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NOTE

Larval cloning in the crown-of-thorns sea star, a keystone coral predator

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ABSTRACT: The crown-of-thorns starfish (COTS), *Acanthaster* cf. *solaris*, is an iconic keystone predator whose population outbreaks have devastating consequences for Indo-Pacific coral reefs. We tested the effects of algal food supply and larval density on the frequency of larval cloning by culturing the early bipinnaria larvae of COTS under variable conditions. Here we show that larval COTS are able to clone themselves in both low and high food conditions, and that the frequency of larval cloning increases with levels of food, but is unaffected by larval density. Across all density treatments (0.3, 1.0 and 3.0 larvae ml^{-1}), the per-capita rate of cloning increased from 4.3 % in low, oligotrophic conditions (0.17 µg chl *a* l^{-1}) to 7.9 % in high food conditions (1.7 µg chl *a* l^{-1}). Larval cloning has the potential to increase both COTS larval supply and the dispersal distance of planktonic larval stages, both of which are critical factors in predicting the timing and location of outbreaks of this species. In addition, the relationship between algal food supply and larval cloning frequency lends support to bottom-up hypotheses (e.g. nutrient enrichment) as predictors of COTS outbreaks. However, cloning was observed even under the oligotrophic conditions characteristic of coral reefs.

KEY WORDS: Cloning · Coral · Crown-of-thorns starfish · Keystone species

1. INTRODUCTION

Predation by crown-of-thorns starfish (COTS), *Acanthaster* cf. *solaris*, is one of the primary drivers of coral mortality in the Indo-Pacific (Pratchett et al. 2014) and is responsible for >40 % of coral loss on the Great Barrier Reef (GBR) over the past 30 yr (De'ath et al. 2012). Outbreaks of COTS are likely stimulated by a number of factors, but one factor frequently invoked to explain outbreaks, and one that has increased since European settlement (McCulloch et al. 2003), is increased nutrient input from terrestrial sources (Birkeland 1982, Brodie et al. 2005, 2012). The vast numbers of feeding larvae produced by COTS (as many as 100 million eggs yr^{-1} from a single female; Babcock et al. 2016b) use phytoplankton as a food source and, while able to develop in oligotrophic conditions, have higher survival under enhanced chlorophyll concentrations (0.5–5 µg chl *a* l^{-1}; Wolfe et al. 2017). It has been hypothesized, therefore, that increased delivery of nutrients from terrestrial sources has supported enhanced phytoplankton concentrations that have, in turn, yielded more successful recruitment events (Brodie et al. 2005) for this keystone coral predator (Paine 1969).

One important and emerging aspect of echinoderm larval biology that has been ignored in prior studies of COTS is the potential for larval cloning, which is

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common in asteroid larvae found in the oligotrophic tropical waters of the Gulf Stream (Bosch et al. 1989, Galac et al. 2016). In addition to increasing the survival of feeding larvae, phytoplankton abundance has also been implicated as a potential cue for larval cloning (reviewed by Allen et al. 2018). In another keystone starfish, *Pisaster ochraceus*, both quality and quantity of phytoplankton food supply were shown to be significant inducers of larval cloning (Vickery & McClintock 2000). Recent studies in echinoids and holothuroids have found similar results (Eaves & Palmer 2003, McDonald & Vaughn 2010) and, taken together, suggest that phytoplankton food availability may be a general inducer of larval cloning across echinoderms.

Asexual reproduction occurs in both the larval and adult life stages of echinoderms through a variety of methods and has been implicated in adjusting population densities with respect to available resources (Mladenov 1996, Lee et al. 2008, Allen et al. 2018). Descriptions of larval cloning exist across echinoderm classes, including the asteroids (Bosch et al. 1989, Balser 1998, Vickery & McClintock 2000, Eaves & Palmer 2003, McDonald & Vaughn 2010), but despite being one of the most intensely studied starfish species on Earth (Pratchett et al. 2017), cloning has not been assessed for COTS. We reared COTS to determine if larval cloning occurred, and then tested 2 factors to determine their role as potential inducers of larval cloning: larval density and algal food supply.

2. MATERIALS AND METHODS

COTS adults were collected on December 1, 2017 at Rib Reef (~18° 29′ S, 146° 52′ E) and immediately transported to Orpheus Island Research Station (OIRS; 18° 37′ S, 146° 29′ E). The sex of adults was determined through visual inspection of gonads through small incisions and then adults were placed into flow-through ambient seawater tanks with coarse filtration (~5 µm). For each experiment, gonads were dissected through small openings made in the body wall. Testes were kept intact in a test tube at ambient temperature until use, while ovaries were placed in 0.45 µm filtered seawater (FSW) with 1 µM 1-methyladenine (1-MA) to induce maturation (Strathmann 1987). Eggs were observed under a stereomicroscope until germinal vesicle breakdown was confirmed (~50 min following immersion in 1-MA), at which point a dilute sperm solution was added to fertilize eggs. The sperm solution was created by macerating the testes and diluting them in FSW in order to activate swimming sperm (active swimming of sperm was confirmed visually under a microscope).

For initial observations of cloning (Expt 1), cultures were started with a single male/female pair on December 6. Larvae were cultured at 28°C in individual glass bowls and beakers (volumes ranging from 0.1 to 1.0 l) that were filled with FSW. Larval density was approximately 3 larvae ml^{-1}. Cultures were hand-stirred and visually inspected 3 times d^{-1} for signs of cloning. These larvae were not fed. Cloning larvae were first found on December 13 (Day 7 post-fertilization) and photographed on Days 8–10 post-fertilization to document regeneration of both larval parts.

For Expt 2, which tested the effects of larval and algal food density on cloning frequency, cultures were generated on December 14 from 3 male/female pairs to create 3 independent families. Larvae were initially cultured as in Expt 1. Larvae were allowed to develop to the early bipinnaria stage and then isolated on December 17 (Day 3). Symmetrical bipinnaria larvae (>90% of larvae fit this criterion) were selected by hand for this experiment to ensure that poorly developed, but uncloned, offspring were not mistakenly used as evidence of cloning events. Larvae were examined for cloning on December 19 (Day 5).

Larvae were maintained in small glass beakers with 40 ml of FSW and randomly assigned to 1 of 3 larval density treatments (3, 1 or 0.3 larvae ml^{-1}) and 2 larval food treatments (N = 3 beakers $treatment^{-1}$). Larvae were fed the unicellular flagellated alga *Isochrysis galbana* at a low (1000 cells ml^{-1}) or high (10 000 cells ml^{-1}) concentration. Algal food concentrations represent chlorophyll concentrations of approximately 0.17 µg chl *a* l^{-1} (range: 0.1–0.28 µg chl *a* l^{-1}) for the low food treatment and 1.7 µg chl *a* l^{-1} (range: 1–2.8 µg chl *a* l^{-1}) for the high food treatments (Alvarez et al. 2017). These low and high chlorophyll levels are commensurate with those found in offshore GBR waters and nearshore coastal waters, respectively (www.bom.gov.au/marinewaterquality/, see Wolfe et al. 2017). To ensure that larvae and algal food remained in suspension, the cultures were stirred by hand several times per day. Larvae from each beaker were observed under a stereomicroscope and individually pipetted out of the beaker and into a new beaker. During this process, each larva was assessed visually for signs of cloning (see Fig. 1), and each larva was counted and assigned as either a clone or an uncloned larva. The proportion of cloning for each replicate beaker was thus assessed, and a 2-way ANOVA was conducted (IBM SPSS version 23) to test the effects of algal food

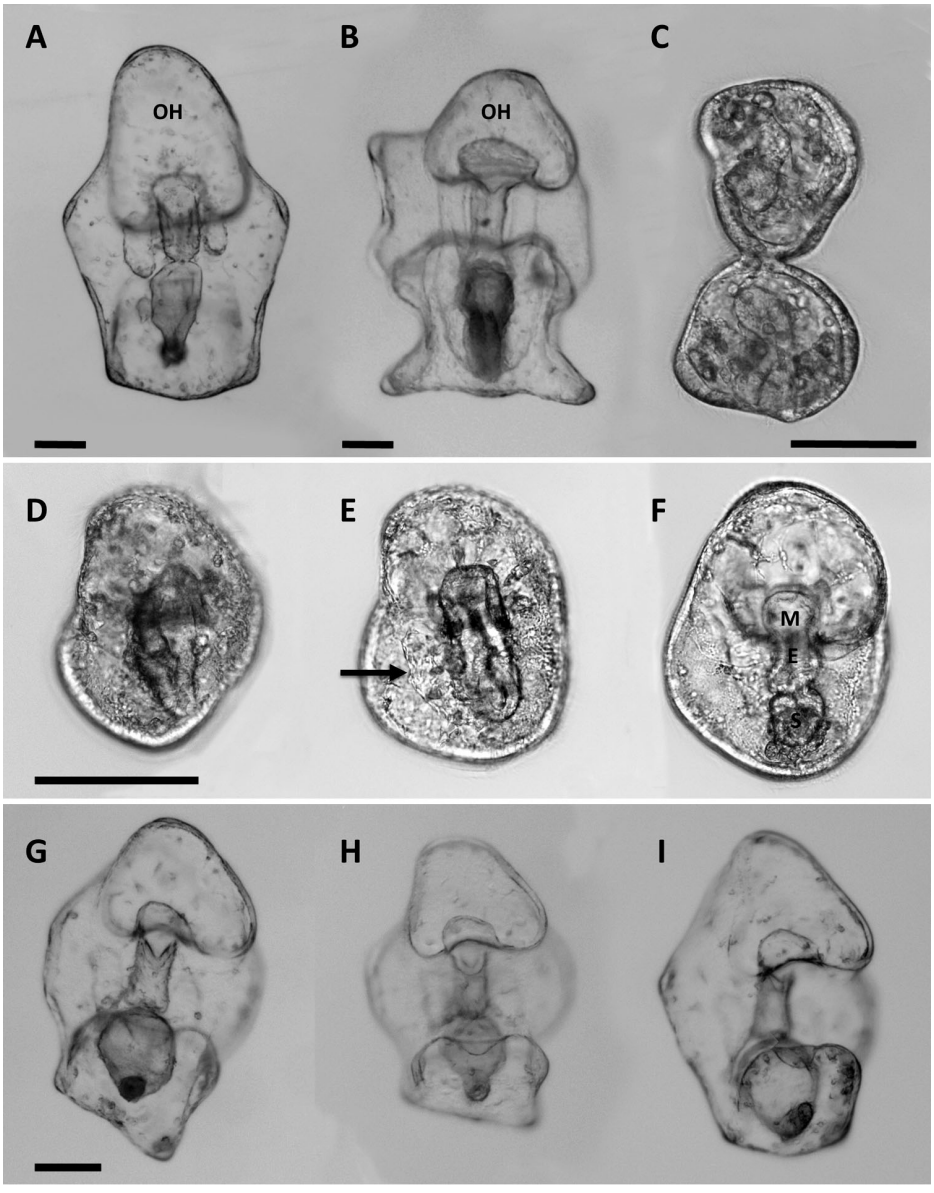

Fig. 1. (A) Crown-of-thorns starfish, *Acanthaster* cf. *solaris*, early bipinnaria larva at the stage at which larvae were sorted into cloning trials. (B) Mid-stage bipinnaria larva that has begun to develop lobes at the base, but has lost part of the oral hood due to a cloning event. (C) Some bipinnaria larvae split relatively evenly into 2 smaller individuals through a bisection event. (D–I) A single larva on Days 8, 9 and 10 of development, following a cloning event on Day 7; the larva bisected into 2 unequal portions, forming (D–F) a smaller 'head' clone which is derived from the oral hood and mouth and (G–I) a larger 'body' clone derived from the remainder of the larval body. Arrow in (E) shows regenerating coelomic sac. Letters in (F) show development of a complete gut in regenerating 'head' clone. The 'body' clone appeared to shrink on Day 9 before regaining size and restoring symmetry on Day 10, suggesting rapid reworking of the larval body post-cloning. OH = oral hood, M = mouth, E = esophagus, S = stomach. All scale bars are 100 µm (scale bar in D also applies to E & F; G also applies to H & I)

supply, larval density and their interaction on the frequency of cloning events. The assumption of equality of variances was tested with Levene's test and the variances were not statistically distinguishable from one another ($p = 0.76$). Residuals of the analysis were tested for normality (another assumption of ANOVA) with a Kolmogorov-Smirnov test and did not deviate significantly from a normal distribution ($p = 0.140$), so no data transformation was performed. To calculate effect size for the interaction between density and food supply, we calculated eta-squared (η^2) by dividing the sum of squares of the effect by the total sum of squares (Lakens 2013). For all analyses, we used an α level of 0.05 as our threshold for significance.

3. RESULTS

We found that larval cloning (Fig. 1) was a frequent occurrence in COTS. Larval cloning was observed starting from the early bipinnaria larval stage (Fig. 1A). While the timing of the onset of larval cloning corresponded to the ability of larvae to feed, cloning was observed in the absence of algal food. We observed a variety of modes of larval cloning, consistent with prior reports of multiple modes of asexual reproduction in starfish larvae (Jaeckle 1994, Allen et al. 2018). The most common mode of cloning observed was spontaneous autotomy of the preoral lobe (Fig. 1B). This method of cloning resulted in unequal splitting of the larva into 2 parts: a smaller anterior portion containing the oral hood and a larger posterior portion containing the mouth, esophagus and stomach. We were able to track and photograph both parts of a single larva to demonstrate that over subsequent days, not only did both parts survive, but also that the smaller anterior portion was able to regrow a complete larval gut (Fig. 1D–F) and coelomic cavity (Fig. 1E). Simultaneously, the larger portion changed in size over time and repaired an asymmetry that was possibly related to an unobserved cloning event (Fig. 1G–I). Less frequently, we observed other modes of cloning, including a bisection of the larval body into 2 roughly equivalent halves (Fig. 1C).

Following confirmation of cloning, we then the tested whether the frequency of larval cloning was affected either by larval density or by the presence of phytoplankton food. Across all treatments, cloning frequencies ranged from 2.5% to 9.6% (Fig. 2). We found that after just 2 d of presentation with algal food, there was a significant effect of algal food supply (2-way ANOVA; $F_{1,12}$ = 8.662; p = 0.012) on larval cloning frequency (high food: 7.91 ± 0.11%; low food: 4.25 ± 0.12%; mean ± SE; Fig. 2). There was no effect of larval density on cloning frequency (high density: 6.59 ± 0.29%; medium density: 4.17 ± 0.17%; low density: 7.50 ± 0.08%; 2-way ANOVA; $F_{2,12}$ = 2.631; p = 0.137), nor was there an interaction between larval density and food supply on cloning frequency (2-way ANOVA; $F_{2,12}$ = 1.759; p = 0.214); however, our small sample size (N = 3 per treatment) limited our statistical power to detect density effects and interactions among treatments. One measure of effect size (η^2) revealed that the interaction between density and food supply accounted for 13% of the variation in cloning frequency.

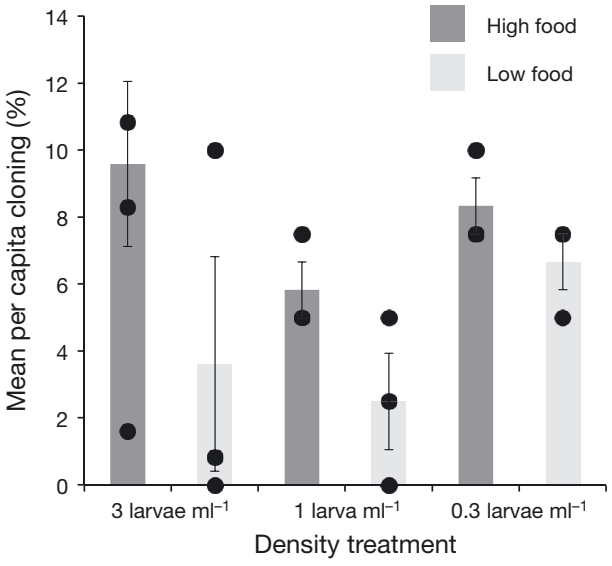

Fig. 2. Effects of larval density (3, 1 or 0.3 larvae ml^{-1}) and algal food supply (fed *Isochrysis galbana*; high food: 10 000 cells ml^{-1} or low food: 1000 cells ml^{-1}) on cloning frequency in larval crown-of-thorns starfish, *Acanthaster* cf. *solaris*. Each treatment combination was replicated in 3 beakers. Bars represent mean ± SE for each treatment; black circles represent per capita cloning frequency for individual beakers. In some cases, only 2 circles are visible because 2 beakers had identical cloning frequencies

4. DISCUSSION

The inherent ability of COTS larvae to clone in oligotrophic conditions, and for this to be enhanced by increased phytoplankton abundance, has potentially significant implications for management of COTS on the GBR. As evident from plankton samples, asexual reproduction by cloning appears to be a normal form of propagation for some tropical asteroids (Bosch et al. 1989, Galac et al. 2016). Asteroids that exhibit larval cloning, including COTS, have a diverse symbiotic bacterial community that includes photosynthetic bacteria that may contribute to the resilience of asteroid larvae in low food conditions (Galac et al. 2016, Carrier et al. 2018).

Our results suggest that not only are COTS larvae surviving (Wolfe et al. 2015, Pratchett et al. 2017) in areas of high chlorophyll concentration (1–2 µg chl *a* l^{-1}), but they may actually multiply asexually. Recent metagenomic surveying of the surface waters of the GBR suggests that COTS larvae form a 'continuous cloud' over the GBR (Uthicke et al. 2015), including areas where outbreaks are not occurring. Our data suggest that, in addition to the tremendous fecundity of this species, asexual reproduction of larvae may contribute to their longevity and widespread dispersal in the plankton. Our data further suggest that

increased phytoplankton enrichment of the normally oligotrophic waters of the GBR contributes not only to increased survival of existing larvae, but may itself be a direct cause of increased numbers of COTS larvae as well.

While larval cloning was thought for many years to be an uncommon but potentially adaptive response to environmental change (Jaeckle 1994), asexual propagation by larvae is now recognized to be widespread among marine invertebrates, particularly among echinoderms (Eaves & Palmer 2003, Allen et al. 2018). Our results match those of prior studies that have identified algal food supply as one inducer of cloning in echinoderms (Vickery & McClintock 2000, McDonald & Vaughn 2010), and suggest that the responsiveness of echinoderm larvae to variations in food supply (including in COTS, see Wolfe et al. 2015) includes enhancing the number of offspring that arise from a single zygote. The frequency of cloning we identify in COTS (~3–10%) is comparable to that demonstrated in other sea stars (e.g. 1–24% in *Pisaster ochraceus*; Vickery & McClintock 2000). The levels of chlorophyll that induced cloning (0.17–1.7 µg chl *a* l^{-1}) are comparable to that which has been shown to be food-limiting in other echinoderm larvae (0.2 µg chl *a* l^{-1}; Fenaux et al. 1994) and include the levels in oligotrophic GBR waters (Wolfe et al. 2017). The levels we used are below those at which growth and survival of COTS are negatively affected by excess phytoplankton (10 µg chl *a* l^{-1}; Wolfe et al. 2015).

The presence of larval cloning both at low food levels and in response to increased food availability is likely to contribute to the success of COTS and has potentially major implications for models of population dynamics in COTS. In particular, current models may be underestimating both the fecundity of this species and the larval dispersal period by ignoring the potential for cloning in the plankton (Rogers-Bennett & Rogers 2008). However, an accurate estimate of the ecological significance of cloned larvae to COTS populations requires demonstration that cloned larvae can reach metamorphosis and estimates of the abundance of adults derived from cloned larvae in existing populations. In future studies, data on not only the frequency of cloning, but also the changes in larval size and time to metamorphosis that are potentially correlated with cloning events should be investigated. In addition, the consequences of these changes in size and development time for larval mortality should be investigated, as planktonic mortality of larvae is known to be a major limitation on recruitment (Rumrill 1990, Lamare & Barker 1999, Vaughn & Allen 2010). Understanding the interactions between environmental inducers of cloning and the fitness consequences for larval cloning will be a fruitful avenue for future work.

Nearly 50 yr ago, Paine (1969) coined the phrase 'keystone species' to refer to the outsized effect that sea stars have on their communities. While widely cited (and often mis-cited, see Lafferty & Suchanek 2016), Paine's (1969) paper is frequently overlooked with regard to his argument that COTS could be considered a keystone species. In fact, Paine (1969) argued that the role of COTS as a keystone species could be very similar to that of *P. ochraceus*, should the 'plague' of COTS be due to factors other than the removal of predators. Recent work has not rendered a final decision on the role of predator removal versus nutrient enrichment in contributing to COTS outbreaks (Babcock et al. 2016a). While the nutrient-enrichment hypothesis has garnered wide support, both it and the predator-removal hypothesis invoked by Paine (1969) have been deemed 'largely unresolved' in a recent assessment of COTS biology (Pratchett et al. 2017).

On one hand, our work shows that COTS larvae clone in the natural low-nutrient conditions of the GBR and, on the other, that cloning is enhanced at elevated nutrient levels. We provide new evidence that the nutrient-enrichment hypothesis may help to explain the occurrence of COTS outbreaks through a novel mechanism, larval cloning, supporting Paine's (1969) suggestion that COTS is indeed a keystone species in the same vein as the more well-known keystone sea star, *P. ochraceus*. Regardless of the descriptors assigned to this species, the regulation of COTS populations is likely to be a key factor in management of rapidly declining coral cover on the GBR, and our work suggests that reduced concentrations of phytoplankton may reduce the frequency of cloning in this species and in turn reduce both recruitment and dispersal of a voracious coral predator.

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