



# The Life Cycle of the *Acropora* Coral-Eating Flatworm (AEFW), *Prosthiosomum acroporae*; The Influence of Temperature and Management Guidelines

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As coral aquaculture is increasing around the world for reef restoration and trade, mitigating the impact of coral predators, pathogens and parasites is necessary for optimal growth. The *Acropora* coral-eating flatworm (AEFW), *Prosthiosomum acroporae* (Platyhelminthes: Polycladida: Prosthiosomidae) feeds on wild and cultivated *Acropora* species and its inadvertent introduction into reef tanks can lead to the rapid death of coral colonies. To guide 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.

**Keywords:** AEFW, *Acropora*, flatworms, reef restoration, coral aquaculture, pest management

## 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* spp. 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 (Tlustý 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* spp. 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 *Prosthiosomum 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). *Prosthiosomum acroporae* is a polyclad flatworm (Platyhelminthes: Polycladida) belonging to the sub-order Cotylea and family Prosthiosomidae. 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).

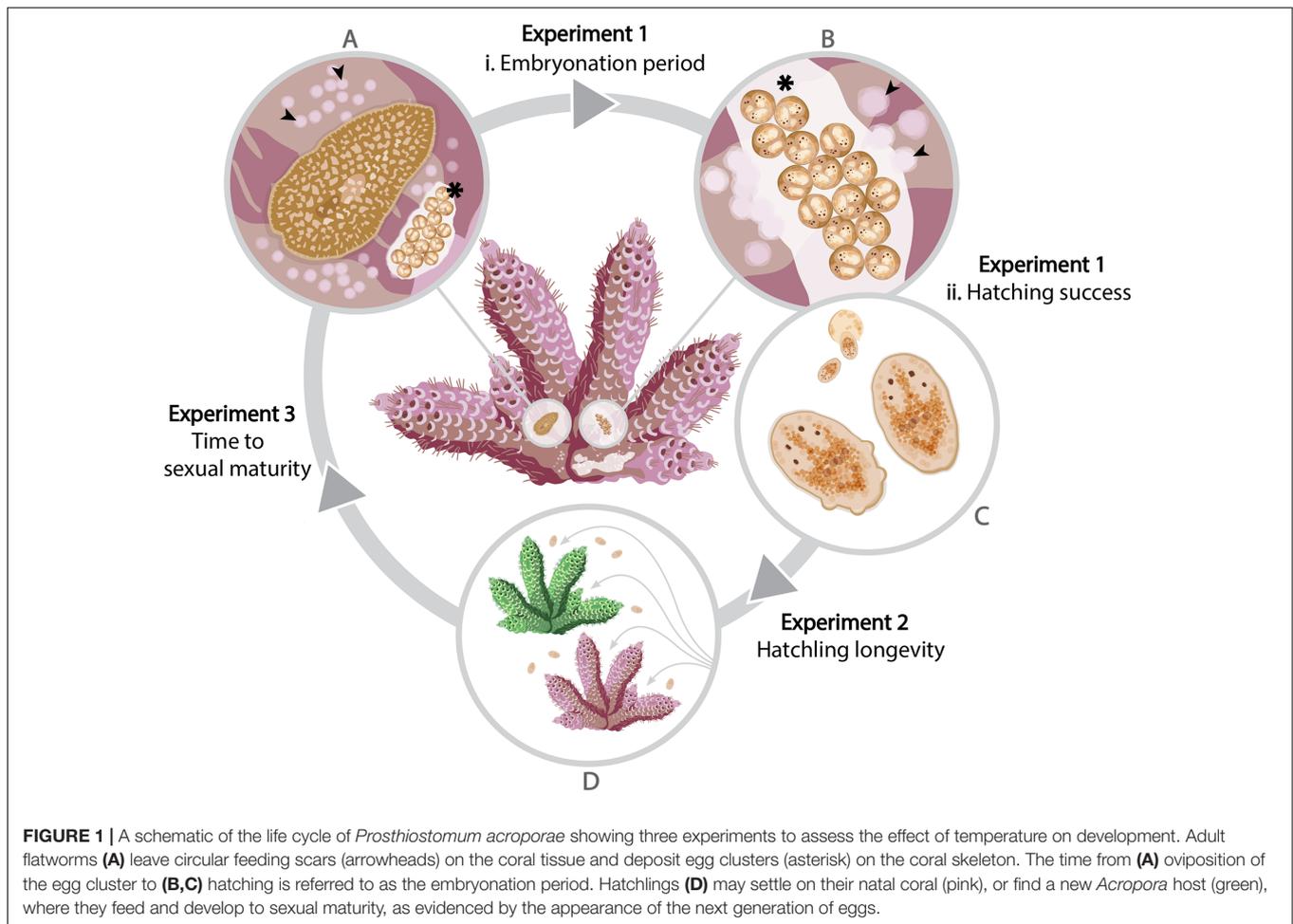
There have been no empirical studies on the management of *P. acroporae* in captivity or in the wild. Effective management of this species requires a comprehensive understanding of its life cycle so that vulnerable life stages can be identified and eradicated through appropriate treatments. The developmental 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 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

### *Prosthiosomum acroporae* Culture

*Prosthiosomum 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° 46.420' S 146° 31.372' E) and mid-shelf reefs (Trunk Reef 18°23'20.4" S 146°48'25.8" E, Davies Reef 18°49'21.6" S 147°39'12.5" E and Rib Reef 18°28'47.1" S 146°52'00.9" 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<sup>-1</sup>. 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 co-culturing flatworms and corals).

*Prosthiosomum acroporae* lay their eggs on bare coral skeleton (**Figure 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 (Sandvik® plastic bags [127 mm × 200 mm]) containing filtered seawater (1 μm). 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

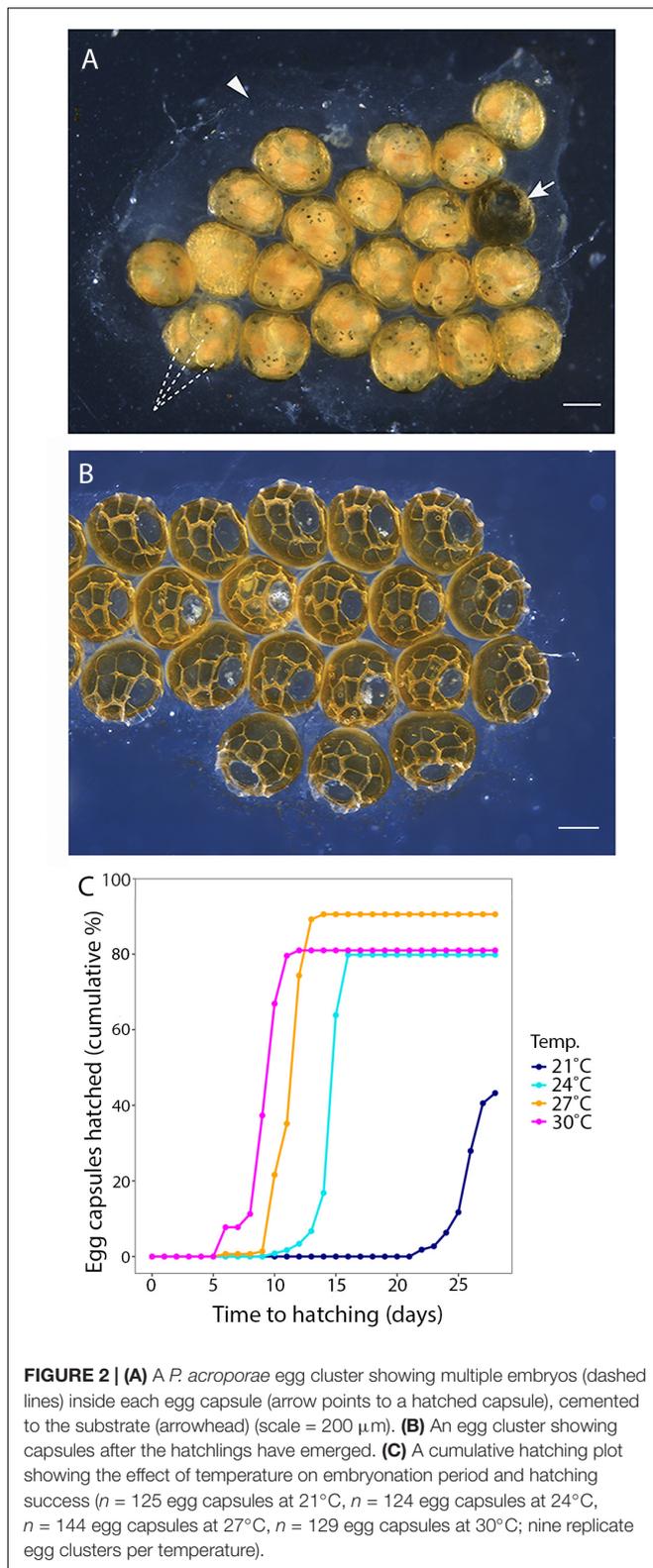
from a single parent. Extraneous egg capsules (not covered by a continuous cement layer) were removed.

## 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)]. 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<sup>1</sup>. 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

<sup>1</sup>weather.aims.gov.au



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  $24 \pm 2 \mu\text{mole m}^{-2} \text{s}^{-1}$  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 post-oviposition 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  $\mu\text{m}$ ) incubated in tanks at 24, 27, or 30°C ( $\pm 0.2^\circ\text{C}$ ), 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.

## 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 1**). Uninfested coral fragments for this experiment were prepared from an *A. millepora* colony collected from

Davies Reef (18°49'21.6" S 147°39'12.5" 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  $24 \pm 0.2$ ,  $27 \pm 0.2$ , or  $30 \pm 0.2^\circ\text{C}$ . 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 bath). 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  $0.8^\circ\text{C}$  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 ( $\sim 4$  mm) to expose the coral skeleton and provide substrate for *P. acroporae* to deposit eggs. Each infestation chamber was fitted with  $60\ \mu\text{m}$  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 +10 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 "nlme" (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  $21\text{--}30^\circ\text{C}$ . 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  $21\text{--}30^\circ\text{C}$ . Similarly, the linear mixed effects model was used to estimate time to sexual maturity at temperatures  $21\text{--}30^\circ\text{C}$  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 re-infestation 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 [ $p < 0.0001$ ;  $1.76 \pm 0.16$  (coefficient  $\pm$  SE); COXME; **Figure 2C**]. Pairwise comparison reveals significant differences between all temperature treatments ( $p < 0.0001$ ). At 21, 24, 27, and  $30^\circ\text{C}$  mean embryonation period for *P. acroporae* egg capsules was 26, 15, 11, and 9 days, respectively (**Figure 2C** and **Table 1**). The first and last day of capsule hatching in all egg clusters at a given temperature is shown in **Figure 2C** and **Table 1**. Predicted hatching probability curves using experimental embryonation data (Kaplan–Meier survival estimates) suggest that the embryonation

**TABLE 1** | Life cycle parameter table for *Prosthlostomum acroporae*.

Temperature (°C)	FH/LH (days) (mean ± SE)	HL (days)	S <sub>min</sub> (days)	S <sub>mean</sub> (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

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, S<sub>min</sub> – minimum time to sexual maturity; S<sub>mean</sub> – mean minimum time to sexual maturity ± SE; LC – minimum time to completion of life cycle (FH + S<sub>min</sub>).

**TABLE 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

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

Temperature also had a significant influence on the hatching success of *P. acroporae* from their egg capsules ( $p < 0.05$ , Chi-squared test; Figure 2C). Pairwise comparison demonstrated that the hatching success of worms from egg capsules in the 21°C treatment [ $43 \pm 4\%$  (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 ( $80 \pm 4\%$ ), 27°C ( $91 \pm 2\%$ ), and 30°C ( $81 \pm 3\%$ ) 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  $14 \pm 1.3$  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.

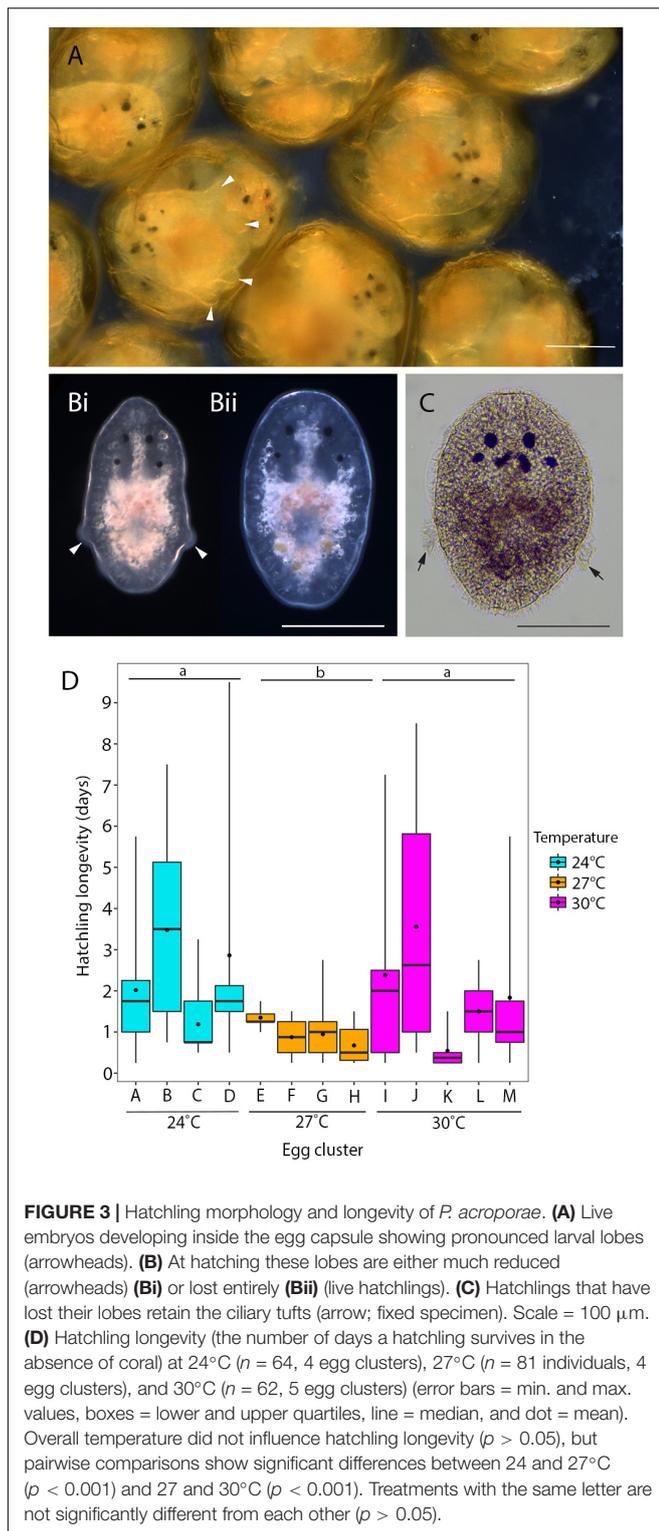
## Experiment 2 – Hatchling Longevity and Morphology

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 Figure 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  $2 (\pm 2.12)$ ,  $1 (\pm 0.52)$ , and  $2 (\pm 2.27)$  at 24, 27, and 30°C respectively (Table 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).

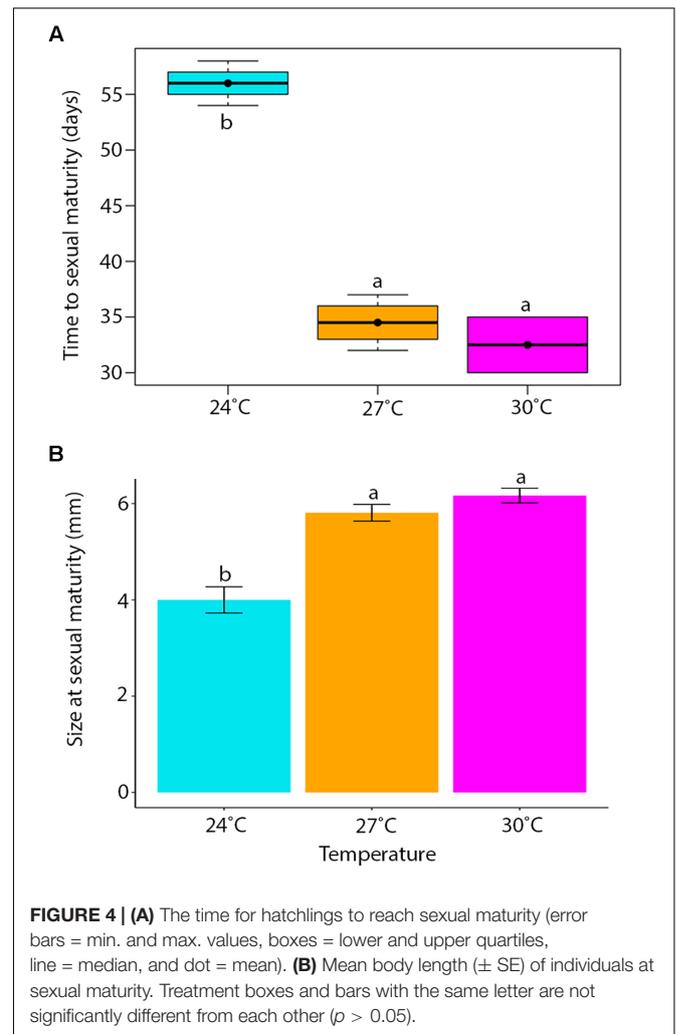
## Experiment 3 – Time to Sexual Maturity and Size at Sexual Maturity

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 4A and Table 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 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  $39.4 \pm 4.97$  days



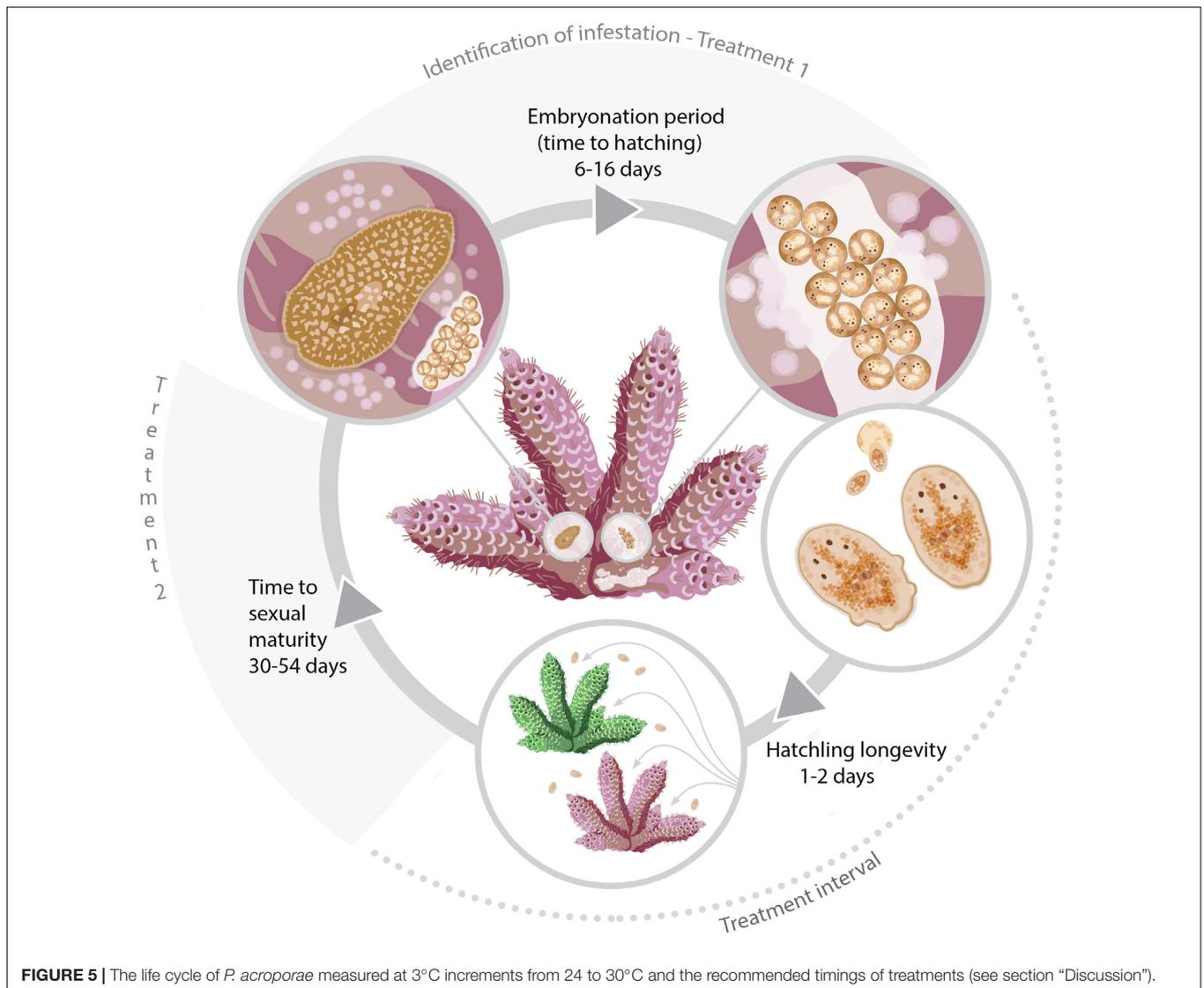
(mean  $\pm$  SE) and was removed from the analysis. Results were limited by the number of replicates that housed sexually mature flatworms, specifically in the 30°C treatment where only two replicates had individuals that reached sexual maturity. One of



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 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  $5.81 \pm 0.82$  and  $6.12 \pm 0.37$  mm (mean  $\pm$  SE) (27 and 30°C respectively) and were larger than flatworms in cooler conditions (24°C) with a body length of  $4.00 \pm 0.27$  mm.

## DISCUSSION

### Advances in Our Understanding of the Life Cycle of *Prosthiosomum 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 *Prosthiosomum 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 *Neobenedenia girellae* (at 26 and 28°C) (Brazenor and Hutson, 2015), and 2–3 weeks for *Macrostomum lignano* at 20°C (Morris et al., 2004; Wudarski et al., 2019), but short compared to 80 days for the trematode flatworm *Schistosoma mansoni* 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 are able to 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 5 and Table 2). For example, *P. acroporae* eggs in an aquarium operating at approximately 28°C would complete embryonation in approximately 11 days (Table 2). Assuming these hatchlings find a susceptible *Acropora* host, they would reach sexual maturity in approximately 31 days at 28°C (Table 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 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.

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. 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 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 *Prosthiosomum 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*. 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<sup>2</sup>.

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 aren't 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<sup>2</sup>; 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 coralivorous 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.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author (Barton, 2019).

## AUTHOR CONTRIBUTIONS

JB, KH, DB, CH, CD, and KR conceived the research and designed the experiments. JB, KR, and CD performed the experiments. JB analyzed the data. JB and KR drafted the manuscript. All authors read, commented on, and agreed upon the content of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2019.00524/full#supplementary-material>

**FIGURE S1** | Variation in *Prosthiosomum acroporae* hatchling morphology between two egg clusters at 27°C. **(A)** Hatchlings have reduced lobes (arrowheads); **(B)** hatchlings have re-absorbed their lobes (scale = 100 μm).

**FIGURE S2** | *Prosthiosomum 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 *Prosthiosomum acroporae* from the surface of an *Acropora* colony. **(C)** 300 μm PVC screening chamber full of *P. acroporae*; with representative specimens circled. **(D)** >100 *P. acroporae* transferred into a plastic beaker for experimentation.

**FIGURE S3** | To maintain *Prosthiosomum acroporae* culture numbers plastic was attached to coral using plastic pegs to provide substrate for egg-laying.

<sup>2</sup>data.AIMS.gov.au

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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