Life Cycle, Prey Capture Ecology, and Physiological Tolerances of Medusae and Polyps of the 'Irukandji' Jellyfish: *Carukia barnesi*

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Thesis Dedication:

I would like to dedicate this thesis to The Lions Foundation of Australia, for their continual financial support dedicated to stinger research, and to Dr. Jack Barnes, for his pioneering work in the field of Irukandji research.

Statement of the Contribution of Others:

Robert Courtney is the primary author of this Thesis and was extensively involved in all aspects of this work under the supervision of: Associate Professor Jamie Seymour; Emeritus Professor Rhondda Jones; and Dr Nik Sachlikidis. Dr Tobin Northfield provided statistical support and assisted with the ecological modeling. Sally Browning assisted with animal husbandry, polyp culture maintenance, and photography. The Lions Foundation of Australia assisted with financial support of this project, providing collection equipment and the research vessel. The Australian Postgraduate Award (APA) scholarship provided financial support for Robert Courtney during this candidature.

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Abstract

This study focuses on the Irukandji jellyfish *Carukia barnesi* Southcott, 1967. Little is known about the general ecology of *C. barnesi*; however, the medusa stage is considered oceanic, planktonic, has been found around coral reefs or islands, and under certain conditions, on beaches. *Carukia barnesi* is a relatively small box jellyfish species (bell size up to 35 mm), that are typically present during the summer monsoonal summer months between November and May in Queensland, Australia, which is commonly referred to as the 'stinger' season. Although not well defined, the distribution of this species is considered along the Great Barrier Reef and adjacent coastline, between Lizard Island and Fraser Island. There is evidence that the length of the Irukandji season in the Queensland region has progressively increased over the last 50 years, based on annual sting records, from 15 days long historically to over 150 days long currently, which has been speculated to be attributed to increased seawater temperatures. Similarly, there have been anecdotal reports that the southern distribution of *C. barnesi* has also increased over the last 50 years.

A sting from *C. barnesi* commonly results in Irukandji syndrome, which is often severely painful, potentially fatal, and frequently requires hospitalization for treatment. The direct cost associated with treating envenomed victims, and the negative impact this species has on the Australian tourism industry through reduced revenue, are substantial (i.e., an estimated 65 million dollars in lost tourism revenue in 2002 alone). Further exacerbating the impact of this species is the simple fact that there are currently no methods in place for mitigating stings when this species is present other than through beach closures. Stinger exclusion nets are commonly used

along the north-eastern coast of Queensland; however, these nets are designed to exclude large cubozoan species, primarily *Chironex fleckeri*, and do not exclude small species such as *C. barnesi*. Also, this species occurs with substantial spatial and temporal variability during the monsoonal summer months. Therefore, understanding the factors that contribute to this variability may facilitate the ability to model, and therefore predict, when and under what circumstances this species may be more prevalent. Currently, the ecological data required to produce a predictive model does not exist. Prior to the commencement of this research project, the early life history of *C. barnesi* had never been observed, or described, and nothing was known about the thermal and osmotic tolerance, or preference, of any of its life stages.

This study first describes the early life history *C. barnesi*, from egg fertilization through to medusa production, and elucidates that this species develops an encapsulated planula stage that remains viable for six days to over six months. The polyps of *C. barnesi* asexually reproduce ciliated swimming polyps and produce medusae through monodisc strobilation. This study resulted in the first verified culture of *C. barnesi* polyps. With the polyp stage of the life cycle in culture, the opportunity to conduct manipulative temperature and salinity experiments were pursued, which provides new insights into potential polyp habitat suitability. Primary findings revealed 100% survivorship in osmotic treatments between 19‰ and 46‰, with the highest proliferation at 26‰. As salinity levels of 26‰ do not occur within the waters of the Great Barrier Reef or Coral Sea, it is concluded that the polyp stage

of *C. barnesi* are probably found in estuarine environments, where these lower salinity conditions commonly occur.

With the relationship of temperature and salinity on the polyp stage known, focus was shifted to exploration of these factors on the medusa stage. The thermal and osmotic tolerance of *C. barnesi* medusae were investigated to determine if environmental parameters drive the marked seasonality of this species. By exploring oxygen consumption over a range of temperatures, the minimum thermal requirement for *C. barnesi* was estimated at 21.5ºC, which does not explain the seasonal occurrence of this species. The optimum temperature for swimming pulse rate was determined to occur between 27.5ºC and 30.9ºC and the optimum temperature was estimated at 29.2ºC, which encompasses the typical summer thermal regime *in situ*. This research concludes that reduced fitness associated with environmental temperatures that departure from optimum may better explain the seasonal pattern of this species. Conversely, departure from optimum temperature did not explain the southern distribution limits of this species, suggesting that *C. barnesi* could theoretically persist further south than their loosely defined southern distribution limits. The optimum salinity of *C. barnesi* medusae was estimated at 35.8‰ and fitness was reduced as salinity levels reduced below 29‰, adding further support that *C. barnesi* medusae are oceanic and cannot persist in estuarine environments, where low salinity conditions commonly occur. The respiration rate of *C. barnesi* was significantly suppressed at night, providing evidence that this species is less active during night conditions, presumably to conserve energy.

Further exploration of the diurnal behavior pattern of *C. barnesi* medusae revealed that during light conditions, this species extends its tentacles and ‘twitches’

them frequently. This highlights the lure-like nematocyst clusters in the water column, which actively attract larval fish that are consequently stung and consumed. This fishing behaviour was not observed during dark conditions, presumably to reduce energy expenditure when they are not luring visually oriented prey. Larger medusae were found to have longer tentacles; however, the spacing between the nematocyst clusters was not dependent on size suggesting the spacing of the nematocyst clusters is important for prey capture. Additionally, larger specimens twitch their tentacles more frequently than small specimens, which correlate with their ontogenetic prey shift from plankton to larval fish. These results indicate that adult medusae of *C. barnesi* are not opportunistically grazing in the water column; instead, they utilize sophisticated prey capture techniques to specifically target larval fish.

This thesis also discusses the results of each of the experiments as a whole, and highlights areas where future research is required to predictively model the occurrence of this species. The overall focus of this thesis was to better understand the ecology and physiological limitations of *C. barnesi* to elucidate the factors that may contribute to the observed seasonal and distributional patterns. This research has also produced the baseline data for future research to build upon, with the expectation that the synthesis of these and future data will facilitate the ability to model, and therefore predict, the occurrence of this species in order to reduce the number of people stung.

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Chapter 1: Cubozoan Ecology

1.1 Introduction

Cubozoans, or box jellyfish, are taxonomically classified within the phylum Cnidaria with polypodiozoans, staurozoans, hydrozoans, anthozoans, and are most similar to scyphozoans, or true jellyfish (Arai, 1997). Cubozoa is further subdivided into two orders: Chirodropida, which have multiple tentacles extending from each pedalia; and Carybdeida, which usually possess only a single tentacle per pedalia and in most cases are comparatively smaller than Chirodropida species (Arai, 1997; Bentlage & Lewis, 2012; Lewis & Bentlage, 2009). There are over 40 described species of Cubozoa globally (Daly et al., 2007; Kingsford & Mooney, 2014; Lewis & Bentlage, 2009), which is variable due to occasional new species discoveries (Gershwin & Ekins, 2015). Furthermore, application of phylogenetic analysis has regrouped some species, such as; *Carybdea alata* Reynaud, 1830, *Alatina moseri* Mayer. 1906 and *Alatina mordens* Gershwin, 2005 have been reclassified as *Alatina alata* Raynaud, 1830 (Lewis et al., 2013).

Cubozoans have a circumglobal distribution spanning all continents except Antarctica, with the southernmost occurrences recorded from the southern tip of Africa (Gershwin & Gibbons, 2009) and New Zealand (Crow et al., 2015) and there have been reports of occurrances as far north as Mexico (Carrette et al., 2012), California (Straehler-Pohl & Matsumoto, 2017) and Japan (Lewis & Bentlage, 2009). However, many species appear to be restricted to warm tropical waters (Carrette et al., 2012; Kingsford & Mooney, 2014). Within Australian waters, there are two main species from the order Chirodropida (i.e., *Chironex fleckeri* Southcott,

1956*, Chiropsella bronzie* Gershwin, 2006) and numerous species from the order Carybdeida (e.g., *Carukia barnesi* Southcott, 1967*, Alatina alata, Morbakka fenneri* Gershwin, 2008, *Carybdea rastonii* Haacke, 1886, *Copula sivickisi* Stiasny, 1926, and *Tripedalia binata* Moore, 1988) (Barnes & Kinsey, 1986; Carrette et al., 2012; Hartwick, 1991a; Hartwick, 1991b; Kinsey & Barnes, 1988; Southcott, 1956; Southcott, 1967). Two cubozoan species, *C. barnesi* and *C. fleckeri*, have a large negative impact on humans in Australia (Barnes & Kinsey, 1986; Carrette, et al., 2012, Kinsey & Barnes, 1988; Southcott, 1956; Southcott, 1967) and seem to be restricted to warm tropical conditions (Carrette et al., 2012; Gershwin et al., 2013; Kingsford & Mooney, 2014).

Australian cubozoans exhibit marked seasonality, arriving in large numbers along the northern coast of Australia during the summer monsoonal months (Barnes & Kinsey, 1986; Burnett et al., 1996; Gordon et al., 2004; Gordon & Seymour, 2009; Hartwick, 1991a, Kinsey & Barnes, 1988; Kingsford & Mooney, 2014). This seasonal occurrence, 'stinger season', November through May along the eastern Australian coast, has been reported to begin earlier (September) and last longer (June) in the warmer waters of the Gulf of Carpentaria (Fenner & Harrison, 2000; Gordon & Seymour, 2012). The factors that drive the strong seasonality of these cubozoans are thought to be a combination of environmental factors, such as temperature and salinity fluctuations during the monsoonal summer months, in combination with their complex life cycle (Gordon et al., 2004; Gordon & Seymour, 2012; Hartwick, 1991a; Kingsford & Mooney, 2014).

Cubozoa have a metagenic life cycle and undergo an alternation of generations passing through a sessile, benthic, asexually reproductive polyp stage and motile, sexually reproductive medusa stage (Arai, 1997; Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Hartwick, 1991a; Werner et al., 1971). Cubozoan polyps typically reproduce through lateral budding of a secondary polyp and temperature, salinity and food availability are known to influence the rate of asexual reproduction of the polyp stage of *A. alata* (Courtney & Seymour, 2013; Klein et al., 2014) and *Carybdea marsupialis* Linnaeus, 1758 (Acevedo et al., 2013; Canepa et al., 2014; Fischer & Hofmann, 2004). This lateral budding often produces a motile secondary morphotype that may be in the form of a creeping stage polyp, which crawls along the substrate with its tentacles prior to settlement (Carrette et al., 2014; Hartwick, 1991a; Straehler-Pohl & Jarms, 2011; Yamaguchi & Hartwick 1980), or a swimming stage polyp, which are ciliated and swim near substrate prior to settlement (Toshino et al., 2013).

The abundance of the medusa stage is thought to reflect the success of the polyp stage, where asexual reproduction of the polyps contributes to the number of polyps able to produce medusae at any one time. However, in most instances, the *in situ* parameters of the polyps are not known due to the unknown location of the polyps. To date, cubozoan polyps have only been discovered *in situ* for two species, *C. fleckeri* and *C. marsupialis* (Cutress & Studebaker, 1973; Fischer & Hofmann, 2004; Hartwick, 1991a; Yamaguchi & Hartwick, 1980). Because of this, laboratory-based polyp cultures that have been produced from wild caught medusae have been a primary source of information regarding this early life stage (Carrette et al., 2014;

Straehler-Pohl & Jarms, 2011). However, of the over 40 described species of cubozoans, the early life history of less than ten cubozoans have been described globally (Arneson & Cutress, 1976; Courtney et al., 2016; Cutress & Studebaker, 1973; Hartwick, 1991a; Hartwick, 1991b; Lewis & Long, 2005; Straehler-Pohl & Jarms, 2011; Toshino et al., 2013; Toshino et al., 2014; Toshino et al., 2015; Werner et al., 1971; Yamaguchi & Hartwick, 1980). This highlights an area where future research is required to further the understanding of this life stage, which could be through further production of laboratory-based polyp cultures and through locating more polyps *in situ*.

The polyp stage of cubozoans is also thought to contribute to the seasonal timing of the medusa stage through synchronous medusa production (Hartwick, 1991a). To synchronise medusa production, many species have been experimentally shown to utilize variations in environmental or food parameters as cues to initiate medusa production, such as temperature (Laska-Mehnert, 1985; Stangl et al., 2002; Toshino et al., 2015; Werner, 1983), a combination of temperature and light intensity (Straehler-Pohl & Jarms, 2011), or reduced food availability (Courtney & Seymour, 2013).

For at least one Australian species, *C. fleckeri*, increasing photoperiod has been suggested as a seasonal cue that initiates the metamorphosis process due to the consistency of the occurrence of the medusae over a six-year period (Gordon & Seymour, 2012). This was established by exploring the daily growth rings of the statoliths of the medusae, and back calculating the time of metamorphosis. Similar techniques were also applied to another Australian species, *Chiropsella bronzie*,

which indicated that multiple cohorts of this species were correlated with rainfall events (Gordon et al., 2004). After medusa production is complete, medusae grow to sexual maturity, spawn, and soon after disappear; presumably dying. The factors that determine the end of the stinger season are currently unknown; however, because the end of the stinger season in Australia coincides with the advent of cooler winter water temperatures, it is plausible that temperature has a direct impact on the medusa stage of cubozoans, and may be a critical factor that determines the end of the stinger season.

1.2 Cubozoan Life Cycle

The life cycle of cubozoans begins through sexual reproduction, which results in egg fertilization (Arai, 1997). This process is variable between species and can be in the form of spawning aggregations of medusae that mass spawn into the water column resulting in external egg fertilization (e.g., *Morbakka virulenta* Kishinouyea, 1910; Toshino et al., 2013), spawning aggregations where internal fertilization occurs (e.g., *Carybdea marsupialis*; Cutress & Studebaker, 1973), paired courtship of medusae, which results in internal fertilization (e.g., *Carybdea sivickisi, Tripedalia cystophora* Conant, 1897, Hartwick 1991b; Lewis & Long, 2005; Werner et al., 1971), or courtship behavior where external fertilization occurs (which has been suggested for *Chironex fleckeri* but never confirmed; Barnes & Kinsey, 1986). In other cases, only experimental artificial external fertilization has been observed (e.g., *Alatina alata*; Carrette et al., 2014).

The next phase of egg development begins with standard cell division to the blastula stage and then development into planulae. This process can take place internally within the adult female medusae (e.g., *T. cystophora*; Werner et al., 1971), externally in an embryo strand (e.g., *Copula sivickisi*; Hartwick, 1991b; Lewis & Long, 2005), or externally without an embryo strand (e.g., *A. alata*; Carrette et al., 2014). Primarily, planulae then hatch as ciliated, free swimming planulae. However, in *M. virulenta*, a blastocyst stage has been described that may hatch as a planula or undergo direct development to the polyp stage, essentially hatching as a polyp (Toshino et al., 2013). Development times of the planula stage are highly variable, ranging from hours to days, and the duration of the planula stage is also highly variable (see Straehler-Pohl & Jarms, 2011).

The primary function of the planula stage seems to be for dispersal and to locate suitable substrate and orientation for metamorphosis from planula to primary polyp. In most instances, a secondary polyp morphotype develops where the planulae settle, described as a creeping stage polyp, which is able to crawl along the substrate to locate a suitable attachment point (Carrette et al., 2014; Hartwick, 1991a; Straehler-Pohl & Jarms, 2011). Other species simply develop primary polyps where the planulae settle (e.g., *Morbakka virulenta*; Toshino et al., 2013). These primary polyps then begin to develop a series of tentacles that, in most cases, have at least one nematocyst within each tentacle tip, with exception noted in *Chironex fleckeri*, that do not seem to develop nematocysts until after the hypostome has formed at the two to four tentacle stage (Hartwick, 1991a; Yamaguchi & Hartwick, 1980).

At the primary polyp stage the hypostome develops and polyps begin to feed by entangling and subduing live prey with their tentacles (Straehler-Pohl & Jarms, 2011). Under laboratory culture conditions, the primary prey consists of rotifers and *Artemia* nauplii due to their ease of culturing (Carrette et al., 2014); however, the primary food source for these polyps *in situ* is expected to be small planktonic animals, such as copepods with an exception noted in *Carybdea morandinii*, which also has photosynthetic zooxanthellae (Straehler-Pohl & Jarms, 2011). As polyps grow, they continue to produce more tentacles with the maximum number variable and species dependent (Straehler-Pohl & Jarms, 2011). At this stage, asexual reproduction begins.

Lateral budding of secondary polyps seems to be the most common form of asexual reproduction (Straehler-Pohl & Jarms, 2011). Lateral budding primarily produces a motile secondary polyp morphotype which is able to move away from the primary polyp. This can be in the form of a creeping polyp as seen in *A. alata, C. fleckeri, C. sivickisi, C. marsupialis* (Carrette et al., 2014; Hartwick, 1991a; Hartwick, 1991b; Toshino et al., 2014), or in the form of a ciliated swimming polyp, as seen in *M. virulenta* (Toshino et al., 2013). Longitudinal fission has also been recorded as a common reproductive strategy in *C. morandinii* (Straehler-Pohl & Jarms, 2011), and intratentacular fission has also been reported for this species (Straehler-Pohl & Jarms, 2011). The rate of asexual reproduction is highly variable and is condition- specific. For example, temperature and salinity have been shown to affect the asexual reproduction rate of *A. alata* polyps where, under suitable temperature and salinity conditions, the number of polyps increased and outside of

this range asexual reproduction ceased (Courtney & Seymour, 2013). Likewise, food availability has been shown to be positively correlated with bud production (Courtney & Seymour, 2013).

The motile polyp morphotypes then attach to the substrate and begin development into a polyp morphotype similar to that of the primary polyp prior to the commencement of asexual reproduction (Straehler-Pohl & Jarms, 2011). Theoretically, this life phase could continue indefinitely provided the conditions are suitable. However, an environmental cue initiates the polyps to produce medusae (Courtney & Seymour, 2013; Laska-Mehnert, 1985; Stangl et al., 2002; Straehler-Pohl & Jarms, 2011; Toshino et al., 2015; Werner, 1983). Medusa production in cubozoans can be in the form of complete metamorphosis of the polyp (Arneson & Cutress, 1976; Carrette et al., 2014; Cutress & Studebaker, 1973; Stangl et al., 2002; Straehler-Pohl & Jarms, 2011; Werner et al., 1971; Yamaguchi & Hartwick, 1980), metamorphosis that leaves behind a small amount of regenerative material, which is able to develop into a polyp (Straehler-Pohl & Jarms, 2005), and the most recently discovered method, monodisc strobilation, that leaves behind a polyp able to continue asexual reproduction of more polyps (Toshino et al., 2015).

1.3 Cubozoan Polyps

Very little is known about cubozoan polyps *in situ* as they have only been located on two occasions. On one occasion, *Carybdea marsupialis* polyps were located attached to dead bivalve shells on the bottom of mangrove channels in Puerto Rico (Fischer & Hoffman, 2004). On the second occasion, polyps of *Chironex*

fleckeri were located in channels on the underside of rocks within an estuarine system in Queensland, Australia (Hartwick, 1991a). *Chironex fleckeri* polyps have been reported to have a distinct preference for the underside of rocks (Hartwick, 1991a). It has been speculated that polyps may choose the underside of structures for a variety of reasons. For example, cubozoan polyps require hard substrate for attachment and choosing the underside of structures allow the polyps to avoid strong currents that may dislodge the polyps (Hartwick, 1991a). There is also the advantage of reduced competition with photosynthetic species that require the upper side of structures to increase solar exposure, such as many cnidarians and algae (Hartwick, 1991a). The underside of structures may also reduce the amount of siltation the polyp may experience (Hartwick, 1991a). Polyps are also thought to hang down into the water column to capture prey and this inverted position may aid in purging undigested material (Hartwick, 1991a).

Further exacerbating the search for cubozoan polyps *in situ* is size; these polyps are very small (1-2 mm) and translucent (see Straehler-Pohl & Jarms, 2011). Structurally, polyps tend to have a simple body plan with few identifiable features that can be used to distinguish between species and, as for most cubozoans, the polyps have not yet been discovered or described. Therefore, for species identification, polyps would usually need to be reared through to medusae for positive identification. Theoretically, the application of genetic identification methods may be implemented for species identification; however, this has only been applied to cubozoan medusae and has not been attempted on cubozoan polyps.

Under laboratory conditions, cubozoan polyps have been found to attach to a number of substrates including coral blocks, glass and polycarbonate containers (Courtney et al., 2013; Straehler-Pohl & Jarms, 2011). These polyps have been shown to attach to the base of containers and on vertical surfaces, however, settlement choice experiments that target material preference and/or orientation preference have not been conducted on any species of cubozoan polyps. This information is required for better understanding the ecology of this important life stage and for predicting how changes to the available substrate may influence the population dynamics of cubozoan polyps (e.g., increasing manmade structures within polyp habitats may be beneficial to the polyps) (Klein et al., 2014). The geographic location of these polyps needs to be discovered to determine what parameters these polyps experience to define the factors that contribute to their distribution, survivorship, reproduction rates and cues for medusa production. This is integral for understanding the ecology of cubozoans.

1.4 Physiological Tolerances of Cubozoan Polyps

Although the geographic location of cubozoan polyps is essentially unknown for most species, experiments that determine the physiological tolerances and/or preferences of the polyps should provide evidence about where these polyps may be located. For example, *Alatina alata* has an oceanic offshore medusa stage and the polyps have been speculated to only survive at depths of less than 300 m due to their thermal tolerance (Courtney & Seymour, 2013). Research on *Carybdea* sp. polyps indicated that these polyps survive in areas with fluctuations in salinity because high

salinity conditions caused increased asexual reproduction and reduced salinity conditions initiated metamorphosis (Canepa et al., 2014). This is particularly interesting as these polyps were of the same species, *Carybdea marsupialis*, and location, Puerto Rico, as the polyps located *in situ* attached to dead bivalve shells in mangrove systems, which are expected to experience variable salinity conditions (Fischer & Hofmann, 2004). The effects of varying temperature and pH conditions have been explored in polyps of *A. alata* to explore how projected ocean acidification scenarios may affect future cubozoan abundance (Klein et al., 2014). This research indicated that although the polyps reproduce at a higher rate under warmer conditions, ocean acidification may impair statolith development during metamorphosis, which may impact negatively on this species. Not surprisingly, feeding frequency has also been shown to be positively correlated with asexual reproduction in *A. alata* (Courtney & Seymour, 2013). Other than these few experiments, very limited experimental data exists on the thermal and/or osmotic tolerance of cubozoan polyps.

There have been some trials that have indicated that polyps may form cysts under unfavorable conditions (Carrette et al., 2014; Hartwick, 1991a; Straehler-Pohl & Jarms, 2011), however, these are not replicated experiments designed to determine the physiological limitations and/or preferences of polyps. On one occurrence, temperature has been shown to affect the creeping stage of *C. morandinii* polyps, which at 20ºC to 25ºC creep for two to three days and above 25ºC this phase is extended to seven to ten days (Straehler-Pohl & Jarms, 2011). In the same study, at temperatures above 25ºC even fully developed polyps could transform back into a

creeping stage, which would detach, leaving behind the periderm collar, and move over the substrate until temperatures dropped to 25ºC (Straehler-Pohl & Jarms, 2011). This suggests that temperature may be an important influence in the settlement choice of this species, and also indicates yet another self-perseverance adaptation to cope with adverse conditions. This is an area that desperately warrants further research (Kingsford & Mooney, 2014).

The polyps for most cubozoan species are undescribed and the geographic location of the polyps is also unknown, therefore, having little understanding of the physiological constraints or preferences of the polyps is of critical concern. For example, currently there is no estimate for the reproductive output of cubozoans (Kingsford & Mooney, 2014). The asexual reproduction rates of cubozoan polyps, and developmental times of all the life stages are thought to be at least partially driven by temperature. However, many other factors such as, salinity, food availability/quality and pH, are expected to also impact on these rates. Furthermore, without understanding of the limitations and preferences of the polyp stage, it is impossible to elucidate many aspects of cubozoan ecology.

1.5 Motile Stages of the Early Life History of Cubozoans

The complex life cycle of cubozoans is often generalized into two primary stages, the sessile benthic polyp stage and the motile planktonic medusa stage (Arai, 1997; Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Hartwick, 1991a; Werner et al., 1971). This would suggest that the medusa stage would be entirely responsible for the distribution of the polyps (e.g., the polyps would be located near

where medusae spawn if this assumption were true). However, there are a few other life stages that do not fit these two categories and may be of great importance to the distribution of the polyps, and therefore the medusae. For example, some cubozoan medusae reproduce through internal fertilization and the medusae theoretically may traverse an unknown distance prior to egg release, while other species release these eggs directly into the water column. One species, *Morbakka virulenta*, releases eggs that enter a blastocyst state for seven to 21 days (Tohsino et al., 2013). Although these blastocysts are not directly motile, currents may move these early life stages great distances depending on the current velocity.

All described cubozoan life cycles then pass through a motile ciliated planula stage, which is the first directly motile stage (Nordstrom et al., 2003), with the exception noted in *M. virulenta* that may remain as unhatched planulae and develop directly into a polyp (Toshino et al., 2013). Cubozoan planulae are ciliated and freely swim in the water column prior to settlement and metamorphosis into a polyp (Hartwick, 1991a; Toshino et al., 2013). This life stage is expected to allow for dispersal and may be important for settlement positioning, with regards to both substrate choice and orientation. Many cubozoan planulae have larval ocelli, photoreceptive eye spots, located on the anterior end, as seen in *Chironex fleckeri*, on the posterior end, as seen in *Tripedalia cystophora* Conant, 1897 or around the central region, as seen in *Chiropsella bronzie* (Nordstrom et al., 2003) and *Copula sivickisi* (Toshino et al., 2014). Other cubozoan planulae, such as *M. virulenta*, lack these structures entirely (Toshino et al., 2013).

Larval ocelli are expected to aid planulae in navigation, either away from or towards light, or to seek certain light intensities (Nordstrom et al., 2003). The differences between the planulae of different species may provide insight into the ecology of this life stage. Some species lack this structure (e.g., *M. virulenta*, Toshino et al., 2013), suggesting that the planulae of some species are well enough equipped to find appropriate substrate and/or orientation for polyp development without visual aid. Alternatively, species with larval ocelli may require simple vision for settlement choice. Because this life stage has never been studied *in situ*, primarily due to their small size and unknown location, very little is known about the ecology of planulae, such as swimming distances, reaction to light at different stages, substrate preference for settlement, orientation preference and tolerances to temperature and salinity. This is another area of cubozoan ecology where experiments that target swimming distances and settlement choice, ideally under variations in light conditions, temperatures, and salinities, is required for a better understanding of the ecology of this life stage.

Cubozoans also poses a motile benthic stage in the form of a creeping stage polyp morphotype (Carrette et al., 2014; Hartwick, 1991a; Straehler-Pohl & Jarms, 2011). Metamorphosis of cubozoan planulae often produce a creeping stage morphotype that moves along the substrate in search of an attachment point (Hartwick, 1991a). However, at least some species opt to either metamorphose into a creeping stage polyp or develop directly as a settled primary polyp (Toshino et al., 2013; Toshino et al., 2014). For example, *C. fleckeri* and *A. alata* polyps are reported to go through a creeping stage (Carrette et al., 2014; Hartwick, 1991a) and *C.*

siviskisi has been reported to do both (Toshino et al., 2014). *Morbakka virulenta* only metamorphoses from a planula directly to a primary polyp (Toshino et al., 2013; Toshino et al., 2015). The primary polyps of cubozoans also bud off motile secondary polyps, which are often in the form of creeping polyps, as seen in *A. alata* (Carrette et al., 2014), *Carybdea morandinii* (Straehler-Pohl & Jarms, 2011), *C. fleckeri* (Hartwick, 1991a), or as swimming stage polyps observed in *M. virulenta* (Toshino et al., 2013; Toshino et al., 2015).

The function of motile morphotypes is once again expected to be for dispersal, however the distances that these motile polyps travel *in situ* remains unknown because they have only been observed under laboratory conditions. Due to this, dispersal distances can only be estimated by the rate of travel and/or the average time span that these polyps remain motile prior to settlement (e.g., millimeters per hour for *C. fleckeri*; Hartwick, 1991a,). This is another area where experiments are required to determine the dispersal distances and substrate choice and orientation preference of this life stage to gain greater insight into the ecology of cubozoans.

1.6 Medusa Production

Arguably one of the most important phases in the life cycle of cubozoans is medusa production and the factors that provide cues that initiate synchronous medusa production. The general consensus is an environmental cue(s) cause the polyps to undergo medusa production, which ultimately contributes to the seasonality of many cubozoan medusae (Laska-Mehnert, 1985; Stangl et al., 2002; Toshino et al., 2015; Werner, 1983). This cue is most likely species specific and

specific to different populations within the same species if they are geographically separated. Cubozoan polyps are expected to react to an environmental trigger that cause the polyps to arrest asexual output and invest into medusa production.

As previously mentioned, these cues have been shown to include increasing temperature (Laska-Mehnert, 1985; Stangl et al., 2002; Toshino et al., 2015; Werner, 1983), increasing temperature in combination with light intensity (Straehler-Pohl & Jarms, 2011), reduced food availability (Courtney & Seymour, 2013), decreasing salinity (Canepa et al., 2014), and speculatively, increasing photoperiod (Gordon & Seymour, 2009). The nature of the specific parameter may influence the outcome. For example, increasing photoperiod may act on an entire group of polyps and produce a very predictable pulse of medusae that is consistent between years. This type of cue has been speculated to cause *Chironex fleckeri* to undergo metamorphosis due to the consistency of the arrival of the medusae over a six-year trial (Gordon & Seymour, 2009). This study concluded that the medusae were produced at the same time each year. This was determined through analysis of age estimates of the medusae (i.e., daily growth rings in the medusa statoliths allow back-calculation to the date of metamorphosis). This theory was based on the evidence that all of the environmental conditions between years, such as temperature, salinity and wind, were highly variable and one of the only factors that may produce this level of consistency (i.e., ± seven days over six years) would be an event that occurs consistently between years; increasing photoperiod (Gordon & Seymour, 2009). However, this has not been determined through manipulative experiments on *C. fleckeri* polyps as no polyps exist in culture currently. Once medusa production in

C. fleckeri is initiated, it seems to continue throughout the medusa season (Gordon & Seymour, 2009).

A seasonally variable cue that triggers medusa production, such as increasing temperature or decreasing salinity, may produce a localized pulse of medusae. Increasing temperature has been experimentally shown to induce synchronous medusa production in some cubozoans. For example, *Tripedalia cystophora* (Laska-Mehnert, 1985; Werner, 1983), *Carybdea marsupialis* (Stangl et al., 2002), *Copula sivickisi* (Toshino et al., 2014) and *Morbakka virulenta* (Toshino et al., 2015) have been shown to produce medusae in response to increasing temperature. Temperature increase in combination with increasing light intensity has also been experimentally shown to induce medusa production in *Carybdea morandinii* (Straehler-Pohl & Jarms, 2011). This result is unique because this is the only cubozoan polyp to be described that possesses symbiotic photosynthetic zooxanthellae (*Symbiodinium* spp.).

Reducing salinity has also been explored as a factor that triggers medusa production in *Carybdea* sp., whereby as salinity is increased, the rate of asexual reproduction increased, and under low salinity conditions asexual reproduction was suppressed and these polyps began to produce medusae (Canapa et al., 2014). Furthermore, these polyps were found to also produce medusae at a faster rate under low salinity conditions (Canapa et al., 2014). A correlation between rain events and medusae abundance has also been shown in *Chiropsella bronzie*, where influxes of juvenile medusae appear approximately 14 days after significant rainfall events, suggesting that once medusa production is initiated, it may be punctuated by low

salinity pulses, caused by rainfall events (Gordon et al., 2004). The effect of low salinity conditions on *C. bronzie* polyps has not been experimentally determined because the polyps of this species have never been seen.

Although not an environmental cue, reduced food availability has also been experimentally shown to induce medusa production in *Alatina alata* polyps (Courtney & Seymour, 2013). However, as this is probably density dependent, and may act on a group of polyps, this would lead to very unpredictable medusa production that would plausibly occur at any given time. *Alatina alata* is a tropical oceanic species and the polyps are presumably located offshore due to the location of the medusae and the thermal and osmotic preferences of the polyp stage (Courtney & Seymour, 2013). During a replicated trial, temperature, salinity, and combinations thereof, did not induce medusa production in this species; however, reduced food did (Courtney & Seymour, 2013).

It has been speculated that offshore tropical waters may have fairly stable temperature and salinity regimes and therefore an environmental cue that initiates synchronous medusa production may not be sufficient (Courtney & Seymour, 2013). For example, continuous asexual reproduction of these polyps would presumably lead to increased polyp density, thus reducing the food availability of each polyp. If individual polyps, or groups of polyps, are effectively starving, then medusa production may be a survival strategy. This type of cue would be expected to lead to medusa production that has the potential to happen at any time. Further supporting this theory, the medusae of *A. alata* are found all year long where they form predictable spawning aggregations eight to ten days after each full moon in the Coral

Sea and near Hawaii (Carrette et al., 2014; Chiaverano et al., 2013; Crow et al., 2015; Lewis et al., 2013; Thomas et al., 2001).

Historically, cubozoans have been described as only able to produce medusae through complete metamorphosis of the polyp into a medusa, which was one of the defining features separating cubozoans from scyphozoans (Werner et al., 1971). Complete metamorphosis occurs when the entire polyp arrests asexual reproduction and undergoes metamorphosis into a medusa which leaves behind no remnant material (i.e., one polyp produces one medusa) (Hartwick, 1991a; Werner et al., 1971). This form of medusa production is still considered the most common, however, there have been three modes discovered so far: complete metamorphosis (Arneson & Cutress 1976; Carrette et al., 2014; Cutress & Studebaker, 1973; Stangl et al., 2002; Straehler-Pohl & Jarms, 2011; Werner et al., 1971; Yamaguchi & Hartwick, 1980); metamorphosis that leaves material behind that develops into a polyp (residuum) (Straehler-Pohl & Jarms, 2005); and monodisc strobilation, which leaves behind a polyp able to continue asexual reproduction (Toshino et al., 2015).

The latter two are considered asexually reproductive events. Of all of the described cubozoan life cycles, complete metamorphosis has been reported to take place in *A. alata* (Arneson & Cutress 1976; Carrette et al., 2014), *C. morandinii* (Straehler-Pohl & Jarms, 2011), *C. marsupialis* from Puerto Rico (Cutress & Studebaker, 1973), *T. cystophora* (Laska-mehnert, 1985; Werner et al., 1971), *C. fleckeri* (Yamaguchi & Hartwick, 1980), and *C. siviskisi* (Toshino et al., 2014), which constitutes the majority of cubozoan life cycle research. One of these cubozoans, *C. marsupialis* from Puerto Rico, has been shown to also adopt a second

strategy of metamorphosis that leaves material behind that develops into a polyp (residuum) (Straehler-Pohl & Jarms, 2005). The polyps of *M. virulenta* have been shown to produce medusa through monodisc strobilation (Toshino et al., 2015). These are the only two cubozoans that have been shown to produce medusa through a method other than complete metamorphosis.

Complete metamorphosis of the polyp is the most common form of medusa production, and the first mode of medusa production discovered, which has led to various theories. The medusa form primarily allows for sexual reproduction, genetic diversity, and likely contributes to the distribution of polyps. Not surprisingly, the medusae are thought to be produced by the polyps during favorable conditions, such as during suitable temperature or salinity regimes (Hartwick, 1991a). Metamorphosis does not leave behind a polyp; therefore, polyps may live in habitats that do not allow for the annual persistence of the polyp stage (Hartwick, 1991a). For example, *C. fleckeri* polyps, which have been found in estuarine systems in Queensland, Australia, are presumed to undergo metamorphosis prior to the summer monsoonal months, where the expected polyp habitats are exposed to high seasonal rainfall events. These rainfall events presumably would decrease temperature and salinity levels drastically and in many areas, produce strong currents. To avoid these conditions, polyps may undergo metamorphosis, to essentially capitalize on two environmental niches, through two body forms, in sequence with the seasons (i.e., persist as polyps during the dry winter months and metamorphose to medusae during the monsoonal summer months; Hartwick, 1991a). Therefore, metamorphosis of the polyps may be linked to a self-perseverance function, whereby the polyps may

survive adverse conditions by undergoing metamorphosis (Hartwick, 1991a). For example, complete metamorphosis would probably be an efficient strategy if the polyp has no chance of survival, as suggested for *C. fleckeri*, where the polyps are expected to be unable to survive the monsoonal inundation (Hartwick, 1991a). Alternatively, *C. marsupialis*, which leaves behind a remnant piece of regenerative material (Straehler-Pohl & Jarms, 2005), may reside in locations where there is some chance that the polyps may persist. *Morbakka virulenta*, which has recently been discovered to undergo monodisc strobilation (Toshino et al., 2015), may inhabit a stable environment where there is a high chance of polyp survival. Understanding the cues for medusa production is expected to provide insight as to what pressures the polyps of a particular species are under, which may elucidate clues as to the ecology of each species.

1.7 Polyp Encystment

Another strategy that cubozoan polyps adopt to persist through periods of adverse conditions is through a process referred to as encystment. Cubozoan polyps are able to contract and produce a mucus layer, which hardens, producing an encapsulated cyst (Straehler-Pohl & Jarms, 2011). Polyps of *Chironex fleckeri* and *Tripedalia cystophora* have been shown to produce these cysts in response to drastic salinity changes, surviving in this state for periods of up to two weeks and reemerge from these cysts when salinity levels were restored (Hartwick, 1991a; Werner, 1975). Polyps of *Carybdea marsupialis* and *Carybdea morandinii* have also been found to form these cysts (Straehler-Pohl & Jarms, 2011). Polyps of *Alatina alata*

have been found to form these cysts in response to starvation, and survive in this state for months (Carrette et al., 2014). *Carybdea morandinii* polyps have been experimentally shown to become encysted in response to unfavorable temperatures (Straehler-Pohl & Jarms, 2011). Additionally, polyps of *C. morandinii* have also been shown to produce mucus anchor strands that attach to substrate, or detach, allowing dispersal (Straehler-Pohl & Jarms, 2011).

1.8 Medusa Development

The medusa stage begins upon completed metamorphosis of the polyp (Hartwick, 1991a), or through medusa detachment from the parent polyp in the case of monodisc strobilation (Toshino et al., 2015). Therefore, as previously mentioned, the polyp stage contributes to the seasonal onset of the medusa stage. Newly detached medusae are presumed to feed primarily on small planktonic invertebrates such as copepods due to the small size of the young medusae. Some cubozoan medusae maintain this diet throughout maturation. For example, adult *Tripedalia cystophora* have been observed *in situ* feeding on swarms of copepods (Buskey, 2003; Stewart, 1996). Some larger cubozoans, such as *Chiropsella bronzie*, maintain an invertebrate diet and feed exclusively on small prawns (*Acetes australis*) (Carrette et al., 2002). Alternatively, the largest cubozoan, *Chironex fleckeri*, is known to primarily consume *A. australis* when juvenile and undergo ontogenetic venom and prey variation during maturation, shifting its diet from invertebrate to vertebrate prey (Carrette et al., 2002). This phenomenon is not restricted to large species; *Carukia*

barnesi are also known to undergo ontogenetic venom and prey variation during maturation (Underwood & Seymour, 2007).

The amount of time it takes for cubozoan medusae to reach sexual maturity, and the lifespan of medusae, is an area where more research is required. The general consensus is that medusae only survive for a maximum of one season. For example, the large cubozoan *C. fleckeri* are primarily small early in the season and much larger and sexually mature late in the season (Barnes & Kinsey, 1986). The time it takes to reach maturity has been difficult to determine based on size alone as cubozoans are able to grow smaller during periods of adversity, which may result in a poor correlation between size and age (Gordon & Seymour, 2009). Some research has avoided this problem by analyzing cubozoan statoliths, which have been shown to develop daily growth rings similar to otoliths in fish (Gordon et al., 2004; Gordon & Seymour, 2009; Kawamura et al., 2003).

The analysis of the daily growth rings in the statoliths provides useful information about cubozoan medusae such as, age, growth rate, estimated time to sexual maturity, back-calculation to the time of medusa production, and may be used to distinguish between cohorts. For example, *Chiropsalmus quadrigatus* Haeckel, 1880, can increase in size at a rate of three millimeters per day (Kawamura et al., 2003). Similar growth rates have been estimated for *C. fleckeri* (Gordon & Seymour, 2009). Another chirodropid, *C. bronzie,* has been shown to have a maximum growth rate of one millimeter per day (Gordon et al., 2004). Over a six-year period, back-calculation to the time of medusa production for *C. fleckeri* has indicated that the commencement of medusa production begins at a similar time each year, suggesting

increasing photoperiod may be a cue that initiates metamorphosis in this species (Gordon & Seymour, 2009). This research has also shown that medusae are continuously produced during the season after metamorphosis has commenced (Gordon & Seymour, 2009).

Some species, such as *Alatina alata*, are known to use reduced food as a cue for medusa production (Courtney & Seymour, 2013). This species may produce medusae throughout the year due to the cue for medusa production not being a seasonal event (i.e., starvation). For example, the medusae of *A. alata* are present all year long (Chiaverano et al., 2013). However, the periodicity of *A. alata* has been demonstrated to be linked to the lunar cycle, where this species forms predictable spawning aggregations eight to 12 days following each full moon (Carrette et al., 2014; Chiaverano et al., 2013; Crow et al., 2015; Lewis et al., 2013; Thomas et al., 2001). In the case of *C. bronzie,* the time of medusa production was back-calculated, demonstrating distinct cohorts arise approximately 14 days post significant rainfall events indicating multiple cohorts within one season occur (Gordon et al., 2004). Exploring daily growth rings in statolith structures have been demonstrated to be useful in exploring the ecology of cubozoan medusae, however, this technique has not been applied to the majority of cubozoans.

The elemental chemistry of *C. fleckeri* statoliths has also been explored through laser ablation. This research has concluded that *C. fleckeri* medusae originate from low salinity conditions (Mooney & Kingsford, 2012), supporting the original theory that *C. fleckeri* polyps are located in estuaries, where polyps have been found *in situ* (Hartwick, 1991a). Conversely, this research has also shown that

not all of these medusae originate from low salinity conditions, suggesting that the polyps may also inhabit other areas, and may not be restricted to low salinity conditions (Mooney & Kingsford, 2012). Further exploration of the elemental chemistry of *C. fleckeri* statoliths through laser ablation has also been able to detect thermal regime variation in the conditions that these medusae have experienced, highlighting another useful technique to explore cubozoan ecology (Mooney & Kingsford, 2016c).

The shape of cubozoan statoliths has also been experimentally explored, revealing the capacity to distinguish between species (i.e., *C. fleckeri, Copula sivickisi, C. barnesi*), and distinguish between geographically separated populations of *C. fleckeri* and *C. sivickisi* but this method was able to distinguish between populations of *C. barnesi* (Mooney & Kingsford, 2016b). This result indicates that distinct populations of *C. fleckeri* and *C. sivickisi* may occur along the coast at spatial scales of tens to hundreds of kilometers (Mooney & Kingsford, 2016b). Conversely, this analysis could not distinguish between populations of *C. barnesi*, suggesting that this species may have larger dispersal distances, increased genetic mixing between populations, or the factors that contribute to morphometric differences in the statoliths of this species are discrete (Mooney & Kingsford, 2016b). Common genetic relatedness analysis techniques may untangle this question, however genetic relatedness experiments have never been conducted on cubozoans. Further research on the elemental chemistry of *C. fleckeri* statoliths revealed the ability of distinguishing between populations at spatial scales of tens of kilometers (Mooney & Kingsford, 2016a). This research has indicated yet another use of

statolith analyses to elucidate the population structure of cubozoans. Surprisingly, this is the only research to date that has explored the population dynamics of any cubozoan.

1.9 Behavior of the Medusa Stage

Cubozoans are described as highly motile, having advanced maneuverability (Garm et al., 2007b; Hamner et al., 1995) and swimming capacities (Colin et al., 2013; Shorten et al., 2005). The large cubozoan *Chironex fleckeri* was monitored through acoustic telemetry and was recorded to cover 10 kilometers over a 26-hour period (Gordon & Seymour, 2009). Smaller species have also been described as having advanced swimming capacity (Shorten et al., 2005). Conversely, some authors claim *Carukia barnesi* medusae are not able to swim (Gershwin, et al., 2013), however; there is no data to support this claim. Cubozoans are routinely reported to exist in structurally complex habitats, such as around the prop roots of mangrove systems (Hamner et al., 1995; Hartwick, 1991a). It is thought that the ability to capitalize on these complex habitats occurs through a combination of swimming capacity and vision (Coates, 2003).

Interestingly, a variety of visual systems are utilised by cnidarians. These range from simple eye spots and pigment cup ocelli to advanced pigment cups with lenses (Blumer et al., 1995; Nilsson et al., 2005; Nordstrom et al., 2003; Singla, 1974; Yamasu & Yoshida, 1973; Yamasu & Yoshida, 1976), with the most advanced visual sensory structures belonging to the medusa stage of the cubozoans (Martin, 2002; Nilsson et al., 2005). Cubozoans have four sets of six ‘eyes’, consisting of a

pair of simple light sensitive pigment cups, a pair of light sensitive pigment slits, and a pair of complex eyes that each has a cornea, a lens, and a retina (Hamner et al., 1995; Martin, 2002; Nilsson et al., 2005; Piatigorsky et al., 1989). However, there has been contention as to the usefulness of these complex eyes, due to the apparent lack of either neural branches to process the information (Gerhart & Kirschner, 1997) or a nervous system able to interpret visual images (Nilsson & Pelger, 1994).

Cubomedusae do however exhibit many vision mediated behaviours (Barnes, 1966; Coates, 2003; Hartwick, 1991b). For example, medusae of *Tripedalia cystophora* are known to target light shafts for feeding on copepods (Buskey, 2003). Other species exhibit obstacle avoidance (Barnes, 1966; Coates, 2003; Garm et al., 2007; Hamner et al., 1995; Hartwick, 1991b), or actively swim away from dark objects (Hamner et al., 1995). Some cubozoans also undergo a diurnal behavioral shift and vision seems to be one of the factors involved in this diurnal differentiation in behavior (Garm et al., 2012; Land, 2012). For example, *C. fleckeri* are active during the day and enter a sleep-like state on the sea floor at night, presumably to conserve energy (Gordon & Seymour, 2009; Seymour et al., 2004). *Tripedalia cystophora* is known to be active during the day (Buskey, 2003; Garm et al., 2012) and other species exhibit a crepuscular pattern as seen in *Carybdea rastonni* (Matsumoto, 1996). Some species, such as *C. sivickisi*, have an opposite pattern where the medusae are active primarily at night (Garm et al., 2012). Vision has also been hypothesized to play a role in prey capture (Buskey, 2003; Matsumoto, 1996; Pearse & Pearse, 1978; Stewart, 1996). However, the extent that vision is used in prey capture by cubozoans is unknown.

1.10 Seasonality of Cubozoan Medusae

There is significant scientific interest in the medusa stage of cubozoans, primarily due to their painful and potentially fatal stings (Pereira et al., 2010), complex venoms (Carrette et al., 2012; Chaousis et al., 2014; Pereira et al., 2010), sophisticated visual structures (Coates, 2003; Garm et al., 2007a; Nilsson et al., 2005), and complex behaviors (Gordon & Seymour, 2009; Seymour et al., 2004). Cubozoan medusae are often highly seasonal, arriving as an influx of many medusae and then disappear for periods of time. For example, Australia cubozoan medusae are present during the warm monsoonal months and absent during the cool dry winter months (Carrette et al., 2012; Gershwin et al., 2013 Kingsford & Mooney, 2014). As previously mentioned, the initiation of the medusa season is driven by the polyp stage, which is thought to use an environmental cue for synchronous medusa production (Laska-Mehnert, 1985; Stangl et al., 2002; Toshino et al., 2015; Werner, 1983). Once medusa production has commenced, it may continue for an unknown period of time, essentially adding more medusae into the system (Gordon et al., 2004; Gordon & Seymour, 2012). The factors that cause the end of the presence of the medusae are far less understood, but correlate with cooling water temperatures during the end of the stinger season in Australia (Barnes & Kinsey, 1986; Kinsey & Barnes, 1988, Hartwick, 1991a). It has been speculated that the stinger season in Australia is influenced by environmental temperature (i.e., the medusae seem to prefer warm summer conditions). During the winter months, it is presumed to be simply too cold for the medusae to persist. Although correlative, no data exists on the thermal tolerance of any cubozoan.

During the monsoonal months in Australia, cubozoans occur with a high amount of temporal and spatial variability (Kingsford et al., 2012). This may be caused by physical forcing, where medusae may be avoiding adverse conditions such as unfavorable thermal or osmotic regimes (Kingsford & Mooney, 2014; Mooney & Kingsford, 2016a). Part of this variability may also be explained by the behavior of cubozoans, which are known to have advanced swimming capacity (Colin et al., 2013; Shorten et al., 2005), obstacle avoidance (Garm et al., 2007b; Hamner et al., 1995), and diurnal behavior patterns (Garm et al., 2012; Seymour et al., 2004). Also, very little is known in most instances about when and where most Australian cubozoan species spawn. These are all areas where future research is urgently required before predictive models of the occurrences of cubozoans are possible.

Understanding the factors that contribute to the occurrence of the medusa stage may allow for projective modeling of these medusae, which is particularly relevant for species that are harmful or fatal to humans. This may provide information to water users that indicate times, or specific conditions, that are of higher risk of the presence of these medusae. This should result in reduced numbers of people being stung. Surprisingly, very little ecological and/or physiological data exist on cubozoan medusae and the ability to create predictive models is not currently possible. Although some authors claim that these medusae can be predicted from wind alone (Gershwin et al., 2013; Gershwin et al., 2014), this has yet to be empirically demonstrated. The following subsections will cover the state of knowledge about cubozoan ecology.

1.11 Study Rationale

This study will focus on the Irukandji jellyfish *Carukia barnesi*. Little is known about the general ecology of *C. barnesi*; however, the medusa stage is considered oceanic, planktonic, has been found around coral reefs or islands, and under certain conditions on beaches (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour 2007). *Carukia barnesi* are typically present during the monsoonal summer months between November and May in Queensland, Australia, and although not well defined, the distribution of this species is considered along the Great Barrier Reef and adjacent coastline, between Lizard Island and Fraser Island.

There is evidence that the length of the Irukandji season in the Queensland region has progressively increased over the last 50 years, based on annual sting records, from 15 days long historically to over 150 days long currently, which has been speculated to be attributed to increased seawater temperatures (Carrette & Seymour, 2013). Similarly, there have been anecdotal reports that the southern distribution of *C. barnesi* has increased over the last 50 years also due to increased sea temperature (Carrette, 2014; Carrette & Seymour, 2013). A sting from *C. barnesi* commonly results in Irukandji syndrome, which is often severely painful, potentially fatal, and frequently requires hospitalization for treatment (Carrette et al., 2012; Little et al., 2003; Pereira et al., 2010). The direct cost in treating envenomed victims is estimated to be between one and three million dollars per year in northern Australia, and the negative impact this species has on the Australian tourism industry through reduced revenue is substantial (Carrette et al., 2012).

Further exacerbating the impact of this species is that there are currently no methods in place for mitigating stings when this species is present other than through beach closures. Stinger exclusion nets are commonly used along the north-eastern coast of Queensland; however, these nets are designed to exclude large cubozoan species, primarily *Chironex fleckeri*, and do not exclude small species such as *C. barnesi*. Also, this species occurs with a high amount of spatial and temporal variability during the monsoonal summer months (Kingsford et al., 2012). Therefore, understanding the factors that contribute to this variability may facilitate the ability to model, and therefore predict, when and under what circumstances, this species may be more prevalent. Currently, the ecological data required to produce a predictive model does not exist. Prior to the commencement of this research project, the early life history of *C. barnesi* had never been observed, or described, and nothing was known about the thermal and osmotic tolerances, or preferences, of any life stage.

1.12 Thesis Description and Aims

Chapter One introduces the consensus of cubozoan ecology and includes the study rationale, the thesis description, and the primary project objectives and aims. Four data chapters then follow; three have been published in peer reviewed journals and one has been submitted for publication. The published versions of the three journal articles are available in Appendices A, B, and C. Each successive chapter consists primarily of an introduction, methods, results, and discussion section. These chapters are briefly described as follows.

Chapter Two focuses on the early life history of *Carukia barnesi*, and describes the life cycle from egg fertilization through to medusa production. This chapter also provides a repeatable method for the production of polyp cultures of this species. This study has resulted in the only verified culture of *C. barnesi* polyps globally.

Chapter Three continues to explore the polyp stage of *C. barnesi*. With the polyp stage of the life cycle in culture, the opportunity to conduct manipulative temperature and salinity experiments was pursued. The primary aim of Chapter Three was to determine the thermal and osmotic tolerances of the polyps by culturing them in a matrix of eight temperatures and ten salinity treatments over a six-week period. These data allowed for discussion as to where these polyps may reside *in situ*, based on their thermal and osmotic preferences. The effect of feeding frequency on asexual reproduction rates was also experimentally pursued. Additionally, combinations of temperature, salinity, and feeding frequency were explored as potential cues for synchronous medusa production.

With the effect of temperature and salinity on the polyp stage known, focus was shifted to exploration of these factors on the medusa stage. As animal husbandry of cubozoans is still in its infancy, it was necessary to develop methods for determining thermophysiological profiles for the medusa stage.

Chapter Four experimentally determines the theoretical thermal tolerance of the medusa stage by measuring oxygen consumption rates and bell contraction frequency, as a measure of fitness, over a range of temperatures. As the osmotic tolerance of the medusa stage was also unknown, bell contraction frequency was also

measured over a range of salinity treatments, to determine their osmotic tolerance. These results are then discussed to elucidate the factors that may contribute to the seasonality and distribution of the medusa stage.

Chapter Five quantifies the diurnal activity pattern of *C. barnesi* medusae and explores the feeding ecology of the medusa stage. Medusae were filmed over replicated 24 hour periods, both under light and dark conditions, to determine diurnal activity patterns to define when this species is more active. The method these medusae implement to capture prey was also explored and different feeding strategies dependent on prey choice and/or medusae size are discussed.

The final chapter, Chapter Six, summerises the results of each of the data chapters as a whole, and highlights areas where future research is required to predictively model the occurrence of *C. barnesi*. The overall focus of this thesis was to better understand the ecology and physiological limitations of *C. barnesi* to elucidate the factors that may contribute to the observed seasonal and distributional patterns. This research has also produced baseline data for future research to build upon, with the expectation that the synthesis of these and future data will facilitate the ability to model, and therefore predict, the occurrence of this species in order to reduce the number of people stung.

The primary aims of this research were to:

i) describe the polyp stage of *C. barnesi* from egg fertilization through to medusa detachment and to provide a repeatable method for producing laboratory-based polyp cultures of this species for scientific research.

ii) determine the thermal and osmotic tolerance of *C. barnesi* polyps, and use these data to discuss where these polyps may live.

iii) explore the effects of feeding frequency on survivorship and asexual reproduction of *C. barnesi* polyps.

iv) determine the effect of thermal and osmotic parameters, and combinations thereof, as well as feeding frequency, as cues for synchronous medusa production.

v) define the thermal and osmotic tolerance of *C. barnesi* medusae in order to determine what factors contribute to the marked seasonality and distribution of this species.

vi) describe the feeding ecology of *C. barnesi* medusae to gain insight into the mechanisms employed by this species to capture prey and to elucidate the diurnal activity pattern of this species.

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Chapter 2:

Early Life History of the 'Irukandji' Jellyfish *Carukia barnesi*

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Chapter 2:

Early life history of the 'Irukandji' jellyfish *Carukia barnesi*

2.1 Abstract

Adult medusae of *Carukia barnesi* were collected near Double Island, North Queensland Australia. From 73 specimens, 8 males and 15 females spawned under laboratory conditions. These gametes were artificially mixed which resulted in fertilized eggs. Post fertilization, most eggs developed to an encapsulated planula stage and then paused for between six days and six months prior to hatching as ciliated planulae. The paused stage planula were negatively buoyant and adhered to substrate. The first planula was produced six days post fertilization, lacked larval ocelli, remained stationary, or moved very slowly for two days prior to metamorphosis into primary polyps. Mature polyps reproduced through asexual reproduction via lateral budding producing ciliated swimming polyps, which in turn settled and developed into secondary polyps. Medusa production for this species was in the form of monodisc strobilation, which left behind polyps able to continue asexual reproduction.

2.2 Introduction

Cubozoans have a metagenic life cycle which alternates between benthic sessile polyps and motile pelagic medusae (Arai, 1997; Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Gordon et al., 2004; Hartwick, 1991a; Toshino et al., 2013; Toshino et al., 2014; Werner et al., 1971). It is this alternation of generations that contributes to the often predictable occurrence of cubozoan medusae (Gordon et al., 2004; Gordon & Seymour, 2012; Hartwick, 1991a; Yamaguchi & Hartwick, 1980). There are approximately 50 described species of Cubozoa and of these the early life histories of only eight have been described to date (Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Hartwick, 1991a; Hartwick, 1991b; Lewis & Long, 2005; Straehler-Pohl & Jarms, 2011; Toshino et al., 2013; Toshino et al., 2014; Toshino et al., 2015; Werner et al., 1971; Yamaguchi & Hartwick, 1980). The polyp stage of cubozoans not only initiates the seasonal onset of medusae, but also allows for population increase through asexual reproduction, which has potential for exponential population growth (Courtney & Seymour, 2013; Fischer & Hofmann, 2004; Hartwick, 1991a; Klein et al., 2014; Straehler-Pohl & Jarms, 2011). Therefore, the success of the polyp stage will drive not only the seasonal timing of medusae but also their abundance.

In northern Australia, cubozoan medusae typically arrive in large numbers associated with increased sea temperatures during the monsoonal months (Carrette et al., 2012; Gordon et al., 2004; Gordon & Seymour, 2012; Hartwick, 1991a; Kinsey & Barnes, 1988). This seasonal cycle in some species has been reported to begin earlier and last longer in areas closer to the equator (Gordon & Seymour, 2012;

Jacups, 2010; Nickson et al., 2009). The seasonal timing of one Australian cubomedusae, *Chironex fleckeri*, has been shown to be initiated by metamorphosis of the polyp stage, which presumably uses increasing photoperiod as a cue for metamorphosis (Gordon & Seymour, 2012). Another highly seasonal Australian cubozoan, both temporally and spatially, is the small carybdeid *Carukia barnesi* (Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967). This species is present in north Queensland waters between November and May, and may be locally abundant or absent for periods of time during the 'stinger season' (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007; Williamson et al., 1996).

Little is known about the general ecology and biology of *C. barnesi*; however, the medusa stage is considered oceanic, planktonic, has been found around coral reefs or islands, and under certain conditions on beaches (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). A sting from *C. barnesi,* as well as several other cubozoans, can cause Irukandji syndrome, which is often severely painful, potentially fatal and may require hospitalization for treatment (Carrette et al., 2012; Little et al., 2003; Pereira et al., 2010). The direct cost in treating envenomed victims is estimated to be between one and three million dollars per year in northern Australia alone, and the negative impact this species has on the Australian tourism industry through reduced revenue is substantial (Carrette et al., 2012). Understanding the general ecology of the polyp stage of *C. barnesi* may allow for the determination of the start of the jellyfish season and elucidate the factors affecting the abundance of medusae

present. This could contribute to decreasing the number of envenomed victims per year and reduce the costs associated with treating these stings.

2.2.1 Aims

The aim of this study was to describe the polyp stage of *C. barnesi* from egg fertilization through to medusa detachment and to provide a repeatable method for producing laboratory based polyp cultures of this species for scientific research.

2.3 Method

2.3.1 Ethics Statement

All specimen collections were conducted in accordance with Permit Numbers: G11/34552.1 and G15/37396.1 (Great Barrier Reef Marine Park Authority).

2.3.2 Specimen Identification

Carukia barnesi is a small species in the family Carukiidae with a bell height of up to 35 mm and tentacles up to 1.2 m long (Bentlage & Lewis, 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). This species has four tentacles, one from each of the four pedalia, a rhopaliar niche with horns, and tentacles that have an alternating pattern of large and small nematocyst crescents that resemble “neckerchiefs” (Bentlage & Lewis, 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). Little is known about the distribution of this species; however, they are present along the north-eastern coast of Australia from Lizard Island to Fraser Island (Bentlage & Lewis, 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007).

2.3.3 Capture Method

Four collection trips were undertaken to capture medusae of *C. barnesi* near Double Island, North Queensland, Australia (16º43.5′S, 145º41.0′E) in 2014 and 2015. The collection trips were undertaken on December 15, 2014, January 4 and 5, 2014, and April 7, 2015, between 1900 and 2300hrs, which resulted in the capture of both male and female medusae that spawned approximately nine hours post capture. Three underwater LED lights (7,000 lumens each) were suspended just below the water level from a 5.8 m vessel. As medusae approached the lights they were captured with a net and transferred into individual 500 ml plastic containers, held at ambient temperature, for transport. The sea surface temperature varied from 27.5ºC to 30ºC with a mean temperature of 29ºC. The salinity at the capture location varied between 34‰ to 35‰ and the water depth varied between three to six meters. Post capture, the specimens were transported to the laboratory and held at a constant temperature of 28ºC ± 0.5ºC in complete darkness.

2.3.4 Fertilization

After the medusae had been held overnight, a total of 8 females and 15 males spawned from a total of 73 medusae, which was determined by the presence of eggs for females and sperm for males evident within the holding vessels. The spawned medusae were removed from the transport containers that contained the gametes and the water was stirred to suspend the eggs. Approximately one third of the mixture was poured into a three-litre container. To this, 100 ml of water that contained male gametes was added. This mixture was stirred briefly and then the three-litre container was filled with filtered sea water to reduce the gamete density. One third of this

mixture was poured into 15 plastic containers of 70 ml volume each. The remaining mixture was then further diluted by approximately 30%, with filtered sea water and poured into 15 additional 70 ml containers. This process was repeated resulting in approximately 250 specimen containers with varying egg densities for each female. The egg densities in the containers ranged from one to 15 eggs per ml. A loose lid was placed on each container to reduce evaporation and the containers were held in darkness at a constant temperature of 28ºC.

2.3.5 Polyp Maintenance

Until the first primary polyps were observed, all containers received a weekly 50% water exchange (taken from the top of the vessels) with filtered sea water and were not fed. Each 70 ml container that contained polyps was fed twice a week with approximately 20 freshly hatched, first instar, decysted *Artemia* nauplii, followed by a 50% water exchange 24 hours later. As the polyp density increased through asexual reproduction, the food density was also increased.

2.3.6 Culturing Polyps

As the polyp numbers increased through asexual reproduction, freshly budded off swimming stage polyps (similar to those described in *Morbakka virulenta* [Toshino et al., 2013; Toshino et al., 2015]) were harvested. These were extracted by using a pipette to stir the contents of the 70 ml containers prior to each 50% water exchange. The waste water was then poured into a larger container where the swimming polyps could be removed and this was repeated post each feeding event. These swimming polyps were transferred into three 30 litre temperature controlled

tanks (28ºC). Once the swimming polyps settled and attached, they were fed live *Artemia* nauplii once per week.

2.3.7 Nematocyst Identification

In order to describe the cnidome of the different developmental stages of *C. barnesi*, a series of nematocyst presses were performed, as described below. The sampled stages were: paused stage unhatched planula; hatched planula; primary polyp; mature polyp; swimming stage polyp; and detached one day old medusa. Each sample was placed on a glass microscope slide and a cover slip was then placed over the sample. In most cases this pressure was sufficient to cause nematocyst discharge. In cases where none of the nematocysts discharged, a small quantity of ethanol was added to the sample. Each sample was then viewed on a stereo microscope, photographed and the nematocyst types were identified using the key developed by Rifkin (see Williamson et al., 1996) then compared to the known nematocyst types present on the adult medusae.

2.4 Results

2.4.1 Egg Fertilization and Development

Unfertilized eggs were translucent and negatively buoyant with a diameter between 0.08 and 0.11 mm (n = 10, $\bar{x}$ = 0.089 mm, SD = 0.009). Approximately two hours post mixing of the gametes, a fertilization membrane was produced. During initial cell division the eggs remained negatively buoyant, with a diameter between 0.08 and 0.11 mm (n = 10, $\bar{x}$ = 0.104 mm, SD = 0.010) (Figure 2.1, I). The first blastula was seen approximately 30 hours post fertilization and the majority of the

eggs were at the blastula stage within 48 hours. Around 90% of the eggs from each batch were fertilized and reached this stage (Figure 2.1, II). During this time, the eggs/blastulae remained negatively buoyant and stuck to the base of the culture containers. The formation of the planula stage took place within the egg capsules and the planulae could be seen slowly rotating within. The planulae then entered a pausal stage and hatched over a minimum of six days and a maximum time of over six months (Figure 2.1, III). The diameter of the unhatched planulae ranged between 0.094 and 0.120 mm (n = 10, $\bar{x}$ = 0.105 mm, SD = 0.009).

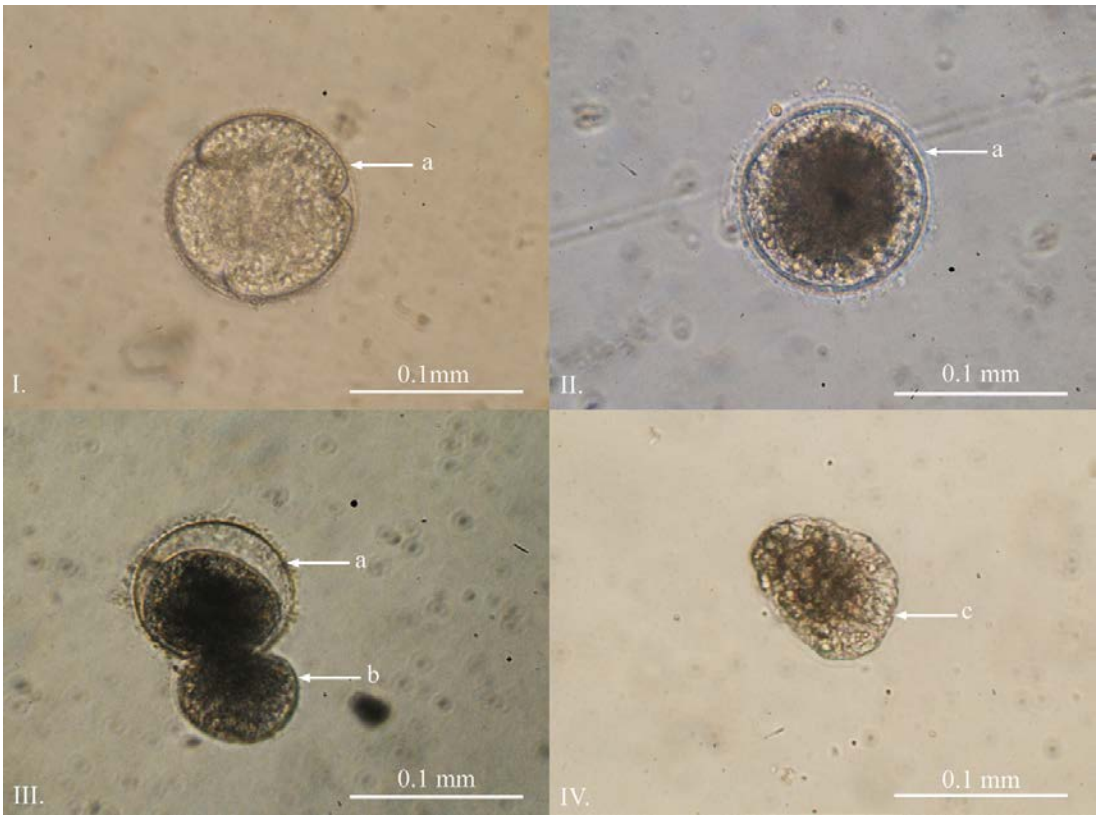

Figure 2.1 Planula development of *Carukia barnesi*: I. Early cell division post fertilisation. **II.** Developed blastula approximately 48 hours post fertilization. **III.** Ciliated planula larvae hatching after a minimum of six days post fertilization. **IV.** Free swimming planula. **Letters indicate:** a) egg capsule; b) emerging planula; c) anterior end.

2.4.2 Planula

Carukia barnesi free swimming planula larvae were first observed six days post fertilization, and lacked larval ocelli. The hatched planulae were heavily ciliated and the beating cilia caused a jittery shaking motion (Figure 2.1, IV). The planulae did not actively swim and appeared stationary, or moved very slowly, during all observations. The planulae were flattened on one end, which was the predominant direction of travel; flat end forward. The length of the planulae ranged from 0.11 to 0.12 mm (n = 10, $\bar{x}$ = 0.115 mm, SD = 0.005), were negatively buoyant and remained on the base of the containers where they eventually settled and metamorphosed into primary polyps after a minimum of two days post hatching.

2.4.3 Primary Polyps

Primary polyps of *C. barnesi* were first observed eight days post fertilization, two days after the first planula was observed. This species appears to lack a creeping polyp stage and instead develops where the planula settles. First a single tentacle and stalk is formed followed by a second tentacle (Figure 2.2, I). The primary polyps are heavily ciliated and, on occasion, were observed slowly swimming near the base of the containers; however, the majority of polyps remained sedentary. During the first week the primary polyps did not feed even when presented with live or finely chopped *Artemia*, rotifers, fine crayfish meat or boiled chicken egg yolk. Nevertheless, the polyps continued to grow and develop a third (Figure 2.2, II) and fourth tentacle (Figure 2.2, III). At the three tentacle stage the polyps captured live rotifers (Figure 2.2, IV) and/or first instar *Artemia* nauplii (Figure 2.2, V). The primary polyps had the following average dimensions reported in millimetres (n = 10): total length ($\bar{x}$ = 0.191, SD = .068); stalk length ($\bar{x}$ = 0.113, SD = 0.038); calyx

length ($\bar{x}$ = 0.078, *SD* = 0.033); calyx width ($\bar{x}$ = 0.075, *SD* = 0.024). The polyps continued to develop until they reached a mature stage (able to asexually reproduce) after approximately 28 days post fertilization (Figure 2.2, VI).

Figure 2.2 Primary polyps of *Carukia barnesi* at different developmental stages: I. Lateral view of a primary polyp at the two tentacle stage. **II.** Vertical view of a three tentacle stage polyp. **III.** Three tentacle stage primary polyp developing a fourth tentacle. **IV.** Three tentacle stage primary polyp feeding on a live rotifer. **V.** Three tentacle stage primary polyp feeding on a live *Artemia* nauplius. **VI.** Four tentacle primary polyp 28 days post fertilization. **Letters indicate:** a) hypostome; b) capitate feeding tentacle; c) stalk region; d) calyx region; e) nematocyst bundle in tentacle tip; f) rotifer; g) *Artemia* nauplius.

The timeline of these processes is highly variable and only the first observation times have been reported. For example, even within one 70 ml specimen jar after 28 days, it was common to see all of these developmental stages at any given time. Even while there were adult polyps undergoing asexual reproduction, there were still unhatched eggs, hatching planulae, free swimming planulae, and newly formed primary polyps in each container. These eggs continued to hatch within the containers for over six months.

2.4.4 Polyps and Asexual Reproduction

Mature polyps first underwent asexual reproduction 28 days post fertilization. The basic polyp anatomy consisted of a calyx region that included a motile hypostome which was surrounded by a single circlet of capitate tentacles. There was a distinct demarcation at the junction between the calyx and the stalk region. The stalk region was thin and contractile with a basal disc at the terminal end which anchored the polyp to substrate. All of the external surfaces of the mature polyps were ciliated. The average size of the mature polyps had the following dimensions reported in millimetres (n = 10): total length ($\overline{x}$ = 0.885, SD = 0.434); stalk length ($\overline{x}$ = 0.560, SD = 0.310); calyx length ($\overline{x}$ = 0.325, SD = 0.142); calyx width ($\overline{x}$ = 0.254, SD = 0.069). The polyps asexually reproduce after having four or more tentacles; however, the number of tentacles on each polyp was highly variable and ranged from four to 24 (n = 78, $\overline{x}$ = 11.12, SD = 5.31).

The primary mode of asexual reproduction observed was through the lateral budding of a ciliated swimming polyp similar to those produced by *M. virulenta* (Toshino et al., 2013; Toshino et al., 2015). This process was first evident by a round

protrusion originating from the side of the calyx (Figure 2.3, I). The bud then develops two tentacles and a stalk and remains attached to the parent polyp along the calyx (Figure 2.3, II and III). This process took approximately four days from the beginning of the bud through to detachment of the free swimming secondary polyp (Fig 2.3, IV). Frequently, polyps were observed producing multiple buds simultaneously, up to five at a time.

Figure 2.3 Asexual reproduction of *Carukia barnesi* polyps: I. The initial stage of lateral budding. **II.** Formed ciliated swimming stage polyp prior to detachment. **III.** Large mature polyp undergoing asexual reproduction. **IV.** Detached swimming stage polyp. **Letters indicate:** a) retracted tentacle; b) swelling of the calyx where the bud begins to form; c) stalk, d) calyx; e) tentacles of the swimming polyp stage; f) attachment point; g) buds produced through asexual reproduction, h) location of nematocysts in the tentacle tips.

The swimming polyps were highly variable in size and ranged from 0.17 to 0.50 mm (n = 10, $\bar{x}$ = 0.298 mm, SD = 0.106), measured along the latitudinal axis from between the tentacles to the terminal end of the stalk, where small polyps produced small swimming polyps and larger polyps produced larger swimming polyps. The polyps swam along the bottom of the containers, and also up the sides, with one tentacle forward in the direction of travel. The swimming polyps moved like this for one to seven days prior to settlement.

2.4.5 Medusa Production

Medusa production was only observed three times and only from the first polyp culture, which was fertilized in January 2014. The quantities and dates of medusa production were; 24th of June 2014 (one medusa, 170 days post fertilization), 26th of August 2014 (approximately 100 medusae, 233 days post fertilization) and 20th of May 2015 (approximately 50, medusae 500 days post fertilization). The polyp culture, both the original cultures and the three 30 l temperature controlled tanks, included more than one million polyps (determined by extrapolation), and due to this, the actual percentage of polyps that underwent medusa production on these dates was minimal.

Medusa production in *C. barnesi* occurred in the form of monodisc strobilation similar to that observed in *M. virulenta* described by Toshino et al. (2015). First, the stalk region contracted and the oral disc widened, the calyx also began to extend (Figure 2.4, I). The tentacles migrated to four equally spaced corners of the forming bell. The tentacles then fused together at the base and a dark pigmentation of the forming rhopalia was visible (Figure 2.4, II). The tentacles were

further reabsorbed and the rhopalia continued development. At this time, the calyx became constricted and tentacles began to develop below the developing medusa. The manubrium then formed, nematocyst warts became visible on the forming bell, small pedalia formed, and there was a further separation between the forming medusa and the original polyp (Figure 2.4, III). The medusa began to pulse approximately two days prior to detachment.

The detached medusae were positively phototactic, congregated on the surface of the containers, and had a bell height that ranged between 0.33 to 0.80 mm (n = 8, $\bar{x}$ = 0.560 mm, SD = 0.140), and a bell width that ranged between 0.45 to 0.83 mm (n = 10, $\bar{x}$ = 0.568 mm, SD = 0.110) (Figure 2.4, V and VI). The nematocyst warts were clearly visible on the external surface of the bell. The rhopalia were developed prior to detachment, and the beginnings of small feeding tentacles were visible. The polyps that were left behind, after strobilation, had between four and eight tentacles (Figure 2.4, IV). These polyps continued to grow and returned to production of swimming polyps. No polyps were observed producing multiple medusae at one time. The medusae were able to consume *Artemia* nauplii soon after detachment; however, survival was limited (five days).

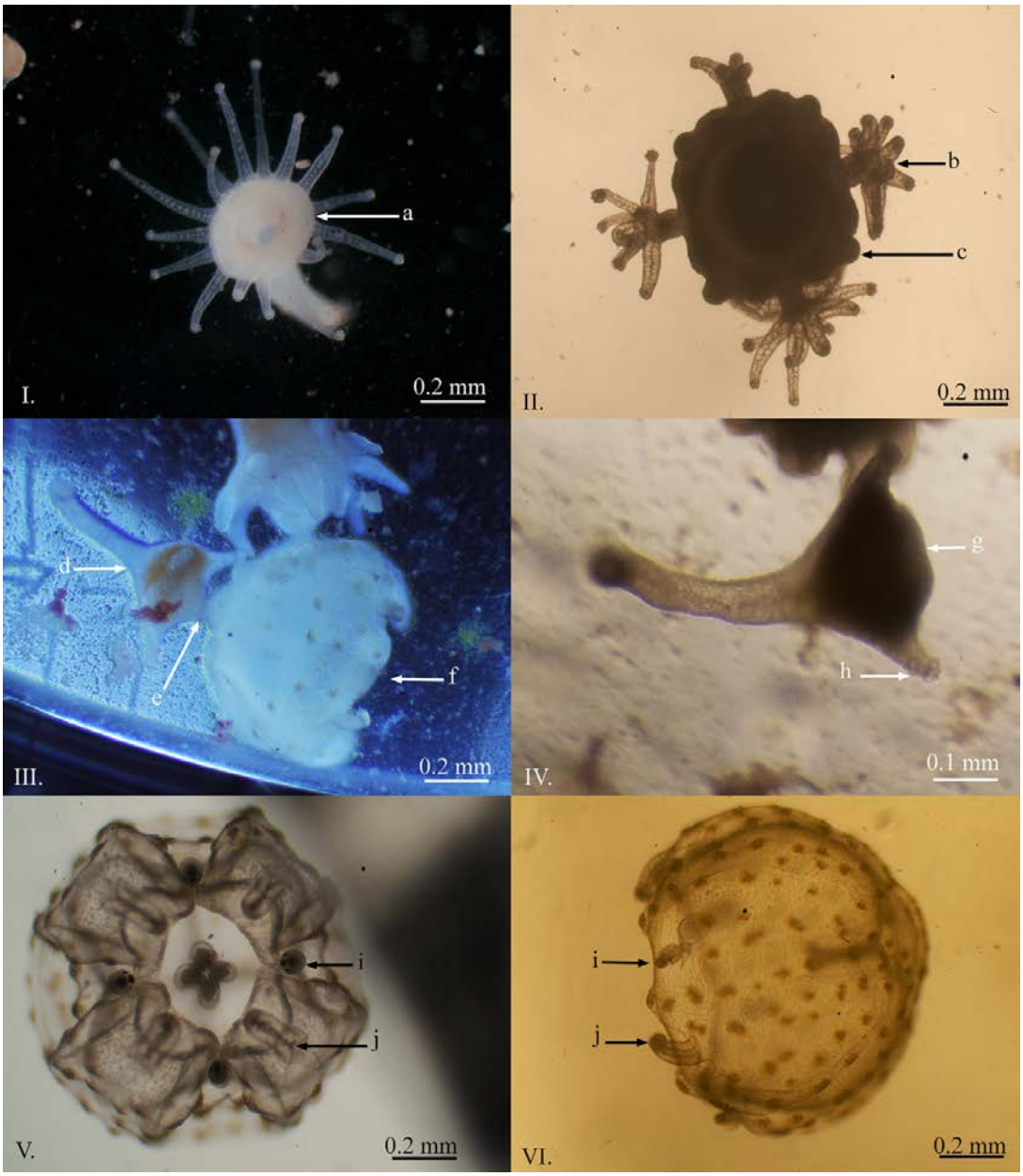

Figure 2.4 Medusa production of *Carukia barnesi*: I. Initial stage of medusa production, note the change in shape of the hypostome region. **II.** The tentacles migrate to four opposing sections of the forming bell, fuse, and begin to form the rhopalia. **III.** The bell begins to pulse and a narrowing divides the bell of the forming medusa from the hypostome of the original polyp. **IV.** The remaining polyp after completed medusa production. **V.** Oral view of a newly detached medusa. **VI.** Lateral view of a newly detached medusa with pigmented nematocyst batteries on the bell. **Letters indicate:** a) change in shape of the hypostome region; b) fusing of tentacles which begin to form rhopalia; c) early development of medusa tentacles; d) polyp producing a medusa through monodisc strobilation; e) connective tissue between the polyp and forming medusa; f) pulsing medusa approximately 24 hours prior to detachment; g) hypostome; h) feeding tentacle; i) formed rhopalia; j) short feeding tentacles.

2.4.6 Nematocysts of Polyps and Early Stage Medusae

There were two different types of nematocysts found in the early developmental and polyp stages, which consisted primarily of homotrichous microbasic tumiteles primarily located within the tentacle tips of the polyps and spherical isorhizas in the body of the polyps. These are the same nematocyst types identified in the medusa stage of this species, which consists primarily of homotrichous microbasic tumiteles on the tentacle nematocyst batteries and spherical isorhizas on the bell nematocyst batteries (Southcott, 1967; Underwood & Seymour, 2007; Williamson et al., 1996). The cnidome of the unhatched and hatched planulae consisted of only homotrichous microbasic tumiteles, whereas both nematocyst types were present in primary polyps, mature polyps, swimming polyps, and newly detached medusae. The size of each nematocyst type, at each life stage, were as follows (with measurements recorded in microns): hatching planula tumiteles (n = 5, 3.1 to 4.0, $\bar{x}$ = 3.6, SD = 0.37); primary polyp tumiteles (n = 7, 11.9 to 13.2, $\bar{x}$ = 12.6, SD = 0.50); primary polyp isorhizas (n = 7, 4.0 to 6.2, $\bar{x}$ = 4.6, SD = 0.80); mature polyp tumiteles (n = 10, 10.1 to 12.8, $\bar{x}$ = 11.2, SD = 0.89); mature polyp isorhizas (n = 7, 4.0 to 5.3, $\bar{x}$ = 4.7, SD = 0.61); swimming polyp tumiteles (n = 8, 11.0 to 11.9, $\bar{x}$ = 11.3, SD = 0.31); swimming polyp isorhizas (n = 5, 6.6 to 7.9, $\bar{x}$ = 7.0, SD = 0.57); freshly detached medusa tumiteles (n = 10, 11.0 to 14.1, $\bar{x}$ = 12.1, SD = 1.00); freshly detached medusa isorhizas (n = 10, 4.8 to 5.3, $\bar{x}$ = 5.1, SD = 0.23).

2.5 Discussion

On four occasions the adult medusae of *C. barnesi* collected from Double Island spawned approximately nine hours post capture while held at a constant temperature of 28ºC in darkness in water collected from the sample site. Although all attempts were made to replicate the conditions at the collection site, it is expected that this was a stress induced spawning event and these specimens may not have spawned *in situ* over the same timeframe. Although *C. barnesi* are routinely collected during the summer months at this location, specimens have never been observed spawning at this, or any other location, nor has it been reported in the literature. This is unlike the known predictable spawning aggregations of *Alatina alata* that occur 8 to 10 days after a full moon in Hawaii (Carrette et al., 2014; Chiaverano et al., 2013; Crow et al., 2015; Lewis et al., 2013; Thomas et al., 2001). Furthermore, the eggs of some cubozoan species are fertilized internally (Toshino et al., 2014; Werner et al., 1971; Hartwick, 1991b), and because the fertilization method implemented in this study was artificial, the possibility that there is more to the reproductive behaviour of *C. barnesi* should not be discounted.

The size and developmental timing of the fertilized egg phase through to gastrulation was similar to other cubozoans (Carrette et al., 2014; Hartwick, 1991a; Hartwick, 1991b; Toshino et al., 2013; Toshino et al., 2014; Toshino et al., 2015). The first notable difference was the dormant phase of the unhatched *C. barnesi* planulae. After the planula developed within the egg capsule, it entered a dormant phase that lasted six days to over six months. During this time the unhatched planulae were negatively buoyant and stuck to the base of the containers. A similar

dormant pause stage has been described in *M. virulenta*, where encysted blastulae lasted seven to 21 days before hatching as either free swimming planulae, or as primary polyps (Toshino et al., 2013; Toshino et al., 2015). The paused stage planula of *C. barnesi* only hatched as free swimming planulae. The function of the encapsulated pause stage is unknown; however, it may stagger the hatching time, potentially reducing the impact of intraspecific competition. There is also the possibility that there is an environmental cue, such as a change in temperature, photoperiod, or substrate, which triggers synchronous hatching.

The hatched planulae of *C. barnesi* were negatively buoyant, lacked larval ocelli, moved very slowly on the base of the containers and had nematocysts. Cubozoan planula have previously been reported as being negatively buoyant (Carrette et al., 2014; Hartwick, 1991a; Hartwick, 1991b; Toshino et al., 2013; Toshino et al., 2014; Toshino et al., 2015), either possessing larval ocelli (Carrette et al., 2014; Nordström et al., 2003; Toshino et al., 2013; Yamasu & Yoshida, 1976), or lacking larval ocelli (Toshino et al., 2013; Toshino et al., 2015), and having nematocysts which were used for attachment to substrate (Hartwick, 1991a). This use of nematocysts in the planula of *C. barnesi* was not observed and the function of the nematocysts during the planula stage is unknown, but is presumably for defence. The lack of larval ocelli and the limited swimming capacity of *C. barnesi* planulae may only provide limited dispersal potential that does not require photoreceptors for orientation or settlement choice.

The primary polyps of *C. barnesi* were able to develop to the four tentacle stage without food. This suggests that the eggs either contain sufficient stores to

reach this stage, or the planulae and/or primary polyps may be able to uptake dissolved organic material directly from the water column. Once the primary polyps began to feed on *Artemia* nauplii they rapidly developed into mature polyps which began asexual reproduction through lateral budding. This process was very similar to the asexual reproduction method seen in *M. virulenta* from Japan, which produced small swimming stage polyps (Toshino et al., 2013; Toshino et al., 2015). The swimming stage polyps of *C. barnesi* were ciliated and swam along the base and up the vertical surfaces of the culture containers prior to settlement and transformation into a secondary polyp. The mobility of the swimming stage polyps may not only provide a dispersal mechanism, but may also allow for increased selectivity in settlement position and/or substrate choice. Also, the size of the swimming polyps was highly variable, where larger polyps produced large swimming polyps and smaller polyps produced small swimming polyps.

The process of medusa production in *C. barnesi* was also very similar to the monodisc strobilation recently discovered in *M. virulenta* (Toshino et al., 2015). Medusa production of cubozoans is highly variable between species ranging from complete metamorphosis of the polyp into a medusa (Arneson & Cutress, 1976; Carrette et al., 2014; Cutress & Studebaker, 1973; Stangl et al., 2002; Straehler-Pohl & Jarms, 2011; Werner et al., 1971; Yamaguchi & Hartwick, 1980), leaving behind a small amount of regenerative material (residuum) (Straehler-Pohl & Jarms, 2005), and monodisc strobilation which may leave behind a developed polyp (with tentacles) able to continue asexual reproduction (Toshino et al., 2015). This is a

reproductive event, which may suggest that the polyps of *C. barnesi* may reside in a location that allows for the annual persistence of the polyp.

The cnidome of the early life stages of *C. barnesi* was found to be identical to the cnidome of the adults. This deviates from other cubozoan species which change their cnidome between polyp and medusa stages (Carrette et al., 2014; Straehler-Pohl & Jarms, 2011).

Cubozoans have been shown to be sophisticated in many areas of their ecology from possessing complex vision capabilities (Coates, 2003; Garm et al., 2007; Nilsson et al., 2005), prey capture techniques (Courtney et al., 2015), behavioural patterns (Gordon & Seymour, 2009; Seymour et al., 2004), and complex venoms (Carrette et al., 2012; Chaousis et al., 2014; Pereira et al., 2010). Not surprisingly, the life cycles of cubozoans also have many species specific complexities (Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Lewis & Long, 2005; Straehler-Pohl & Jarms, 2011; Toshino et al., 2013; Toshino et al., 2014; Toshino et al., 2015; Yamaguchi & Hartwick, 1980). In order to investigate cubozoan life cycles, production of lab-based polyp cultures is essential. From these cultures the effects of varying parameters such as temperature, salinity, and feeding frequency can be explored to elucidate the most likely habitats that support polyp proliferation. This could then be used to determine where the polyp colonies may reside *in situ*. These parameters could also be experimentally pursued to elucidate cues for medusa production which may give better insight into what factors drive the strong seasonal occurrence of this, and other, cubozoan species.

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Chapter 3:

Thermal and Osmotic Tolerance of 'Irukandji' Polyps: Cubozoa; *Carukia barnesi*

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Chapter 3:

Thermal and Osmotic Tolerance of 'Irukandji' Polyps: Cubozoa; *Carukia barnesi*

3.1 Abstract

This research explores the thermal and osmotic tolerance of the polyp stage of the Irukandji jellyfish *Carukia barnesi*, which provides new insights into potential polyp habitat suitability. The research also targets temperature, salinity, feeding frequency, and combinations thereof, as cues for synchronous medusa production. Primary findings revealed 100% survivorship in osmotic treatments between 19 and 46‰, with the highest proliferation at 26‰. As salinity levels of 26‰ do not occur within the waters of the Great Barrier Reef or Coral Sea, we conclude that the polyp stage of *C. barnesi* are probably found in estuarine environments, where these lower salinity conditions commonly occur, in comparison to the medusa stage, which is oceanic. Population stability was achieved at temperatures between 18 and 31ºC, with an optimum temperature of 22.9ºC. We surmise that *C. barnesi* polyps may be restricted to warmer estuarine areas where water temperatures do not drop below 18ºC. Asexual reproduction was also positively correlated with feeding frequency. Temperature, salinity, feeding frequency, and combinations thereof, did not induce medusa production, suggesting that this species may use a different cue, possibly photoperiod, to initiate medusa production.

3.2 Introduction

Tropical Australian cubozoans are highly seasonal, with the medusa stage usually arriving along the tropical coastlines during the monsoonal summer months (Carrette et al., 2012; Gordon et al., 2004; Gordon & Seymour, 2012; Hartwick, 1991a; Kingsford et al., 2012; Kinsey & Barnes, 1988; Williamson et al., 1996). This 'stinger season' in Australia is typically between November and May, which has been reported to begin earlier, and last longer, in warmer areas closer to the equator such as in the Gulf of Carpentaria (Gordon et al., 2004; Jacups, 2010; Nickson et al., 2009). At least part of the observed seasonality of the medusa stage is thought to be driven by the complex life cycle of cubozoans.

Cubozoans have a metagenic life cycle that alternates between benthic polyps, which reproduce asexually, and pelagic medusae, that reproduce sexually (Arai, 1997; Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Gordon et al., 2004; Hartwick, 1991a; Hartwick, 1991b; Toshino et al., 2013; Toshino et al., 2014; Werner et al., 1971). Medusa production in cubozoans is variable and can be in the form of complete metamorphosis of the polyp into a medusa (Arneson & Cutress, 1976; Carrette et al., 2014; Cutress & Studebaker, 1973; Stangl et al., 2002; Straehler-Pohl & Jarms, 2011; Werner et al., 1971; Yamaguchi & Hartwick, 1980), metamorphosis that leaves behind a small amount of regenerative material which is able to develop into a polyp (residuum) (Straehler-Pohl & Jarms, 2005), and monodisc strobilation that leaves behind a polyp able to continue asexual reproduction of more polyps (Courtney et al., 2016a; Toshino et al., 2015). Some cubozoan polyps are known to use environmental factors, such as temperature

(Laska-Mehnert, 1985; Stangl et al., 2002; Werner, 1983), a combination of temperature and light intensity (Straehler-Pohl & Jarms, 2011), photoperiod (Gordon & Seymour, 2012), or reduced food availability (Courtney & Seymour, 2013), as a cue to induce synchronous medusa production. It is thought that once medusa production is initiated, it can be punctuated by significant rainfall events, for example *Chiropsella bronzie*, or is continuous, as in *Chironex fleckeri* (Gordon et al., 2004; Gordon & Seymour, 2012). Therefore, medusa production by the polyps is considered to be one factor that drives the seasonal fluctuation in abundance of the medusa stage (Gordon et al., 2004; Gordon & Seymour, 2012; Hartwick, 1991a; Yamaguchi & Hartwick, 1980).

The medusa stage of *Carukia barnesi*, the Irukandji jellyfish, often causes painful and potentially fatal stings (Carrette et al., 2012; Kinsey & Barnes, 1988; Little et al., 2003; Pereira et al., 2010; Williamson et al., 1996). A sting from *C. barnesi* is also renowned for causing Irukandji syndrome which often requires hospitalization for treatment (Carrette et al., 2012; Little et al., 2003; Pereira et al., 2010). Considerable costs are associated with treating sting victims and lost revenue to the tourism industry annually is believed to be substantial (Carrette et al., 2012). Although only the medusa stage of this cubozoan poses a threat to humans, the polyp stage is expected to contribute to the seasonal periodicity through the synchronous timing of medusa production causing the start of the stinger season. Similarly, the abundance of medusae present during the season may be reflective of the success of the polyp stage.

The polyp stage also contributes to the abundance of medusae present by asexually reproducing more polyps, thus increasing the number of polyps able to produce medusae at any one time. Thermal, osmotic and food availability parameters are known to influence the rate of asexual reproduction of the polyp stage of *Alatina* nr *mordens* (Courtney & Seymour, 2013; Klein et al., 2014) and *Carybdea marsupialis* (Acevedo et al., 2013; Canepa et al., 2014; Fischer & Hofmann, 2004). However, in most instances, the *in situ* parameters of the polyps are not known due to the unknown location of the polyps, and to date, cubozoan polyps have only been discovered *in situ* for two species: *C. fleckeri* and *C. marsupialis* (Cutress & Studebaker, 1973; Fischer & Hofmann, 2004; Hartwick, 1991a; Yamaguchi & Hartwick, 1980). Because of this, laboratory-based cultures of cubozoan polyps are essential for exploring the factors that affect polyp survivorship and proliferation in order to deduce where the polyps may reside, which may also shed light on the factors affecting the seasonal influx of medusae.

Carukia barnesi, like other Australian cubozoans, exhibits a marked seasonality. Little is known about the ecology of the early life stages of this species (Courtney et al., 2016a), even though it was discovered as causing Irukandji syndrome over 50 years ago (Fenner, 2006; Kinsey & Barnes, 1988; Southcott, 1967). The medusa stage of *C. barnesi* is considered oceanic and is typically found around coral reefs and islands and under certain condition along beaches (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). However, nothing is known about the thermal and osmotic preference or the location of the polyp stage *in situ*. Therefore, a series of

temperature, salinity, and feeding frequency experiments were conducted on the polyp stage in order to gain a better understanding of the ecology of this species.

The primary aims of this research were: 1) to determine the thermal and osmotic tolerance of the polyps, and use these data to discuss where these polyps may live; 2) to explore the effects of feeding frequency on survivorship and asexual reproduction and discuss intrinsic population growth rates under different feeding regimes; and 3) to determine the effect of thermal and osmotic parameters, and combinations thereof, as well as feeding frequency, as cues for medusa production.

3.3 Method

3.3.1 Specimen Identification

Carukia barnesi is a small species in the family Carukiidae. The medusa stage has a bell height of up to 35 mm and tentacles up to 1.2 m long (Bentlage & Lewis, 2012; Courtney et al., 2015; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007) with a single tentacle that extends from each of the four pedalia; rhopaliar niches with horns; and tentacles that have an alternating pattern of large and small nematocyst crescents that resemble "neckerchiefs" (Bentlage & Lewis, 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). The polyps of *C. barnesi* average 0.9 mm in length and have 11 capitate tentacles on average. The polyps asexually reproduce ciliated swimming polyps and produce medusae through monodisc strobilation (see Courtney et al., 2016a). Little is known about the distribution of this species; however, medusae are

often present along the north-eastern coast of Australia during the warm monsoonal months (November to May) between Lizard Island and Fraser Island (Bentlage & Lewis, 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007).

3.3.2 Experimental Design and Pre-treatment

This project consisted of two separate experiments in which polyps of *C. barnesi* were exposed to different temperature, salinity and/or feeding frequency treatments that were monitored for survivorship, population increase through asexual reproduction, and medusa production following a previously published method (Courtney & Seymour, 2013). The *C. barnesi* polyps were derived from the laboratory-based culture outlined in Courtney et al. (2016a), which was established approximately six months prior to these experiments from wild caught adult medusae. This polyp culture was maintained at a constant temperature of 28ºC ± 0.5ºC and a salinity level of 33‰ ± 0.5‰.

Both of the experiments presented here were conducted in 24 well sterile micro-plates and only 12 of the wells within each plate were used to allow for blank wells between different treatments. Each well had a surface area of 11.5 cm^2 and a volume of 3.5 ml. The mature polyps of *C. barnesi* produce swimming stage polyps through lateral budding (Courtney et al., 2016a). These swimming polyps were harvested from the primary cultures and transferred into the 24 well micro-plates at a density of approximately 15 swimming polyps per well. As these polyps settled and developed into secondary polyps, they were fed freshly hatched *Artemia* sp. nauplii

to satiation (approximately 20 *Artemia* sp.) every seven days. A complete water exchange was carried out 24 hours post each feeding event with filtered artificial seawater. The water quality parameters were maintained at the original culture parameters (28ºC ± 0.5ºC and 33‰ ± 0.5‰) for four weeks prior to any experimental trials to allow the polyps to acclimate, grow to maturity, and begin asexual reproduction. During this time, and during all experiments, a photoperiod of 13 hours light : 11 hours dark was maintained. These polyps were then exposed to one of the conditions outlined below; polyps were only exposed to one treatment, there was no mixing of water between replicates or treatments, and all treatments in each experiment were conducted simultaneously.

The first experiment consisted of 80 thermal and osmotic treatments (see Method: 4.3.3 Thermal and Osmotic Effects on Survivorship and Asexual Reproduction), which were monitored using a binocular dissection microscope, for polyp survival, asexual reproduction, and medusa production over a six-week period. Each treatment comprised six independent replicates and each replicate consisted of an average of 18.63 polyps at the beginning of the experiment (i.e., $\bar{x}$ = 18.63, *SD* = 5.87 polyps; housed in each of 480 wells). Each replicate (well) was fed approximately 20 *Artemia* nauplii once per week and this quantity was increased as the polyp numbers increased (i.e., approximately one *Artemia* per polyp per week). Each feeding event was followed by a complete water exchange with artificial seawater of the same parameters as each experimental treatment (temperature and salinity).

The second experiment required a further five treatments to determine the effects of feeding frequency on asexual reproduction, survivorship, and medusa production (see Method: 4.3.4 The Effects of Feeding Frequency on Survivorship and Asexual Reproduction). This experiment was conducted at the original polyp culture conditions of 28ºC ± 0.5ºC and 33‰ ± 0.5‰ over a six-week period. Each treatment comprised six replicates and each replicate consisted of an average of 20.5 polyps at the beginning of the experiment (i.e., $\bar{x}$ = 20.50, *SD* = 2.79 polyps; housed in each of 30 wells). Each replicate (well) was fed approximately 20 *Artemia* sp. nauplii per feeding event and this quantity was increased as the polyp numbers increased (i.e., one *Artemia* per polyp per feeding event).

3.3.3 Thermal and Osmotic Effects on Survivorship and Asexual Reproduction

To determine the thermal and osmotic tolerances of *C. barnesi* polyps, and the effects these treatments had on survival, asexual reproduction and medusa production, replicates of polyps (as described above) were exposed to an 80 combination matrix of eight temperatures (11, 14, 18, 21, 25, 28, 31, and 34ºC ± 0.5ºC) and ten salinities (16, 19, 22.5, 26, 29, 33, 36, 39, 42.5, and 46‰ ± 0.5‰) over a period of six weeks. Each incubation temperature was maintained by partially submerging each test plate into one of eight temperature controlled water baths for the duration of the experiment. The range of temperatures selected encompasses the potential thermal regime a polyp may experience in the Coral Sea (temperatures commonly below 11ºC at depths of approximately 500 m) and along the adjacent Australian coastline including estuarine environments (temperatures commonly

occur above 34ºC in shallow estuarine pools). The salinity range tested encompasses common salinities found in the Coral Sea and also included both hypersaline and hyposaline conditions that are commonly found in estuarine environments. These environmental parameters were explicitly targeted to deduce the thermal and osmotic tolerance of the polyp stage. Prior to the beginning of this experiment each replicate was photographed and the number of polyps in each well was determined. Every seven days during the six-week experiment, each replicate was fed and water exchanged (as described above). Each replicate was again photographed and the number of polyps in each well was determined in seven-day intervals during the six-week experiment.

To determine the thermal and osmotic tolerance of polyps, the change in population numbers was determined in seven-day intervals during the six-week experiment. To allow for comparisons between treatments, a model was fitted across all treatments that described final polyp density as a function of initial density, temperature, and salinity. We assumed that the final polyp density was proportional to the initial density, functions of temperature $F(T)$ and salinity $G(S)$, and a parameter a which describes the maximum population change at optimal conditions such that,

$$P_{final} = aF(T)G(S)P_{initial}. \quad (1)$$

Thus, we assume independent effects of temperature and salinity on growth rates. Preliminary analyses suggested including interactive effects between temperature and salinity did not qualitatively change the results. We model $F(T)$ and $G(S)$ as

asymmetrical modified Gaussian functions centered around the optimal temperature and salinity, respectively such that,

$$F(T) = \begin{cases} \exp\left[\frac{-(T_{opt}-T)^2}{{\sigma_{t,l}}^2}\right], for\ T < T_{opt} \\ \exp\left[\frac{-(T_{opt}-T)^2}{{\sigma_{t,h}}^2}\right], for\ T > T_{opt} \end{cases} \quad (2)$$

$$G(S) = \begin{cases} \exp\left[\frac{-(S_{opt}-S)^2}{{\sigma_{s,l}}^2}\right], for\ S < S_{opt} \\ \exp\left[\frac{-(S_{opt}-S)^2}{{\sigma_{s,h}}^2}\right], for\ S > S_{opt} \end{cases}, \quad (3)$$

where T_{opt} and S_{opt} describe the optimal temperature and salinity, respectively. The $\sigma_{i,j}^2$ parameters represent the slope for independent variable i (temperature or salinity) and j, the side of the curve (low or high). We assume that the final polyp density follows a negative binomial distribution, and fit the models and calculated the log-likelihood, maximum likelihood estimates and 95% maximum likelihood profile confidence limits using the bbmle package, version 1.0.18 (Bolker & R Development Core Team, 2016), in the statistical package R, version 3.2.4 (Bolker, 2008; R Core Team, 2016). The raw data file and R code has been included in the published version of this chapter (see Supporting Information; S1 Dataset and S1 R Code, in Courtney et al., 2016b). To evaluate the effects of salinity and temperature, we used AIC values (Akaike Information Criterion) to compare the full model with models containing only temperature or salinity (i.e., $P_{final} = aF(T)\ P_{initial}$ or $P_{final} = aG(S)P_{initial}$). Here, large changes in AIC (presented as ΔAIC) with removal of temperature or salinity suggest the importance of the respective environmental component for reproduction and/or survival. Although there are no standard cut-off

values for AIC, like there are for *p*-values, we used a conservative value of ΔAIC = 7, which corresponds to significance levels in the vicinity of 0.003 for nested models (Murtaugh, 2014).

3.3.4 Effects of Feeding Frequency on Survivorship and Asexual Reproduction

In order to determine the effect of feeding frequency on survivorship and asexual reproduction, polyps were exposed to five feeding regimes over a six-week period while held at the original culture conditions of 28ºC ± 0.5ºC and 33‰ ± 0.5‰. The five feeding regimes consisted of feeding every 1, 3, 7, or 14 days and a no food treatment over a six week period. Each feeding event consisted of food saturation for 24 hours (approximately one *Artemia* sp. nauplii per polyp per feeding event as outlined above) followed by a 100% water exchange 24 hours post feeding event with artificial pre acclimated sea water of 28ºC ± 0.5ºC and 33‰ ± 0.5‰. In the 14-day and no food treatments, water exchanges were conducted every seven days during the six-week experiment. Each replicate was photographed and the number of polyps in each replicate was determined at the start and in seven-day intervals during the experiment. Polyp density was analysed with a generalised linear mixed model with feeding frequency, time and the interaction between time and feeding frequency as fixed effects and replicate number as a random effect. This was analysed via proc glimmix in SAS, version 9.4 (SAS Institute Inc., 2015), using the ar(1) covariance matrix to describe temporal autocorrelation, and we assumed polyp density followed a negative binomial distribution with a log-link function, typical for count data (Bolker et al., 2009). We evaluated statistical inference tests with Wald F

tests conducted in proc glimmix. The raw data file has been included in the published version of this chapter (see Supporting Information; S2 Dataset, Courtney et al., 2016b).

3.3.5 Environmental Cues for Medusa Production

To explore feeding frequency, temperature, salinity, and combinations thereof, as potential cues for medusa production, each of the previously described six-week experiments was also monitored for medusa production. Medusa production for this species is in the form of monodisc strobilation (Courtney et al., 2016a) and each polyp was monitored for a change in body shape, formation of statoliths, attached and free swimming medusae. At the end of the six-week experiments each replicate of each treatment (80 temperature and salinity treatments and five feeding frequency treatments) was rapidly returned (shocked) back to the original culture condition of 28ºC ± 0.5ºC and 33‰ ± 0.5‰ and were monitored for a further four weeks (i.e., six weeks in treatment and four weeks post treatment). During this additional four-week period, the polyps were fed once per week followed by a complete water exchange 24 hours post-feeding event.

3.4 Results

3.4.1 Thermal and Osmotic Effects on Survivorship and Asexual Reproduction

Polyp survivorship and asexual reproduction rates significantly decreased as environmental values moved away from the optimal temperature and salinity, and removal of parameters describing the effects of each environmental variable suggested each variable was important (effect of removing temperature parameters by setting $F(T) = 1$: $\Delta AIC = 594$, and effect of removing salinity parameters by setting $G(S) = 1$: $\Delta AIC = 164$). These ΔAIC values are distinctly higher than our conservative baseline of $\Delta AIC = 7$, and suggest that temperature and salinity alone are not sufficient to explain the data, and polyp survival and/or reproduction is indeed a function of both temperature and salinity. Summary statistics describing the data used to generate the model are provided in Appendix D. The optimal temperature and salinity for polyp proliferation occurred at 22.9ºC and 26.0‰, respectively, where the population increased by over 10 times during the six-weeks (see Figure 3.1 and Table 3.1) compared to 100% mortality associated with both high and low temperatures and very low salinity levels. The temperature and salinity range that allowed for population stability (i.e., equal to or greater than 100% of the starting population) consisted of thermal treatments between 18ºC and 31ºC at salinity levels between 19‰ and 46‰. Although asexual reproduction was high at temperatures that commonly occur in the waters of the Great Barrier Reef, this was not the case with salinity, where the highest polyp proliferation was not encompassed by salinity levels that occur in the waters of the Great Barrier Reef (see Figure 3.1). While variation in population growth surrounding the optimum temperature and

salinity was relatively symmetrical with respect to temperature (σ_{tl} = 3.47, σ_{th} = 5.01), population reproduction showed a steeper decrease with lower salinity (σ_{sl} = 5.19) than higher salinity (σ_{sh} = 31.07).

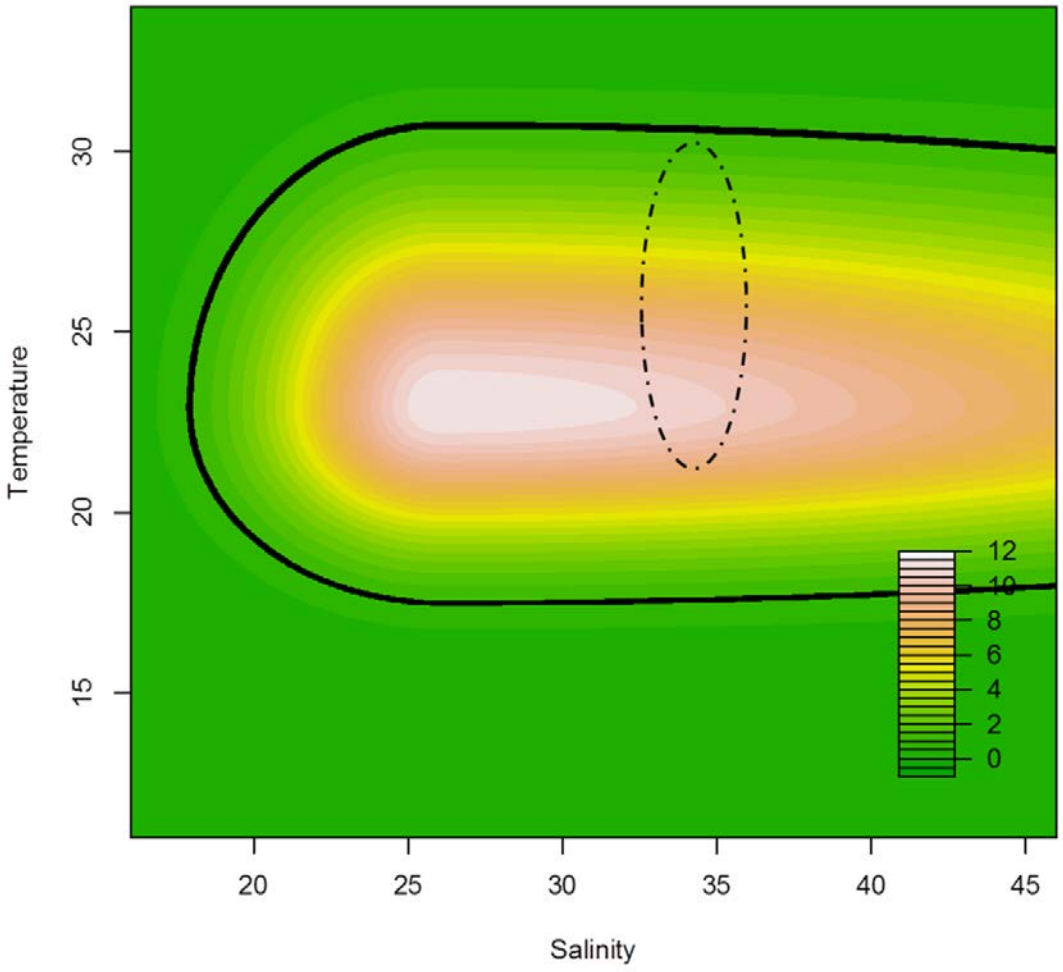

Figure 3.1 Maximum likelihood estimated proportional change in polyp density after six weeks plotted as a function of temperature and salinity from best-fit model. Model output describes proportional change in polyp density for different temperature and salinity values as described in equation 1. Solid black line represents values of 1, where polyp density is expected to remain constant. The area encompassed by the dashed line indicates sea surface temperature and salinity levels that commonly occur within the waters of the Great Barrier Reef (Australian Institute of Marine Science, 2016; Ban et al., 2012). Models were fit to data collected from a matrix of eight temperature and ten salinity treatments, each replicated six times. Summary statistics describing the data used to generate the model are provided in Appendix D.

Table 3.1 Maximum likelihood estimates for parameters describing proportional change in polyp density of *Carukia barnesi*, after six weeks, modelled as a function of temperature and salinity.

Symbol	Description	Value	2.5% CL	97.5% CL
A	Maximum proportional change	11.48	9.00	14.95
σ_{tl}	Temperature curve low	3.47ºC	2.91ºC	4.02ºC
σ_{th}	Temperature curve high	5.01ºC	4.39ºC	5.72ºC
T_{opt}	Optimum Temperature	22.91ºC	21.92ºC	23.86ºC
σ_{sl}	Salinity curve low	5.19‰	4.39‰	6.22‰
σ_{sh}	Salinity curve high	31.07‰	21.70‰	89.95‰
S_{opt}	Optimum Salinity	26.04‰	24.54‰	27.85‰

Model output describes proportional change in polyp density for different temperature and salinity values as described in equation 1, 2, and 3. Models were fit to data collected from a matrix of eight temperature and ten salinity treatments, each replicated six times. Estimated parameter values are based on the best fit parameters and 95% maximum likelihood profile confidence limits are provided.

3.4.2 Effects of Feeding Frequency on Survivorship and Asexual Reproduction

Feeding frequency significantly increased asexual reproduction ($F_{4, 25}$ = 16.76, $p < 0.001$). In addition, there was significant temporal variation in polyp proliferation ($F_{6, 149} = 78.65$, $p < 0.001$) and the positive effect of feeding frequency became incrementally stronger over time ($F_{24, 149} = 5.21$, $p < 0.001$). Polyps that were fed daily increased mean population numbers by over three times during the six-weeks, and polyps that were unfed increased in population by 0.5 times over the same time frame (Figure 3.2).

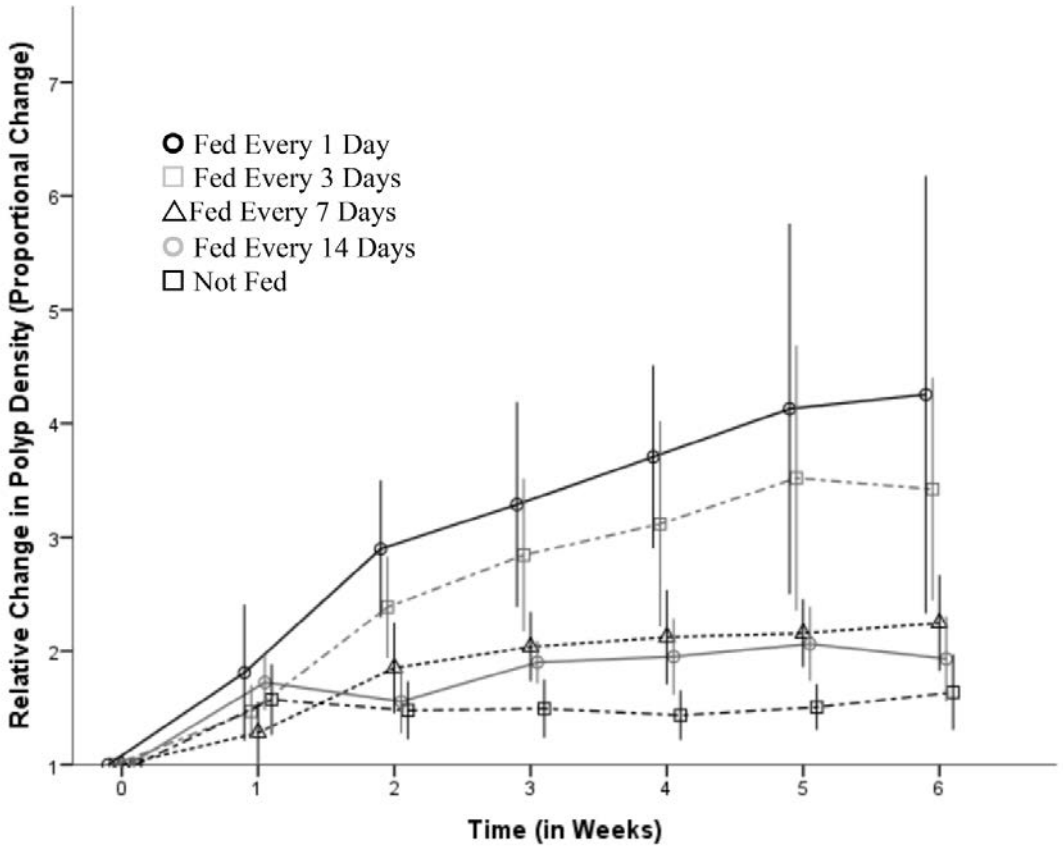

Figure 3.2 The relative change in *Carukia barnesi* polyp density, over six weeks of exposure, to five feeding regimes. The feeding levels consisted of: fed every day (O); fed every three days (□); fed every seven days (△); fed every 14 days (○); unfed for 42 days (□). Each of the five feeding treatments consisted of six independent replicates, each of which consisted of a mean staring polyp density of 20.5 polyps per well (n = 30, $\bar{x}$ = 20.5, SD = 2.8). The values for each time sequence were calculated as the proportional change in polyp density from the starting population density at time zero, where values of one indicate no population change and values above one indicate polyp proliferation through asexual reproduction. Values are reported as means and error bars represent 95% CI assuming a normal distribution. All counts were conducted in seven-day intervals however the values have been graphed offset on the time axis for clarity.

3.4.3 Environmental Cues for Medusa Production

Temperature, salinity, and feeding frequency, and variations thereof, did not trigger medusa production during the six-week experiments or during the four-week post treatment monitoring period. Only one polyp went through medusa production

during the ten weeks. This occurred in the 31ºC and 46‰ treatment and was identified during week five of the experiment. As a percentage, one polyp undergoing medusa production during this experiment was not significant. Returning the polyps to the original culture conditions of 28ºC ± 0.5ºC and 33‰ also did not cause medusa production over the four-week post experiment monitoring period.

3.5 Discussion

Carukia barnesi polyps had a high tolerance to osmotic treatments with 100% survivorship from treatments between 18 and 46‰ (Figure 3.1), which indicates that this species may inhabit areas with a high degree of osmotic variation. The osmotic treatment that yielded the highest degree of polyp proliferation was 26‰. Salinity levels this low do not occur in the waters of the Great Barrier Reef or in the Coral Sea, however these low salinity conditions are a common occurrence in estuarine systems (Cyrus & Blaber, 1992; Mao & Ridd, 2015; Wolanski & Elliott, 2015; Yu et al., 2014). These results indicate that although the polyp stage does not seem restricted to low salinity waters, the observed increase in asexual reproduction under low salinity conditions suggests that the polyp stage is probably an estuarine inhabitant. Cubozoans that have coastal or estuarine polyp stages have previously been reported for two species; *C. fleckeri* polyps were located *in situ* attached to the underside of rocks near a river mouth in Queensland (Hartwick, 1991a), and polyps of *C. marsupialis* were once located attached to bivalve shells in a mangrove habitat in Puerto Rico (Cutress & Studebaker 1973). To date, these are the only two

discoveries of cubozoan polyps *in situ* globally, however at least one other Australian cubozoan polyp, those of *C. bronzie,* has also been suggested to be estuarine (Gordon et al., 2004). The possibility that *C. barnesi* has an estuarine polyp stage has not previously been considered primarily due to their distinctly oceanic medusa stage (Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967) compared to species which are known to be primarily coastal, such as *C. fleckeri* and *C. bronzie* (Barnes & Kinsey, 1986; Gordon et al., 2004; Gordon & Seymour, 2012; Mooney & Kingsford, 2016; Southcott, 1956).

There have been no reported sightings, or documented stings caused by *C. barnesi* from estuaries; however this does not discount the possible presence of medusae in these areas when they are newly detached and small. It is currently unknown how the polyps enter low salinity habitats and nothing is known about the osmotic tolerance of the medusa stage or where or when the medusae spawn. In speculation, due to there being no reports of adult *C. barnesi* medusae within estuarine systems, it is plausible that the eggs, that are known to have a long encapsulated planula stage from six days to six months (Courtney et al., 2016a), are transported inshore on currents. Future research is required on the medusa stage, such as determining their osmotic tolerance, to better understand how the life cycle is completed *in situ*.

Temperature also affected both the survivorship and asexual reproduction rate of *C. barnesi* polyps. There was notable symmetry between the high and low temperature curves with a modelled optimum temperature of 22.9ºC (Table 3.1). As the thermal treatments moved away from optimum the amount of polyp proliferation

through asexual reproduction was reduced. The minimum and maximum thermal constraints of the polyps were between 18ºC and 31ºC, which indicates that the polyp stage has a suitable operating envelope of approximately 13 degrees, whereby positive population growth is possible over a period of six weeks.

The southern distribution limit of the medusa stage of *C. barnesi* is not well established. However, at present it is considered to be near Fraser Island based on sting and capture records (Seymour unpublished). The winter (July) sea surface temperature near Fraser Island typically averages 20ºC and increases to 27ºC during the summer (February) (Ban et al., 2012), which is within the thermal capacity range for population growth of the polyp stage and encompasses their thermal optimum of 22.9ºC. Although the temperature profile of *C. barnesi* polyps seems to fit well with the water temperature near Fraser Island, it is expected that the water temperature in estuarine systems near river mouths, which are predominately large river systems along the eastern coast of Queensland, to be significantly lower in temperature than the adjacent open ocean. For example, temperatures near the mouth of the Noosa Rivers (approximately 100km south of Fraser Island) are known to average as low as 18ºC during the winter (Coles & Greenwood, 1983) compared to 20ºC in the open ocean near Fraser Island (Ban et al., 2012). Therefore, the polyp stage of *C. barnesi* may be restricted to warmer estuarine areas where the winter water temperature does not drop as low as 18ºC north of Fraser Island. This suggests that the medusa stage may limit the southern distribution of this species, though no data exists on the thermal tolerance of the medusa stage to confirm this.

The observed rate of asexual reproduction of *C. barnesi* during these experiments was high compared to another cubozoan polyp, *A.* nr *mordens* (Courtney & Seymour, 2013). For example, a maximum polyp population increase of 50% was recorded for *A.* nr *mordens* over six weeks (Courtney & Seymour, 2013) compared to over 1000% in *C. barnesi* during this replicated experiment (Figure 4.1). This high rate of reproduction is expected to increase the number of polyps able to produce medusae at any one time and in turn influence the abundance of medusae present. Highly reproductive populations are expected to make use of rapid environmental changes (Chevin et al., 2010; Merilä & Hendry, 2014; Sommer et al., 2016). Purely in speculation, and assuming these polyps are estuarine and require hard substrate for attachment similar to other cnidarian polyps (Brewer, 1976; Brewer, 1978; Brewer, 1984; Holst & Jarms, 2006), increasing manmade structures within marine environments (e.g., marinas, boat ramps and mooring platforms), may increase the available hard substrate for polyp attachment. This anthropogenic effect has been suggested to positively impact on scyphozoan polyp populations (Duarte et al., 2012; Purcell, 2012; Purcell et al., 2007; Richardson et al., 2009; Feng et al., 2015). Not surprisingly, increased asexual reproduction was correlated with increased feeding frequency. Therefore, changes in estuarine trophic dynamics, primarily eutrophication, may lead to increased copepod density (Nittrouer et al., 1995; Suchy et al., 2016), presumably a primary food source for polyps and early stage medusae, which may influence polyp proliferation and in turn affect medusae abundance.

Some cubozoan polyps are known to use environmental factors, such as temperature (Laska-Mehnert, 1985; Stangl et al., 2002; Toshino et al., 2015; Werner, 1983), or reduced food availability (Courtney & Seymour, 2013), as a cue for medusa production. However, temperature, salinity, feeding frequency, and combinations of these factors, did not trigger medusa production in *C. barnesi*. This suggests that the polyps may use a different cue for synchronous medusa production. Because of the consistency of the first arrival of the medusa stage over the last 50 years (Carrette & Seymour, 2013), it is possible that this species uses increasing photoperiod as a cue due to the interannual variation in environmental parameters such as temperature and salinity. This type of cue has also been suggested for *C. fleckeri* due to the consistency of occurrence during a seven-year study (Gordon & Seymour, 2012). There is also evidence that the stinger season length has increased over the last 50 years from 15 days to 151, which has been speculated to be caused by increasing global sea temperatures (Carrette & Seymour, 2013). Therefore, it is possible that *C. barnesi* polyps use photoperiod to initiate synchronous medusa production but continue to produce medusae throughout the season until the winter water temperature becomes too low.

Future research should pursue determining the thermal and osmotic parameters of the medusa stage of *C. barnesi*, and experimentally determine possible environmental cues for medusa production. Understanding the contributing factors that lead the spatial and temporal variability in medusae abundance may in turn prove valuable to predicting future *C. barnesi* abundance, and/or distributional range extensions, under projected sea temperature rise scenarios.

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Chapter 4:

Thermal and Osmotic Tolerance of the Medusa Stage of 'Irukandji' Jellyfish: Cubozoa; *Carukia barnesi*

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Chapter 4:

Thermal and Osmotic Tolerance of the Medusa Stage of 'Irukandji' Jellyfish: Cubozoa; *Carukia barnesi*

4.1 Abstract

This research explores the thermal and osmotic tolerance of the medusa stage of the Irukandji jellyfish *Carukia barnesi* to determine if environmental parameters influence the marked seasonality of this species. By exploring oxygen consumption over a range of temperatures, the minimum thermal requirement for *C. barnesi* was estimated at 21.5ºC, which does not explain the seasonal occurrence of this species. The optimum temperature for swimming pulse rate was determined to fall between 27.5ºC and 30.9º, which encompassed the typical summer thermal regime *in situ*. This research concludes that the reduced fitness associated with environmental temperatures that depart from optimum may better explain the seasonal pattern of this species than the theoretical thermal limits. Conversely, departure from optimum temperature did not explain the southern distribution limits of this species, suggesting that *C. barnesi* could theoretically persist further south than their loosely defined southern distribution limits. The optimum salinity of *C. barnesi* medusae was estimated to occur at salinity levels above 33.6‰. Salinity levels below 29‰ reduced fitness and became lethal below 25‰. This result supports the hypothesis that *C. barnesi* medusae are oceanic and cannot persist in estuarine environments where low salinity conditions commonly occur. Furthermore, the respiration rate of *C. barnesi* was significantly suppressed at night, providing evidence that this species is less active during night conditions, presumably to conserve energy.

4.2 Introduction

Cubozoans are complex gelatinous animals with: sophisticated visual structures (Coats, 2003; Garm et at., 2007; Hamner et al., 1995; Martin, 2002; Nilson et al., 2005; Piatigorski et al., 1989); complex prey capture techniques (Buskey, 2003; Courtney et al., 2015); venoms (Carrette et al., 2012; Chaousis et al., 2014; Little et al., 2003; Pereira et al., 2010; Williamson et al., 1996); diurnal behavior patterns (Gordon & Seymour, 2009; Seymour et al., 2004); elevated swimming capacities (Colin et al., 2013; Shorten et al., 2005); and metagenic life cycles (Arai, 1997; Arneson & Cutress, 1976; Courtney et al., 2016a; Cutress & Studebaker, 1973; Hartwick, 1991a; Toshino et al., 2014; Werner et al., 1971; Yamaguchi & Hartwick, 1980). The medusa stage of many species show high levels of seasonality, however, in most cases little is known as to the factors that contribute to, or cause, the seasonal patterns (Carrette & Seymour, 2013, Chiaverano et al., 2013; Crow et al., 2015; Kingsford & Mooney, 2014; Kingsford et al., 2012; Kinsey & Barnes, 1988; Mooney & Kingsford, 2016). Although previous research has focused on many aspects of cubozoan ecology, some areas that have received far less attention are the thermal and osmotic tolerances of the medusa stage. Exploring these factors may give insight into why some species are highly seasonal and determine if this seasonality is driven by annual temperature and/or salinity fluctuations. The thermal ecology of cubozoans may also explain the latitudinal distribution limits with many species restricted to tropical regions (Carrette et al., 2012, Kingsford & Mooney, 2014). Understanding the thermal limitations of cubozoans, particularly those known

to be dangerous to humans, would be beneficial to predicting future distributional range changes under current and projected sea temperature rise scenarios.

One oceanic, venomous, tropical Australian cubozoan that is known to occur along the eastern coast between Lizard Island and Fraser Island, that demonstrates marked seasonality, is the 'Irukandji' jellyfish *Carukia barnesi* (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967). This species is typically located near reefs, islands, and under certain circumstances, along beaches during the summer monsoonal months between November and May, with a high amount of spatial and temporal variability (Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). This species, as with all other cubozoans, has a metagenic life cycle, which alternates between benthic sessile polyps that reproduce asexually, and motile planktonic medusae that reproduce sexually (Arai, 1997; Arneson & Cutress, 1976; Cutress & Studebaker, 1973; Gordon et al., 2004; Hartwick, 1991a; Hartwick, 1991b; Toshino et al., 2013; Toshino et al., 2014; Werner et al., 1971).

The annual arrival of many tropical Australian cubozoans is typically correlated with increasing sea temperature during the summer monsoonal months. This has led to the general consensus that the medusa stage of many tropical species (e.g., *Chiropsella bronzie, Chironex fleckeri*) requires warm temperatures for persistence and subsequently disappears when the temperature becomes too low during the winter months, presumably dying (Barnes & Kinsey, 1986; Gordon et al., 2004; Gordon & Seymour, 2012; Kingsford & Mooney, 2014; Kinsey & Barnes 1988). Although this pattern is routinely observed, very little data exist on the

thermal tolerance of cubozoan medusae. One factor that makes determining the thermal tolerance of cubozoan medusae difficult is the inability to maintain specimens in laboratory conditions for extended periods of time in order to measure the effects of different temperatures, primarily due to limitations in animal husbandry practices. Many measures that are used to determine the effect of temperature in other taxonomic groups, primarily insects, are experiments conducted on developmental times, survivorship, growth rates, reproductive output, metabolic rate, or motor control, at different temperatures (Damos & Savopoulou-Soultani, 2012; Roy et al., 2002). Of these measures, respiration rate (often used as a surrogate measure of metabolism) and motor control, such as bell contraction frequency in the case of jellyfish, may provide an avenue for determining a thermophysiological profile by means that do not require maintaining specimens in artificial environments for extended periods (Mangum et al., 1972). Respiration rate studies are not new to research on gelatinous species, where the respiration rate of many scyphozoans have been determined, primarily to estimate the impact of jellyfish on trophic food webs (see review, Purcell et al., 2010). Likewise, relationships between body size and respiration rate have been investigated (Ikeda, 2014; Lilley & Lombard, 2015), but very few studies have been conducted that explicitly target minimum and maximum thermal tolerances, based on the thermophysiological profiles of jellyfish.

One area of thermophysiological research on ectothermic invertebrates is routinely conducted in the field of entomology, whereby understanding the effect of temperature on developmental times of insects is paramount in predicting when particular insects will be at certain life stages for effective pest management

interventions (Fisher et al., 1999; Huffaker et al., 1999; Roy et al., 2002). This requires precise estimation of when the environmental temperature is too low, or too high, for a species to operate, usually determined as theoretical biological zero, where the measured function, often developmental time, respiration rate, or reproductive rate, theoretically ceases due to unfavorable environmental temperatures (see review, Damos & Savopoulou-Soultani, 2012). The value of biological zero is then used as the basis for projecting developmental times *in situ* based on the amount of time the environmental temperature is above this minimum temperature threshold (usually reported as degree days) (Damos & Savopoulou-Soultani, 2012; Ikemoto & Takai, 2000). Compaitively, this extensive body of knowledge does not exist for cubozoans or scyphozoans.

The primary aim of this project was to determine the thermal tolerance of the medusa stage of *C. barnesi* by measuring oxygen consumption rate over a range of temperatures in order to define the thermal limits of this species. Additionally, the effect of temperature on bell contraction frequency, which is essentially a measure of swimming capacity and assumed to be a proxy for fitness (Mangum et al., 1972), was explored to better define the thermal tolerance of this species. Finally, the effect of varying salinity levels on bell contraction frequency was determined to gain insight into the possible environmental parameters that may affect the survival of *C. barnesi* medusae.

4.3 Method

4.3.1 Specimen Identification

Carukia barnesi is a small species in the family Carukiidae with a bell height of up to 35 mm and tentacles up to 1.2 m long (Bentlage & Lewis, 2012; Courtney et al., 2015; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007). Some of the identifying features of this species include: a single tentacle that extends from each of the four pedalia (i.e., four tentacles in total, one extending from each pedalia); rhopaliar niches with horns; and tentacles that have an alternating pattern of large and small nematocyst crescents that resemble 'neckerchiefs', and lack of gastric cirri (Bentlage & Lewis, 2012; Kinsey & Barnes, 1988; Southcott, 1967; Underwood & Seymour, 2007).

4.3.2 Capture Method

Adult medusae of *C. barnesi* were collected near Double Island, North Queensland, Australia (16º43.5′S, 145º41.0′E), at night, following a previously published method (Courtney et al., 2015, Courtney et al., 2016a). The sea surface temperature varied from 27.5ºC to 30ºC; with an average salinity of 35‰. In brief, specimens were attracted to a small vessel with underwater lights. The specimens were collected with a net and transferred into individual 500 ml containers for transport. Prior to experimental trials, all specimens were held at a constant 28ºC ± 0.5ºC in a constant temperature controlled cabinet overnight (Courtney et al., 2015, Courtney et al., 2016a). All specimen collections were conducted in accordance with Permit Numbers: G11/34552.1 and G15/37396.1 (Great Barrier Reef Marine Park Authority).

4.3.3 Experimental Overview

This project consisted of three separate experiments in which the medusa stage of *C. barnesi* were exposed to varying thermal and osmotic conditions to determine their tolerance to these parameters. The first experiment consisted of exposing medusae to thermal treatments (experimental methods explained in detail below) and measuring oxygen consumption to determine the relationship between temperature and respiration rate, which is often used as a surrogate measure of metabolism (Ikeda, 2014). The second experiment exposed *C. barnesi* medusae to a range of thermal treatments to determine how temperature affects the swimming pulse rate (i.e., bell contraction frequency), as a measure of fitness, to indicate critical temperature thresholds. The third experiment was designed to explore the osmotic tolerance of this species, where bell contraction frequency was measured over a range of salinity levels to determine the suitable salinity range of the medusae to elucidate their upper and lower osmotic tolerance. As the salinity levels of the waters of the Great Barrier Reef are considered reasonably stable, and do not change with latitude, respiration rate trials were not conducted at different salinities.

4.3.4 Experimental Equipment

The ratio between respiration rate chamber volume, and test animal wet weight (mass), has been shown to affect the respiration rate of scyphozoan medusae, resulting in a new recommendation of a ratio of > 50 ml of water per gram of test animal, wet weight, and ideally 100 ml of water per gram, wet weight, has been suggested (Purcell et al., 2010). This ratio has been suggested to minimize the effects of reduced oxygen levels and restriction of movement during respiration rate

experiments on jellyfish. To abide by these recommendations, respiration rate chambers of 70 ml volume were used to accommodate *C. barnesi* medusae that ranged in mass between 0.1 g and 0.7 g wet weight.

A StrathKelvin 929 six-chamber respirometer unit, fitted with polyographic membrane-type probes, was used for real-time dissolved oxygen measures at a rate of two measures per second in each chamber. Each respiration measure consisted of six chambers, each of 70 ml in volume: five chambers, each containing one specimen, and one blank chamber used as a control in order to compensate for intrinsic factors, such as background respiration, and extrinsic factors, such as atmospheric and thermal variation. From this, respiration rate was calculated through linear regression as the change in oxygen concentration per hour minus the control. All probes were calibrated prior to each experiment using a dual-point calibration method. Zero calibration solution was obtained by mixing a 4% solution of Na_2SO_3, and oxygen saturation was obtained by aeration of the test water with room air. Temperature, salinity, and atmospheric pressure was recorded and compensated for during calibration. The experimental temperatures were controlled by conducting the respiration experiments in a temperature controlled water-bath which maintained each test temperature ± 0.3ºC.

4.3.5 The Influence of Temperature on Respiration Rate

To determine the effect of temperature on the respiration rate of *C. barnesi*, 90 specimens were exposed to one of seven thermal treatments between 22 and 33ºC. During preliminary data analysis, a distinct pattern arose between respiration rate trials that were conducted during the day and those conducted during the night, even

though the night trials were conducted under full laboratory lighting. As evidence for a diurnal activity changes have been previously noted in *C. fleckeri* (Seymour et al., 2004), and shown in *C. barnesi* (Courtney et al., 2015), the time of the metabolic run (night or day) was included as a second variable for analysis (e.g., seven thermal treatments between 22ºC and 33ºC during the day; 22, 23.5, 25, 27.5, 30, 31.5 and 33ºC) and four additional thermal treatments between 24ºC and 33ºC at night; 24, 27.5, 30 and 31.5ºC). These treatments were chosen because they encompass the temperatures this species is likely to experience during the summer months in the waters of the Great Barrier Reef.

Each specimen was transferred from the 28ºC temperature controlled cabinet to the test temperature (between 22 and 33ºC) at a rate of 4ºC per hour and were held for one hour at the testing temperature to reach equilibrium with the incubation temperature prior to each experimental trial. Each specimen was then transferred into a respiration rate chamber, which contained oxygen saturated seawater of the same water quality parameters at the desired test temperature. Each respiration trial was conducted over one to two hours and each trial was terminated prior to reaching 80% oxygen saturation in order to reduce the assumed deleterious effects of low oxygen levels. This method was repeated at each test temperature. The respiration rate trials that were conducted during the night were still run under full laboratory lighting and were not designed to test the effects of light and dark, but rather to test for circadian or endogenous rhythm. In order to compensate for the displacement of each specimen within each chamber, which changes the effective water volume, the wet mass of each specimen was subtracted from the known volume of each chamber (70

ml − wet mass g). Jellyfish are known to have a high water content (Purcell et al., 2010; Vinogradov, 1953) and the largest *C. barnesi* exposed to experiments in this study was approximately 0.7g (e.g., displaced a maximum of 1% of the chamber volume).

To compare the respiration rate of specimens of different sizes, it was necessary to explore the influence of mass on oxygen consumption. Dry mass is one of the most stable measures of mass for species with high water content (Lucas, 1996); therefore, dry mass was regressed against oxygen consumption at the near capture site average temperature of 30ºC. The up-scaling respiration mass exponent for cubozoans has not previously been determined, and due to the small variation in mass range in these experiments the relationship was analysed through linear regression. To compensate for the influence of mass statistically in further analysis, oxygen consumption was divided by the dry mass of the specimen (e.g., μmol O_2 g^{-1} $^{\text{Dry Mass}}$ h^{-1}).

Data analysis then consisted of a two-way ANOVA with replication to test the effect of temperature and day/night on the mass adjusted respiration rate of *C. barnesi*, which was followed by a post-hoc LSD to indicate significant differences. As these data were essentially linear between 22ºC and 30ºC, these data were further explored through linear regressiextrapolate to theoretical biological zero. Oxygen consumption was regressed against thermal treatments between 22ºC and 30ºC during measures conducted during the day. The equation of best fit was determined and the intercept with zero was calculated along with 95% confidence intervals.

4.3.6 The Influence of Temperature on Swimming Pulse Rate

To determine how temperature affects the bell contraction rate of *C. barnesi,* 23 specimens were exposed to thermal treatments between 16 and 38ºC ± 0.5ºC in two degree increments. Each specimen was removed from the 28ºC temperature controlled cabinet in which they were housed in individual 500 ml containers. These containers were then placed into a water-bath which maintained each experimental temperature ±0.5ºC. Beginning at 28ºC, the specimens were left at each temperature for one hour in order to reach equilibrium with the test temperature prior to measuring the bell contraction rate. Each specimen was monitored for 30 to 60 seconds and the number of bell contractions was recorded as beats per minute. Measures were only taken while the specimens were actively swimming or pulsing. After each measure, the temperature was ramped down 2ºC over one hour, and was then held constant for an additional hour, prior to each pulse rate measure. This process was then repeated with new specimens where the temperature was ramped up in 2ºC increments starting at 28ºC following the same method as previously stated.

Specimen size was expected to influence the bell contraction frequency, where smaller specimens were assumed to have a higher standard pulse rate than larger specimens. Therefore, it was necessary to explore the relationship between bell size and pulse rate prior to analysis. To do this, the pulse rates at near capture conditions were examined from this trial and 'the influence of salinity on swimming pulse rate trial' (explained below) of 28ºC and 35‰. Bell size was determined as niche bell height, a longitudinal measure from the center of the rhopaliar niche to the

apex of the bell (NB). Pulse rate was then regressed against bell size to determine the relationship between these two factors.

We used a non-linear mixed effect modeling approach to evaluate the effect of temperature on the bell contraction frequency (pulse rate measured as beats per minute) of the medusae. The model, $F(T)$ describes the decline in pulse rate as temperatures depart from optimum as asymmetrical reparameterized polynomials, with monotonic decreases in pulse rate as temperature either increases or decreases from optimum. We allowed increases in temperature to have a different effect on pulse rate than decreases in temperature from optimal such that the performance curve is asymmetrical. Expected pulse rates range from the maximum rate, described by parameter A at the optimal temperature (T_{opt}) to zero at the estimated critical thermal minimum (CT_{min}) and maximum (CT_{max}). Because it is unrealistic to have pulse rates with negative values, the model was constrained outside of the inhabitable temperature range, such that temperatures less than the critical temperature minimum and greater than the critical temperature maximum (CT_{min} and CT_{max} respectively) were set to zero. The specific parameterization is described as,

$$F(T) = A * \begin{cases} 0 + {e_o}^2 & for\ T < CT_{min} \\ 1 - \left(\frac{T_{opt} - T}{T_{opt} - CT_{min}}\right)^2 + {e_j}^2 + {e_o}^2 & for\ CT_{min} \leq T \leq T_{opt} \\ 1 - \left(\frac{T - T_{opt}}{T_{opt} - CT_{max}}\right)^2 + {e_j}^2 + {e_o}^2 & for\ T_{opt} \leq T \leq CT_{max} \\ 0 + {e_o}^2 & for\ T > CT_{max} \end{cases} \quad (1).$$

The parameter ${e_j}^2$ describes the distribution of pulse rates among jellyfish, and follows a Gaussian distribution with a mean of zero and a variance of ${\sigma_j}^2$ (i.e., a

random effect of individual medusae). This random effect accounts for repeated sampling of the same specimen in different treatments and also accounts for variations in pulse rates between individuals, such as bell size. The parameter e_o^2 describes the observation error, which we assume follows a Gaussian distribution with a mean of zero and a variance of σ_o^2. The random effect parameter e_j^2 is only included for values where positive pulse rates are expected. This necessitates the following two assumptions. First, the variation between jellyfish only occurs within the inhabitable range, and second, the total variation for values with non-zero expected values ($\sigma_j^2 + \sigma_o^2$) is more than the variation for data with values expected to be zero (σ_o^2) (i.e., unequal variance). Evaluation of other distributions for the observation error either provided poorer fits or would not converge due to inability to integrate over the two types of distributions (i.e., the Gaussian jellyfish random effect and a non-Gaussian experimental error) (Bolker et al., 2009). The log-likelihood, maximum likelihood estimates and 95% maximum likelihood confidence limits were calculated for general non-linear mixed effect model described above using the nlmixed procedure in SAS, version 9.4 (SAS Institute Inc., 2015).

4.3.7 The Influence of Salinity on Swimming Pulse Rate

To determine how salinity affects the bell contraction rate of *C. barnesi*, which is essentially a proxy for fitness (Mangum et al., 1972), 120 medusae were exposed to one of eight salinity treatments (i.e., 15 replicates of each salinity treatment) of 20, 23, 26, 29, 32, 35, 38 and 41‰ ± 0.5‰ at 29ºC ± 0.5 ºC (ambient temperature). This trial was conducted in 500 ml plastic containers and the salinity levels were lowered by adding reverse osmosis water to seawater collected from the

capture site (salinity at the capture site was 35‰ ± 0.5‰). Salinity was increased by adding aquarium grade sea salt. Each specimen was removed from its transport container and placed directly into the specific salinity treatment. This experiment was designed to test the response to shock salinity changes, which may happen near river mouths when rainfall events occur. The pulse rate of each individual was then measured after 24 hours exposure. All pulse rate measures were conducted following the same protocol presented above.

To determine the affect of salinity on the bell contraction frequency of *C. barnesi* medusae, the data were interpreted through piecewise regression with two change points. We assumed a negative binomial distribution to account for overdispersion typical of count data (Bolker et al., 2009) (i.e., the pulse rate data were measured as integers). We also assumed the slope before and after the change points to be zero. The slope between the two change points was determined by the change point values. This approach was compared to various linear and non-linear models, and the piecewise regression provided the best explanation of these data (data not shown). We further evaluated the best fit model by comparing it to an alternative model that assumed a constant pulse rate irrespective of salinity and analysed the model fits through a likelihood ratio test assuming a chi-squared approximation of the log-likelihood ratio. These analyses were conducted in the statistical package R, version 3.2.4 (Bolker, 2008; R Core Team, 2016).

4.4 Results

Regression analysis revealed that wet mass was positively correlated with dry mass of *C. barnesi* (R^2 = 0.986, $F_{1,\ 131}$ = 8962.71, p < 0.001) with wet mass explaining 98.6% of the variance of dry mass. The linear equation of best fit was [Wet Mass (g) = (9.8×10^{-3}) + 18.11 × Dry Mass (g)] (see Figure 4.1). The water content of *C. barnesi* was also calculated as the percentage change between wet mass and dry mass for each specimen and the average water content was 94.3% water (n = 133, $\bar{x}$ = 94.3%, SD = 0.6%).

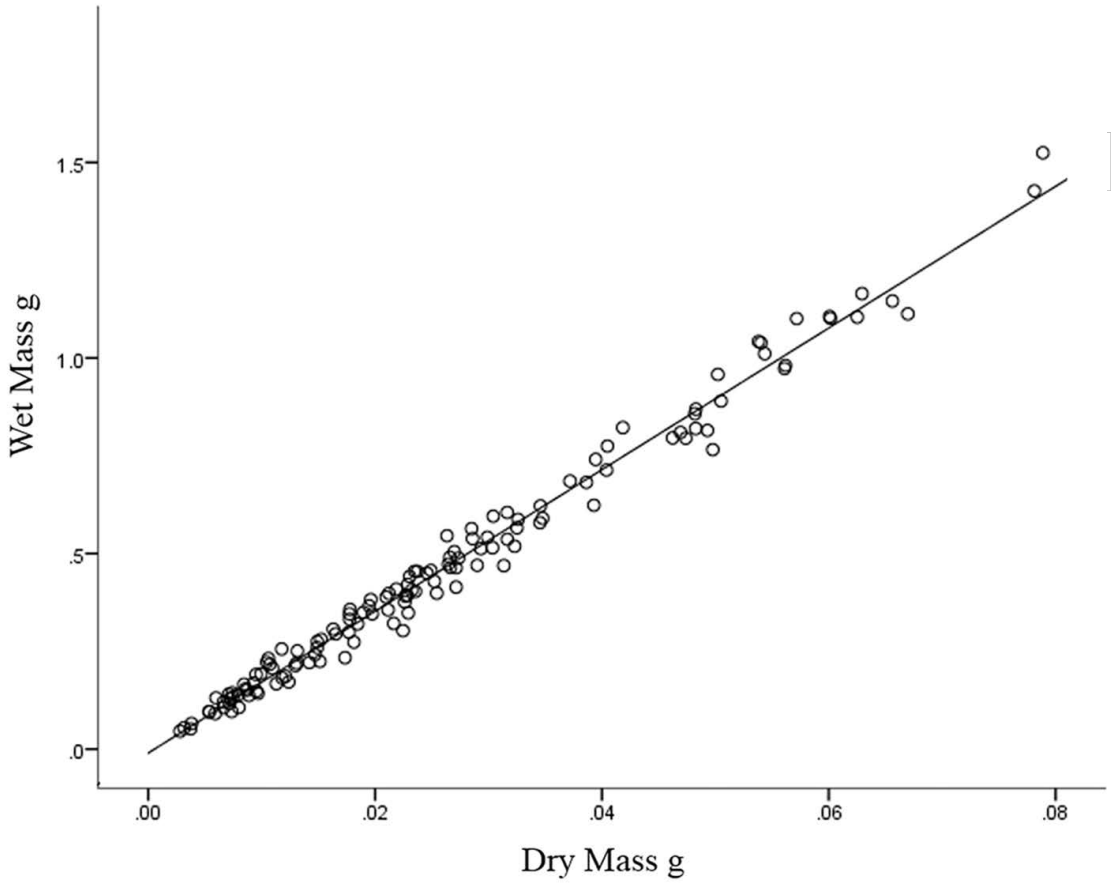

Figure 4.1 The relationship between wet mass and dry mass for *Carukia barnesi* medusae. Linear regression explained 98.6% of the variance between wet mass and dry mass (n = 133).

Dry mass was positively correlated with bell size. Curve estimation revealed the equation of best fit was a power curve, specifically [Dry Mass (g) = (2.44×10^{-4}) (bell size$^{1.97}$ Nb mm)] (see Figure 4.2). The curvilinear relationship was statistically significant ($R^2 = 0.867$, $F_{1, 131} = 851.68$, $p < 0.001$) with dry mass explaining 87% of the variance of bell size.

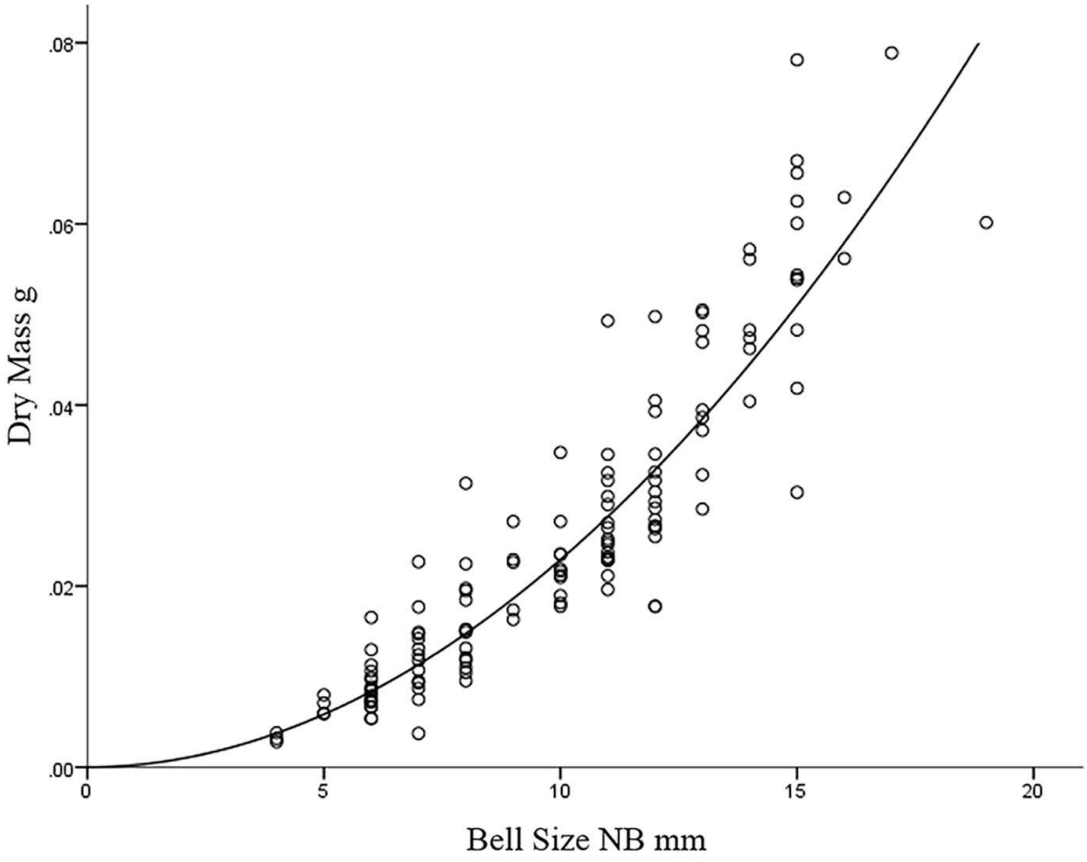

Figure 4.2 The curvilinear relationship between dry mass and bell size for *Carukia barnesi* medusae. Curve estimation revealed that a power curve explained 87% of the variance between dry mass and bell size (n = 133). Bell size was determined as niche bell height, a longitudinal measure from the center of the rhopaliar niche to the apex of the bell (NB).

The relationship between oxygen consumption and dry mass was explored through linear regression, which revealed a statistically significant positive relationship (R^2 = 0.559, $F_{1, 11}$ = 13.961, p = 0.003). This indicates that mass

explains 56% of the variance on oxygen consumption, over the tested size range, and is described by the equation [Oxygen Consumption (μmol O_2 h^{-1}) = 0.55 + 93.91 × Dry Mass (g)] (see Figure 4.3).

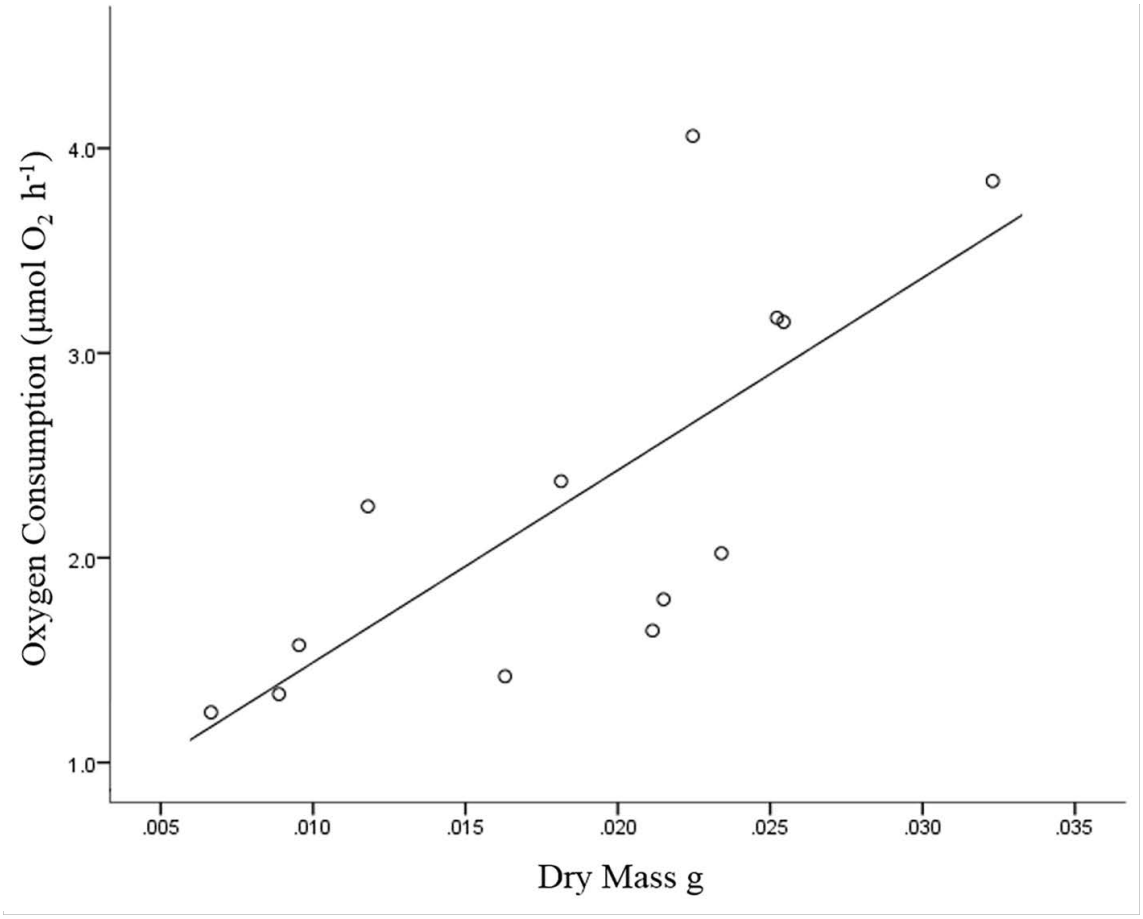

Fig 4.3 The relationship between oxygen consumption and dry mass of *Carukia barnesi* medusae. The oxygen consumption of 13 *Carukia barnesi* medusae of different dry mass.

4.4.1 The Influence of Temperature on Respiration Rate

A two-way ANOVA with replication was conducted to explore the relationship between temperature, time of measure (day/night), and their interaction, on the respiration rate of *C. barnesi* medusae. Oxygen consumption per gram dry

weight was significantly affected by temperature ($F_{7, 77} = 10.925$, $p < 0.001$), time of measure (day/night) ($F_{1, 77} = 8.405$, $p = 0.005$) and their interaction ($F_{2, 77} = 7.876$, $p = 0.001$) (see Figure 4.4). The respiration rates were similar between the day and night measures between 25ºC and 27.5ºC, however, they were dissimilar between 30ºC and 32ºC where the night measures were significantly suppressed compared to those recorded during the day. Post-hoc analysis also revealed the highest respiration rates were recorded between 30 and 33ºC during the day.

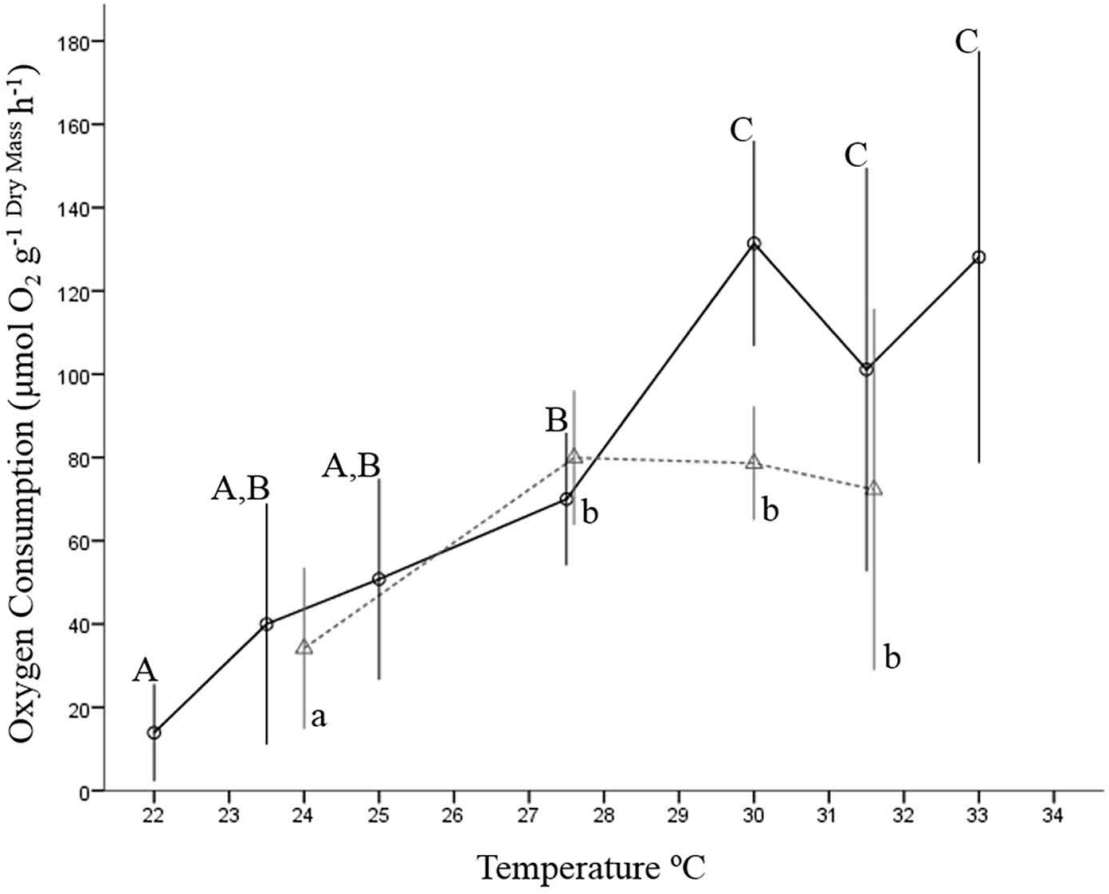

Figure 4.4 Mass adjusted oxygen consumption of *Carukia barnesi* medusae at different temperatures. Respiration rate trials were conducted during the day (Black Circles) and at night (Grey Triangles). Corresponding letters indicate non significant differences at alpha 0.05. Overlapping error bars at 27.5ºC and 31.5ºC have been offset horizontally for error bar presentation. Error bars represent 95% confidence intervals.

To determine the theoretical minimum thermal threshold of *C. barnesi* medusae, the mass adjusted oxygen consumption was further analysed by selecting only respiration measures obtained during the day and from treatments of less than 30ºC. These data were then analysed via linear regression, which revealed a statistically significant positive linear relationship between oxygen consumption and temperature ($R^2 = 0.606$, $F_{1, 38} = 58.454$, $p < 0.001$) (see Figure 4.5). The equation that describes this relationship is [Oxygen Consumption (μmol O_2 $g^{-1\ \text{Dry Mass}}$ h^{-1}) = −305 + 14.2 × Temperature (ºC)], which was used to extrapolate to the *x*-axis intercept as theoretical biological zero (21.48ºC). Although this value has no biological significance to the animal, it may be used as an approximation of temperatures which are becoming too cold for long term persistence (Lucas, 1996).

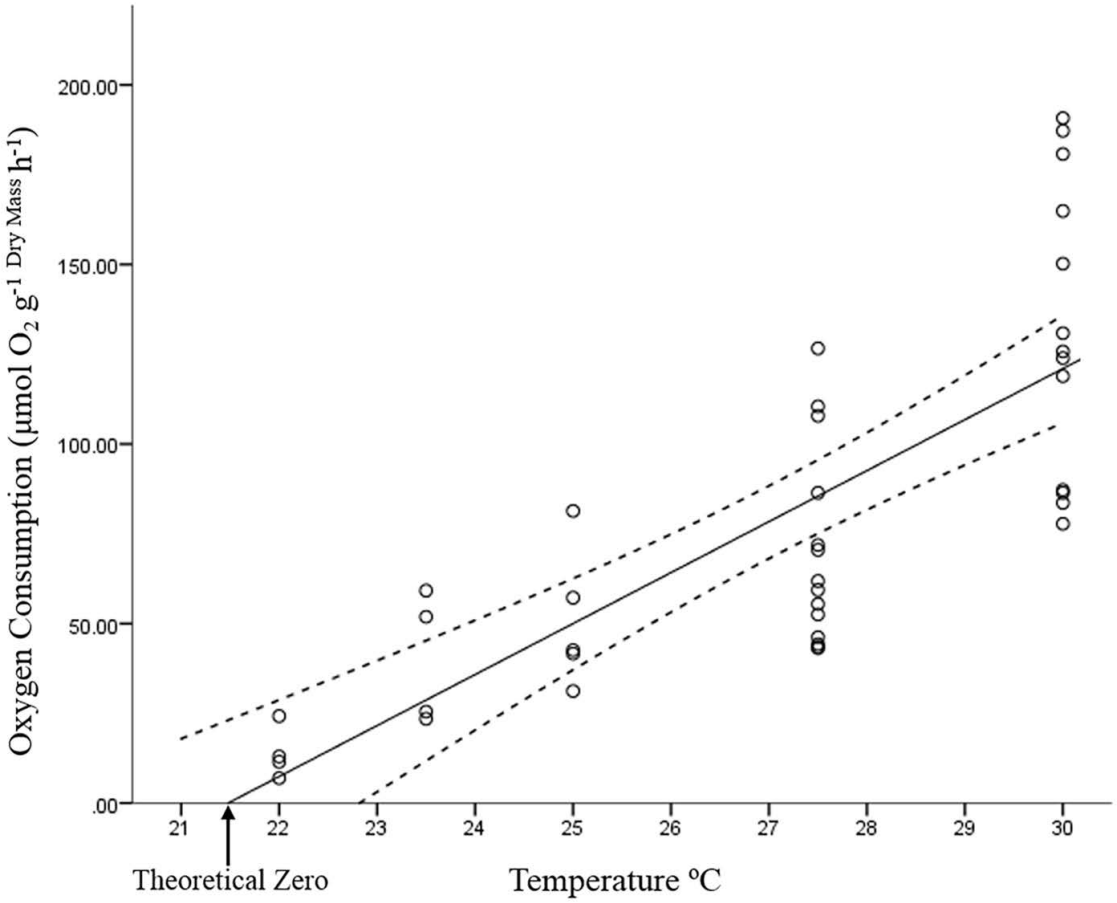

Figure 4.5 Mass adjusted oxygen consumption of *Carukia barnesi* medusae at different temperatures during the day. Raw values used to generate the fit line to theoretical biological zero which is explained by the equation [Oxygen Consumption (µmol O_2 $g^{-1\ Dry\ Mass}$ h^{-1}) = −305 + 14.2 × Temperature (ºC)], which has an *x*-axis intercept of 21.48ºC. Linear regression explains 61% of the variation. Dashed lines indicate 95% confidence limits.

4.4.2 The Influence of Bell Size on Pulse Rate

A linear regression was conducted to explore the relationship between bell contraction frequency and bell size. There was a statistically significant negative relationship between the pulse rate of *C. barnesi* medusae and size ($R^2 = 0.157$, $F_{1, 46} = 8.55$, $p = 0.005$), where small specimens had a higher standard pulse rate than larger specimens (see Figure 4.6). Over the tested size range, 16% of the variation in pulse rate was explained by bell size, and this relationship was explained by the equation [Pulse Rate (BPM) = 130 − 3.3 × Bell Size (NB mm)].

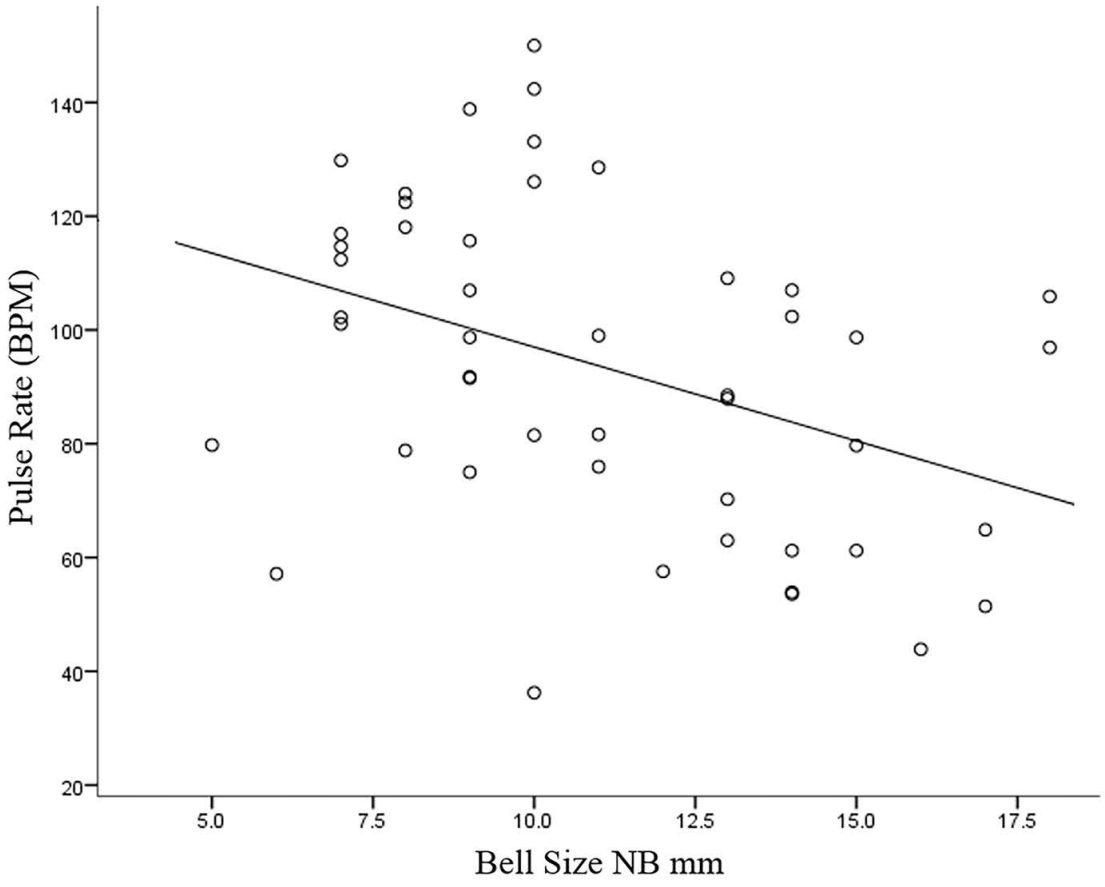

Figure 4.6 The relationship between pulse rate and bell size for *Carukia barnesi* medusae. Pulse rate was recorded as beats per minute (BPM) for 48 *Carukia barnesi* medusae to determine the relationship between pulse rate and bell size. Bell size was determined as niche bell height, a longitudinal measure from the center of the rhopaliar niche to the apex of the bell (NB).

4.4.3 The Influence of Temperature on Pulse Rate

The optimum temperature for bell contraction frequency was estimated as 29.18ºC where maximum pulse rate was estimated at 102 beats per minute. The 95% confidence limits associated with the optimum temperature indicates that the optimum temperature is between 27.46ºC to 30.9ºC (see Table 4.1 in conjunction with Figure 4.7). The bell contraction frequency of *C. barnesi* medusae decreased as temperature deviated from optimum reaching zero at the critical lower thermal threshold (CT_{min}) at 14.96ºC and the critical upper thermal threshold (CT_{max}) at 38.11ºC. Temperatures above CT_{max} were associated with 100% mortality. There was no significant variation in the pulse rate between individual experimental medusae (σ_j^2 as described in Equation 1), which also implies that after accounting for temperature; individual characteristics, such as bell size, did not contribute to significant variation in pulse rate.

Table 4.1 Maximum likelihood estimates and confidence intervals for parameters describing the bell contraction frequency of *C. barnesi* medusae modelled as a function of temperature.

Parameter	Estimate	2.5% CL	97.5% CL	DF	*t* Value	*p*
A	101.98 (BPM)	95.03 (BPM)	108.93 (BPM)	22	30.43	<0.0001
T_{opt}	29.18ºC	27.46ºC	30.91ºC	22	35.01	<0.0001
CT_{min}	14.96ºC	14.06ºC	15.86ºC	22	35.51	<0.0001
CT_{max}	38.12ºC	37.37ºC	38.86ºC	22	105.80	<0.0001
σ_j^2	27.78	−35.10	90.66	22	0.92	0.3695

Model output describes the change in bell contraction frequency (pulse rate reported as beats per minute; BPM) for different temperatures as described in Equation 1. Parameter descriptions derived from equation 1: A describes maximum pulse rate; T_{opt} indicates the estimated optimum temperature; CT_{min} and CT_{max} describe the estimated lower and upper critical temperatures respectively; σ_j^2 describes the variation in pulse rate among individual experimental medusae, which implicitly accounts for variation in pulse rate influenced by bell size. Estimated parameter values are the best fit parameters and 95% maximum likelihood profile confidence limits are provided. Degrees of freedom are indicated by DF; p and t values describe results from the associated t test.

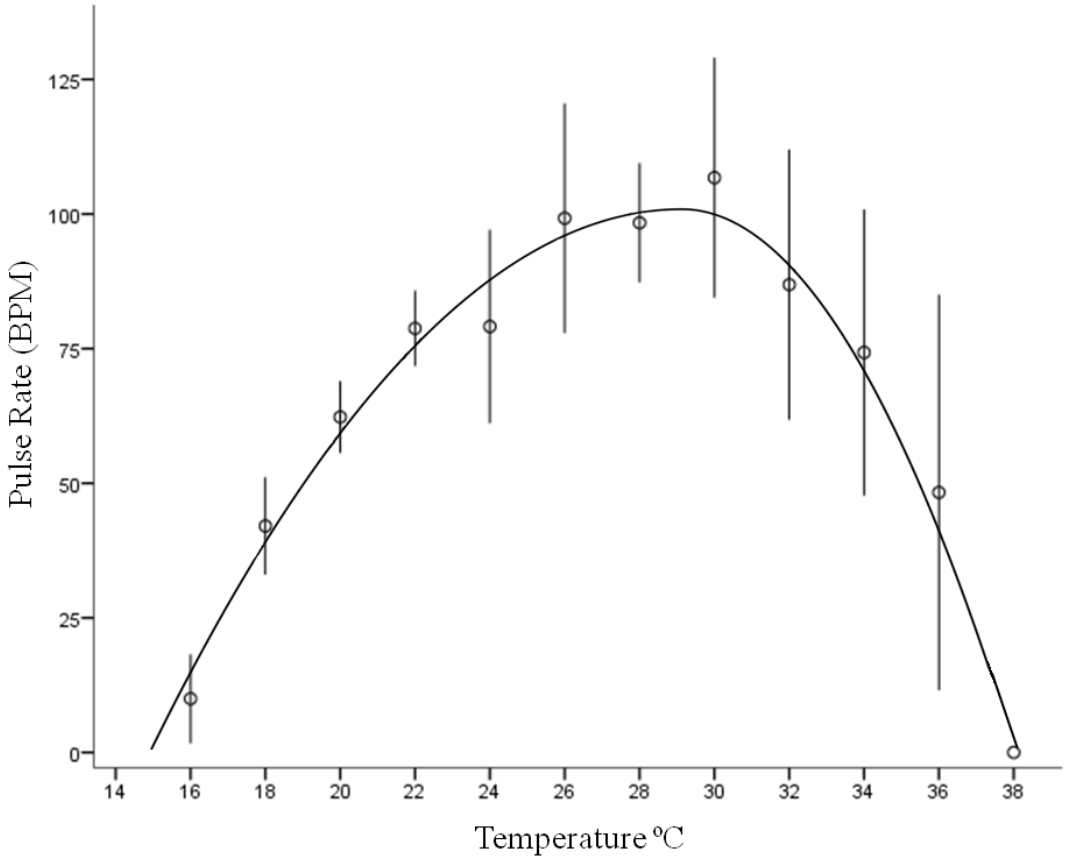

Figure 4.7 The influence of temperature on the pulse rate of *Carukia barnesi* medusae. Values are presented as means and error bars represent 95% confidence intervals. Model output describes pulse rate as a function of temperature as described in Equation 1. The decline in pulse rates as temperatures depart from optimum was modelled as asymmetrical reparameterized polynomials, with the particular polynomial dependant on whether temperature was increasing or decreasing from optimum as described in Equation 1.

4.4.4 The Influence of Salinity on Pulse Rate

Salinity had a statistically significant affect on the pulse rate of *C. barnesi* medusae. The best fit model suggests a positive linear relationship between pulse rate and salinity between the modelled change point values of 25‰ and 33.6‰. Within the range, the pulse rate increased from zero beats per minute at 25‰ to 59.4

beats per minute at 33.6‰. The model suggests a low point pulse rate of zero beats per minute at salinity levels below 25‰ and a high point pulse rate of 59.4 beats per minute at salinity levels above 33.6‰ (see Figure 4.8). The best fit model was also compared to an alternative model that assumed pulse rate does not depend on salinity through a likelihood ratio test assuming a chi-squared approximation of the log-likelihood ratio ($X^2_{3,\ n\ =\ 120} = 50.619$, $p < 0.001$). This result indicates that salinity influences the pulse rate of *C. barnesi* medusae and that the best fit model describes this relationship better than a straight, horizontal line.

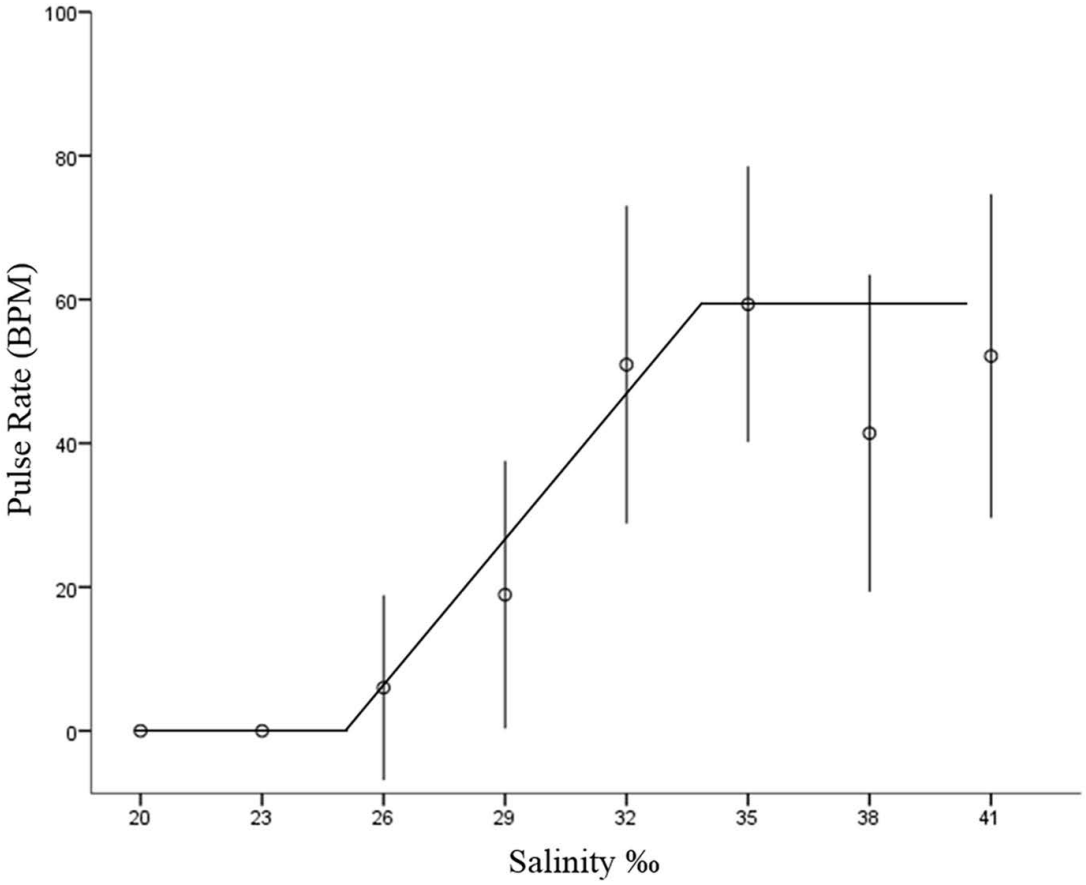

Figure 4.8 The influence of salinity on the pulse rate of *Carukia barnesi* medusae. Values are presented as means and error bars represent 95% confidence intervals assuming a normal distribution. Model output describes the pulse rate as a function of salinity.

4.5 Discussion

The maximum mean respiration rate of *C. barnesi* medusae, at the average capture site ambient temperature of 30ºC, was approximately an order of magnitude higher than previously reported respiration rates of scyphozoans recorded under ambient conditions (see review, Purcell et.al., 2010). This may indicate the elevated metabolic cost of cubozoans which are known to have: fast growth rates (Gordon et al., 2004; Gordon & Seymour, 2012; Kawamura et al., 2003); elevated swimming speeds (Colin et al., 2013; Shorten et al., 2005); complex sensory systems (Coates, 2003; Garm et al., 2007; Nilsson et al., 2005) and behaviors (Gordon & Seymour, 2009; Seymour et al., 2004); and relatively toxic venoms (Carrette et al., 2012; Chaousis et al., 2014; Pereira et al., 2010), compared to scyphozoans. Temperature had a significant effect on the metabolic rate of *C. barnesi* and maximum oxygen consumption occurred at 30ºC, which was not significantly different from higher temperatures between 31.5ºC and 33ºC (Figure 4.4). Not surprisingly, oxygen consumption was significantly reduced as temperature was reduced. The theoretical minimum thermal requirement for this species, biological zero, was calculated at 21.5ºC (Figure 4.5), suggesting that this species could theoretically persist at temperatures above this. As the oxygen consumption experiments did not include thermal treatments high enough to significantly suppress the respiration rate, it was not possible to evaluate the theoretical thermal maximum based on these respiration rate data alone. However, when the respiration rate results are compared in conjunction with results from the effect of temperature on the pulse rate of *C. barnesi* medusae, a notable similarity exists. The optimum temperature for maximum

pulse rate was determined to occur between 27.5ºC and 30.9ºC (Table 4.1), essentially mirroring the results from the respiration rate trials. Outside of this range, both higher and lower, the pulse rate was significantly reduced, reaching zero at 38ºC and near zero at 16ºC (Figure 4.7).

It should be noted that the presented temperature experiments were conducted as shock exposure type experiments, with very short acclimation times, which may have confounding effects when extrapolated to *in situ* applications. There is little doubt that if given the opportunity to extend pre-experimental acclimation times, which is currently not possible for cubozoan medusae due to animal husbandry limitations, the thermal range presented here may be underestimated. Therefore, we believe the critical upper and lower thresholds presented here to be conservative, and do not discount the potential presence of this species in thermal conditions below the theoretical thermal minimum of 21.5ºC. Furthermore, the specimens examined during this study were captured in the Cairns region, which is well within the loosely estimated distribution of this species, between Lizard Island and Fraser Island.

With the primary thermal limitations of this species theoretically determined, it is possible to compare the laboratory based results with *in situ* temperature conditions. The theoretical suitable thermal range based on oxygen consumption occurs between 21.5ºC (theoretical zero) to over 32ºC, which not surprisingly includes all temperatures that occur during the monsoonal summer months (November to May) in the Cairns region where the specimens were captured (The Queensland Government Coastal data system, Cairns, 2016). Unexpectedly, this also

encompassed the winter thermal regime in the Cairns region, suggesting that the seasonality of the medusa stage is not driven by the theoretical lower thermal limitations alone. During the winter months, the sea surface temperature rarely, if ever, falls below 21.5ºC (The Queensland Government Coastal data system, Cairns, 2016). This suggests that the seasonality of these medusae may not be driven by thermal extremes at either end of the summer, but may be driven by reduced fitness as the environmental conditions depart from optimum. For example, the optimum temperature for *C. barnesi* medusae falls between 27.5ºC to 30.9ºC, and was estimated at 29.2ºC (see Table 4.1), which are typical summer conditions during the monsoonal months *in situ*.

These results indicate that the local oceanic temperature and salinity regimes during the Irukandji season are encompassed by the thermal and osmotic preference of the medusae. However, during the winter months, the water temperature falls outside of the thermal preference range of the medusae, which theoretically causes the end of the Irukandji season. Conversely, temperature does not explain the consistent timing of the start of the Irukandji season. It would seem that the water temperature is sufficiently within the thermal range of the medusae well before the beginning of the Irukandji season (late October). We believe that the beginning of the Irukandji season is not driven by temperature, and the polyp stage may use an environmental cue, such as photoperiod, to induce synchronous medusa production (Courtney et al., 2016b), which may explain the consistent periodicity of the beginning of the Irukandji season (Carrette & Seymour, 2013).

Although temperature explains part of the seasonal timing of the medusae, there seems to be a mismatch between the thermal preference of the medusae and thermal regimes near their proposed southern distribution limit (i.e., Fraser Island), as summer water temperatures are sufficiently within the preferred range of the medusae at least 167 km south of this location (i.e., Brisbane) (The Queensland Government Coastal data system, Brisbane, 2016). Vital future research is now required to ground-truth these results, which could be conducted by sampling for this species near, and south of, Fraser Island. Although the physiological tolerances have been explored under laboratory conditions, there is little doubt these parameters may be further refined *in situ*. Further defining the factors that limit the southern distribution of *C. barnesi* is essential as there is anecdotal evidence that the southern distribution of this species has extended south over the last 50 years (Carrette, 2014). If this species is extending its distribution south, it will be shifting from rural regions with low populations, into the most heavily populated coastal areas of Queensland. For example, if the southern distribution of this species increases by just 167 km south of Fraser Island, it will include the Sunshine Coast and Brisbane; Australia's third largest city. If this occurs, it is expected to have a substantial negative impact on the Queensland tourism industry as currently there are no procedures to deal with this species other than beach closures.

The pulse rate of *C. barnesi* was also significantly affected by salinity and the optimum salinity was estimated to occur at salinity levels above 33.6‰. Decreases in salinity below this point reduced fitness, eventually leading to lethal dilution at 23‰. Furthermore, the minimum tolerable salinity was estimated to occur at 25‰ (Figure

4.8). This low tolerance to low salinity conditions is expected to exclude the medusae from inshore estuarine areas near river mouths where low salinity conditions commonly occur. This result also strengthens the hypothesis that the medusae of this species are oceanic, where the salinity in the waters of the Great Barrier Reef are relatively stable and rarely drop below 33‰ or rise above 36‰.

Previous research on the feeding ecology of *C. barnesi* demonstrated that this species actively targets larval fish during the day and not at night, presumably to conserve energy (Courtney et al., 2015). This type of resting behavior has also been documented in *C. fleckeri*, which have been shown to rest at night (Seymour et al., 2004). Here we have shown that the respiration rate of this species is suppressed at higher temperatures at night, which provides further evidence of a circadian diurnal activity pattern. This period of rest may be necessary to sustain the elevated respiration rate recorded during the day.

As research into the thermal ecology of cubozoans is in its infancy, the thermal and osmotic tolerance of additional cubozoan species is required. From this, a better understanding of the factors that drive the strong seasonal periodicity of many cubozoans can be determined. After the physiological constraints cubozoans are known, they can be modeled, and ground-truthed, against the observed species occurrence records. The empirical understanding of the physiological limitations of cubozoans is required before accurate projections of distributional, or seasonal, range changes are possible.

4.6 References

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Chapter 5:

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Chapter 5:

Prey Capture Ecology of the Cubozoan *Carukia barnesi*

5.1 Abstract

Adult *Carukia barnesi* medusae feed predominantly on larval fish; however, their mode of prey capture seems more complex than previously described. Our findings revealed that during light conditions, this species extends its tentacles and 'twitches' them frequently. This highlights the lure-like nematocyst clusters in the water column, which actively attract larval fish that are consequently stung and consumed. This fishing behaviour was not observed during dark conditions, presumably to reduce energy expenditure when they are not luring visually oriented prey. We found that larger medusae have longer tentacles; however, the spacing between the nematocyst clusters is not dependent on size suggesting the spacing of the nematocyst clusters is important for prey capture. Additionally, larger specimens twitch their tentacles more frequently than small specimens, which correlate with their ontogenetic prey shift from plankton to larval fish. These results indicate that adult medusae of *C. barnesi* are not opportunistically grazing in the water column; instead utilize sophisticated prey capture techniques to specifically target larval fish.

5.2 Introduction

Cnidarians utilize a diverse array of food acquisition/prey capture strategies ranging from reliance on symbiotic zooxanthellae and filter feeding, to active prey capture with nematocyst laden tentacles (Arai, 1997; Grossowicz & Benayahu, 2012; Mapstone, 2014; Venn et al., 2008). Those that use nematocysts may implement simple prey capture strategies which rely on size and tentacle structure to opportunistically graze within the water column (Brewer, 1989), while others use propulsion and induced swimming kinematics to increase potential prey and food particle contact with trailing tentacles (D'Ambra et al., 2001). Others, such as cubozoans, are highly mobile and possess complicated visual structures, which have been hypothesized to play a role in prey capture (Buskey, 2003; Matsumoto, 1996; Pearse & Pearse, 1978; Stewart 1996).

Perhaps the most extreme prey capture strategy recorded so far is seen in siphonophores, which use modified tentacles as 'lures' in a form of aggressive mimicry (Purcell, 1980). They actively attract and lure specific prey types, either through resembling schooling conspecifics, or by mimicking the prey items of the targeted species (Haddock, 2005; Mapstone, 2014; Pugh & Haddock, 2010; Purcell, 1980). Many siphonophore lures not only mimic the appearance of other species but also their movements. Specifically, these lures are motile and are often moved using a 'jigging' or 'twitching' motion, which resembles the movements of specific prey types (Mapstone, 2014; Purcell, 1980).

In many cnidarians, the diurnal light-dark cycle often mediates a condition-specific behavioral response. For example, numerous anthozoan and siphonophore

species extend their feeding tentacles only during the day while others only at night (Purcell, 1981; Sebens & DeRiemer, 1977; Sorek et al., 2014). Similarly, many species of hydromedusae and scyphomedusae use light to facilitate vertical migrations in the water column to locate food, while others laterally migrate with the sun to increase solar exposure for symbiotic zooxanthellae (Graham et al., 2001; Hamner & Hauri, 1981). Cubozoans also undergo a diurnal behavioral shift (Gordon & Seymour, 2009; Seymour et al., 2004), and vision seems to be one of the factors involved in this diurnal differentiation in behavior (Garm et al., 2012; Land, 2012).

Interestingly, a variety of visual systems are utilised by cnidarians. These range from simple eye spots and pigment cup ocelli to advanced pigment cups with lenses (Blumer et al., 1995; Nilsson et al., 2005; Nordstrom et al., 2003; Singla, 1974; Yamasu & Yoshida, 1973; Yamasu & Yoshida, 1976), with the most advanced visual sensory structures belonging to the medusa stage of the cubozoans (Martin, 2002; Nilsson, 2005). However, there has been contention as to the usefulness of these complex eyes, due to the apparent lack of either neural branches to process the information (Gerhart & Kirschner, 1997), or a nervous system able to interpret visual images (Nilsson & Pelger, 1994).

Cubomedusae do however exhibit many light mediated behaviours (Barnes, 1966; Coates, 2003; Hartwick, 1991b). These include targeting light shafts for feeding (Buskey, 2003), obstacle avoidance (Garm et al., 2007), actively swimming away from dark objects (Hamner et al., 1995), or decreased activity at night (Seymour et al., 2004). However, the extent that vision is used in prey capture by cubozoans is unknown. One highly venomous cubozoan that possesses sophisticated

visual organs is *Carukia barnesi* Southcott, 1967 (Coates, 2003; Nilsson et al., 2005; Piatigorsky et al., 1989; Underwood & Seymour, 2007). The general ecology and biology of *C. barnesi* is not well understood. The medusa stage of this species is seasonally present coastally along north-eastern Australia typically from November to May each year. This species is considered oceanic and is found around coral reefs and islands, and under certain conditions, on beaches (Barnes, 1964; Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988). Juvenile *C. barnesi* feed predominantly on crustaceans and during maturation undergo an ontogenetic venom change, correlated with a prey shift, from planktonic invertebrates to larval fish (Underwood & Seymour, 2007).

Carukia barnesi have four sets of six 'eyes', which is typical among cubozoans, consisting of a pair of simple light sensitive pigment cups, a pair of light sensitive pigment slits, and a pair of complex eyes that each has a cornea, a lens, and a retina (Hamner et al., 1995; Martin, 2002; Nilsson et al., 2005; Piatigorsky et al., 1989). However, the acuity and use of these eyes is unknown. The complete lifecycle and feeding ecology of this species is poorly understood and has not been described to date. This study describes part of the feeding ecology of the cubozoan *C. barnesi* and aims to understand the mechanisms employed by this species to capture its prey.

5.3 Method

5.3.1 Species Description

Carukia barnesi is a small (approximately 20 mm bell-width), oceanic, planktonic carybdeid that inflicts a potentially fatal sting that causes Irukandji Syndrome (Barnes, 1964; Carrette et al., 2012; Kingsford et al., 2012; Kinsey & Barnes, 1988; Pereira et al., 2010). This species has four tentacles in total and each extends from a pedalia attached to each corner of the bell. These tentacles are up to 750 mm long and have an alternating pattern of large and small nematocyst clusters often referred to as nematocyst-bearing rings or crescents (Bentlage & Lewis, 2012; Southcott, 1967; Underwood & Seymour, 2007). These are referred to in this paper as large and small 'nematocyst clusters'. The bell sizes of the specimens used in these experiments ranged from 8 to 21 mm niche bell (Nb) height (a longitudinal measure from the center of the rhopalial niche to the apex of the bell).

5.3.2 Specimen Collection

No specific permissions were required for the collection locations/activities as the species involved is not endangered or protected and the collection site did not require permits. Medusae of *C. barnesi* were collected near Double Island, North Queensland, Australia (16º43.5′S, 145º41.0′E) during November 2013, between 1900 and 2200 h. To attract medusae, high-powered LED lights were submerged on each side of a small (five meter) research vessel. Medusae were captured as they approached the light and were transferred into individual 500 ml plastic containers. The sea surface temperature varied from 27.5ºC to 30ºC with an average salinity of

35‰. The water depth at the capture sites varied between three to six meters. Post capture, specimens were transported to the laboratory and placed in a constant temperature controlled cabinet set at 28ºC for a minimum of six hours prior to the commencement of experimental trials.

5.3.3 Experimental Tank

Specimens were housed in a purpose-built plankton kreisel (a circular tank [1170 mm X 400 mm wide with an effective volume of ~ 375 liters] in which seawater rotates vertically). Seawater was maintained at 35‰ and 28ºC, to mimic oceanic conditions at the specimen capture site. A photoperiod of 13 h light:11 h dark was maintained, with the light period occurring from 0600 to 1900 h to simulate the local November photoperiod. The illumination cycle was achieved by fixing lights on each side of the kreisel that provided an average light intensity of 21µmol photons/s/m^2, and dark, achieved by turning the lights off with a timer. An infra-red sensitive digital video camera was positioned approximately one meter from the face of the kreisel. Five infra-red spotlights, which remained on continuously, were positioned around the kreisel to allow for filming in darkness.

5.3.4 Size Dependent Tentacle Morphology

To determine the relationship between medusa bell size and tentacle length, two *C. barnesi* were placed into the kreisel around midday and allowed to acclimate for approximately six hours prior to each experimental trial. The specimens were then filmed for 24 hours (i.e., a full 13:11 light:dark cycle) beginning with the dark cycle. This was repeated three times, with newly captured specimens, resulting in 24 hour video sequences for six *C. barnesi* medusae. Recorded video sequences were

subsequently analysed in 30 to 60 minute increments, where each tentacle was measured from the pedalia to its terminal end. As identifying individual tentacles on each specimen between time sequences was impossible, all tentacles at any one time were measured and the mean tentacle length of each specimen was calculated. In order to elucidate whether larger specimens, with larger bells, had longer tentacles, regression analysis was used to determine the relationship between bell size (niche bell height mm) and the maximum recorded mean tentacle length of each specimen over 24 hours. Similarly, to quantify the relationship between bell size and the distance between the large nematocyst clusters, the distance between six consecutive large nematocyst clusters on an individual extended tentacle were measured to the nearest millimetre for each specimen. The mean large nematocyst cluster distance for each specimen was then calculated and regressed against animal size (niche bell height mm), to determine the relationship between bell size and the distance between the large nematocyst clusters.

5.3.5 The Influence of Light on Tentacle Extension

The effect of the light on tentacle extension (i.e., zero percent extension = shortest and 100% extension = longest tentacle length of each specimen) was determined at 0, 30, 60, 120, 240 and 360 minutes of exposure to both light and dark treatments. These values were arcsine square root transformed (to normalize proportional data) prior to analysis, which consisted of a two-way repeated measures ANOVA to determine if: a) light or dark; or b) the amount of time exposed to light or dark; or c) an interaction between the two, affected the percent tentacle extension. All statistical analyses were conducted using the statistics package IBM SPSS.

5.3.6 The Effect of Bell Size, Light and Tentacle Extension on 'Twitch Rate'

During the previous experiments, the extended tentacles would frequently contract in a jerking or 'twitching' motion, and then relax. To quantify the occurrence of these twitches, one minute sections of the video footage were analysed in nine approximately 30-minute intervals for each specimen (i.e., 6 specimens measured 9 times each). The number of these contractions, or 'twitches', during each one minute sample was recorded. A 'twitch' was counted any time a tentacle rapidly contracted in a distinct pulse during each one minute measure, resulting in a value for 'twitch rate per minute' (a short video clip was included in the published version of this chapter [see S1 Video, Courtney et al., 2015]). This was conducted for each specimen, and only footage from the light treatment was analysed as twitching was not recorded in dark conditions (refer to Experimental Tank section). Linear regression was performed to determine if the rate of these twitches was affected by the state of a specimens tentacles (i.e., percent extended). Following this, to elucidate whether this relationship was driven by a specimen size effect (bell size), percent tentacle extension was pooled into 10 percent groups (i.e., 0-10%, 11-20%, etc.) and an ANCOVA conducted to determine if tentacle extension influenced the twitch rate, with bell size set as the covariate.

5.3.7 Prey Capture

On three occasions, two *C. barnesi* medusae were housed in the kreisel with approximately ten larval/juvenile fish (*Acanthochromis* sp.; approximately 10-15mm in length). The larval/juvenile fish were reared, housed and used as outlined in the ethics approval "Approval for Animal Based Research for Teaching - A2061" (granted to Dr. Jamie Seymour by the James Cook University Animal Ethics Committee). Breeding pairs of *Acanthochromis* sp. were held in a 3,000 litre tank and the larval/juvenile fed naturally on crustaceans, such as copepods and amphipods, which occur within the 50,000 litre recirculating aquarium system. All fish used were free feeding at the beginning of this study and were only held with the *C. barnesi* for approximately 10 minutes per feeding event. Fish that were stung were consumed by the *C. barnesi* and any fish that were not consumed were returned to the larval/juvenile fish tank. During these feeding events the fish were seen to rapidly move towards the twitching tentacles and were subsequently envenomed and caught. This process was captured on video on numerous occasions and still images were extracted to investigate the prey capture method implemented by *C. barnesi*.

5.4 Results

5.4.1 Size Dependent Tentacle Morphology

There was a statistically significant positive relationship between bell size (niche bell height mm) and tentacle length, where larger specimens had longer tentacles than smaller specimens (R^2 = 0.796, $F_{1, 4}$ = 16.642, p = 0.017) (see Figure 5.1). Conversely, the distance between the large nematocyst clusters was not correlated with bell size (R^2 = 0.003, $F_{1, 4}$ = 0.13, p = 0.916), with a mean distance of 30 mm (± 6 mm 95% Cl) on extended tentacles.

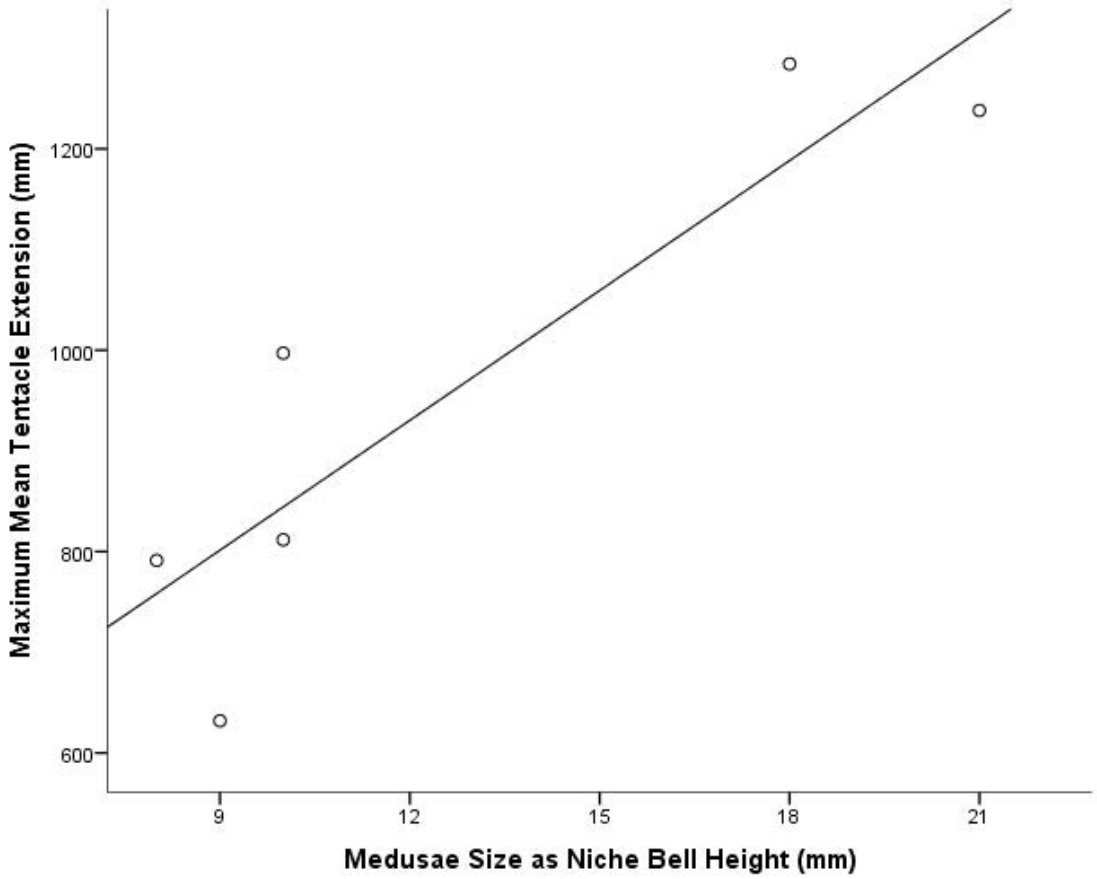

Figure 5.1 Maximum mean recorded tentacle lengths (mean length mm, *n* = 6) for *Carukia barnesi* over a range of medusa bell sizes (niche bell height mm).

5.4.2 The Influence of Light on Tentacle Extension

Carukia barnesi tentacles were significantly longer during the light treatment than in the dark treatment ($F_{1, 5} = 7.112$, $p = 0.045$), while time had no significant effect ($F_{5, 25} = 0.216$, $p = 0.952$). However, there was a significant interaction effect between the light/dark treatments and time ($F_{5, 25} = 10.100$, $p < 0.001$). Tentacles contracted as the dark treatment progressed reaching a contracted state of less than 20% after approximately two hours. After the lights were turned on, tentacles began to extend reaching maximum mean extension of approximately 80% after approximately six hours exposure to the light treatment (see Figure 5.2).

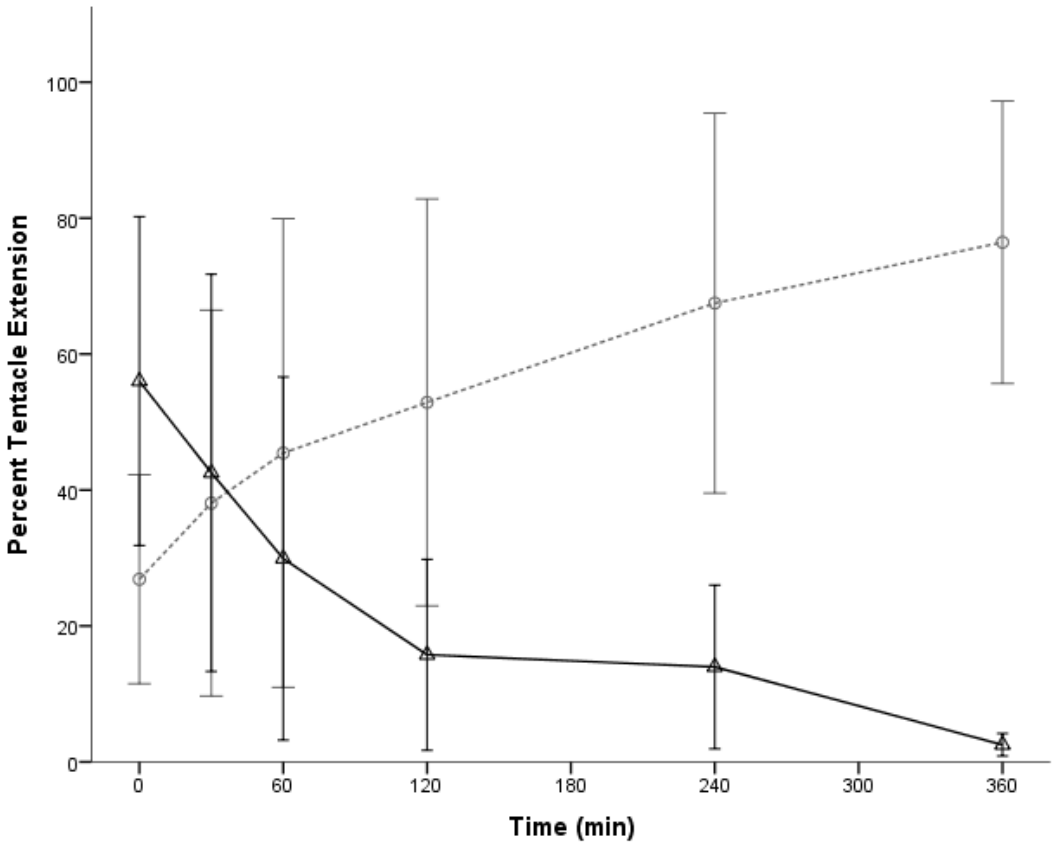

Figure 5.2 *Carukia barnesi* mean tentacle extension, as a percentage, over 360 minutes, exposed to light (○, dashed line) and dark (△, solid line) treatments. Error bars represent 95% confidence intervals ($n = 6$).

5.4.3 The Influence of Bell Size, Light and Tentacle Extension on 'Twitch Rate'

There was a significant positive relationship between tentacle extension and twitch rate, with elongated tentacles twitching more frequently (twitches per minute) than retracted tentacles (R^2 = 0.492, $F_{1, 53}$ = 50.397, p < 0.001) (see Figure 5.3), while bell size also effected the twitch rate ($F_{4, 54}$ = 2.718, p = 0.040), where larger animals 'twitch' their tentacles more frequently than small specimens (see Figure 5.4). The average twitch rate during the light was 6.3 twitches per minute however no twitching was recorded during the dark.

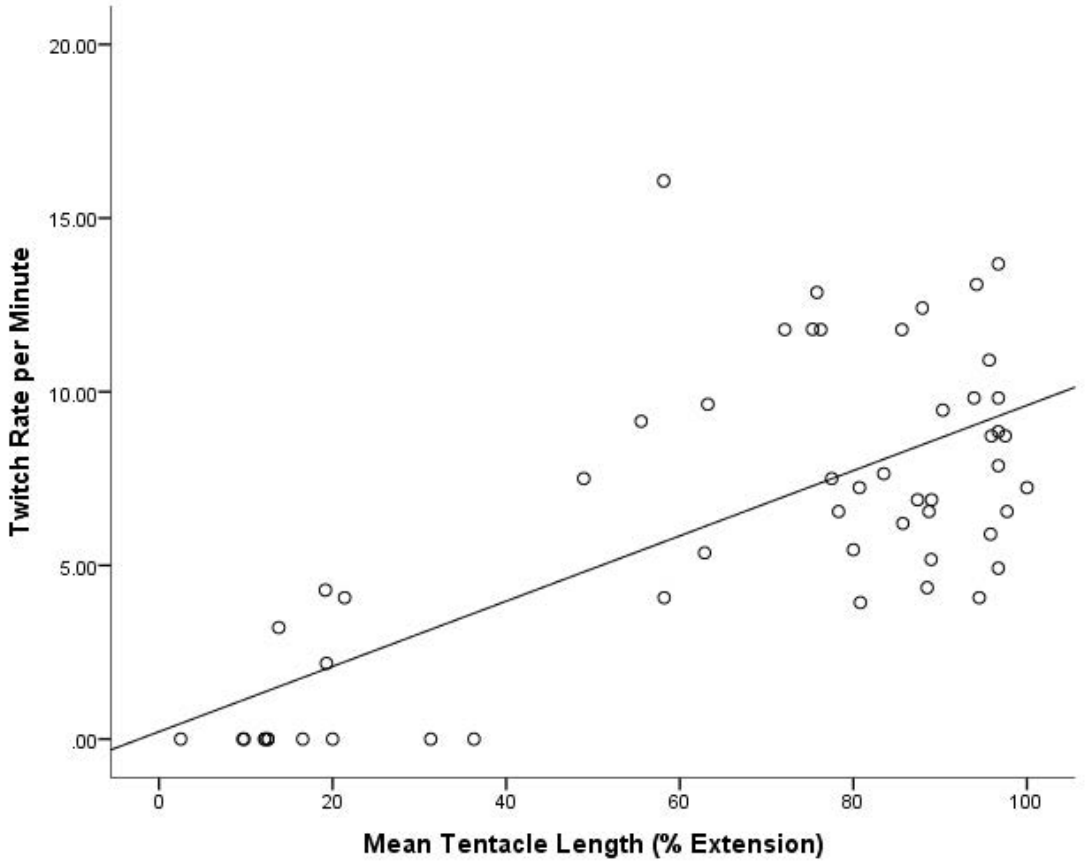

Figure 5.3 The number of tentacle twitches recorded for *Carukia barnesi*, over one minute intervals (twitch rate per minute), plotted against different mean (*n* = 6) tentacle extensions (percent extension). Video footage was analysed in approximately 30 minute intervals (i.e. 6 specimens measured 9 times each).

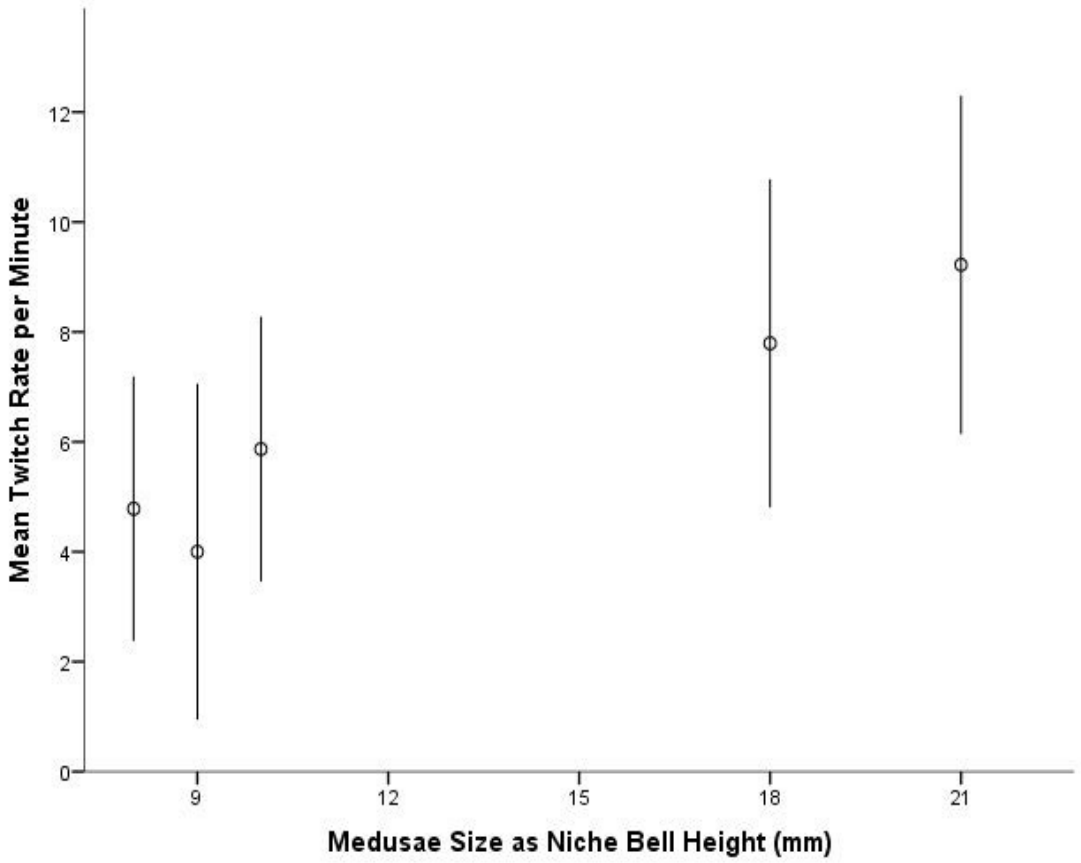

Figure 5.4 The mean number of tentacle twitches recorded for *Carukia barnesi*, over one minute intervals (twitch rate per minute) plotted against medusa bell size in millimeters. Error bars represent 95% confidence intervals (n = 6).

5.4.4 Prey Capture

Larval fish (*Acanthochromis* sp.) were often attracted to the nematocyst clusters on the extended fishing tentacles of *C. barnesi* especially when they were being 'twitched'. Fish would pursue these clusters and become 'stung' around the mouth region or head, resulting in death (see Figure 5.5). A short video clip was included in the published version of this chapter (see S2 Video, Courtney et al., 2015).

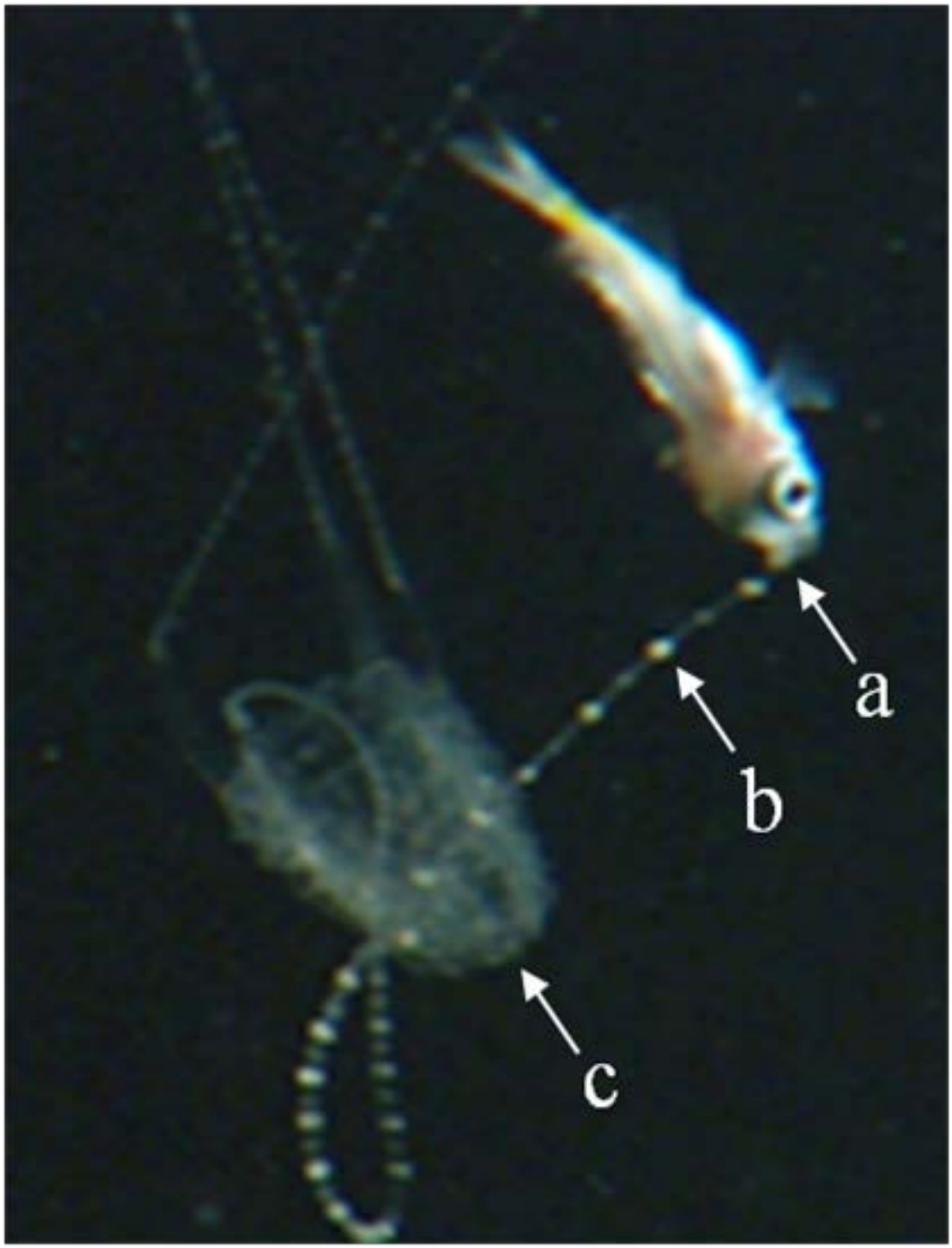

Figure 5.5 Envenomation of a larval fish (*Acanthochromis* sp.) that was captured by a twitching tentacle of an adult *Carukia barnesi*; a: envenomation site; b: nematocyst cluster; c: bell. The bell size of this specimen is approximately 15 mm in height and the fish is approximately 10 mm in length.

5.5 Discussion

Adult *C. barnesi* are known to feed almost exclusively on larval fish (Underwood & Seymour, 2007); however, their mode of prey capture seems more complex than previously described. Our findings suggest that *C. barnesi* are active predators that capture visually orientated prey, in this case larval fish, by using a lure-like system to simulate the size and movements of the fish's prey (e.g., small plankton). This method of prey capture has previously been described in some siphonophores (Mapstone, 2014; Purcell, 1980), and has been considered unique compared to other cnidarians. Many larval fish are visual hunters and because of this feed predominately during light conditions (Puvanendran & Brown, 2002), and this correlated well with the luring behaviour seen in *C. barnesi* that occurs only during daylight hours. Luring at night would be less efficient resulting in reduced prey capture and contracting their tentacles at night would decrease swimming induced drag, thus reducing energy expenditure. This suggested feeding cycle is consistent with the diurnal feeding cycle observed in another cubozoan (i.e., *Chironex fleckeri*, Southcott, 1956), which is known to become inactive at night to conserve energy (Seymour et al., 2004).

The nematocyst clusters along the extended tentacles are also motile, where *C. barnesi* 'jig' or 'twitch' these tentacles frequently. Fish, including larval fish, are known to be attracted to prey by movement, and may preferentially attack prey items of specific sizes (Hunter, 1980). In order to increase catch rates, twitching, or movement of the nematocyst clusters, would appear to serve this purpose, where movement of the nematocyst clusters would highlight these lures in the water

column. Once larval fish are attracted to these nematocyst clusters they are consequentially 'stung' and consumed. Furthermore, larger *C. barnesi* were found to twitch their tentacles more frequently than smaller specimens, which may be related to their recent ontogenetic transition from planktonic to vertebrate prey (Underwood & Seymour, 2007). Smaller medusae (under 8 mm) have a preference for plankton, and these prey items are almost certainly captured in a similar manner as used by other cnidarian medusae, that is, by haphazardly encountering prey in the water column. As such, twitching of tentacles, which presumably increases energy consumption, would be inefficient for the capture of small plankton, which may explain the lower twitch rate observed in the smaller specimens (8-10 mm).

Not surprisingly, larger medusae were found to have longer tentacles, which would presumably increase their chance of prey capture and correlates with the change in prey preference from plankton to larval fish. However, it was surprising that the distance between the nematocyst clusters, or lures, was similar regardless of the length of their tentacles. This suggests that the distance between the nematocyst clusters are important for the visual stimulation of prey and/or to optimize prey capture (i.e., the lures are set at an optimum distance for prey capture). The function of the alternation between large and small nematocyst clusters along the tentacles is unknown; however, they may be used to target different sizes of larval fish by presenting a choice of lure/food particle sizes. Further research is required to determine the specific function of the large and small nematocyst clusters.

Cubomedusae have been shown to be more sophisticated in many areas of their ecology than most other cnidarians. For example, they have elevated swimming

speeds (Colin et al., 2013; Shorten et al., 2005), greater vision capabilities (Coates, 2003; Garm et al., 2007; Nilsson et al., 2005), more sophisticated behaviours (e.g., sleeping) (Gordon & Seymour, 2009; Seymour et al., 2004) and highly toxic venoms (Carrette et al., 2012; Chaousis et al., 2014; Pereira et al., 2010). In conclusion, this research has demonstrated that *C. barnesi* utilise sophisticated prey capture techniques to actively lure prey. Future investigation into other species of cubomedusae is now required to determine if they too employ sophisticated prey capture mechanisms.

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Chapter 6: General Discussion

Carukia barnesi are small cubozoans that exhibit marked seasonality and distribution. Prior to the commencement of this project there were numerous knowledge gaps in regard to the ecology of *C. barnesi* and the factors that contribute to the observed seasonal occurrence and distribution limits of this species were unknown. Understanding these abundance patterns is essential for determining when, where, and under what circumstances the medusae may be more prevalent. This was not previously possible primarily because information regarding the early life history of *C. barnesi* did not exist and there had been no previous research exploring the thermal and/or osmotic tolerance of this species at any life stage. Also, the feeding ecology and diurnal activity patterns of the medusa stage had not previously been explored. The overall focus of this thesis was to better understand the ecology and physiological limitations of *C. barnesi* to elucidate the factors that may contribute to the observed seasonal and distributional patterns. This project has also produced the baseline data for future research to build upon with the expectation that the synthesis of these and future data will facilitate the ability to model, and therefore predict, the occurrence of this species in order to reduce the number of people stung.

The primary aims of this research were to:

i) describe the polyp stage of *C. barnesi* from egg fertilization through to medusa detachment and to provide a repeatable method for producing laboratory based polyp cultures of this species for scientific research.

ii) determine the thermal and osmotic tolerance of *C. barnesi* polyps, and use these data to discuss where these polyps may live.

iii) explore the effects of feeding frequency on survivorship and asexual reproduction of *C. barnesi* polyps.

iv) determine the effect of thermal and osmotic parameters, and combinations thereof, as well as feeding frequency, as cues for synchronous medusa production.

v) define the thermal and osmotic tolerance of *C. barnesi* medusae in order to determine what factors contribute to the marked seasonality and distribution of this species.

vi) describe the feeding ecology of *C. barnesi* medusae to gain insight into the mechanisms employed by this species to capture prey and to elucidate the diurnal activity pattern of this species.

This research project has led to the only laboratory-based polyp culture of *C. barnesi* globally. Producing this culture from wild caught adult medusae allowed for detailed documentation of the life cycle from egg fertilization through to medusa detachment, which has elucidated some key areas where future research should focus. Beginning at the start of the life cycle, *C. barnesi* has an unhatched dormant encapsulated planula stage that lasts from six days to over six months. This pause stage requires further investigation to determine how long this stage can persist, under different environmental conditions, to determine the purpose of this stage. These dormant planula should also be investigated through temperature, salinity, and light intensity experiments to determine if there is a cue that causes these planulae to hatch. This may be of ecological importance if there is an environmental cue that causes synchronous planula production, which may give insight into the location of

the polyps *in situ*. These experiments could be conducted by monitoring these unhatched planulae over time and/or by exposing these unhatched planulae to different temperature, salinity or light regimes and monitoring hatch rate.

These planulae become motile after hatching and research should also explore how far the planulae swim prior to settlement and the preference of substrate choice and or orientation may also provide clues as to where these polyps may be located. Pre-settlement swimming distances, substrate preference and settlement orientation of the swimming stage polyps could also provide valuable ecological information about this species, which may also give insight into where these polyps may be. These experiments could be conducted by video recording the hatching planulae and tracking the distance they travel or by providing a range of substrate choices (e.g., rock, coral, bivalve shell, sand and mangrove root) and orientations (e.g., on top, underneath or vertical surfaces) and quantifying substrate and orientation preferences. This type of experimentation should also be conducted on the ciliated swimming stage secondary polyps. These experiments may provide clues as to the composition of the microhabitat where these polyps survive (e.g., under rocks or on bivalve shell; Cutress & Studebaker, 1973; Fischer & Hofmann, 2004; Hartwick, 1991a; Yamaguchi & Hartwick, 1980).

The beginning of the Irukandji season is caused by the release of medusae by the polyps and over the last 50 years the Irukandji season has increased in length from 15 days to over 150, which has speculatively been attributed to increased sea temperature (Carrette & Seymour, 2013). Interestingly, the beginning of the Irukandji season has remained relatively consistent over the last 50 years based on

sting records, with the first sting most years occurring on the 31st of October (± 7 days) at Palm Cove, Cairns, Queensland (Carrette, 2014). This thesis experimentally explored temperature, salinity, and combinations thereof, along with feeding frequency, as cues for synchronous medusa production. During these trials no significant medusa production occurred. This provides strong evidence that this process is not driven by these factors alone. As there is high interannual variation in most environmental parameters, such as increasing temperature or changing salinity levels, very few cues for synchronous medusa production would lead to the observed consistency of the arrival of *C. barnesi* medusae other than a parameter that is consistent between years, such as increasing photoperiod. This is a clear area where future research is required that specifically targets potential cues for synchronous medusa production, such as increasing photoperiod, photo-intensity, and/or combinations of these factors, coupled with temperature or salinity. These experiments should expose polyps to different photoperiod regimes and monitor medusa production and inclusion of additional environmental parameters should be considered. These experiments are essential for understanding the factors that drive the timing of the Irukandji season and are therefore integral to producing predictive models of the occurrence of this species.

This thesis also explored the thermal and osmotic tolerances of the polyp stage of *C. barnesi* and the results of these experiments clearly indicate that the polyps of this species prefer low salinity conditions that do not occur in the waters of the Great Barrier Reef or the Coral Sea. These conditions only occur inshore; in estuarine systems. The medusa stage of *C. barnesi* is considered oceanic and having

an inshore estuarine polyp stage was not previously considered. Research is now required to ground truth these results, and to do that, polyps need to be found *in situ*. Globally, cubozoan polyps have only been located *in situ* for two species *C. fleckeri* and *Carybdea marsupialis* (Cutress & Studebaker, 1973; Fischer & Hofmann, 2004; Hartwick, 1991a; Yamaguchi & Hartwick, 1980), which were located through extensive manual searching, and at least for *C. fleckeri*, have not been located since. This method of benthic sampling is difficult, expensive, and time consuming. However, with recent advances in forensic genetic analysis, future exploration through environmental DNA may provide an effective procedure to detect this species. This could be conducted through benthic layer sampling from areas that have environmental parameters that are similar to the thermal and osmotic preference of *C. barnesi* polyps, (low salinity areas within estuarine systems), and then screening the samples for genetic material from *C. barnesi*.

Determining where these polyps reside is integral to understanding the ecology of the polyp stage of this species. For example, without knowledge as to where these polyps are, it is impossible to determine how factors (e.g., temperature, salinity, food availability, or substrate availability for attachment) may affect the population dynamics of the polyps. As the abundance of the medusa stage is thought to be reflective of the success of the polyp stage, understanding the environmental parameters these polyps experience is essential. Research should now focus on locating the polyps and monitoring the environmental conditions where the polyps are located. Understanding how the polyps respond to variations in these parameters,

which could be experimentally determined in the laboratory, is essential for producing predictive models that couple environmental and physiological data.

In order to produce predictive models that work outside of the local area, the effect of these environmental parameters will need to be determined for polyps that originate from different geographic locations. Although not well defined, the distribution of this species is considered to occur between Lizard Island and Fraser Island (Kingsford et al., 2012), with Cairns being near the centre of this geographic range. It is expected that specimens that occur in warmer or colder areas on the boundaries of this range will have some amount of within species plasticity or adaptation to local thermal, and/or osmotic regimes. Therefore, future research should explore the development times and thermal and osmotic tolerance of *C. barnesi* polyps, replicating the experiments presented here, with polyps that originate from medusae that are captured from different locations, preferably from cooler locations, such as near Fraser Island. This will allow for a better understanding of the factors that may limit the distribution of these polyps and provide the required data for modelling these polyps while accounting for latitudinal variation, particularly in temperature.

Access to *C. barnesi* polyps also provides other future research possibilities that could further the understanding of this life stage. For example, research should explore the metabolic rate of these polyps at different temperatures as another avenue of determining the preferred thermal range of the polyps. This could also be used to assess other parameters, such as food type or quantity, and water quality parameters, such as water turbidity or pH on the respiration rate of the polyps. This

may also provide a method to evaluate the thermal preference of other life stages, such as swimming stage polyps or planulae. None of these factors have been explored in *C. barnesi* polyps.

Although the onset of medusa production by the polyps causes the timing of the beginning of the stinger season, both the polyps and the medusae are believed to contribute to the length of the stinger season. Once medusa production is initiated, this process can be continuous throughout the season as shown in *C. fleckeri* (Gordon & Seymour, 2012), or punctuated by significant rainfall events as shown in *Chiropsella bronzie* (Gordon et al., 2004), leading to multiple cohorts of medusae during the stinger season. This was discovered by exploring the statoliths of these species to determine the age of the specimens (i.e., similar to fish otoliths, cubozoan statoliths can be examined for daily growth rings for age determination; Gordon et al., 2004; Gordon & Seymour, 2012; Kawamura et al., 2003). This experimental method should now be applied to *C. barnesi* to determine; i) how old individual medusae are, ii) if there are multiple cohorts within each season, and iii) when medusa production begins and ends each season. These experiments would provide critical information necessary for determining what factors contribute to the seasonal abundance and distribution of this species. For example, if the age of a medusa was known, then an estimate of how far a medusa could travel from the polyp colony could be estimated, and the time of medusa production could be calculated. Understanding if there are multiple cohorts, or a single cohort, is essential for determining the factors that contribute to the length of the Irukandji season. For example, if there is only one cohort, then the length of the season should not be

longer than the lifespan of the medusa, however if there are multiple cohorts within each season, then the length of the season may be determined by environmental factors that either do not allow for the persistence of the medusae or cause the polyps to stop producing medusae.

The elemental chemistry of these statoliths should also be analysed to determine the water quality parameters the medusae have experienced (e.g., low salinity conditions). This method has previously been applied to *C. fleckeri* (Mooney & Kingsford, 2016), and should be considered for *C. barnesi* to determine where these medusae originate. Although these medusae are expected to begin in low salinity habitats, based on the osmotic preference of the polyp stage, exploring the elemental chemistry of the statoliths would provide further evidence as to where the polyps are. This could be coupled with population genetic analysis to indicate the relatedness between individuals within and between times/seasons/locations, which may give insight to the population dynamics of this species and provide further information as to where these medusae originate from.

This thesis also explored the thermal and osmotic tolerance of the medusa stage of *C. barnesi* and the results of these experiments indicate that the local oceanic temperature and salinity regimes during the Irukandji season are, not surprisingly, encompassed by the thermal and osmotic preference of the medusae. However, during the winter months, the water temperature falls outside of the thermal preference range of the medusae, which theoretically causes the end of the Irukandji season. Therefore, the Irukandji season may continue to increase in length under proposed sea temperature rise scenarios. Conversely, there seems to be a

mismatch between the thermal preference of the medusae and thermal regimes near their proposed southern distribution limit (i.e., Fraser Island), as summer water temperatures are sufficiently within the preferred range of the medusae at least 167 km south of this location (i.e., Brisbane). Vital future research is now required to ground truth these results, which could be conducted by sampling for this species near, and south of, Fraser Island. Although the physiological tolerances have been explored under laboratory conditions, there is little doubt these parameters may be further refined *in situ*. Further defining the factors that limit the southern distribution of this species is essential as there is anecdotal evidence that the southern distribution of this species has extended south over the last 50 years (Carrette, 2014). Of high importance is if this species is extending its distribution south it will be shifting from rural regions with low populations, into the most heavily populated coastal areas of Queensland. For example, if the southern distribution of this species increases by just 167 km south of Fraser Island it will include the Sunshine Coast and Brisbane, Australia's third largest city. If this happens, it is expected to be devastating to the Queensland tourism industry as currently there are no procedures to deal with this species other than beach closures.

With no known environmental barriers restricting the southern distribution of this species, it is theoretically possible that this species may be able to persist further south than they have previously been recorded. To date, there have been no reported sightings, or stings, reported from areas south of Fraser Island. Therefore, there may be another limiting factor, such as the actual *in situ* location of the polyps, coupled with the maximum lifetime distance travelled by the medusae, which sets the

southern distribution limit. That being said, it is plausible, that the southern distribution of the medusae may extend south under current conditions. As this species is known to be oceanic, and the Eastern Australian Current has been documented to be increasing in temperature and velocity (Ridgway, 2007), advection of the medusae further south should be considered. Also, it is expected that specimens that occur in colder areas on the boundaries of this range will have some amount of within species plasticity or adaptation to local thermal, and/or osmotic regimes. Therefore future research should replicate the thermal tolerance experiments presented here with medusae that are captured from different locations, preferably from cooler locations such as near Fraser Island.

Prior to this research project, very little was known about numerous aspects of the ecology of *C. barnesi*, from short scale diurnal activity patterns to the strong annual seasonal influx in abundance pattern. Over short time scales, this project has shown that this species is more active during the day and actively target visually oriented prey through a form of aggressive mimicry not previously described in cubozoans. It has also been shown that this species is less active at night, presumably to conserve energy, when 'fishing' for visually oriented prey, in this case larval fish, would be less efficient. Understanding the diurnal activity patterns of *C. barnesi*, and/or times of increased activity *in situ*, may allow for improved future management of areas where this species is known to be present. This is of concern because stinger net enclosures are not designed to exclude this small species of jellyfish, and currently the only method of mitigating stings is through beach closures when *C. barnesi* are detected. Experiments that explore these diurnal

behaviour patterns further are now required to pursue times of increased or decreased activity, and/or position in the water column, to identify if there are finer scale activity patterns present.

This species is known to be motile and occur with a high amount of temporal and spatial variability (Kinsey & Barnes 1988; Kingsford et al., 2012). However, the factors that drive this variability in occurrence remain unknown. Future experiments should also explore how or if this species uses vision to find prey and/or navigate around obstacles as demonstrated in other cubozoans (Barnes, 1966; Coates, 2003; Garm et al., 2007; Hamner et al., 1995; Hartwick 1991b). These experiments could be conducted under laboratory conditions by exposing the specimens to obstacles or food and monitoring their behaviour. Also, increased sampling effort for this species *in situ* may determine when, where, and under what circumstances this species is more abundant. Additionally, increased sampling effort may expose behavioural patterns, or preferences, which may identify if these animals change their behaviour in response to oceanic conditions, such as rough weather and/or heavy rain (e.g., are fewer specimens captured during rough weather). Through increased sampling effort and further behavioural experiments, it is expected that a better understanding of this species will emerge, and with that more possibilities for Irukandji management. For example, if this species has a preference for calm water, a certain beach shape, or swim away from objects of particular colours, then this may be capitalised on by shifting the stinger nets to lower risk sections of the beach, pre-emptive beach closures under specific conditions, or possibly adding structure to the nets that deter *C. barnesi*. Definitive answers to these questions are required before predictive

models can be developed with the accuracy and resolution that would allow the ability to predict lower or higher risk days, or times of the day, for beach usage during the Irukandji season.

Future research is now required to further our understanding of the factors that drive the temporal and spatial variability in abundance exhibited by *C. barnesi*. Condition modeling these parameters may allow for prediction of *C. barnesi* occurrences. This could be used as an early warning system to mediate stinger net closures and in turn reduce the number of people stung each year. The use of ecological and physiological data in modeling cubozoan patterns remain in its infancy. However, inclusion of these parameters in future models seems paramount for accuracy. In order to produce predictive models, a more refined model of oceanic conditions is also required that focuses on temperature and salinity modeling in the waters of the Great Barrier Reef, including the reef lagoon, estuarine areas including the river mouths, and a model that has the capacity to incorporate monsoonal inputs and upwelling events would be advantageous. Producing high resolution predictive models of this species will require a cross disciplinary integrated approach to couple real-time oceanographic and weather data with physiological and behavioural data of *C. barnesi*. With recent advances in satellite temperature and salinity data acquisition, and increased computational power, the capacity to conduct condition modelling in dynamic marine environments for many parameters already exists. Through the synthesis of current and future physiological and behavioural data of *C. barnesi*, it will be theoretically possible to predict when and where this species may be more abundant. Even if these models only have short range predictive power (e.g.,

hours to days), they will still provide an ample amount of time for lifeguards to pre-emptively close beaches within the affected areas. Also, numerous people are stung on the Great Barrier Reef (Carrette & Seymour, 2013) and these predictive models could be used by commercial dive boat operators to choose dive sites that are lower risk under the predicted circumstances.

In conclusion, this research has shown that the diurnal behavioral patterns of *C. barnesi* are complex and are partially driven by light. The life cycle of *C. barnesi* is similar to other cubozoans with several notable exceptions; primarily, dormant encapsulated planulae, swimming stage polyps, and medusa production through mono-disc strobilation. The polyp stage of *C. barnesi* seems to be an estuarine inhabitant based on their preference to low salinity conditions. Furthermore, the polyp stage has the thermal capacity to proliferate throughout the year theoretically as far south as Fraser Island. Variations in temperature and salinity did not induce synchronous medusa production in this species. This indicates that increasing summer water temperature does not initiate the beginning of the Irukandji season. Therefore, due to the consistency of the beginning of the season, a cue such as increasing photoperiod may initiate medusa production, as suggested in other species (Gordon & Seymour, 2012). The thermal preference of these medusae was well correlated with the summer oceanic conditions in the waters of the Great Barrier Reef. However, the winter conditions are well below the thermal preference of the medusa stage, suggesting that the end of the Irukandji season is driven by temperature. Conversely, water temperatures in the Brisbane area are within the thermal preference range of the medusae during the summer months. This indicates

that the medusae could theoretically survive further south than they have previously been documented under current conditions. Future research is now desperately required to ground truth these results to determine if this is true.

Future research should also consider applying the methods presented here to other cubozoan species. For example, understanding the thermal tolerance of *C. fleckeri* would be significantly important for management of this dangerous species, and may also give insight as to how the stinger season length and distribution may be reflected by other species under projected sea temperature rise scenarios. The polyp stage of cubozoans is also expected to be important in contributing to the seasonal timing of the medusae, the abundance of the medusae, and possibly the distribution of the medusae. For these reasons, future research should focus on producing polyp cultures of more cubozoan species, which could be utilized for physiological tolerance experiments and could be explored for potential cues for medusa production.

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APPENDIX A

Early Life History of the 'Irukandji' Jellyfish *Carukia barnesi*

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RESEARCH ARTICLE

Early Life History of the 'Irukandji' Jellyfish *Carukia barnesi*

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Abstract

Adult medusae of *Carukia barnesi* were collected near Double Island, North Queensland Australia. From 73 specimens, 8 males and 15 females spawned under laboratory conditions. These gametes were artificially mixed which resulted in fertilized eggs. Post fertilization, most eggs developed to an encapsulated planula stage and then paused for between six days and six months prior to hatching as ciliated planulae. The paused stage planulae were negatively buoyant and adhered to substrate. The first planula was produced six days post fertilization, lacked larval ocelli, remained stationary, or moved very slowly for two days prior to metamorphosis into primary polyps. Mature polyps reproduced through asexual reproduction via lateral budding producing ciliated swimming polyps, which in turn settled and developed into secondary polyps. Medusae production for this species was in the form of monodisc strobilation, which left behind polyps able to continue asexual reproduction.

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Introduction

Cubozoans have a metagenic life cycle which alternates between benthic sessile polyps and motile pelagic medusae [1–8]. It is this alternation of generations that contributes to the often predictable occurrence of cubozoan medusae [4,5,9,10]. There are approximately 50 described species of Cubozoa and of these the early life history of only eight have been described to date [2,3,5–8,10–15]. The polyp stage of cubozoans not only initiates the seasonal onset of medusae, but also allows for population increase through asexual reproduction, which has potential for exponential population growth [5,14,16–18]. Therefore, the success of the polyp stage will drive not only the seasonal timing of medusa but also their abundance.

In northern Australia, cubozoan medusae typically arrive in large numbers associated with increased sea temperatures during the monsoonal months [4,5,9,19,20]. This seasonal cycle in some species has been reported to begin earlier and last longer in areas closer to the equator [9,21,22]. The seasonal timing of one Australian cubomedusae, *Chironex fleckeri*, has been shown to be initiated by metamorphosis of the polyp stage, which presumably uses increasing photoperiod as a cue for metamorphosis [9]. Another highly seasonal Australian cubozoan,

both temporally and spatially, is the small carybdeid *Carukia barnesi* [20,23,24]. This species is present in north Queensland waters between November and May, and may be locally abundant or absent for periods of time during the 'stinger season' [19,20,23–26].

Little is known about the general ecology and biology of *C. barnesi*; however, the medusae stage is considered oceanic, planktonic, has been found around coral reefs or islands, and under certain conditions on beaches [19,20,23,24,26]. A sting from *C. barnesi*, as well as several other cubozoans, can cause Irukandji syndrome, which is often severely painful, potentially fatal and may require hospitalization for treatment [19,27,28]. The direct cost in treating envenomed victims is estimated to be between one and three million dollars per year in northern Australia alone, and the negative impact this species has on the Australian tourism industry through reduced revenue is substantial [19]. Understanding the general ecology of the polyp stage of *C. barnesi* may allow for the determination of the start of the jellyfish season and elucidate the factors affecting the abundance of medusae present. This could contribute to decreasing the number of envenomed victims per year and reduce the costs associated with treating these stings.

Aims

The aim of this study was to describe the polyp stage of *C. barnesi* from egg fertilization through to medusa detachment and to provide a repeatable method for producing laboratory based polyp cultures of this species for scientific research.

Method

Ethics Statement

All specimen collections were conducted in accordance with Permit Numbers: G11/34552.1 and G15/37396.1.

Specimen Identification

Carukia barnesi is a small species in the family Carukiidae with a bell height of up to 35 mm and tentacles up to 1.2 m long [20,24,26,29]. This species has four tentacles, one from each of the four pedalia, a rhopaliar niche with horns, and tentacles that have an alternating pattern of large and small nematocyst crescents that resemble "neckerchiefs" [20,24,26,29]. Little is known about the distribution of this species; however, they are present along the north-eastern coast of Australia from Lizard Island to Fraser Island [20,23,24,26,29].

Capture Method

Four collection trips were undertaken to capture medusae of *C. barnesi* near Double Island, North Queensland, Australia (16°43.5′S, 145°41.0′E) in 2014 and 2015. The collection trips were undertaken on January 4 and 5, 2014, December 15, 2014, and April 7, 2015, between 1900 and 2300hrs, which resulted in the capture of both male and female medusae that spawned approximately nine hours post capture. Three underwater LED lights (7,000 lumens each) were suspended just below the water level from a 5.8 m vessel. As medusae approached the lights they were captured with a net and transferred into individual 500 ml plastic containers, held at ambient temperature, for transport. The sea surface temperature varied from 27.5°C to 30°C with a mean temperature of 29°C. The salinity at the capture location varied between 34‰ to 35‰ and the water depth varied between three to six meters. Post capture, the specimens were transported to the laboratory and held at a constant temperature of 28°C ± 0.5°C in complete darkness.

Fertilization

After the medusae had been held overnight, a total of 8 females and 15 males spawned from a total of 73 medusae, which was determined by the presence of eggs for females and sperm for males evident within the holding vessels. The spawned medusae were removed from the transport containers that contained the gametes and the water was stirred to suspend the eggs. Approximately one third of the mixture was poured into a three litre container. To this, 100 ml of water that contained male gametes was added. This mixture was stirred briefly and then the three litre container was filled with filtered sea water to reduce the gamete density. One third of this mixture was poured into 15 plastic containers of 70 ml volume. The remaining mixture was then further diluted by approximately 30%, with filtered sea water and poured into 15 additional 70 ml containers. This process was repeated resulting in approximately 250 specimen containers with varying egg densities for each female. The egg densities in the containers ranged from one to 15 eggs per ml. A loose lid was placed on each container to reduce evaporation and the containers were held in darkness at a constant temperature of 28°C.

Polyp Maintenance

Until the first primary polyps were observed, all containers received a weekly 50% water exchange (taken from the top of the vessels) with filtered sea water and were not fed. Each 70 ml container that contained polyps was fed twice a week with approximately 20 freshly hatched, first instar, decysted *Artemia* nauplii, followed by a 50% water exchange 24 hours later. As the polyp density increased through asexual reproduction, the food density was also increased.

Culturing Polyps

As the polyp numbers increased through asexual reproduction, freshly budded off swimming stage polyps (similar to those described in *Morbakka virulenta* [6,15]) were harvested. These were extracted by using a pipette to stir the contents of the 70 ml containers prior to each 50% water exchange. The waste water was then poured into a larger container where the swimming polyps could be removed and this was repeated post each feeding event. These swimming polyps were transferred into three 30 litre temperature controlled tanks (28°C). Once the swimming polyps settled and attached, they were fed live *Artemia* nauplii once per week.

Nematocyst Identification

In order to describe the cnidome of the different developmental stages of *C. barnesi*, a series of nematocyst presses were performed, as described below. The sampled stages were: paused stage unhatched planula; hatched planula; primary polyp; mature polyp; swimming stage polyp; and detached one day old medusae. Each sample was placed on a glass microscope slide and a cover slip was then placed over the sample. In most cases this pressure was sufficient to cause nematocyst discharge. In cases where none of the nematocysts discharged, a small quantity of ethanol was added to the sample. Each sample was then viewed on a stereo microscope, photographed and the nematocyst types were identified using the key developed by Rifkin [see 25] then compared to the known nematocyst types present on the adult medusae.

Results

Egg Fertilization and Development

Unfertilized eggs were translucent and negatively buoyant with a diameter between 0.08 and 0.11 mm ($n = 10$, $\bar{x} = 0.089$ mm, $SD = 0.009$). Approximately two hours post mixing of the

gametes, a fertilization membrane was produced. During initial cell division the eggs remained negatively buoyant, with a diameter between 0.08 and 0.11 mm (n = 10, $\bar{x}$ = 0.104 mm, SD = 0.010) (Fig 1I). The first blastula was seen approximately 30 hours post fertilization and the majority of the eggs were at the blastula stage within 48 hours. Around 90% of the eggs from each batch were fertilized and reached this stage (Fig 1II). During this time, the eggs/blastulae remained negatively buoyant and stuck to the base of the culture containers. The formation of the planula stage took place within the egg capsules and the planulae could be seen slowly rotating within. The planulae then entered a pausal stage and hatched over a minimum of six days and a maximum time of over six months (Fig 1III). The diameter of the unhatched planulae ranged between 0.094 and 0.120 mm (n = 10, $\bar{x}$ = 0.105 mm, SD = 0.009).

Planula

Carukia barnesi free swimming planula larvae were first observed six days post fertilization, and lacked larval ocelli. The hatched planulae were heavily ciliated and the beating cilia caused a jittery shaking motion (Fig 1IV). The planulae did not actively swim and appeared stationary, or moved very slowly, during all observations. The planulae were flattened on one end, which was the predominant direction of travel; flat end forward. The length of the planulae ranged from 0.11 to 0.12 mm (n = 10, $\bar{x}$ = 0.115 mm, SD = 0.005), were negatively buoyant and remained on the base of the containers where they eventually settled and metamorphosed into primary polyps after a minimum of two days post hatching.

Primary Polyps

Primary polyps of *C. barnesi* were first observed eight days post fertilization, two days after the first planula was observed. This species appears to lack a creeping polyp stage and instead develops where the planula settles. First a single tentacle and stalk is formed followed by a second tentacle (Fig 2I). The primary polyps are heavily ciliated and, on occasion, were observed slowly swimming near the base of the containers; however, the majority of polyps remained sedentary. During the first week the primary polyps did not feed even when presented with live or finely chopped *Artemia*, rotifers, fine crayfish meat or boiled chicken egg yolk. Nevertheless, the polyps continued to grow and develop a third (Fig 2II) and fourth tentacle (Fig 2III). At the three tentacle stage the polyps captured live rotifers (Fig 2IV) and/or first instar *Artemia* nauplii (Fig 2V). The primary polyps had the following average dimensions reported in millimetres (n = 10): total length ($\bar{x}$ = 0.191, SD = .068); stalk length ($\bar{x}$ = 0.113, SD = 0.038); calyx length ($\bar{x}$ = 0.078, SD = 0.033); calyx width ($\bar{x}$ = 0.075, SD = 0.024). The polyps continued to develop until they reached a mature stage (able to asexually reproduce) after approximately 28 days post fertilization (Fig 2VI).

The timeline of these processes is highly variable and only the first observation times have been reported. For example, even within one 70 ml specimen jar after 28 days, it was common to see all of these developmental stages at any given time. Even while there were adult polyps undergoing asexual reproduction, there were still unhatched eggs, hatching planulae, free swimming planulae, and newly formed primary polyps in each container. These eggs continued to hatch within the containers for over six months.

Polyps and Asexual Reproduction

Mature polyps first underwent asexual reproduction 28 days post fertilization. The basic polyp anatomy consisted of a calyx region that included a motile hypostome which was surrounded by a single circlet of capitate tentacles. There was a distinct demarcation at the junction between the calyx and the stalk region. The stalk region was thin and contractile with a basal

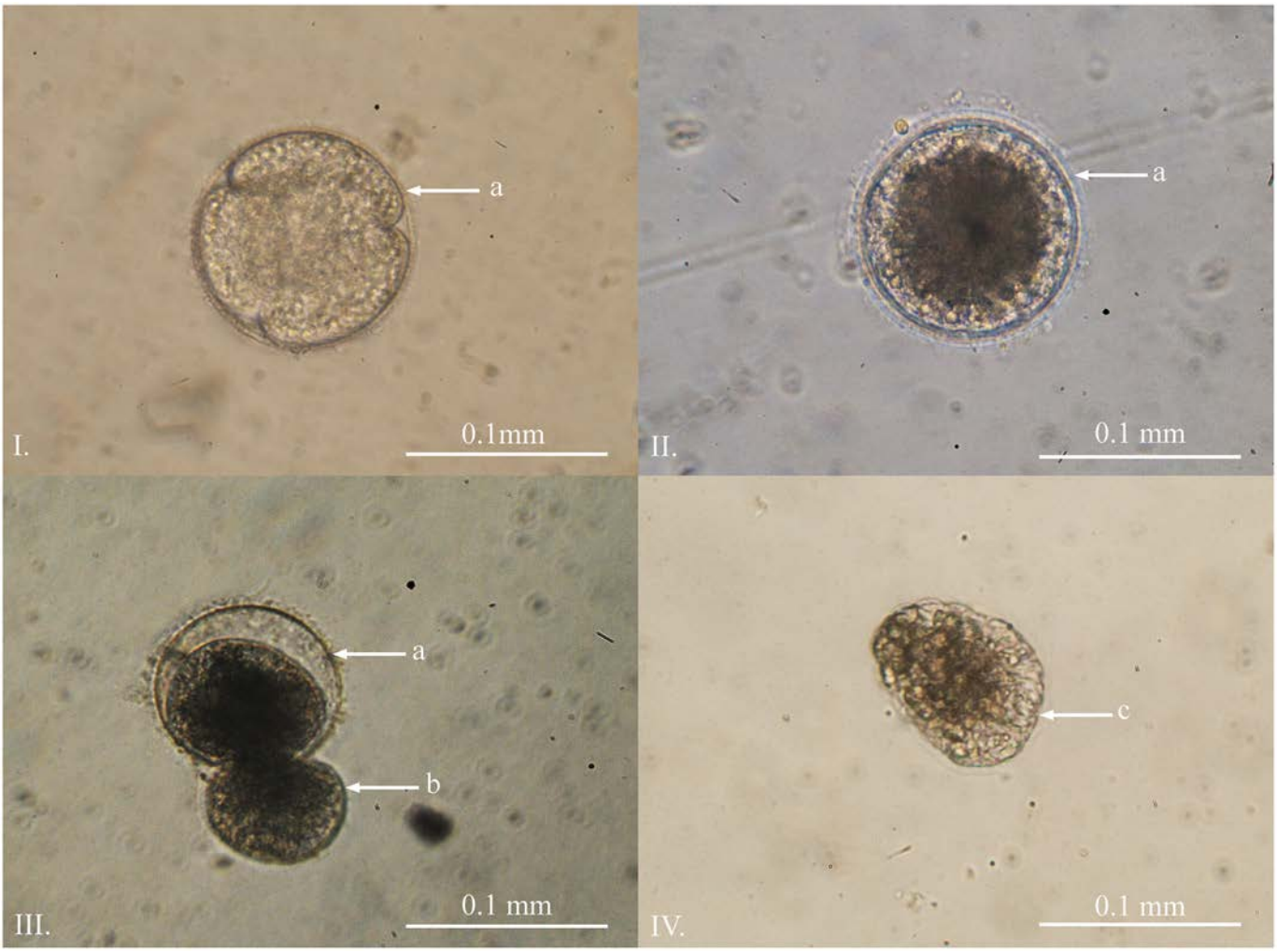

Fig 1. Planula development of *Carukia barnesi*. I. Early cell division post fertilisation. **II.** Developed blastula approximately 48 hours post fertilization. **III.** Ciliated planula larvae hatching after a minimum of six days post fertilization. **IV.** Free swimming planula. **Letters indicate:** a) egg capsule; b) emerging planula; c) anterior end.

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disc at the terminal end which anchored the polyp to substrate. All of the external surfaces of the mature polyps were ciliated. The average size of the mature polyps had the following dimensions reported in millimetres (n = 10): total length ($\bar{x}$ = 0.885, SD = 0.434); stalk length ($\bar{x}$ = 0.560, SD = 0.310); calyx length ($\bar{x}$ = 0.325, SD = 0.142); calyx width ($\bar{x}$ = 0.254, SD = 0.069). The polyps asexually reproduce after having four or more tentacles; however, the number of tentacles on each polyp was highly variable and ranged from four to 24 (n = 78, $\bar{x}$ = 11.12, SD = 5.31).

The primary mode of asexual reproduction observed was through the lateral budding of a ciliated swimming polyp similar to those produced by *M. virulenta* [6,15]. This process was first evident by a round protrusion originating from the side of the calyx (Fig 3I). The bud then develops two tentacles and a stalk and remains attached to the parent polyp along the calyx (Fig 3II and 3III). This process took approximately four days from the beginning of the bud through to detachment of the free swimming secondary polyp (Fig 3IV). Frequently, polyps were observed producing multiple buds simultaneously, up to five at a time.

Fig 2. Primary polyps of *Carukia barnesi* at different developmental stages. I. Lateral view of a primary polyp at the two tentacle stage. **II.** Vertical view of a three tentacle stage polyp. **III.** Three tentacle stage primary polyp developing a fourth tentacle. **IV.** Three tentacle stage primary polyp feeding on a live rotifer. **V.** Three tentacle stage primary polyp feeding on a live *Artemia* nauplius. **VI.** Four tentacle primary polyp 28 days post fertilization. **Letters indicate:** a) hypostome; b) capitate feeding tentacle; c) stalk region; d) calyx region; e) nematocyst bundle in tentacle tip; f) rotifer; g) *Artemia* nauplius.

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Fig 3. Asexual reproduction of *Carukia barnesi* polyps. I. The initial stage of lateral budding. **II.** Formed ciliated swimming stage polyp prior to detachment. **III.** Large mature polyp undergoing asexual reproduction. **IV.** Detached swimming stage polyp. **Letters indicate:** a) retracted tentacle; b) swelling of the calyx where the bud begins to form; c) stalk, d) calyx; e) tentacles of the swimming polyp stage; f) attachment point; g) buds produced through asexual reproduction, h) location of nematocysts in the tentacle tips.

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The swimming polyps were highly variable in size and ranged from 0.17 to 0.50 mm ($n = 10$, $\bar{x} = 0.298$ mm, $SD = 0.106$), measured along the latitudinal axis from between the tentacles to the terminal end of the stalk, where small polyps produced small swimming polyps and larger polyps produced larger swimming polyps. The polyps swam along the bottom of the containers, and also up the sides, with one tentacle forward in the direction of travel. The swimming polyps moved like this for one to seven days prior to settlement.

Medusa Production

Medusa production has only been observed three times to date and only from the first polyp culture, which was fertilized in January 2014. The quantities and dates of medusa production were; 24th of June 2014 (one medusa, 170 days post fertilization), 26th of August 2014 (approximately 100 medusae, 233 days post fertilization) and 20th of May 2015 (approximately 50, medusae 500 days post fertilization). The polyp culture, both the original cultures and the three

30 l temperature controlled tanks, included more than one million polyps (determined by extrapolation), and due to this, the actual percentage of polyps that underwent medusa production on these dates was minimal.

Medusa production in *C. barnesi* occurred in the form of monodisc strobilation similar to that observed in *M. virulenta* described by Toshino et al. [15]. First, the stalk region contracted and the oral disc widened, the calyx also began to extend (Fig 4I). The tentacles migrated to four equally spaced corners of the forming bell. The tentacles then fused together at the base and a dark pigmentation of the forming rhopalia was visible (Fig 4II). The tentacles were further reabsorbed and the rhopalia continued development. At this time, the calyx became constricted and tentacles began to develop below the developing medusa. The manubrium then formed, nematocyst warts became visible on the forming bell, small pedalia formed, and there was a further separation between the forming medusa and the original polyp (Fig 4III). The medusa began to pulse approximately two days prior to detachment.

The detached medusae were positively phototactic, congregated on the surface of the containers, and had a bell height that ranged between 0.33 to 0.80 mm ($n = 8$, $\bar{x} = 0.560$ mm, $SD = 0.140$), and a bell width that ranged between 0.45 to 0.83 mm ($n = 10$, $\bar{x} = 0.568$ mm, $SD = 0.110$) (Fig 4V and 4VI). The nematocyst warts were clearly visible on the external surface of the bell. The rhopalia were developed prior to detachment, and the beginnings of small feeding tentacles were visible. The polyps that were left behind, after strobilation, had between four and eight tentacles (Fig 4IV). These polyps continued to grow and returned to production of swimming polyps. No polyps were observed producing multiple medusae at one time. The medusae were able to consume *Artemia* nauplii soon after detachment; however, survival was limited (five days).

Nematocysts of Polyps and Early Stage Medusae

There were two different types of nematocysts found in the early developmental and polyp stages which consisted primarily of homotrichous microbasic tumiteles primarily located within the tentacle tips of the polyps and spherical isorhizas in the body of the polyps. These are the same nematocyst types identified in the medusae stage of this species, which consists primarily of homotrichous microbasic tumiteles on the tentacle nematocyst batteries and spherical isorhizas on the bell nematocyst batteries [24–26]. The cnidome of the unhatched and hatched planulae consisted of only homotrichous microbasic tumiteles, whereas both nematocyst types were present in primary polyps, mature polyps, swimming polyps, and newly detached medusae. The sizes of each nematocyst type, at each life stage, are as follows (with measurements recorded in microns): hatching planula tumiteles ($n = 5$, 3.1 to 4.0, $\bar{x} = 3.6$, $SD = 0.37$); primary polyp tumiteles ($n = 7$, 11.9 to 13.2, $\bar{x} = 12.6$, $SD = 0.50$); primary polyp isorhizas ($n = 7$, 4.0 to 6.2, $\bar{x} = 4.6$, $SD = 0.80$); mature polyp tumiteles ($n = 10$, 10.1 to 12.8, $\bar{x} = 11.2$, $SD = 0.89$); mature polyp isorhizas ($n = 7$, 4.0 to 5.3, $\bar{x} = 4.7$, $SD = 0.61$); swimming polyp tumiteles ($n = 8$, 11.0 to 11.9, $\bar{x} = 11.3$, $SD = 0.31$); swimming polyp isorhizas ($n = 5$, 6.6 to 7.9, $\bar{x} = 7.0$, $SD = 0.57$); freshly detached medusa tumiteles ($n = 10$, 11.0 to 14.1, $\bar{x} = 12.1$, $SD = 1.00$); freshly detached medusa isorhizas ($n = 10$, 4.8 to 5.3, $\bar{x} = 5.1$, $SD = 0.23$).

Discussion

On four occasions the adult medusae of *C. barnesi* collected from Double Island spawned approximately nine hours post capture while held at a constant temperature of 28°C in darkness in water collected from the sample site. Although all attempts were made to replicate the conditions at the collection site, it is expected that this was a stress induced spawning event and these specimens may not have spawned *in-situ* over the same timeframe. Although, *C. barnesi*

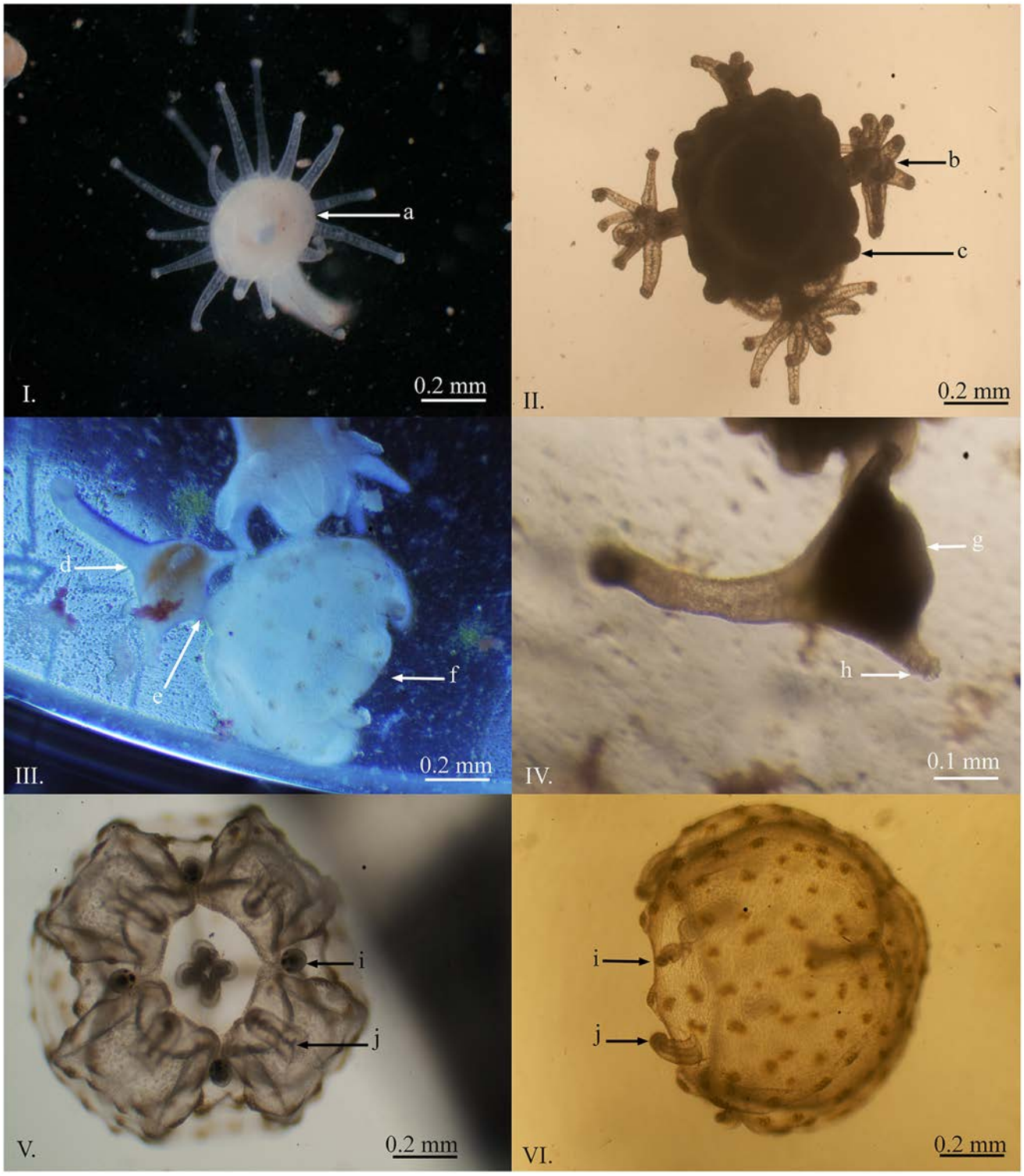

Fig 4. Medusae production of *Carukia barnesi*. I. Initial stage of medusae production, note the change in shape of the hypostome region. **II.** The tentacles migrate to four opposing sections of the forming bell, fuse, and begin to form the rhopalia. **III.** The bell begins to pulse and a narrowing divides the bell of the forming medusae from the hypostome of the original polyp. **IV.** The remaining polyp after completed medusae production. **V.** Oral view of a newly detached medusa. **VI.** Lateral view of a newly detached medusa with pigmented nematocyst batteries on the bell. **Letters indicate:** a) change in shape of the hypostome region; b) fusing of tentacles which begin to form rhopalia; c) early development of medusa tentacles; d) polyp producing a medusa through monodisc strobilation; e) connective tissue between the polyp and forming medusa; f) pulsing medusa approximately 24 hours prior to detachment; g) hypostome; h) feeding tentacle; i) formed rhopalia; j) short feeding tentacles.

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are routinely collected during the summer months at this location, specimens have never been observed spawning at this, or any other location, nor has it been reported in the literature. This is unlike the known predictable spawning aggregations of *Alatina alata* that occur 8 to 10 days after a full moon in Hawaii [11,30–33]. Furthermore, the eggs of some cubozoan species are fertilized internally [7,8,12], and because the fertilization method implemented in this study was artificial, the possibility that there is more to the reproductive behaviour of *C. barnesi* should not be discounted.

The size and developmental timing of the fertilized egg phase through to gastrulation was similar to other cubozoans [5–7,11,12,15]. The first notable difference was the dormant phase of the unhatched *C. barnesi* planulae. After the planula developed within the egg capsule, it entered a dormant phase that lasted six days to over six months. During this time the unhatched planulae were negatively buoyant and stuck to the base of the containers. A similar dormant pause stage has been described in *M. virulenta*, where encysted blastulae lasted seven to 21 days before hatching as either free swimming planulae, or as primary polyps [6,15]. The paused stage planulae of *C. barnesi* only hatched as free swimming planulae. The function of the encapsulated pause stage is unknown; however, it may stagger the hatching time, potentially reducing the impact of intraspecific competition. There is also the possibility that there is an environmental cue, such as a change in temperature, photoperiod, or substrate, which triggers synchronous hatching.

The hatched planulae of *C. barnesi* were negatively buoyant, lacked larval ocelli, moved very slowly on the base of the containers and had nematocysts. Cubozoan planula have previously been reported as being negatively buoyant [5–7,11,12,15], either possessing larval ocelli [6,11,34,35], or lacking larval ocelli [6,15], and having nematocysts which were used for attachment to substrate [5]. This use of nematocysts in the planula of *C. barnesi* was not observed and the function of the nematocysts during the planula stage is unknown, but is presumably for defence. The lack of larval ocelli and the limited swimming capacity of *C. barnesi* planulae may only provide limited dispersal potential that does not require photoreceptors for orientation or settlement choice.

The primary polyps of *C. barnesi* were able to develop to the four tentacle stage without food. This suggests that the eggs either contain sufficient stores to reach this stage, or the planulae and/or primary polyps may be able to uptake dissolved organic material directly from the water column. Once the primary polyps began to feed on *Artemia* nauplii they rapidly developed into mature polyps which began asexual reproduction through lateral budding. This process was very similar to the asexual reproduction method seen in *M. virulenta* from Japan, which produced small swimming stage polyps [6,15]. The swimming stage polyps of *C. barnesi* were ciliated and swam along the base and up the vertical surfaces of the culture containers prior to settlement and transformation into a secondary polyp. The mobility of the swimming stage polyps may not only provide a dispersal mechanism, but may also allow for increased selectivity in settlement position and/or substrate choice. Also, the size of the swimming polyps was highly variable, where larger polyps produced large swimming polyps and smaller polyps produced small swimming polyps.

The process of medusa production in *C. barnesi* was also very similar to the monodisc strobilation recently discovered in *M. virulenta* [15]. Medusa production of cubozoans is highly variable between species ranging from complete metamorphosis of the polyp into a medusa [2,3,8,10,11,14,36], leaving behind a small amount of regenerative material (residuum) [37], and monodisc strobilation which may leave behind a developed polyp (with tentacles) able to continue asexual reproduction [15]. This is a reproductive event, which may suggest that the polyps of *C. barnesi* may reside in a location that allows for the annual persistence of the polyp.

The cnidome of the early life stages of *C. barnesi* was found to be identical to the cnidome of the adults. This deviates from other cubozoan species which change their cnidome between polyp and medusa stages [11,14].

Cubozoans have been shown to be sophisticated in many areas of their ecology from possessing complex vision capabilities [38–40], prey capture techniques [41], behavioural patterns [42,43], and complex venoms [19,28,44]. Not surprisingly, the life cycle of cubozoans also have many species specific complexities [2,3,6,7,10,13–15]. In order to investigate cubozoan life cycles, production of lab-based polyp cultures is essential. From these cultures the effects of varying parameters such as temperature, salinity, and feeding frequency can be explored to elucidate the most likely habitats that support polyp proliferation. This could then be used to determine where the polyp colonies may reside *in-situ*. These parameters could also be experimentally pursued to elucidate cues for medusae production which may give better insight into what factors drive the strong seasonal occurrence of this, and other, cubozoan species.

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Author Contributions

Conceived and designed the experiments: RC JS. Performed the experiments: RC SB JS. Analyzed the data: RC SB JS. Contributed reagents/materials/analysis tools: RC SB JS. Wrote the paper: RC JS. Photography: SB.

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APPENDIX B

Thermal and Osmotic Tolerance of 'Irukandji' Polyp Cubozoa: *Carukia barnesi*

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RESEARCH ARTICLE

Thermal and Osmotic Tolerance of 'Irukandji' Polyps: Cubozoa; *Carukia barnesi*

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Abstract

This research explores the thermal and osmotic tolerance of the polyp stage of the Irukandji jellyfish *Carukia barnesi*, which provides new insights into potential polyp habitat suitability. The research also targets temperature, salinity, feeding frequency, and combinations thereof, as cues for synchronous medusae production. Primary findings revealed 100% survivorship in osmotic treatments between 19 and 46‰, with the highest proliferation at 26‰. As salinity levels of 26‰ do not occur within the waters of the Great Barrier Reef or Coral Sea, we conclude that the polyp stage of *C. barnesi* is probably found in estuarine environments, where these lower salinity conditions commonly occur, in comparison to the medusa stage, which is oceanic. Population stability was achieved at temperatures between 18 and 31°C, with an optimum temperature of 22.9°C. We surmise that *C. barnesi* polyps may be restricted to warmer estuarine areas where water temperatures do not drop below 18°C. Asexual reproduction was also positively correlated with feeding frequency. Temperature, salinity, feeding frequency, and combinations thereof did not induce medusae production, suggesting that this species may use a different cue, possibly photoperiod, to initiate medusae production.

Introduction

Tropical Australian cubozoans are highly seasonal, with the medusa stage usually arriving along the tropical coastlines during the monsoonal summer months [1–7]. This 'stinger season' in Australia is typically between November and May, which has been reported to begin earlier, and last longer, in warmer areas closer to the equator such as in the Gulf of Carpentaria [3,8,9]. At least part of the observed seasonality of the medusa stage is thought to be driven by the complex life cycle of cubozoans.

Cubozoans have a metagenic life cycle that alternates between benthic polyps, which reproduce asexually, and pelagic medusae, that reproduce sexually [2,4,10–16]. Medusa production in cubozoans is variable and can be in the form of complete metamorphosis of the polyp into a

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medusa [11,12,16–20], metamorphosis that leaves behind a small amount of regenerative material which is able to develop into a polyp (residuum) [21], and monodisc strobilation that leaves behind a polyp able to continue asexual reproduction of more polyps [22,23]. Some cubozoan polyps are known to use environmental factors, such as temperature [19,24,25], a combination of temperature and light intensity [18], photoperiod [3], or reduced food availability [26], as a cue to induce synchronous medusa production. It is thought that once medusa production is initiated, it can be punctuated by significant rainfall events, for example *Chiropsella bronzie*, or is continuous, as in *Chironex fleckeri* [2,3]. Therefore, medusae production by the polyps is considered to be one factor that drives the seasonal fluctuation in abundance of the medusae stage [2–4,20].

The medusa stage of *Carukia barnesi*, the Irukandji jellyfish, often causes painful and potentially fatal stings [1,6,7,27,28]. A sting from *C. barnesi* is also renowned for causing Irukandji syndrome which often requires hospitalization for treatment [1,27,28]. Considerable costs are associated with treating sting victims and lost revenue to the tourism industry annually is believed to be substantial [1]. Although only the medusa stage of this cubozoan poses a threat to humans, the polyp stage is expected to contribute to the seasonal periodicity through the synchronous timing of medusae production causing the start of the stinger season. Similarly, the abundance of medusae present during the season may be reflective of the success of the polyp stage.

The polyp stage also contributes to the abundance of medusae present by asexually reproducing more polyps, thus increasing the number of polyps able to produce medusae at any one time. Thermal, osmotic and food availability parameters are known to influence the rate of asexual reproduction of the polyp stage of *Alatina* nr *mordens* [26,29] and *Carybdea marsupialis* [30–32]. However, in most instances, the *in situ* parameters of the polyps are not known due to the unknown location of the polyps, and to date, cubozoan polyps have only been discovered *in situ* for two species: *C. fleckeri* and *C. marsupialis* [4,12,20,32]. Because of this, laboratory-based cultures of cubozoan polyps are essential for exploring the factors that affect polyp survivorship and proliferation in order to deduce where the polyps may reside, which may also shed light on the factors affecting the seasonal influx of medusae.

Carukia barnesi, like other Australian cubozoans, exhibits a marked seasonality. Little is known about the ecology of the early life stages of this species [22], even though it was discovered as causing Irukandji syndrome over 50 years ago [6,33,34]. The medusa stage of *C. barnesi* is considered oceanic and is typically found around coral reefs and islands and under certain condition along beaches [1,5,6,34,35]. However, nothing is known about the thermal and osmotic preference or the location of the polyp stage *in situ*. Therefore, a series of temperature, salinity, and feeding frequency experiments were conducted on the polyp stage in order to gain a better understanding of the ecology of this species.

The primary aims of this research were: 1) to determine the thermal and osmotic tolerance of the polyps, and use these data to discuss where these polyps may live; 2) to explore the effects of feeding frequency on survivorship and asexual reproduction and discuss intrinsic population growth rates under different feeding regimes; 3) to determine the effect of thermal and osmotic parameters, and combinations thereof, as well as feeding frequency, as cues for medusae production.

Method

Specimen Identification

Carukia barnesi is a small species in the family Carukiidae. The medusa stage has a bell height of up to 35 mm and tentacles up to 1.2 m long [6,34–37] with a single tentacle that extends

from each of the four pedalia; rhopaliar niches with horns; and tentacles that have an alternating pattern of large and small nematocyst crescents that resemble "neckerchiefs" [6,34–36]. The polyps of *C. barnesi* average 0.9 mm in length and have 11 capitate tentacles on average. The polyps asexually reproduce ciliated swimming polyps and produce medusae through monodisc strobilation (see [22]). Little is known about the distribution of this species; however, medusae are often present along the north-eastern coast of Australia during the warm monsoonal months (November to May) between Lizard Island and Fraser Island [5,6,34–36].

Experimental Design and Pre-treatment

This project consisted of two separate experiments in which polyps of *C. barnesi* were exposed to different temperature, salinity and/or feeding frequency treatments that were monitored for survivorship, population increase through asexual reproduction, and medusae production following a previously published method [26]. The *C. barnesi* polyps were derived from the laboratory-based culture outlined in Courtney et al. [22], which was established approximately six months prior to these experiments from wild caught adult medusae. This polyp culture was maintained at a constant temperature of 28°C ± 0.5°C and a salinity level of 33‰ ± 0.5‰.

Both of the experiments presented here were conducted in 24 well sterile micro-plates and only 12 of the wells within each plate were used to allow for blank wells between different treatments. Each well had a surface area of 11.5 cm^2 and a volume of 3.5 ml. The mature polyps of *C. barnesi* produce swimming stage polyps through lateral budding [22]. These swimming polyps were harvested from the primary cultures and transferred into the 24 well micro-plates at a density of approximately 15 swimming polyps per well. As these polyps settled and developed into secondary polyps, they were fed freshly hatched *Artemia* sp. nauplii to satiation (approximately 20 *Artemia* sp.) every seven days. A complete water exchange was carried out 24 hours post each feeding event with filtered artificial seawater. The water quality parameters were maintained at the original culture parameters (28°C ± 0.5°C and 33‰ ± 0.5‰) for four weeks prior to any experimental trials to allow the polyps to acclimate, grow to maturity, and begin asexual reproduction. During this time, and during all experiments, a photoperiod of 13 hours light: 11 hours dark was maintained. These polyps were then exposed to one of the conditions outlined below; polyps were only exposed to one treatment, there was no mixing of water between replicates or treatments, and all treatments in each experiment were conducted simultaneously.

The first experiment consisted of 80 thermal and osmotic treatments (see Method: Thermal and Osmotic Effects on Survivorship and Asexual Reproduction), which were monitored using a binocular dissection microscope, for polyp survival, asexual reproduction, and medusae production over a six-week period. Each treatment comprised six independent replicates and each replicate consisted of an average of 18.63 polyps at the beginning of the experiment (i.e., $\bar{x}$ = 18.63, *SD* = 5.87 polyps; housed in each of 480 wells). Each replicate (well) was fed approximately 20 *Artemia* nauplii once per week and this quantity was increased as the polyp numbers increased (i.e., approximately one *Artemia* per polyp per week). Each feeding event was followed by a complete water exchange with artificial seawater of the same parameters as each experimental treatment (temperature and salinity).

The second experiment required a further five treatments to determine the effects of feeding frequency on asexual reproduction, survivorship, and medusae production (see Method: The Effects of Feeding Frequency on Survivorship and Asexual Reproduction). This experiment was conducted at the original polyp culture conditions of 28°C ± 0.5°C and 33‰ ± 0.5‰ over a six-week period. Each treatment comprised six replicates and each replicate consisted of an average of 20.5 polyps at the beginning of the experiment (i.e., $\bar{x}$ = 20.50, *SD* = 2.79 polyps;

housed in each of 30 wells). Each replicate (well) was fed approximately 20 *Artemia* sp. nauplii per feeding event and this quantity was increased as the polyp numbers increased (i.e., one *Artemia* per polyp per feeding event).

Thermal and Osmotic Effects on Survivorship and Asexual Reproduction

To determine the thermal and osmotic tolerance of *C. barnesi* polyps, and the effects these treatments had on survival, asexual reproduction and medusae production, replicates of polyps (as described above) were exposed to an 80 combination matrix of eight temperatures (11, 14, 18, 21, 25, 28, 31, and 34°C ± 0.5°C) and ten salinities (16, 19, 22.5, 26, 29, 33, 36, 39, 42.5, and 46‰ ± 0.5‰) over a period of six weeks. Each incubation temperature was maintained by partially submerging each test plate into one of eight temperature controlled water baths for the duration of the experiment. The range of temperatures selected encompasses the potential thermal regime a polyp may experience in the Coral Sea (temperatures commonly below 11°C at depths of approximately 500 m) and along the adjacent Australian coastline including estuarine environments (temperatures commonly occur above 34°C in shallow estuarine pools). The salinity range tested encompasses common salinities found in the Coral Sea and also included both hypersaline and hyposaline conditions that are commonly found in estuarine environments. These environmental parameters were explicitly targeted to deduce the thermal and osmotic tolerance of the polyp stage. Prior to the beginning of this experiment each replicate was photographed and the number of polyps in each well was determined. Every seven days during the six-week experiment, each replicate was fed and water exchanged (as described above). Each replicate was again photographed and the number of polyps in each well was determined in seven-day intervals during the six-week experiment.

To determine the thermal and osmotic tolerance of polyps, the change in population numbers was determined in seven-day intervals during the six-week experiment. To allow for comparisons between treatments, a model was fitted across all treatments that described final polyp density as a function of initial density, temperature, and salinity. We assumed that the final polyp density was proportional to the initial density, functions of temperature $F(T)$ and salinity $G(S)$, and a parameter a which describes the maximum population change at optimal conditions such that,

$$P_{final} = aF(T)G(S)P_{initial}. \quad (1)$$

Thus, we assume independent effects of temperature and salinity on growth rates. Preliminary analyses suggested including interactive effects between temperature and salinity did not qualitatively change the results. We model $F(T)$ and $G(S)$ as asymmetrical modified Gaussian functions centered around the optimal temperature and salinity, respectively such that,

$$F(T) = \begin{cases} \exp\left[\dfrac{-(T_{opt} - T)^2}{{\sigma_{t,l}}^2}\right], \textit{for } T < T_{opt} \\ \exp\left[\dfrac{-(T_{opt} - T)^2}{{\sigma_{t,h}}^2}\right], \textit{for } T > T_{opt} \end{cases} \quad (2)$$

$$G(S) = \begin{cases} \exp\left[\frac{-(S_{opt} - S)^2}{\sigma_{s,l}{}^2}\right], \text{for } S < S_{opt} \\ \exp\left[\frac{-(S_{opt} - S)^2}{\sigma_{s,h}{}^2}\right], \text{for } S > S_{opt} \end{cases}, \quad (3)$$

where T_{opt} and S_{opt} describe the optimal temperature and salinity, respectively. The $\sigma_{i,j}{}^2$ parameters represent the slope for independent variable i (temperature or salinity) and j, the side of the curve (low, or high). We assume that the final polyp density follows a negative binomial distribution, and fit the models and calculated the log-likelihood, maximum likelihood estimates and 95% maximum likelihood profile confidence limits using the bbmle package, version 1.0.18 [38], in the statistical package R, version 3.2.4 [39,40]. The raw data file and R code has been included in Supporting Information (S1 Dataset and S1 R Code). To evaluate the effects of salinity and temperature, we used AIC values (Akaike Information Criterion) to compare the full model with models containing only temperature or salinity (i.e., $P_{final} = aF(T)\ P_{initial}$ or $P_{final} = aG(S)P_{initial}$). Here, large changes in AIC (presented as ΔAIC) with removal of temperature or salinity suggest the importance of the respective environmental component for reproduction and/or survival. Although there is no standard cut-off value for AIC, like there is for *p*-values, we used a conservative value of ΔAIC = 7, which corresponds to significance levels in the vicinity of 0.003 for nested models [41].

Effects of Feeding Frequency on Survivorship and Asexual Reproduction

In order to determine the effect of feeding frequency on survivorship and asexual reproduction, polyps were exposed to five feeding regimes over a six-week period while held at the original culture conditions of 28°C ± 0.5°C and 33‰ ± 0.5‰. The five feeding regimes consisted of feeding every 1, 3, 7, or 14 days and a no food treatment over a six week period. Each feeding event consisted of food saturation for 24 hours (approximately one *Artemia* sp. nauplii per polyp per feeding event as outlined above) followed by a 100% water exchange 24 hours post feeding event with artificial pre acclimated sea water of 28°C ± 0.5°C and 33‰ ± 0.5‰. In the 14-day and no food treatments, water exchanges were conducted every seven days during the six-week experiment. Each replicate was photographed and the number of polyps in each replicate was determined at the start and in seven-day intervals during the experiment. Polyp density was analysed with a generalised linear mixed model with feeding frequency, time and the interaction between time and feeding frequency as fixed effects and replicate number as a random effect. This was analysed via proc glimmix in SAS, version 9.4 [42], using the ar(1) covariance matrix to describe temporal autocorrelation, and we assumed polyp density followed a negative binomial distribution with a log-link function, typical for count data [43]. We evaluated statistical inference tests with Wald F tests conducted in proc glimmix. The raw data file has been included in Supporting Information (S2 Dataset).

Environmental Cues for Medusae Production

To explore feeding frequency, temperature, salinity and combinations thereof as potential cues for medusae production, each of the previously described six-week experiments was also monitored for medusae production. Medusae production for this species is in the form of monodisc strobilation [22] and each polyp was monitored for a change in body shape,

formation of statoliths, attached and free swimming medusae. At the end of the six-week experiments each replicate of each treatment (80 temperature and salinity treatments and five feeding frequency treatments) was rapidly returned (shocked) back to the original culture condition of 28°C ± 0.5°C and 33‰ ± 0.5‰ and were monitored for a further four weeks (i.e., six weeks in treatment and four weeks post treatment). During this additional four-week period, the polyps were fed once per week followed by a complete water exchange 24 hours post feeding event.

Results

Thermal and Osmotic Effects on Survivorship and Asexual Reproduction

Polyp survivorship and asexual reproduction rates significantly decreased as environmental values moved away from the optimal temperature and salinity, and removal of parameters describing the effects of each environmental variable suggested each variable was important (effect of removing temperature parameters by setting $F(T) = 1$: $\Delta AIC = 594$, and effect of removing salinity parameters by setting $G(S) = 1$: $\Delta AIC = 164$). These ΔAIC values are distinctly higher than our conservative baseline of $\Delta AIC = 7$, and suggest that temperature and salinity alone are not sufficient to explain the data, and polyp survival and/or reproduction is indeed a function of both temperature and salinity. Summary statistics describing the data used to generate the model are provided in Supporting Information (S1 Table). The optimal temperature and salinity for polyp proliferation occurred at 22.9°C and 26.0‰, respectively, where the population increased by over 10 times during the six-weeks (see Table 1) compared to 100% mortality associated with both high and low temperatures and very low salinity levels. The temperature and salinity range that allowed for population stability (i.e., equal to or greater than 100% of the starting population) consisted of thermal treatments between 18°C and 31°C at salinity levels between 19‰ and 46‰. Although asexual reproduction was high at temperatures that commonly occur in the waters of the Great Barrier Reef, this was not the case with salinity, where the highest polyp proliferation was not encompassed by salinity levels that occur in the waters of the Great Barrier Reef (see Fig 1). While variation in population growth surrounding the optimum temperature and salinity was relatively symmetrical with respect to temperature ($\sigma_{tl} = 3.47$, $\sigma_{th} = 5.01$), population reproduction showed a steeper decrease with lower salinity ($\sigma_{sl} = 5.19$) than higher salinity ($\sigma_{sh} = 31.07$).

Table 1. Maximum Likelihood Estimates for Parameters Describing Proportional Change in Polyp Density of *Carukia barnesi*, After Six Weeks, Modelled as a Function of Temperature and Salinity.

Symbol	Description	Value	2.5% CL	97.5% CL
a	Maximum proportional change	11.48	9.00	14.95
σ_{tl}	Temperature curve low	3.47°C	2.91°C	4.02°C
σ_{th}	Temperature curve high	5.01°C	4.39°C	5.72°C
T_{opt}	Optimum Temperature	22.91°C	21.92°C	23.86°C
σ_{sl}	Salinity curve low	5.19‰	4.39‰	6.22‰
σ_{sh}	Salinity curve high	31.07‰	21.70‰	89.95‰
S_{opt}	Optimum Salinity	26.04‰	24.54‰	27.85‰

Model output describes proportional change in polyp density for different temperature and salinity values as described in Eqs 1, 2 and 3. Models were fit to data collected from a matrix of eight temperature and ten salinity treatments, each replicated six times. Estimated parameter values are based on the best fit parameters and 95% maximum likelihood profile confidence limits are provided.

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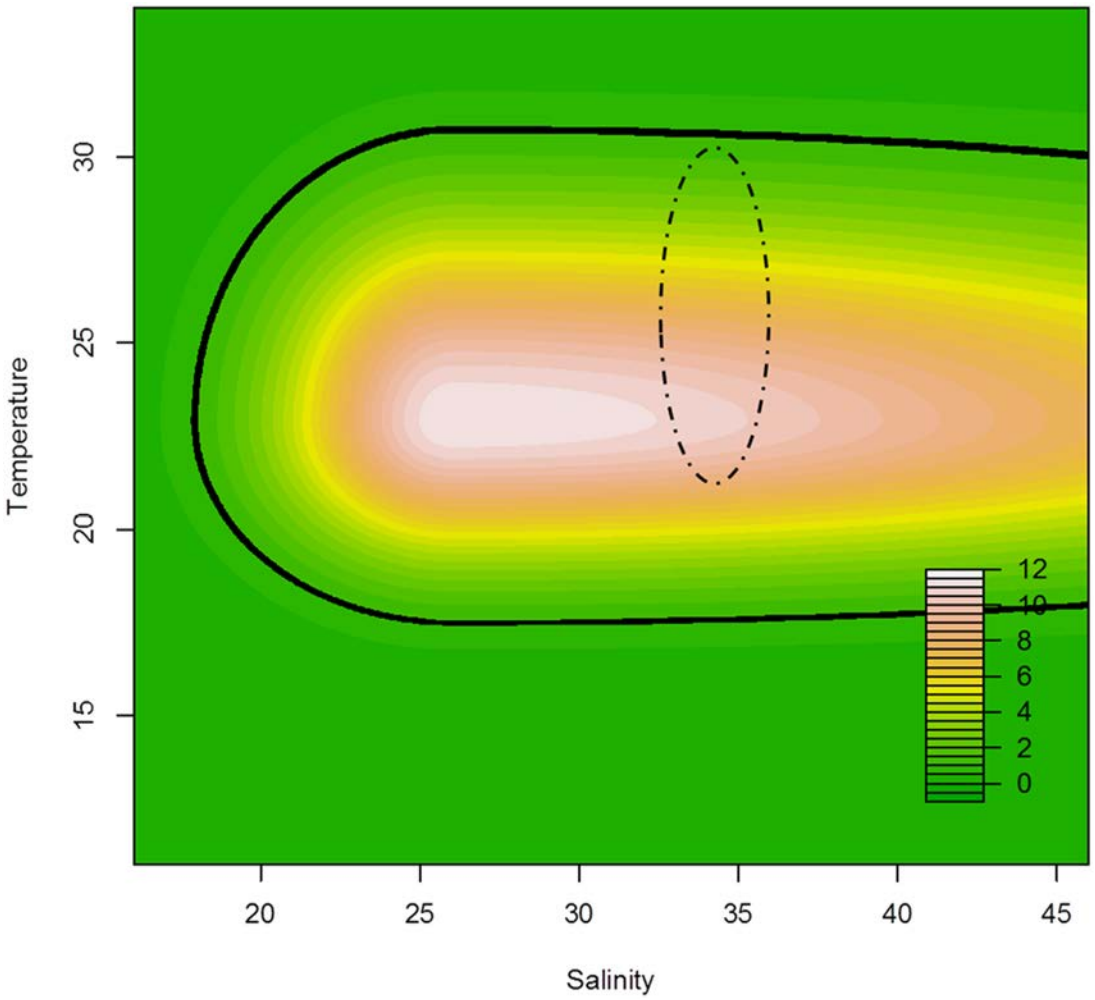

Fig 1. Maximum likelihood estimated proportional change in polyp density after six weeks plotted as a function of temperature and salinity from best-fit model. Model output describes proportional change in polyp density for different temperature and salinity values as described in Eq 1. Solid black line represents values of 1, where polyp density is expected to remain constant. The area encompassed by the dashed line indicates sea surface temperature and salinity levels that commonly occur within the waters of the Great Barrier Reef [44,45]. Models were fit to data collected from a matrix of eight temperature and ten salinity treatments, each replicated six times. Summary statistics describing the data used to generate the model are provided in Supporting Information (S1 Table).

doi:10.1371/journal.pone.0159380.g001

Effects of Feeding Frequency on Survivorship and Asexual Reproduction

Feeding frequency significantly increased asexual reproduction ($F_{4,\ 25} = 16.76$, $p < 0.001$). In addition, there was significant temporal variation in polyp proliferation ($F_{6,\ 149} = 78.65$, $p < 0.001$) and the positive effect of feeding frequency became incrementally stronger over time ($F_{24,\ 149} = 5.21$, $p < 0.001$). Polyps that were fed daily increased mean population numbers by over three times during the six-weeks, and polyps that were unfed increased in population by 0.5 times over the same time frame (Fig 2).

Environmental Cues for Medusae Production

Temperature, salinity, and feeding frequency, and variations thereof, did not trigger medusae production during the six-week experiments or during the four-week post treatment monitoring

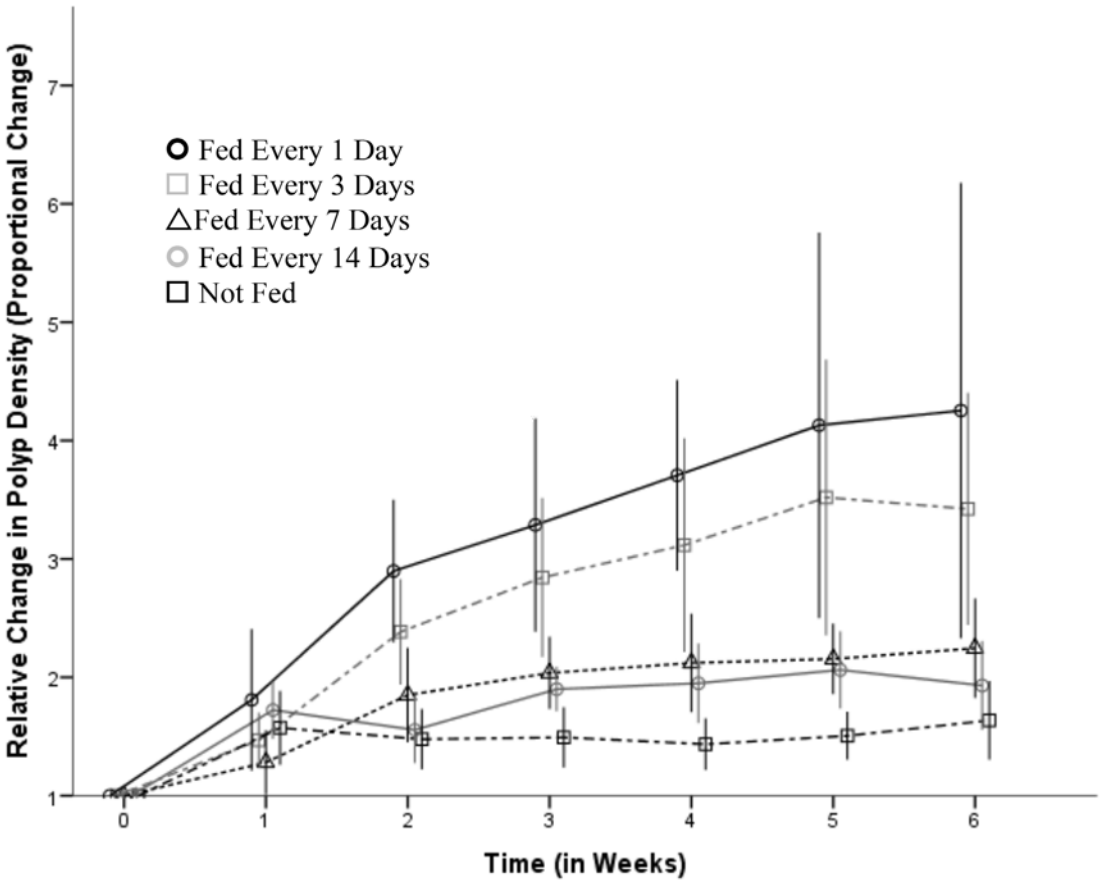

Fig 2. The relative change in *Carukia barnesi* polyp density, over six weeks of exposure, to five feeding regimes. The feeding levels consisted of: fed every day (dark circles); fed every three days (light squares); fed every seven days (dark triangles); fed every 14 days (light circles); unfed for 42 days (dark squares). Each of the five feeding treatments consisted of six independent replicates, each of which consisted of a mean staring polyp density of 20.5 polyps per well ($n = 30$, $\bar{x} = 20.5$, $SD = 2.8$). The values for each time sequence were calculated as the proportional change in polyp density from the starting population density at time zero, where values of one indicate no population change and values above one indicate polyp proliferation through asexual reproduction. Values are reported as means and error bars represent 95% CI assuming a normal distribution. All counts were conducted in seven-day intervals however the values have been graphed offset on the time axis for clarity.

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period. Only one polyp went through medusae production during the ten-weeks. This occurred in the 31°C and 46‰ treatment and was identified during week five of the experiment. As a percentage, one polyp undergoing medusae production during this experiment was not significant. Returning the polyps to the original culture conditions of 28°C ± 0.5°C and 33‰ also did not cause medusae production over the four-week post experiment monitoring period.

Discussion

Carukia barnesi polyps had a high tolerance to osmotic treatments with 100% survivorship from treatments between 18 and 46‰ (Fig 1), which indicates that this species may inhabit areas with a high degree of osmotic variation. The osmotic treatment that yielded the highest degree of polyp proliferation was 26‰. Salinity levels this low do not occur in the waters of the Great Barrier Reef or in the Coral Sea, however these low salinity conditions are a common occurrence in estuarine systems [46–49]. These results indicate that although the polyp stage does not seem restricted to low salinity waters, the observed increase in asexual reproduction

under low salinity conditions suggests that the polyp stage is probably an estuarine inhabitant. Cubozoans that have coastal or estuarine polyp stages have previously been reported for two species; *C. fleckeri* polyps were located *in situ* attached to the underside of rocks near a river mouth in Queensland [4], and polyps of *C. marsupialis* were once located attached to bivalve shells in a mangrove habitat in Puerto Rico [12]. To date, these are the only two discoveries of cubozoan polyps *in situ* globally, however at least one other Australian cubozoan polyp, those of *C. bronzie*, has also been suggested to be estuarine [2]. The possibility that *C. barnesi* has an estuarine polyp stage has not previously been considered primarily due to their distinctly oceanic medusa stage [5,6,34] compared to species which are known to be primarily coastal, such as *C. fleckeri* and *C. bronzie* [2,3,50–52]. There have been no reported sightings, or documented stings caused by *C. barnesi* from estuaries; however this does not discount the possible presence of medusae in these areas when they are newly detached and small. It is currently unknown how the polyps enter low salinity habitats and nothing is known about the osmotic tolerance of the medusa stage or where or when the medusae spawn. In speculation, due to there being no reports of adult *C. barnesi* medusae within estuarine systems, it is plausible that the eggs, that are known to have a long encapsulated planula stage from six days to six months [22], are transported inshore on currents. Future research is required on the medusa stage, such as determining their osmotic tolerance, to better understand how the life cycle is completed *in situ*.

Temperature also affected both the survivorship and asexual reproduction rate of *C. barnesi* polyps. There was notable symmetry between the high and low temperature curves with a modelled optimum temperature of 22.9°C (Table 1). As the thermal treatments moved away from optimum the amount of polyp proliferation through asexual reproduction was reduced. The minimum and maximum thermal constraints of the polyps were between 18°C and 31°C, which indicates that the polyp stage has a suitable operating envelope of approximately 13 degrees, whereby positive population growth is possible over a period of six weeks.

The southern distribution limit of the medusa stage of *C. barnesi* is not well established. However, at present it is considered to be near Fraser Island based on sting and capture records (Seymour unpublished). The winter (July) sea surface temperature near Fraser Island typically averages 20°C and increases to 27°C during the summer (February) [45], which is within the thermal capacity range for population growth of the polyp stage and encompasses their thermal optimum of 22.9°C. Although the temperature profile of *C. barnesi* polyps seems to fit well with the water temperature near Fraser Island, it is expected that the water temperature in estuarine systems near river mouths, which are predominately large river systems along the eastern coast of Queensland, to be significantly lower in temperature than the adjacent open ocean. For example, temperatures near the mouth of the Noosa Rivers (approximately 100km south of Fraser Island) are known to average as low as 18°C during the winter [53] compared to 20°C in the open ocean near Fraser Island [45]. Therefore, the polyp stage of *C. barnesi* may be restricted to warmer estuarine areas where the winter water temperature does not drop as low as 18°C north of Fraser Island. This suggests that the medusa stage may limit the southern distribution of this species, though no data exists on the thermal tolerance of the medusa stage to confirm this.

The observed rate of asexual reproduction of *C. barnesi* during these experiments was high compared to another cubozoan polyp, *A.* nr *mordens* [26]. For example, a maximum polyp population increase of 50% was recorded for *A.* nr *mordens* over six weeks [26] compared to over 1000% in *C. barnesi* during this replicated experiment (Fig 1). This high rate of reproduction is expected to increase the number of polyps able to produce medusae at any one time and in turn influence the abundance of medusae present. Highly reproductive populations are expected to make use of rapid environmental changes [54–56]. Purely in speculation, and

assuming these polyps are estuarine and require hard substrate for attachment similar to other cnidarian polyps [57–60], increasing manmade structures within marine environments (e.g., marinas, boat ramps and mooring platforms), may increase the available hard substrate for polyp attachment. This anthropogenic effect has been suggested to positively impact on scyphozoan polyp populations [61–65]. Not surprisingly, increased asexual reproduction was correlated with increased feeding frequency. Therefore, changes in estuarine trophic dynamics, primarily eutrophication, may lead to increased copepod density [66,67], presumably a primary food source for polyps and early stage medusae, which may influence polyp proliferation and in turn affect medusae abundance.

Some cubozoan polyps are known to use environmental factors, such as temperature [19,23–25], or reduced food availability [26], as a cue for medusae production. However, temperature, salinity, feeding frequency, and combinations of these factors, did not trigger medusae production in *C. barnesi*. This suggests that the polyps may use a different cue for synchronous medusae production. Because of the consistency of the first arrival of the medusae stage over the last 50 years [68], it is possible that this species uses increasing photoperiod as a cue due to the interannual variation in environmental parameters such as temperature and salinity. This type of cue has also been suggested for *C. fleckeri* due to the consistency of occurrence during a seven-year study [3]. There is also evidence that the stinger season length has increased over the last 50 years from 15 days to 151, which has been speculated to be caused by increasing global sea temperatures [68]. Therefore it is possible that *C. barnesi* polyps use photoperiod to initiate synchronous medusae production but continue to produce medusae throughout the season until the winter water temperature becomes too low.

Future research should pursue determining the thermal and osmotic parameters of the medusa stage of *C. barnesi*, and experimentally determine possible environmental cues for medusae production. Understanding the contributing factors that lead the spatial and temporal variability in medusae abundance may in turn prove valuable to predicting future *C. barnesi* abundance, and/or distributional range extensions, under projected sea temperature rise scenarios.

Supporting Information

S1 Dataset. *Carukia barnesi* Polyp Count Data Associated With "Thermal and Osmotic Effects on Survivorship and Asexual Reproduction". Variable list/description: Sample identification number; Well number; Treatment number; Temperature (°C); Salinity (‰); Time (weeks) (e.g., Time 0 indicates start of experiment and Time 6 indicates after 6 weeks; Total (total number of polyps counted); Proportion (proportional change in polyp density), this variable was calculated as Total polyps at Time 6/Total polyps at Time 0.
(CSV)

S2 Dataset. *Carukia barnesi* Polyp Count Data Associated With "Effects of Feeding Frequency on Survivorship and Asexual Reproduction". Variable list/description: Sample identification number; Time (weeks) (e.g., Time 0 indicates start of experiment and Times 1–6 indicate after 1–6 weeks; Well number; Temperature (°C); Salinity (‰);Total (total number of polyps counted); Proportion (proportional change in polyp density), this variable was calculated as Total polyps at Times 1–6/Total polyps at Time 0.
(CSV)

S1 R Code. The R Code Used for Analysis of "Thermal and Osmotic Effects on Survivorship and Asexual Reproduction".
(TXT)

S1 Table. Mean Values and Standard Errors of the Number of Polyps Present after Six Weeks Exposure to a Matrix of Eight Temperatures and Ten Salinity Treatments. All values were calculated as the relative change in polyp density and are presented as proportional change, where values above one indicate population increase through asexual reproduction and values below one indicate polyp mortality. There were six independent replicates for each treatment, therefore $n = 6$ for all means presented below and standard errors were calculated assuming a normal distribution.
(RTF)

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Author Contributions

Conceived and designed the experiments: RC JS. Performed the experiments: RC SB. Analyzed the data: RC TN JS. Contributed reagents/materials/analysis tools: RC JS. Wrote the paper: RC JS.

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APPENDIX C

Prey Capture Ecology of the Cubozoan *Carukia barnesi*

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RESEARCH ARTICLE

Prey Capture Ecology of the Cubozoan *Carukia barnesi*

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Abstract

Adult *Carukia barnesi* medusae feed predominantly on larval fish; however, their mode of prey capture seems more complex than previously described. Our findings revealed that during light conditions, this species extends its tentacles and 'twitches' them frequently. This highlights the lure-like nematocyst clusters in the water column, which actively attract larval fish that are consequently stung and consumed. This fishing behavior was not observed during dark conditions, presumably to reduce energy expenditure when they are not luring visually oriented prey. We found that larger medusae have longer tentacles; however, the spacing between the nematocyst clusters is not dependent on size, suggesting that the spacing of the nematocyst clusters is important for prey capture. Additionally, larger specimens twitch their tentacles more frequently than small specimens, which correlate with their recent ontogenetic prey shift from plankton to larval fish. These results indicate that adult medusae of *C. barnesi* are not opportunistically grazing in the water column, but instead utilize sophisticated prey capture techniques to specifically target larval fish.

Introduction

Cnidarians utilize a diverse array of food acquisition/prey capture strategies ranging from reliance on symbiotic zooxanthellae and filter feeding, to active prey capture with nematocyst laden tentacles [1–4]. Those that use nematocysts may implement simple prey capture strategies which rely on size and tentacle structure to opportunistically graze within the water column [5], while others use propulsion and induced swimming kinematics to increase potential prey and food particle contact with trailing tentacles [6]. Others, such as Cubozoans, are highly mobile and posses complicated visual structures, which have been hypothesized to play a role in prey capture [7–10].

Perhaps the most extreme prey capture strategy recorded so far is seen in Siphonophores, which use modified tentacles as 'lures' in a form of aggressive mimicry [11]. They actively

attract and lure specific prey types, either through resembling schooling conspecifics, or by mimicking the prey items of the targeted species [3,11–13]. Many Siphonophore lures not only mimic the appearance of other species but also their movements. Specifically, these lures are motile and are often moved using a 'jigging' or 'twitching' motion, which resembles the movements of specific prey types [3,11].

In many Cnidarians, the diurnal light-dark cycle often mediates a condition-specific behavioral response. For example, numerous Anthozoan and Siphonophore species extend their feeding tentacles only during the day while others only at night [14–16]. Similarly, many species of Hydromedusae and Scyphomedusae use light to facilitate vertical migrations in the water column in order to locate food, while others laterally migrate with the sun to increase solar exposure for symbiotic zooxanthellae [17,18]. Cubozoans also undergo a diurnal behavioral shift [19,20], and vision seems to be one of the factors involved in this diurnal differentiation in behavior [21,22].

Interestingly, a variety of visual systems are utilised by Cnidarians. These range from simple eye spots and pigment cup ocelli to advanced pigment cups with lenses [23–28], with the most advanced visual sensory structures belonging to the medusa stage of the Cubozoans [24,29]. However, there has been contention as to the usefulness of these complex eyes, due to the apparent lack of either neural branches to process the information [30], or a nervous system able to interpret visual images [31].

Cubomedusae do however exhibit many light mediated behaviors [32–34]. These include targeting light shafts for feeding [7], obstacle avoidance [35], actively swimming away from dark objects [36], or decreased activity at night [20]. However, the extent that vision is used in prey capture by Cubozoans is unknown. One highly venomous Cubozoan that possesses sophisticated visual organs is *Carukia barnesi* Southcott, 1967 [24,33,37,38]. The general ecology and biology of *C. barnesi* is not well understood. The medusa stage of this species is seasonally present coastally along north-eastern Australia typically from November to May each year. This species is considered oceanic and is found around coral reefs and islands, and under certain conditions, on beaches [39–42]. Juvenile *C. barnesi* feed predominantly on crustaceans and during maturation undergo an ontogenetic venom change, correlated with a prey shift, from planktonic invertebrates to larval fish [38].

Carukia barnesi have four sets of six 'eyes', which is typical among Cubozoans, consisting of a pair of simple light sensitive pigment cups, a pair of light sensitive pigment slits, and a pair of complex eyes that each has a cornea, a lens, and a retina [24,29,36,37]. However, the acuity and use of these eyes is unknown. The complete lifecycle and feeding ecology of this species is poorly understood and has not been described to date. This study describes part of the feeding ecology of the Cubozoan *C. barnesi* and aims to understand the mechanisms employed by this species to capture its prey.

Method

Species Description

Carukia barnesi is a small (approximately 20 mm bell-width), oceanic, planktonic Carybdeid that inflicts a potentially fatal sting that causes Irukandji Syndrome [39–43]. This species has four tentacles in total and each extends from a pedalia attached to each corner of the bell. These tentacles are up to 750 mm long and have an alternating pattern of large and small nematocyst clusters often referred to as nematocyst-bearing rings or crescents [38,44,45]. These are referred to in this paper as large and small 'nematocyst clusters'. The bell sizes of the specimens used in these experiments ranged from 8 to 21 mm niche bell (Nb) height (a longitudinal measure from the center of the rhopalial niche to the apex of the bell).

Specimen Collection

No specific permissions were required for the collection locations/activities as the species involved is not endangered or protected and the collection site did not require permits. Medusae of *C. barnesi* were collected near Double Island, North Queensland, Australia (16°43.5′S, 145° 41.0′E) during November 2013, between 1900 and 2200 h. To attract medusae, high-powered LED lights were submerged on each side of a small (five meter) research vessel. Medusae were captured as they approached the light and were transferred into individual 500ml plastic containers. The sea surface temperature varied from 27.5°C to 30°C with an average salinity of 35‰. The water depth at the capture sites varied between three to six meters. Post capture, specimens were transported to the laboratory and placed in a constant temperature controlled cabinet set at 28°C for a minimum of six hours prior to the commencement of experimental trials.

Experimental Tank

Specimens were housed in a purpose-built plankton kreisel (a circular tank [1170 mm X 400 mm wide with an effective volume of ~ 375 liters] in which seawater rotates vertically). Seawater was maintained at 35‰ and 28°C, to mimic oceanic conditions at the specimen capture site. A photoperiod of 13 h light:11 h dark was maintained, with the light period occurring from 0600 to 1900 h to simulate the local November photoperiod. The illumination cycle was achieved by fixing lights on each side of the kreisel that provided an average light intensity of 21μmol photons/s/m^2, and dark, achieved by turning the lights off with an electronic timer. An infra-red sensitive digital video camera was positioned approximately one meter from the face of the kreisel. Five infra-red spotlights, which remained on continuously, were positioned around the kreisel to allow for filming in darkness.

Size Dependent Tentacle Morphology

In order to determine the relationship between medusae bell size and tentacle length, two *C. barnesi* were placed into the kreisel around midday and allowed to acclimate for approximately six hours prior to each experimental trial. The specimens were then filmed for 24 hours (i.e., a full 13:11 light:dark cycle) beginning with the dark cycle. This was repeated three times, with newly captured specimens, resulting in 24 hour video sequences for six *C. barnesi* medusae. Recorded video sequences were subsequently analyzed in 30 to 60 minute increments, where each tentacle was measured from the pedalia to its terminal end. As identifying individual tentacles on each specimen between time sequences was impossible, all tentacles at any one time were measured and the mean tentacle length of each specimen was calculated. In order to elucidate whether larger specimens, with larger bells, had longer tentacles, regression analysis was used to determine the relationship between bell size (niche bell height mm) and the maximum recorded mean tentacle length of each specimen over 24 hours. Similarly, in order to quantify the relationship between bell size and the distance between the large nematocyst clusters, the distance between six consecutive large nematocyst clusters on an individual extended tentacle were measured to the nearest millimeter for each specimen. The mean large nematocyst cluster distance for each specimen was then calculated and regressed against animal size (niche bell height mm), to determine the relationship between bell size and the distance between the large nematocyst clusters.

The Influence of Light on Tentacle Extension

The effect of the light on tentacle extension (i.e., zero percent extension = shortest and 100% extension = longest tentacle length of each specimen) was determined at 0, 30, 60, 120, 240 and 360 minutes of exposure to both light and dark treatments. These values were arcsine square root transformed (to normalize proportional data) prior to analysis, which consisted of a two-way repeated measures ANOVA to determine if: a) light or dark; or b) the amount of time exposed to light or dark; or c) an interaction between the two, affected the percent tentacle extension. All statistical analyses were conducted using the statistics package IBM SPSS.

The Effect of Bell Size, Light and Tentacle Extension on 'Twitch Rate'

During the previous experiments, the extended tentacles would frequently contract in a jerking or 'twitching' motion, and then relax. To quantify the occurrence of these twitches, one minute sections of the video footage were analyzed in nine approximately 30-minute intervals for each specimen (i.e., 6 specimens measured 9 times each). The number of these contractions, or 'twitches', during each one minute sample was recorded. A 'twitch' was counted any time a tentacle rapidly contracted in a distinct pulse during each one minute measure, resulting in a value for 'twitch rate per minute' (see S1 Video). This was conducted for each specimen, and only footage from the light treatment was analyzed as twitching was not recorded in dark conditions (refer to Experimental Tank section). Linear regression was performed in order to determine if the rate of these twitches was affected by the state of a specimens tentacles (i.e., percent extended). Following this, to elucidate whether this relationship was driven by a specimen size effect (bell size), percent tentacle extension was pooled into 10 percent groups (i.e., 0–10%, 11–20%, etc.) and an ANCOVA conducted to determine if tentacle extension influenced the twitch rate, with bell size set as the covariate.

Prey Capture

On three occasions, two *C. barnesi* medusae were housed in the kreisel with approximately ten larval/juvenile fish (*Acanthochromis* sp.; approximately 10–15 mm in length). The larval/juvenile fish were reared, housed and used as outlined in the ethics approval "Approval for Animal Based Research or Teaching—A2061" (granted to Dr Jamie Seymour by the James Cook University Animal Ethics Committee). Breeding pairs of *Acanthochromis* sp. were held in a 3,000 liter tank and the larval/juvenile fish feed naturally on crustaceans, such as copepods and amphipods, which occur within the 50,000 liter recirculating aquarium system. All fish used were free feeding at the beginning of this study and were only held with the *C. barnesi* for approximately 10 minutes per feeding event. Fish that were stung were consumed by the *C. barnesi* and any fish that were not consumed were returned to the larval/juvenile fish tank. During these feeding events the fish were seen to rapidly move towards the twitching tentacles and were subsequently envenomed and caught. This process was captured on video on numerous occasions and still images were extracted to investigate the prey capture method implemented by *C. barnesi*.

Results

Size Dependent Tentacle Morphology

There was a statistically significant positive relationship between bell size (niche bell height mm) and tentacle length, where larger specimens had longer tentacles than smaller specimens ($R^2 = 0.796$, $F_{1,4} = 16.642$, $p = 0.017$) (see Fig 1). Conversely, the distance between the large

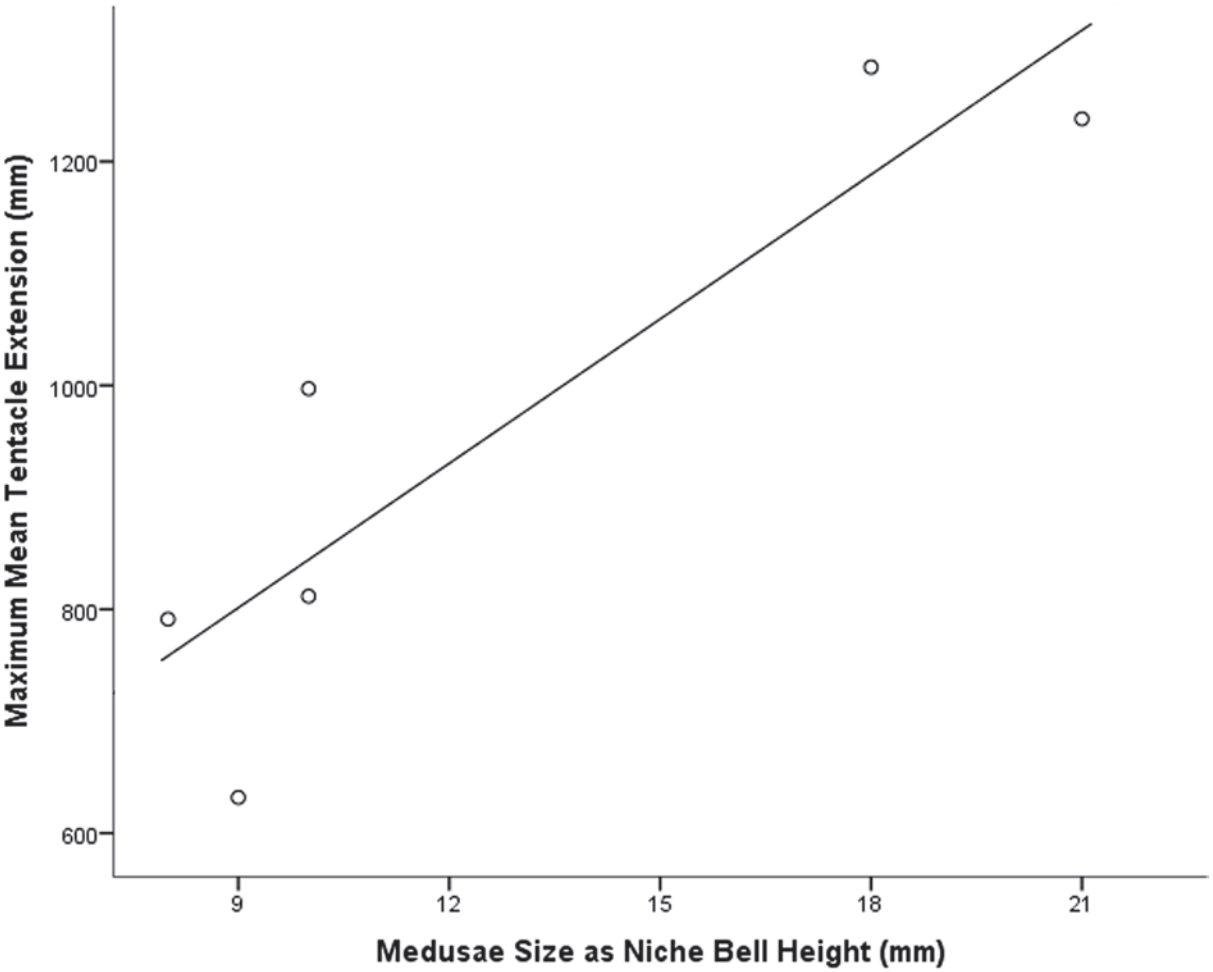

Fig 1. Maximum mean recorded tentacle lengths (mean length mm, n = 6) for *Carukia barnesi* over a range of medusae bell sizes (niche bell height mm).

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nematocyst clusters was not correlated with bell size ($R^2 = 0.003$, $F_{1, 4} = 0.13$, $p = 0.916$), with a mean distance of 30 mm (± 6 mm 95%Cl) on extended tentacles.

The Influence of Light on Tentacle Extension

Carukia barnesi tentacles were significantly longer during the light treatment than in the dark treatment ($F_{1, 5} = 7.112$, $p = 0.045$), while time had no significant effect ($F_{5, 25} = 0.216$, $p = 0.952$). However, there was a significant interaction effect between the light/dark treatments and time ($F_{5, 25} = 10.100$, $p < 0.001$). Tentacles contracted as the dark treatment progressed reaching a contracted state of less than 20% after approximately two hours. After the lights were turned on, tentacles began to extend reaching maximum mean extension of approximately 80% after approximately six hours exposure to the light treatment (see Fig 2).

The Influence of Bell Size, Light and Tentacle Extension on 'Twitch Rate'

There was a significant positive relationship between tentacle extension and twitch rate, with elongated tentacles twitching more frequently (twitches per minute) than retracted tentacles ($R^2 = 0.492$, $F_{1, 53} = 50.397$, $p < 0.001$) (see Fig 3), while bell size also effected the twitch rate

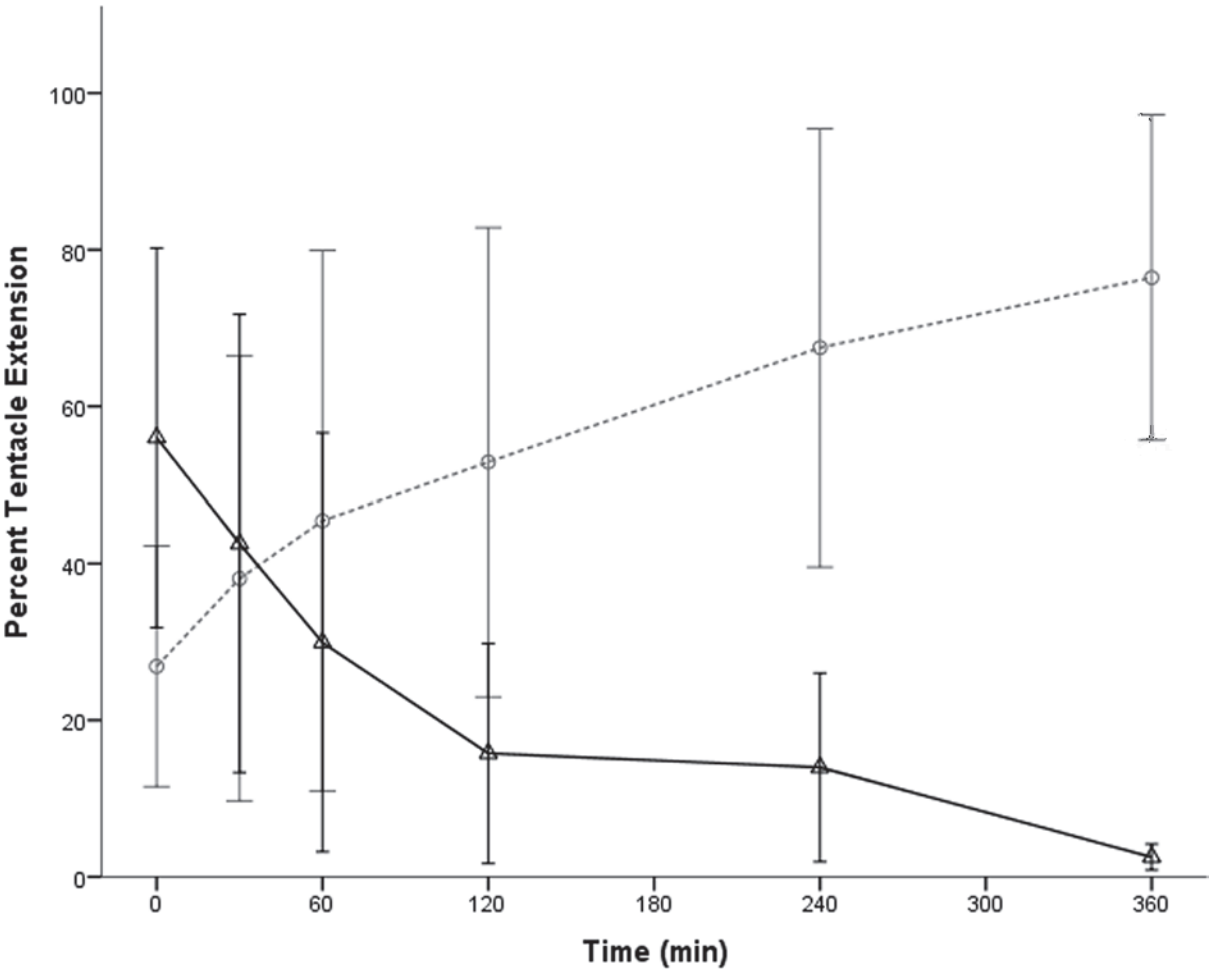

Fig 2. *Carukia barnesi* mean tentacle extension, as a percentage, over 360 minutes, exposed to light (○, dashed line) and dark (△, solid line) treatments. Error bars represent 95% confidence intervals (n = 6).

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($F_{4, 54} = 2.718$, $p = 0.040$), where larger animals 'twitch' their tentacles more frequently than small specimens (see Fig 4). The average twitch rate during the light was 6.3 twitches per minute however no twitching was recorded during the dark.

Prey Capture

Larval fish (*Acanthochromis* sp.) were often attracted to the nematocyst clusters on the extended fishing tentacles of *C. barnesi* especially when they were being 'twitched'. Fish would pursue these clusters and become 'stung' around the mouth region or head, resulting in death (see Fig 5 and S2 Video).

Discussion

Adult *C. barnesi* are known to feed almost exclusively on larval fish [38]; however, their mode of prey capture seems more complex than previously described. Our findings suggest that *C. barnesi* are active predators that capture visually orientated prey, in this case larval fish, by using a lure-like system to simulate the size and movements of the fish's prey (e.g., small plankton). This method of prey capture has previously been described in some Siphonophores

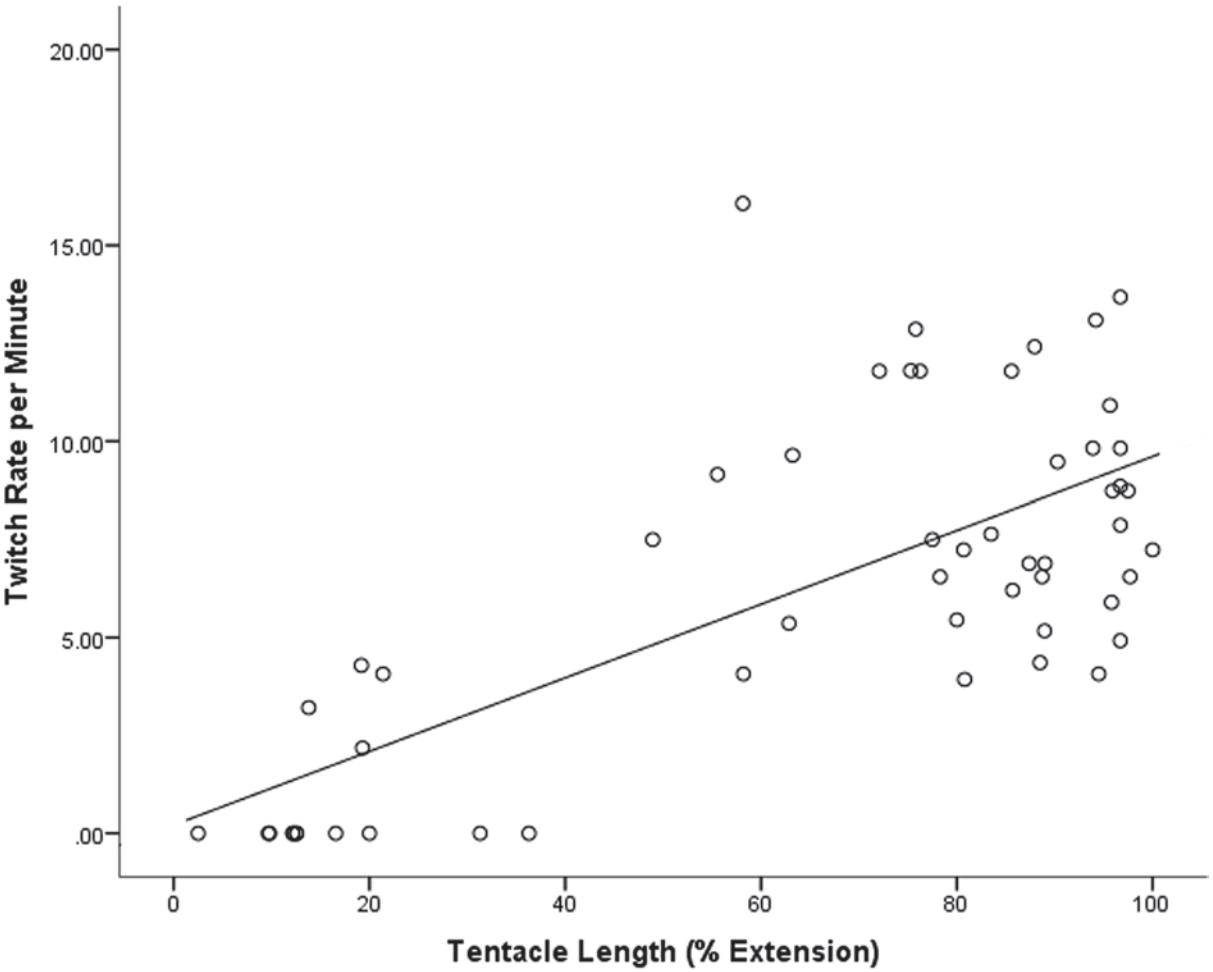

Fig 3. The number of tentacle twitches recorded for *Carukia barnesi*, over one minute intervals (twitch rate per minute), plotted against different tentacle extensions (percent extension). Video footage was analyzed in approximately 30 minute intervals (i.e. 6 specimens measured 9 times each).

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[3,11], and considered unique compared to other Cnidarians. Many larval fish are visual hunters and because of this feed predominately during light conditions [46], and this correlated well with the luring behavior seen in *C. barnesi* that occurs only during daylight hours. Luring at night would be less efficient resulting in reduced prey capture and contracting their tentacles at night would decrease swimming induced drag, thus reducing energy expenditure. This suggested feeding cycle is consistent with the diurnal feeding cycle observed in another Cubozoan (i.e., *Chironex fleckeri*, Southcott, 1956), which is known to become inactive at night to conserve energy [20].

The nematocyst clusters along the extended tentacles are also motile, where *C. barnesi* 'jig' or 'twitch' these tentacles frequently. Fish, including larval fish, are known to be attracted to prey by movement, and may preferentially attack prey items of specific sizes [47]. In order to increase catch rates, twitching, or movement of the nematocyst clusters, would appear to serve this purpose, where movement of the nematocyst clusters would highlight these lures in the water column. Once larval fish are attracted to these nematocyst clusters they are consequentially 'stung' and consumed. Furthermore, larger *C. barnesi* were found to twitch their tentacles more frequently than smaller specimens, which may be related to their recent ontogenetic transition from planktonic to vertebrate prey [38]. Smaller medusae (under 8 mm) have a

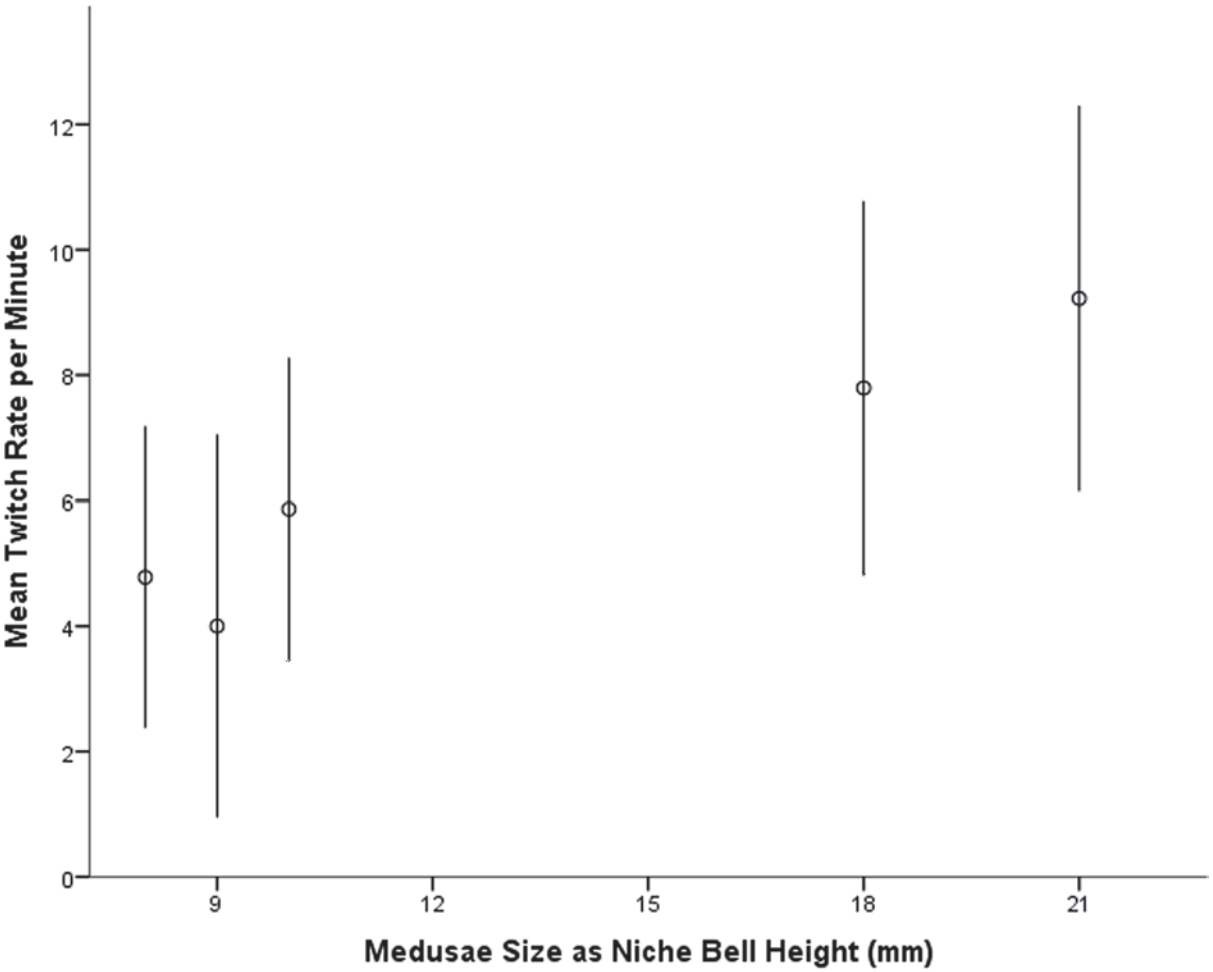

Fig 4. The mean number of tentacle twitches recorded for *Carukia barnesi*, over one minute intervals (twitch rate per minute) plotted against medusae bell size in millimeters. Error bars represent 95% confidence intervals (n = 6).

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preference for plankton, and these prey items are almost certainly captured in a similar manner as used by other Cnidarian medusae, that is, by haphazardly encountering prey in the water column. As such, twitching of tentacles, which presumably increases energy consumption, would be inefficient for the capture of small plankton, which may explain the lower twitch rate observed in the smaller specimens (8–10 mm).

Not surprisingly, larger medusae were found to have longer tentacles, which would presumably increase their chance of prey capture and correlates with the change in prey preference from plankton to larval fish. However, it was surprising that the distance between the nematocyst clusters, or lures, was similar regardless of the length of their tentacles. This suggests that the distance between the nematocyst clusters are important for the visual stimulation of prey and/or to optimize prey capture (i.e., the lures are set at an optimum distance for prey capture). The function of the alternation between large and small nematocyst clusters along the tentacles is unknown; however, they may be used to target different sizes of larval fish by presenting a choice of lure/food particle sizes. Further research is required to determine the specific function of the large and small nematocyst clusters.

Cubomedusae have been shown to be more sophisticated in many areas of their ecology than most other Cnidarians. For example, they have elevated swimming speeds [48,49], greater

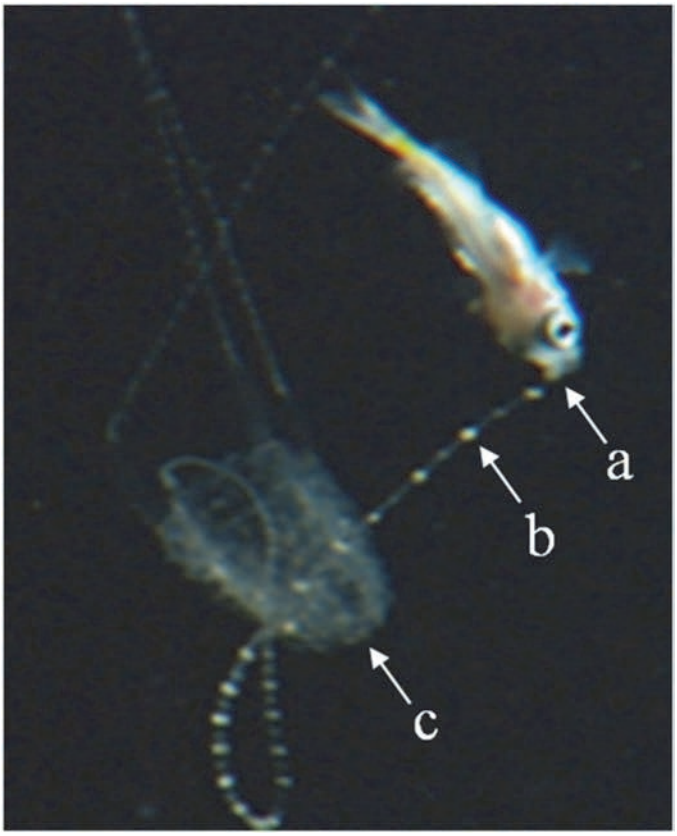

Fig 5. Envenomation of a larval fish (*Acanthochromis* sp.) that was captured by a twitching tentacle of an adult *Carukia barnesi*. a: envenomation site; b: nematocyst cluster; c: bell. The bell size of this specimen is approximately 15 mm in height and the fish is approximately 10 mm in length.

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vision capabilities [24,33,50], more sophisticated behaviors (e.g., sleeping) [19,20] and highly toxic venoms [40,43,51]. In conclusion, this research has demonstrated *that C. barnesi* utilize sophisticated prey capture techniques to actively lure prey. Future investigation into other species of Cubomedusae is now required to determine if they too employ sophisticated prey capture mechanisms.

Supporting Information

S1 Video. Quantification of a 'twitch' of the tentacles of *Carukia barnesi*. Note the two distinct twitch events during this 11 second video sequence. The first twitch event occurs after approximately one second of elapsed time. The second twitch event occurs eight seconds later.
(MP4)

S2 Video. Envenomation of a larval fish (*Acanthochromis* sp.) that was captured by a twitching tentacle of an adult *Carukia barnesi*. The bell size of this specimen is approximately 15 mm in height and the fish is approximately 10 mm in length. Note the envenomation site is on the head region of the fish.
(MP4)

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Author Contributions

Conceived and designed the experiments: RC NS JS. Performed the experiments: RC JS. Analyzed the data: RC RJ JS. Contributed reagents/materials/analysis tools: RC JS. Wrote the paper: RC NS RJ JS.

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APPENDIX D

S1 Table. **Mean Values and Standard Deviations of the Number of Polyps Present after Six Weeks Exposure to a Matrix of Eight Temperatures and Ten Salinity Treatments.** All values were calculated as the relative change in polyp density and are presented as proportional change, where values above one indicate population increase through asexual reproduction and values below one indicate polyp mortality. There were six independent replicates for each treatment, therefore $n = 6$ for all means presented below and standard errors were calculated assuming a normal distribution.

	11ºC	14ºC	18ºC	21ºC	25ºC	28ºC	31ºC	34ºC
16‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.20$ $SE = 0.11$	$\bar{x} = 0.76$ $SE = 0.11$	$\bar{x} = 0.01$ $SE = 0.01$	$\bar{x} = 0.01$ $SE = 0.01$	$\bar{x} = 0.00$ $SE = 0.00$
19‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.45$ $SE = 0.11$	$\bar{x} = 1.36$ $SE = 0.17$	$\bar{x} = 1.25$ $SE = 0.09$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$
22.5‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 1.65$ $SE = 0.22$	$\bar{x} = 3.66$ $SE = 0.38$	$\bar{x} = 8.96$ $SE = 1.32$	$\bar{x} = 1.85$ $SE = 0.21$	$\bar{x} = 1.56$ $SE = 0.43$	$\bar{x} = 0.00$ $SE = 0.00$
26‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 1.76$ $SE = 0.19$	$\bar{x} = 3.78$ $SE = 1.06$	$\bar{x} = 7.29$ $SE = 0.96$	$\bar{x} = 1.84$ $SE = 0.19$	$\bar{x} = 2.17$ $SE = 0.12$	$\bar{x} = 0.00$ $SE = 0.00$
29‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 2.86$ $SE = 0.24$	$\bar{x} = 4.19$ $SE = 0.34$	$\bar{x} = 3.05$ $SE = 1.01$	$\bar{x} = 1.85$ $SE = 0.20$	$\bar{x} = 1.83$ $SE = 0.29$	$\bar{x} = 0.00$ $SE = 0.00$
33‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 3.14$ $SE = 0.50$	$\bar{x} = 4.17$ $SE = 0.86$	$\bar{x} = 3.13$ $SE = 1.00$	$\bar{x} = 2.25$ $SE = 0.16$	$\bar{x} = 2.03$ $SE = 0.40$	$\bar{x} = 0.00$ $SE = 0.00$
36‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 3.09$ $SE = 0.37$	$\bar{x} = 2.26$ $SE = 0.61$	$\bar{x} = 4.92$ $SE = 0.31$	$\bar{x} = 1.84$ $SE = 0.14$	$\bar{x} = 1.77$ $SE = 0.20$	$\bar{x} = 0.00$ $SE = 0.00$
39‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 2.94$ $SE = 0.28$	$\bar{x} = 3.48$ $SE = 0.52$	$\bar{x} = 4.63$ $SE = 1.09$	$\bar{x} = 1.76$ $SE = 0.21$	$\bar{x} = 1.50$ $SE = 0.24$	$\bar{x} = 0.00$ $SE = 0.00$
42.5‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 1.66$ $SE = 0.42$	$\bar{x} = 4.84$ $SE = 0.74$	$\bar{x} = 3.76$ $SE = 0.95$	$\bar{x} = 1.94$ $SE = 0.10$	$\bar{x} = 1.65$ $SE = 0.19$	$\bar{x} = 0.00$ $SE = 0.00$
46‰	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 0.00$ $SE = 0.00$	$\bar{x} = 2.16$ $SE = 0.35$	$\bar{x} = 2.00$ $SE = 0.40$	$\bar{x} = 3.14$ $SE = 0.27$	$\bar{x} = 1.72$ $SE = 0.11$	$\bar{x} = 1.03$ $SE = 0.23$	$\bar{x} = 0.00$ $SE = 0.00$