aquaculture efforts, and the destructive nature of the *Acropora*-eating flatworm, *Prosthiostomum acroporae*, we investigated the potential of 2 biological control organisms in marine aquaria for the control of *P. acroporae* infestations. *Acropora millepora* fragments infested with adult polyclad flatworms (5 flatworms fragment^{-1}) or single egg clusters laid on *Acropora* skeleton were cohabited with either sixline wrasse *Pseudocheilinus hexataenia* or the peppermint shrimp *Lysmata vittata* and compared to a control (i.e. no predator) to assess their ability to consume *P. acroporae* at different life stages over 24 h. *P. hexataenia* consumed 100% of adult flatworms from A. *millepora* fragments (n = 9; 5 flatworms fragment−1), while *L. vittata* consumed 82.0 ± 26.76% of adult flatworms (mean ± SD; n = 20). *Pseudocheilinus hexataenia* did not consume any *Prosthiostomum acroporae* egg capsules, while *L. vittata* consumed 63.67 ± 43.48% (mean ± SD; n = 20) of egg capsules on the *Acropora* skeletons. Mean handling losses in controls were 5.83 ± 10.77% (mean ± SD; shrimp system) and 7.5 ± 13.92% (fish system) of flatworms, and 1.0 ± 2.99% (shrimp system) and 2.39 ± 3.84% (fish system) of egg capsules. Encounters between *L. vittata* and *P. hexataenia* result in predation of *P. acroporae* on an *Acropora* coral host and represent viable biological controls for reducing infestations of *P. acroporae* in aquaculture systems.

Introduction

Biological control utilizes living organisms (control agents) to suppress the population density and subsequent impact of a specific pest organism by leveraging ecological interactions through predation, parasitism, herbivory, or other natural mechanisms (Eilenberg et al. 2001). Biological controls are used extensively in agriculture, where the tactical release of parasites or predators is used to reduce insect pest species of economic importance (Smith and Basinger 1947; Simmonds et al. 1976; Greathead 1994; Eilenberg et al. 2001). In aquaculture, high stocking densities of cultured organisms can facilitate transmission of pathogens and parasites, requiring analogous approaches for disease management (Deady et al. 1995; Tully et al. 1996; Maeda et al. 1997; Powell et al. 2018). In the northern hemisphere, cleaner fishes (e.g. ballan wrasse *Labrus bergylta* Ascanius, 1767 and, more recently, lumpfish *Cyclopterus lumpus* Linnaeus, 1758) are bred in captivity and subsequently cohabited with farmed salmon (primarily *Salmo salar* Linnaeus, 1758) to remove ectoparasitic copepods such as salmon louse (e.g. *Lepeophtheirus salmonis* [Krøyer, 1837]; Tully et al. 1996). This nonchemical approach to pest management is preferable to costly treatments, which stress cultured fish and reduce appetite (Skiftesvik et al. 2013; Powell et al. 2018). Within coral aquaculture and the marine ornamental trade, the peppermint shrimps *Lysmata wurdemanni* (Gibbes, 1850), *L. seticaudata* (Risso, 1816), *L. boggessi*, and *L. ankeri* Rhyne and Lin, 2005, as well as the nudibranch *Berghia sp.* are used for biological control of anemones *Aiptasia* sp. (Rhyne et al. 2004; Calado et al. 2005; Rhyne and Lin 2006). The reef fishes saddle wrasse *Thalassoma duperrey* (Quoy and Gaimard, 1824) and threadfin butterflyfish *Chaetodon auriga* Forsskål, 1775 are also potential candidates to mitigate infestations of the corallivorous nudibranch *Phestilla sibogae* Begh, 1905 in captivity (Gochfeld and Aeby 1997).

Control of *Acropora* coral pests is highly desired, given that it is the most represented genus imported into many countries globally (Rhyne et al. 2014), and that *Acropora* are commonly used in coral aquaculture to support reef restoration efforts (Barton et al. 2017). A problematic coral pest,

Prosthiostomum acroporae (Rawlinson, Gillis, Billings, and Borneman, 2011), commonly known as the

Acropora-eating flatworm, has plagued hobbyist aquaria for many years (Delbeek and Sprung 2005). *P.*

acroporae is an obligate associate of *Acropora* sp*.* and actively consumes coral tissue, which results in

characteristic ~1 mm circular pale feeding scars, often resulting in coral tissue necrosis. Infestations are

associated with colonial mortality at high densities in captivity (Nosratpour 2008). *P. acroporae*

infestations are challenging to detect because of their highly cryptic nature, which facilitates their

spread into new systems undetected. Infestations impact coral health through reduction of host coral

fluorescence over time and hinder the coral's ability to photoacclimate to changes in lighting conditions

(Hume et al. 2014). Infestations are often not detected until compromised host health is observed

through visual signs, at which point flatworm population density is high and colonial mortality of the

coral may occur. There is no current empirical evidence to support effective treatment or prevention

measures for *P. acroporae* infestations, although Barton et al. (2019) examined the life cycle under a

range of temperature conditions and suggested timed intervention to disrupt the life cycle.

The aim of the present study was to evaluate the potential of two biological controls to reduce

infestation by the *Acropora*-eating flatworm *P. acroporae* on *Acropora* coral. Biocontrol candidates

included the peppermint shrimp *L. vittata* (Stimpson, 1860), which has been previously reported to

remove parasites on fish and in the environment (Vaughan et al. 2017, 2018a,b), and the wrasse

Pseudocheilinus hexataenia (Bleeker, 1857), based on anecdotal evidence from the hobbyist community

that it may reduce *P. acroporae* populations in aquaria through active foraging (Delbeek and Sprung

2005). This study examined the efficacy of potential biocontrols on adults and eggs of *P. acroporae* in

captive systems over a 24 h period in vivo.

Materials and methods

Species selection, husbandry, and culture

Twenty *Lysmata vittata* and 10 *Pseudocheilinus hexataenia* were purchased from Cairns Marine, Cairns, Australia, and maintained for 1 mo before any experimentation. Because of space limitations, shrimps were housed together in one 50 l flow-through aquarium system (10 turnovers d^{-1}) with approximately 5 kg of 'live' rock for hiding and protection between molts. *P. hexataenia* were housed individually in 50 l flow-through aquarium systems (10 turnovers d^{-1}) with a 60 mm PVC tee (3-way junction) each for shelter. Filtered seawater (0.04 μm nominal pore size) at 27°C was used to supply the system. Shrimps and fish were fed twice daily to satiation with a mixture of thawed Tasmanian mysid shrimp, Ocean Nutrition® Marine Fish Eggs, Ocean Nutrition® Cyclopods, and Vitalis® Platinum formulated feed. Animals were fed the morning prior to the commencement of each experimental trial but not during their trial period. Adult *Prosthiostomum acroporae* were collected from a culture of infested captive *Acropora* sp. colonies. Flatworms were maintained in culture using established methods (see Barton et al. 2019).

Coral fragment preparation, infestation, and egg collection

To provide *A. millepora* for biological control trials, 96 *A. millepora* fragments (approximately 50 mm height; 30 mm width) were generated from donor colonies harvested from 2 colonies sourced from Davies Reef, Australia (harvested September 2017; GBRMPA Permit: G12/35236.1), and 5 captive colonies originating from Orpheus Island, Australia (harvested May 2016; G14/36802.1). A combination of bone cutters and a band saw (Gyrphon® Aquasaw XL) was used to prune *A. millepora* fragments, which were then fixed onto aragonite coral plugs (32 mm diameter) with cyanoacrylate glue.

To infest *A. millepora* fragments with *P. acroporae*, fragments were housed temporarily in individual 5 l containers. Before the start of each experimental trial, 5 *P. acroporae* individuals, approximately 3 mm in size, were directly pipetted onto each *A. millepora* fragment. After 60 s, each fragment was gently shaken to ensure *P. acroporae* had laterally appressed themselves to the host coral's tissue and were not stuck in the coral mucus (flatworms can dislodge if stuck in mucus). Any worms that detached were attempted to be reattached once and then discarded for another specimen if unsuccessful.

Egg capsules were naturally laid on *Acropora* skeleton in the *P. acroporae* culture and then harvested using bone cutters to remove the section of skeleton with these eggs. The underside of each subsequent skeletal fragment was glued onto clean aragonite disks or 'frag plugs' with cyanoacrylate glue. The number of eggs per cluster was determined by counting them under a dissecting microscope (Leica EZ4, 10–40x magnification) while immersed in seawater to prevent desiccation. Only fragments of coral skeleton bearing unhatched and undamaged egg capsules were selected for experimentation.

Lysmata vittata experiments

Experiments with *L. vittata* were conducted on 4 separate trial days (i.e. 6 control and 6 treatment replicates per trial; n = 24 control; 24 treatment). On the day before each *L. vittata* trial, a random number generator was used to designate treatments and controls to aquaria. PVC blocks ($80 \times 80 \times 25$ mm; 32 mm diameter depression with central 10×15 mm hole to hold 32 mm diameter aragonite plugs in all replicates) were placed in each aquarium (3.5 l) before each trial. After their morning feeding, 6 *L. vittata* were haphazardly caught from their holding system using a 500 ml wide−mouth container and placed into their respective experimental tanks. *L. vittata* were given a minimum of 2 h to acclimate to their surroundings in the replicate experimental flow-through aquaria (5 l h^{-1}) maintained at 27 ± 0.1°C. *L. vittata* were considered acclimated once they settled on the bottom of each aquarium.

A. millepora fragments (1 per aquarium) infested with 5 *P. acroporae* each were introduced to each of the 3.5 l aquaria (treatment and control) for 24 h to determine if the presence of *L. vittata* (treatment) influenced the number of remaining flatworms on each coral fragment. The number of flatworms remaining was determined using a seawater screening method (Barton et al. 2019). In addition, the PVC blocks and clear tanks were inspected for flatworms with the naked eye after each trial, with any flatworms found added to the remaining total of flatworms. Experiments examining the influence of *L. vittata* on *P. acroporae* egg capsules were conducted using the same approach, with the exception of egg capsules being counted before and after the trial under a stereo microscope (Leica EZ4, 10–40× magnification). Skeletal fragments (n = 48) were divided equally across treatments and controls (i.e. n = 24 control, 24 treatment) in *L. vittata* trials with 47.27 ± 19.09 (mean ± SD) egg capsules per fragment. *L. vittata* do not forage immediately before or after molting (D. Vaughan pers. comm.), therefore any shrimps that molted during the 24 h trial was excluded (i.e. 4 replicates were removed due to molting; n = 20).

Pseudocheilinus hexataenia experiments

Pseudocheilinus hexataenia (n = 9) were acclimated for approximately 2 wk to their randomly allocated flow-through aquaria at 27 ± 0.1°C with PVC blocks in place. The 50 l aquaria (n = 9 with wrasse, 9 without) were separated by black plastic because of the acute eyesight and territorial behavior of *P. hexataenia*. After acclimation, each fish regularly accepted food and did not exhibit signs of physical or behavioral stress. Following morning feeding of *P. hexataenia*, infested *A. millepora* fragments (5 flatworms each) were introduced to each 50 l aquarium and left for a duration of 24 h to assess if the presence of the wrasse influenced the number of flatworms remaining on each coral fragment. Flatworms were recovered using an established screening method (Barton et al. 2019). The surfaces of the aquaria and the PVC blocks holding the fragment plugs were inspected visually for any remaining worms, which were added to the total remaining flatworms if present. Experiments examining the

influence of *P. hexataenia* on *P. acroporae* egg capsules were conducted similarly, but egg capsules were counted before and after inspection with a stereo microscope (Leica EZ4, 10–40× magnification). The 18 skeletal fragments used in *P.* hexataenia trials (n = 9 treatment, 9 controls) had 42.33 ± 16.95 (mean ± SD) egg capsules per skeletal fragment.

Statistical analysis

Binomial generalized linear mixed models (GLMMs) and generalized linear models (GLMs) were generated in RStudio (Version 1.0.143; R packages 'car,' Fox and Weisberg 2019, and 'lme4,' Bates et al. 2015) to assess the effect of *L. vittata* treatments on *P. acroporae* egg capsules and individual flatworms. Treatment was considered a random effect and trial identity a fixed effect in the model to ensure that there were no effects that changed the results significantly (p < 0.05) between *L. vittata* trials. Lacking any significant effects from trial identity in both experiments testing *L. vittata* egg and individual consumption, the GLM with pooled data denoted any significant effects (p < 0.05) of treatment on consumption for each experiment. Four replicates were removed from statistical analysis of the *L. vittata* vs. egg capsule experiment because these replicates molted during the experimental trial. Kruskal-Wallis tests were used to assess the results of *P. hexataenia* experiments with a significance threshold of α = 0.05.

Results and discussion

The peppermint shrimp *Lysmata vittata* consumed both settled flatworm individuals and egg capsules laid on coral skeleton. The presence of *L. vittata* significantly reduced (GLM; p < 0.001) *Prosthiostomum acroporae* infestations over 24 h, with 82.0 ± 26.76% of the flatworms consumed (mean ± SD; n = 20; Fig. 1). Control tanks (n = 24) showed a loss of 5.83 ± 10.77% (n = 24; Figure 5-1). This indicates that

94

approximately 94% of flatworms were recovered using the screening method, which is consistent with

previous use (Barton et al. 2019). *L. vittata* also significantly reduced *P. acroporae* egg capsules (GLM; p

< 0.05), with 63.7 ± 43.48% (n = 20) of the egg capsules removed compared to only 1.0 ± 2.99% (n = 24)

in the control (Figure 5-1).
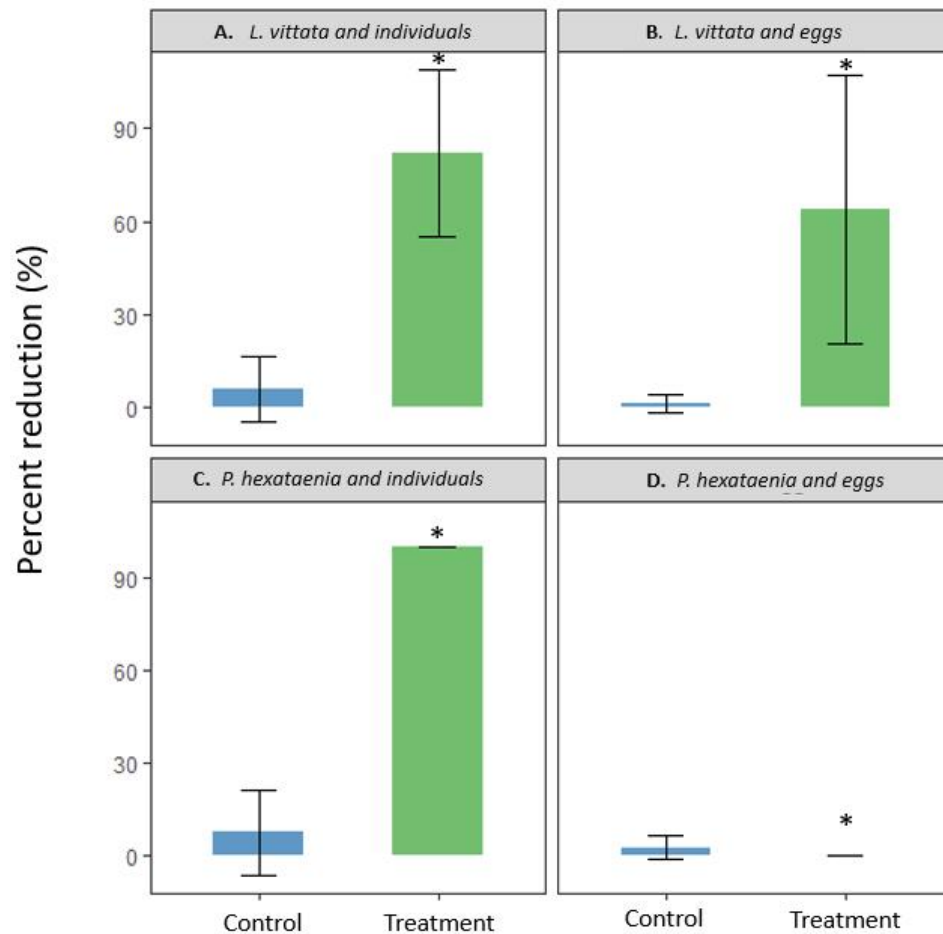
Figure 5-1: Mean proportion of *Acropora*-eating flatworm individuals and egg capsules removed (error bars: ± SD) in the presence and absence of biocontrols.

(A) *Lysmata vittata* and flatworm individuals (n = 24), (B) *L. vittata* and flatworm eggs (n = 20 egg clusters), (C) *Pseudocheilinus hexataenia* and flatworms (n = 9), and (D) *P. hexataenia* and flatworm eggs (n = 9 egg clusters). *: statistical significance between treatments and controls. Photos: = *L. vittata* and *P. hexataenia*. (*P. hexataenia* photo credit: creative commons license istockphoto.com user: marrio31 id#471448553)

Lysmata shrimps use their setae-covered antennules to detect chemical cues (via cuticular sensilla) from their environment and locate suitable prey items (Zhu et al. 2011; Caves et al. 2016). Because they do not use visual mechanisms to locate and capture prey, *L. vittata* predation on *P. acroporae* is not hindered by the camouflage of these flatworms. However, *L. vittata* must physically encounter *P. acroporae* eggs or individuals while foraging to consume them, thus potentially limiting their ability to control *P. acroporae* populations in larger aquaria (aquaria >3.5 L were not tested in this study), where the probability of a direct encounter would be limited by proximity and the availability of alternate food sources (*L. vittata* were not fed during the trials). Despite this possible limitation, *L. vittata* remain useful as a potential treatment of *P. acroporae* infestations because intimate co-habitation with *Acropora* enables shrimp to scavenge among coral branches and consume *P. acroporae* individuals and egg capsules. *L. vittata* are also an aggregating species and can be kept in high numbers when provided with sufficient food and shelter (Vaughan et al. 2018b), rendering them potentially useful to clean heavily infested corals introduced to a high density of shrimp as a sort of coral cleaning station. Future research could examine diet preferences of *L. vittata* and their performance at different densities (e.g. in larger aquaria), which may alter their efficacy in removing flatworms from *Acropora* colonies (e.g. Grutter and Bshary 2004).

Experimental trials with *Pseudocheilinus hexataenia* demonstrated that these fish are effective at reducing the *P. acroporae* population, with their presence having a significant effect on flatworm abundance remaining on *A. millepora* fragments (Kruskal-Wallis; $p < 0.001$). All *P. acroporae* exposed to *P. hexataenia* were removed over 24 h (100%; n = 9), compared to a loss of 7.5 ± 13.92% of flatworms (mean ± SD; n = 9) in controls. In contrast, all egg capsules were recovered intact in the experimental treatments (100%; n = 9) when cohabited with *P. hexataenia*. In the control, 2.39 ± 3.84% egg capsules (mean ± SD; n = 9) were not recovered, resulting in significant differences between treatment and

control (Kruskal-Wallis; p < 0.05), likely from incidental mechanical damage to egg capsules through handling.

These results indicate that *P. hexataenia* is highly efficient at eating flatworms using well-developed eyesight (Gerlach et al. 2016) but does not interact with the hard exterior of flatworm egg capsules. The implementation of *P. hexataenia* as biological controls must consider their ecology and husbandry requirements. In the wild, these fish actively forage in their established territory (Geange and Stier 2009; Geange 2010), generally only coming together for mating purposes (Kuwamura 1981). While their foraging behavior appears similar in captivity, the solitary and territorial nature of *P. hexataenia* renders keeping more than 1 individual in smaller aquaria (e.g. <1000 l) problematic. More than 1 individual could be kept in aquaculture systems large enough to avoid territorial confrontation, but the 'patrol' range of this territory may remain relatively constant. It is for this reason, combined with the fact that this fish does not interact with flatworm egg capsules, that they may not be as suitable for treating acute infestations of *P. acroporae* compared to *L. vittata*. However, their performance in our trials suggests that this colorful labrid is a useful tool for consuming adult flatworms, thus mitigating the chronic impacts of a given *P. acroporae* infestation by removing or reducing the *P. acroporae* density to non-lethal levels for the *Acropora* host. Future research which examines efficacy of *P. hexataenia* as a biological control in larger systems (>400 L) could help us understand their true potential at reducing *P. acroporae* abundance in large aquaculture systems with more space and alternative food sources.

P. hexataenia and *L. vittata* identify prey items in different ways while foraging, which has implications for how they are used in the captive environment and their ecological roles in native ecosystems. Little is understood about the dynamics of wild *P. acroporae* populations, although our results may provide further understanding of the trophic relationships between *P. acroporae* and natural predators in reef ecosystems. *P. acroporae* are cryptic and there are no documented infestations causing colonial mortality of *Acropora* colonies in the wild. It does remain likely that some proportion of wild mortality of

Acropora colonies attributed to other causes (e.g. sedimentation and algal competition) is instead experiencing negative secondary effects on coral health from *P. acroporae* infestation. However, the presence of natural predators of *P. acroporae* (e.g. *P. hexataenia* and *L. vittata*) may reduce incidences of mortality in wild *Acropora* colonies.

In captive systems, pairing both of these biological control organisms with the manual removal *of P. acroporae* egg clusters is likely to be highly effective in reducing the overall infestation within a given aquarium system. Any reduction in *P. acroporae* abundance would theoretically prevent energy loss by the *Acropora* host to repair tissue damage from *P. acroporae* feeding, leading to healthier *Acropora* corals utilizing *P. hexataenia* and *L. vittata* in the long term. However, consideration must be given to the sustainable supply of the organisms if used as biological controls. *L. vittata* are available through the ornamental trade and can be bred in captivity. Although peppermint shrimp species from other regions (e.g. *L. wurdmenii, L. boggessi*, Rhyne and Lin 2006) were not investigated in the present study, they could also be examined for their ability to interact analogously with *P. acroporae* and could be supplied sustainably for biocontrol of flatworm infestations. Although *P. hexataenia* is categorized as Least Concern (Bertoncini 2010; IUCN Red List 2010), overharvesting for use as biological controls in the ornamental trade could impact local populations. Lessons should be taken from the Scandinavian salmonid industry, where harvesting of wrasse broodstock used for biological control of sea lice parasites has exerted considerable pressures upon wild populations (Brooker et al. 2018; Powell et al. 2018).

In summary, this study provides the first empirical evidence of potential biological control organisms for *P. acroporae* in captivity. The ability of both *L. vittata* and *P. hexataenia* to consume *P. acroporae* renders them useful preventative measures of infestation in addition to potentially being used to treat colonies infested with adult flatworms and thereby drastically reducing the impact of this pest on captive colonies. While *P. hexataenia* had no apparent interest in *P. acroporae* egg capsules, *L. vittata*

displayed the added benefit of consuming egg capsules through their foraging activities, with

encounters with the egg clusters likely to further control the flatworm populations in captive systems.

The addition of sustainable biological control organisms adds a valuable tool for flatworm control, which

is suitable for both aquarium hobbyists and large-scale coral aquaculture facilities.

Chapter 6: Efficacy of chemical treatments for *Acropora*-eating flatworm infestations

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Abstract

Outbreaks of pests and pathogens can be financially devastating to aquaculture operations, where high stocking densities can facilitate rapid spread and high stock losses. Chemical treatments are widely used as a management tool in aquaculture systems both prophylactically and in response to acute outbreaks of parasites and disease. While prophylactic chemical treatments are used to some degree in the prevention of coral pests by coral collectors and in coral aquaculture, no evaluation of the efficacy of these dips have been conducted on one of the most impactful coral pests, the *Acropora* eating-flatworm, *Prosthiostomum acroporae.* Furthermore, no study has investigated the consequences these treatments may have on coral host growth and survivorship. We investigated the efficacy of anthelmintics levamisole and praziquantel for the removal of *Acropora*-eating flatworms from *A. millepora* using one-hour chemical immersions and assessed if these treatments negatively impacted coral growth and/or caused bleaching. Coral fragments (194 total) were spread across eight treatments; levamisole infested (LI), levamisole uninfested (LU), praziquantel (in EtOH) infested (PI), praziquantel (in EtOH) uninfested (PU), handling control infested (HCI), handling control uninfested (HCU), EtOH control (EC), control with no handling (NHC). To test the efficacy of flatworm removal by short, one-hour chemical immersions, *A. millepora* fragments were manually infested (three *P. acroporae* per fragment) and immersed separately to uninfested *A. millepora* fragments (60 total). All fragments were shaken in in a bath of seawater following immersion, then mechanically screened to recover any flatworms not removed from either immersion or shaking to determine the removal efficacy of the treatments. Furthermore, coral fragments (194 total) were photographed before treatment and four weeks following treatments to compare coral basal growth and visual signs of bleaching between infested and uninfested fragments. Levamisole and praziquantel immersions removed significantly more flatworms from *A. millepora* fragments (93% ± 3.8 and 95.0% ± 2.6 respectively; mean ± SE; $p < 0.05$) compared to the handling control (26% ± 7.5%). Chemical treatments had no significant effect on basal growth, with

fragments across all treatments (including controls) increasing basal area by 73.31 ± 3.82% (mean ± SE).

Furthermore, bleaching was not observed for any *A. millepora* fragments across the treatments and

controls. Results from this study demonstrate that levamisole and praziquantel used in conjunction with

water movement were effective at removing >90% of *Acropora* eating-flatworms with no observable

negative impacts on coral health on treated coral fragments relative to controls.

Introduction

Pest management is critical for aquaculture operations, as high stocking density and stress can facilitate

the rapid spread of parasites and pathogens (Shinn et al., 2015). For example, platyhelminth (flatworms)

parasites in marine environments (e.g. monogeneans infecting fishes) warrant prophylactic chemical

treatment of animals entering aquarium or aquaculture facilities (often during quarantine; see Hadfield

& Clayton, 2011). This is necessary to prevent parasite outbreaks, which can heavily impact productivity

(Liu and Bjelland, 2014; Shinn et al., 2015). While chemical treatments can be effective in managing

aquaculture pests (Reed et al., 2009; Shinn and Bron, 2012; Yamamoto et al., 2011), these treatments

are expensive and can be associated with reduced growth (Paladini et al., 2017; Powell et al., 2018;

Shinn and Bron, 2012).

Anthelmintics are used to combat parasitic platyhelminths in agriculture, aquaculture and human

medicine (Doenhoff et al., 2008; Park and Marchant, 2019; Pax et al., 1996). Anthelmintics use variable

modes of action (Martin, 1997). The commonly applied anthelmintic levamisole inhibits enzymatic

activity by acting as a nicotinic acetylcholine receptor agonist, causing continuous stimulation of

platyhelminth muscle and subsequent paralysis (e.g. levamisole; Camacho et al., 1995; Martin, 1997;

Martin et al., 1997; Pax et al., 1996; Ribeiro et al., 2005). Another commonly used anthelmintic,

praziquantel, is thought to disrupt tegument homeostasis (Staudt et al., 1992; Martin, 1997; Martin et

al., 1997) with an increased influx of Ca^{2+} and subsequent paralysis (Doenhoff et al., 2008). Praziquantel is used extensively to treat schistosomiasis in humans (Doenhoff et al., 2008; Park and Marchant, 2019), and has considerable potential for application in aquaculture to manage platyhelminths, but is currently not approved for non-prescription use (Bader et al., 2018; Power et al., 2019; Shinn and Bron, 2012). In aquatic organisms praziquantel can be administered orally (Forwood et al., 2016), via the bloodstream (Justine et al., 2009), or in therapeutic bath immersions (dosage varies between 2 mg L^{-1} and 10 mg L^{-1}). The duration of treatments typically lasts a few hours to several days (Bader et al., 2019; Hadfield and Clayton, 2011; Paladini et al., 2017; Reed et al., 2009; Sharp et al., 2004).

Coral aquaculture is a burgeoning industry to support the demand of the marine ornamental trade, scientific research and reef restoration practices (Barton et al., 2017). Corals are associated with a variety of invertebrates (Stella et al., 2010), some of which can be harmful, especially in captivity (Barton et al., 2020a). The *Acropora*-eating flatworm, *Prosthiostomum acroporae* (Rawlinson, Gillis, Billings, and Bourneman 2011), is a polyclad flatworm that has been reported to be associated with corals at sites on the Great Barrier Reef (Rawlinson and Stella, 2012) and in captive aquaria (Nosratpour, 2008; Carl, 2008; Rawlinson et al., 2011; Hume et al., 2014; Barton et al., 2019; 2020). Its high fecundity and cryptic nature often result in rapid proliferation in captive environments, where it can cause colonial mortality of infested *Acropora*. Barton et al. (2019a) described the life cycle of *P. acroporae* and suggested that chemical treatment intervals of 2-3 weeks are potentially effective at breaking the life cycle between 24-30°C.

Prophylactic treatments for coral are commonly applied in the aquarium trade and come in the form of chemical immersions (commonly referred to as 'dips') of therapeutic solutions to treat a variety of ailments. Another anthelmintic, ivomectin, is used in chemical immersions (2mg L^{-1} over 5 hours) to treat the coral pest, *Waminoa* sp. (Winsor, 1990) (Leewis et al., 2009; Osinga et al., 2012). To date, levamisole HCl is the only chemical immersion suggested in the literature for the treatment of

platyhelminth infestation of corals (Carl, 2008; Nosratpour, 2008). While Carl (2008) suggested a dose of 40 mg L^{-1} levamisole for one hour, no empirical evidence of the efficacy of this treatment for removal of *P. acroporae* from infested *Acropora* hosts was provided. Furthermore, Carl (2008) indicated that concentrations above 40 mg L^{-1} could cause bleaching or tissue loss in *Acropora*, and that consistent exposure can leave corals more susceptible to bleaching, however little is known about the impact on coral growth. Bleached corals are undesirable in the marine ornamental trade, and are likely to have compromised survivorship as evidenced by mortality and susceptibility to disease following bleaching events in the natural environments (Anthony et al., 2009; Baird and Marshall, 2002; Miller et al., 2009; Sakai et al., 2019).

The aim of this study was to assess the efficacy of two anthelmintics, levamisole HCl and praziquantel, for the removal of *P. acroporae* individuals from infested *Acropora* colonies. We also examined the growth and bleaching of treated corals following exposure to these chemical treatments. Identification of effective treatments that remove *P. acroporae* without compromising coral quality, is valuable to the coral aquaculture community for pest management. Furthermore, a treatment regime can subsequently be coordinated to target specific stages of the *P. acroporae* life history.

Materials and methods

Coral fragment preparation

Acropora millepora colony fragments were collected in June 2019 at depths between 2-10 m from Davies Reef (18°49'21.6"S 147°39'12.5"E) located in the central Great Barrier Reef Australia (GBRMPA Permit G12/3236.1). Corals were transported to flow-through aquaria (24 °C) at the National Sea Simulator (Australian Institute of Marine Science) under natural light. Fragments were then screened using filtered seawater rinses to remove all adult *P. acroporae* and visually inspected for removal of egg clusters with tweezers to ensure subsequent infestation with a known number of flatworms (see Barton

et al., 2019a). After screening, *Acropora* colonies were broken into smaller fragments (~30 x 50mm; width x height; n=194;) using coral cutters and a coral saw (Gryphon Aquasaw XL). Each fragment was then mounted on an aragonite base with cyanoacrylate glue.

Coral fragments were transferred indoors to three 250L flow-through systems and allowed to acclimate to experimental conditions for four weeks. Coral aquaria were supplied with filtered seawater (1 μm) at approximately 3 L min^{-1} and were slowly acclimated from 24 to 26°C over a period of 24 days. Fragments were illuminated by four AquaIllumination Hydra® 52 lights per tank to provide uniform light intensity of approximately 100 μmol m^{-2} s^{-1}. Although *A. millepora* can accommodate higher light intensity, to avoid adverse effects of excessive irradiance, light acclimation was achieved through raising irradiance to approximately 150 μmol m^{-2} s^{-1} over four weeks. A Maxspect® Gyre XF250 unit in each tank was used to provide internal water circulation to the coral fragments.

Fragments of *A. millepora* were screened a second time for flatworms after two weeks to remove any potential *P. acroporae* present (one individual flatworm was removed that may have hatched post the first screening). Additionally, corals were housed in a 250L flow-through aquarium with a single *Pseudocheilinus hexataenia* Bleeker, 1857, a known predator of adult *P. acroporae* (Barton et al., 2020) providing an additional safeguard against infestation. An assortment of herbivores including *Trochus* Linneaus, 1758, *Stomatella* Lamarck, 1816 and a lavender tang *Acanthurus nigrofuscus* Forsskål, 1775 were used to control undesirable algae growth on coral fragment bases during acclimation.

Prosthiostomum acroporae culture

Prosthiostomum acroporae were cultured *in vivo* to obtain known quantities of worms for experiments in this study. Flatworms were propagated following the methodology outlined in Barton et al. (2019a),

using long term cultures of *P. acroporae* maintained on host *Acropora* colonies in three 250L flow

through aquaria. In brief, various *Acropora* spp. (including *A. millepora, A. spathulata*, *A. loripes*, *A.*

selago, *A. latistella* and *A. muricata*) were infested with *P. acroporae* via the introduction of egg capsules

collected from other infested corals in culture. The temperature of the culture was adjusted from 27°C

down to 26°C (the experimental temperature) over a period of three weeks prior to experiments.

Each *A. millepora* fragment for experimental infestation (54 fragments), was placed in a 2.5 L aquarium

with seawater and *P. acroporae* individuals (three per fragment; 162 flatworms in total) were pipetted

directly onto the coral fragments. The supply of flatworms from the culture was exhausted during the

experiment so that the last treatment (control) was unable to be conducted with the 20 infested *A.*

millepora (n = 14). Any flatworm that immediately moved off the coral were detectable by eye and

pipetted back on once more. Flatworms that moved off the coral a second time were discarded. After

five minutes each submerged coral fragment was gently shaken by hand to ensure flatworms were

attached and could not be easily dislodged.

Treatment preparation and immersion

Coral fragments (n = 194) were spread across eight treatments (Figure 6-1); levamisole infested (LI),

levamisole uninfested (LU), praziquantel infested (in EtOH) (PI), praziquantel uninfested (in EtOH) (PU),

handling control infested (HCI), handling control uninfested (HCU), EtOH control (EC), control with no

handling (NHC). Three treatments (PI, LI, and HCI) had 20 coral fragments each with the exception of HCI

(n = 14; 54 total) infested with *P. acroporae* to compare the removal efficacy of the chemical immersion

process, while PU, LU, and HCU had 20 uninfested fragments each (60 total). EC and NHC had 40

uninfested coral fragments each (80 total) which remained in the recovery system (see section 2.4)

untouched to discern if the treatment, temporary infestation, or handling (e.g. flatworm screening

method) of coral fragments had any effects on coral growth or visual bleaching.

Immersions were prepared for each treatment (excluding NHC) and added to 2 L replicate beakers. Levamisole HCl (CAS Number: 16595-80-5) is highly soluble in seawater and therefore could be prepared directly in seawater and a 25 g L^{-1} stock was diluted to 40 mg L^{-1} in 1 µm filtered seawater in all levamisole replicates. The poor solubility of praziquantel (CAS Number 55268-74-1) in seawater required preparation of a 50 g L^{-1} stock solution in 100% ethanol added to 1 µm filtered seawater in the associated experimental replicates (PI, PU) to a final concentration of 50 mg L^{-1} praziquantel. An ethanol control treatment (EC; 0.01% Ethanol in 1 µm filtered seawater) was incorporated into the experimental design to differentiate any effects of praziquantel/ethanol versus ethanol on coral metrics (basal growth and visual bleaching signs). Handling control replicates (HC) consisted of filtered seawater (1 µm).

Once immersions were prepared, coral fragments mounted on a PVC base (to keep fragments upright), were added to their specified treatments consisting of a 2L aerated beaker (aerated with coarse bubbles through acrylic tubes). After one-hour immersion duration, fragments and the associated PVC base were removed from their respective 2 L beaker and given a vigorous five second shake in their respective rinse container containing filtered seawater, later referred to as the 'shake step'. The number of flatworms removed from the coral during the immersion and after the shake step were recorded, along with any flatworms adhering to the PVC base. Both the individual *A. millepora* fragment and the respective PVC base were rinsed in filtered seawater (26°C) to mechanically remove *P. acroporae* individuals remaining and ensure all worms were accounted for before placing corals in one of the three recovery tanks. Each tank was stocked with fish and snails in the same manner as the acclimation aquarium (*Acanthurus nigrofuscus*, *Pseudocheilinus hexataenia*, *Trochus* sp., and *Stomatella* sp.) to control algae and additionally safeguard against *P. acroporae*. The removal efficacy was calculated as the total number of flatworms removed by the treatment, including the associated shake step, divided

by the total number of flatworms initially added (three per fragment). The mortality of *P. acroporae* was

not measured in this study.
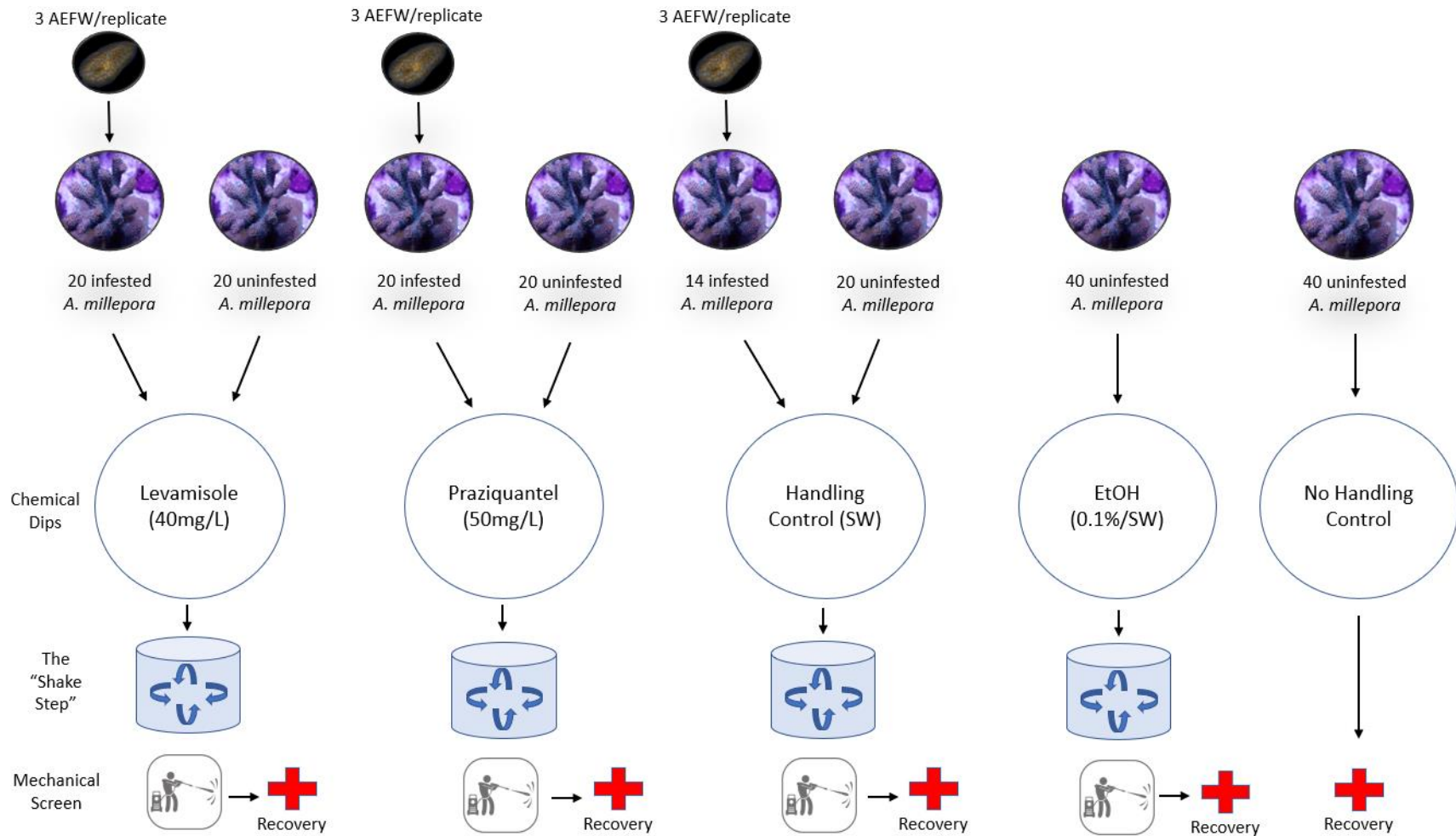
Figure 6-1: Schematic showing immersion procedure with all treatments (levamisole, praziquantel, handling control, EtOH control, and no handling).

Monitoring coral recovery

Color change and basal growth were measured as proxies for coral recovery following chemical exposure for one month. After mechanical removal, coral fragments were placed into one of the three identical pre-conditioned 250 L aquaria in a randomly assigned position in each tank. A random number generator was used to determine the recovery tank and position within the tank before treatment. For each tank, lighting was provided by four Aqua Illumination Hydra® 52 lights (12:12 light: dark; 1 hour ramp up/down; ~150 µmol m^{-2} s^{-1}) and one Maxspect® Gyre XF250 unit. Each aquarium was fed daily with *Artemia* nauplii at a rate of 0.35 nauplii mL^{-1} for corals. Manual handling of coral fragments was as limited as possible, with the only handling during weekly photo capture.

All corals were photographed (from the top) on their associated trays prior to chemical immersion (day 0), again the day following treatment (day 1) and weekly thereafter until day 28. For consistency, photos were taken in the dark using a computer-controlled (MSI Adora24G) photo cart equipped with a Nikon® DSLR D810, four Ikelite® DS161 strobes, and manually adjustable x-y stage (Figure 6-2). Camera settings remained consistent for all photos (shutter speed 1/8 sec, aperture f/11).

The C clade section of a CoralWatch Coral Health Chart was used to assess the color change in each coral fragment following the methods of Siebek et al. (2006). Images were first converted to greyscale in ImageJ to remove the influence of luminosity on photo color and to allow the export of mean grey values (MGV; 1-250; higher values are lighter) for each CoralWatch Coral Health category (C1 to C6) and each coral fragment in each photograph. Comparison of MGV of the coral watch color standards between time points (initial and four weeks later) indicated the consistency of photographs. Before comparison of coral fragment MGV, these raw values were corrected by 7% to reflect the uniform change in the color standards between time points. Corals that shifted two or more color categories

lighter were considered bleached, while all others were not considered to have bleached.
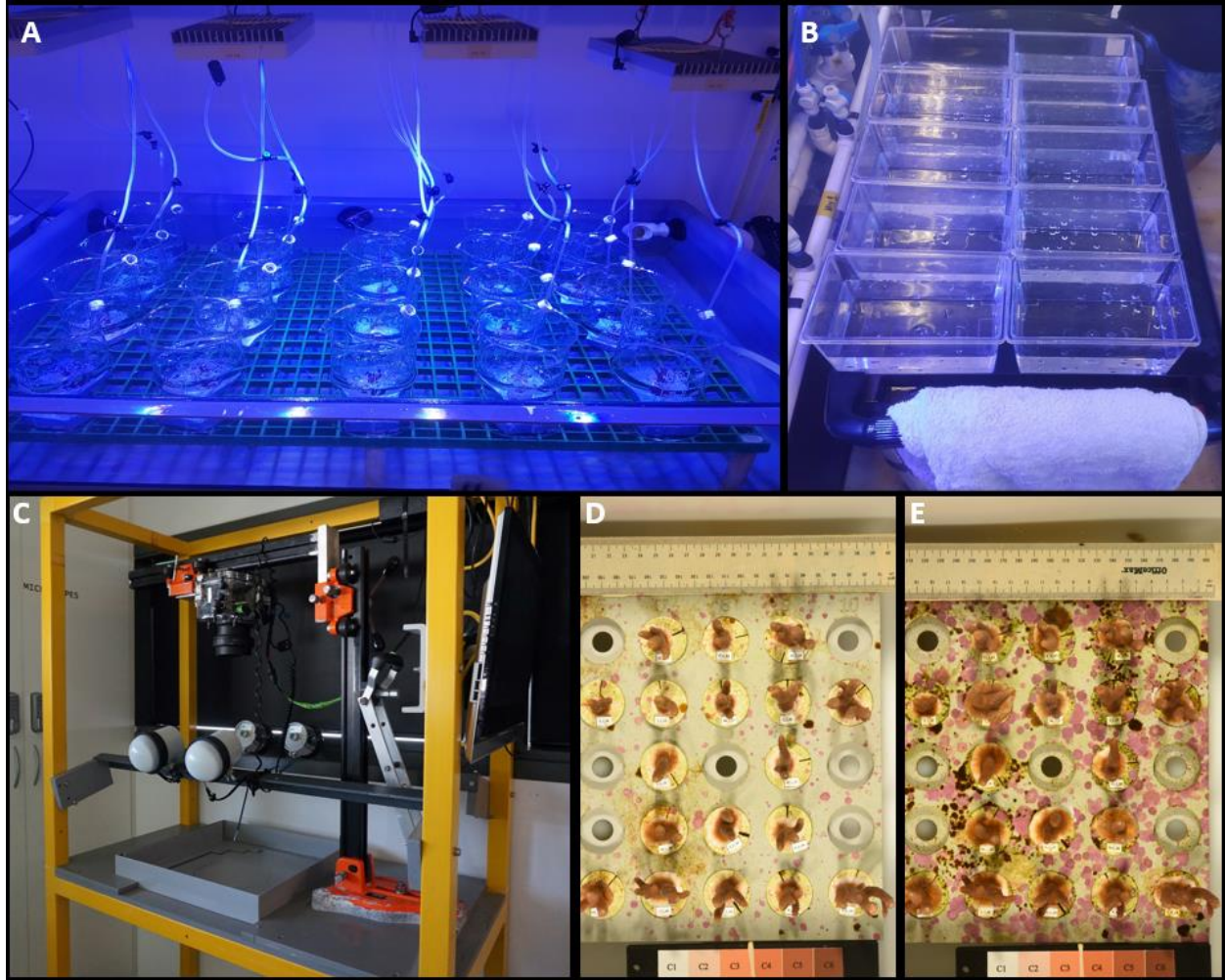
Figure 6-2: Photographs of experimental design: A. *Acropora millepora* fragments in their respective 2 L beakers during a one-hour chemical immersion, B. Containers with filtered seawater use for the 'shake step' after chemical immersion, C. Camera cart used for taking photos of *A. millepora* fragments, D. Initial photo (before chemical immersion) taken of *A. millepora* fragments, E. Day 28 photo taken of the same tray of *A. millepora* fragments.

ImageJ (FIJI ImageJ; Schneider et al., 2012) was used to measure the lateral area and circumference of each coral fragment, allowing comparison of photographs taken the day after chemical immersion and on day 28 to calculate the percentage increase in basal growth area (mm^2) (similarly to Forsman et al., 2015; Page et al., 2018) and circumference (mm) of each coral fragment. Basal growth is not only

associated with overall growth in *A. millepora*, but it is required for attachment to substrate post fragmentation. This is relevant to the marine ornamental trade, where basal growth onto substrate is a sought-after feature of coral fragments examined by consumers as a qualitative indication of fragment health at the time of purchase. The implications of uncompromised basal growth are also relevant to reef restoration, where the leading cause of fragment mortality is detachment (Shafir et al., 2006; Shaish et al., 2008; Smith and Hughes, 1999).

Statistical analysis

All analyses were run using RStudio (version 3.5.1). Flatworm removal efficacy from the immersion step alone and efficacy after the shake step (includes removal from immersion step) were modeled separately because the shake step results are dependent on the immersion step removal. Binomial generalized linear mixed-effects models (GLMM; R package "lme4" Bates et al., 2015) were run with tray identification as a fixed effect and treatment as a random effect. This model was fitted with the "glmer" function. Normality of all the data was assessed using QQnorm and Shapiro–Wilk tests. A Tukey post-hoc test (R package "emmeans" Lenth, 2016) was used for pairwise comparison of all treatments with $P < 0.05$ as the significance threshold. For coral basal growth, lme was used to compare area data from each fragment. Bleaching response was measured as the proportional change in MGV using glm and Kruskal-Wallis, followed by a post-hoc Dunn Test. Data was visualized using (R package "ggplot2" Wickham, 2016).

Results and Discussion

Immersion efficacy

Treatment had a significant influence on flatworm removal for the immersion and immersion + shake step ($p < 0.05$; GLMM). Praziquantel treatments (PI) removed 90 ± 3.4% of flatworms (percent ± SE)

compared to 75 ± 6.61% and 7.1 ± 3.1% removed by levamisole treatments (LI) and the handling control

(HC), respectively, during chemical immersion (Figure 6-3). The shake step increased the efficacy of

flatworm removal for LI from 75 ± 6.61% (immersion only) to 93.33% ± 3.80 (immersion and shake;

Figure 6-3). Similarly, praziquantel removal increased from 90.0 ± 3.4% (immersion only) to 95.0 ± 2.66%

(immersion and shake), while 7.1 ± 3.1% and 33.33 ± 7.52% of flatworms were removed from the

handling control (Figure 6-3). Both chemical treatments were effective at removal of *P. acroporae*

individuals from infested *Acropora millepora* fragments, but there was no difference between the

efficacy of levamisole and praziquantel (p > 0.05; Tukey Post Hoc). The removal observed from the

'shake step' in all treatments suggests that immersions of levamisole or praziquantel, with only aeration

providing water movement, may not always remove flatworms from the coral host. These results

emphasize the importance of additional water movement to improve the efficacy of chemical

immersions to treat corals infested with *P. acroporae*. Therefore, we suggest that a combined

application of chemical treatment immersion with a small wavemaker would increase the efficacy of

flatworm removal. For commercial applications, this would be necessary to ensure water circulation

while treating multiple coral fragments and/or colonies concurrently. Water circulation may also be

helpful to ensure turbulence reaches the center of larger colonies (>10 cm) or those with complex

branching structure such as *Acropora loripes*, in order to remove flatworms which may be present there.

While there were no significant differences in flatworm removal between levamisole and praziquantel (P

> 0 .05; Tukey Post Hoc), we observed many flatworms in the levamisole treatment adhered to the

beaker once removed from the coral, while in praziquantel, worms were clearly paralyzed and unable to

adhere to treatment beakers. Furthermore, flatworms removed during the shake step after immersion

in levamisole appeared to recover and adhere to the surface of shake containers, suggesting potential

rapid recovery from levamisole exposure by *P. acroporae*. Praziquantel may have a more pronounced

paralytic effect on *P. acroporae* because worms remained curled and unattached in their containers during immersion and after the shake step. Hirazawa et al. (2000) similarly observed immediate muscle contraction and subsequent removal of the monogenean *Heterobothrium okamotoi* Ogawa, 1991 treated with praziquantel, compared to a five minute delay using levamisole HCl in therapeutic immersions to treat the tiger puffer, *Takifugu rubripes* (Temminck & Schlegel, 1850). While future development of in situ treatments for *P. acroporae* are desirable, the toxicity of levamisole and the rapid degradation of praziquantel in seawater (Thomas et al., 2016) are likely to hinder these efforts. Dedicated treatment areas where corals can be immersed in praziquantel and shaken or rinsed in clean seawater baths may have best results with prophylactic, single preventative dips to remove flatworms before entering quarantine systems.
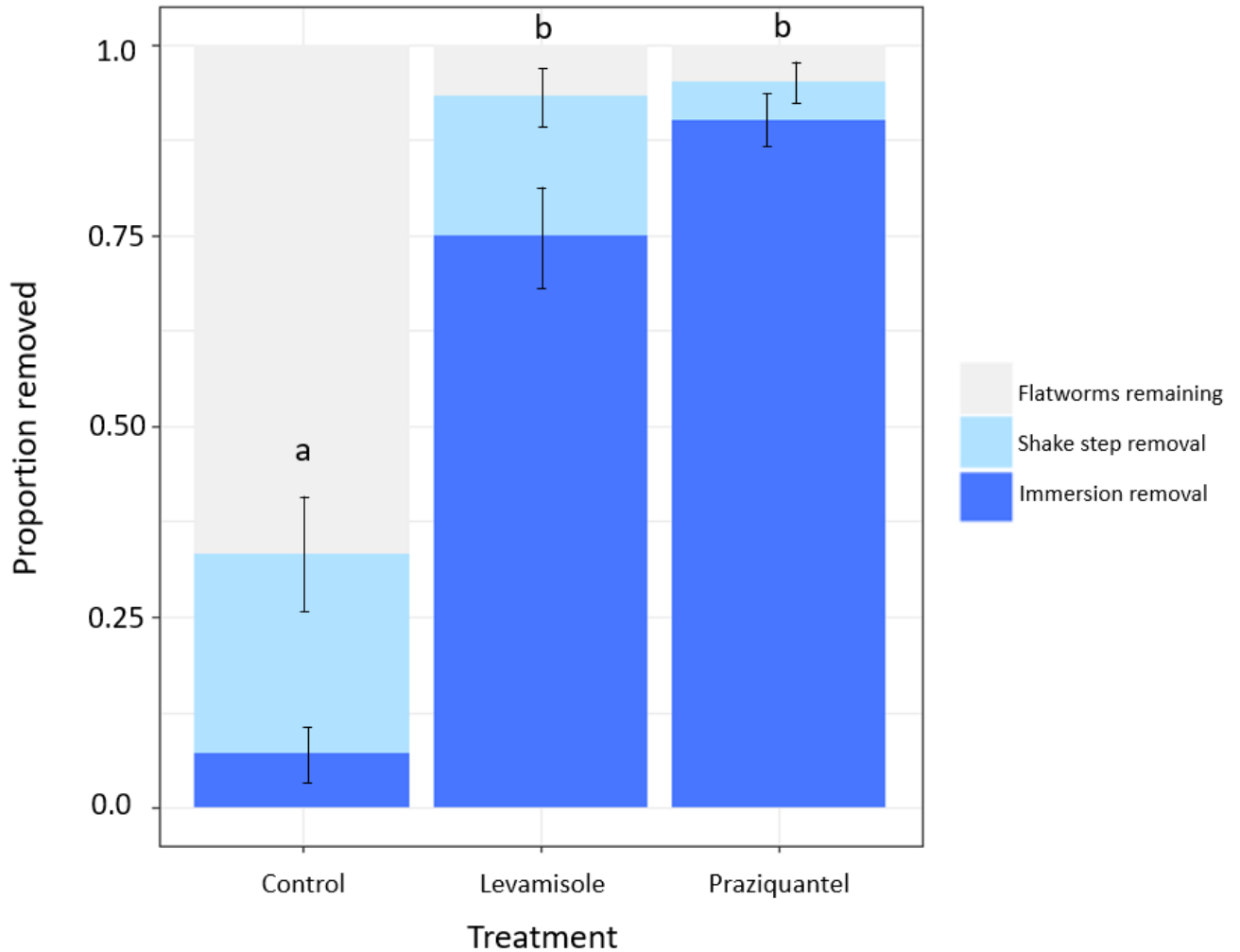
Figure 6-3: Stacked bar plot showing the mean percentage (±SE) of *P. acroporae* recovered from *A. millepora*

fragments from each associated chemical treatment (Handling control, Levamisole, and Praziquantel) from each

immersion, shake step, and the mechanical screening step to recover remaining flatworms. The letters (a) and (b)

indicate treatments with statistical differences from each other.

3.2 Coral health metrics following chemical treatment

There was no mortality observed in any coral fragments except for the partial colonial mortality in a

single fragment during the first week following immersion in levamisole, with the fragment later

showing no further signs of tissue necrosis. Chemical treatment had no effect on the mean grey value

(MGV) of all *A. millepora* fragments in the experiment, irrespective of whether corals were infested or

not before being treated (P > 0.05; GLMM). No fragments in any treatment of the experiment were

considered bleached relative to their initial MGV. These results provide evidence that the use of

levamisole HCl and praziquantel did not advance observable bleaching during the four weeks following

treatment. Treatment also had no significant effect (p > 0.05; GLMM) on the basal growth of *Acropora*

millepora fragments during the experiment, with mean basal area increasing by 73.31 ± 3.82% (mean ±

SE) across all treatments (Figure 6-4). While coral fecundity, the coral microbiome, vertical growth, and

photosynthetic efficiency of treated coral was not investigated in this study, our results suggest that the

prophylactic use of levamisole or praziquantel to treat corals does not result in reduced basal growth or

any observable bleaching in the short-term. This is important because growth inhibition would increase

the associated cost of therapeutic treatment (Shinn et al., 2015).
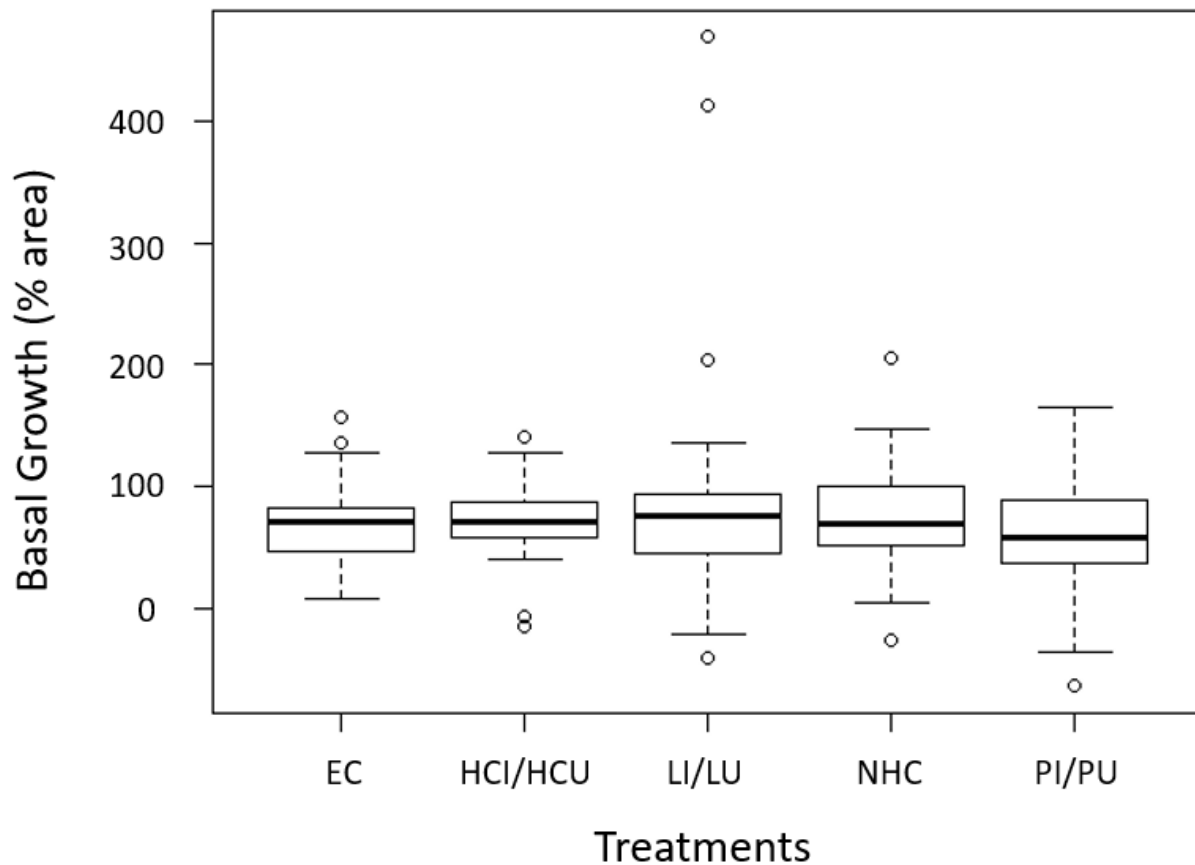
Figure 6-4: Box and whisker plot demonstrating the percentage basal growth of *Acropora millepora* in each treatment (**EC**: ethanol control, **HCI/HCU**: handling control infested and uninfested, **LI/LU**: levamisole infested and uninfested, **NHC**: no handling control, and **PI/PU**: praziquantel infested and uninfested.) after four weeks, with straight lateral lines demoting means, whiskers showing quartiles.

3.3 Treatment cost and availability

While we validate the use of praziquantel in high concentration (50 mg L^{-1}) and low duration (i.e., immersion over one hour for the treatment of *P. acroporae* infestation on corals), availability of praziquantel may vary between different countries, while levamisole is readily available globally and in use as a universal de-wormer of cattle (Varady and Čorba, 1999). Both praziquantel and levamisole are regarded as cost-effective treatments in the context of finfish aquaculture (Alves et al., 2019). Based on the average cost of 5 g of each chemical from three major suppliers (Sigma-Aldrich, Tokyo Chemical

Industry (TCI), and Fisher Scientific), the cost per L of treatment solution are $0.74USD L^{-1} for praziquantel (50 mg L^{-1}) compared to $0.48USD L^{-1} for levamisole HCl (40 mg L^{-1}), making levamisole HCl marginally more cost-effective. It should be noted that praziquantel is less toxic to human and environmental health than levamisole, which is reflected in the Australian Poisons Standard (February 2020), and examination of associated safety data sheets (Sigma-Aldriich®; L9756, P4668) in accordance with risk assessment for use in the laboratory. While praziquantel appears safe for vertebrates (Mitchell and Hobbs, 2007), further research is required to understand the toxicity of praziquantel to other organisms, and how drug resistance may be induced with increased use of praziquantel (Bader et al., 2018). Furthermore, the permitting and governance of chemical use for coral aquaculture is currently lagging as evidenced by the absence of levamisole approval for therapeutic use for corals, although it is approved for use in ornamental fish, birds, dogs, and cattle in Australia (Poisons Standard February 2020). Depending on the country, the use and disposal of either of these chemicals in coral aquaculture require education, regulation, and ethical use to ensure environmental responsibility.

5. Conclusion

We show that levamisole HCl and praziquantel can be used in chemical immersions in conjunction with water movement to remove >90% of flatworms from infested corals. A chemical treatment interval (time between treatments) of approximately three weeks (variable with temperature; see Barton et al. 2019), should remove the majority of flatworms from the host. Less than 100% removal efficiency of flatworms from infested corals in this study indicates the need to optimize the administration of levamisole and praziquantel treatments. Mechanical screening following chemical removal as conducted in the present study should increase flatworm removal efficacy. This protocol is suitable for treatment of infestations in an established coral aquaculture system, or as preventative treatment of *Acropora* in quarantine.

Chapter 7: General Discussion

Corals of the genus *Acropora* are not only important to coral reefs and their diversity (Munday et al. 1997; Alvarez-Filip et al. 2009; Stella et al. 2011) but are also primary targets for coral aquaculture. It is the most represented genera in coral reef restoration research to date and species are highly sought after by the ornamental trade (Barton et al. 2017). The *Acropora*-eating flatworm, *Prosthiostomum acroporae* is one of the most destructive pests of scleractinian corals in captivity (Chapter 2) and is only found on *Acropora* corals. Flatworms feed directly on coral tissue eventually compromising coral health and often resulting in colonial mortality in captivity (Chapter 3). The cryptic nature and high fecundity of *P. acroporae* often lead to infestations going unnoticed until populations have proliferated to harmful densities on captive *Acropora* that cause colonial mortality (Norsratpour 2008)*.* Furthermore, the typical high stocking density of *Acropora* in aquaculture systems further facilitates widespread infestation. Given our findings that more than half of wild *Acropora* on inshore areas of the Great Barrier Reef are infested with *P. acroporae* (Chapter 4), there is a risk of introducing corals infested with *P. acroporae* into captive systems from wild collections. This is especially true without established biosecurity strategies. A management plan for appropriate detection, prevention, diagnosis, and treatment (Chapter 6 and 7) will help to support research activities, reef restoration and the marine ornamental trade that maintain *Acropora* in captivity.

Integrated Pest Management

The findings of this thesis can be incorporated into an Integrated pest management (IPM) model, which provides a holistic approach by which preventative measures are applied first, followed by responses aligned with organism biology and ecology in the event of infestation (Kogan 1998; Zehnder et al. 2007; Baker et al. 2020). This management framework is practiced in part to prevent complete reliance on

pesticide use, instead first using cultural practices such as crop rotation and strategic introduction of

natural predators or parasites (i.e., biocontrols) which suppress pest species' abundance. While this

framework was popularized by agricultural practices (Zhender et al. 2007; Baker et al. 2020), applying an

IPM framework to pest control in coral aquaculture can provide managers with a valuable toolbox to

prevent, accurately identify and diagnose, and treat infestations. Here we synthesize an IPM framework

for control of *P. acroporae* on captive *Acropora* corals (Figure 7-1). We identify appropriate prevention

methods including screening incoming corals, quarantine procedures, rapid real-time diagnostic

methods for visual identification of adult worms and eggs, transmission mitigation behaviors, biological

controls, chemical treatments, and timed exclusion or fallowing methods. For each of these key steps

we consider site-specific challenges of different stakeholders for captive coral husbandry.

Prevention methods

Component 1: Processing incoming corals

It is nearly always more efficient to prevent a pest from entering a system than taking retrospective

action to remove or control them. The most obvious risk of pest entry is when new corals enter an

aquaculture facility, either from the wild or from another facility. For this purpose, we developed a

mechanical screening technique to detect infested *Acropora* colonies by using jets of seawater to

remove flatworms (see Chapter 4). This protocol provides stakeholders with the ability to bypass the

camouflage of *P. acroporae* and be alerted to infested corals entering a facility which will require

isolation during quarantine. This method has been shown to be >95% effective at removing flatworms

from *Acropora* hosts (Chapter 5. We recommend the initial mechanical screening to occur before any

corals are introduced to incoming quarantine systems. This process not only identifies infested corals

but is also a first step in removal of *P. acroporae*.

Incoming coral colonies should also be visually inspected for the presence of egg capsules (Chapter 3). While eggs are often present at the base of colonies or on the underside of branches, egg capsules can also appear at the site of fresh breakage of *Acropora* branches where *P. acroporae* can deposit eggs in transit. These can be removed using a sharp implement such as a scalpel or forceps. Extraneous material (rock, biofouling, dead skeleton etc.) can be removed from coral bases with an appropriate tool (coral size dependent; E.g., Gemini® XT ringsaw or Gryphon® band saw) to remove substrate which may house egg capsules or other pest species.

Following mechanical screening and visual inspection for eggs, we recommend corals enter quarantine systems that are segregated corresponding to their screening result. Screening may not be 100% effective, especially with large colonies with compact inner branches (e.g. *Acropora loripes*) where it can be difficult to access these branches with direct jets of seawater to remove adults and scrape egg capsules from inner branches. The initial separation of perceived infested and uninfested individuals reduces the likelihood of lateral infestation of 'clean' corals. Indeed, newly hatched flatworms can swim or crawl to spread to new corals through an aquarium system (Chapter 3). Because of the risk of lateral transmission in incoming coral systems, we recommend corals be introduced to systems in a single cohort, and then managed as a single unit to mitigate lateral transmission of motile flatworms between corals at different stages of quarantine.

Component 2. Quarantine dynamics

After incoming corals are screened for *Prosthiostomum acroporae*, we recommend careful quarantine practices. Quarantine systems are commonly isolated from non-quarantine systems to limit the potential transmission of infestation between systems (Miller-Morgan et al. 2012). Miller-Morgan et al. (2012) advocate that quarantine systems should be physically separated from any wholesale, retail, or

import systems with separate equipment for effective quarantine. Furthermore, quarantine systems with dedicated signage and restricted access can educate husbandry technicians and mitigate the risk of infestation transmission during the quarantine process (Miller-Morgan 2012). Because of the motility of newly hatched *P. acroporae* (Chapter 3), we recommend flow-through aquariums if a constant water supply is possible, to reduce the probability of hatched *P. acroporae* finding new *Acropora* hosts because some will be flushed out of the system incidentally as water is exchanged. In contrast, *P. acroporae* hatching in recirculating systems will have more opportunity to settle on new *Acropora* hosts if individuals survive filtration and re-enter the coral holding area. While some individuals may be killed by filtration equipment in recirculating aquaria, previous work (Chapter 3) found new hatchlings able to bypass 100µm filter material (*P. acroporae* hatchlings are ~250 x 150 µm (length x width) and can squeeze through), which is considerably finer than ≥ 200 µm filter socks commonly used in coral aquaria (JB pers. obs.). Utilizing filters such as 0.01 µm or 0.02 µm Sawyer® Mini water filtration units may provide solutions for the exclusion of *P. acroporae* hatchlings in closed systems. It can also be expected that the use of UV sterilization would reduce the rate of infestation in recirculating aquaculture/aquarium systems (RAS). Similarly, the treatment of effluent in this manner will mitigate the biosecurity risk of introducing newly hatched *P. acroporae* to proximal inshore reefs in open systems.

While the priority of quarantine systems is to mitigate the risk of infestation to other corals in a facility, consistent, high-quality coral husbandry is required to have the most robust animals leaving quarantine. For *Acropora* spp., this includes water quality, water flow, feeding and light acclimation to keep corals as healthy as possible during the quarantine process. Hume et al. (2014) demonstrated that corals will struggle to photoacclimate when being fed on by *P. acroporae*. If corals are infested, measured photoacclimation should therefore be practiced where possible to mitigate oxidative stress in host tissues (Hume et al. 2014). We recommend that the duration of quarantine be as long as the projected

time to sexual maturity of flatworms at the given system temperature whenever possible (Chapter 3). It

is not recommended that corals leave quarantine if any egg capsules have been observed in the

quarantine systems. We suggest that weekly mechanical screening of the corals continue during the

quarantine period. More frequent screening could result in tissue loss from the screening process,

negatively impacting coral health. The presence of eggs suggests that sexually mature flatworms remain

in the system, compromising all corals in the system. Furthermore, it is not recommended that any

corals leave quarantine until all colonies are screened and determined to be free of flatworms. Initial

data from weekly screening suggests that a *P. acroporae* population can be eliminated after three

consecutive weeks of screening (Chapter 4).

Component 3: Transmission mitigation behaviors

Infestation prevention also includes mitigating the risk of pest introduction from one system to another.

Generally, good practices to avoid transmission include using maintenance equipment (e.g., algae

scrapers, siphon hoses) specifically designated for a given aquarium system/room (see Miller-Morgan et

al. 2012). Like optimal quarantine practices for ornamental fish (Miller-Morgan et al. 2012), the traffic of

animals through a facility is recommended to be one way to mitigate transmission of *P. acroporae*

infestations. Measures can also be taken to minimize risk by workflow management. For example,

prioritizing work with uninfested corals before work with corals in quarantine to reduce the risk of

incidental transfer between systems or hosts, or even having technicians specifically working on

quarantine and not in grow out areas. We recommend that personal hygiene of husbandry technicians

be a standardized practice, where technicians enter a system or area using foot baths and assigned

coveralls to limit spread of infestations. Similarly, where after a user has their hands in an aquarium they

clean their hands with freshwater and spray the applicable areas (hands/arms) with 70% EtOH solution

to kill any *P. acroporae* which may be present in the water column and adhere to skin or clothing.

Showers or changing of clothes after work with infested colonies may also be considered in some

circumstances. Strong mitigation procedures can reduce human error which can proliferate *P. acroporae*

abundance through lateral transmission from infested systems to corals in clean systems.

Ongoing staff training can aid the identification of *P. acroporae* outbreaks as they occur and enable

effective and early identification of infestation. Often, infested *Acropora* look healthy while infested

with low densities of *P. acroporae*. We recommend that all husbandry personal be trained to identify

signs of infestation including the characteristic ~1 mm circular pale feeding scars on tissue, occurring

especially under and between coral branches and at the base of corals, as well as the brown egg clusters

found on exposed coral skeleton. If heavily infested corals are discovered, it is likely that 'subclinical'

corals with low densities of infestation are also present within a system. Practicing regular inspection

and mechanical screening of perceived uninfested corals around a facility can identify these pests as

well as vulnerabilities in the integrated pest management plan.

Infestation Treatment

Prosthiostomum acroporae deposit egg capsules on unfouled substrates. This includes but is not limited

to snail shells, coral mounting bases, and even the bottom or sides of an aquarium system. We

recommend that systems which become infested to the point where egg capsules are evident on the

benthos are fallowed to allow for the eradication of the infestation; either by removal of *Acropora* for

one-month (other corals may stay because of the specificity of *P. acroporae* for *Acropora*), complete

draining, or even acid washing before more *Acropora* are introduced. Fallowing is practiced extensively

in finfish aquaculture (Delabbio et al. 2004; Bui et al. 2019) and has been used frequently on sea cages in

salmonid aquaculture to mitigate sea lice infestations (Bron et al. 1993; Marty et al. 2010; Werkman et

al. 2011). Fallowing in the context of *P. acroporae* requires removing all *Acropora* or even water from a system to allow for any potential hatchlings to die and adults to be without host for a duration dictated by the life cycle of *P. acroporae*. A wholesaler for instance could use alternate holding systems for each cohort, allowing certain tanks which house *Acropora* to be without *Acropora* colonies for a given duration (embryonation period + time to hatchling starvation Chapter 2) to allow for eggs to hatch in the absence of hosts and for those hatchlings to starve. This must consider temperature which strongly influences the embryonation period of egg capsules.

Aquarium systems known to house *Acropora* currently or previously infested with *P. acroporae* can be subjected to either a fallow period or acid washing before a new group of corals are added, to allow hatching of remaining flatworms and starvation of resulting adults, thus preventing them from affecting newly added corals. Our results demonstrate that temperature manipulation could also be used to encourage egg hatching and shorten the adult starvation time required to fallow a given system. If a given system can be heated to 30°C, all eggs should hatch within one week, and all hatchlings should starve without finding a host after another week (Chapter 3). However, some systems may lack the ability to increase the temperature to 30°C, in which case to the fallow period of the system will be longer because of the influence temperature has on the embryonation period ; the cooler the water temperature, the longer the embryonation period and time taken to attain sexual maturity (Chapter 3).

Component 4: Biological control

Biological control organisms provide an additional tool for integrated pest management of *Prosthiostomum acroporae*. We found the sixline wrasse *Pseudochielinus hexataenia* and peppermint shrimp *Lysmata vittata* consume *P. acroporae* (see Chapter 5). *P. hexataenia* consumed 100% of adult flatworms on *Acropora millepora* fragments, though did not eat flatworm egg capsules. *L. vittata*

consumed most adult flatworms and egg capsules (82.0 ± 26.36% and 63.67 ± 43.48% respectively; mean ± SD; Chapter 5).

L. vittata are an aggregating species with poor eyesight that must encounter the flatworms to consume them. Coral colonies which enter the facility heavily infested or are discovered in rearing systems could be placed into a shrimp treatment chamber (Figure 7-3, which houses several *L. vittata* in a single aquarium just large enough for the infested colony. Such a chamber would use the chamber size (relative to the treated corals) to foster a high frequency of predative interactions between the shrimp and *P. acroporae* individuals and egg capsules, to clean the infested colony. *L. vittata* would be able to access areas of the *Acropora* colony which has egg clusters which are unable to be removed by a husbandry technician (Chapter 5). While shrimp performance in this context has not been validated, our preliminary research indicates a high propensity of this species to feed on adult flatworms and their egg capsules in confined environments. Moreover, the life cycle of *L. vittata* has been closed and this shrimp can be bred in captivity.

In contrast to *L. vittata*, *P. hexataenia* are territorial and presumably use their keen eyesight (Gerlach et al. 2016) to hunt flatworms. Because *P. hexataenia* actively forage for flatworms, we recommend they be housed in perceived infested and uninfested quarantine systems to consume *P. acroporae* individuals they encounter (Chapter 5). *L. vittata* could also be included if they are large enough to not be eaten by *P. hexataenia.* Despite large proportions of infested *Acropora* colonies in the wild (Chapter 4), flatworm populations appear to be kept to densities that do not significantly impact coral health. In a similar fashion, *P. hexataenia* appear well-suited to mitigate *P. acroporae* population growth by continuous grazing on *P. acroporae*. The differing ecology of these two biocontrol organisms influence the recommendation for their use in integrated pest management. We suggest the limited foraging range and aggregating nature of *L. vittata* makes this species more suited to treatment and quarantine

applications, while the territorial nature of *P. hexataenia* make these species more appropriate for holding and grow-out scenarios in larger tanks.

Component 5: Chemical treatment

Chemical treatments can be used proactively as a prophylactic treatment for *Acropora* corals entering a facility or following positive identification of *Prosthiostomum acroporae* infestation. For ideal efficacy, the chemical treatment process can follow mechanical removal of any rock or dead skeleton not part of the coral, in addition to physical removal of egg capsules present. Indeed, eggs are generally unaffected by chemical treatments (JB pers. obs.) which results in the need for multiple chemical treatments. We examined the efficacy of two anthelmintic chemical immersions to remove *P. acroporae* from infested *Acropora* fragments. We found praziquantel at 50 mg L^{-1} removed 95 ± 2.6% (mean ± SE) and levamisole HCl (40mg L^{-1}) removed 93 ± 3.4% of flatworms after one-hour immersions compared to 33.3 ± 7.5% in the seawater control (Chapter 6). Furthermore, chemical treatments had no measured effect on coral growth, nor were they associated with bleaching of the treated corals. A second chemical treatment approximately three weeks following the initial dip should ensure that any *P. acroporae* recruits are removed before reaching sexual maturity (Chapter 3; 6) if all egg capsules are removed from the system. If treating a confirmed or suspected infestation, models from Chapter 3 can be used to inform an ideal chemical treatment interval (time between first and second treatment) for a given temperature aquarium. For example, if one were to treat infested *Acropora* with a praziquantel immersion on day 0 when infestation is first noticed in a 25 °C aquarium, the following midpoint formula can be used to calculate an ideal treatment interval:

$$\frac{(Embryonation\ period + Time\ to\ sexual\ maturity)}{2} = Ideal\ treatment\ interval$$

$$\frac{(14\ days + 47\ days)}{2} = 30.5\ day$$

Using equation 1 (95% CI), the projected ideal interval is 30.5 days. The appeal of using the midpoint between the embryonation period and time to sexual maturity period is that you are insulated from the risk of outliers which may mature at a rate which fosters re-infestation if chemical treatments are applied too close to either the predicted embryonation period or time to sexual maturity. This also protects against a situation where treatments are not 100% efficacious at removing eggs and adults. Additionally, no new corals should enter an aquarium with corals being treated. Future studies could focus on optimizing the efficacy of chemical immersion techniques, and the development of chemical treatments able to be administered *in situ* without harming other inhabitants of the treated aquarium would provide additional chemical treatment options for control of captive *P. acroporae* infestations, especially for use in a hobbyist capacity.
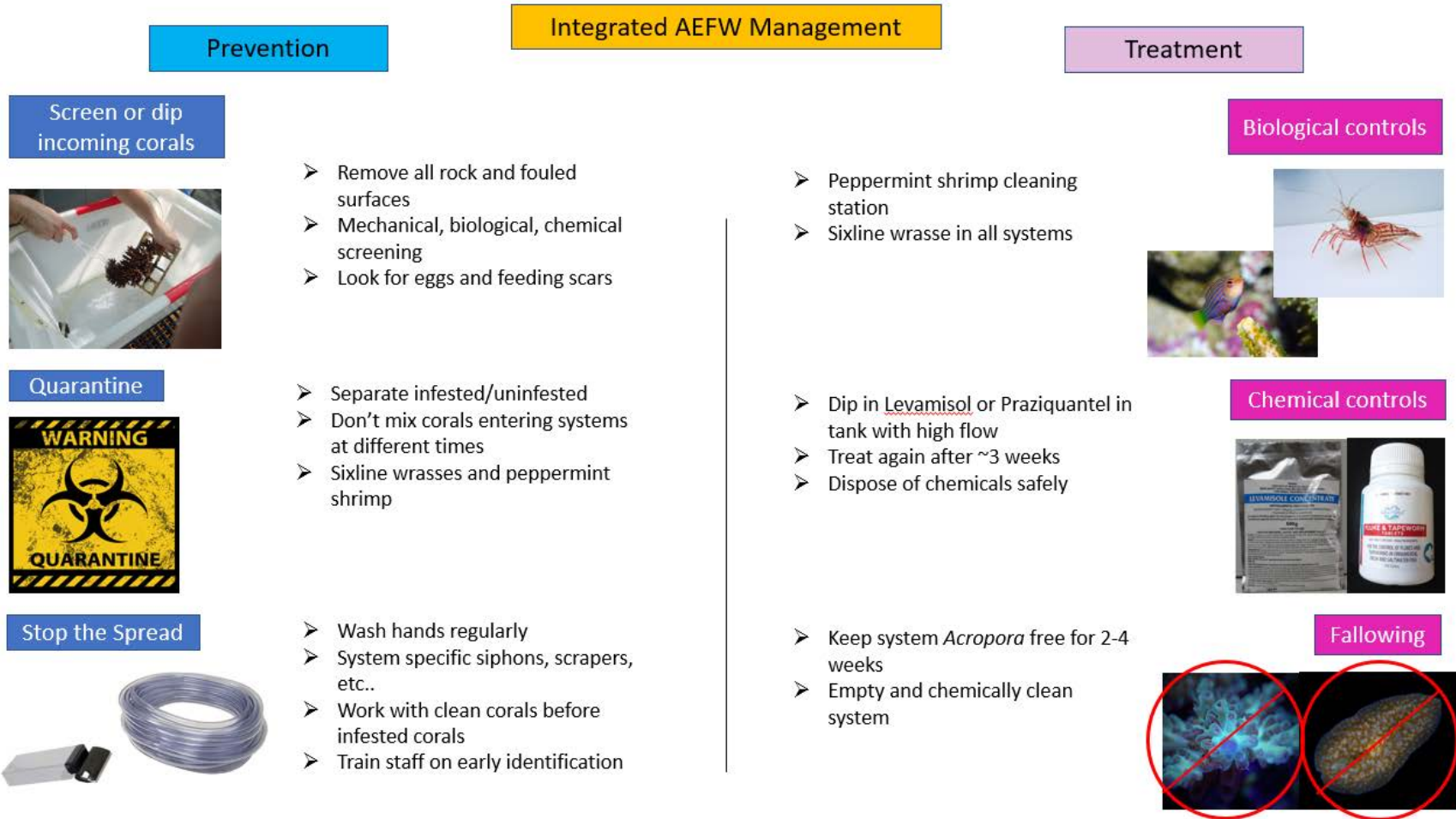
Figure 7-1: Integrated pest management plan for control of *Prosthiostomum acroporae* (AEFW) in captivity.

Implementation considerations and challenges

IPM and Reef Restoration

The relevance of suggested IPM procedures to reef restoration is dependent on both the mode of propagation, sexual or asexual, of the corals to be transplanted, and where the nursery phase (if any) occurs. For instance, reef restoration efforts which directly release larvae onto the reef will not be affected by *P. acroporae* in captivity because of the absence of a nursery phase for propagated corals (see Villanueva et al. 2012). *P. acroporae* are of considerable concern to corals propagated in *ex situ* facilities (land based; Osinga et al. 2011), where it is possible for infestation to transfer from captive raised corals to wild *Acropora* populations proximal to the reef being restored (Hume et al. 2014). While corals grown *in situ* could transfer *P. acroporae* between the nursery and the transplant reefs, the natural recruitment of other organisms to these nurseries (Abelson 2006; Shafir and Rinkevich, 2010; Frias-Torres et al. 2015) may suppress *P. acroporae* density through predation. The recruitment of natural predators combined with the open nature of these nurseries should make serious infestations rare in comparison to *ex situ* systems. Subsampling of *Acropora* within these nurseries by mechanical screening can identify any widespread infestation that requires intervention, without massive labor expenditure to screen every *Acropora* within the nursery. Opportunities may exist in the future to treat coral nurseries *in situ* with anthelmintics in a similar manner to those used as off-label treatments in sea cage aquaculture, or alternatively facilitate the introduction of native biological control organisms. With permitting not yet established for extensive reef restoration in some countries (e.g., Australia), the approval of chemical treatments for *in situ* applications will likely have to wait until significant research is undertaken to understand the ecological consequences of these treatments in the context of coral reef environments.

IPM and research

While the goal of coral research is to answer research questions ethically, taking measures to prevent infestation of a research system is time consuming. Despite the allure of circumventing management protocols, it is in the best interest of the researcher to methodically care for their *Acropora* corals before and during experiments. It is recommended that experiments not be conducted with compromised corals, or those which are declining in health. Unlike *Acropora* colonies progressing through the ornamental supply chain, *Acropora* in research environments likely only need to acclimate to experimental conditions. This process should ideally start during the quarantine procedure after incoming corals are screened to prevent the introduction of pests into experimental systems. If components of *P. acroporae* IPM are missed, the researcher may expose corals to the risk of *P. acroporae* infestation, which may compromise the integrity of research results.

Mechanical screening of coral colonies before and after fragment generation can best mitigate the risk of infestations. While removal efficacy is reduced in larger coral colonies where pressurized water cannot effectively dislodge flatworms in some areas, the removal of flatworms from coral fragments is significantly more efficacious resulting in a greater than 95% removal rate (Barton et al. 2020). Therefore, confidence that specimens intended for experimentation are clean is increased when corals are screened as fragments. In the case of coral recruits used for research, the likelihood that they will be infested by *P. acroporae* is highly unlikely. The time at which they are at the greatest risk of infestation is during inoculation with Symbiodiniaceae, where it is common practice for new recruits to share aquaria with larger colonies to initiate endosymbiont infection (Petersen et al. 2008). In the case of older >3mo sexually propagated corals, the risk of infestation is purely from lateral transmission. If they are housed in 'clean' systems, the risk infestation is greatly reduced. If they are sharing their tanks with other *Acropora*, or they are plumbed into a common sump which connects them to other *Acropora* holding

systems, it is recommended that these corals are screened to ensure they are uninfested before transfer into experimental systems.

IPM and the coral collector

By the nature of wild collection, the coral collector will have the highest risk of handling infested coral colonies. As the first line of defense, these collectors bear the largest responsibility in ensuring that corals leaving their facility will be pest free. Infestation prevention should involve initial mechanical screening before prophylactic dipping of all incoming *Acropora* colonies. A strict quarantine procedure is recommended to ensure corals are pest free before sale onto wholesalers. While coral collectors may prophylactically dip their corals (no standard practices in place), the business model favors rapid turn-over of coral. Because of their sensitive nature, *Acropora* can quickly lose color in holding systems not designed for the long-term growth and survival of these animals. If the coral collector provides lackluster or 'dull' colored corals to wholesalers (Ellis and Ellis 2002), these corals are likely to sell for less onto the next wholesaler or local retail store. Essentially, the quicker *Acropora* move through the supply chain of the marine ornamental trade, the brighter they are likely to be when they reach the consumer. Thus, there is a lack of incentive for coral collectors to adequately quarantine stock before allowing it to enter the supply chain.

Currently coral collectors have nearly unlimited ability to collect *Acropora* in places like the Great Barrier Reef World Heritage Area by permit, where *Acropora* are covered under ton year^{-1} quotas (Pratchett et al. 2020). With the current, practically unlimited supply, it is likely that collectors will only prophylactically chemically immerse their corals rather than apply rigorous quarantine procedures, effort, and capital to maintain corals under ideal conditions. They can afford a certain level of loss as per unit it is relatively cheap to harvest, and they can just collect more. This practice is questionable,

especially in the face of continuous loss of coral cover and recent widespread bleaching on the Great

Barrier Reef. The landscape of governance around wild harvest would need to change considerably

moving forward to influence the practices of coral collectors and achieve sustainability. Perhaps, if or,

when coral fisheries begin to only grant licenses for collection of *Acropora* for aquaculture purposes

(e.g., broodstock establishment), collectors will be inclined to prioritize long term coral husbandry

(including the IPM of *P. acroporae*) as the price per unit of *Acropora* become more valuable.

IPM and the wholesaler

IPM in the wholesale setting presents similar problems to those which are faced by the coral collector in

terms of pest control. The wholesaler is at the mercy of the coral collector who provided the corals and

must not assume that corals come into their facility pest free. Quarantine and prophylactic dipping are

especially important here. Because of the large volume of corals moving through a given wholesale

facility, they are at risk of infestation from a variety of pest species coming from suppliers worldwide.

Any given wholesaler likely receives corals from different countries worldwide, and even multiple

suppliers from within those countries. If proper quarantine and treatment protocols are not observed

here, there is a high risk of lateral infestation. Corals which come into a facility pest free only require

exposure to *P. acroporae* individuals from one hatched egg capsule to potentially become infested.

Furthermore, they may not show signs of infestation until they reach the next line in the ornamental

supply chain (Figure 7-2). Because *P. acroporae* can lay egg capsules on any clean exposed substrate, egg

capsules can be deposited cryptically on racks holding corals for sale.

Similarly, to the coral collector, the coral wholesaler is in the business of selling colorful corals but

generally not growing them. *Acropora* require more rigorous husbandry and associated equipment to

prevent devaluation through loss of color and maintain optimal health. Another financial consideration

is that while *Acropora* are sold in high volume, they only sell for ~$20 USD per medium colony fragment

(~8 cm diameter) compared to *Homophyllia,* which sell for considerably more (between $50 and $300

USD), occupy a smaller space (4-7 cm diameter) in holding systems, and are much more resilient to

variable holding conditions. As it stands with wild collection of *Acropora*, the wholesaler has no

incentive to observe any quarantine protocols outside of a simple prophylactic treatment.

In contrast to the sale of wild corals, *Acropora* corals which are cultured commercially and not simply

fragmented, as they move through the supply chain to consumers (common for wild corals to be

fragmented into many pieces and marketed as cultured), there is financial incentive to ensure that pests

are managed in order to protect donor colonies and fragment grow out in aquaculture systems. Unlike

the trade in ornamental fish (freshwater and marine), it is less common that countries impose

biosecurity protocols on corals entering a country which could bear any number of pests.

There is potential within coral aquaculture for the use of biocontrols to have a mitigating effect on *P.*

acroporae abundance during the potentially brief stay of *Acropora* in facilities. *Pseudocheilinus*

hexataenia are constantly grazing and would benefit coral holding systems by consuming

Prosthiostomum. acroporae which they encounter. While these should be limited to one per holding

tank <1000L, they can mitigate the impacts of *P. acroporae* by significantly reducing numbers to levels

that have minimal impact on coral health. Peppermint shrimp could also be beneficial in a similar

manner. Suspected infested corals can be placed in small aquaria holding several peppermint shrimps

for incidental cleaning to occur. Because peppermint shrimp also consume *Aiptasia* anemones, they also

make good candidates as grazers in coral holding runways, where they can forage on anemones or *P.*

acroporae egg capsules encountered.

IPM and socioeconomic status

Coral aquaculture facilities are unlikely to be the same, which is highlighted by the variability of operations which occur across different socioeconomic regions (Salayo et al. 2012). For instance, coral farmers growing high volumes of corals in lagoons to sell to domestic wholesalers are less likely to have the same infrastructure support as a western operation with a large budget for top of the line equipment to produce a smaller amount of high value fragments grown *ex situ*. While growers may have every intention of ensuring all *in situ* aquacultured corals are pest free, it remains highly unlikely that they would allow their profit margin to be affected by the additional cost of screening and quarantine of corals in captivity. This is especially true for *Acropora* which will quickly deteriorate in non-optimal conditions. Coral aquaculture taking place in low socioeconomic regions may not have the resources to prophylactically immerse corals in chemicals like praziquantel or levamisole HCl because of the additional costs of these treatments. In such operations, mechanical screening is the most practical means of pest screening or removal and could be achieved through using simple tools such as a garden pressure sprayer or squirt bottle.

IPM and the local fish store

Local fish stores (LFS) or aquarium shops, are effectively the last line of defense for the prevention of pests associated with corals before the hobbyist receive them. The origin of corals sold in LFS are variable globally, with both wild and aquacultured specimens available (Rhyne et al. 2012; 2014; Tlusty et al. 2013. Depending on the business model of the operation, the LFS may sell wild corals as quickly as possible to limit the operational costs per coral sold (labor and utilities) and reduce the risk of corals losing color while in store. In such cases, pest control will likely be limited to the prophylactic chemical treatment of corals and perhaps the inclusion of biological controls. In contrast, some LFS will supply

locally propagated corals and propagate some proportion of their corals sold in store (within or outside

retail areas). While it would be ideal for all LFS to have dedicated quarantine systems, LFS which

prioritize propagation as part of their business model have more financial incentive to allocate resources

to pest control for the long-term wellbeing of corals. While staff may not be hired with these husbandry

skills, the education of these to quickly identify signs of *P. acroporae* infestation is independent to the

origin of sold coral and is recommended as a priority to prevent and quickly respond to infestations if

they do occur. Similarly, while hobbyists learn coral husbandry practices from a variety of sources

(Facebook, online forums, personal experience, etc.), interface with educated staff and sharing of pest

control methods could reduce the incidence of *P. acroporae* infestations at the hobbyist level.

IPM and the hobbyist aquarium

The lack of incentive for quarantine protocols for *Acropora* throughout the supply chain of corals in the

marine ornamental trade underlines the importance of quarantine and pest control at the hobbyist

level. Even if corals are prophylactically dipped at every step of the supply chain, it only takes one egg

capsule to seed an infestation at one of these levels. Hobbyists must acknowledge the risk they take

every time a new *Acropora* specimen is introduced into their aquarium without quarantine. While

quarantine systems for corals may be commonplace in research facilities and aquaculture operations, it

is quite uncommon for reef aquarium hobbyists to have them. While a variety of factors may influence

the absence of quarantine systems, the cost of running a dedicated quarantine system and the space

requirement of such a system are likely contributing factors. Another factor is a general lack of patience

in the marine ornamental trade. Hobbyists often want to put their new coral fragment in their aquarium

and immediately share a picture to social media. This culture can foster a lack of patience at the

individual level which may not be beneficial to pest control efforts. Additionally, there is little emphasis

on education of the practice of sound biosecurity and general pest management in the marine

ornamental trade to ensure sustainability, by mitigating the unnecessary mortality of infested corals.

While the appetites of the reef hobbyist will vary, a quarantine system does not have to be large to

accommodate the biosecurity needs of the general coral enthusiast.

Relatively simple and cost-effective options for a hobbyist quarantine system are the variety of all-in-

one aquarium sold worldwide. The limited footprint combined with a comparatively low financial

expenditure (compared to larger systems) required to provide adequate light to all-in-one cubes are

relatively minimal. If quarantine is not practiced and infestation does occur, the control of *P. acroporae*

in the hobbyist's aquarium can be more difficult than treatment in aquaculture applications. By nature,

hobbyists look to create their vision of a coral reef which almost invariably includes the use of rocky

substrates (harvested reef rock or artificial). The use of 'live rock' as coral substrate makes it difficult to

ensure that all living *Acropora* tissue is removed from the aquarium when removing corals for chemical

immersion treatment. In the case of removal for chemical immersion, one may be effective at removing

flatworms from the removed *Acropora* colony, but tissue that encrusts on the rock is often very difficult

to remove. The consequence of this residual tissue is that it provides a refuge for *P. acroporae* egg

capsules and individuals. After coral colonies are returned to their aquarium following treatment, the

likelihood of lateral transmission is quite high if egg capsules or individuals remain on living tissue.

Propagation systems are comparatively easier to treat because these systems generally do not rely on

live rock as a substrate for *Acropora* attachment, instead using a variety of substrates to maintain donor

colonies, coral fragments, or recruits. These include a variety of plastics, PVC, fiberglass reinforced

plastic (FRP), aragonite and ceramics. The benefit of these substrates is they allow the entire coral to

enter either chemical dips, be isolated, or biocontrol reactors (peppermint shrimps) with considerably

reduced risk of leaving behind infested tissue or egg-bearing skeleton in their respective systems. Until

in situ or 'in tank' treatments are developed and validated, treating *P. acroporae* outbreaks in hobbyist

aquaria will continue to be challenging. The biodiversity within these aquariums may be visually

appealing, but the sensitive invertebrates which comprise this diversity must be considered when

developing in tank treatments to avoid adverse consequences (e.g. unintended mortality) to these

organisms.
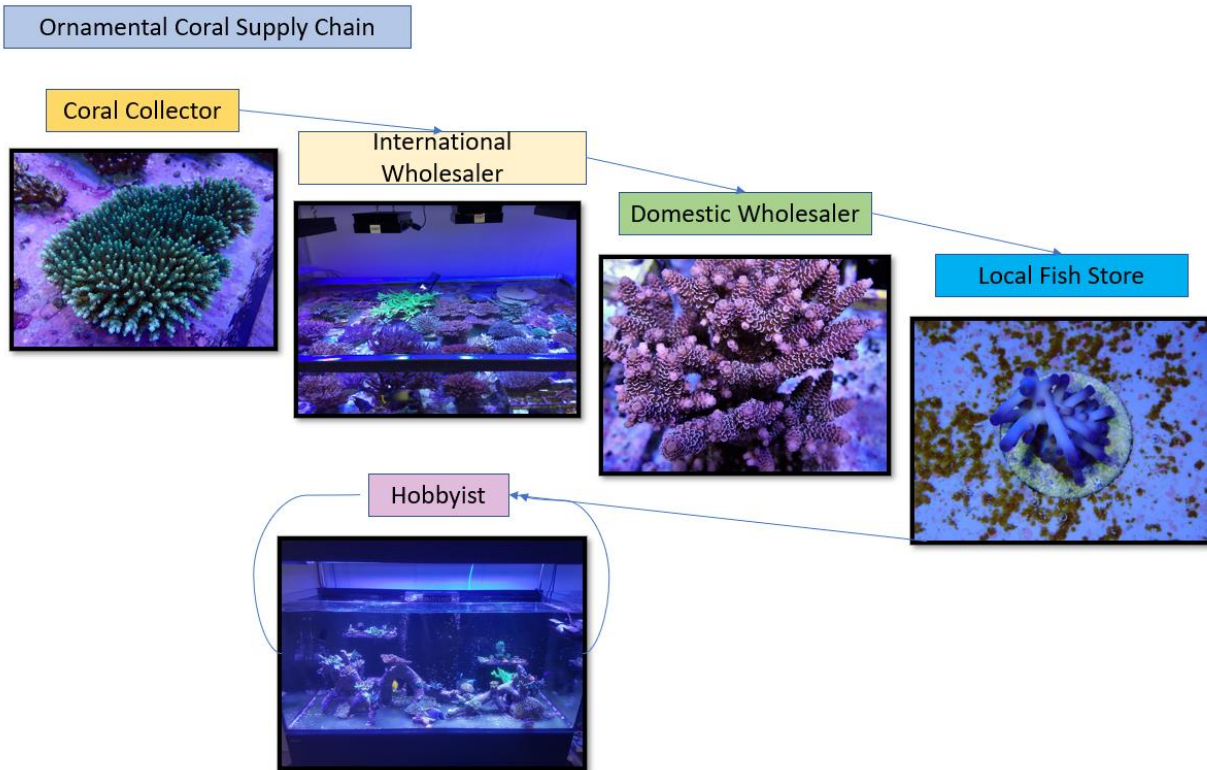
Figure 7-2: A simplified example of the ornamental supply chain of *Acropora* collected from the Greater Barrier

Reef for the ornamental trade.

Free Support Tools (Mobile Application)

To provide educational information and real time treatment interval durations, we developed a web

application using ShinyApp in Rstudio (Figure 7-4). In this application, users can input the temperature of

their aquarium system and are given recommended treatment intervals based on the relationship

between temperature and *P. acroporae* development (as described in Chapter 3). Additionally,

photographs of *P. acroporae* egg capsules, feeding scars, and individuals on host *Acropora* to assist

stakeholders in infestation detection. The application is in a beta phase, with more information and

aesthetics to follow in the future. The application is hosted at the following link:

https://cawthron.shinyapps.io/acropora_eating_flatworm/
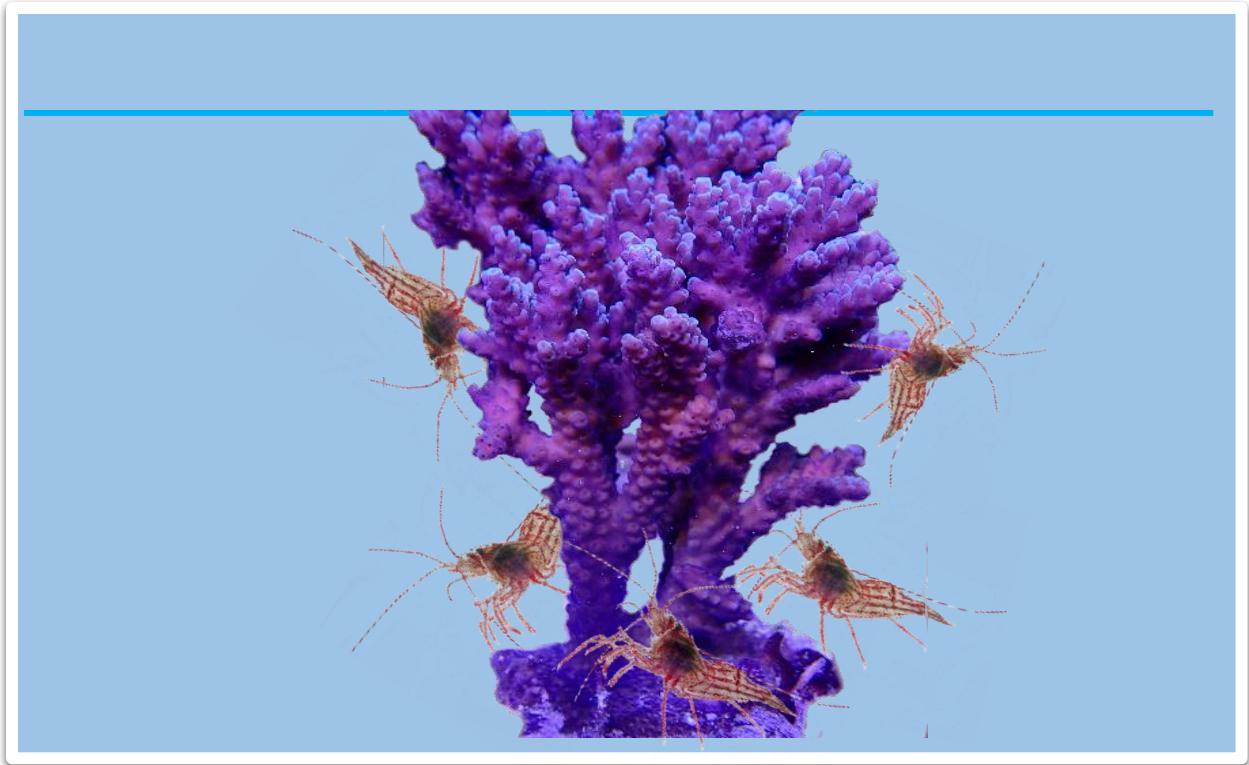
Figure 7-3: Shrimp treatment chamber housing infested *Acropora* colony and peppermint shrimp *Lysmata vittata* to consume *Prosthiostomum acroporae* egg capsules and individuals from infested colony.

Figure 7-4. Screenshot showing homepage of a web-based decision support tool for the diagnosis, mitigation and treatment of *Acropora*-eating flatworms in captivity.

In conclusion

This thesis examined evidence of coral-associated invertebrates causing harm to captive scleractinian corals, and identified which key characteristics make these organisms harmful; the direct consumption of coral tissue, association with disease or colonial mortality, high reproductive capacity, and evidence that their populations proliferate in captive environments. We demonstrated the *Acropora*-eating flatworm *Prosthiostomum acroporae* to be particularly harmful to coral health, often associated with colonial mortality of infested hosts, and sought to address the considerable knowledge gaps to inform management decisions. Our examination of the life cycle of *P. acroporae* provided this data to inform treatment intervals and fallowing periods required to break their life cycle in captivity. Furthermore, the widespread finding of *P. acroporae* along the Great Barrier Reef and a related polyclad afflicting *Montipora* spp. suggested to be *Prosthiostomum montiporae*, alert stakeholders to the high likelihood of introduction of this pest to captive systems in the absence of management protocols. We validated the use of both biological control organisms (*Lysmata vittata* and *Pseudochielinus hexataenia*) and chemical treatments (levamisole HCl and praziquantel) as part of the management toolbox to treating *P. acroporae* infestation in captive systems. The results of this thesis are synthesized as the first management guidelines for a coral pest in captivity, which can serve as a management framework for coral pests in captivity moving forward, whether for coral collectors, coral aquaculture, or coral reef hobbyists moving forward.

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Supplementary Material

Chapter 3

The Supplementary Material for this article can be found online at:

https://www.frontiersin.org/articles/10.3389/fmars.2019.00524/full
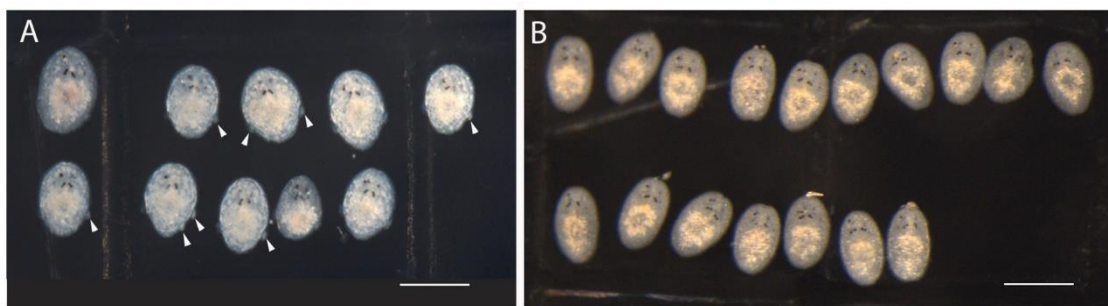
Figure A-1 Variation in *Prosthiostomum acroporae* hatchling morphology between two egg clusters at 27 C. (A)

Hatchlings have reduced lobes (arrowheads); (B) hatchlings have presumably re-absorbed their lobes (scale = 100

mm).
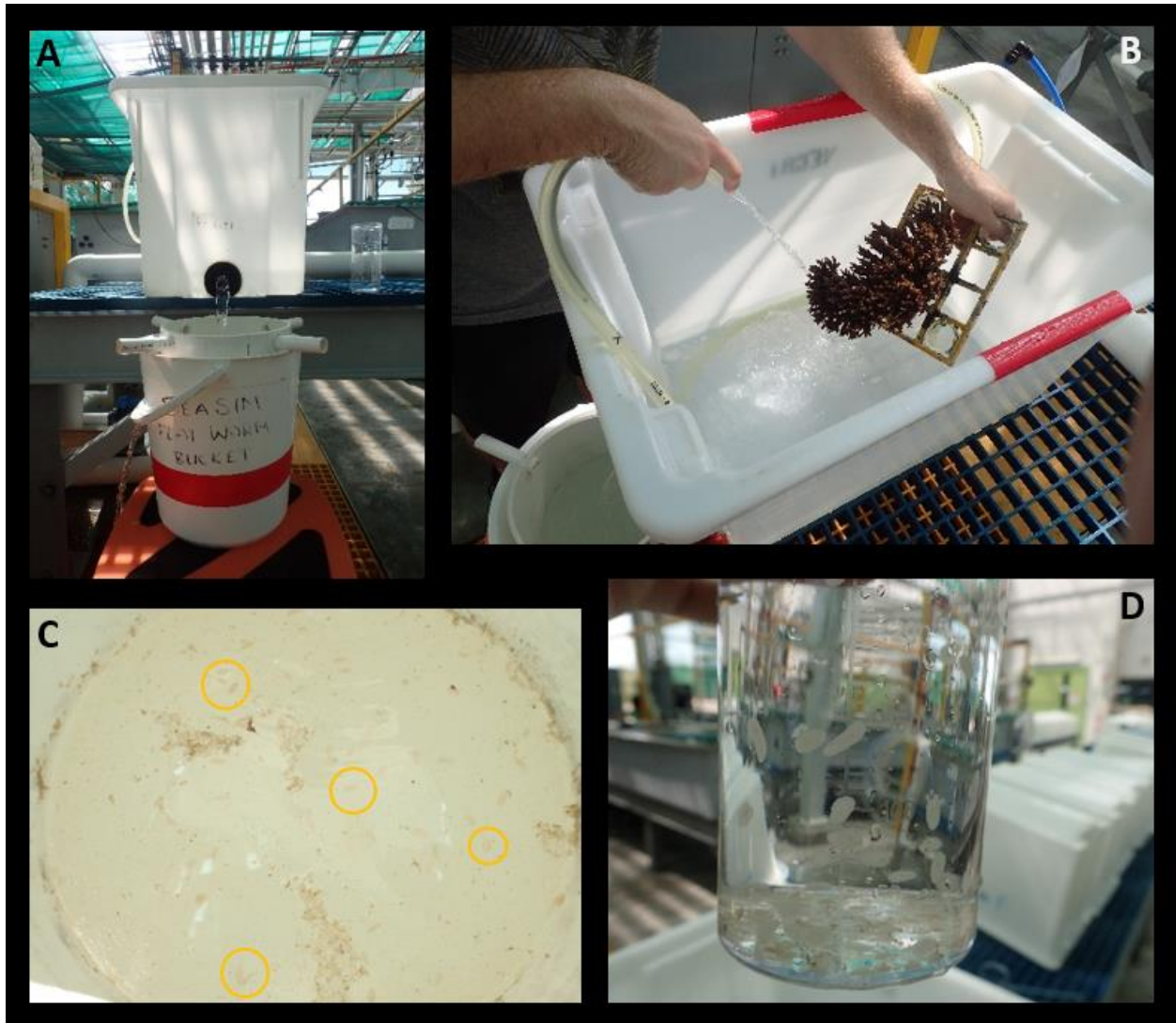
Figure A-2 Prosthiostomum acroporae screening method. (A) Photo of the entire screening system [Nally bin, 300-micron PVC screening chamber (arrow), and 20 L bucket]. (B) Inside the Nally bin, a jet of filtered seawater is used to physically remove Prosthiostomum acroporae from the surface of an Acropora colony. (C) 300 mm PVC screening chamber full of P. acroporae; with representative specimens circled. (D) >100 P. acroporae transferred into a plastic beaker for experimentation.

Figure A-3 To maintain Prosthiostomum acroporae culture numbers plastic was attached to coral using plastic pegs to provide substrate for egg-laying.

Chapter 4

Table A1: Details of all flatworms collected from wild and captive corals that were preserved in either 95% ethanol (EtOH) or 4% frozen paraformaldehyde

(PFA) for molecular or morphological analyses, respectively.

Specimen	Collection site	Coral host	Wild or Captive	Coordinates (if wild)	Temperature (°C)	Collection Date	Preservation	GenBank ID	Notes
P. acroporae 1	SeaSim	*Acropora millepora*	C	X		4/4/2016	EtOH	MT668955	Infestation in coral system for several months
P. acroporae 2	SeaSim	*Acropora millepora*	C	X		4/4/2016	EtOH	MT668963	Infestation in coral system for several months
P. acroporae 3	SeaSim	*Acropora millepora*	C	X		4/4/2016	EtOH	MT668964	Infestation in coral system for several months
P. acroporae 4	SeaSim	*Acropora loripes*	C	X		4/4/2016	EtOH	MT668957	Infestation in coral system for several months
P. acroporae 5	West Pelorus Island	*Acropora* sp.	W	18°33'14.5"S 146°29'18.4"E	27.8	1/5/2016	EtOH	MT668966	5-10m depth
P. acroporae 6	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668958	Samples taken from *P. acroporae* cultures
P. acroporae 7	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668965	Samples taken from *P. acroporae* cultures
P. acroporae 8	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668967	Samples taken from *P. acroporae* cultures
P. acroporae 9	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668968	Samples taken from *P. acroporae* cultures
P. acroporae 10	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668969	Samples taken from *P. acroporae* cultures
P. acroporae 11	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668959	Samples taken from *P. acroporae* cultures

P. acroporae 12	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668960	Samples taken from *P. acroporae* cultures
P. acroporae 13	SeaSim	*Acropora millepora*	C	X		4/4/2018	EtOH	MT668971	Samples taken from *P. acroporae* cultures
P. acroporae 14	Backnumbers Reef	*Acropora millepora*	W	18°29'13.5"S 147°10'31.1"E	26.3	5/12/2017	EtOH	MT668961	5-10m depth
P. acroporae 15	Geoffrey Bay, Magnetic Island	*Acropora tenuis*	W	19°9' 7.2648" S 146° 52' 32.466" E	28.0	22/11/2017	EtOH	MT668962	Longer than 10mm
P. acroporae 16	Geoffrey Bay, Magnetic Island	*Acropora tenuis*	W	19°9' 7.2648" S 146° 52' 32.466" E	28.0	22/11/2017	EtOH	MT668972	Longer than 10mm
P. acroporae 17	Geoffrey Bay, Magnetic Island	*Acropora tenuis*	W	9°9'7.2648" S 146° 52' 32.466" E	27.4	3/11/2016	EtOH	MT668954	Regular size 4-5mm
P. acroporae 18	Trunk Reef	*Acropora millepora*	W	18°23'20.4"S 146°48'25.8"E	28.0	16/6/2016	EtOH	MT668970	5-10m depth
P. acroporae 22	Little Pioneer Bay Orpheus Island	*Acropora spathulata*	W	18°35'53.6"S 146°29'25.0"E	27.8	1/5/2016	EtOH	MT668973	5-10m depth
P. acroporae 24	Pioneer Bay, Orpheus Island	*Acropora* sp.	W	18°36'18.7128" S 146°28'59.7792" E	27.6	2/5/2016	EtOH	MT668956	5-10m depth
Prosthiostomum sp. 1	SeaSim	*Montipora digitata*	C	X		10/7/2018	EtOH	MT668974	Approximately 7 months in captivity
Prosthiostomum sp. 2	SeaSim	*Montipora digitata*	C	X		10/7/2018	EtOH	MT668974	Approximately 7 months in captivity
Prosthiostomum sp. 3	SeaSim	*Montipora digitata*	C	X		10/7/2018	EtOH	MT668974	Approximately 7 months in captivity
Prosthiostomum sp. 4	Pioneer Bay, Orpheus Island	*Acropora* sp.	W	18° 36' 18.7128" S146° 28' 59.7792" E	27.6	2/5/2016	EtOH	MT668975	< 5 m of water; large individual >11 mm
Prosthiostomum sp. 6	Coral collection facility,	*Montipora tuberculosa*	C	X	26.8	14/1/2019	EtOH	Failed sequence	Infested fragments in coral growout system for > 7 weeks,

	Mackay, Australia								subsequent mortality of fragments
Prosthiostomum sp. 5	Coral collection facility, Mackay, Australia	*Euphyllia glabrescens*	C	X	28.2	23/11/2018	EtOH	MT668976	No feeding scars or egg cluster present on *E. glabrescens*
Prosthiostomum sp. 6	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 7	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 8	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 9	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 10	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 11	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 12	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 13	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 14	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomum sp. 15	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity
Prosthiostomid z 1	SeaSim	*Montipora digitata*	C	X		10/7/2018	EtOH	MT668977	Approximately 7 months in captivity
Prosthiostomid z 2	SeaSim	*Montipora digitata*	C	X		10/7/2018	EtOH	MT668977	Approximately 7 months in captivity
Prosthiostomid z 3	SeaSim	*Montipora digitata*	C	X		10/7/2018	PFA		Approximately 7 months in captivity

| Prosthiostomid z 4 | SeaSim | *Montipora digitata* | C | X | | 10/7/2018 | PFA | | Approximately 7 months in captivity |
| Prosthiostomid z 5 | SeaSim | *Montipora digitata* | C | X | | 10/7/2018 | PFA | | Approximately 7 months in captivity |

Table A-2: A list of the ingroup (Prosthiostomidae) and outgroup (Cotyleans/Acotyleans) 28s rDNA sequences of

Polycladida from GenBank used for phylogenetic analysis.

Taxa	Organism	Source	Accession #
Ingroup *Prosthiostomids*	*Enchiridium evelinae* Marcus, 1949	Brazil	KY263683
Cotyleans	*Enchiridium periommatum* Bock, 1913	Panama	MH700300
	Enchiridium sp.	Heron Island, Great Barrier Reef, Australia	MH700303
	Echiridium japonicum Kato, 1943	Eilat, Israel	MH700298
	Euprosthiostomum mortenseni	Parish, Jamaica	MH700304
	Prosthiostomum lobatum	Florida, USA	MH700372
	Prosthiostomum sp.	Eilat, Israel	MH700375
	Prosthiostomum siphunculus	Mataró, Spain	HQ659012
	Prosthiostomum cynarium	St. John, US Virgin Islands	MH700371
	Prosthiostomum utarum	Carmabi Beach, Piscadera Baai, Curacao	MH700377
	Prosthiostomum milcum	Old Dan Bank, Long Key, Florida, USA	MH700373
	Prosthiostomum purum	Gulf of Aqaba, northern Red Sea,	MH700374
	Prosthiostomum vulgaris	Japan	LC100091
	Prosthiostomum trilineatum	Ritidian Point, Guam	MH700376
	Prosthiostomum grande	Ebisu Island, Japan	LC100090.1
	Prosthiostomum torquatum	Tanabe Bay, Japan	LC504234.1
	Prosthiostomum acroporae	Lizard Island, Austrlia	JQ791553
	Prosthiostomum acroporae	New York, UsaA	HQ659010
Outgroup	*Prostheceraeus crozieri*	Long Key, Fl, USA	HQ659013
Cotyleans	*Pericelis cata*	?	EU679114
	Cestoplana rubrocincta	Phillip Island, Australia	HQ659009
Acotyleans	*Hoploplana californica*	California, US,	KC869850
	Imogine zebra	Massachusetts, USA	MH700313
	Notoplana atomata	New Hampshire, USA	MH700330

Table A-3: Comparison of morphology of *P. montiporae* (as described by Poulter 1975) and Prosthiostomum sp.

Morphological character	MEFW (Hawaii)	Notes
Eye Arrangement	Paired cerebral eye clusters form roughly coniform groups (~22 per group); Marginal eyes form a band which extends posteriorly to be level with first cerebral eyes.	Paired cerebral eye clusters form roughly coniform groups (~18 per group); Difference in number of eyes per group is thought to be size dependent.
Pharynx	Barrel-shaped pharynx occupies a length equivalent to ~10 percent of body length and lies 12% of the body length behind the anterior margin. Pharynx is a tubular structure modified by a deep longitudinal cleft, overlapping edges rolled loosely, scroll-like; the base of the cleft terminates in a flat helical pattern.	Pharynx length when unfurled not measured but the morphology appears to be the same.
Sucker	Sucker 3.9 mm (42 percent of body length) behind anterior margin; diameter, 0.3 mm. Epithelium 30 ftm in height. Muscular development of the sucker surpasses that of the adjacent body wall.	
Male reproductive structures	Ventral orientation of testes, with spermiducal vesicles forming rows, one on each side of the main intestine, posterior to the male copulatory apparatus	
	Medially directed sperm ducts join each respective row of spermiducal vesicles, with a terminal spermiducal vesicle lying anterior to and on each side of the ovate seminal vesicle.	Medial direction of sperm ducts could explain not seeing them clearly in saggital section.

A sperm duct passes posteriorly from each terminal spermiducal vesicle, penetrates the muscle wall of the seminal vesicle, proceeds posteriorly just under the luminal epithelium, and opens independently into the posterior portion of the seminal vesicle. Seminal vesicle ovate. The wide ejaculatory duct issues from the dorsoanterior extent of the seminal vesicle. It narrows anteriorly, detours one accessory vesicle, and enters the penis papilla.

The two orbiculate accessory vesicles are stacked one atop the other; they are apposed but not bound: The ventral vesicle lies adjacent to the ventroanterior face of the seminal vesicle, apposed but not bound. A duct from the lumen of each accessory vesicle passes anteriorly through the nonnucleated hull, roughly paralleling the ejaculatory duct, and becomes confluent with the ejaculatory duct in the penis base.

The penis appears to lie in a penis pouch but is not protruding into the male atrium.

Terminal portion of male system located close behind posterior termination of pharynx. Male gonopore 2.4 mm (26 percent of body length) behind anterior margin. Length of terminal portion of male system including seminal vesicle to penis pouch,

Female reproductive structures

Ovaries dorsal, but maturing ova may occupy the entire dorsoventral space between the musculature of the body wall. The uteri lie on each side of main intestine anterior and posterior to the female copulatory apparatus, joining posteriorly under the main intestine at 65 percent of body length from the anterior margin.

An oviduct from each uterus converges anterior to the terminal portion of female copulatory apparatus, forming a large common ova fill-chamber that joins the vagina at its most ventroposterior extent. Vaginal epithelium tall, thicker in close proximity to the uterus, with extremely long cilia.

Vagina opens into dorsoventrally compressed cement pouch. The cement pouch receives extensive cement gland secretion, opens by a narrow aperture into the female antrum (also with a pouch) which is broader and more compressed than usual. Female gonopore 60 ftm in diameter, located 3.0 mm (32 percent of body length) behind the anterior margin equidistant between male gonopore and sucker.

Supplementary Methods

Screening acroporid corals for the *Acropora*-coral eating flatworm *Prosthiostomum acroporae*

Flatworms were removed from coral using a jet of filtered seawater which mechanically removes *P. acroporae* individuals (Figure A-4B; method adapted from Rawlinson and Stella, 2012). This method exhibited >95% efficiency following screening using a blind survey of infested coral fragments. In brief, a total of fifty flatworms were placed in known numbers on five coral fragments and were then screened and counted by a person with no prior knowledge of the number of worms per fragment. During screening, corals were held within a Nally bin (60 x 39 x 37 cm) (Figure A-4B) which drained into a 20L bucket containing a fabricated PVC screening chamber with 300 μm mesh (Figure A-4C). The mesh of the PVC screening chamber was positioned below the water level in the 20L bucket to prevent exposure of specimens to air. Once removed from the coral, flatworm specimens were dislodged from the surface of

the Nally bin or mesh with a directed jet of water from a transfer pipette and transferred to a container

with fresh seawater using a second, wide-mouthed transfer pipette.

Appendix B

Associated Literature

Publication completed immediately prior to the commencement of my candidature:

Barton JA, Willis BL, Hutson KS (2017) Coral propagation: a review of techniques for ornamental trade and reef restoration. *Reviews in Aquaculture* 9: 238-256. doi:10.1111/raq.12135

Coral propagation: a review of techniques for ornamental trade and reef restoration

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Abstract

Aquaculture of coral offers an alternative to wild harvest for the ornamental trade and shows considerable promise for restoring reefs and preserving biodiversity. Here, we compare advantages and disadvantages of asexually derived fragments versus sexually derived propagules and *in situ* versus *ex situ* nursery phases for the ornamental trade and reef restoration. Asexual propagules, sourced from a donor coral colony that is cut into smaller parts and attached to artificial substrate, are most commonly used. The most suitable corals are typically branching species, although fragments from species with other growth forms can be successful, albeit slower growing. Sexually derived propagules are collected from the wild or from colonies in aquaria during spawning, with an artificial substrate provided for settlement. The timing of spawning is known for many broadcast spawning corals, but opportunities for collection of gametes are generally limited to only once or a few times per year. Brooding species with multiple periods of larval release provide better options for culture of sexually derived propagules. Propagation techniques have developed considerably over the past 20 years, yielding faster growth rates, reduced mortality and reduced detachment from substrates. Simple and cost-effective propagation techniques can be used to restore denuded reefs, preserve endangered species, provide live corals to the international ornamental trade, enable livelihood diversification for coastal communities and provide experimental materials for marine research. This review provides a comprehensive synthesis of recent developments in aquaculture propagation techniques for the purpose of ornamental trade and coral reef restoration, including asexual and sexual propagation, nursery and transplantation stages.

Key words: coral aquaculture, coral fragments, coral propagation, coral spawning, ornamental trade, reef restoration.

Introduction

Coral reefs are the largest living structures built by modular, colonial organisms in the world. Corals are home to incredible biodiversity and are paramount in providing the structural habitat, food sources and settlement cues that many marine organisms depend on to survive and reproduce (Stella *et al.* 2011). The structural complexity of coral reefs is positively associated with biomass and density of fish species (Graham & Nash 2013) and plays a significant role in mitigating the effects of habitat disturbances on the structure of reef fish communities (Emslie *et al.* 2014).

Corals (Anthozoa: Cnidaria: Scleractinia) typically represent colonies comprised of many individual polyps. Colonies grow by budding new polyps, a process of modular iteration. Over many generations, a scleractinian coral colony creates a large calcium carbonate skeleton that is characteristic of the taxa. The majority of corals breed sexually by broadcast spawning (Baird *et al.* 2009), and typically colonies of the same species release gametes simultaneously over a period of one to several nights following a full moon, generally in late spring (Babcock *et al.* 1986). Although some corals catch plankton and small fish using nematocysts or trap particulate matter through mucus or