ECOLOGY, SYMBIOSIS AND COMMUNITY IN SCYPHOZOANS WITH AN EMPHASIS ON THE FLORIDIAN *CASSIOPEA*

A Dissertation

by

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ABSTRACT

True jellyfishes of the class Scyphozoa exhibit a range of unique biological traits and are intrinsically linked to the ecosystems in which they reside. Despite this, their ecology, systematics and biology have long been understudied. Examining the photosynthetic jellyfish *Cassiopea*, I connect the biology, systematics and microbial ecology that inform the broader ecology of the genus. In this work, I demonstrate that *Cassiopea xamachana* ephyrae (the more motile immature stage) can survive for upwards of 6 weeks unfed when provided with light, a trait that may allow for higher dispersion. Examining wild *Cassiopea*, I identify the endemic species *C. xamachana* cooccurring with the non-native *C. andromeda* based on mitochondrial *COI* and *16S*, as well as correct problems with the previously published *Cassiopea* global phylogenies. Using these same wild medusae, I describe for the first time the wild microbiome of *Cassiopea xamachana* within the Florida Keys. While the external microbiome of *Cassiopea* medusae is similar to the benthic community around it but enriched in *Endozoicomonas*, the internal community is low diversity with multiple possible stable states primarily enriched in *Endozoicomonas, Mycoplasma* and *Vibrio.* In addition to my work on *Cassiopea*, I provide a thorough review of associative interactions previously reported between jellyfish and crustaceans, covering 211 distinct associations, in order to facilitate future work on community ecology in jellyfishes. Altogether, this work provides new information on the ecological value in jellyfishes overall and greatly improves knowledge of the ecology of *Cassiopea* specifically.

DEDICATION

This work is dedicated to all who have raised me, both as a scholar and as a person:

To my mother and father—You encouraged my love of cnidarian biology from an early age and encourage me still.

To Drs. Allen Collins and Cheryl Lewis Ames— You provided me with my first exposure to research and to the genus *Cassiopea* and continue to collaborate with me and support my growth into a better scientist. Thank you for everything.

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Contributors

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Collaborator Joleen Aulgur assisted with data collection for Chapter 3. Professor Jessica Labonté assisted with method development and execution for Chapter 5.

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NOMENCLATURE

GVC	Gastrovascular cavity

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1. INTRODUCTION

1.1. General

Invertebrate-invertebrate and invertebrate-microbiome interactions play a central role in the world's oceans[1–3]. Despite this, comparatively little scientific attention has been devoted to their study. Scyphozoans (phylum Cnidaria) and other soft pelagic invertebrates are difficult to catch undamaged, require specialized containment, and have little economic value (aside from a few edible jellyfish of the order Rhizostomeae)[4]. Given these traits, work on their fine-scale ecologies has been limited. The lack of work in this area has obscured the degree to which scyphozoans are providing habitat, interacting with their environments, and fostering unique microbial communities.

Scyphozoans are present throughout both coastal and pelagic marine habitats. In these ecosystems, they provide utility to other organisms in a variety of ways. Best understood among these is the use of adult scyphozoans as nursery habitat, though this can come in many forms[5]. For example, in the case of juvenile carangid fishes and *Chrysaora*, the fish receive protection, then gradually consume their host [6]. In juvenile Walleye Pollock, use of *Chrysaora* for protection is limited to the daytime [7]. *Gadus* and *Caranx* juvenile fish target the gonads of *Cyanea* while living as ectoparasites [8].

Beyond nursery habitat, medusae are parasitized by ophioroids, *Ophiocnemis marmorata,* barnacles, *Alepas* spp. and lobster phyllosoma[8–11]. Medusae are now known to be consumed by fishes and birds in high numbers[12–14]. In some locations, gelatinous zooplankton constitute a primary component of the local food web through predation[15].

In addition to macroscale trophic interactions, medusae have profound impacts on plankton, biogeochemical cycling and microbial community. Carrion falls of *N. nomurai* transports large amounts of organic matter and changes local microbial communities[16–18]. For *Aurelia*, decomposition promotes a new bacterial community, and all documented medusae have distinct microbial communities from the water column surrounding them[19,20]. Here as well we have few representative individuals and few species on which to base our understanding of scyphozoan species' relevance.

Basics of ecology, including symbiosis, commensal interaction, microbial interaction, recruitment, and systematics have been understudied for Scyphozoa, despite their relevance as key factors in the survivorship and ecologies of better studied vertebrates. This knowledge gap can only be closed through a concerted effort to fully understand the ecologies of some of the most common scyphozoan genera. One genus whose widespread distribution lends itself to easy study is *Cassiopea.*

1.2. Symbiosis within Scyphozoa

Symbiosis is present throughout Cnidaria and found frequently within the coastal representatives of the Medusozoa. Scyphomedusae such as *Cotylorhiza tuberculata*, *Linuche unguiculata* and *Cassiopea andromeda* all harbor photosymbionts, but they are not unique [21–23]. Up to 25% percent of scyphozoan species may be facultative or obligate symbiont hosts [24]. For most of these medusae, the symbiosis is not obligate, with the exception of the *Cassiopea* species complex. These symbioses are largely formed with individuals in the polyp stage (excluding *Linuche*) and thought to provide the medusae with the majority of their carbon budgets [24].

Within the Kolpophorae (a Rhizostome medusa clade including the genera *Cassiopea*, *Mastigias* and *Versuriga* along with others), symbiosis is acquired through endodermal acquisition and cells are stored in mesoglear amoebocytes (mesoglea being the extracellular matrix that comprises the internal support structure of cnidarians) during polyp, ephyra and medusa stages[24]. Medusae have distinct internal microenvironments modified symbiosis[25,26]. In genera like *Mastigias*, behavioral modifications like diel migration and shadow avoidance help to optimize both photosynthetic potential and off-hour heterotrophic nutrient acquisition[27].

These symbiotic jellyfishes remain underexplored relative to asymbiotic species, despite their unique adaptations and capacities. These symbioses likely have strong connections to geographic region, systematic position, and microbial communities of the medusae.

1.3. Microbial interaction within Scyphozoa

Within the simple blind cnidarian gut and tissues, a distinct array of bacteria persist. In samples from East Asian waters, *Aurelia* (a Semaeostome) has *Vibrio*-dominated tissue microbiome, while *Mycoplasma* was more common in *Nemopilema* and *Rhopilema* samples (both Rhizostome jellyfish)[28]. In waters off of the northeastern coast of the US however, sampled *Aurelia* were *Mycoplasma*-dominated[29]. Even at smaller scales, microbial communities associated with a genus are often geographically isolated. For instance, *Mastigias* medusae within the Kakaban, Haji Buang, and Tanah Bamban lakes of Palau varied in their primary fraction, with high *Endozoicomonas* abundances in individuals from Tanah Bamban but not Kakaban Lake[30]. Despite this,

many key players are found repeatedly, namely *Vibrio, Tentibaculum, Endozoicomonas* and *Mycoplasma,* but limited replicates make broad statements on community composition difficult [20]. With the exception of *Tentibaculum,* the genera listed above are also all common components of previously sequenced coral microbial communities[31].

These microbial communities are core to scyphozoan development. Some species will not settle without bacterial cues, others will develop with deformities in the polyp itself or in the strobila. This has been demonstrated in both *Cassiopea* and *Aurelia*[32,33]. Altered microbial communities result in both increased polyp mortality and transcriptional changes related to defense in *Aurelia*[33,34]. Modifications to microbial community and nutrient availability in *Aurelia* ephyrae significantly improves limb regeneration[35]. Unfortunately, little work has been done on identification and experimental modification of adult scyphozoan microbiomes, so their importance is less well established. Nevertheless, the microbial communities associated with jellyfish impacts at least their growth and development, making microbial community analysis crucial to better understand the dynamics of jellyfish growth, development and bloom formation. As much recent work on microbial community has focused on *Aurelia,* a non-Semaeostome test case for gut microbiome is required to better understand the 10 to 25% of jellyfishes who are facultatively or obligate symbiotic. *Cassiopea* is an example of an accessible and unique genus that can be used for this approach.

1.4. The genus *Cassiopea*

Within the Florida Keys, South Florida and throughout Central America and the Caribbean, *Cassiopea* medusae are sporadically present *en masse* on shorelines[36]. The

family *Cassiopea* belongs to (Cassiopeidae) includes an unknown number of largely morphologically cryptic species who all sit apex-down on near-shore substrates (often less than 2 m below the surface) and produce a large amount of their necessary nutrition through a long term obligate symbiosis with various Symbiodiniaceae clades[37]. These jellyfish are popular in the aquarium trade and may contribute to pore water flux in the areas in which they reside[38].

Cassiopea spp. are mangrove-dwelling scyphozoans present throughout the globe's tropics and hold particular interest for study. They exhibit a unique biology and an extremely specialized ecological niche. Recent research has shown that species of *Cassiopea* are potential bioindicators in mangrove ecosystems and have an array of notable physiological and genetic traits, such as "sleep-like" nighttime behaviors[39], ever-extending telomeres[40], autonomous conglomerations of stinging cells capable of incapacitating prey in the surrounding water[41], and exceptionally plastic relationships with endosymbiont *Symbiodinium*[42]. Despite these novel traits, the taxonomy of the genus *Cassiopea* and its species is unexplored for the continental United States. The genome of *Cassiopea xamachana* from Florida has been published[43], however there is some indication that Florida hosts several cryptic species[37]. The only published phylogeny of the genus, Holland et al. 2004, included a small sample size and a primary focus on samples collected from Oahu, Hawaii[37] and depicted a messy taxonomy with current species designations largely unrelated to genetic closeness. Furthermore, *Cassiopea* has recently been reported as introduced along many coastlines, with reports in the Mediterranean, Brazil, New South Wales and additional

locations[36,44,45]. Significant research in the US is conducted on nominal *C. xamachana*, *C. andromeda* or unidentified *C.* spp., usually without genetic barcoding to be used for later reference. This implies that scientists may be publishing on a range of species, potentially a mix of native and invasive, while treating them as a single cohesive taxonomic unit.

Cassiopea's symbiosis is obligate, the adults are unable to retain mass without either *Symbiodinium* or *Breviolum*[46,47]. They have high photoplasticity, capable of both highly irradiated and low light areas[42]. Additionally, they are easy to maintain in their symbiotic state within low-cost lab tanks[38]. Despite this, there is little information on the importance of this symbiosis to medusa growth and development. In lab settings, symbiotic state has been directly tied to microbial communities through internal and mucosal studies[48,49]. This may also be the mechanism through which *Cassiopea* control symbiont access to resources[50]. As symbiosis and microbial community are tied within *Cassiopea*, the absence of any data on wild microbial community hampers improved understanding of symbiosis within the species.

This genus, omnipresent in the Florida Keys, also provides a study subject for microbial, symbiosis ecology and population structure work on a non-seasonal scyphozoan.

1.5. Broader Impacts and Connections

Years of tautology held jellyfishes and other soft-bodied gelatinous zooplankton in low regard, as trophic dead-ends or inconsequential[51]. This is not an accurate

representation of their importance to the ecosystems in which they reside, they are prey[52], predator[53], nursery[54], and vector[16].

This dissertation completes four primary tasks that expand information on the class Scyphozoa and adds ecological context for future work. First, I aggregate information on crustacean/pelagic cnidarian commensal interactions reported from the past 150 years into one accessible datasheet (Chapter 2). Second, I demonstrate the impact of symbiotic state on development in model *C. xamachana* (Chapter 3). Third, I demonstrate that there are multiple species in the same niche within the Floridian shallow-water *Cassiopea* (Chapter 4). Fourth, I describe the microbial community associated with *Cassiopea*, a community closely related to other symbiotic cnidarians, in the first region-level examination of microbial community from any scyphozoan (Chapter 5). These chapters all tackle questions of symbiosis and ecology in jellyfishes differently, but they all operate as useful building blocks towards future research within scyphozoan ecology.

2. PLANKTONIC ASSOCIATIONS BETWEEN MEDUSAE (CLASSES SCYPHOZOA AND HYDROZOA) AND EPIFAUNAL CRUSTACEANS[*]

2.1. Question and Hypothesis

Question: What basic services and use cases are gained by crustaceans from medusae in natural environments?

Hypothesis: Medusa-crustacean interactions exhibit a pattern of use that includes a large proportion of the near-shore gelata species but provides primarily short-term amorphous benefits for coastal and epipelagic species.

2.2. Background

An increased focus on ocean climate research in the past twenty years has made clear the fragility of the world's oceans and the organisms that live within them. The rate at which species are disappearing, undergoing climate-related range fluctuations, and experiencing developmental and behavioral changes is unlike anything seen in the time of record[55–57]. Attempts to model changes in populations, species, and ecosystems have laid bare the degree to which dynamics among many marine invertebrates remain unknown and poorly understood[52,58,59]. This problem is especially apparent in jellyfish of the phylum Cnidaria, which are chronically understudied and poorly categorized[17,60–62]. Long considered a pure pest, the last decade has demonstrated an increasing number of

[*] As published in Muffett, K., & Miglietta, M. P. (2021). Planktonic associations between medusae (classes Scyphozoa and Hydrozoa) and epifaunal crustaceans. PeerJ, 9, e11281. https://doi.org/10.7717/peerj.11281

ways in which jellyfish are critical components of the ecosystems they reside in[63,64]. While they are best known for the vertebrates that depend on them for nutrition, including turtles and birds, they provide a host of ecosystem services unrelated to a "prey" designation. Reef and non-reef fish juveniles readily congregate around large scyphozoans, some hiding within the bell or between tentacles when disturbed[7,65,66]. Large jellyfish can reach sizes that allow them to support independent encrusting organisms, like barnacles and brittle stars[10,67,68].

While research has expanded around services jellyfish provide[5], much of this research focuses on benefit and harm to vertebrates[7,63,69]. However, the relationships between scyphomedusae, hydromedusae and other invertebrates are currently poorly characterized. A prime invertebrate group to analyze through this lens is Crustacea. Crustaceans are some of the most visible and well-studied marine invertebrates. They are present in every region and are integral components of food webs, including species of high commercial value and known ecological significance[2]. Ecological processes that impact them are thus relevant to humans. However, studies focusing on epifaunal crustaceans and jellyfish interactions have been scarce, incomplete, and taxonomically imprecise. Moreover, such studies are often narrowly focused accounts of interactions with single individuals[10,70,71]. Some early communications discuss these interactions as common knowledge that has, however, failed to be recorded in the scientific literature[72]. This review provides a list of documented crustacean epibionts on medusae of the orders Scyphozoa and Hydrozoa. This work aims to assess the breadth and depth of jellyfish-crustacean interaction and develop a resource for further studies.

2.3. Methodology

Four independent sets of searches were conducted in Google Scholar using keywords, as described in fig. 2.1. All four searches were conducted in early November 2019 and were revisited in January 2021 to include all results through the end of 2019. Searches were performed in English, and as such, only papers published in or with an available translation to English were included. The number of papers yielded by each of the four searches is shown in Fig. 2.1, ranges from 4,840 articles (for keywords Crustacea, Scyphozoa) to 13,300 (for keywords Crustacea, Jellyfish) (See Fig. 2.1 for details). Only papers in which the primary focus was associations between medusae (Hydrozoa and Scyphozoa) and crustaceans were further selected.

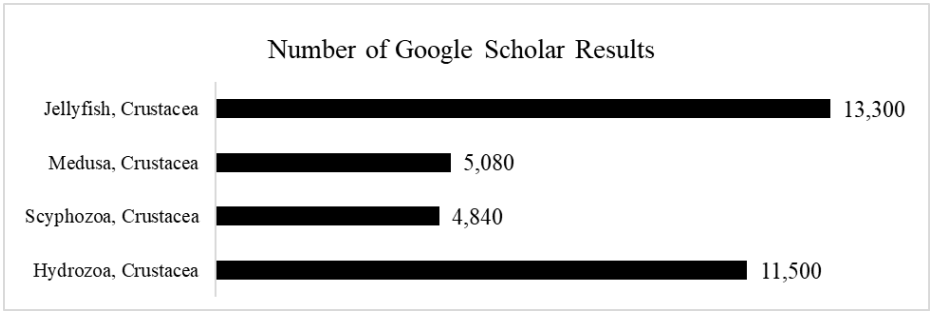

Figure 2.1 Search Results Analyzed. The number of results reported by Google Scholar Advanced Search where both "Crustacea" and one of the four medusa describer terms was included ("Hydrozoa", "Scyphozoa", "medusa", or "jellyfish") and at least one of the following terms was included (Association, Associated, Symbiotic, Symbiosis, Commensal, Epifaunal, Harboring, Parasitic, Parasitoid, Epibiont or Epibiotic).

The four searches performed returned many invariable results. All titles and abstracts were checked for relevance. Results from 161 papers were obtained initially and then narrowed to 81, after excluding repeat papers mistakenly included

multiple times and papers on cubomedusae, ctenophores, ascidians, and non-crustacean epibionts. Also, results from six relevant literature reviews were included[9,73–77]. These reviews account for 40 interactions from 29 sources (Appendix A: Table 2.1). The inclusion of the literature reviews was deemed essential to include results from earlier sources and non-English sources not available on Google Scholar. Results from literature reviews that had no information on the nature of the interaction between the medusa and crustaceans (such as taxa identification, location, etc.) were eliminated. Records were also analyzed for taxon validity using the World Register of Marine Species (WoRMS). Seven papers within the database that referred to invalid taxa with no valid synonymized name in WoRMS were removed. Results from 97 unique sources (68 articles from the Google Scholar search and 29 from literature reviews) were kept. From these 97 sources, 211 distinct interactions were extracted. Details provided by each paper were recorded in Table 2.1.

2.4. Results and Discussion

The final table produced by this review process includes 211 recorded interactions between hydrozoan or scyphozoan medusae and crustaceans, extracted from 97 papers on a variety of topics (available as Table 1 in [http://doi.org/10.7717/peerj.11281]). For both cnidarians and crustaceans, order, family, genus, and species are included in Supplementary Materials available at [https://doi.org/10.7717/peerj.11281]. Results that lacked taxonomic identification (at least Family level) were not included. The full table (available as Table 1 in [http://doi.org/10.7717/peerj.11281]) provides sampling information, such as year and month of sampling, sampling method, and region of

sampling. For crustaceans, records include the life stage involved in the interaction, sex of the epibiont, location on the hosts, and additional notes, if available. In most studies, fewer data were available on the cnidarian hosts, reducing the degree to which these interactions could be analyzed in terms of hydromedusan or scyphomedusan life stage. In the next paragraphs, we discuss the jellyfish-crustacea interactions through all of the categories included.

2.4.1. Diversity

2.4.1.1. Diversity of scyphozoan hosts

A supermajority of records (70%, or 148/211) involves Scyphomedusae, with 53 records involving just the five most common scyphozoan species: *Lychnorhiza lucerna (*Haeckel, 1880), *Catostylus mosaicus* (Quoy & Gaimard, 1824), *Stomolophus meleagris (*Agassiz, 1860), *Cyanea capillata (*Linnaeus, 1758) and *Rhopilema hispidum* (Vanhöffen, 1888). These records are heavily concentrated in the upper water column. Deeper water collections (ROV/HOV) were dominated by hydromedusae (69%, or 27/39), while records involving the upper water column (0-30 m) were more common and dominated by scyphomedusae (78%, or 83/106). Sixty-seven records included no specific sampling depth. These records were generally more than 50 years old. Although they are likely near-surface sampling records and mainly report known shallow-water species, they cannot be verified as such because of the lack of explicit information. Most of these (87%, or 58/67) are records of scyphomedusae. Overall, the diversity of scyphomedusae was low, with only 39 species from 27 genera represented in records (Fig 2.2a). The genus *Chrysaora* had the largest contingent of accounts, with 21

individual records of associations across at least seven *Chrysaora* species. This genus has been reported to interact with 16 different epifaunal crustaceans. The genera *Chrysaora*, *Lychnorhiza*, and *Catostylus* accounted for a third of scyphozoan records. These records originate mainly from the upper water levels of various locations (i.e., the east coast of the United States, the southeast of Brazil, the southern Australian coast, and the western Philippines, Japan and Pakistan).

2.4.1.2. Diversity of hydrozoan hosts

Twenty-six genera, and six Hydrozoan orders were reported in association with Crustacea in 63 records (Fig 2.2b). The order Leptothecata included the greatest number of records (18), with 17 records of Siphonophorae and 12 of Narcomedusae. The diversity of Hydrozoa was significantly limited by region, with 45 of the 63 records (71%) from the Gulf of California. Additionally, those from the Gulf were acquired from primarily deep water ROV missions. The medusae recorded belonged to 28 known species, with twelve records unable to provide higher resolution than genus and a single Prayid siphonophore only identified to the family level. *Rosecea cymbiformis (*Delle Chiaje, 1830) *(*4), *Aegina citrea (*Eschscholtz, 1829) (5), and *Aequorea coerulescens (*Brandt, 1835) *(*6) were the three most common species.

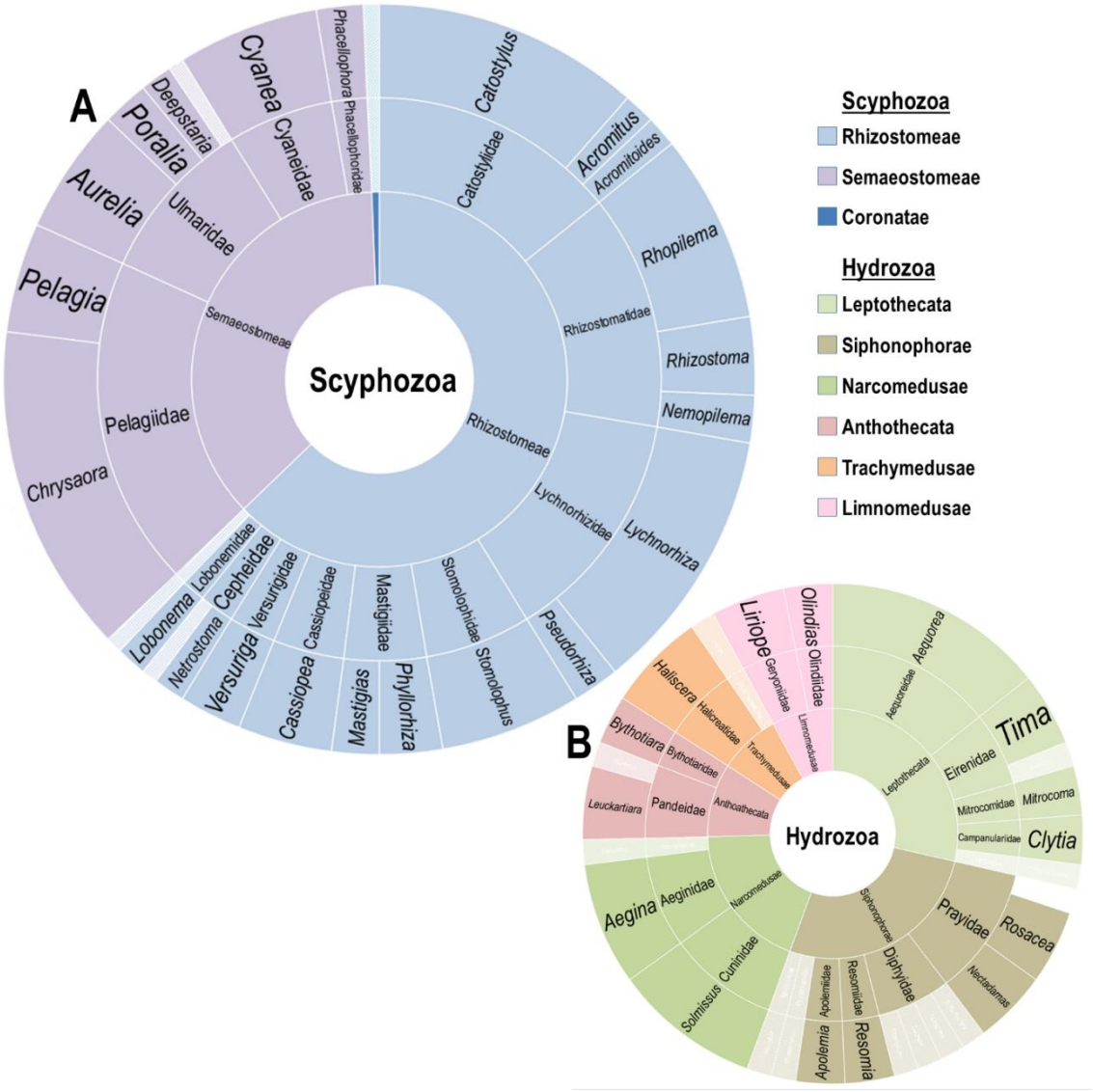

Figure 2.2 Diversity of Scyphozoa and Hydrozoa species. Rings from innermost to outermost are order, family, genus in the classes Scyphozoa (2a) and Hydrozoa (2b) as distributed by number of accounts including a host in that group. Families and genera with single reports are whitened.

2.4.1.3. Diversity of crustacean epibionts

The crustaceans included Hexanauplia (reported in 37 discrete observations), Malacostraca (173), and a single representative of Branchiopoda (*Evadne* sp.) (Fig. 2.3). Recorded Hexanauplia consisted of mainly specialist groups known to be obligate

epibionts and had overall low species resolution, with 13 of the 23 documented associations lacking a species name. The Macrochironidae, a group of known scyphozoan parasites, makes up 12 of the copepod epibiont records. Outside of this family, no additional Hexanauplia epibiont was recorded more than twice. The single reported case of a medusa with *Evadne* sp. occurred in a broad analysis of items found on a *Catostylus* medusae[78]. As this was not replicated throughout medusae within the study, or in other studies, it is unlikely this is a common or genuine association.

The bulk of the associations involve crustaceans of the class Malacostraca. These 173 records include amphipods and decapods in equal proportion (47%, or 81/173 each), isopods (5%, or 9/173), and mysids (1%, or 2/173). The amphipods are dominated by the parasitic family Hyperidae, recorded in 32 separate encounters. Members of the family of Hyperidae are present across 22 identified scyphozoan and hydrozoan species, making them the most widely distributed family. *Hyperia galba* (Montagu, 1813) is present in nine records from both surface and deep-water samples, making it the single most plentiful within the amphipods. Outside of the family Hyperidae, *Tryphana malmii* (Boeck, 1871) is recorded six times in association with deep-sea jellyfish. Most amphipod species recorded were recorded on multiple host species.

Decapod associations (81 records) are separated among twelve families, Epialidae (17), Portunidae (14), Palaemonidae (12), Hippolytidae (14), Scyllaridae (11) Cancridae (6), Chlorotocellidae (2), Scyllaridae (1), Luciferidae (1), Penaeidae (1), Varunidae (1), and Grapsoidea (1). No decapod was found in association with hydrozoans or in deep-sea records. The representatives of Epialtidae are comprised exclusively of multiple species

of the genus *Libinia.* The Portunidae records are mainly composed of the commercially valuable *Charybdis feriata (*Linnaeus, 1758) (11 records), *Charybdis annulata* (Fabricius, 1798) (1) and two *Callinectes, Calinectes sapidus (*Rathbun, 1896) and an unidentified *Callinectes* specimen (1). *Periclimenes paivai* (Chace, 1969) is the most common Palaemonidae, representing three of the twelve records, with six additional *Periclimenes* species, two *Ancylomenes* species and one *Leander paulensis (*Ortmann, 1897). All Hippolytidae associations were between a specimen of *Latreutes anoplonyx (*Kemp, 1914) or *Latreutes mucronatus (*Stimpson, 1860) and one of an array of different scyphomedusae in Asia, Australia, and the Arabian Sea-Persian Gulf corridor. The families Scyllaridae and Scyllarinae include seven *Ibacus*, three *Scyllarus,* and *Eduarctus martensii* (Pfeffer, 1881). These associations were all exclusively larval. The majority (4) of Cancridae records involve *Metacarcinus gracilis* (Dana, 1952) with two unknown *Cancer* species. These crabs were found on *Chrysaora* medusae and one *Phacellophora camtschatica (*Brandt, 1835). Two *Chlorotocella gracilis* (Balss, 1914) (Chlorotocellidae) were found on Japanese rhizostomes, both in somewhat limited encounters. The last three accounts include a *Cyrtograpsus affinis* (Dana, 1851) (Family: Varunidae), *Lucifer* sp. *(*Family: Luciferidae), and a juvenile Grapsoidea of unknown genus and species. The account of *Lucifer* sp. was of a record of one specimen on a medusa in New South Wales, and is not likely a common or genuine association[78]. *Cyrtograpsus affinis* and the juvenile of the family Grapsoidea were also one-off reports found in single medusae[79,80].

Associations that involved mysids or isopods were far fewer than those involving decapods and amphipods. The isopod records include only four species, including the deep-sea parasite *Anuropus* associated with *Deepstaria enigmatica* (Russell, 1967). Besides the *in situ* accounts of the *Deepstaria* scyphomedusae with an attached *Anuropus,* three Isopoda species were found in association with upper water column medusae. These are *Cymodoce gaimardii* (H. Milne Edwards, 1840) and *Synidotea marplatensis* (Giambiagi, 1922), each recorded three times, and *Cymothoa catarinensis* (Thatcher et al., 2003), found once in association with *Chrysaora lactea* (Eschscholtz, 1829). Within the order Mysida, the two species *Mysidopsis cathengelae* (Gleye, 1982) and *Metamysidopsis elongata (*Holmes, 1900) were recorded on *Chrysaora* during a bloom in the Southern California Bight[81].

Three species of cirripeds were recorded 15 times in association with jellyfish, *Alepas pacifica* (Pilsbry, 1907) accounting for twelve of such records, *Conchoderma virgatum* (Spengler, 1789) accounting for two, and a single report of an unidentified *Anelasma* epibiont on a *Pelagia noctiluca* (Forsskål, 1775) from 1902. *Alepas pacifica* has been found on seven separate host species, all scyphozoans. The vast majority of these records came from a single literature review included within an extensive paper from Vader[73]. None of these species were found in deep-sea records.

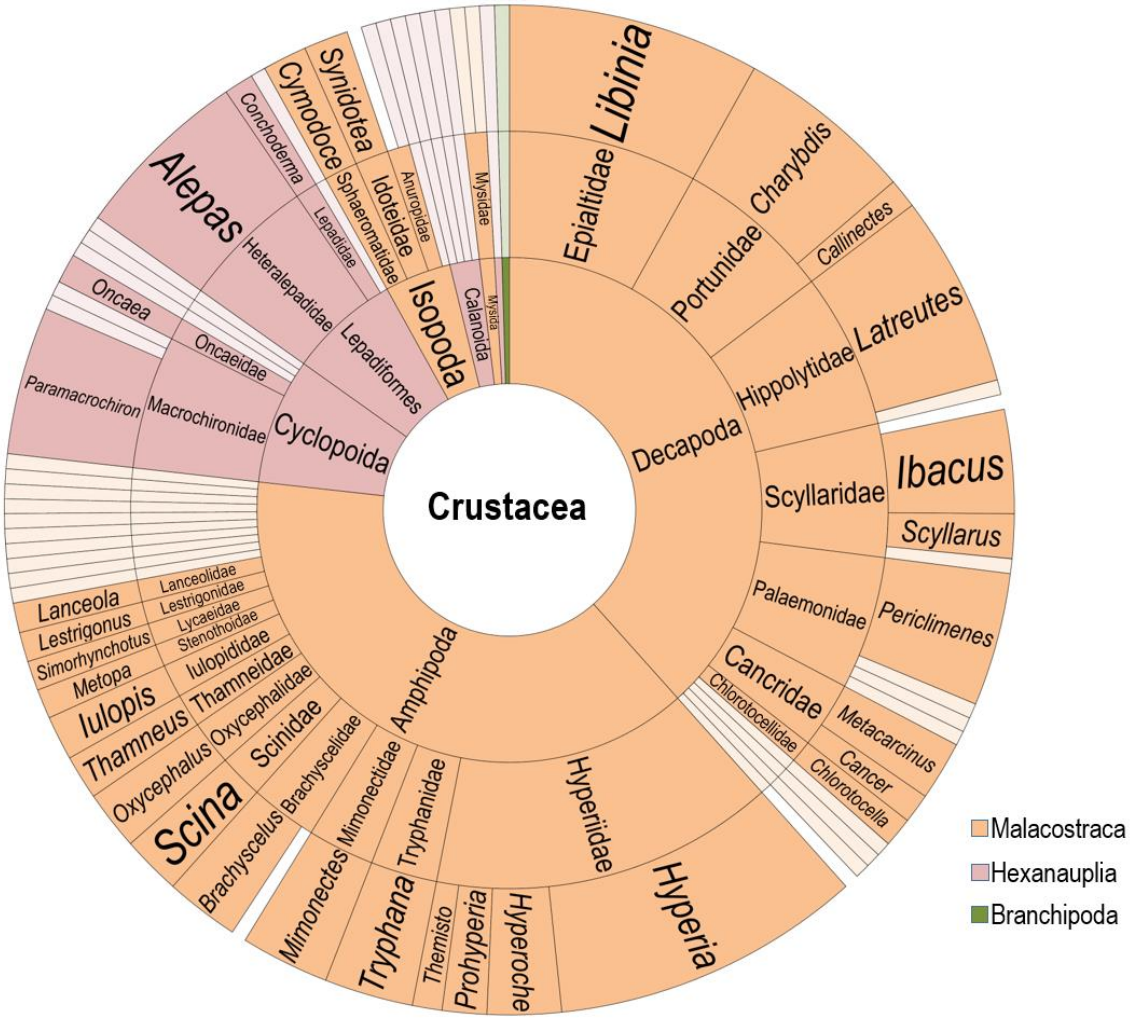

Figure 2.3 Diversity of Crustacean epibionts. From innermost ring to outermost ring: Subphylum, Order, Family, *Genus*. Color coded by classes Malacostraca (orange), Hexanauplia (pink), and Brachipoda (green). Families and genera reported only once are whitened.

2.4.2. Field Collections

Only 58 papers included some explicit method of capture of the jellyfish and its epibiont (Fig 2.4). Between 1862 and 1962, only seven of the twenty records reported a method of capture. From 1963 to 1989, this increased to 64%, with 25 of 39 records including the collection method. Since 1990, there have been only seven failures to

report collection methods out of 140 accounts. The most common method of collection, used in 31 of the papers, is "by hand", defined as using handheld dip nets, buckets, plastic bags, and, in limited cases, collection of carcasses from beaches. Trawling was first used in 1968 and has remained in use until recently, reported in 17 of the 33 associations after 2010. Although 38 records were obtained through deep water methods (HOV and ROV), these were used scarcely before 1999. Some studies employed multiple methods, with divers and ROV, or dip net and trawl capture, such that it was unclear which associations were found by each collection method. These were listed as "multi-method" and include four papers.

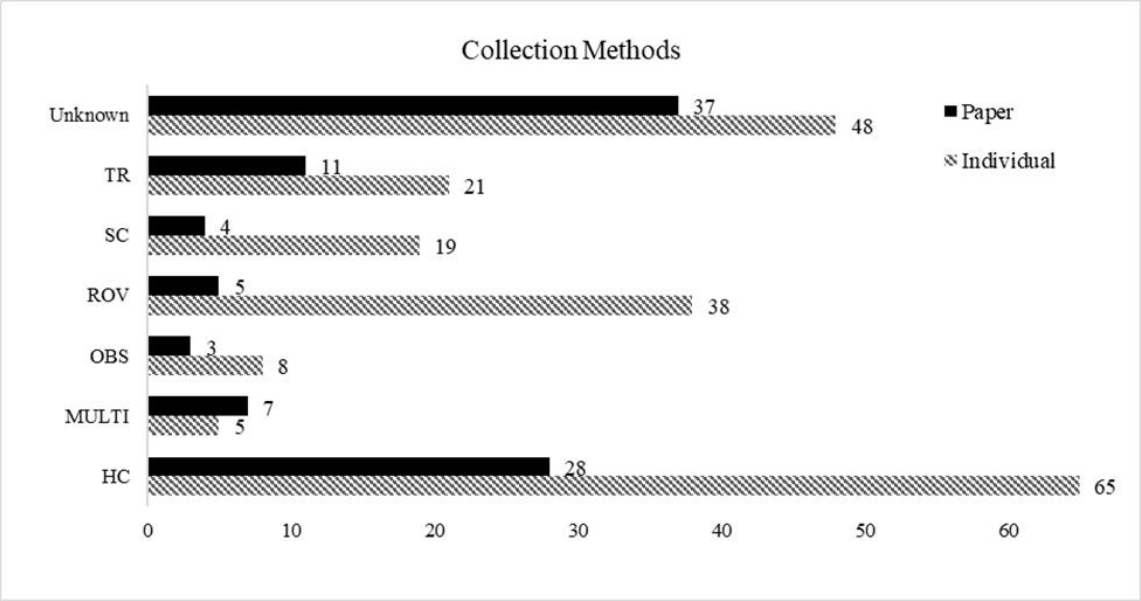

Figure 2.4 Collections information for both number of papers using a collection method and number of associations reported from this collection type. Types are collection by hand (HC), multiple methods (MULTI), ring net (RN), scuba diving (SC), trawling (TR), in situ observation (OBS) or unknown (Unknown). Individual records from papers with multiple methods where specific methods are known are categorized under the known method.

The larger proportion of scyphozoan hosts to hydrozoan hosts may be a sampling artifact. The vast majority of the papers discussed here were only analyzing interactions in the top 30 m of the water column. A fair number, especially earlier texts, involve serendipitous encounters at the water's edge or within sight of the surface[72,73,81,82]. The larger, more visible nature of surface water scyphozoans of the rhizostomes and semaeostomes makes them an easier collection target than deep water species. Note that only a single scyphozoan of the order Coronatae, which has no large shallow representatives, was recorded as well. Many elements of the sampling methods impact the scope of this data, and the preeminence of hand collection and papers written on chance occurrences, as opposed to prolonged study, result in a picture that heavily weights organisms more frequently seen or interacted with by humans.

The oldest records of jellyfish-crustacean interaction involved hand collection with buckets and nets, often from shore. These include first accounts of hyperiid amphipod-jellyfish associations from the Chesapeake Bay[82]. Buckets and nets have remained mainstays, with hand collection accounting for 34 of the 108 post-2000 records and 32 of the 55 pre-2000 records. Buckets and plastic bags are likely preferable to nets, as they may reduce chances of epibiont detachment and medusa damage.

Trawling (by ring nets, otter nets, and bottom trawls), while reported in twelve papers, has been a prominent capture method in South America for the last two decades. However, trawling provides an additional threat, as epibionts may detach, get caught in the bell of a medusa, or move to a different location within the carcass. Given the damage sustained by gelatinous bodies during trawls, and the inability to capture more

delicate associations, this is the methodology that seems most likely to provide low-quality relationship information. A focus on a lower number of medusae examined in more detail, may provide more useful information on the ecology of the interaction between jellyfish and their epibionts. Notably, Greer et al.[11] uses a combination of *in situ* imaging (with an automatic ISIIS imaging system) and trawls. Trawls were used to verify the identity of organisms seen in the captured images. Such a protocol should be considered for future quantitative and qualitative work.

66% of the records (136/211) are from known surface encounters. 18% of the records (38/211) involve deep water accounts using either an ROV/HOV. These records are distributed unevenly across depths with few records below the mesopelagic zone (Fig 2.5). Most of these records fail to provide epibiont location on the jellyfish but provide the only available information on deep water scyphomedusa and hydromedusa hosts. Most of the deep water records are from the Gulf of California. While this sampling method is useful, the high cost and difficulty of use of ROV and HOV equipment make it unrealistic for the vast majority of researchers. The limited number of deep-water accounts and the novelty of many of the findings on each dive can be attributed mainly to these limitations[83–86].

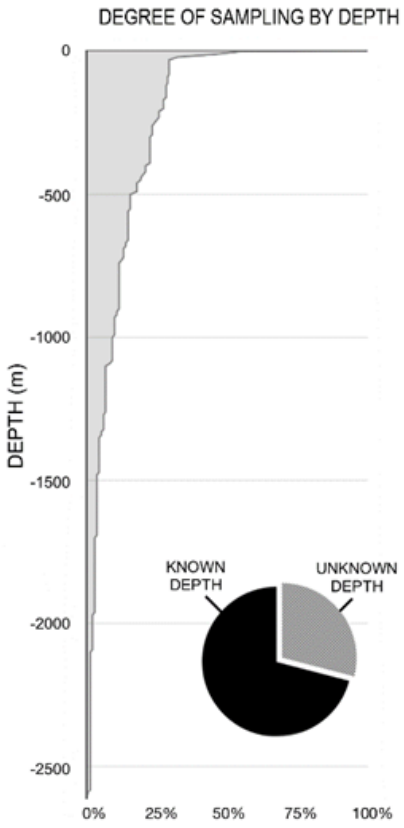

Figure 2.5 Percent of sampling by depth. 68% of samplings had known depth data (pie chart). 74.4% of sampling was done above 30 m.

Given the fragility of scyphozoan and hydrozoan medusae, as well as the delicacy of the interaction with their epibionts, the most precise picture of the jellyfish-crustacean associations has been achieved from dip net, plastic bag, bucket, or other by-hand collection methods. These are not only a cost-effective strategy requiring little additional equipment, they also maintain maximum integrity of the organisms. Hand collection, however, is restricted to analyzing associations that are close to the surface. Trawl sampling provides a reliable way to collect many medusae offshore but sacrifices sample integrity. ROV is an imperfect sampling method, often failing to record epibiont

positioning, but allows for the only viewing, documentation, and collection of deep water associations, thereby being uniquely important, especially for hydromedusa research. Moreover, the majority of the records document all symbionts on the target host species, often with little data beyond a name or tentative classification for the epibiont. This lack of closer examination leads to an inability to correctly categorize the nature of the relationship, including positioning, feeding behaviors, and duration of the interaction.

In conclusion, the overall best sampling results come from observation-first methodologies such as collection by-hand while snorkeling and diving, as in Mazda et al.[87], ROV/HOV *in situ* underwater photography, as employed by Gasca et al.[84], or imaging and supplemental trawling as in Greer et al.[11]. Obtaining underwater pictures of medusae and epibiont is crucial to the understanding of the associate placement in relation to host and its behavior. It is also more informative than post hoc in-lab examinations and analysis of trawl contents, because the stress of collection and sampling may impact the epibiont position within the host[75]. As waterproof video equipment becomes less expensive, options like a simple GoPro may provide clear enough imaging to allow novel *in situ* observations. Adding an underwater imaging component to sampling may also enable collectors to revisit the ecological context of the association.

2.4.3. Life stages

Age classes and sex, where available, are reported in the full table. 63% of all records (133/211) reported an age class for the crustacean. 65% of the interactions with

a listed age class (65%, or 86/133) reported crustacean juveniles, eggs, larval stages, copepodites, megalopae, or other immature forms. For a minority of records (37%, or 73/211), no information on the crustaceans' age class and sex was available. When individuals were described as "male" or "female" without any qualifier attached, they were catalogued and treated as adult specimens. Megalopae were noted only nine times out of the 106 records that reported an age class for the crustacean associate (8%). In these nine records, the megalopae belonged to the genera *Callinectes, Periclimenes, Metacarcinus, Cancer,* and *Charybdis*, and were all in association with Scyphomedusae (Orders: Rhizostomeae and Semaeostomeae). In addition to megalopae, phyllosoma larvae of the families Scyllaridae and Scyllarinae were reported 12 times. The occurrence of larvae of this type associated with medusae and, more generally, with gelatinous zooplankton is well known, especially along the Japanese coast[77]. Within and upon the host, juvenile crustaceans were often coexisting with adult forms. Eighty-one of the associations include juveniles (excluding megalopae, eggs, and copepodites), sometimes embedded in host tissue[10,74,87–89]. The presence of eggs and ovigerous females was reported in 39 cases from 23 different species. In at least three papers, females and ovigerous females were present in exceptionally high proportions relative to adult males[87,90,91]. Records of megalopae of the commercial crab, *Charybdis feriata* were reported in substantial numbers on two separate hosts[92,93]. In other reports, associations between juvenile *Metacarcinus gracilis (*Dana, 1852) and medusae are hypothesized to be beneficial to the crab as the medusae supply means of transport and food acquisition, which may be similar across juvenile decapod-scyphozoan associations[74].

2.4.4. Nature of the Associations

There is no agreement between authors on the degree to which medusae and crustaceans' interactions are parasitic, commensal, or otherwise. In the case of the scyphozoan *Phacellophora camtschatica* and the decapod *Metacarcinus gracilis (*Dana, 1852), the interaction may involve a mutualistic cleaning relationship as *M. gracilis* graduates into adulthood[74]. Other reports of megolopae do not suggest any parasitization of the medusae. Weymouth (1910) also indicates that this is a commensal relationship important to *M. gracilis* megalopae until they reach ~20 mm. In other cases, such as the shrimp *Perimincles paivai*, the commensals seemed to be feeding on the mucus, not the host tissue[78,90]. Dittrich[94] demonstrates an aggressive parasitoidism by *Hyperia galba* in which a large subset of host medusae was so reduced by predation as to lose almost all morphological features. While the ultimate death of these hosts is not recorded within the text, the loss of all tentacular structure and non-mesoglear tissue would make survival nearly impossible. The numbers in which *Hyperia* can be found on some of the recorded medusae, occasionally upwards of 100 amphipods engaging in host consumption, may lend credence to the parasitoid rather than classically parasitic nature of this relationship in many hosts[73,74,94]. However, additional reports on the same species and other hyperiids reported that this group engages in cradle positioning, facing outwards from the medusa, into the water column with no reported predation, or engage in only limited predation of the gonadal tissue or mesogleal tissue[82,83,88]. Based on this information, it seems likely that the family Hyperidae includes a variety of strategies,

and the family *Hyperia* itself may also encompass non-aggressive parasitism, aggressive parasitism, and parasitoidism. In part, this may be due to temporal behavioral differences within species, with more extreme predation in summer and autumn and limited parasitism in spring as populations raise and fall[82,94]. "Inverted cradle" positioning is a recurring feature of amphipod associates[82,95]. While some of the crustaceans fed on the medusae themselves, Towanda and Thuesen (2006) primarily recorded crustaceans engaging in theft of prey collected by medusae. Many crustaceans that were reported feeding on the medusae were feeding entirely or in part on the highly regenerative gonadal tissue[9,74,96] or engaging in the excavation of small pits in the host mesoglea[72,88,97]. Reports of *Libinia dubia* (H. Milne Edwards, 1834) have the greatest agreement on the parasitic nature of the species' interactions with their medusa host[15,72,79].

The largest exception to the above patterns of limited consumption or longer-term residence is the scholarship surrounding phyllosoma larvae on gelatinous zooplankton. These larvae have been reported to stab a pair of pereiopods through the exumbrella or exterior of a nectophore and use the medusa as propulsion and food source. This is a common occurrence both in the northern Gulf of Mexico and at various locations along the Japanese coast[11,77]. In the review on the subject by Wakabayashi et al.[77], it is hypothesized that the flattened body and ventral mouth of these phyllosoma larvae is ideal for consumption of gelatinous zooplankton while attached. The exact length of this parasitoid association is unknown, though it is likely generally ended by the medusa's eventual death as the larva eats its way through.

The degree to which crustaceans engage in host consumption may be in part obscured by the speed with which medusae regenerate tissues, especially gonadal and oral arm tissues[74]. The number of associates (at least eight crustacean species) found residing within the bell and around the gonads, suggests that gonadal tissue may be common nourishment even when bell and arm tissue is not consumed. Overall, the relationships of crustaceans with their medusa hosts remain largely uncharacterized and require additional study. Few papers have analyzed the gut contents of the epibionts, which would be a helpful tool in determining whether inverted positioning on hosts was actually a signal of lack of consumption, or simply a break from such[9,73,74,91]. Detailed records of the diets of such organisms are difficult to reconstruct. However, specific searches for nematocysts in digestive tract and excretions or stable isotope analysis have proven successful at identifying cnidomedusae as possible food sources[76,98]. Expanding future works to include both these practices, photographs of the host medusae, and notes on swimming strength, tentacular loss and other signs of deterioration would improve our understanding of how detrimental these relationships actually are. This sort of documentation of host condition is impossible when specimens are collected via trawl.

In addition to consumption, the issue of host choice and host specificity has been analyzed only sparsely. There is evidence in multiple studies that while some individual jellyfish host symbionts, others in the same area lack them due to their size or species[74,75,93]. While exotic species often have lower amounts of parasitization in their introduced range[99], the degree to which epibionts in medusae are affected by host or epibiont endemicity is unknown. The high number of cryptic species, a history of

misidentification, and poor understandings of historical ranges compound issues with sparse research on the topic[36,100–102].

Only one study provides an indication of how nuanced the relationship between gelatinous zooplankton hosts and epibionts may be; six years of monthly observation showed that single adult females of the amphipod *Oxycephallus clausi* (Bovallius, 1887) had a broad range of gelatinous hosts, but shifted to primarily *Ocyropsis fusca (*Rang, 1827), a lobate ctenophore, during brood release[87]. While ctenophores are not the focus of this review, it shows that the nature of interactions may change during the crustacean lifecycle. These sorts of long-term analyses are hard to pursue but provide a fascinating look at the range of information that can be collected with observational methods. Uneven sex ratios, such as those seen in the case of *Oxycephallus clausi* (97% female), are present across many associations[87,90,91,95]. The most common explanation for this higher ratio of females and often ovigerous females is use of scyphozoan and hydrozoan hosts primarily as nursery habitat for movement and protection of juveniles[80,87,103]. Potential territoriality in some females, like those of *P. paivai*, may help ensure more resources for their brood, and is in line with other symbiont crustaceans[104]. For deep sea crustaceans, such as *Pseudolubbockia dilatata* (Sars, 1909), more even sex ratios would be expected, as there is evidence of long-term resident brooding pairs, and mate scarcity is a feature of deep-sea life. Evidence for long-term association and pairing has not been found for other deep water crustaceans, although understanding these deep sea interactions is generally hampered but small sample sizes and difficulty of observation[86,104,105].

2.4.5. Years and locations

The oldest records examined were only available from earlier literature reviews[9,74,76]. The first record is the Bate (1857) account of the amphipod *Iphimedia eblanae* on the scyphozoan *Rhizostoma pulmo (*Macri, 1778) from 1862, also reported in the Vader (1972) review on amphipod associations with medusae. Thiel[106] refers to older records from as far back as 1791. Overall, the number of records detailing interactions has risen over time but has not exceeded ten papers during any five years. While these numbers are increasing modestly, the number of distinct interactions that any given paper reports have increased. Pre-1990s articles, on average put forward information on 1.24 associations per paper. In contrast, the average number of associations reported in papers published from 1990 to 2018 increased more than twofold (an average of 2.83 records per paper). These surveys provide useful records of separate associations found in one area or on one organism and are informative of ecosystem features on a regional level. Still, given the studies' breadth, they often lack depth, not characterizing relationships between individual host species and their associates.

Records were unevenly distributed globally, with Africa and Europe completely devoid of records from the past thirty years with the exception of a single note on an accidental observation from Gran Canaria, Spain. The eastern coast of North America (one record since 1984[107] and China (no direct records), as well as West Africa (one record from 1972[108]) and the Mediterranean Sea (last collections 1985[94] also lack records from the last 30 years. The areas consistently covered by recent papers are Australia (1968-2009), the Philippines (2014, 2018), the eastern coast of South America

(1980-2016), and the western United States (1966-2015). Japanese records represent the longest continuity over time, with 33 records between 1902 and 2019. The association that consistently appears throughout time is that of *Alepas pacifica (*Thoracica, Lepadiformes) with Nomura's Jellyfish (*Nemopilema nomurai*)[9,10]. The first record of this association was in 1902[9], and the most recent in 2015[10]. Phyllosoma larvae of multiple species, *Chlorotocella gracilis* (Balss, 1914), and *Latreutes* spp. also have records spanning multiple decades and papers.

It is worth mentioning that the uneven geographic distribution of associations reported herein may be an artifact of lack of readily available English translations of works from some areas. Reports from Japan and China of crustacean and gelatinous zooplankton associations are mentioned by Hayashi et al.[75] and Wakabayashi et al.[77], but were not available in English and therefore are not accounted for in this review. Similarly, European records may be underestimated, as non-English records are absent. Other locations' lack of records may be a more accurate representation of a gap in academic knowledge. Africa's west and eastern coasts are known to be understudied ecosystems, and so the missing research here is likely not just untranslated[109]. As in other ecological inquiries, the expansion of Local Ecological Knowledge into the study of gelatinous zooplankton should be considered, as fishermen and coastal communities often have a deep knowledge of organisms and their associations[109]. Fishermen are often well acquainted with specific gelatinous zooplankton species and know their harms, and may have knowledge of symbionts living upon or within them[110].

2.4.6. Commercial species

Many commercial crustaceans and jellyfish were found to have associations that may be of ecological and commercial importance. Twelve records reported the edible jellyfish *Rhopilema* spp. as hosts[9,67,74,93,111–113]. The commercially harvested shrimp, *Penaeus stylirostris* (Stimpson, 1871), was found on *Stomolophus meleangris*[5]. Notably, young *Callinectes sapidus*, the Chesapeake Blue Crab, was reported by Jachowski (1963) as regularly found on *Chrysaora quinquecirrha (*Desor, 1848) medusae without consuming them. This association was reported again briefly in the Mississippi Sound by Phillips et al.[15]. This interaction between a jellyfish and the blue crab has never been corroborated further except for a nonspecific report of a *Callinectes* sp. associated with jellyfish reported by Towanda et al.[74] as unpublished data. The commercially valuable crab, *Charybdis feriata,* has been reported in association with ten jellyfish species[67,74,76,93,111,114]. These reports involve juveniles[74,76,92,93,115] and megalopae[92,93] of *C. feriata*, and this association has been recorded in Hong Kong, Japan, the Philippines, Mozambique, and Indonesia, suggesting a consistent pattern over time (first record in 1965[76] and last record by Boco & Metillo[93]) and across their range.

Slipper lobster larvae of the genera *Scyllarus* and *Ibacus* have been reported many times across various hosts[77]. Some slipper lobsters are commercially fished for consumption, and a large number of these larvae (40% in the Gulf of Mexico) have been shown to live attached to gelatinous zooplankton[11].

The consumption of some Scyphozoan hosts, such as *Catostylus mosaicus* and *Rhopilema* spp., makes their records valuable as well. The fishing pressures on the

jellyfish populations may significantly impact the crustaceans that rely on their oral arms and bells for transport and nourishment of their juvenile stages. Further understanding of these relationships may be especially important in cases where both the medusae (e.g., *Rhopilema* spp., *Lobonemoides robustus (*Stiasny, 1920) and *Catostylus* spp.) and crustacean (*Charybdis feriata)* are subject to fishing[92,93,114]. Finally, current information on *Callinectes sapidus* and its relationship to and frequency of interaction with host jellyfish is needed, as the blue crab represents a commercially valuable fishery in the Gulf of Mexico and along the Atlantic Coast of the USA.

Understanding the nature of the relationships between economically valuable species of Crustacea and common scyphozoans and hydrozoans can improve fisheries practices and regulation, as already acknowledged for economically important fish and their jellyfish hosts[66]. The importance of maintaining juvenile communities for commercially sized adult populations to recruit from is well established and a frequent impetus for marine protection areas. The fishing of medusae is different from most modern vertebrate fishing. It is temporally highly variable, and blooms, when found, are fished as intensely as possible by local fishermen. It is also comparatively new as an export industry, especially in Southeast Asia[116]. Additional regulation and management should be considered for jellyfish species known to harbor juveniles of commercially viable crustaceans. It is clear that many crustaceans, fish, and other organisms live in, upon and around medusae, thus indiscriminate efforts to remove or destroy blooms of endemic species are likely unwise[5,66].

2.5. Conclusion

Many of the interactions we reviewed are fragmented and not comprehensive. Studies covering timing and breadth of infection of commercially valuable crustaceans on marine scyphozoans are scarce, but may be valuable information to fully understand the complexity of their life cycle, and thus the species' vulnerability at each life cycle stage. The general picture of the commensal relationships that arise from this review is complex and emphasizes the diversity of jellyfish and crustaceans' relationships. Any attempt to paint them as uniformly parasitic fails to acknowledge the diversity of crustacean host-use strategies. While some seem to be parasitic or parasitoid, others are life-stage dependent commensals reliant on medusae for transportation. Some deep water crustaceans may be lifelong commensals[86]. In each of these cases, the work thus far is far from exhaustive. Additional research on seasonality, maternal care, territoriality, impact on host and other such matters should be further pursued.

The scyphozoans and hydrozoans studied here represent only a small proportion of the globally recognized species. Even shallow water coastal species are poorly covered. This research has been restricted to a small selection of near-shore sites over the past 50 years, leaving inadequate coverage even in regions with a significant scyphozoan research presence (i.e., the Mediterranean, western Europe, China, northeastern North America). Because much of the published research focused on single occurrences, this paper's overall results do not necessarily capture the broader ecology of the species involved[72,75,82,117]. Similarly, species descriptions that mention an association

without details on the conditions in which it was found offer little insight on the frequency and ecological role of such interactions [71,97,108,118–121].

Best practices moving forward should include some of the following elements: *in situ* imaging pre-collection, observations on medusa health, analysis of epibiont gut contents when possible, preferential use of non-destructive collection methods, observations on symbiont placement within or upon the medusa, and frequency, geographical and temporal variation of the association.

With this review, we highlight a significant knowledge gap and a lack of formal study on the ecology of the crustaceans residing on and around jellyfish, as well as a glimpse of the ecological complexity of these interactions. We provide easy access to a century of ecological research and a framework for analyzing and contextualizing future research on this topic**.**

3. IMPACTS OF LIGHT AND FOOD AVAILABILITY ON EARLY DEVELOPMENT OF *CASSIOPEA* MEDUSAE[†]

3.1. Question and Hypothesis

Question: How do light and feeding levels impact *Cassiopea xamachana* morphology and survival in the first six weeks post-release?

Hypothesis: Increasing light and feeding contribute to higher growth and increased survivorship.

3.2. Introduction

Much work has been done on the invasive potential of species of the phylum Cnidaria, especially scyphozoans and hydrozoans[36,101,122]. Cnidarians have spread throughout the world in a pattern largely consistent with ship traffic[37,123]. Much of this spread is considered a result of extremely hardy polyp stages[124]. Polyps present a clear invasion concern as potential foulers on ship hulls and other surfaces, but other life stages should be considered, especially motile ephyrae. For example, ephyrae of the semaeostome *Aurelia* spp. are known to persist for months (as long as 100 days) without feeding[125]. Starvation tolerance and robustness in the motile ephyra stage may contribute to a species' capability to survive long journeys in cargo ships or along coastlines and thus to its potential as an invasive species.

[†] As published in: Muffett, K. M. K., Aulgur, J., & Miglietta, M. P. (2022). Impacts of Light and Food Availability on Early Development of *Cassiopea* Medusae. *Frontiers in Marine Science*, 8(January), 1–11. https://doi.org/10.3389/fmars.2021.783876

Cassiopea is a rhizostome genus of Scyphozoa, with an epibenthic adult form that has successfully spread throughout the tropics and subtropics of the world[36,37]. This spread includes a large natural distribution and an even larger invasive distribution[36]. *Cassiopea* medusae live in shallow waters and rely on a combination of nutrition from *Symbiodinium* cells housed within their tissues and active consumption of prey material. Light is an essential environmental parameter for *Cassiopea,* given its reliance on *Symbiodinium*'s photosynthetic capacity[42,126]. The known compensation light level for *Cassiopea* medusae is 50 μmol/m2/s PAR in adults[127]. However, recent work has shown that these medusae exhibit remarkable capacity for photosynthetic plasticity[42]. Despite *Cassiopea*'s long-term presence in aquaria and more recent spotlight as a model system and a potential food source[38], little work exists on the ecology and development of its ephyrae and the role of light on their development.

Scyphozoans are phenotypically plastic in response to external conditions[128]. While it is evident that starvation induces aberrant morphologies in *Aurelia* jellyfish, many other stressors that impact jellyfish developmental trajectories are poorly understood[125,129]. This work aims to understand how development and survivorship in non-clonal *Cassiopea xamachana* are affected by starvation and sub-compensation light conditions. Development was analyzed in one hundred and eight newly released ephyrae, split into low-light and darkness groups. Within both low-light and darkness treatments, multiple feeding levels were implemented to investigate the survival rates of the ephyrae and their morphological and developmental response to these regimes. Growth, zooxanthellae

concentration, and aberrant body form are used as indicators of the hardiness of a known invasive species during one of its most motile stages.

3.3. Materials and Methods

3.3.1. Animal Sourcing, Care, and Experimental Parameters

Eight adult *Cassiopea* were acquired from the Florida Keys through an independent fisherman as breeding stock. Polyps that rooted along the sides of the tank were transferred to a polyp holding tank.

Polyp colony was raised at 22℃ in 35 ppt in a low light (30 μmol/m^2/s PAR) 5-gallon tank in the Sea-Life Facility at Texas A&M University at Galveston. This culture was fed twice weekly, with water filtered continuously. To induce strobilation, indomethacin dissolved in DMSO was introduced gradually to the colony over the course of one week to reach a peak concentration of 40 mM [130]. One week after exposure, the first visible indications of strobilation occurred. Water was carefully exchanged each day following exposure to DMSO, and ephyrae released in the 24 hrs prior were removed. Individuals released from April 3, 2021 to April 7, 2021 were isolated. On April 3,4, and 5, all ephyrae between 2 and 5 mm diameter were included in the experiment (1, 4, and 25 ephyrae, respectively). On April 6 and 7, 60 and 18 ephyrae between 2 and 5 mm were used, all excess individuals were removed. At intake, each ephyra was moved to clean artificial seawater, then photographed using Leica Acquire. After photographing, all ephyrae were placed in inverted individual plastic containers of 150 mL of artificial seawater and placed within an incubator held stable at 24.5℃ for light hrs and 23℃ for dark hrs. Viparspectra P-1000 full-spectrum lights were shaded and adjusted such that

the mean PAR within a container was 41 μmol/m2/s with a deviation of 15 μmol/m^2/s PAR. Lights were on in 12 hour light/dark cycles each day. To maintain a mean of 41 μmol/m^2/s PAR lighting and even heating, containers were rotated through positions throughout each week. Each day, each ephyra was individually removed from the holding container and placed in a Petri dish for photography (image capture completed using Leica MDG41 microscope and Leica MC170 camera using Leica Acquire application version 3.4.1 running on Mac computer). While each ephyra was on the microscope stage, 50 mL of artificial seawater were removed from the holding container and replaced with new artificial seawater, along with the relevant treatment dose of 20-hr *Artemia* shrimp. Before water removal, all remaining living and dead *Artemia* accounting for greater than 50% of an *Artemia* body were counted, recorded, and removed individually. Smaller fragments of *Artemia* were removed but not counted. Ephyra was then carefully returned to the container with a pipette, the container was inverted slowly and returned to the incubator. During each photography session, ambient room conditions were kept at 22-23°C with 7 μmol/m^2/s PAR, with the exception of the 0.5 to 1 minute spent on the light stage, where light was between 10 and 79 μmol/m2/s PAR. Zero-light treatment individuals (see below for treatment descriptions) were held under blackout conditions within the incubation chamber except during photography and food introduction. Every seven days, a complete 150 mL water change was done with surface cleaning and wiping. The above procedure was repeated each day from day 1 to day 40. Upon day 41, all procedures continued as described above, but without feedings.

All feedings and photography took place daily during daylight hrs between 8 am and 12 pm.

3.3.2. Dark Starvation Control

On the final day of ephyra release, ten newly released ephyrae were placed in zero light conditions. These ephyrae were removed only for water change and photography as a double negative control for light and prey. Medusae were counted and photographed daily to monitor survival rate and body modifications. Body measurements (see below) were performed from pictures using ImageJ software. After four weeks (28 days), these individuals were moved to low light conditions and provided 8 *Artemia*/day. The move occurred after the mean bell size for living individuals fell below 2.2, a ⅓ reduction in size from day 0. Ephyrae were followed for the remaining 14 days to determine growth after 28 days of complete starvation. Given the change in treatment at day 28, statistics for this group are reported separately from the other treatments. For this group, measurements of bell diameter were taken instead of rhopaliar radius measurements. As the ephyrae shrank, their rhopalia became increasingly difficult to differentiate from the surrounding tissue, and the center of the manubrium was often indistinguishable from the bell. Bell diameter was well correlated ($r = 0.89$) with weight in other treatment groups, and as such, the replacement was deemed acceptable.

3.3.3. Treatments

Ninety-eight ephyrae (<24 hrs old) were divided randomly into six experimental groups (Table 3.1). Four treatment groups of 17 individuals each placed in 41 μmol/m^2/s PAR

light for 12 hrs/day (blackout conditions for the remaining 12 hrs). Within this group, an Unfed Light group (L0) was not given *Artemia*; the Light 2 group (L2) was given two 18-hour *Artemia* nauplii per day; the Light 4 group (L4) was given four 18-hour *Artemia* nauplii per day; the Light 8 group (L2) was given eight 18-hour *Artemia* nauplii per day.

Table 3.1 Distribution of living and dead individuals by treatment group with details on light level and feeding regimen.

Group Name	Light Level	Feeding level	Group Size	Indv. removed	Effective Group Size	Living (Day 42)	Dead (Day 42)
Unfed Light (L0)	41 umol x 12hr/day, <1 umol x 12hr/day	Not Fed	17	0	17	12	5
Light 2 (L2)	41 umol x 12hr/day, <1 umol x 12hr/day	2 *Artemia* per day	17	1	16	15	1
Light 4 (L4)	41 umol x 12hr/day, <1 umol x 12hr/day	4 *Artemia* per day	17	1	16	16	0
Light 8 (L8)	41 umol x 12hr/day, <1 umol x 12hr/day	8 *Artemia* per day	17	1	16	16	0
Dark 4 (D4)	<1 umol x 24hr/day	4 *Artemia* per day	15	0	15	14	1
Dark 8 (D8)	<1 umol x 24hr/day	8 *Artemia* per day	15	1	14	11	3
Dark Starvation Control	<1 umol x 24hr/day	Not Fed	10	0	10	5	5

Two treatment groups of 15 individuals each were held in zero-light. The Dark 4 treatment group was held in blackout conditions 23.5 hrs/day (the last half hour representing a generous estimate of the time to feed, photograph, and change water each day) and fed four 18-hour *Artemia* nauplii per day. The Dark 8 treatment group was fed 8 18-hour *Artemia* nauplii per day.

The unfed light group (L0) operates as a control for size increase from feeding, and the dark 8 (D8) group operates as a control for light. The Dark Starvation control group experienced declines too intense for one-to-one comparisons with other groups and were not included in downstream analyses with other groups.

Each ephyra in each treatment was photographed daily following the protocol described in 2.1.

3.3.4. Processing

At the end of each treatment (on day 42), oral and aboral photographs of each surviving ephyrae were taken. Individuals were then processed for wet weight and zooxanthellae concentrations following a modified protocol from Zamoum and Furla[131]. First, each individual was dried lightly with a Kimtech wipe on the oral and aboral sides for 2 seconds. Next, each ephyra was placed in a weigh boat, weighed to the nearest tenth of a milligram (+/- 0.1 mg), and then vivisected to remove a complete radial segment, weighing roughly 10 mg (between half and an eighth of the medusa in most cases). The segment was placed directly into 500uL of 4 M NaOH. The remaining tissue was weighed separately and placed directly into 500 uL 100% EtOH for storage. Tissue placed in NaOH was incubated at 37°C for 60 minutes, vigorously vortexed for 15 seconds every 15 minutes. After one hour, the sample was vortexed again, and 40 uL of fluid was immediately drawn from the vial and placed on a Hausser Scientific hemacytometer for cell counting. Cell counts were standardized by original tissue volume in each sample for comparability[131].

3.3.5. Removal of damaged and dead ephyra

Ninety-eight specimens were initially included in this experiment. All individuals in the fed treatment groups that failed to consume a minimum of one *Artemia* nauplii within the first seven days were removed from the experiment. Each of these individuals died within ten days of release. One individual was removed from the experiment due to an error that led to bell damage of this specimen. In total, 4 out of 98 ephyrae were removed.

All ephyrae that died during the experiment were flash-frozen and stored for later use. Given the difficulty of diagnosing the actual point of death in *Cassiopea* ephyrae, removal due to death required meeting two of the following three conditions 1) bell diameter reduction to below 1.6 mm, 2) cessation of pulsing, and 3) significant deterioration of bell margin features. While we recognize there may be a slim chance that ephyrae in these states could recover, we deemed it unlikely. Once two criteria were met, individuals were frozen and eliminated from the experiment.

3.3.6. Measurements and data analyses

A total of 4587 photographs were produced during the experiment. All photographs associated with this project were labeled by specimen and day. ImageJ was used to measure bell diameter, rhopaliar radius, average oral arm length, and number of palp buds in each photograph and compiled for all specimens (see example measurements in Fig. 3.1). These measurements represented the quantitative factors that can be gleaned from images of living non-anesthetized ephyrae. Bell diameter and oral arm length fluctuated each day as no chemical relaxant was used on ephyrae, and level

of relaxation and tension in each ephyra varied by day. Predation success was averaged by week and measured as the number of *Artemia* nauplii provided minus *Artemia* nauplii collected after each feeding session. While not a meaningful metric for comparison between individuals, color changes within individuals were noted over the course of the experiment.

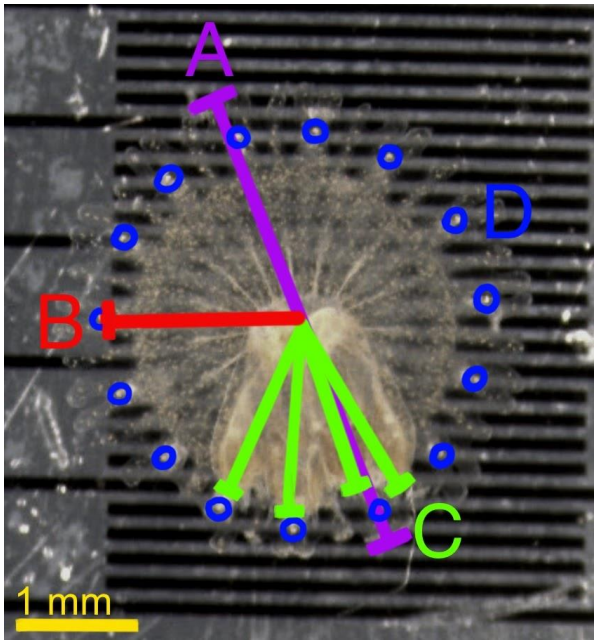

Figure 3.1 Measurement examples on an example ephyra. A) (purple) Bell diameter, B) (red) Rhopaliar radius, C) (green) oral arm length, reported as average of all oral arms, D) (blue) Rhopalia, added together for rhopaliar number

Correlations between bell diameter, rhopaliar radius, average oral arm length and wet weight were calculated for each photographed medusa. Rhopaliar radius was used as the standardized size proxy in all analyses as it was well correlated at day 42 with wet weight ($r = 0.91$) and less variable over time within the same individual than bell diameter (see Supplemental Fig. 3.1).

Unplanned comparisons were run using R[132]. Difference in growth by day and group was computed using the lme package and adjusted using Bonferroni adjustments for all against all[133]. Planned comparisons were run on consumption, growth and palp development between lit and unlit groups, and planned comparisons were run on growth between groups within lit treatment groups.

3.4. Results

3.4.1. Mortality rates in treatments

Four of the six experimental groups (L0, L2, D4, D8) experienced some mortality. No group's death rate reached the level of statistical significance. Survivorship in the L0 treatment group (70.6%) was the lowest, however medusae held in the dark and provided eight nauplii/day (D8) were also below 80% survivorship (78.6%). The four other treatment groups all had survivorship of greater than 90% (L2: 93.8%, L4: 100%, L8: 100%, D4: 93.3%). No tested random factors (date of addition, starting container position, rhopaliar number, coloring at strobilation) except for starting bell width explained mortality. As there was no significant difference in starting bell size among groups, this was not a confounding factor. Seven of the ten deaths were in individuals with a starting bell size of under 3.2 mm in diameter (mean starting bell size overall was 3.65 mm). At an alpha= 0.1 threshold, the mortality rate of L0 was not significantly higher than that of the fed groups. The average death date for the ten ephyrae who died before day 42 was day 28.3, with a range of days 15 to 37. For all further analyses, only surviving medusae were used.

3.4.2. Growth

Changes in rhopaliar radius per day were distinct between most groups and were best explained by polynomial (Fig. 3.2) or logarithmic growth curves. All groups except L0 experienced an increase in size over the course of the experiment.

From day 0 to day 42, L0 saw a decrease of rhopaliar radius of 19%. D4 and D8 saw an average growth of 40% and 86%, respectively. The fed groups held in light saw roughly a 60% increase in growth relative to one another with each doubling of food availability (L2=58% growth, L4=115% growth, L8= 179% growth). As these rates all leveled off before day 42, size at day 42 likely represents the maximum size sustainable under each set of conditions. While L0 experienced slight size reduction, oral arm tissue shrank in volume if not length (see supplementary materials). Periods of increasing size were commensurate with feeding conditions, and growth lasted longest in the L8 and D8 groups (Fig 3.2) as proxied by point of polynomial inversions. D4 and L0 groups had extremely short growth periods, both averaging 11 days of growth (in the case of L0 very weak growth) before slight size reduction and stabilization. However, individual trajectories within every group varied widely.

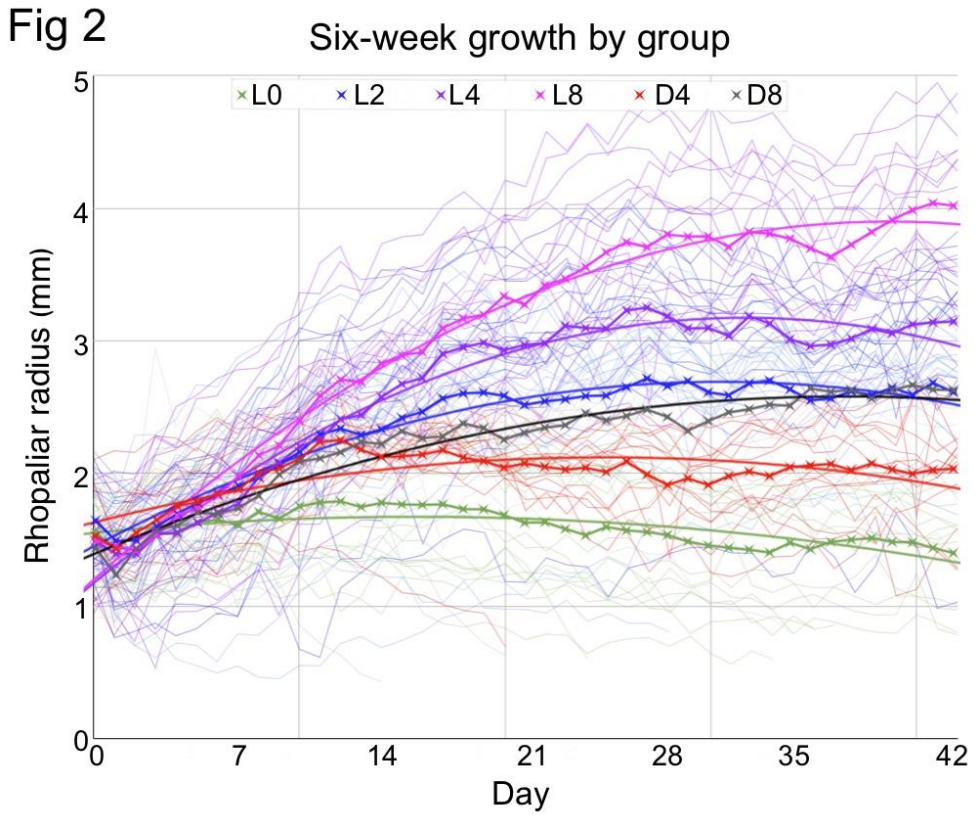

Figure 3.2 Change in rhopaliar radius from day 0 to day 42 in millimeters. Bolded lines with crosses are averages of each group, accompanied by polynomial trend lines

Figure 3.3 shows the average increase in rhopaliar radius between days 0 and 42. Only L4 and L8 groups grew enough for confidence intervals that exclude 1 (no change in size). The growth rate of D4 and D8 groups were both significantly different from that of the light at the same feeding level (L4 and L8) (D4: 1.40 vs. L4: 2.15 and D8: 1.86 vs. L8: 2.79, p=<0.05) (Fig. 3.3). While the growth of L2 was significantly higher than L0 (1.58±0.59 vs. 0.81±0.27), D4 had a non-significant (though visible) increase in growth over L0 and a non-significant difference from D8 (L0: 0.81±0.27, D4:1.40±1.04, D8: 1.86±0.97). The most comparable growth between dark and light groups was between

L2 and D8, despite the dramatic difference in feeding levels and light conditions (Fig. 3.3).

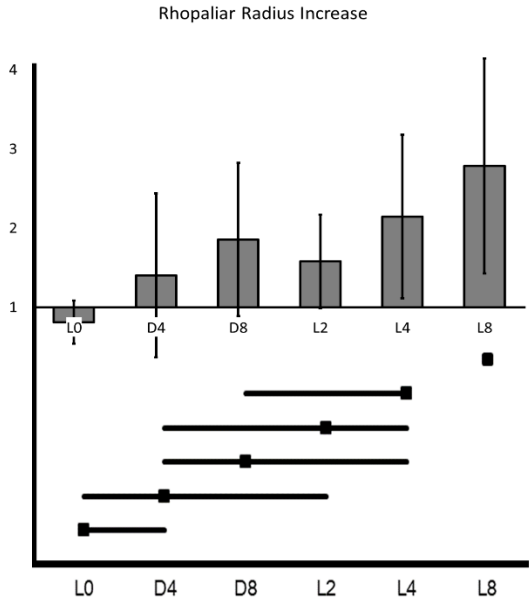

Figure 3.3 Ratio of day 42 to day 0 rhopaliar radius averaged by group. X-axis represents growth multiplier in radius from day 0 to 42. Error bars represent one standard deviation. Horizontal bars represent nonsignificance of comparisons of the means at a= .05

There was a highly significant interaction between Light and Feeding levels in growth (p=1.7e-8), linked to a reduction in predation success in groups held in the dark. This trend is visible in this clustering of individuals by averaged real consumption (Fig. 3.4).

L2 consumed an average of 1.72 ± 0.27 *Artemia* per day. L4 consumed 3.52 ± 0.51 *Artemia* per day, slightly higher than D4. D4 individuals averaged 0.259 fewer *Artemia* nauplii consumed per day than their lit counterparts, while D8 averaged 0.347 fewer per day than L8. Within every group (except for L0), an increase in real

consumption was associated with a trend towards higher average rhopaliar radiuses and higher maximum rhopaliar radiuses.

Oral arm to bell ratio showed no significant difference by group, and average oral arm length tracked fairly closely with rhopaliar radius.

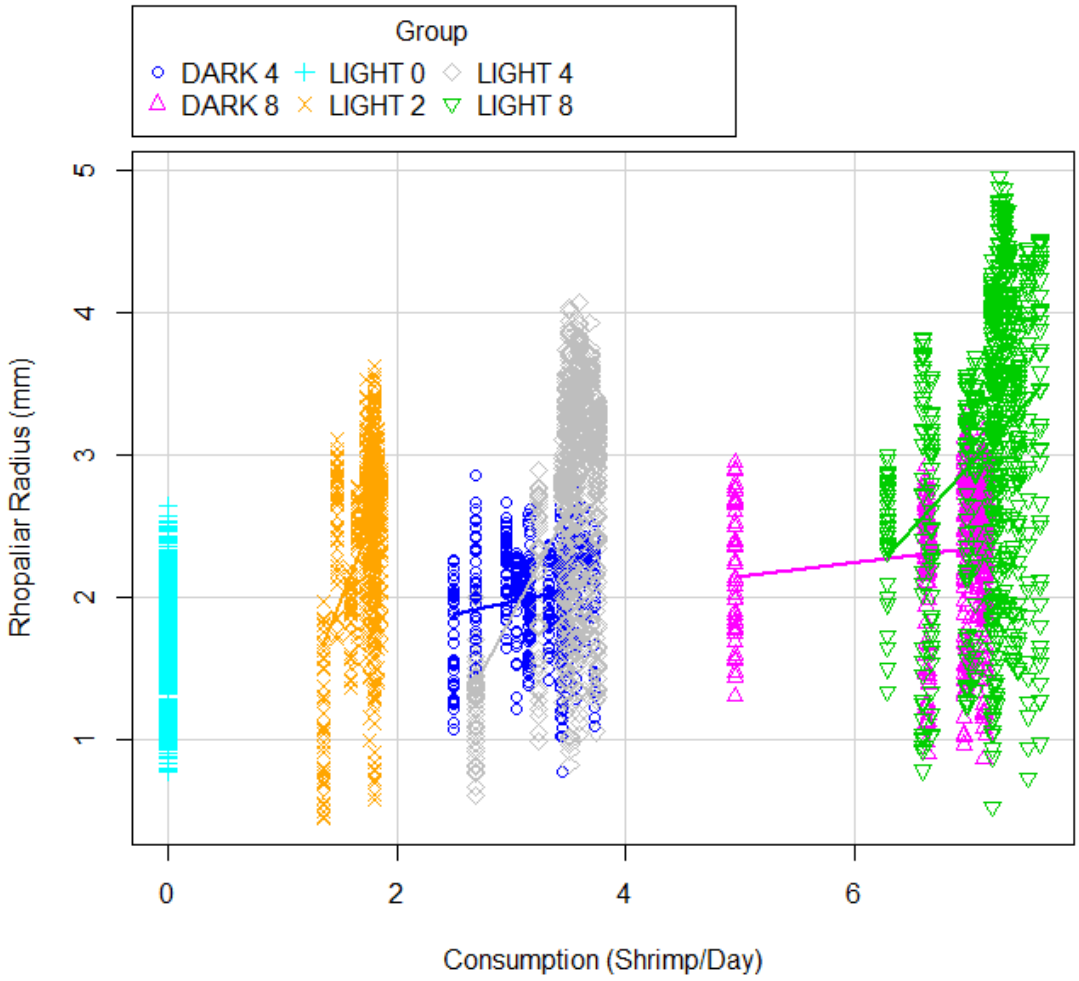

Figure 3.4 Rhopaliar radius explained by consumption averaged over the course of the experiment. Each direct vertical represents a single individual, all groups are color-coded.

3.4.3. Mass

Final weights ranged from 0.2 mg to 50.6 mg of tissue with a heteroscedastic distribution of weights. The median weight was 9.7 mg. Average weights were 1.66 mg (L0); 9.04 mg (L2); 17.48 mg (L4); 36.30 mg (L8); 3.74 mg (D4); 9.12 mg (D8). The weight range for L8 was far larger than all other groups (11.7 to 50.6 mg) (Fig. 3.5). The single lowest weight (0.2 mg) and the lowest average weight (1.7 mg) were in the L0 group.

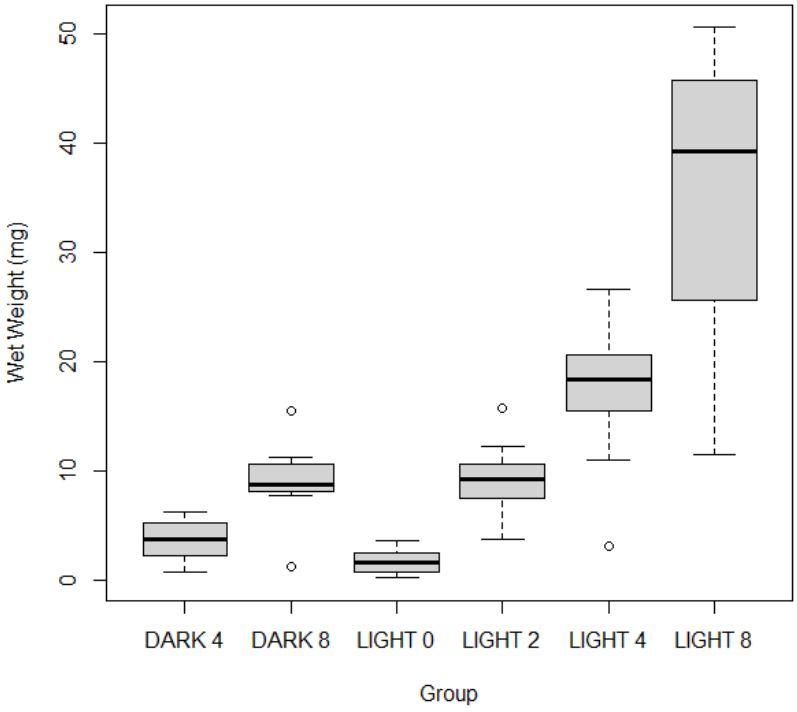

Figure 3.5 Weights (mg) by group. Boxplot of wet weights of individuals by group in milligrams.

3.4.4. Symbionts

Symbiont cell densities were normalized by tissue mass and were not statistically significant between experimental groups within the same light conditions. However, differences were significant ($p<2e-16$) between light and dark groups (Fig 3.6). All dark individuals (25) had below 1400 cells/mg, and the majority (15/25) had too few for symbiont cells to be present in the hemacytometer sampling (recorded as zero). These individuals were all visibly blue.

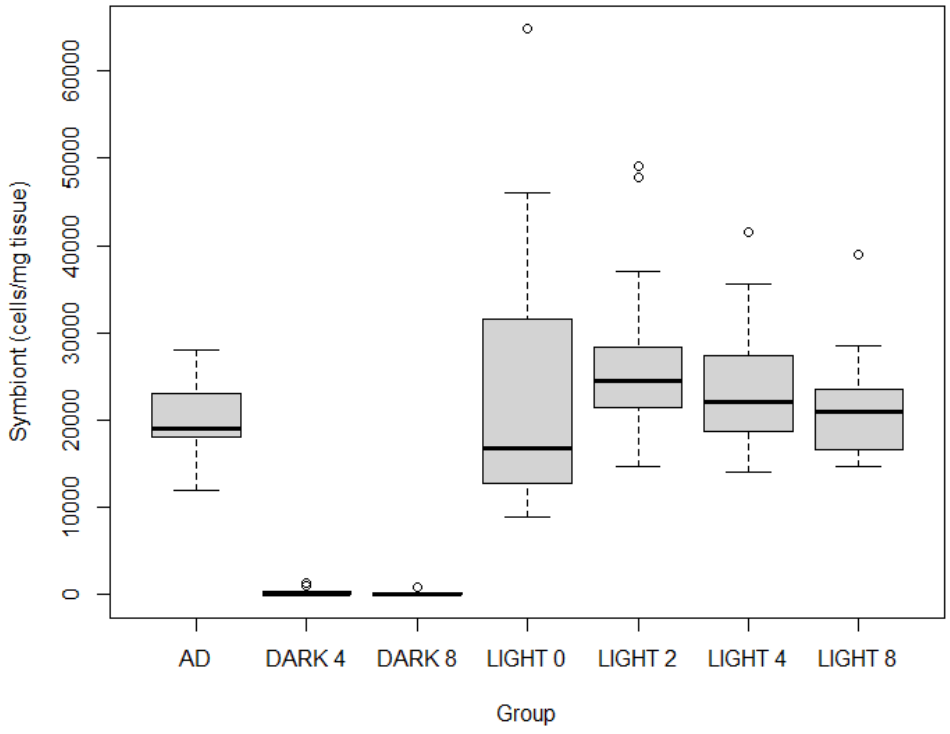

Figure 3.6 Symbiont cell densities per mg tissue. "AD" group represents digested segments from 12 adults between 8 and 31 mm in diameter from the same polyp colony held in standard tank light conditions (350 PAR).

In the D groups, shifts toward bleached coloration within the bell began within the first week of life and continued throughout the experiment. The average time to first

reduction in color intensity was 6.5 days in the D4 group and 5.6 days in the D8 group. About half of the 25 surviving individuals in the D groups started bleaching between days 4 and 6. The earliest bleaching involved a D4 group ephyra that had little coloration at day 0 and was entirely devoid by day 5. The latest was a D4 group ephyra that did not begin losing pigmentation until day 34. Four D4 individuals and one D8 individual were not bleached by day 42. The average time to plateau (the point at which visible reduction in pigmentation stopped, even if a small number of visible spot clusters remained) was 33 days, with 50% plateauing between 26.1 and 39 days.

Six out of 98 individuals had exceptionally little pigmentation at day zero (entirely transparent oral arms). Bleaching was observed in 4 (out of 29) ephyra in the D treatments and 2 (of 65) in L treatment. The two bleached or near bleached ephyrae in the light group slowly gained full coloration over the course of the experiment. There was no increased mortality associated with the bleaching (one of the six died) or any difference in growth rates compared to their peers.

3.4.5. Palp development

Palp numbers relative to size were far higher in the D groups (Fig. 3.7). Over the six weeks of the study, tissue nodules resembling small versions of the floating palps usually found on adult *Cassiopea* developed on almost all individuals (in D and L treatments), except those in the unfed group (L0). New tissue nodules generally appeared between days 9 and 15 and expanded in size or shrank as the medusae expanded or shrank. These resemble the floating palps of adult *Cassiopea*. However, some may be the beginning stages of the oral arm compartments holding cassiosomes

(free-floating nematocyst-laden stinging structures) in adults [41]. When controlling for rhopaliar radius, the number of palps was significantly higher per mm radius in zero-light groups than their counterparts in low light.

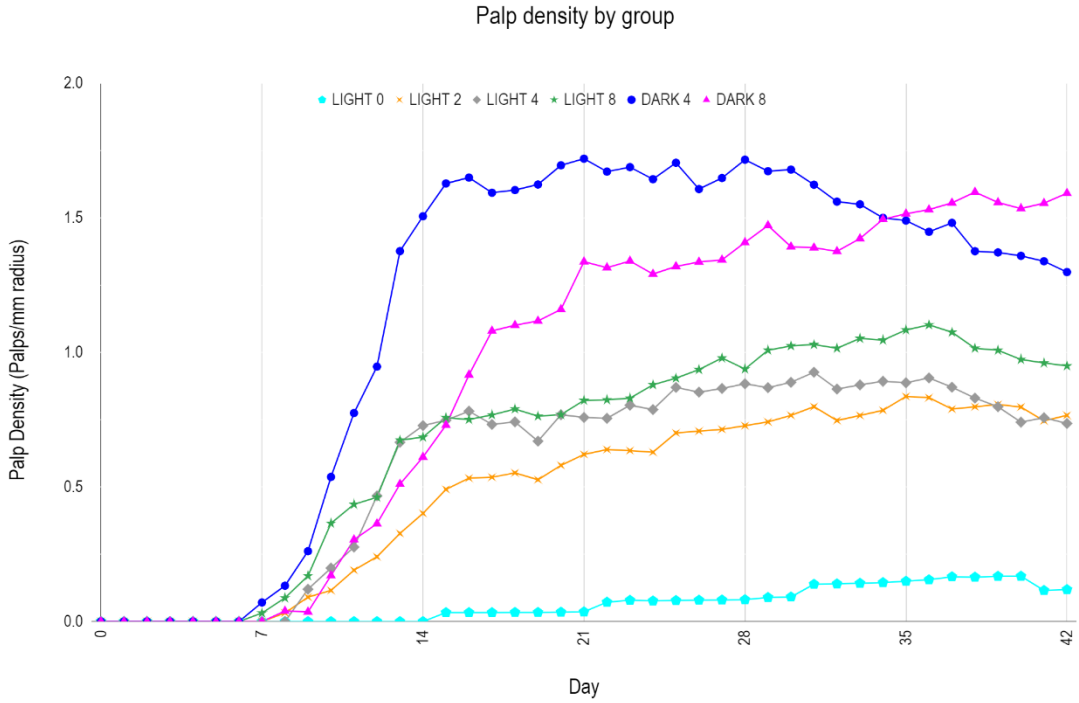

Figure 3.7 Palp density (number of palps per mm rhopaliar radius) averaged by group over the 42 days.

3.4.6. Starvation Dark Control

To determine *Cassiopea* tolerance to starvation and absence of light, ten additional ephyrae were placed under completely lightless conditions without feeding for four weeks. They were then moved into lit conditions with high food availability (8 *Artemia*/day) to determine whether they were recoverable. Two out of the ten individuals died during starvation (day 13 and day 18), and three individuals died during the

recovery (day 36, day 36, day 39). Generally, individuals who fell close to or below 1.5 mm bell diameter were unable to consume *Artemia* nauplii and/or began disintegrating. Half of the individuals (5/10) survived starvation and recovery. The surviving ephyrae gained back more than their lost size within two weeks post-starvation (surviving ephyrae were 20% larger at day 42 than day 0). The surviving ephyrae had an average diameter of 3.46 mm on day 0. The nadir in average diameter for this group was 2.17 mm on day 29, less than two-thirds their size at release. After a two-week recovery, the average diameter rose to 4.17 mm, nearly double their size two weeks prior (Fig. 3.8).

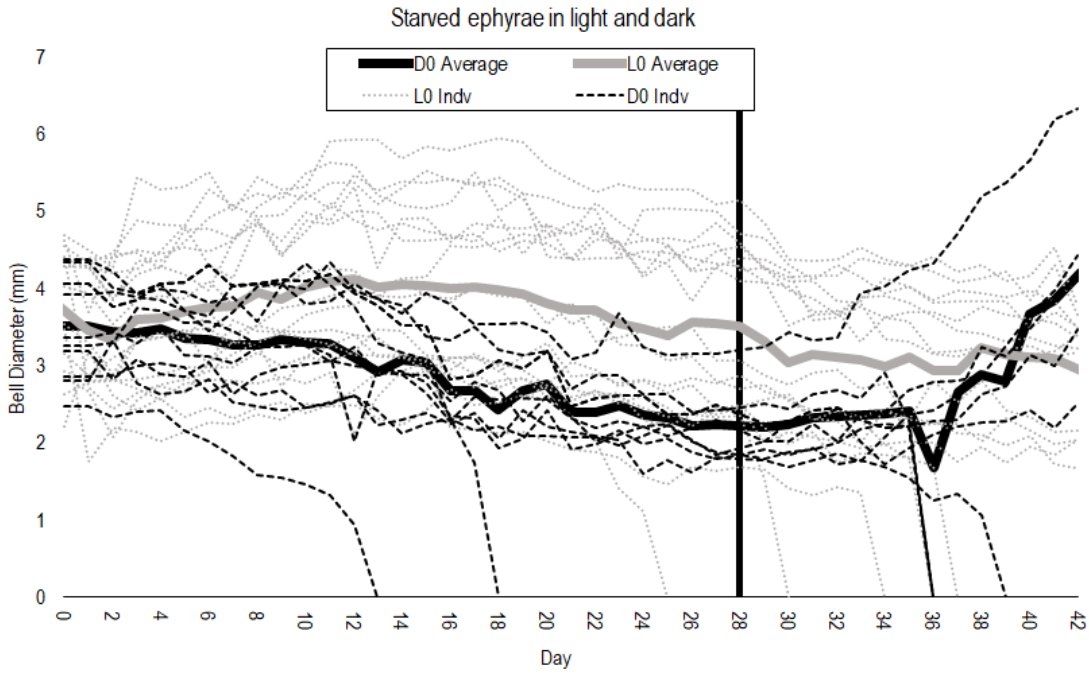

Figure 3.8 Bell diameter of starved ephyrae. Bell diameter of starved light (grey) and dark (black) individuals. Vertical line at removal from starvation (day 28) for dark individuals only. Dotted lines are averages for each group.

3.4.7. Aberrant Body Forms

Four individuals (two in L2, one in L4, and one in L8) developed oversized oral arms relative to bell size. The two individuals in the L2 also displayed inverted bells in the first two weeks of the experiment. Nine individuals across different treatments regularly had tensed bells in the first 14 days of the experiment, causing them to be laid on their sides during daily recording. These individuals mostly returned to normal pulsing patterns within ten days. All three individuals were removed early for failure to feed (see section 2.5) and had some visible abnormality by day 3. Specimen 3 (L4) and 26 (L2) tightly contracted their bells into a near sphere, while specimen 66 (D8) inverted its bell before ceasing to pulse altogether. Specimen 17 (D4) developed two especially large palps, extending well over the bell margin despite relatively small oral arms. All aberrant body forms and positionings can be seen in the supplementary photographs available on figshare [https://figshare.com/projects/Cassiopea_Light_and_Development/123538].

3.5. Discussion

We observed 108 ephyrae of *Cassiopea* respond to seven combinations of light conditions and food regimes for 43 days. While starved *Aurelia* ephyrae show a reduction in bell size[125], *Cassiopea* ephyrae's response to starvation in light (L0 group) is a reduction in the size of the manubrium with a statistically insignificant reduction in bell size. Starved *Cassiopea* ephyrae kept at below compensation light levels (treatment L0) were able to survive six weeks, with only a few showing a reduction to the size at which ability to feed is hindered (< 2 mm diameter). Starved *Cassiopea* ephyrae in dark

conditions (D0) were also able to survive for 28 days and showed remarkable recovery capability when feeding and light exposure were reinstated. Our results on survivability in the dark and absence of food are overall similar to those observed in *Aurelia* ephyra (*Cassiopea:* 62.5% recovery at 28 days starvation in 24℃, *Aurelia*: 60% advancement at 33 days in 15℃)[125].

Starvation work on *Cassiopea* is not new, and many of the changes observed in early authors on the subject holdshold true here. The mouthparts of the starved ephyrae experienced clear retreat and many stressed individuals did experience bell inversion as reported by Mayer 1914[134]. Hatai (1917) and Mayer (1914) both report severe shrinking in small starved *Cassiopea*, so it is both possible that these works signal a difference between adults and ephyrae, and that these works occurred in darker environments than 41 PAR[134,135].

As documented in corals, marginally sub-compensation level light was not an impetus for bleaching in *Cassiopea* ephyra, but near-zero light exposure was (D4 and D8) (Figure 3.7)[136]. Growth in zero-light ephyrae was significantly reduced compared to their unbleached counterparts kept at a light level of ~41 uE/m^2/s for 12 hrs/day (L0, L2, L4, L8). All ephyra in light treatments (L0, L2, l4, L8) maintained similar symbiont densities to those of healthy adults, while zero-light groups had an average symbiont density less than 1.5% the average symbiont density of their peers in light groups, most with densities too low to be detected (Figure 3.7). The variability in photosynthesis to respiration rates reported by past work on *Cassiopea* may be in part due to the large differences in *Symbiodinium* cell counts between individuals—while

the *Symbiodinium* densities reported here for day zero and adult controls and all light groups are in overlapping ranges, they include individuals with double the cell/mg zooxanthellae density of others [137].

Within light conditions (Dark or Light), final weight was correlated with the amount of food, with weight increasing from L0 to L8 and from D4 to D8. Ephyrae in L8 weighed on average 21.9 times more than L0. The mean weight of ephyrae kept in the dark and fed with four nauplii/day was less than half (41%) that of ephyra kept in lit conditions and fed with two nauplii/day. In *Cassiopea* specifically, bleaching has been suggested to have an impact on wet weight in adults [46]. Within this study we see a marked differential in individuals allowed light access and not, likely exacerbated by the starting condition of the individuals (i.e., newly released, less tissue).The weight of the ephyra kept in the dark and fed eight nauplii a day was nearly indistinguishable from the weight of ephyra kept in lit conditions and fed with two nauplii/day (9.11 vs. 9.04 mg). Weight comparison between ephyrae kept at similar feeding regimes but with or without light (D8 vs. L8 and D4 vs. L4) indicates that the presence of light was responsible for a fourfold increase in weight.

Oral arm appendages developed early (week 2) in most ephyrae and in larger numbers (relative to size) in those fed in the dark (D4 and D8). However, when not controlling for ephyra size, D8/L8 and D4/L4 had similar trajectories, possibly indicating that oral arm appendage development is influenced by consumption regardless of ephyra size. While the exact reason is unclear, the higher number of appendages

represents the only morphological difference, beyond bleaching itself, observed between ephyrae kept in dark and light conditions.

The ephyrae photographed demonstrated high variability between individuals in coloration, number of rhopalia, and growth outcomes. A sizable number developed irregularly as well. Beyond enlarged oral arms, our photographs also captured irregular coloration development, oral arm structure changes, bell patterning, and other features of growth not enumerated here. We encourage the full exploration and use of the over four thousand photographs available in the figshare archive for this project [https://figshare.com/projects/Cassiopea_Light_and_Development/123538]. A time series of one individual from each group is pictured below (Figure 3.9).

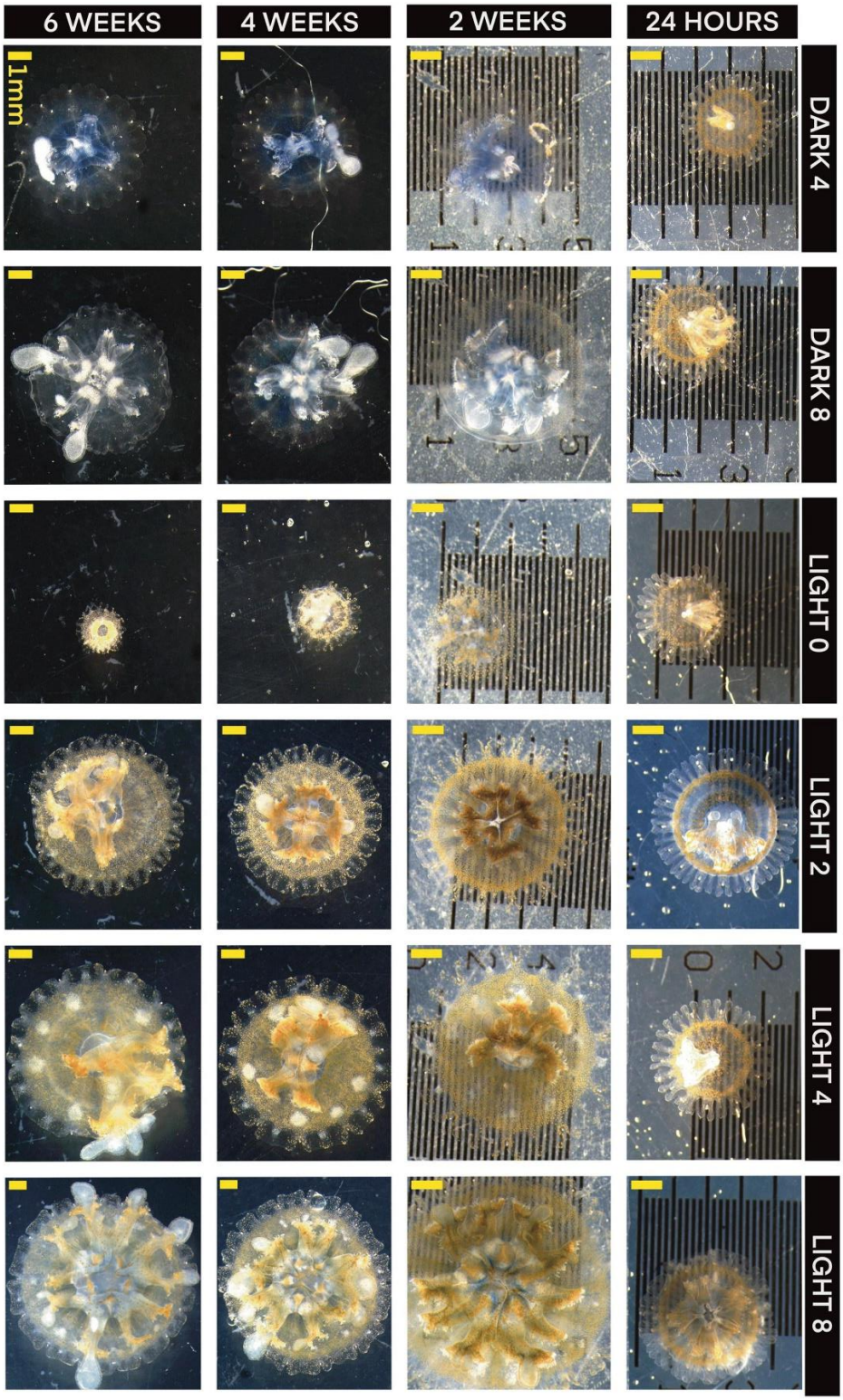

Figure 3.9 Time series for growth of a representative individual from each group.

In summary, we show that ephyrae of *Cassiopea xamachana* are tolerant of food and light stress and may be a sturdy life cycle stage with potential for species introduction. Furthermore, we show that they may survive in environments that are suboptimal in both light and food requirements, and that *Cassiopea* ephyrae can retain stable zooxanthellae levels in response to changes in feeding regime.

While polyps, planula, podocysts and planulocysts, are still the more likely long-distance spreading life cycle stages, our data join a recent body of knowledge that shows ephyrae have a remarkable ability to withstand extreme conditions and can possibly survive weeks-long journeys in ballast water or on currents to new locations.

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4. DEMYSTIFYING *CASSIOPEA* SPECIES IDENTITY IN THE FLORIDA KEYS: *CASSIOPEA XAMACHANA* AND *CASSIOPEA ANDROMEDA* COEXIST IN SHALLOW WATERS[‡]

4.1. Question and Hypothesis

Question: Are *Cassiopea* within the shallow water of the Florida Keys one species? Is there genetic divergence across sites across the length of the Florida Keys?

Hypothesis: The Florida Keys *Cassiopea* population includes phylogenetic diversity.

4.2. Introduction

Marine invertebrates have a wealth of cryptic lineages[138,139]. Although our understanding of the diversity within cryptic marine taxa has grown, precise distributions of species are often challenging to assess with common sampling approaches. Scyphozoan phylogenies have frequently been sampled across large geographic areas but with few individuals at each site[37,62]. In some pelagic systems, such as *Aurelia* or *Chrysaora*, this approach may be sufficient[140,141]. However, in systems with a strong invasion potential and limited natural dispersion capabilities, like the genus *Cassiopea*, this shallow or single-site sampling may result in inadequate coverage to identify all species present.

As in other scyphozoan lineages, *Cassiopea* suffers from poor phylogenetic clarity. This is consequential because *C. xamachana,* and the genus *Cassiopea* more

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[‡] As published in: Muffett, K., & Miglietta, M. P. (2023). Demystifying *Cassiopea* species identity in the Florida Keys: *Cassiopea xamachana* and *Cassiopea andromeda* coexist in shallow waters. *PloS One*, 18(3), e0283441. https://doi.org/10.1371/journal.pone.0283441

broadly, have become an emergent model system for research in symbiosis, behavior, and regeneration[21,38,46,48,50]. Recent work has demonstrated that morphological, symbiosis and ecological differences exist between *Cassiopea* species[47,142], however, most non-taxonomic research is done on unspecified *Cassiopea*[40,143]. This lack of clarity on the identity of the *Cassiopea* used in research, is problematic as it may lead to confounded, unreproducible, or less comparable experimental results.

High morphological heterogeneity within populations and apparent crypsis across species make *Cassiopea* difficult to identify[37,38,47]. *Cassiopea* species identification and species boundaries are even more blurred in the Florida Keys, one of the main collection grounds for *Cassiopea* research in the United States[47]. *Cassiopea* from Florida are often arbitrarily assigned one of three names: *C. xamachana*, *C. andromeda* or *C. frondosa*. *C. xamachana,* originally described in Jamaica by Bigelow 1892, theoretically represents the dominant morphotype in the Florida Keys and Caribbean[144]. *C. xamachana*'s description is distinct from *C. andromeda (*Forskål, 1775), a Red Sea native and an invasive documented in Brazil, the Mediterranean, and Hawai'i, though many of the characters on which that distinction was made are variable[36,37,44]. The separation of *C. xamachana* and *C. andromeda* is a point of contention, and the two species have been sometimes considered synonymous. When synonymized, *C. xamachana* was considered a population of introduced *C. andromeda*[38,145]. This synonymization was originally a product of Gohar and Eisawy's efforts to reduce the many described *Cassiopea* species into only three species—*C. dieuphila (*species name not revisited), *C. andromeda* and *C. frondosa*-- based solely on

one morphological character, the rhopaliar number[145]. Rhopaliar number is now recognized as having high intraspecific variability and limited interspecific variability and thus not suitable for species delimitation[37,142]. Four decades later, genetic sampling of *Cassiopea* by Holland et al. (2004) across the globe upended this oversimplistic morphological assessment and significantly expanded the known species diversity in the genus but found no evidence of a "*C. xamachana*" lineage. The "*C. xamachana*" individuals (n=4) collected from Bermuda and the Florida Keys by Holland et al. 2004 were not distinct from other global *C. andromeda* populations[37]. The *C. frondosa* species, a distinctive deeper water *Cassiopea*, and a more distant relative also found within the Keys, has remained a stable clade throughout various phylogenies and as such we did not sample these medusae[37,47]. Since Holland et al. (2004), some of the *Cassiopea* species invalidated by Gohar and Eisawy (1960) were validated and characterized morphologically, however the *C. xamachana*/*C. andromeda* distinction was not reevaluated[142], as such we have not met the need for an updated and more in-depth phylogeny of the shallow water Florida Keys *Cassiopea*.

Here, using a concerted sampling of eight shallow water sites in the Florida Keys, and using mitochondrial and nuclear genes, as well as morphological data, we address the presence and the phylogenetic status of *C. xamachana* and *C. andromeda* in the Florida Keys.

4.3. Materials and Methods

4.3.1. Collection

In August 2021, 55 *Cassiopea* were collected by hand in eight sites along the length of the Florida Keys. All sites were from near-shore water under 2 m depth (Table S1). Each *Cassiopea* was photographed, diameter measured, and small tissue samples were preserved in ethanol (95%) at room temperature. Six small individuals were fully preserved in 95% EtOH, these samples were later used for limited morphological analysis (Appendix 1 for list of samples). Medusa density was estimated in area of collection by marking out one square meter with a tape measure and counting medusae within the area. Permitting for these specimens was waived by Florida Fish and Wildlife Conservation Commision.

4.3.2. DNA Sequencing

DNA extractions from the 55 specimens were performed according to a salting out protocol (see full protocol[146]). Mitochondrial Cytochrome c oxidate subunit I (*COI*) and mitochondrial 16S ribosomal RNA were amplified using *Cassiopea*-specific protocols and primers[142]. Nuclear *28S* was amplified using the *Cassiopea*-specific primers and protocols in Daglio et al. 2017[62]. All products were purified using ExoSap and sanger sequenced at Texas A&M-Corpus Christi Genomics Core (Corpus Christi, Texas) or GeneWiz Azenta (Plainsfield, New Jersey). Fifty-five 514 bp segments of *COI*, 34 575 bp segments of the 16S rRNA gene and 18 822 bp segments of *28S* were assembled on Geneious and cleaned of low quality bases.

4.3.3. Data Analysis

4.3.3.1. *C. xamachana* and *C. andromeda* Clade Validation Dataset

Newly produced *COI* and 16S rRNA gene sequences (55 and 38 sequences respectively) were aligned using Geneious 2022.2.2 (https://www.geneious.com) to *COI* and 16S rRNA gene sequences from the *C. xamachana* and *C. andromeda* published genomes. Provisional sequence identity was assigned through agreement >98% to either the *C. xamachana* genome[43] or the *C. andromeda* mitogenome[147]. All sequences were uploaded to Genbank (see Table S1 for complete list of accession numbers).

4.3.3.2. Combined COI and 16S rRNA *Cassiopea* Phylogeny Dataset

A subset of 11 representative *COI* sequences from individuals collected in this study (see Table 4.1 and Table S1) were aligned with *Cassiopea COI* GenBank sequences from Holland et al. 2004, Morandini et al. 2016, Daglio et al. 2017, Abboud et al. 2018 and Gamero-Mora et al. 2022 (see table) using MAFFT 7 (L-INS-i)[148]. The final *COI* dataset was composed of 89 sequences, 11 of which were produced here, all trimmed to 514bp. The remaining 78 GenBank sequences belonged to the following species: *C. andromeda, C. xamachana, C. ornata, C.* sp. 1, *C.* sp. 2, *C.* sp. 3, *C. culionensis, C. mayeri, C. frondosa,* and outgroups *Mastigias papua* and *Versuriga anadyomene*.

16S rRNA gene sequences from 10 representative individuals across the Florida Keys (see Table 4.1) collected in this study were aligned with *Cassiopea* 16S rRNA gene GenBank sequences from Gamero-Mora 2022 and Daglio et al. 2017 and trimmed to 544 bp. The final 16S rRNA gene dataset was composed of 22 sequences, ten from *C.*

xamachana and *C. andromeda* from this study (same individuals as *COI* minus one) and 12 GenBank sequences belonging to *C. andromeda, C. xamachana, C. culionensis, C. mayeri, C. frondosa, C. ornata,* and outgroup taxa *M. papua* and *V. anadyomene.* 16S rRNA gene sequences were aligned using MAFFT (E-INS-i).

Models for all *COI* codons and 16S rRNA gene dataset were chosen by AICc from MEGA X 11[149] model tester (*COI* codon position 1: TN93+I, *COI* codon position 2: TN93+I, *COI* codon position 3: TN93+G+I, 16S: GTR+G+I). A dataset combining *COI* and the 16S rRNA gene was run in IQtree 1.6 under an ML framework with support from 1000 aLRT (approximate likelihood ratio test) and 1000 non-parametric bootstraps. Bayesian support was generated in BEAST 1.8[150] with 10 million steps. All phylogenetic trees were edited with Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 4.1 *COI* and 16S rRNA gene sequences used for combined tree generation, with both original identity from source and identity post-analysis. Isolates with the name *C. andromeda** were collected as *C. xamachana* then redefined as *C. andromeda* in Holland et al. 2004 but as of this writing appear in GenBank as *C. xamachana*. Used In: * denotes *COI* alignment, ^ denotes 16S rRNA gene alignment, + denotes *COI* haplotype network, # denotes 16S rRNA gene haplotype network.

Species	Reported species upon sequence publication	Locality	*COI* Accession	16S Accession	Dataset Used in	Source
C. andromeda	-	Cudjoe Key, FL, USA	OP503345	OP503932	*^+#	This study
C. andromeda	-	Key Largo, FL, USA	OP503353	OP503938	*^+#	This study
C. andromeda	-	Key West, FL, USA	OP503325	OP503913	*^+#	This study
C. andromeda	*C. andromeda*	Hilton lagoon, Waikiki, leeward O'ahu, Hawai'i, USA	AF231109	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	Hilton lagoon, Waikiki, leeward O'ahu, Hawai'i, USA	AY319448	-	*+	Holland et al. 2004

C. andromeda	*C. andromeda*	Hilton lagoon, Waikiki, leeward O'ahu, Hawai'i, USA	AY319449	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	Hilton lagoon, Waikiki, leeward O'ahu, Hawai'i, USA	AY319450	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	Kainaone fish pond, Moloka'i, Hawai'i, USA	AY319453	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	Kainaone fish pond, Moloka'i, Hawai'i, USA	AY319454	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	El Ghardaqa, Egypt	AY319458	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda*	French Polynesia	JN700934	JN700934	*^+#	Kayal et al. 2013
C. andromeda	*C. andromeda*	Brazil	KC464458	-	*+	Morandini et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610551	KY610609	*^+#	Daglio et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610552	-	*+	Daglio et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610553	-	*+	Daglio et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610554	-	*+	Daglio et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610555	-	*+	Daglio et al. 2017
C. andromeda	*C. andromeda*	Isla San José, Baja California Sur, Mexico	KY610556	-	*+	Daglio et al. 2017
C. andromeda	-	Key Largo, FL, USA	OP503367	OP503939	*^+#	This study
C. andromeda	C. sp.	Walsingham Pond, Hamilton, Bermuda	MF742175	-	*+	Abboud et al. 2018
C. andromeda	C. sp.	Mo'orea, Windward Islands, French Polynesia	MF742213	-	*+	Abboud et al. 2018
C. andromeda	C. sp.	Mo'orea, Windward Islands, French Polynesia	MF742214	-	*+	Abboud et al. 2018
C. andromeda	C. sp.	Mo'orea, Windward Islands, French Polynesia	MF742215	-	*+	Abboud et al. 2018
C. andromeda	*C. andromeda**	Walsingham Pond, Bermuda	AY319463	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda**	Richardson Bay, Bermuda	AY319464	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda**	Richardson Bay, Bermuda	AY319465	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda**	Walsingham Pond, Bermuda	AY319466	-	*+	Holland et al. 2004
C. andromeda	*C. andromeda**	Key Largo, Florida, USA	AY319468	-	*+	Holland et al. 2004

C. xamachana	*C. frondosa*	Bahia Delfines, Bocas del Toro, Panama	KY610557	-	*+	Daglio et al. 2017
C. xamachana	*C. frondosa*	Bahia Delfines, Bocas del Toro, Panama	KY610558	-	*+	Daglio et al. 2017
C. xamachana	*C. frondosa*	Bahia Delfines, Bocas del Toro, Panama	KY610559	KY610614	*^+#	Daglio et al. 2017
C. xamachana	*C.* sp.	Key Largo, Florida, USA	MF742149	-	*+	Abboud et al. 2018
C. xamachana	*C.* sp.	Cassiopea Lake, Koror State, Palau	MF742166	-	*+	Abboud et al. 2018
C. xamachana	-	Marathon Key, FL,USA	OP503314	OP503902	*^+#	This study
C. xamachana	-	Tavernier, FL, USA	OP503334	OP503922	*^+#	This study
C. xamachana	-	Lobster Walk, Monroe County, FL, USA	OP503341	OP503929	*^+#	This study
C. xamachana	-	Cudjoe Key, FL, USA	OP503343	OP503931	*^+#	This study
C. xamachana	-	Big Pine Key, FL, USA	OP503317	OP503907	*^+#	This study
C. xamachana	-	Big Pine Key, FL, USA	OP503320	-	*+	This study
C. xamachana	-	Key West, FL, USA	OP503326	OP503914	*^+#	This study
C. xamachana	*C. xamachana*	Panama	JN700936	JN700936	*^+#	Kayal et al 2013
C. xamachana	*C. xamachana*	Bahia Delfines, Bocas del Toro, Panama	KY610560	-	*	Daglio et al. 2017
C. xamachana	*C. xamachana*	Bahia Delfines, Bocas del Toro, Panama	KY610561	-	*	Daglio et al. 2017
C. xamachana	*C. xamachana*	Bahia Delfines, Bocas del Toro, Panama	KY610562	-	*	Daglio et al. 2017
C. culionensis	*C. culionensis*	Monterey Bay Aquarium, USA	KF683387	-	*	Mellas et al 2014
C. culionensis	*C. culionensis*	Philippines	MW160923	MW164879	*^	Gamero-Mora 2022
C. culionensis	*C. culionensis*	Philippines	MW160930	MW164886	*^	Gamero-Mora 2022
C. frondosa	*C. frondosa*	Key Largo, Florida, USA	AY319467	KY610617	*^	Holland et al. 2004 and Daglio et al. 2017
C. frondosa	*C. frondosa*	San Blas Islands, Panama	AY319469	-	*	Holland et al. 2004
C. frondosa	*C. frondosa*	San Blas Islands, Panama	AY319470	-	*	Holland et al. 2004
C. mayeri	*C. mayeri*	Japan	MW160931	MW164859	*^	Gamero-Mora 2022
C. mayeri	*C. mayeri*	Philippines	MW160934	MW164863	*^	Gamero-Mora 2022
C. mayeri	*C.* sp.	Sorido Bay, Kri, Papua	MF742205	-	*	Abboud et al. 2018
C. ornata	*C. ornate*	Short Drop Off, Palau	AY319456	-	*	Holland et al. 2004
C. ornata	*C. ornata*	Kakaban, Kalimantan, Indonesia	AY319472	AB720918	*^	Holland et al. 2004 and Gamero-Mora 2022

C. ornata	*C. ornate*	Kakaban, Kalimantan, Indonesia	AY319473	-	*	Holland et al. 2004
C. ornata	*C.* sp.	Risong Cove, Auluptagel Island, Koror State, Palau	MF742179	-	*	Abboud et al. 2018
C. ornata	*C.* sp.	Risong Cove, Auluptagel Island, Koror State, Palau	MF742193	-	*	Abboud et al. 2018
C. sp. *3*	*C.* sp. *3*	Kahuku windward, Oahu, Hawai'i, USA	AY319452	-	*	Holland et al. 2004
C. sp.	*C.* sp.	Coombabah Creek, Queensland, Australia	MF742133	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Coombabah Creek, Queensland, Australia	MF742135	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Kakaban, Berau, Kalimantan Timur, Indonesia	MF742139	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Kakaban, Berau, Kalimantan Timur, Indonesia	MF742140	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Kakaban, Berau, Kalimantan Timur, Indonesia	MF742141	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Kakaban, Berau, Kalimantan Timur, Indonesia	MF742142	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Haji Buang, Maratua, Berau, Kalimantan Timur, Indonesia	MF742143	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Haji Buang, Maratua, Berau, Kalimantan Timur, Indonesia	MF742148	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Danau Hidden Gam, Papua	MF742150	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Danau Hidden Gam, Papua	MF742151	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Mascot Channel, New Ireland, Papua New Guinea	MF742165	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Ongael Lake, Koror State, Palau	MF742183	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Lake Alexander, Northern Territory, Australia	MF742190	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Lake Alexander, Northern Territory, Australia	MF742191	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Lake Alexander, Northern Territory, Australia	MF742192	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Mascot Channel, New Ireland, Papua New Guinea	MF742209	-	*	Abboud et al. 2018
C. sp.	*C.* sp.	Mascot Channel, New Ireland, Papua New Guinea	MF742212	-	*	Abboud et al. 2018
C. sp. *1*	*C.* sp. *1*	Port Douglas, Queensland, Australia	AY319471	-	*	Holland et al. 2004

C. sp. *2*	*C.* sp.	Papua New Guinea	MF742198	-	*	Abboud et al. 2018
C. sp. *2*	*C.* sp.	Papua New Guinea	MF742199	-	*	Abboud et al. 2018
C. sp. *2*	*C.* sp. *2*	Observation Point, Papua New Guinea	AY319459	-	*	Holland et al. 2004
C. sp. *2*	*C.* sp. *2*	Observation Point, Papua New Guinea	AY319460	-	*	Holland et al. 2004
C. sp. *3*	*C.* sp.	Kagoshima Bay, Nagasuiro, Japan	MF742162	-	*	Abboud et al. 2018
C. sp. *3*	*C.* sp.	Nggatokae Mangroves, Western Solomon Islands	MF742189	-	*	Abboud et al. 2018
C. sp. *3*	*C.* sp. *3*	Emona, Papua New Guinea	AY319461	-	*	Holland et al. 2004
C. sp. *3*	*C.* sp. *3*	Emona, Papua New Guinea	AY319462	-	*	Holland et al. 2004
C. sp. *3*	*C.* sp. *3*	Wedding Chapel, windward O'ahu, Hawai'i, USA	AY331594	-	*	Holland et al. 2004
C. sp. *3*	*C.* sp. *3*	Kualoa Ranch, windward O'ahu, Hawai'i, USA	AY331595	-	*	Holland et al. 2004
C. xamachana	*C. xamachana*	eDNA Key Largo, FL, USA	-	MT709260	#	Ames et al. 2021
Unverified *Cassiopea* sequence	*C. andromeda*	eDNA Key Largo, FL, USA	-	MT709258	#	Ames et al. 2021
Mastigias papua	*Mastigias papua*	Mekeald Lake, Palau	KU901434	KY610621	*^	Swift et al. 2016[128]
Versuriga anadyomene	*Versuriga anadyomene*	Beibu Gulf, South China Sea	KX904853	KX904852	*^	Sun et al. 2019[151]

4.3.3.3. *28S* Dataset

The *28S* dataset consisted of 28 sequences-- 18 newly produced *Cassiopea* sequences, eight Genbank sequences of *C. frondosa*, *C. andromeda*, *C. ornata*, and two sequences belong to outgroups *M. papua* and *V. anadyomene* (see Appendix 2 for all accession numbers). All 822 to 846 nucleotide *28S* sequences were aligned using MAFFT (G-INS-i). *28S* phylogenetic trees were run on IQtree under ML framework with support from aBayes, 1000 SH-aLRT (approximate likelihood ratio test) and 1000 non-parametric bootstraps under the Mega X model tester suggested model (TN93 + G) and edited in Figtree v1.4.

4.3.4. Haplotype Networks

All 55 *COI* sequences from this study and 29 additional GenBank *COI* sequences belonging to *C. xamachana* and *C. andromeda* were used to generate a *COI* haplotype network. 38 16S rRNA gene sequences from this study and seven additional GenBank 16S rRNA gene sequences belonging to *C. xamachana* and *C. andromeda* were used to generate a 16S rRNA gene haplotype network. Two additional 16S rRNA gene sequences produced from water samples in the Florida Keys for eDNA purposes (from Ames et al. 2021) were included in the 16S rRNA gene haplotype map as these represented the only *Cassiopea* 16S rRNA gene data from the Florida Keys outside of the sequenced *C. xamachana* genome (see Table 4.1 for a complete list of sequences used). Haplotype networks were built using PopART v1.7[152].

4.3.5. Morphometric Data

Cassiopea bell diameter were recorded for each medusa and compared using a one-way analysis of variance of each of these features between sites and between mitotypes performed in R v. 4.0.3[132].

4.4. Results

4.4.1. Genetic Identity

Of the 55 *Cassiopea COI* sequences collected in the Florida Keys, 49 had a *C. xamachana* mitochondrial haplotype and six had a *C. andromeda* haplotype, as determined by agreement with published *C. xamachana* and *C. andromeda* mitogenomes[43,147]. The *COI* divergence between the two species was approximately

~7%, as previously reported[147], with no intraspecific divergence within the *C. xamachana* or *C. andromeda* collected (Fig 4.1).

The 16S rRNA gene and *COI* combined dataset, with sequences from GenBank, and rooted using sequences of *M. papua* and *V. anadyomene*, showed *C. xamachana* and *C. andromeda* as well supported sister taxa (posterior probability: 1, aLRT: 100%, bootstrap: 100%), and together as sister to a low supported clade that contains *C. ornata* from the Western Pacific , *C.* sp 1 from Australia, *C. culionensis*, *C.* sp 2 and *C. mayeri* from the Western Pacific. *C.* sp. 3 (from Papua New Guinea and Hawai'i) and *C. frondosa* are at the base of the tree (posterior probability: 1, aLRT: 99.5%, bootstrap: 100%) (Fig 4.1). Fourteen sampled individuals of undescribed *Cassiopea* from the Western Pacific (Abboud et al 2018) remain external to known *Cassiopea* taxa. Tree topology is in agreement with previous phylogenies[37,142].

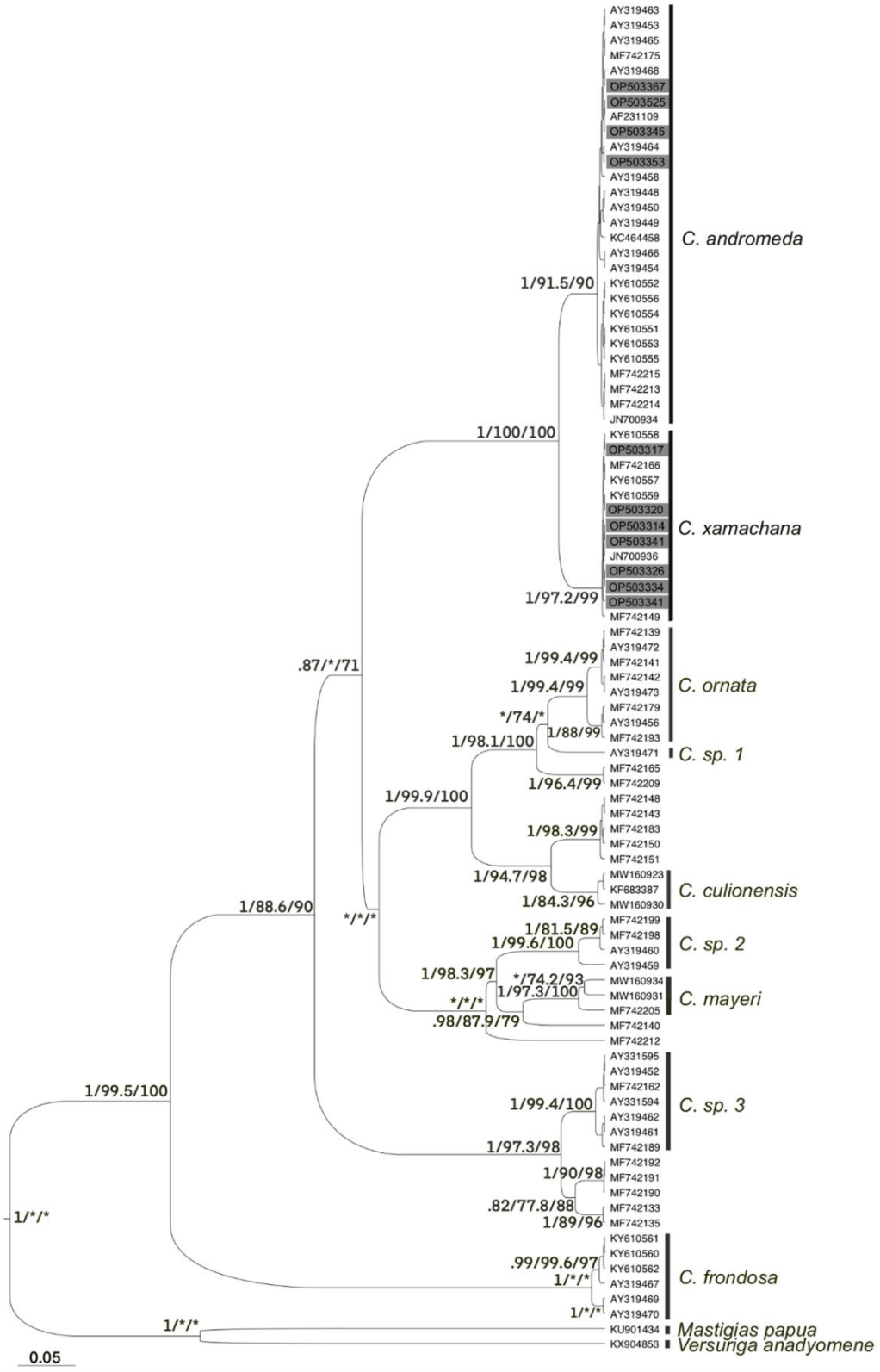
AY319463
AY319453
AY319465
MF742175
AY319468
OP503367
OP503525
AF231109
OP503345
AY319464
OP503353
AY319458
AY319448
AY319450
AY319449
KC464458
AY319466
AY319454
KY610552
KY610556
KY610554
KY610551
KY610553
KY610555
MF742215
MF742213
MF742214
JN700934
C. andromeda
KY610558
OP503317
MF742166
KY610557
KY610559
OP503320
OP503314
OP503341
JN700936
OP503326
OP503334
OP503341
MF742149
C. xamachana
MF742139
AY319472
MF742141
MF742142
AY319473
MF742179
AY319456
MF742193
C. ornata
AY319471
C. sp. 1
MF742165
MF742209
MF742148
MF742143
MF742183
MF742150
MF742151
MW160923
KF683387
MW160930
C. culionensis
MF742199
MF742198
AY319460
AY319459
C. sp. 2
MW160934
MW160931
MF742205
C. mayeri
MF742140
MF742212
AY331595
AY319452
MF742162
AY331594
AY319462
AY319461
MF742189
C. sp. 3
MF742192
MF742191
MF742190
MF742133
MF742135
KY610561
KY610560
KY610562
AY319467
AY319469
AY319470
C. frondosa
KU901434
Mastigias papua
KX904853
Versuriga anadyomene
1/91.5/90
1/100/100
1/97.2/99
1/99.4/99
1/99.4/99
/74/
1/88/99
1/98.1/100
1/96.4/99
.87/*/71
1/99.9/100
1/98.3/99
1/94.7/98
1/84.3/96
1/88.6/90
//*
1/81.5/89
1/99.6/100
1/98.3/97
*/74.2/93
1/97.3/100
//*
.98/87.9/79
1/99.5/100
1/99.4/100
1/97.3/98
1/90/98
.82/77.8/88
1/89/96
1/*/*
.99/99.6/97
1/*/*
1/*/*
1/*/*
0.05

Figure 4.1 Combined *COI* and 16S rRNA gene tree of *Cassiopea*. Maximum likelihood *COI*+ 16S tree with posterior probability, aLRT, and bootstrap supports. Individuals in grey were sequenced in this study. All accession numbers are for *COI*, see 16S accession numbers in Table 4.1**.**

The clade of *C. xamachana*, as defined by the published genome, is composed by sequences from the Florida Keys and Atlantic Panama, and one isolate from Palau (reported by Abboud et al. 2018). According to the analyzed dataset, except for the single Palau sequence, the *C. xamachana* mitotype is restricted to the West Atlantic (Fig 4.2a). *COI* sequences identified as "*C. frondosa"* and collected in Panama (Atlantic), also fell within the *C. xamachana* clade. A third common species, *C. andromeda,* includes sequences from specimens collected in Hawai'i, Mexico, Bermuda, Brazil, Florida and the Red Sea (Fig 4.2a & 4.2b).

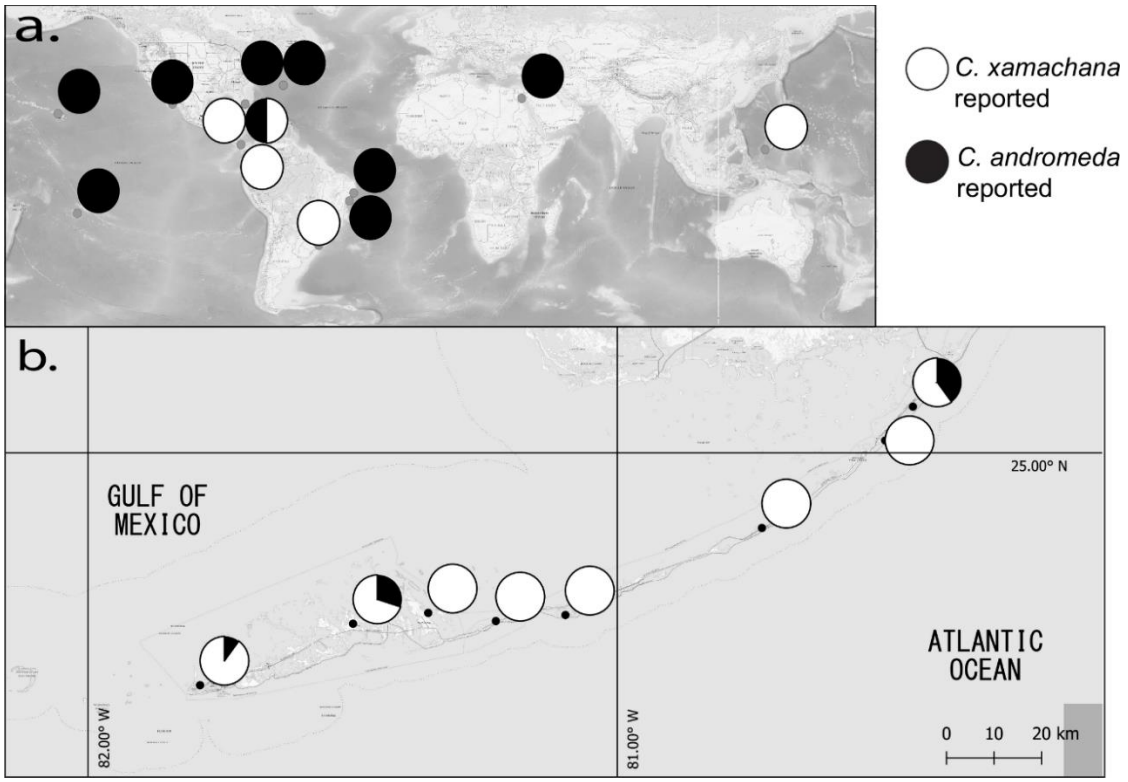

Figure 4.2 Distribution of *C. xamachana* and *C. andromeda*. (a) Global *C. andromeda* (black) and *C. xamachana* (white) distributions from sequences published in relevant

scyphozoan or *Cassiopea* specific phylogenies from 2004–2022. Locations with both recorded as black-and-white. (b) *C. andromeda* (black) and *C. xamachana* (white) isolates from the Florida Keys from this study, pie chart indicates proportion of specimens that were *C. xamachana* and *C. andromeda* at each site.

C. xamachana and *C. andromeda* had ~3.1% divergence in 16S rRNA gene sequences, lower than that calculated for *COI*. The six 16S sequences from collected *C. andromeda* showed no intraspecific diversity. The 32 *C. xamachana* 16S rRNA gene sequences showed low intraspecific diversity (d=0.0006) (Fig 4.3b).

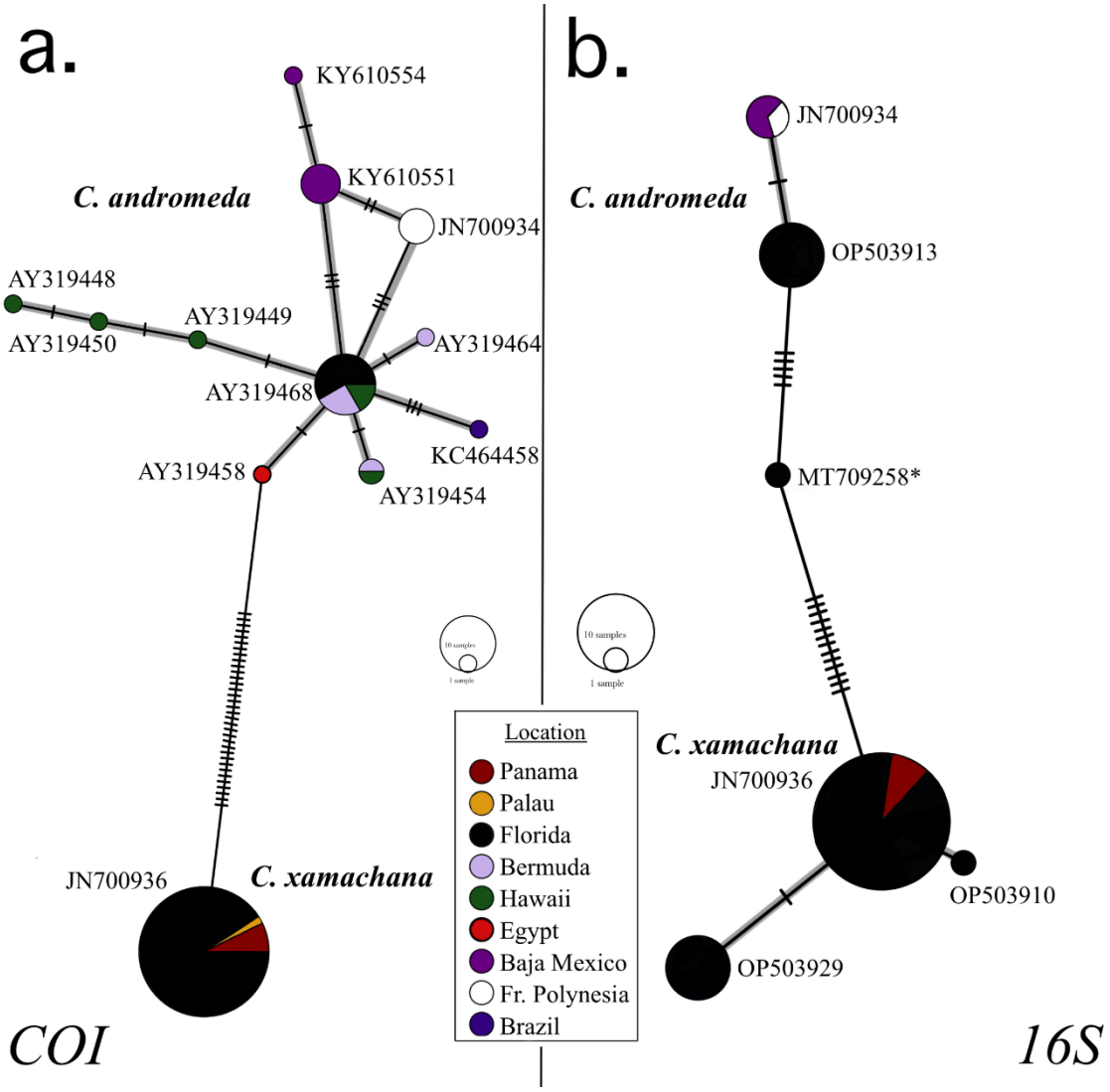

Figure 4.3 Haplotype network for *C. xamachana* and *C. andromeda*. (a) *COI* haplotype network and (b) 16S rRNA haplotype network. One accession number that is consistent with the haplotype is displayed next to each group. Sequence MT709258* is a product of eDNA work and may not represent a genuine haplotype. Grey highlight connects all sequences from each species.

When considering the *COI* dataset used for haplotype network building, *C. andromeda* showed 11 total haplotypes and *C. xamachana* a single haplotype (Fig 4.3a). *C. xamachana* showed three 16S haplotypes, and *C. andromeda* two, though only one was from specimens collected in this study (Fig 4.3b). The most common *C. xamachana*

16S rRNA gene haplotype was present in both the Keys and Panama. The second largest 16S rRNA haplotype was present only in upper Keys sites, in conjunction with a single individual from Big Pine Key showing a single nucleotide change. In the 16S haplotype network, there is one intermediate between *C. xamachana* and *C. andromeda* individuals, however this sequence was collected as part of an eDNA project and may not represent a mitochondrial haplotype present in the system[153].

The nuclear *28S* sequences (n=18) from all sampled *Cassiopea* showed polymorphism, and tree topology was incongruent with mitochondrial genes sequenced. Specifically, sequences belonging to specimens collected in this study identified as *C. xamachana* and *C. andromeda* showed no differentiation. Genbank sequences of *C. andromeda* from Baja California (Genbank Acc. KY611005-7) included gaps not found in the sequences from the Keys and were closely related to two GenBank sequences of "*C. frondosa*" from Panama (likely *C. xamachana*) (Genbank Acc. KY611002-3). *C. ornata* from Palau and true *C. frondosa* from Key West (GenBank Acc. HM194838 and HM194872) were divergent from the *C. xamachana*/*C. andromeda* clade and each other.

4.4.2. Geographic distribution of *C. andromeda* and *C. xamachana* within the Florida Keys

We collected 55 samples in 8 localities along the Florida Keys (Appendix 1). With 49/55 individuals, *C. xamachana* was more frequently found in samples (Fig 4.2b). *C. xamachana* was found in all sites and *C. andromeda* in three of eight sampling sites. In the three sites that hosted the two species, *C. xamachana* was more abundant than *C. andromeda* (proportion of *C. andromeda*: 3/10 on Cudjoe Key, 1/10 on Key West, 2/5

on Key Largo). Two of the five monospecific *C. xamachana* sites were shallow lagoons (< 1m depth). The other two were low coverage tidal oceanic sites with low density and large individuals. The three sites of cohabitation were densely populated sites (>10 medusae/m2) with calm water but direct oceanic exposure. Both the Key Largo site and the Key West site were at marinas.

C. andromeda have been found on both the Atlantic and Gulf sides of Key Largo, as well as on the Gulf side of Cudjoe Key and Key West.

4.4.3. Size and color

At each location, *C. andromeda* and *C. xamachana* presented the same color type and similar morphology, with no apparent character that could distinguish between the two species. Overall, individuals with the *C. andromeda* mitotype had somewhat smaller diameters (mean= 4.8 cm) than *C. xamachana* (mean= 7.8 cm) but not to a significant degree (ANOVA, $F(2,47) = 3.69$, $p= 0.061$). Two of the sites where *C. andromeda* were found (Cudjoe Key and Key Largo Ocean Bay Marina) had primarily small individuals, and site was the most important factor in size determination (ANOVA, $F(1,47) = 5.08$, $p= 0.029$). As all specimens were preserved in ethanol as opposed to formalin, no in-depth comparative morphological analyses were performed. A general inspection showed that *C. andromeda (*n=2) and *C. xamachana* (n=3), when devoid of symbionts, had different bell markings, however, with such a limited dataset, no conclusions could be drawn.

With regard to coloration, *Cassiopea* collected from this work were blue, white, purple, pink, brown, and green. This is a well-known phenomenon in *Cassiopea,* and the

exact dynamics of color are poorly understood. Color profiles were generally consistent within sites but were highly variable between sites (Fig 4.4). There was no consistent difference in color bell, oral arm or paddle coloration between species within each site at which the mitotypes were cooccurring.

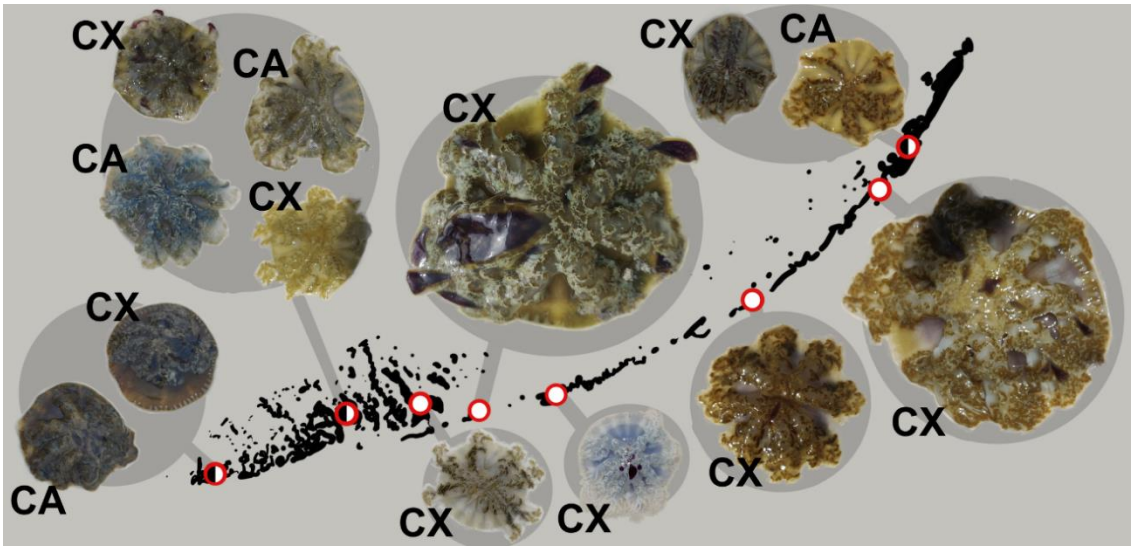

Figure 4.4 Color and morphological variability in collected *Cassiopea* by site in the Florida Keys. "CX" for *Cassiopea xamachana* and "CA" for *Cassiopea andromeda*.

4.5. Discussion

C. xamachana, has been genetically undersampled in the Florida Keys relative to its use in research and laboratory work[43,48,143,154]. A belief that *C. xamachana* was a *C. andromeda* subpopulation likely contributed to this lack of focus on dense geographic sampling[37,141]. Moreover, in the last 20 years *C. xamachana COI* isolates have occasionally been misidentified as *C. frondosa*, a very distantly related *Cassiopea*, further adding to the taxonomic confusion. Our data support the notion that *C. xamachana* and *C. andromeda* mitotypes are now both found in the Florida Keys. Although mitochondrial DNA suggests these two species are reciprocally monophyletic

clades, additional nuclear genes are necessary to confirm their monophyly. The *Cassiopea* collected here were not distinguishable in the field and they inhabited the same shallow water, sometimes coexisting side by side in the same location. 89% of our samples matched *C. xamachana*. *C. xamachana* was also sampled in 100% of sampling sites (8/8), while *C. andromeda* was sampled in 38% (3/8). We thus show that in our sampling effort, the *C. xamachana* mitotype is more abundant both in terms of number of jellyfish and locations where it is found.

Our results also confirm some findings of recent eDNA analyses conducted in the same area that recorded 16S rRNA gene residues of both species in the Florida Keys[153]. While we find the *C. xamachana* and *C. andromeda* mitotypes in our data, we fail to find the intermediate 16S rRNA gene signature found by Ames et al. 2021, a sequence that may have been an interspecific chimera. Despite having more representatives in this study, there was no diversity within the *C. xamachana COI* sequences and little diversity in their 16S profiles. This may represent a higher degree of continuity across the Florida Keys and Panama than expected. Further study of the exact boundaries that impact *Cassiopea* genetic populations is needed. While *C. xamachana* has been found in Brazil and Palau[141,155], it does not yet have the non-native range demonstrated by the *C. andromeda* clade.

While there has been no demonstrated divergence in behavior or tolerance to environmental factor between *C. xamachana* and *C. andromeda*, there has also been no study formally comparing them. Our data show that wild-caught *Cassiopea,* even from single locations present clear hazards for comparative analysis if not properly

identified[62]. This brings into foreground the inadvisability of treating results from Floridian samples and other locations as representatives of one clade without genetic evidence. In addition to genetic study, the Keys population would benefit from careful morphological and ecological analysis within mixed assemblage sites to parse whether these cooccurring populations have distinct diagnostic morphometrics or ecological features.

The mitochondrial markers analyzed in this work present evidence of historical genetic separation between *C. xamachana* and *C. andromeda*, the nuclear marker (*28S*), however, does not. As *Cassiopea* has very few published *28S* sequences, some of which certainly suffer from the same issues of misidentification as the *COI* isolates, firm conclusions cannot be drawn as to the usefulness of *28S* for species delimitation. Given the low mitochondrial divergence relative to a 10% benchmark[138], the *28S* results may indicate introgression and hybridization between *C. andromeda* and *C. xamachana* . In-depth study of a larger array of nuclear markers is needed to parse the hybridization potential or degree of *C. xamachana* and *C. andromeda* within the Florida Keys (Fig 4.5).

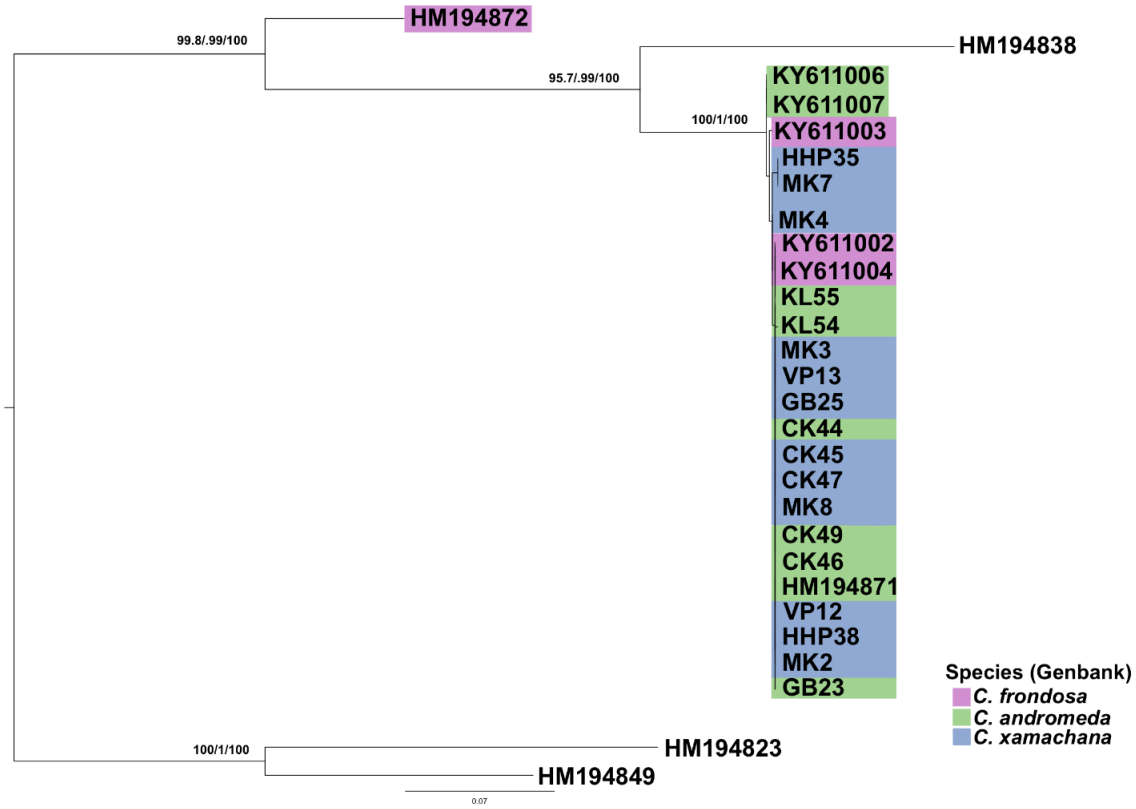

Figure 4.5 *28S* tree with all *C. frondosa, C. andromeda* and *C. ornata* sequences from Genbank. KY611002-4 are likely misidentified *C. xamachana*. Support is organized as SH-aLRT/aBayes/bootstrap**.**

As only 11% of individuals found in the Keys in this sampling effort were *C. andromeda*, only relatively dense sampling (either large collections from multiple locations or environmental sequencing) could identify the mix. In Brazil, multiple *Cassiopea* invasions have resulted in both *C. xamachana* and *C. andromeda* populations, but these were found in separate sampling efforts[36,155,156]. Locations with *Cassiopea*, especially those with a paucity of sequences, may present the sort of assemblages already identified here in the Florida Keys, Hawai'i, Brazil, the Philippines and Palau, and may require multiple rounds of sampling to parse[37,141,142]. Some Pacific *Cassiopea* populations remain unidentified (see the sequences without species identity in fig 4.1),

further hampering our understanding of invasion history within the genus. Greater sampling numbers are needed to further characterize species distributions within the Keys and elsewhere.

Finally, in constructing the phylogeny, we found instances of *Cassiopea* misidentification in GenBank. Three *COI* sequences identified as *Cassiopea frondosa* were instead *C. xamachana*. Additionally, five sequences identified as *C. xamachana* were instead *C. andromeda,* despite their originating text correctly identifying their species affinity[37]. This accounts for five of sixteen total identified *C. andromeda* sequences. This indicates a general problem with GenBank sequences and is a result of the taxonomic confusion that has surrounded *Cassiopea* species.

4.6. Conclusion

Cassiopea in the Florida Keys has long been defined as two species, the deeper-water, distinctive *C. frondosa* and the shallow-water *C. xamachana*[47]. In 1960 and again in 2004, *C. xamachana* was relegated to a junior synonym of *C. andromeda*[37,145]. Using a phylogenetic approach, we show that *C. andromeda* and *C. xamachana* mitochondrial genotypes are both found in sympatry in the Florida Keys, showing no obvious morphological differences. We show that it is difficult to determine the population history of *Cassiopea* collected in shallow water in the Keys without proper molecular barcoding. This is relevant because a wealth of research has been performed with various *Cassiopea* without a proper assessment of the species it was conducted on. We also found evidence that *Cassiopea* research has suffered from frequent species misidentification. This paper calls for deeper sampling of jellyfish assemblages within

Cassiopea and other highly cryptic scyphozoan genera. It also indicated that a proper species identification that involves molecular barcoding is essential for any work on *Cassiopea*, especially from Florida. This even more crucial as *Cassiopea* continues to successfully establish itself as an emerging model system for physiological studies and as a proxy for investigations on zooxanthellae-Cnidaria interaction. Caution should be exercised in generalizing result from published studies that assumed *Cassiopea* identity without explicitly investigating species identification with molecular tools.

5. MICROBIOME OF *CASSIOPEA* IN THE FLORIDA KEYS

5.1. Question and Hypothesis

Question: Do the internal and external microbial community of *Cassiopea* differ from the surrounding environment? Does the microbial community of *Cassiopea* stay consistent across the Keys?

Hypothesis: *Cassiopea* within the Florida Keys have a distinct internal microbiome that is different from the surrounding environment. Core components of these communities are present across sites.

5.2. Introduction

Cnidarian-photosymbiont interactions are central to ecosystem health in coral reefs and beyond. Photosymbionts are present in the well-known stony corals, octocorals and anemones, but also play important roles in a variety of lesser-known zooxanthellate jellyfishes, chief among them the genus *Cassiopea* [24,157–159]. For Hexacorallia, microbial communities play a key role in maintaining health within this photosymbiont partnership, with distinct corals demonstrating cophylogeny with their bacterial communities [160]. While this is well established, few analyses have documented the stability of host-microbiome associations within zooxanthellate jellyfishes.

Within jellyfish microbial studies, many involve the microbiome of lab-reared specimens [48,161] or limited sample sizes [28,30,162]. While lab studies may be able to track state changes across life stage [161] and between condition types (symbiotic/aposymbiotic) [48], there is little guarantee that the microbial communities demonstrated in laboratory conditions translate to *in situ* conditions. Recent *in situ* studies provide insights into

microbial taxa associated with some scyphozoans ("true jellyfish") but have not included species of the genus *Cassiopea*. In the moon jellyfish, *Aurelia* spp., individuals from the Chinese coast are *Vibrionaceae*-dominated, while samples from the northwestern Atlantic are *Mycoplasma*-dominated (Daley et al., 2016; Peng et al., 2021). Between the Indonesian marine lakes Kakaban, Haji Buang, and Tanah Bamban, *Mastigias* vary significantly in composition by lake, but maintain *Oceanospirillales* (primarily *Endozoicomonas*-like)[30]. In samples across taxa, *Mycoplasma* is a component of the identified gut and tissue microbiome of jellyfishes, though its function remains unknown [29,163–167]. Pathogenesis is often a focus of scyphozoan microbiome discovery. *Tenacibaculum* has been identified in several species, e.g *Cotyllorhiza tuberculata*, *Aurelia aurita*, and *Pelagia noctiluca*, and, as a fish disease, *Tenacibaculum* has important fisheries ramifications [29,163,165,168]. As most studied jellyfish populations occur in coastal waters only sporadically, disentangling time and bloom specific effects requires work on coastal non-blooming species, like the many representatives of the Kolpophorae [24].

The Upside-Down Jellyfish, *Cassiopea* spp., is a model organism for cnidarian-photosymbiont interactions [38,169]. These medusae are residents of tropical and subtropical waters globally and can survive in water that is frequently shallow (as little as 8 cm depth) and hot (up to 33˚C for *C. andromeda*) [42,170]. *Cassiopea* obligatorily hosts zooxanthellae symbionts, losing mass even when well fed while aposymbiotic [46]. As in corals and other scyphozoans, mucus plays a central role for *Cassiopea*, presenting a sticky interface between the medusae and their benthic environment [41,171].

Cassiopea spp. presents an opportunity to act as both a comparison group to the microbial communities associated with other scyphozoans and those of symbiotic anthozoans. The stable nature of *Cassiopea* assemblages allows the examination of differences in the microbial community of one population (Florida Keys *Cassiopea*) at one time point across a broad geographic area. As these photosymbiotic near-shore benthic jellyfishes are both plentiful and present for much of the year, they are ideal to study how features of an individual (size, density, and location) impact internal and external microbial communities.

The aims of this work were to identify the core components of the internal and external mucosal microbiomes of *Cassiopea* and compare the internal *Cassiopea* microbial communities of sites across the Florida Keys.

5.3. Methods

5.3.1. Sampling

Table 5.1: List of collections with latitud, longitude, salinity, pH, surface temperature. Average diameter of collected medusae from the site and date of collection. (*)Estimated medusa density was performed only in the area of collection with a simple meter stick, and may not have been representative of the entire site.

Site Name	Latitude	Longitude	Salinity (ppt)	pH	Temp (C)	Average depth (m)	Average col. medusa diam (cm)	Est. density (medusae/ m2)*	Date (AM/PM)
Key West	24.561526	-81.7881727	36	8.2	30.4	0.56	6.62	100	15.8.2021 PM
Cudjoe Key	24.6775378	-81.4991819	36	8.4	32.6	0.36	5.91	30	17.8.2021 AM
Big Pine Key	24.6978731	-81.3572574	35	7.7	29.1	0.15	4.43	30-50	15.8.2021 PM
Bahia Honda Key TP1	24.6825793	-81.2293673	37	8.3	31.8	0.86	12.41	1	13.8.2021 PM
Bahia Honda Key TP2	24.6825793	-81.2293673	36	8	29.4	0.51	10.28	2	15.8.2021 AM

Marathon Key	24.69396	-81.0980507	31	8.1	35.2	0.08	6.53	5	11.8.2021 PM
Lower Matacumbe Key	24.8582157	-80.7267927	35	8.1	32.9	0.89	11.40	5	16.8.2021 PM
Tavernier	25.0233783	-80.4940316	31	8.1	33.3	0.35	12.26	7	16.8.2021 AM
Key Largo	25.0872104	-80.4415797	35	7.8	32.0	0.56	4.30	10	18.8.2021 AM

Samples were collected by wading from eight sites along the length of the Florida Keys in late August 2021 over the course of 8 days. At each site and in between sampling efforts, all equipment was cleaned with ethanol and bleach. A 1 L water sample was collected from above each medusa aggregation and filtered (0.2um pore size, Thermo Scientific Cat No.09-740-30G) with a manual sampler as described in the USGS manual water sampling protocol [172], replicate filters were placed in 15 mL of ethanol and dimethyl sulfoxide ethylenediamine tetraacetic acid saturated salt storage solution (1 L pH 7.5: 93.06 g EDTA, 60 mL 20 % NaOH solution, 20 mL 25 % HCl, 40 mL DMSO, 800 mL water, NaCl to oversaturation; see ref. Pavlovska 2021) (commonly referred to as DESS). At each *Cassiopea* medusa aggregation, a scraping of replicate ~10 g of sediment collections (benthic sample) were placed in 15 mL of ethanol and DESS storage solution.

At each site, coordinates, temperature, time of day, depth, salinity, and pH were recorded (Table 5.1). Salinity was measured with a refractometer and temperature and pH were measured with an Apera A1209.

Each *Cassiopea* medusa was collected from shallow waters <1.5 m from within a single assemblage at each site. Medusae were grasped by the oral arms, lifted from the water, swabbed along the apex of the bell, then placed bell-down onto a pre-prepared dissection plate (Fig 5.1). External bell swabs were done in replicate and placed in 3 mL

DESS and ethanol. Medusa oral arm base was bisected between oral arm groups and replicate swabs were inserted and circled the digestive cavity. One internal swab was placed in DESS, the other in ethanol, as with external swab. A section of bell tissue was then collected and placed in ethanol for species confirmation. Size of each medusa was taken and individual medusae were photographed. As sampling was done in a nonsterile environment, blanks for buffer with swab and buffer with filter were taken as well.

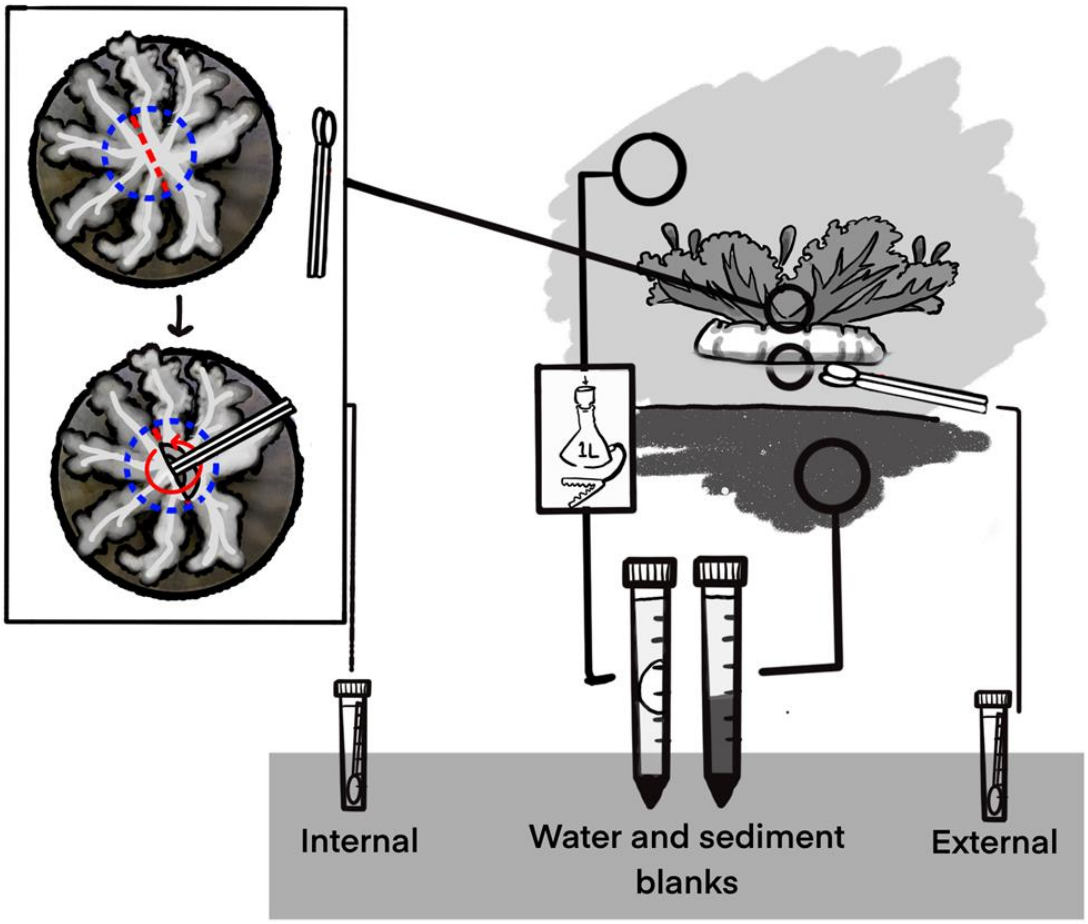

Figure 5.1 Sampling strategy for medusae collection onsite. Pictured right is one medusa in standard orientation. Locations from which all related samples are taken. Internal swabs were collected through an incision in the oral arms (pictured left).

5.3.2. Extraction and Sequencing

Samples in DESS and EtOH were maintained at room temperature for between three and ten days, then frozen. DESS can be stored for at least thirty days at room temperature without significantly compromising community integrity [173]. All samples were then extracted using Zymobiomics DNA Kits (PN# D4300T). 16S V4 amplifications were done for all samples. Only samples that produced visible bands were sent for 16S V3-V4 prep and sequencing. While DESS preserved samples yielded good amplification results, EtOH-preserved samples largely failed to produce adequate amplification. As such only a subset of EtOH-preserved samples were sent for sequencing. Sequencing was performed by the University of Texas Medical Branch Sequencing Lab. Library was prepared with Zymo Quick 16S V3-V4 kit (PN #D6400) in 20 uL reactions (10 uL Quick-16S qPCR premix, 4 uL Quick-16S V3-V4 primers (341f: (CCTACGGGDGGCWGCAG, CCTAYGGGGYGCWGCAG) and 806r: (GACTACNVGGGTMTCTAATCC)), 4 uL water and 2 uL sample). qPCR was run using Roche LightCycler 480 Instrument II (10min 95C, [30sec 95C, 30sec 55C, 3min 72C]x20). PCRs were enzymatically cleaned according to kit protocols and barcodes were added in a secondary reaction at 20 uL (10 min 95˚C, [30 sec 95˚C, 30 sec 55˚C, 3 min 72˚C]x5). Plates included two microbial community standards and two PCR negative controls. Samples were loaded at 10 pM, with 15% 10 pM PhiX sequencing control and run on Illumina MiSeq with a 600 cycle v3 kit.

5.3.3. Data analysis

Data generated was run through the mothur v1.47 [174] pipeline with only limited deviations from the published standard mothur MiSeq protocols (full pipeline can be found in Appendix 3) [175]. Sequences attributable to Chloroplast, Mitochondria, Eukaryota and unattributable sequences, were removed ("remove.seq"). All genera found in the negative controls (4 PCR controls, 5 collection and extraction controls) at a rate of greater than 1% were removed using "remove.lineage". Number of sequences removed at each of these filtering steps can be found in Appendix 4. Based on the sequenced microbial community positive control, ASVs were oversplit and the amplification was biased towards Gram positive bacteria. Given this oversplitting, analysis for 16S V3-V4 data was lumped by classification at the genus level. Genera with five or fewer sequences were removed.

5.3.4. Statistical analyses

Refraction curves, principal component analysis, nmds, diversity metrics and analysis of molecular variance were computed in MOTHUR. NMDS plot was visualized using R 4.0.1 (R Core Team, 2021).

5.4. Results

5.4.1. Data description

The dataset included 13.27 million sequences after merging ("make.contigs"), 7.17 million after screening for homopolymers and length ("screen.seqs": maxlength=480, maxhomop=8). Unique sequences were aligned to the 16S small subunit V3-V4 reference sequence from *E. coli* ("pcr.seqs") and filtered again to produce 3.66 million unique sequences that were clustered into ASVs ("pre.cluster":

diffs=4). After chimera removal ("chimera.vsearch"), 2.27 million unique sequences were classified according to the Silva reference library v.132 ("classify.seqs"). After quality filtering, there were 2405 genera and taxonomic units (eg Protobacteria unclassified) present. After removing rares there were 1824 genera present.

5.4.2. Distribution of sequences in full dataset

Of the 5715156 final sequences, almost all were Bacteria as opposed to Archaea (98.96% to 1.04%). Of these Bacteria, 70.27% were Proteobacteria, 10.32% were Bacteroidetes, 8.81% were Tenericutes and 2.74% were Cyanobacteria. No other phylum had greater than 2% relative abundance. At the class level, Gammaproteobacteria comprised 49.20% of all bacterial sequences. Alphaproteobacteria, the next most abundant, comprised 12.13%. Bacteriodia (9.20%), Mollicutes (8.42%), Deltaproteobacteria (5.46%), Oxyphotobacteria (2.53%), Verrucomicrobiae (1.35%) and Campylobacteria (1.09%) were the other classes with an relative abundance above 1%. The majority of Archaea within the dataset were from the classes Halobacteria (40.17%) and Woesearchaeia (33.94%).

5.4.3. Distribution of sequences within gastrovascular cavity (GVC) samples

Within gastrovascular cavity samples, 70.41% of the 2485633 total sequences were Proteobacteria, 17.89% Tenericutes, 4.02% Bacteriodetes, 1.75% Cyanobacteria and 1.19% Chlamydiae. 67.73% of all sequences from gastrovascular cavities belonged to just three orders, Oceanospirillales (42.02%), Mycoplasmatales (17.80%) and Vibrionales (13.38%). The Oceanospirillales sequences identified within the gastrovascular cavity were monotypic, 98.07% were identified to the genus *Endozoicommonas.* This was true

of the Mycoplasmatales as well, 99.96% were identified as sequences from the genus *Mycoplasma.* Vibrionales was primarily *Vibrio* (77.43%) but included unclassified Vibrionaceae sequences as well (18.68%).

5.4.4. Distribution of sequences within external mucus samples

Of the 2011849 total sequences from external mucosal swabs, 65.58% were Proteobacteria, 12.62% Bacteriodea, 3.17% Cyanobacteria and 2.54% Planctomycetes. At the order level 19.34% of sequences were Oceanospirillales, 6.65% unclassified Gammaproteobacteria, 6.33% Rhodobacterales, 4.89% Desulfobacterales, 4.78% Chromatiales, 4.30% Flavobacteriales, with fourteen additional orders comprising between 1 and 2.5% of abundance each. 84.40% of external swab Oceanospirillales sequences were places within the family Endozoicomonadaceae. The families Rhodobacteraceae (6.33%), Chromatiaceae (4.29%), Flavobacteriaceae (3.22%), and Desulfobacteraceae (3.35%) also had abundances greater than 2%.

5.4.5. Benthic and water samples

Benthic samples were primarily dominated by Proteobacteria (51.28%) and Bacteroidetes (19.77%). At the order level, Desulfobacterales (9.67%), unclassified Gammaproteobacteria (7.50%), Rhodobacterales (7.16%), Flavobacteriales (5.96%), and Chromatiales (4.16%) were most common.

Proteobacteria (68.26%) and Bacteroidetes (14.91%) were the primary phyla of water samples. Primary orders within water samples were Oceanospirillales (15.30%), Rhodobacterales (14.96), SAR11 (11.92%), Flavobacteriales (10.76%), and Chromatiales (7.41%). 1.35% of these Oceanospirillales sequences were assigned to *Endozoicommonas.*

5.4.6. Diversity

Evenness and species richness were both lower in the gastrovascular cavity samples than in external mucus, benthic or water samples. The evenness of gastrovascular cavity communities was low (Shannon diversity H= 1.91+/-2.13) when compared to external mucus (H= 4.37+/-1.74) and environmental samples (Water: H=3.59+/-1.47; Benthic: H=4.84+/-0.35).

Species richness was low in gastrovascular cavity samples (Inverse Simpson: 1/D= 5.12+/-13.09) and high in external mucus samples (1/D= 36.92 +/-39.93), similar to benthic samples (1/D= 48.11+/-14.75)(Figure 5.2). On average, 291 (+/-332) genera were observed in gastrovascular samples and 500 (+/- 428) were observed in external samples.

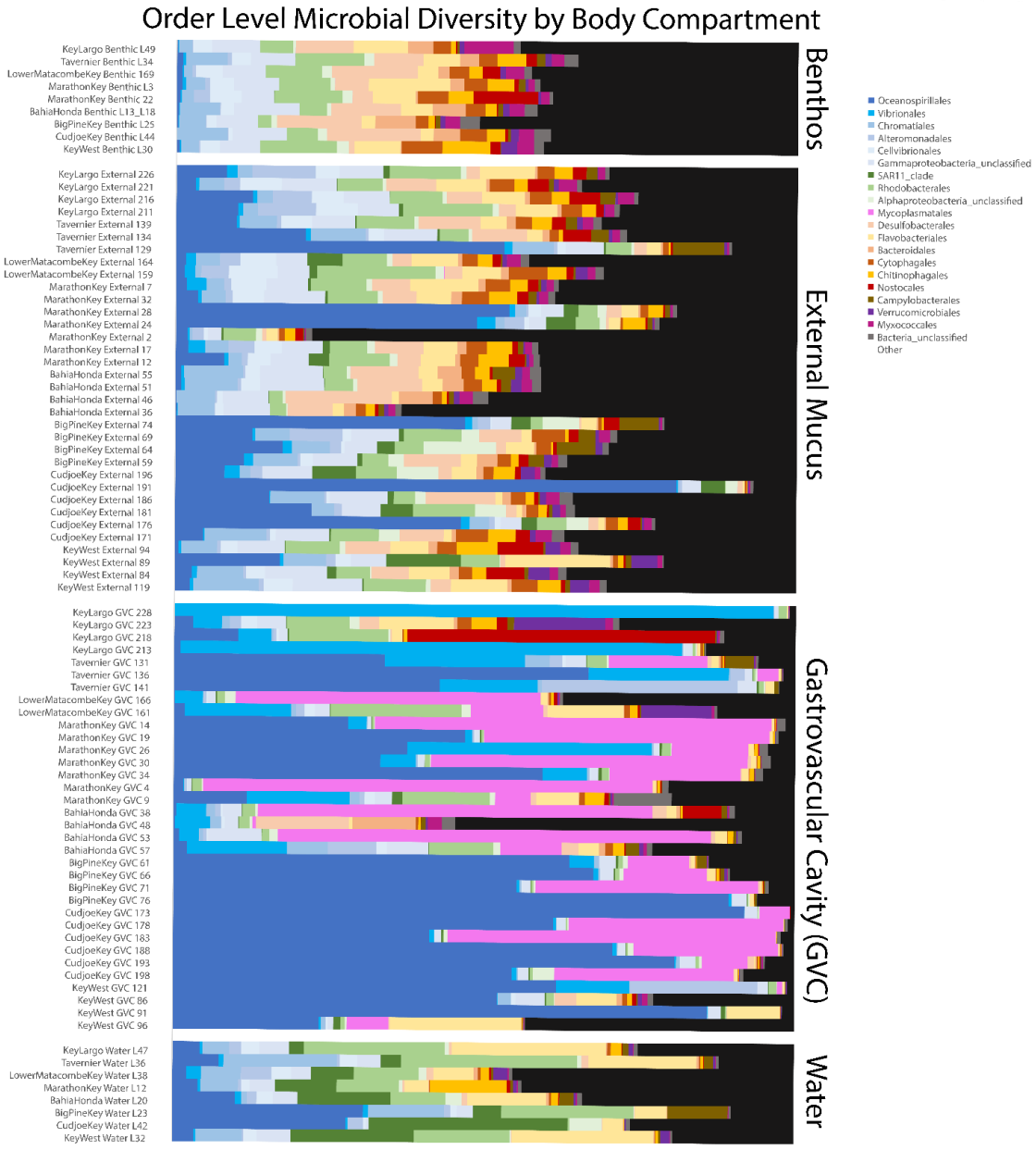

Figure 5.2. Order level diversity of samples including the top 20 most common orders by name. Grouped by sample type.

5.4.7. Community identity

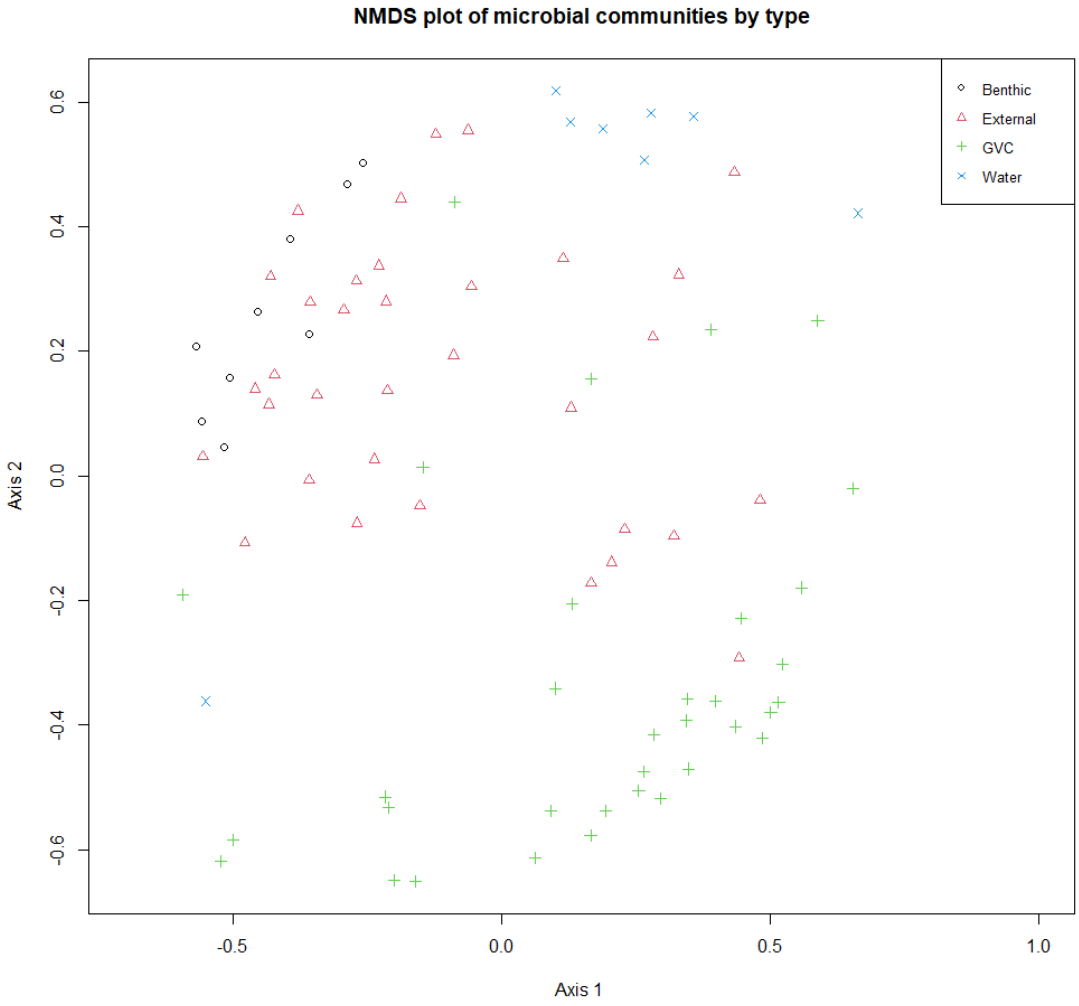

Figure 5.3 Two dimension NMDS plot segregated by sample type. Stress = 0.269974; R-squared for configuration: 0.718595.

External and internal communities were distinct from each other (AMOVA p-value: <0.001*). GVC communities were distinct from benthic and water comparison groups as well (AMOVA p-value: <0.001*) (Fig. 3). External mucus communities were distinct from water (AMOVA p-value: <0.001*) but not statistically significant from benthic samples (AMOVA p-value: 0.01). Clustering difference was most distinct between GVC and environmental samples.

Within gut microbial communities, Dirichlet multinomial mixture analysis defined two clades, high Endozoicomonas with high Mycoplasma (Profile 2) and low *Endozoicomonas* with higher *Vibrio* and unclassified Rhodobacteriaceae (Profile 1). These profile types were not evenly distributed across sites (to see microbial communities grouped by site, refer to fig S4).

Profile 1 sites were Lower Matacumbe Key, Bahia Honda Key and Key Largo. Sites that included a mix of Profile 1 and 2 individuals were Tavernier (P2:P1=2:1) and Marathon Key (P2:P1=4:3). The Lower Keys sites, Key West (P2:P1=3:1), Cudjoe Key (P2:P1=6:0) and Big Pine Key (P2:P1=4:0), were primarily Profile 2. Comparative star plots of community components separated by site can be seen below (Figure 5.3).

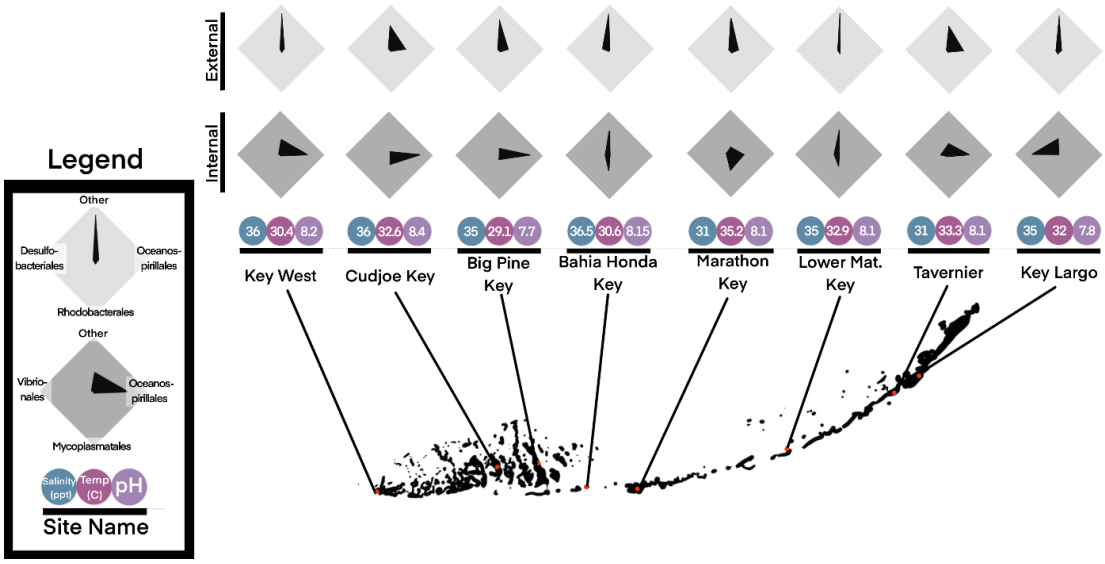

Figure 5.4 Star plots of community composition for internal and external mucus swabs at each site separated by order (three most prominent orders and "other"). Each spoke of the star plot ranges from 0% (center) to 100% (edge of shaded diamond). External group spokes (going clockwise): Other, Oceanospirillales, Rhodobacterales, and Desulfobacterales. Internal group: Other, Oceanospirillales, Mycoplasmatales, and Vibrionales. Major condition information (salinity, water surface temperature and pH) for

each site are presented below star plots. Sites are mapped onto their location within the Florida Keys.

5.4.8. Core Microbiome

There was no core microbial group present at a rate of > 0.2% of reads in all the 34 gastrovascular cavity samples. The most common clade, *Endozoicomonadaceae*, was present in 32 of the 34 samples at this level. For *Mycoplasma,* 26/34 samples met a 0.2% threshold, the same proportion as for *Vibrio.* There were no medusae where all three of these genera were absent from the gastrovascular cavity, and three medusae in which two of the three were absent.

5.4.9. Species Identity

Four *Cassiopea andromeda* mitotype individuals from Key Largo (1), Cudjoe Key (2) and Key West (1) were included in the broader sample. The gastrovascular communities of these individuals were not different from *C. xamachana* individuals (AMOVA pval >0.05).

5.5. Discussion

Cnidarians exhibit varied patterns of microbial assembly. The low diversity (in both richness and evenness) state present within the *Cassiopea* internal community has been demonstrated in multiple other scyphozoans—notably *Cotylorhiza tuberculata* [165]. The regional variation seen across the eight sites sampled within this project is potentially lower than those demonstrated in other species, such as *Aurelia, Mastigias,* and *Tipedalia,* however the sites chosen are all within a contiguous tract [30,164].

5.5.1. *Endozoicomonas*

Endozoicomonas, *Mycoplasma* and *Vibrio* are all commonly associated with coral microbial communities. The large proportion of *Endozoicomonas* in internal and external samples fits within a broader framework of both *Endozoicomonas* and known cnidarian-associated microbial communities;· *Endozoicomonas* strains are present in a variety of invertebrates, including many corals, sponges and sea anemones, but also scyphozoans and cubozoans [20,176–179]. In some hosts, multiple *Endozoicomonas* distinct genotypes found in some specific hosts, different *Endozoicomonas* clades may provide different metabolic services to a host (e.g., Vitamin B12 metabolism) [180]. The group is not restricted to symbiotic taxa, but is more commonly found in healthy scleractinian corals than bleached and diseased scleractinian corals [181]. For these taxa it may play a role in a variety of fundamental symbiont-host interactions, including nutrient transport and symbiont cell breakdown [180]. If the relationship between *Cassiopea* and their *Endozoicomonas* strains are as close as those found in some corals, host tissues may induce beneficial behaviors, however it is not as clear in other taxa that *Endozoicomonas* is always a feature of a healthier microbiome [158,179]. In coral, this bacterial group is found in close proximity to *Symbiodinium,* and the same may be true of the association in *Cassiopea* medusae [180]. This provides an explanation for the high *Endozoicomonas* proportion on many of the *Cassiopea* bell swabs analyzed.

Endozoicomonas is abundant in the microbial community of the rhizostome scyphozoan *Mastigias* [30]. *Mastigias*, like *Cassiopea*, host zooxanthellae from the clade

Symbiodiniaceae, however not all symbiotic rhizostomes appear to harbor the microbe, and not all taxa with the microbe are symbiotic [30,165]. *Endozoicomonas* may be more common across medusae, but the difficulty of culturing some strains may have excluded them from the results of culture-based analyses [179,180]. Until greater clarity is available on the function of *Endozoicomonas* for the medusae, all that can be said is that it may be beneficial.

5.5.2. *Mycoplasma*

Mycoplasma, as with *Endozoicomonas*, is present in studied coral and scyphozoan microbiomes [29,163,165,177]. While intracellular parasitism is common for *Mycoplasma*, those associated with cnidarians may be different [158]. *Mycoplasma* sp. is noted as a potential endosymbiont in lab-raised *Aurelia* polyps, while adults from many jellyfish populations are known to have them [163,164,166]. Whether this group represents a beneficial endosymbiont, a result of prey capture or a parasite, its exclusivity to the gastrovascular tract in *Cassiopea* sampled within the Keys is notable, as other studies have not demonstrated body cavity localization [164,166].

5.5.3. *Vibrio*

Vibrio is both a core part of coral microbiomes [177,182] and known to facilitate metamorphosis within *Cassiopea* [32,183]. Its restriction to a limited subset of *Cassiopea* internal microbial communities was surprising, given its known role in life transition. While *Vibrio* spp. are found in gut communities of presumed healthy scyphozoans [29,163], they can also be associated with senescence and disease (Kramar et al., 2019; Tinta et al.,

2012). For *Cassiopea* specifically, an increase in *Vibrio* may be associated with an aposymbiotic or stressed state [49]. In the single site (Key Largo) with *Vibrio*-dominated microbial communities, the medusae were visibly symbiotic, but small. There are different *Vibrio* strains, so genus wide statements on the beneficial or harmful nature of the genus within *Cassiopea* gastrovascular cavities should not be made.

5.5.4. Caveats: Seasonality and Size

Seasonal changes are a feature of some cnidarian microbial communities [166,185]. In an outdoor laboratory setting, this is true of *Cassiopea* as well [49]. As these samples were taken during the hottest time of the year in the Florida Keys (August), this community may not be representative of other timepoints. Winter communities may harbor different taxa, or taxa in different proportions [185]. As the internal microenvironment of *Cassiopea* alters over the course of a day with regards to light and dark cycles, it is reasonable to expect that it may become more or less hospitable to some microbiota as temperature and light cycles adjust throughout the year [25].

As *Cassiopea* medusae grow at rates commensurate with their feeding, there is no guarantee that the size of medusae in this study is representative of their age. Developmental changes are known to facilitate changes to microbial communities within other scyphozoans, but size of adult has not been tested [33,161,164]. As medusa size and site were not independent (smaller medusae and larger medusae were not usually seen in mixed assemblages), we cannot determine whether size class had an impact.

5.5.5. Laboratory cultures

Cassiopea are primarily used for in-lab studies on symbiosis [42,46,143]. Some of these use wild-caught individuals and others use lab-bred cultures, many of which have been held for years. The lab-raised *Cassiopea* used in Rothig et al. 2021 appear to have a completely different microbiome than the wild individuals caught here [48]. When using wild-caught or captive-bred individuals, this potential discrepancy should be kept in mind, as captive-bred symbiosis and tolerance levels may differ from medusae in the wild. This discrepancy is found in many model organisms, including *Exaiptasia,* so does not decrease *Cassiopea's* viability as a model organism [157].

5.5.6. Diversity and Consistency

The highest diversity sites were at Key Largo marina, a Bahia Honda Key oceanic site, and a large protected cove in Lower Matacombe Key. The internal microbial community of the Key Largo marina stands out for its variation, with *Vibrio*-dominated and *Nostocales*-dominated medusae. All medusae seen at this site were small, and the assemblage at this site included both *C. xamachana* and *C. andromeda* (as did Cudjoe Key and Key West). As these medusae were not in obvious distress, it seems likely that *Cassiopea* has many stable states that may be able to meet its functional requirements.

Across the system there was no core microbiome. The external microbial community of *Cassiopea* from the Florida Keys adheres fairly closely to the epibenthic microbial community at each site, with the addition of *Endozoicomonas* for most individuals. *Endozoicomonas* may be a primary mucosal component and extend to the exterior of the body through mucus exchange, as it is less prevalent on the external surface

than internal for most individuals. Notably, the abundant *Mycoplasma* and *Vibrio* from the gastric cavity of *Cassiopea* are not present in any external mucus samples. *Oceanospirillales* (the order in which *Endozoicomonas* is contained) is the only bacterial order that external samples are enriched in relative to benthic communities. The bell microbiome demonstrated by this data is far higher diversity than the reported mucosal and umbrellar diversity from planktonic species, such as *Rhizostoma pulmo* that have been previously reported [167]. Despite the diverse external communities, *Cassiopea* retain very little microbial diversity in their gastrovascular cavities.

Microbial communities across sites were not significantly different from each other, as gastrovascular cavity components remained dominated by a narrow range of taxa throughout the Keys. This suggests that while lacking a formal "core" microbiome, *Cassiopea* gastric cavities may naturally rely on some combination of several microbial key taxa.

5.6. Conclusion

The microbiome of *Cassiopea* within the Florida Keys demonstrates strong body compartment discrimination and a central group of genera overrepresented in the gut and external microbiome. All of these genera, *Endozoicomonas, Vibrio,* and *Mycoplasma* are found in other medusae and also in corals. Despite this overrepresentation of some microbial taxa, *Cassiopea* lacks a core microbiome. As we grow our understanding of cnidarian microbial communities, and especially medusae, this consistency across the Florida Keys demonstrates that specific taxa are strongly associated with *Cassiopea,* and

this can extend across many sites with diverse physical conditions. While previous jellyfish microbial community analyses often relied on few individuals, this shows a higher degree of temporal and regional continuity in medusa gut microbiota than previously described.

6. CONCLUSIONS

Seminal work has painted the class Scyphozoa with a broad brush that suggests scyphozoans to be a net negative for ecosystems and a scourge brought on by humans and climate change [186]. This interpretation, however, may dramatically undersell the degree to which jellyfishes are part of larger ecosystems and exist in community beneficially or non-antagonistically with their surroundings, as well as the degree to which some jellies too can be susceptible to other nonnative species.

With my second chapter, I review the records of a specific class of epibionts and host use patterns throughout the Hydrozoa and Scyphozoa. Within this work, I demonstrate that pelagic cnidarian host-use by crustaceans is widespread and common. Our understanding of the importance of pelagic cnidarians within food webs is poor, likely hampered by the inadequacy of capturing gelatinous zooplankton with traditional techniques[187]. By collecting over 200 accounts of crustacean-gelatinous zooplankton interactions, I provide one clear resource from which to draw support for future work on these associations. Especially valuable would be new work on the specificity and longevity of associative behaviors between medusae and commercially harvested crustaceans. As many of the accounts reported were over a hundred years old and/or written in another language, some of this data might have been neglected by current researchers, to the detriment of the field. The global span of this literature review allows a picture of a world in which the soft-bodied and understudied Scyphozoa and Hydrozoa provide nursery and lifelong habitat for crustaceans, a major *acknowledged* driver of marine food webs.

To hone questions of ecosystem mechanics down further, I examined development, systematics, symbiosis and microbial community within a Gulf of Mexico scyphozoan—the Upside-Down Jellyfish, *Cassiopea*.

In my third chapter, I narrow in on one specific species often considered a pest. *Cassiopea xamachana* exhibits a complex symbiosis with algae of the clade Symbiodiniaceae. For this species, we determined developmental response to stress stimuli, to both better understand the nature of the symbiosis, and to calibrate our understanding of potential life stage spread mechanics. I found that *Cassiopea* ephyrae were tolerant to both starvation and lack of light, and that the presence of light was equivalent to a doubling of their daily food intake, despite the light exposure remaining below published adult compensation levels. Our data show highly resilient and adaptive ephyrae when provided access to light, potentially increasing population connectivity and invasion success, as this stage is more capable of long distance movement than the bulky near-shore adults.

To test whether Florida Keys *Cassiopea* display population structuring within their range, I collected 55 *Cassiopea* from across the Florida Keys and analyzed two mitochondrial (cytochrome oxidase subunit 1 or COI, and 16S rRNA gene) and one nuclear gene (28S). I show that there was no variability in *COI* and little variation in *16S* within *C. xamachana*, these genes showed no indicators of genetic isolation at sites within the Florida Keys. While this can be closely tied to my earlier work, this is not in agreement with previous estimates for *Cassiopea*, considered to be genetically distinct populations at even small scales [141].This misconception has likely stemmed in part from

a cryptic additional species, the non-native *C. andromeda,* which I demonstrate to be present at multiple sites within the Florida Keys. This revision of *Cassiopea* species identity within the Florida Keys has implications for the use of wild-caught specimens moving forward, as the distinctions between these groups remain unclear. The case of *C. andromeda* highlights the difficult of identifying and understanding native scyphozoan populations, this collection provided a chance to correct a key area of disagreement in the phylogeny for the genus.

To both 1) identify the core components of the microbial communities associated with wild *Cassiopea* and 2) test whether these microbial communities were stable across different localities, I employed microbial 16S V3-V4 sequencing to determine the composition of the *Cassiopea* internal and external microbiomes. The profile produced demonstrates that while *Cassiopea* lack a core microbiome, they are consistently high in *Mycoplasma*, *Endozoicomonas*, and *Vibrio*. This consistency is true across much of the Florida Keys, with most sites being heavily weighted towards these three genera of bacteria. This also demonstrates a continuity between *Cassiopea* and the better-known symbiotic cnidarians, specifically coral. If the easy to collect *Cassiopea* uses *Endozoicomonas* for symbiosis-related nutrient transport, as is hypothesized in coral, they could provide an ideal system in which to examine this connectivity.

While jellyfish in aggregate are poorly understood, my work helps to develop a holistic understanding of how to treat *Cassiopea* as a management unit. Firstly, *Cassiopea* ephyrae can live through starvation and darkness or an extended period of time in a way that may increase spread. Secondly, *Cassiopea* within the keys do not

seem to demonstrate closed populations but do co-occur with non-native *Cassiopea.* Thirdly, *Cassiopea* have a low diversity internal microbial community that has likely interplay with their photosymbiosis, and that holds consistent throughout much of the Florida Keys.

Overall, my thesis builds a solid foundation on which future *Cassiopea* ecological research can be constructed, and focuses questions on the nature their symbiotic relationships as well as on the role of their associated microbiome. For cnidarian ecologists and biologists, these results also provide new information on collection and laboratory methods that will move the field forward.

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APPENDIX 1: CHAPTER 4 SPECIMEN COLLECTION LIST

Specimen collection list. Specimen number is provided in first column, location is provided with approximate latitude (lat) and longitude (long). Salinity (in ppt), pH (to the nearest 0.1), surface temperature at the site in Celcius (Temp), date and time of collection, accession numbers for genes sequenced, whether a complete specimen was retained (Preserved) are reported for each medusa. Additionally, diameter of the medusa and species designation (CX for *C. xamachana* and CA for *C. andromeda*) are reported.

Specimen	**Lat**	**Long**	**Salinity (ppt)**	**pH**	**Temp (C)**	**Accession Numbers**	**Date**	**Pres-erved**	**Species**	**Time**	**Diameter (cm)**
1	24.69396	-81.0980507	31	8.1	35.2	*COI*: OP503313	11.8.2021	N	CX	12:03	6.13
2	24.69396	-81.0980507	31	8.1	35.2	*COI*: OP503314 *16S*: OP503902 *28S*: OP738399	11.8.2021	N	CX	12:19	8.527
3	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503315 *28S*: OP738400	11.8.2021	N	CX	16:52	4.288
4	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503354 *16S*: OP503903 *28S*: OP738413	11.8.2021	Y	CX	NR	1.9
5	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503316 *16S*: OP503904	11.8.2021	N	CX	17:19	6.951
6	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503355 *16S*: OP503905	11.8.2021	N	CX	17:32	8.898
7	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503356 *16S*: OP503906 *28S*: OP738415	11.8.2021	N	CX	17:55	6.984

8	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503357 *28S*: OP738401	11.8.2021	N	CX	18:14	8.5
9	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503358	11.8.2021	N	CX	NR	NR
10	24.69396	-81.0980507	30	8.3	35.7	*COI*: OP503359 *16S*: OP503906	11.8.2021	N	CX	NR	NR
11	24.6825793	-81.2293673	37	8.3	31.8	*COI*: OP503317 *16S*: OP503907	13.8.2021	N	CX	17:17	8.8
12	24.6825793	-81.2293673	37	8.3	31.8	*COI*: OP503318 *16S*: OP503908 *28S*: OP738409	13.8.2021	N	CX	17:54	15.66
13	24.6825793	-81.2293673	37	8.3	31.8	*COI*: OP503319 *28S*: OP738402	13.8.2021	N	CX	18:28	12.773
14	24.6825793	-81.2293673	36	8	29.4	*COI*: OP503360	15.8.2021	N	CX	9:32	9.348
15	24.6825793	-81.2293673	36	8	29.4	*COI*: OP503361 *16S*: OP503909	15.8.2021	N	CX	9:49	11.226
16	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503320	15.8.2021	N	CX	12:18	5.601
17	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503321	15.8.2021	N	CX	12:26	4.827
18	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503322 *16S*: OP503910	15.8.2021	N	CX	12:40	5.716
19	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503362 *16S*: OP503911	15.8.2021	Y	CX	NR	1.5

20	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503363	15.8.2021	N	CX	NR	NR
21	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503323	15.8.2021	N	CX	13:09	4.694
22	24.6978731	-81.3572574	35	7.7	29.1	*COI*: OP503324 *16S*: OP503912	15.8.2021	N	CX	13:18	4.743
23	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503325 *16S*: OP503913 *28S*: OP738412	15.8.2021	N	CA	16:30	5.609
24	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503326 *16S*: OP503914	15.8.2021	N	CX	16:36	5.283
25	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503327 *16S*: OP503915 *28S*: OP738403	15.8.2021	N	CX	16:48	9.306
26	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503328 *16S*: OP503916	15.8.2021	N	CX	16:52	8.816
27	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503329 *16S*: OP503917	15.8.2021	N	CX	16:59	5.025
28	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503330 *16S*: OP503918	15.8.2021	N	CX	17:06	5.264
29	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503331 *16S*: OP503919	15.8.2021	N	CX	NR	NR
30	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503332 *16S*: OP503920	15.8.2021	N	CX	17:15	7.184

31	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503333 *16S*: OP503921	15.8.2021	N	CX	17:22	6.482
32	24.561526	-81.7881727	36	8.2	30.4	*COI*: OP503364 *16S*: OP503922	15.8.2021	N	CX	NR	NR
33	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503334 *16S*: OP503922	16.8.2021	N	CX	11:22	12.34
34	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503335 *16S*: OP503924	16.8.2021	N	CX	11:32	17.904
35	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503336 *16S*: OP503925 *28S*: OP738416	16.8.2021	N	CX	11:47	6.996
36	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503337 *16S*: OP503926	16.8.2021	N	CX	12:06	15.388
37	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503338 *16S*: OP503927	16.8.2021	N	CX	12:41	10.237
38	25.0233783	-80.4940316	31	8.1	33.3	*COI*: OP503339 *16S*: OP503928 *28S*: OP738410	16.8.2021	N	CX	12:56	10.7
39	24.8582157	-80.7267927	35	8.1	33.0	*COI*: OP503340	16.8.2021	N	CX	17:21	8.096
40	24.8582157	-80.7267927	35	8.1	33.0	*COI*: OP503341 *16S*: OP503929	16.8.2021	N	CX	17:31	14.72
41	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503342	17.8.2021	N	CX	10:31	6.169

						16S: OP503930					
42	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503343 *16S*: OP503931	17.8.2021	N	CX	10:45	6.207
43	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503344	17.8.2021	N	CX	10:59	8.338
44	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503345 *16S*: OP503932 *28S*: OP738404	17.8.2021	N	CA	11:19	7.128
45	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503346 *28S*: OP738405	17.8.2021	N	CX	11:31	4.204
46	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503347 *16S*: OP503933 *28S*: OP738406	17.8.2021	N	CA	11:48	6.417
47	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503348 *16S*: OP503934 *28S*: OP738407	17.8.2021	N	CX	12:04	9.64
48	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503349	17.8.2021	N	CX	12:22	8.5
49	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503365 *16S*: OP503935 *28S*: OP738408	17.8.2021	Y	CA	NR	1.9
50	24.6775378	-81.4991819	36	8.4	32.6	*COI*: OP503366	17.8.2021	Y	CX	NR	1.8
51	25.0872104	-80.4415797	35	7.8	32.0	*COI*: OP503350	18.8.2021	N	CX	11:06	3.979

						16S: OP503936					
52	25.0872104	-80.4415797	35	7.8	32.0	*COI*: OP503351	18.8.2021	N	CX	NR	NR
53	25.0872104	-80.4415797	35	7.8	32.0	*COI*: OP503352 *16S*: OP503937	18.8.2021	N	CX	11:24	6.079
54	25.0872104	-80.4415797	35	7.8	32.0	*COI*: OP503353 *16S*: OP503938 *28S*: OP738411	18.8.2021	N	CA	11:34	5.441
55	25.0872104	-80.4415797	35	7.8	32.0	*COI*: OP503367 *16S*: OP503939 *28S*: OP738414	18.8.2021	Y	CA	NR	2.4

APPENDIX 2: CHAPTER 4 *28S* SEQUENCES

Sequences used for *28S* tree. All species IDs are as presented in GenBank. For individuals collected in this study, specimen ID is included in location in parentheses.

Species	Location	Accession Number	Source
C. frondosa	Key West, FL, USA	HM194872	Bayha 2010
C. ornata	Koror, Palau	HM194838	Bayha 2010
C. andromeda	Key West, FL, USA	HM194871	Bayha 2010
C. frondosa	Bocas del Toro, Panama	KY611002	Daglio et al. 2017
C. frondosa	Bocas del Toro, Panama	KY611003	Daglio et al. 2017
C. frondosa	Bocas del Toro, Panama	KY611004	Daglio et al. 2017
C. andromeda	Isla San Jose, Baja California, Mexico	KY611006	Daglio et al. 2017
C. andromeda	Isla San Jose, Baja California, Mexico	KY611007	Daglio et al. 2017
C. andromeda	(GB23) Garrison Bight, Key West, FL, USA	OP738412	This Study
C. andromeda	(CK44) Cudjoe Key, FL, USA	OP738404	This Study
C. andromeda	(CK46) Cudjoe Key, FL, USA	OP738406	This Study
C. andromeda	(CK49) Cudjoe Key, FL, USA	OP738408	This Study
C. andromeda	(KL54) Key Largo, FL, USA	OP738411	This Study
C. andromeda	(KL55) Key Largo, FL, USA	OP738414	This Study
C. xamachana	(MK2) Marathon, FL, USA	OP738399	This Study
C. xamachana	(MK3) Marathon, FL, USA	OP738400	This Study
C. xamachana	(MK4) Marathon, FL, USA	OP738413	This Study
C. xamachana	(MK7) Marathon, FL, USA	OP738415	This Study
C. xamachana	(MK8) Marathon, FL, USA	OP738401	This Study

C. xamachana	(VP12) Veterans Park, Bahia Honda, FL, USA	OP738409	This Study
C. xamachana	(VP13) Veterans Park, Bahia Honda, FL, USA	OP738402	This Study
C. xamachana	(GB25) Garrison Bight, Key West, FL, USA	OP738403	This Study
C. xamachana	(CK45) Cudjoe Key, FL, USA	OP738405	This Study
C. xamachana	(CK47) Cudjoe Key, FL, USA	OP738407	This Study
C. xamachana	(HHP35) Harry Harris Park, Tavernier, FL, USA	OP738416	This Study
C. xamachana	(HHP38) Harry Harris Park, Tavernier, FL, USA	OP738410	This Study
Versuriga anadyomene	Cemetery Reef, Palau	HM194823	Bayha 2010
Mastigias papua	Ongael Lake, Palau	HM194849	Bayha 2010

APPENDIX 3: MOTHUR PIPELINE COMMANDS FOR CHAPTER 5 SEQUENCES

List of ordered commands used for microbial data used in chapter 5.

mothur commands used in order

make.file
make.contigs
screen.seqs(maxambig=0, maxlength=480, maxhomop=8)
unique.seqs
summary.seqs(fasta=silva.v3v4_v132.align)
align.seqs(reference=silva.v3v4_v132.align)
screen.seqs(start=1, end=18929)
filter.seqs(vertical=T, trump=.)
unique.seqs
pre.cluster(diffs=4)
chimera.vsearch(dereplicate=t)
classify.seqs(reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax)
remove.lineage(taxon=Chloroplast-Mitochondria-unknown-Eukaryota)

Commands below used to download contaminant genera for manual flagging:
###phylotype
###make.shared(label=1)
###classify.otu(label=1)

Continues from above hashtagged section:
remove.lineage(taxon=Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;-Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus;-Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Escherichia-Shigella;-Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Micrococcaceae;Rothia;-Bacteria;Bacteroidetes;Bacteroidia;Sphingobacteriales;Sphingobacteriaceae;Sphingobacterium;-Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Paracoccus;-Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Corynebacteriaceae;Corynebacterium_1;-Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;-Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus;-Bacteria;Proteobacteria;Alphaproteobacteria;Micavibrionales;Micavibrionaceae;Micavibrio;-Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Corynebacteriaceae;Corynebacterium;-Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acineto

bacter;-
Bacteria;Proteobacteria;Alphaproteobacteria;Acetobacterales;Acetobacteraceae;Asaia;-
Bacteria;Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae;
Burkholderia-Caballeronia-Paraburkholderia;-
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Micrococcaceae;Micrococcus;-
Bacteria;Bacteroidetes;Bacteroidia;Flavobacteriales;Weeksellaceae;Chryseobacterium;-
Bacteria;Firmicutes;Bacilli;Bacillales;Family_XI;Gemella;-
Bacteria;Acidobacteria;Acidobacteriia;Solibacterales;Solibacteraceae_(Subgroup_3);Ca
ndidatus_Solibacter;-
Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Veillonella;-
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Aeribacillus;-
Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Mycobacteriaceae;Mycobacte
rium;-Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella_7;-
Bacteria;Firmicutes;Clostridia;Clostridiales;Heliobacteriaceae;Hydrogenispora;-
Bacteria;Cyanobacteria;Oxyphotobacteria;Synechococcales;Cyanobiaceae;Synechococc
us_CC9902;-
Bacteria;Proteobacteria;Alphaproteobacteria;Reyranellales;Reyranellaceae;Reyranella;)
remove.groups(groups=(controls, EtOH samples, fresh swab))
phylotype
make.shared(label=1)
classify.otu(label=1)

Analysis**:**
sub.sample (size=20562)
dist.shared (calc=braycurtis-jclass,subsample=t)
nmds(braycurtis)
amova(braycurtis)
homova(braycurtis)
pcoa(braycurtis)
corr.axes(method=spearman)
get.communitytype
get.communitytype(GVC samples only)
summary.single(calc=nseqs-coverage-sobs-shannon-invsimpson, subsample=T)

APPENDIX 4: CHAPTER 5 SEQUENCE REMOVAL DETAILS

Table 5.2 Number of sequences conserved in each sample dataset after each filtering step in the mothur pipeline.

Samp #	Site	Type	Start-ing contig #	After seque-nces scree-ned	After sequ-ences alig-ned	Align-ment filtrat-ion	Chi-mera remo-val	Off target and unknown sequence removal	Contam-ination removal	Rares remo-val
#119	GB	External	134881	76790	75995	75995	72185	64396	64252	64231
#12	MK	External	99056	42813	42254	42254	40863	37690	35505	35503
#121	GB	GVC	167417	101916	101428	101428	95916	95437	95350	95350
#129	HHP	External	187388	107087	106271	106271	101739	97410	97119	97112
#131	HHP	GVC	95369	53326	52841	52841	50852	50616	50395	50385
#134	HHP	External	144608	79601	78616	78616	76194	57166	55570	55552
#136	HHP	GVC	127762	71493	71193	71193	69694	69625	69576	69574
#139	HHP	External	126046	67178	66312	66312	64023	49811	49578	49562
#14	MK	GVC	79234	43990	43106	43106	42884	42719	42587	42579
#141	HHP	GVC	163285	99177	98584	98584	93400	92865	92597	92592
#159	LW	External	124957	63630	62449	62449	59678	38078	37382	37357
#161	LW	GVC	127202	72271	71591	71591	69479	65189	64437	64422
#164	LW	External	161641	84765	83585	83585	80651	72153	65109	65106
#166	LW	GVC	111681	57310	56396	56396	55738	54489	54291	54289
#169	LW	Benthic	136570	73116	71567	71567	67349	62936	62636	62602
#17	MK	External	100021	51192	50711	50711	48920	41777	38801	38787
#171	CK	External	134223	69255	68476	68476	66368	54358	54291	54277
#173	CK	GVC	170555	101924	101463	101463	101342	101328	101308	101307
#176	CK	External	194511	106555	105562	105562	101470	86329	85102	85100
#178	CK	GVC	148597	89927	88932	88932	88530	88457	88420	88418
#181	CK	External	158857	80217	79350	79350	75722	74853	72828	72826
#183	CK	GVC	154405	88499	87346	87346	86859	86771	86722	86717
#186	CK	External	140142	73301	72337	72337	69686	68417	68149	68143
#188	CK	GVC	138561	75004	74429	74429	74070	73990	73970	73969
#19	MK	GVC	92749	49661	48913	48913	48679	48256	47848	47846
#191	CK	External	197434	119727	118887	118887	116912	116312	114132	114130
#193	CK	GVC	160033	96065	95426	95426	94781	94541	94509	94506
#196	CK	External	156252	79319	77960	77960	74971	71284	68454	68454
#198	CK	GVC	127620	74161	73376	73376	72871	72661	72614	72609
#2	MK	External	139063	64531	64171	64171	62167	60304	59440	59353

#211	KL	External	183297	97687	96778	96778	93721	76767	70631	70626
#213	KL	GVC	169601	102559	102067	102067	100309	98761	98473	98470
#216	KL	External	118156	63465	62894	62894	59845	42236	42132	42122
#218	KL	GVC	149243	68773	67804	67804	66317	62222	61013	61012
#22	MK	Benthic	90502	45215	44854	44854	43381	34874	34466	34456
#221	KL	External	144275	81115	80114	80114	75389	62525	62201	62189
#223	KL	GVC	132452	73046	71978	71978	69910	60465	59902	59893
#226	KL	External	159209	87690	86623	86623	83647	62540	58595	58591
#228	KL	GVC	200594	123018	122632	122632	122393	122214	122164	122163
#24	MK	External	135965	70178	69690	69690	66882	63640	58290	58287
#26	MK	GVC	109474	58167	57704	57704	57113	56638	55541	55534
#28	MK	External	110294	59635	59076	59076	57732	48526	46315	46296
#30	MK	GVC	64033	34809	34320	34320	33781	33456	33046	33045
#32	MK	External	64239	27857	27514	27514	26738	21065	20592	20562
#34	MK	GVC	132983	74759	73972	73972	73259	72249	70168	70160
#36	VP	External	111034	59866	59052	59052	56056	52790	52536	52415
#38	VP	GVC	111380	59686	58319	58319	57293	56528	56251	56248
#4	MK	GVC	142379	70963	69631	69631	68899	68500	68003	67990
#46	VP	External	120027	62985	61716	61716	58537	57522	57151	57070
#48	VP	GVC	121003	61932	60524	60524	58582	57514	57361	57338
#51	VP	External	124349	68573	67595	67595	64171	59861	59453	59382
#53	VP	GVC	98420	54331	53456	53456	53084	52649	52365	52355
#55	VP	External	110781	52742	51757	51757	49867	47602	47382	47362
#57	VP	GVC	135538	75470	74589	74589	70421	67280	66623	66586
#59	BPK	External	145600	74266	73280	73280	70181	48441	47559	47556
#61	BPK	GVC	160783	97102	96320	96320	93132	92633	92241	92235
#64	BPK	External	180895	98250	97281	97281	95211	89915	83159	83158
#66	BPK	GVC	125138	64840	64309	64309	62804	62272	61988	61983
#69	BPK	External	192167	108289	107153	107153	103097	71818	70857	70846
#7	MK	External	130995	69828	69184	69184	67304	49510	48043	48027
#71	BPK	GVC	145688	88352	87317	87317	86851	86587	86499	86492
#74	BPK	External	144282	78158	76783	76783	73878	71744	68337	68322
#76	BPK	GVC	193779	117075	116591	116591	116037	115880	115418	115418
#84	GB	External	111625	59681	58718	58718	55818	46377	45718	45713
#86	GB	GVC	138227	79003	78208	78208	76013	74767	74630	74616
#89	GB	External	143371	76386	75219	75219	72422	69048	64692	64689
#9	MK	GVC	92215	49155	48146	48146	46732	43083	40383	40374
#91	GB	GVC	189196	116576	115973	115973	115316	114738	114528	114525

#94	GB	External	110316	58546	57878	57878	55889	43311	43158	43143
#96	GB	GVC	122659	66592	66055	66055	65365	64893	64635	64633
#L12	MK	Water	104134	48926	48551	48551	46911	45243	39315	39300
#L13_L18	VP	Benthic	117887	61239	60110	60110	53988	53553	53328	53290
#L20	VP	Water	95481	52561	51917	51917	50284	49526	49074	49041
#L23	BPK	Water	224511	122984	122440	122440	120061	118647	115918	115913
#L25	BPK	Benthic	129366	70567	69155	69155	60405	59614	59454	59420
#L3	MK	Benthic	113461	62098	61409	61409	56134	55178	54913	54855
#L30	GB	Benthic	94549	51225	50721	50721	47139	36254	36080	36065
#L32	GB	Water	98642	54301	53757	53757	51369	50724	50634	50619
#L34	HHp	Benthic	117535	62081	60969	60969	52680	50642	50500	50468
#L36	HHP	Water	93021	49415	49003	49003	46958	46648	46474	46463
#L38	LW	Water	115069	60915	60309	60309	58220	52838	41346	41318
#L42	CK	Water	130033	72403	71980	71980	70190	70121	70116	70105
#L44	CK	Benthic	117006	62199	61580	61580	57934	57858	57845	57833
#L47	KL	Water	94689	49218	48935	48935	47303	44826	44345	44315
#L49	KL	Benthic	135811	75442	74099	74099	67915	66971	66903	66889
Mean #			133499.8	73011.71	72206.31	72206.306	69821.894	65085.90588	63902.54	63887.45882
Min #			64033	27857	27514	27514	26738	21065	20592	20562
Max #			224511	123018	122632	122632	122393	122214	122164	122163