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Spawning time of *Acanthaster cf. solaris* on the Great Barrier Reef inferred using qPCR quantification of embryos and larvae: do they know it's Christmas?

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Abstract

Outbreaks of crown-of-thorns seastars (CoTS; *Acanthaster* spp.) are a major contributor to degradation of Indo-Pacific coral reefs. Understanding the dispersal and fate of planktonic life stages is crucial to understand and manage outbreaks, but visual detection of CoTS larvae is challenging. We apply a quantitative PCR (qPCR) assay to enumerate CoTS larvae in a 3-year time series of plankton samples from two reefs (Agincourt and Moore Reefs) on the Great Barrier Reef. Plankton surveys were complemented with settlement assays, and benthic surveys of juvenile and adult densities over time. Only one out of 109 plankton samples from Agincourt Reef had detectable CoTS mtDNA compared to 41 out of 575 samples from Moore Reef. This may be explained by differences in adult densities, or differences in connectivity and larval retention. Detections of larval CoTS were restricted to summer (November–February), with first detections each year coinciding with water temperatures reaching 28 °C and peak detections late December. A disproportionate number of larval detections occurred in 7 days around full moon. Complementary sampling of settlement and post-settlement life stages confirmed that elevated densities of CoTS larvae at Moore Reef translated to high rates of settlement adding to infestations at this reef. Moreover, there were declines in the detection of larvae, as well densities of juvenile and adult CoTS at Moore Reef, in 2017 and 2018. This study demonstrates that qPCR for genetic identification and quantification of larvae can assist to elucidate life history parameters of nuisance species difficult to obtain with other tools.

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Introduction

Coral reef species of crown-of-thorns seastars (CoTS; *Acanthaster* spp.) are renowned for having boom and bust life cycles (Uthicke et al. 2009), where very high densities of adult seastars (which feed predominantly on reef-building corals) cause extensive disturbance and degradation to coral reef ecosystems (Pratchett et al. 2014). New and recurring outbreaks of CoTS have occurred throughout the Indo-Pacific in recent years, often causing severe, if not widespread, coral loss (French Polynesia: Kayal et al. 2012; Indonesia: Baird et al. 2013; Japan: Nakamura et al. 2014; Chagos Archipelago: Roche et al. 2015; Maldives: Saponari et al. 2015). In Australia's Great Barrier Reef (GBR), 42% of the ~ 50% coral loss observed between 1985 and 2012 was attributed to predation by the Pacific CoTS, *Acanthaster cf. solaris* (De'ath et al. 2012). Since 2012, there has been further pressure on GBR coral assemblages due to the progression of the fourth documented CoTS outbreak (Pratchett et al. 2014; Uthicke et al. 2015b), which was further

compounded by climate change-induced coral bleaching in 2016 and 2017 (Hughes et al. 2019).

Extreme fluctuations in the local abundance of CoTS are at least partially attributable to vagaries in larval supply, development and settlement (Uthicke et al. 2009; Caballes and Pratchett 2014; Wilmes et al. 2018). Like many large asteroids with planktotrophic larvae, *Acanthaster* spp. are extremely fecund (Babcock et al. 2016). However, realisation of their exceptional reproductive potential may be constrained by a range of extrinsic and intrinsic factors, including sex ratios, conspecific densities, spawning synchrony, and environmental conditions during spawning (Caballes and Pratchett 2014; Rogers et al. 2017). Improved understanding of population dynamics for CoTS is therefore, fundamentally dependent on establishing spatial and temporal patterns in reproduction, dispersal and fate of larvae.

Current knowledge of CoTS larvae (including development rates, dietary preferences, and environmental sensitivities) comes mainly from laboratory-based experimental studies (Lucas 1982; Okaji et al. 1997; Wolfe et al. 2015b; Johansson et al. 2016; Uthicke et al. 2018b) and surprisingly little research has been conducted on CoTS larvae in the field (Pratchett et al. 2017). One reason for this lack of field studies relates to difficulties in the detection and discrimination of CoTS larvae. Morphological discrimination of CoTS larvae from other asteroid and holothuroid larvae is near impossible (Uthicke et al. 2015a), and phenotypic plasticity in larval development and form (Wolfe et al. 2015a) further impedes visual discrimination of *Acanthaster* spp. However, genetic markers have considerable potential to overcome this limitation. In echinoderms, genetic methods to detect larvae have been described for asteroidea (Deagle et al. 2003; Richardson et al. 2016) and holothuroidea (Medeiros-Bergen et al. 1995). Qualitative genetic markers for CoTS have been developed for Okinawa, Japan (Suzuki et al. 2016) and the GBR (Uthicke et al. 2015a). Recently, we developed a quantitative PCR (qPCR) based method to quantify CoTS larvae and detected CoTS larvae (up to ~ 84 m^{-3}) in many samples of the Northern GBR in December 2014 (Doyle et al. 2017).

Effective detection of CoTS larvae using specific genetic markers and quantitative methods opens the way for much greater insights into the timing of spawning, as well as planktonic larval duration (Uthicke et al. 2015a). Serendipitous observations of CoTS spawning have occurred mostly during summer months (summarised in: Babcock and Mundy 1992; Pratchett et al. 2014). Likewise, seasonal changes in gonad indices or gonad morphology for adult CoTS suggests that spawning occurs predominantly, if not exclusively, during summer (Babcock and Mundy 1992; Yasuda et al. 2010). Babcock and Mundy (1992) recorded the seasonal changes in the proportional weight of gonads for *Acanthaster* cf. *solaris* based on periodic collections at Davies Reef in the central GBR from October 1990 to February 1991. The proportional weight of gonads increased gradually throughout October and November, but dropped abruptly in early December, coinciding with direct observations of synchronous spawning on December 7th (Babcock and Mundy 1992). A more recent study (Caballes 2017) suggests that there may be inter-annual variability in both the extent to which spawning is protracted or curtailed, and the specific timing of peak spawning.

The purpose of this study was to demonstrate the utility of specific and quantitative genetic screening to refine estimates of spawning and embryo or larval (hereafter referred to as larval) occurrence for *A.* cf. *solaris* in the northern GBR. Herein, we present data from 684 plankton samples collected over three putative spawning seasons from two reefs in the Northern GBR experiencing current CoTS outbreaks. Samples were analysed with qPCR to quantify CoTS larvae with the aim to identify the time-window and peak of larval occurrences, and thus inferred time of spawning. In addition, settlement collectors were analysed for the 2016/2017 summer and we conducted intensive searches for juveniles from 2014 to 2018 on one of the reefs to test if larval presence translates to recruitment. Changes in larval and juvenile densities over time were put into context by comparing them to changes in adult densities over 7 years.

Materials and methods

Sample collection and quantitative PCR

Sampling sites were located at Moore Reef (16.84742°S, 146.22705°E) approximately 27 nautical miles east of Cairns, and Agincourt Reef (15.98289°S, 145.82220°E) approximately 55 nautical miles north of Cairns on the GBR. Plankton samples at Moore Reef and Agincourt Reef were collected over three summers by tourist operators (Reef Magic Cruises and Quicksilver, respectively) that visit fixed pontoons almost daily. Plankton samples were collected via bottom to surface vertical zooplankton hauls using a 0.5 m diameter plankton net (75 μm mesh). Plankton samples were washed and concentrated using a 40-μm mesh to remove most of the water and then transferred to a 50 ml tube. The final concentrated plankton volume was no greater than 10 ml in the 50 ml tube which was then topped up with 100% ethanol to preserve DNA.

Generally, three plankton samples were collected per sampling occasion on a fixed site at each pontoon. Out of these, we analysed two samples genetically. The third sample was kept as spare, or, in some instances, to microscopically validate positive genetic detections (see below). For presentation, data were averaged over the two duplicates, raw data for individual duplicates are given in Supplementary Table 2. However, on a few occasions samples were

collected twice a day, i.e. during two discrete (separated by > 1 h) time periods. In these cases, we present averages of four individual samples. A total of 54 sampling days (109 individual samples) spread over 2 summer seasons (2015/16) were sampled on Agincourt Reef, and 277 sampling days (575 samples) through 3 summer seasons (2015/16, 2016/17, 2017/18) on Moore Reef.

DNA extraction and quantitative PCR (qPCR) were performed following Doyle et al. (2017). A standard for qPCR was derived from a bulk extraction of laboratory reared CoTS larvae. Approximately 20 late stage CoTS bipinnaria larvae were extracted using a Qiagen DNeasy Blood and Tissue Kit. Extracts were combined to produce a bulk extraction.

A fivefold dilution series was prepared and sent to the National Measurement Institute (Australia) for exact copy number determination using digital droplet PCR. Aliquots of this same dilution series were then utilised to produce a qPCR standard curve in each run from which the qPCR-based cycle threshold values were converted to copy numbers.

Verification by microscopy and PCR

A total of nine plankton samples from Moore Reef, including three summer samples from 2015, four summer samples from 2016 and two winter samples from 2016 with negative detections were selected for sorting microscopically and subsequent PCR of individual larvae. Plankton samples were sorted using a Leica M165C microscope under 40× magnification. Individual larvae were photographed using a Nikon D810 SLR camera with microscope adapter. Individual larvae were transferred into 1.5 ml Eppendorf screw top tube into 100% ethanol using fine tweezers and stored at 4 °C. DNA extraction was carried out using Qiagen DNeasy Blood and Tissue Kit extraction kit following manufactures protocol with final elution in 3×50 ml 10 mM Tris pH 8.0. PCR reaction used 5 µl template DNA in 20 µl reactions. PCR reagents: 400 nM each of CoTS specific forward and reverse primer (126 bp amplicon length) (Doyle et al. 2017), AmpliTaq gold 360 2 × master mix. Thermal cycling conditions used an initial HotStart Taq activation at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s with a final 10 min extension of 72 °C. PCR products were run on 2% agarose gels stained with ethidium bromide and were visualized under UV light.

Field sampling for CoTS juveniles

Random belt transects (60 m × 6 m) were carried out adjacent to a tourism platform at Moore Reef between 2014 and 2018. The transect tape was used to mark the middle of the belt and 3 m each side of the tape was searched by a Scuba diver. Juvenile CoTS are often located by feeding scars (white patches) on live coral. Juvenile CoTS were classed as individuals smaller than 150 mm in diameter, which are difficult to detect (MacNeil et al. 2016). Given the large size of the survey area we did not focus on small (< 20 mm) juveniles likely representing the last summer's spawning (Wilmes et al. 2017), thus the majority of juveniles likely present 1 + year individuals.

Recruitment onto the Reef–*Settlement collectors*

27 settlement collectors were deployed 19 October 2016 at Moore Reef followed by another 27 between 8 and 12 December 2016, and retrieved in 2–11 February and 12–13 March 2017 respectively. Three replicate collectors were set up at three different depths (crest = 2–4 m, slope = 5–7 m, base = 8–9 m) at each of three different sites (Site A: 16.88681°S, 146.19190°E; Site B: 16.88943°S, 146.18809°E; Site C: 16.84607°S, 146.22440°E). Settlement collectors consisted of two leaf baskets filled with approximately 60–70 aquarium filter bio balls. Each collector was secured to the substrate with a 400 mm long stainless-steel stake and cable allowing it to float approximately 1 m above the substrate.

Upon retrieval, each collector was covered in a fine meshed cotton bag and held in a large flow through tub on the vessel until processing. To collect all organisms from each sample, a single bagged collector was placed in a 20 L bucket. Water was allowed to drain through a 100 × 100 mm window with fine mesh (120 microns) towards the bottom of the bucket. The leaf baskets, cotton bags and each individual bio-ball were hosed down with salt water and any remaining organisms were removed using fine tweezers. The salt water was then drained from the sample, which was transferred into a 600 ml jar filled with 100% ethanol.

For DNA extractions, the settlement collector samples were drained of all ethanol and placed in a freezevac (Dyna-Vac) for 12–36 h to remove any remaining ethanol. The samples were homogenised by transferring them individually into a high-speed blender (NutriBullet) cup and blitzed until a fine powder was obtained. Between each sample, blender cups and blades were soaked for 30 min in a 10% bleach solution, thoroughly rinsed and dried to avoid any cross contamination.

DNA was extracted from a small subsample (0.025–0.050 g) in a 2 mL Eppendorf tube. 750 µL TES buffer (100 mM Tris pH 9.0, 100 mM EDTA pH8, 1% SDS, 100 mM NaCl) was added. Samples were vortexed and incubated at 65 °C for 60 min, and 187.5 µL of 5 M KOAc added. Subsequently, samples were vortexed and incubated on ice for further 10 min before being spun at maximum speed (16100 rcf) for 20 min at room temperature. The supernatant was transferred to a new 2 mL Eppendorf tube

to which 600 μL isopropanol was added to precipitate the DNA. The solution was gently mixed and allowed to stand for 5 min at room temperature before being spun for 15 min at maximum speed. The supernatant was carefully decanted and 600 μL 70% ethanol was added to each tube. The tubes were briefly vortexed and spun at maximum speed for 5 min. This step was repeated a 2nd time after which the ethanol was removed using a pipette. The DNA pellet was allowed to air–dry for 5 min to evaporate the majority of the ethanol before resuspending the DNA pellet in 150 μL of 10 mM Tris pH9.

Polymerase chain reactions (PCR) were run using 10 μL assay volumes containing 1 μL DNA template, 5 μL AmpliTaq gold 360 × 2 master mix and 400 nM forward and reverse CoTS primers (COTS-COI_F_1321, COTS-COI_R_1446, see above). The PCR run consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 30 s each at 95 °C, 60 °C and 72 °C, and a final annealing at 72 °C for 10 min. Due to the variability in our starting material, PCRs of all samples were repeated with higher DNA concentrations (4 μl instead of 1 μl template) and 1 μl DNA diluted at a 1:10 ratio. PCR results were checked and visualised on a 2% agarose gel with ethidium bromide.

Adult numbers culled

The Great Barrier Reef Marine Park Authority (GBRMPA) directs and supports extensive culling of CoTS across the GBR and requires that contractors provide detailed information pertaining to the number and size of CoTS that are killed. We considered CoTS culling data from the GBRMPA's Eye on the Reef program for the two reefs investigated under the Creative Commons License. During control cruises, individual operators record the number of CoTS killed by lethal injection with bile salt and the total person hours spent searching during each day of operation. This data was summarised as an indication for CoTS presence on these reefs for each year between 2015 and 2017 for Agincourt Reef and 2012–2018 for Moore Reef.

Temperature data

Temperature logger data from Moore Reef and two nearby locations (Arlington Reef, Green Island Reef were downloaded from the AIMS Sea Temperature Observing System [Australian Institute of Marine Science (AIMS) 2017] and daily averages calculated.

Statistical analysis

We used χ^2 statistics to test whether positive samples were over represented near full moon (defined here as 3 days before full moon, full moon and 3 days after full

moon = 7 days out of 29.52 days lunar cycle). This test probes whether the observed number of positive samples during and outside that period deviated significantly from the expected frequencies under random distribution. Initial data inspection with frequency histograms demonstrated no bias of sampling effort towards specific lunar phases, so we assumed the expected probability for a sample to be in the 7 days period as 7/29.52 = 0.237, and outside that period as 0.763. Statistical analyses and graphs were prepared in R version 3.5.2 (R-Core-Team 2018).

Results

Time series

Only one individual sample out of 109 collected on Agincourt Reef (10/01/17) had CoTS DNA above the detection limit using qPCR. However, the copy number detected in that sample was less than that equivalent to one larva per tow (Fig. 1). A total of 41 individual samples out of the 575 collected on Moore Reef had CoTS DNA concentrations above the detection limit which resulted in
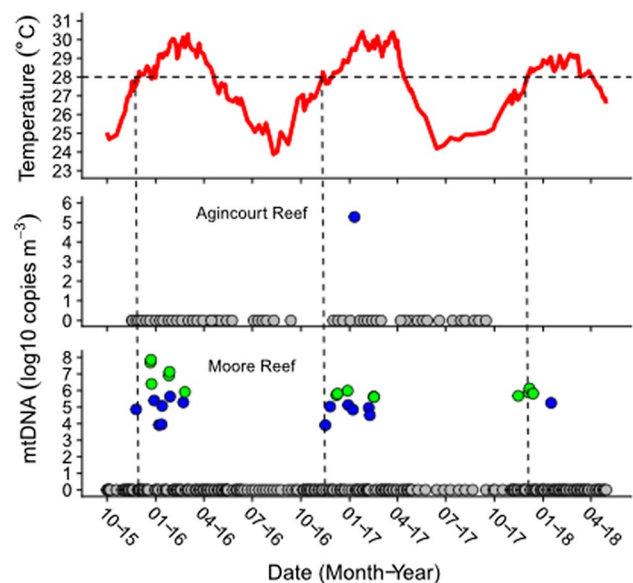

Fig. 1 Mitochdrial DNA (cytochrome oxidase subunit I) copy numbers of *Acanthaster* cf. *solaris* on Agincourt and Moore Reef in the Northern GBR. All data were averaged over a minimum of two duplicate samples per sampling occasion. Green dots: at least one of the duplicates amounted to >1 larvae per plankton tow (estimated as the maximum number), blue dots: CoTS DNA detected above assay detection limit, but neither of the individual samples converts to >1 larvae per plankton tow. Grey dots: both duplicates had DNA concentrations below the detection limit. The top graph represents water temperatures measured on Moore Reef and nearby locations with similar water depth. Dashed lines highlight dates when water temperatures reach 28 °C

29 sampling dates with positive detections (Fig. 1). The earliest annual detection was observed on 15 November 2017, and the latest on 25 February 2016. Comparisons to the average temperature suggest that larval detections generally occur once water temperatures reached 28 °C.
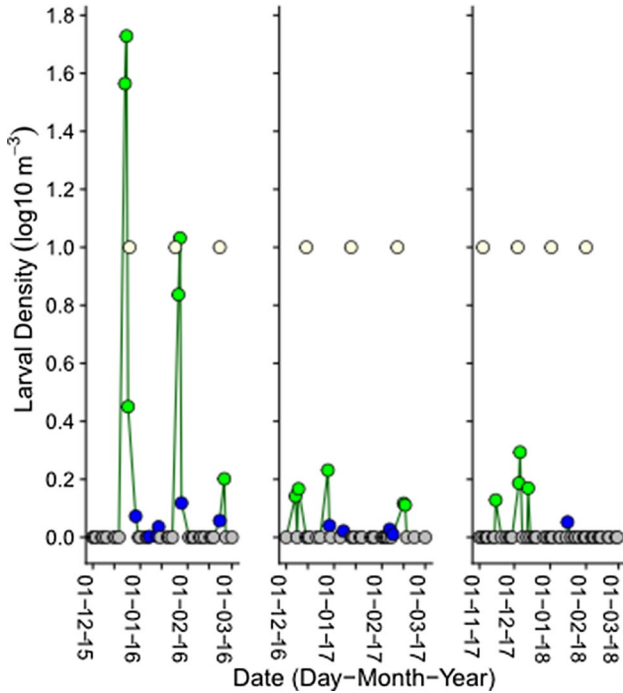

Fig. 2 Larval densities of *Acanthaster* cf. *solaris* during the summer months at Moore Reef. The yellow circle symbolises the date of full moon. Green dots: at least one of the duplicates amounts to > 1 larvae per plankton tow (estimated as the maximum number), blue dots: CoTS DNA detected above assay detection limit, but neither of the individual samples converts to > 1 larvae per plankton tow. Grey dots: both duplicates had DNA concentrations below the detection limit

DNA copy and larvae numbers observed in 2015/16 were much higher than in 2016/17 or 2017/18 (Fig. 1).

Focusing on the larvae detected in the plankton near Moore Reef during the three summer periods suggested some correlation of the detection of positive samples around the full moon (Fig. 2). This match seems particularly distinct in 2015/16, whereas exceptions to these observations existed in the other spawning periods. However, positive samples were significantly over-represented in the 7 days around the full moon period for all positive samples (12/29, $\chi^2 = 5.16$, $df = 1$, $p = 0.0051$) or samples with at least one duplicate being above the value representing one larva per tow (8/15 samples, $\chi^2 = 7.53$, $df = 1$, $p = 0.0009$).

Based on average mtDNA copy number, estimates of larval densities in 2015/16 reached a maximum of 14.6 Ind. m^{-3} (minimum estimate for the same sample: 9.4 Ind. m^{-3} maximum: 32.9 Ind. m^{-3}), with smaller numbers (maximum estimates < 1 Ind. m^{-3}) observed in the following years (details in Supplementary Tables 1, 2). Corresponding to the decline in densities, we also observed a steady decline of sampling dates with positive mtDNA detection from 13 in 2015/16 to 11 in 2016/17 and only 5 in 2017/18.

Verification by microscopy and PCR

No asteroid larvae were detected microscopically in the two winter samples selected. In the summer samples chosen for microscopic analyses, we found 222 putative asteroid embryos or larvae and 45 of these amplified with CoTS specific primers (Table 1). We detected larval densities in the same order of magnitude as detected using qPCR. Developmental stages consisted predominantly (70%) of Gastrula

Table 1 Plankton samples sorted for potential asteroid embryos and larvae (Picked) and individual confirmation of *Acanthaster* cf. *solaris* embryos using PCR (Confirmed CoTS)

Season	Date	ID	Range qPCR (No.)	Picked (No.)	Confirmed CoTS (No.)	Developmental stage (%)			
						Gast.	Dp.	Early Bp.	Bp.
Summer	22/12/15	273	20.9–87.6	52	18	89.0	5.5	5.5	0
	23/12/15	276	33.1–120.8	133	24	78.9	10.5	10.5	0
	24/12/15	279	1.2–4.1	7	2	0	0	50.0	50.0
	9/12/16	785	0.3–1.1	20	1	100.0	0	0	0
	28/12/16	803	0.3–2.0	5	0	–	–	–	–
	16/12/16	794	0	2	0				
	16/2/17	1060	0.0–1.4	3	0	–	–	–	–
Winter	8/6/16	650	0	0	0	–	–	–	–
	12/7/16	671	0	0	0	–	–	–	–

For comparison, the table also lists the average estimate of CoTS embryos or larvae as determined via qPCR in the two other replicates at the same points in time (Range qPCR). For these samples, we give the range between the minimum estimate of the lower duplicate and the maximum of the higher

Developmental stages are—*Gast.* Gastrula, *Dp.* Dipleurula, *Bp.* Bipinnaria

Fig. 3 Examples of *Acanthaster* cf. *solaris* larvae identified through microsopy and PCR verification **a** Gastrula larvae (sample 263), **b** Dipleurula larvae (273), **c** early Bipinnaria (279) and **d** Bipinnaria (279). Scale bar represents 200 μm
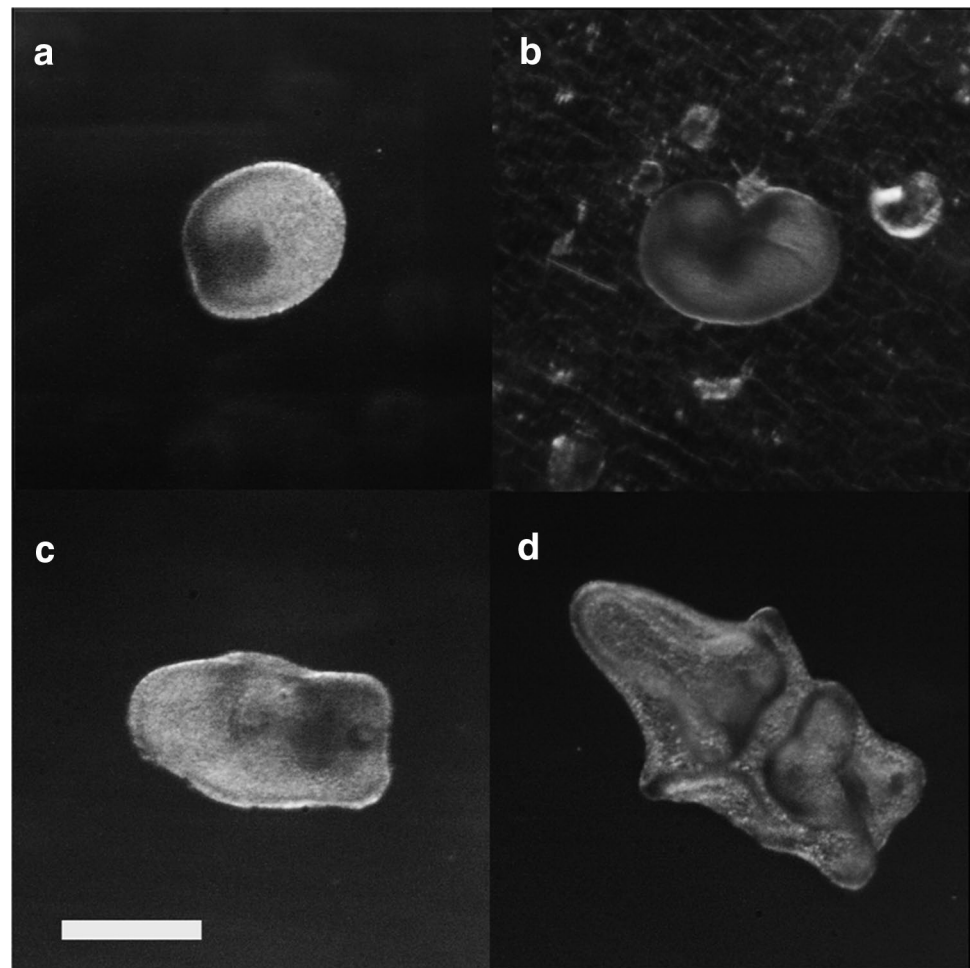

Table 2 Summary of PCR results showing the number of settlement collectors retrieved in January and March 2017 on Moore Reef in which *Acanthaster* cf. *solaris* DNA was detected on a 2% agarose gel (yes = strong band, maybe = weak band, no = no band)

A. *cf. solaris* DNA present	Feb 2017	Mar 2017
Yes	4	10
Maybe	2	4
No	21	13

and no larvae were found further than Bipinnaria stage (Fig. 3).

Recruitment onto the Reef—Settlement collectors

Settlement collectors deployed at Moore Reef during the 2016/2017 summer revealed settlement of CoTS in several traps. Using standard PCRs, *Acanthaster* cf. *solaris* DNA was clearly detected in 14 settlement trap samples as evidenced by a strong band on the 2% agarose gel, with potentially 6 more detections (weak bands) representing 25.9% or 37% of the samples and suggesting *A.* cf. *solaris* larvae

have recruited to the collectors (Table 2). There was a temporal difference in the number of collectors containing *A.* cf. *solaris* DNA, with more collectors containing *A.* cf. *solaris* settlers in March 2017 (10 plus 4 possible) than in February 2017 (4 plus 2 possible). This suggests that some settlement occurred between October 2016 and February 2017, but the settlement pulse was stronger between December 2016 and March 2017.

Juveniles and adult numbers culled

Over 10,000 (10,452) juvenile CoTS were counted and collected on Moore Reef between 2014 and 2018. Densities of juvenile CoTS were > 100 Ind. ha^{-1} from 2014 to 2016 (Table 3), but were markedly lower in 2017 (66.2) and 2018 (20.8).

The number of CoTS culled by control operations provided a measure of adult CoTS present on the reefs investigated. Less effort was allocated to the Agincourt Reef complex. The number killed per hour in 2015–2016 was distinctly less than in the same period at Moore Reef. The number culled per hour sharply declined on Agincourt Reef

Table 3 *Acanthaster* cf. *solaris* juvenile (<150 mm) counts on Moore Reef during five survey years

Year	Effort		Individuals collected	Density (Ind. ha^{-1})
	Transects (No.)	Area (ha)		
2014	788	28.37	3016	106.3
2015	659	23.72	2729	115.0
2016	675	24.30	3125	128.6
2017	406	14.60	967	66.2
2018	822	29.60	615	20.8

Table 4 A summary of *Acanthaster* cf. *solaris* culled at two target reefs in the Cairns Section of the Great Barrier Reef Marine Park

Reef	Year	Effort (min.)	*A.* cf. *solaris*	
			Total culled	Culled h^{-1}
Agincourt Reef	2015	720	96	8.0
	2016	8680	956	6.6
	2017	1400	2	0.1
	Total culled:		1054	
Moore Reef	2012	7551	585	4.6
	2013	43,057	4278	6.0
	2014	44,600	6427	8.6
	2015	36,765	7145	11.7
	2016	25,248	4609	11.0
	2017	29,972	2130	4.3
	2018	9921	58	0.4
	Total culled:		25,232	

The effort is given as total person search time, the number culled per hour can be regarded as a catch per unit effort

in 2017. The number culled per unit effort on Moore Reef increased from 2012 to 2016 and thereafter dropped to distinctly lower values. In total more than 25,000 *A.* cf. *solaris* were culled on Moore Reef between 2014 and 2018 (see Table 4).

Discussion

Extensive plankton sampling over three consecutive years has revealed inter-annual variability in larval abundance of *Acanthaster* cf. *solaris* at two reefs (Moore Reef and Agincourt Reef) in the northern GBR, which is presumably related to changes in local abundance and reproductive capacity of adults. Accordingly, benthic surveys at Agincourt and Moore Reefs revealed sustained declines in the abundance of both adult and juvenile CoTS through the course of this study. Despite these inter-annual differences, the timing of peak larvae detections was consistent among years. In all years studied (2015/16, 2016/17 and 2017/18),

clear spawning peaks were detected in the second half of December. More specifically, peak detections of larvae in plankton samples coincided with increasing water temperatures to 28 °C and were concentrated around the full moon.

Based on observed spawning events and changes in reproduction condition, previous studies, suggested that *Acanthaster* spp. spawns in summer, and mainly when water temperature is > 27 °C (Pratchett et al. 2014). In Japan, Yasuda et al. (2010) demonstrated that *A.* cf. *solaris* start to spawn once water temperatures reach 28 °C, which is consistent with our finding for the northern GBR. Aside from potentially inducing spawning in CoTS (Caballes and Pratchett 2017) temperature also has a major influence on early development. Embryonic and larval development for *A.* cf. *solaris* increases with increasing temperature above 28 °C (Hoegh-Guldberg and Pearse 1995; Uthicke et al. 2015b) up to a maximum of 31 °C (Lamare et al. 2014). Increasing temperature is therefore, likely, to promote increased gonad development, but may not necessarily represent the cue for when individuals spawn.

Caballes and Pratchett (2017) were able to induce spawning in male CoTS by not only increasing phytoplankton concentrations, but also by introducing sperm from other males. It is also possible that CoTS use other biophysical spawning cues, e.g., lunar phases as described for scleractinian corals (Babcock et al. 1986; Tanner 1996) to synchronise spawning at large scales. In our study, there was disproportionate detection of larvae 3 days before to 3 days after the full moon. This may be in indication for lunar cues in CoTS spawning, but this hypothesis requires further investigation. However, Babcock and Mundy (1992) found no link between CoTS spawning and lunar phases or tidal cycles. Similarly, close examination of a spawning observations collated by Pratchett et al. (2014, Table 1) does not indicate any alignment of spawning with lunar phases. Given the limited persistence of unfertilised eggs and spatial constraints to fertilization success (Babcock et al. 1994), it is likely that CoTS rely on very specific spawning cues to ensure localised spawning synchrony, rather than committing to synchronous spawning at larger scales. This means that the role of lunar phases may be obscured by other more localised cues and spawning patterns.

Larval densities in plankton tows at Moore Reef (up to ~ 14 Ind. $\times$ m^{-3}) were in the range of those reported in December 2014 for the GBR (Doyle et al. 2017) and June 2013 in Japan (Suzuki et al. 2016). We detected hardly any larvae at Agincourt Reef. The reasons for this are unresolved, but lower adult numbers on that and adjacent reefs and different hydrological conditions may be involved. We detected the highest larval density in the 2015/16, with declining numbers and incidence of positive samples in the following years. Interestingly, the reduction in larval numbers was reflected in reduced juvenile numbers. If we accept

that juveniles investigated here are 1+ year old, the 2015/16 larvae correspond to juveniles observed in 2017. The decline in larvae and juvenile numbers was also mirrored by a distinct decline in adults culled on the reefs. During previous outbreak cycles (Vanhatalo et al. 2017), outbreaks on the northern reefs generally declined after 5+ years, most likely due to a depletion of their food source (corals). Driven by prevailing currents moving the larvae, previous outbreaks moved south at rates of about 60 km year^{-1} (Vanhatalo et al. 2017). This seems a similar pattern during this fourth outbreak.

Microscopic examination of plankton samples showed most life stages identified were larvae in early developmental stages, and mainly gastrulae and early bipinnaria. In experimental cultures, gastrulae were generally observed 24–48 h post fertilisation, with early bipinnaria occurring after 3–4 days (Keesing et al. 1997; Lamare et al. 2014). This indicates that many of these larvae in the plankton indeed result from spawning on Moore Reef or nearby reefs. Given the time to settlement is at least 11 days and may be up to 22 days (Lucas 1982; Uthicke et al. 2015b; Pratchett et al. 2017), the proportion of self-recruitment, i.e. the amount of the observed larvae actually settling on Moore Reef, is unclear. Thus, we cannot be certain how many of the settlers and juveniles observed on Moore Reef are derived from the larvae we detected in the plankton tows.

Despite limited detections of late stage larvae in plankton tows at a time when settlement was likely to occur, settlement collectors confirm that there was effective settlement during the summer of 2016/2017. CoTS were detected (based on DNA) in ~25% of settlement collectors deployed on Moore Reef. Recruitment and juveniles are rarely observed in echinoderms, but reviews by Ebert (1983) and Balch and Scheibling (2001) found several examples of larvae and recruitment studies in echinoidea and ophiuroidea, whereas not many examples exist for asteroidea (but see: Sewell and Watson 1993). Given the difficulty to directly observe settlement of echinoderms to coral reefs due to their minute size and cryptic nature, settlement collectors are commonly used to detect recruitment, including in *Acanthaster* spp. (Keesing et al. 1993). The results of two staggered deployment periods over the 2016/2017 summer suggest that settlement was greater between December to March than October to February.

This study builds on qPCR methods we developed previously and tested with samples collected during a single sampling event (December 2014) in the Northern section of the GBR (Uthicke et al. 2015a; Doyle et al. 2017). Methods utilising qPCR have been developed for quantification of several marine planktonic species such as larvae of barnacles (Endo et al. 2010), crabs (Jensen et al. 2012), copepods (Jungbluth et al. 2013), algae (Kon et al. 2015) and other starfish (Richardson et al. 2016). Although we have demonstrated

CoTS eDNA is detectable in small volume (2 lt) water samples (Uthicke et al. 2018a), we believe this would have a negligible effect on the quantification of CoTS larvae as a single CoTS larvae at any of the possible life history stages has several orders of magnitude more target gene copies. In addition, not a single sample in the present study had positive CoTS DNA detection outside the spawning season. These facts confirm that contamination through free eDNA is no concern for larval quantification. We verified genetic findings by microscopy and amplification of individual larvae in a subset of plankton samples. In a previous study some larvae picked as putative CoTS larvae returned DNA sequences of other asteroids or holothuroids (Uthicke et al. 2015a). Similarly, in the present study many larvae picked as potential CoTS did not amplify with CoTS specific primers and are likely other echinoderms. However, some samples appeared as faint PCR bands and were excluded from the counts, making our microscopy-based estimates conservative. Despite this, larval concentrations found in comparative samples analysed with qPCR were mostly congruent, even though they were from separate plankton tows. Thus, microscopic and genetic analyses corroborate each other, indicating the range determined for the qPCR analysis estimated in our previous study (Doyle et al. 2017) was realistic.

In summary, intensive plankton sampling and genetic identification allowed us to refine the apparent spawning times for *A.* cf. *solaris* and suggests that lunar phases may be important in predicting when spawning will occur. The declining abundance of adult and juvenile CoTS in the Northern GBR during our study period reflects the natural demise of population outbreaks. This decline may also be accelerated by intensive culling and collections of juvenile CoTS. Our study further reveals the utility of qPCR in tracking temporal changes in larval supply and population dynamics. Comparison of the data across life stages clearly suggested that declining abundance of adults was directly linked to reduced larvae abundance, which further contributed to the local demise of CoTS populations. Understanding causes initiating this downward spiral would be crucial for managing future CoTS outbreaks.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest and consent was obtained from all participants.

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