UNIVERSITY OF CALIFORNIA, MERCED

Genetic and Behavioral Variation in Motile Marine Holobionts (Scyphozoa-Symbiodiniaceae)

A dissertation submitted in partial satisfaction for the degree of Doctor of Philosophy

in

Quantitative and Systems Biology

by

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To my mutualistic partner and our endearing little parasites.

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Acknowledgements

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Karly Higgins-Poling, et al. (in prep) *Deconstructing complex behaviors in gelatinous zooplankton: the behavioral and morphological foundations of jellyfish blooms and other things jellyfish do and don't do*

Karly Higgins-Poling, et al. (in prep) *Environmentally driven population phylogenomics of Mastigias papua in Palau*

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ABSTRACT

Genetic and Behavioral Patterns in Motile Marine Holobionts (Scyphozoa-Symbiodiniaceae)

by

Karly Higgins-Poling

Doctor of Philosophy in Quantitative and Systems Biology
University of California, Merced, 2024
Professor Chris Amemiya, Chair

Marine symbiosis has become an increasing focus in biology as symbiosis continues to challenge existing paradigms. Intra-interspecific diversity, genomic mechanisms, and behavioral consequences are central topics of symbiosis in the light of changing climates and oceans. It can be difficult to define and describe the intertwined existence of holobionts, i.e. hosts and their closely associated symbionts. These relationships are further complicated with the integrative nature of genomic and behavioral studies in remote pelagic marine systems. My thesis aims to tease apart the complexities of the role of symbiosis in Scyphozoa–Symbiodiniaceae genomics and behavior from a single population to class level analysis. To begin, I developed a PacBio reference genome for *Mastigias papua*, a photosymbiotic scyphozoan. The genome was then applied to a multispecies comparison between other symbiotic and nonsymbiotic Scyphozoa revealing the presence of candidate genes for maintaining symbiosis. In the subsequent chapter, the genome was used to design a probe set for targeted sequencing of ultraconserved elements (UCEs) in scyphozoan species and their endosymbionts to assess coevolution. While phylosymbiosis was unsupported, the UCEs demonstrated utility as an alternative to physical separation for sequencing host and endosymbiont tissue. I then applied and redefined an ethological framework for scyphozoan behavior emphasizing the need for additional studies, particularly those with a focus on symbiosis. Finally, I completed an observational study of migratory behavior during a rare syntopic co-occurrence of symbiotic and aposymbiotic *M. papua* medusae. This study suggested a role of the endosymbiont in what has previously been described as host behavior. The culmination of this work has provided novel conclusions, methodologies, and conceptual frameworks to advance scyphozoan biology and the study of pelagic symbiotic marine systems.

Ch. 1 Comparative genomics of scyphozoans reveal characteristics of pelagic photosymbiosis

1.1 Abstract

Photosymbiotic relationships across marine phyla are bleaching in response to the threats of climate change. A well-developed reference of comparisons between symbiotic and nonsymbiotic species will help uncover genetic mechanisms sustaining symbiosis in changing oceans. Cnidaria offers a variety of symbiotic and nonsymbiotic species, occupying ecologically impactful niches globally. Most cnidarian species comparisons within symbiosis has focused on benthic systems, especially scleractinians. Here we consider the understudied pelagic systems, particularly scyphozoan jellyfishes. We sequenced genomes of the scyphozoan *Mastigias papua* to study genetically and phenotypically diverse conspecific populations and make comparisons with other symbiotic and non-symbiotic scyphozoans. I utilized PacBio sequencing to produce a 19X, 0.657Mb N50, 409Mb contig-level genome assembly and annotation with 84% BUSCO completeness. I then employed OrthoVenn, Orthofinder and Interproscan to compare GO Term Enrichments of the *M. papua* genome to one other symbiotic species — *Cassiopea xamachana* — and four non-symbiotic species (*Aurelia aurita, Chrysaora quinquecirrha, Rhopilema esculentum*, and *Sanderia malayensis*). I found qualitative and quantitative differences between species including orthogroup similarity and the presence of 13 significant (p-value < 0.05) candidate genes for symbiosis between *M. papua* and *C. xamachana*.

1.2 Introduction

Invertebrates represent over 92% of marine biodiversity but in the race against extinction to describe Earth's biodiversity (Novacek & Cleland 2001) are often overlooked for more charismatic organisms (Chen 2021). The study of corals bucks this trend due to their ecological importance for reef health (Hoek & Bayoumi 2017). Corals, like various other cnidarians, have adapted complex symbiotic associations with mutualistic Symbiodiniaceae, or zooxanthellae, that can be impacted by climatic change (LaJeunesse et al. 2018). Mutualistic symbiosis with zooxanthellae can foster metabolic exchange (McCloskey et al. 1994), the potential for thermal tolerance in select few species (Bellantuono et al. 2012), and life cycle transitions (Djeghri 2019). The variety of interactions can convolute descriptions of host ecology and evolution in taxa that traverse million-year timescales like cnidarians (Boero et al. 2007). Tools of the omics era can mitigate the complexity of studying cnidarian symbiotic relationships (McKenna et al. 2021). Genomic and transcriptomic datasets may inform ecologically relevant

responses to changing oceans such as coral reef bleaching (Savary et al. 2021) or pelagic scyphozoan blooms (Hamner & Dawson 2009).

Class Scyphozoa includes the orders Coronatae, Semaeostomeae, and Rhizostomeae (Bayha et al. 2010). Coronatae and Rhizostomeae contain taxa symbiotic with zooxanthellae but Semaeostomeae do not. All families in the rhizostome suborder Kolpophorae are symbiotic, with few exceptions across individual populations and genera (Dawson et al. 2001, Graham et al. 2003). Semaeostomeae, and Rhizostomeae medusae can sexually reproduce via planulae that settle and grow into polyps. Polyps reproduce asexually through strobilation, or budding into ephyrae that later metamorphosize into medusae (Ceh et al. 2015). Rhizostome typical strobilation is monodisc, single budding ephyrae, particularly in those that have symbiotic forms (Helm 2018). Semaeostome typical strobilation is polydisc with multiple budding ephyrae (Helm 2018). Rhizostome species benefit from zooxanthellae for strobilation alongside controlled temperature (Sugiura 1965). The complete process of strobilation without zooxanthellae is unclear (Hofmann & Kremer 1981, Djeghri et al. 2019). Zooxanthellae are horizontally transmitted, consumed from the environment (Enrique-Navarro et al. 2022) at the polyp and medusae stage, and can be vertically transmitted from infected polyps. These general patterns of scyphozoan symbiosis can break down in some species leading to bleaching, such as *Mastigias papua* (Fig. 1.1A), offering genomic insight into scyphozoan symbiosis in natural systems.

Testing hypotheses of cnidarian divergence and symbiosis benefit from enclosed systems. *M. papua* in ancestral oceanic and isolated marine lake populations in Palau (Fig. 1.1C, 1.1D) provide a natural laboratory for evolutionary and ecological hypotheses (Swift et al. 2016). *M. papua* is primarily known for its populations of millions of medusae in marine lakes including the tourist hotspot Ongeim'l Tketau, commonly called 'Jellyfish Lake'. *M. papua* can also occur at much lower densities in coves, lagoons, and open pelagic environments of the Indo-Pacific (Fig. 1.1C, 1.1C) (Dawson & Hamner 2003, Rocha de Souza & Dawson 2018). In established Palauan marine lake communities, *M. papua* diverges rapidly, displaying a unique suite of morphologies (Dawson & Hamner 2003; Dawson 2005), behavioral patterns (Dawson & Hamner 2003) and genetic diversity, including subspecies (Swift et al. 2016, Swift & Dawson 2020). There are currently five identified subspecies corresponding to five of the distinct marine lake populations in Palau (Fig. 1.1D) (Dawson 2005). The ecological roles of *M. papua* are multiple—as predator and prey of other invertebrates yet intertwined with nutrient exchange from their endosymbionts: the photosynthetic zooxanthellae *Cladocopium*—altering marine lake limnology (McCloskey et al. 1994). *M. papua*-*Cladocopium* has been observed to bleach during environmental perturbations such as the El Niño Southern Oscillation and can thus exist in a symbiotic and temporary non-symbiotic form (Fig. 1.1A,B) (Dawson et al. 2001).

Generating a reference genome is crucial for multiple lines of investigation. For example, to study past and future evolutionary patterns of *M. papua* populations, and coevolutionary potential of their endosymbionts. The genome will establish a foundation for a variety of future studies assessing *M. papua* responses to climatic change in these populations that are sensitive to environmental perturbations (Dawson et al. 2001). The results will allow for applications to other organismal responses to changing climates such as blooming scyphozoans. With a newly available reference genome further exploration is possible of the population structure and life history—including symbioses, evolutionary mechanisms, and ecological genomics—of the subspecies in Palau and beyond. Herein I make an intraspecific genomic comparison between a lagoon and lake *M. papua* individual to showcase the improvement of the genome with sequencing capabilities and address areas of variation. I then develop interspecific comparisons to another symbiotic Rhizostomeae (*Cassiopea xamachana*), and non-symbiotic species from Rhizostomeae (*Rhopilema esculentum*), and Semaeostomeae (*Aurelia aurita, Sanderia malayensis,* and *Chrysaora quinquecirrha*). I hypothesize *C. xamachana* and *M. papua* to have the highest shared gene content along with presence of candidate genes deemed important to Symbiodiniaceae maintenance (Gonzalez-Pech et al. 2017).

1.3 Methods

1.3.1 Biological Materials

Mastigias papua medusae were chosen at random and collected using live capture netting from a Palauan lagoon and marine lake site. Following collection, they were shipped live to California, where upon arrival they were dissected into oral arm, temporal lobe, and bell tissues. Tissues were flash frozen using liquid nitrogen and shipped to Dovetail Genomics where extraction, sequencing and assembly were completed. The initial genome M1 specimen was collected 4 August 2017 from Ngermeuangel Cove, Koror, Palau and aposymbiotic oral arm tissue was sent to Dovetail. Due to the low quality genome produced from M1, a subsequent azooxanthellate specimen for genome M2 was collected on 16 July 2018 from Clear Lake, Mecherchar, Palau. Oral arm and terminal club tissue were sent to Dovetail. This specimen, M2, was re-extracted with remaining available tissue for PacBio sequencing.

1.3.2 Nucleic Acid Extraction

Tissue input was 500mg ground down with 10mL CTAB plus 50µL BME, incubated at 68ºC for 15 minutes. 10µL Protease K and 1µL RNaseA was added and incubated at 60ºC for 30 minutes, then extracted twice with phenol, and once with chloroform. DNA was precipitated from the aqueous phase from these extractions with 0.7x isopropyl alcohol. The precipitated DNA was washed with 75% EtOH and dissolved in 100µL TE. The A260/280 ratio was 1.94 and the A260/230 ratio was 2.27.

1.3.3 Library Prep, Sequencing & Assembly of M1 & M2

Library preparation and Illumina sequencing (M1& M2)

Briefly, ~500 ng of HMW gDNA was reconstituted into chromatin in vitro and fixed with formaldehyde. Fixed chromatin was digested with DpnII, the 5' overhangs filled in with biotinylated nucleotides, and then free blunt ends were ligated. After ligation, crosslinks were reversed, and the DNA was purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library.

Assembly (M1& M2)

The input data for the de novo assembly consisted of 669.6 million read pairs sequenced from paired-end libraries (totaling 193.3 Gbp). Reads were trimmed for quality, sequencing adapters, and mate-pair adapters using Trimmomatic (Bolger et al 2015). A de novo assembly was constructed for M1 and M2 separately using the paired-end (mean insert size ~390 bp) library. De novo assembly was performed using Meraculous [version 2.2.4] (Chapman et al, 2011) with a kmer size of 61. A Chicago library was prepared for M1 and M2 separately (Putnam et al, 2016) producing 142 million 2x150 bp paired-end reads. A Dovetail HiC library (Erez Lieberman-Aiden et al., 2009) was prepared producing 144 million 2x150 bp paired-end reads. The input de novo assembly, Chicago library reads, and Dovetail HiC library reads were used as input data for the HiRise pipeline to scaffold genome assemblies (Putnam et al, 2016). An iterative analysis was conducted. First, Chicago library sequences were aligned to the draft input assembly using a modified SNAP read mapper (http://snap.cs.berkeley.edu). The separations of Chicago read pairs mapped within draft scaffolds were analyzed by HiRise to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative misjoins, to score prospective joins, and make joins above a threshold. After aligning and scaffolding Chicago data, Dovetail HiC library sequences were aligned, and scaffolded following the same method.

PacBio Sequencing & Assembly (M2)

DNA samples from M2 tissues were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The PacBio SMRTbell library[ies] (~20kb) for the PacBio Sequel were constructed using SMRTbell Template Prep Kit 1.0 (PacBio, Menlo Park, CA, USA) using the manufacturer's protocol. The pooled library was bound to polymerase using the Sequel Binding Kit 2.0 (PacBio) and loaded onto PacBio Sequel using the MagBead Kit V2 (PacBio). Sequencing was performed on four PacBio Sequel SMRT cells, using Instrument Control Software v5.0.0.6235, Primary analysis software v5.0.0.6236, and SMRT Link v5.0.0.6792. Chicago, Dovetail HiC, and a HiRise assembly were completed as above.

1.3.4 Annotation

For genome annotation with BRAKER2 (Hoff et al. 2016, Hoff et al. 2019, Bruna et al. 2021), we designed a custom database of known protein sequences from fifteen Cnidaria and other taxonomically closely related species. We downloaded the respective '*_protein.faa.gz' files from https://ftp.ncbi.nlm.nih.gov (*Acropora digitifera*GCF_000222465.1, *Acropora millepora* GCF_013753865.1, *Actinia tenebrosa*, GCF_009602425.1, *Dendronephthya gigantea* GCF_004324835.1, *Enteromyxum leei* GCA_001455295.2, *Exaiptasia diaphana*GCF_001417965.1, *Henneguya salminicol* GCA_009887335.1, *Hydra vulgaris* GCF_000004095.1, *Kudoa iwatai*GCA_001407235.2, *Myxobolus squamalis* GCA_010108815.1, *Nematostella vectensis* GCF_000209225.1, *Orbicella faveolata* GCF_002042975.1, *Pocillopora damicornis* GCF_003704095.1, *Stylophora pistillata* GCF_002571385.1, *Thelohanellus kitauei* GCA_000827895.1). Since this dataset did not contain close relatives of *M. papua*, we decided to *de novo* annotate four Cnidaria genomes (*Aurelia aurita* complex sp. Pacific GCA_004194395.1, *Chrysaora chesapeakei* GCA_011763395.1, *Nemopilema nomurai* GCA_003864495.1, *Rhopilema esculentum*GCA_013076305.1) with the Metazoa fraction of OrthoDB v10 (Kriventseva et al. 2019) using BRAKER2, to obtain more closely related reference protein for annotating *Mastigias*. In short, BRAKER2 executes GeneMark-ES (Lomsadze et al. 2005, Ter-Hovhannisyan et al. 2008) to collect seeds that are then rapidly searched for sequence similarity to the protein database with DIAMOND (Buchfink et al. 2015). Subsequently, hits are spliced-aligned with Spaln2 (Gotoh 2008a, Gotoh 2008b, Iwata & Gotoh 2012). The resulting evidence was used for training and prediction of gene structures by GeneMark-EP/EP+ (Bruna et al. 2020). The resulting gene set was filtered and used for training the gene finder AUGUSTUS (Stanke et al. 2006, Stanke et al. 2008), to predict genes with the proteins as evidence. We concatenated the protein sequences from our custom database, the predicted protein sequences from the four genomes, and the *Metazoa* OrthoDB fraction to run BRAKER2 on the softmasked *M. papua* genome. An assembly hub for the *M. papua* genome on the UCSC Genome Browser was generated with MakeHub (Hoff 2019).

1.3.5 Comparative Analyses

Intraspecific

Statistics were generated to compare the quality of all three *M. papua* genomes using gVolante with BUSCOv5 and Metazoa genes (Table 1.1). The *M. papua* PacBio genome was aligned to M1 and M2 separately with GSAlign (Lin & Hsu 2020) to compare improvements in quality and estimate single nucleotide variations.

Interspecific

Completeness of the genomes from *Cassiopea xamachana* (Ohdera et al. 2019), *Rhopilema esculentum* (Li et al. 2020), *Aurelia aurita* (Gold et al. 2019), *Chrysaora quinquecirrha* (Haorong 2020), and *Sanderia malayensis* (Nong et al. 2020), were

assessed using gVolante with BUSCOv5 and Metazoa genes to ensure an equitable comparison between all genomes could occur. The OthoMCL algorithm was employed on Orthovenn3 (Sun et al. 2023) to compare orthologous gene clusters between the genomes and obtain GO Term enrichments for symbiotic vs. non-symbiotic groups. The significant GO Terms were searched for candidate genes found important to the maintenance of Symbiodiniaceae symbiosis (Gonzalez-Pech et al. 2017). Orthofinder v2.3.8 (Emms & Kelley 2019) was then used to get more specific statistics on orthologous genes of the above genomes. The species tree outputs from Orthovenn3 and Orthofinder were compared to ensure phylogenetic patterns were consistent across alternate orthogroup analyses.

1.4 Results

1.4.1 Assembly & Annotation

The first *M. papua* genome assembly, M1, had a size of 237.82 Mb and was highly fragmented, with 25,112 scaffolds, the longest being 160,879 bp, and an estimated coverage of 0.38X. The M2 genome assembly had a size of 238.59Mb and an estimated 40.88X coverage with 4,617 scaffolds, the longest being 4,196,601bp. The final *M. papua*genome was PacBio sequenced at 19X coverage and assembled with Dovetail HiRise Scaffolding Software producing a size of 409.39Mb with 2,002 scaffolds, the longest being 3,287,832bp. The BUSCO stats of this assembly showed 7.55% missing, 8.28% partial and 84.17% complete. Annotation of the *M. papua* genome produced 33,845 total genes with BUSCO stats of 85.9% complete, 9% missing and 5.1% partial (Fig. 1.2, Table 1.2).

1.4.2 Comparative Analyses

Intraspecific

The M1 (lagoon) to PacBio GSAlign alignment identified 2,362,595 SNVs, 107,828 insertions, and 118,584 deletions. The M2 (lake) to PacBio GSAlign alignment identified 1,637,351 SNVs, 75,167 insertions, and 80,484 deletions.

Interspecific

M. papua had an above average (82.09%) BUSCOv5 completeness percentage at 84.17%. OrthoVenn GO term enrichment produced 85 significant (p-value < 0.05) GO terms shared by the symbiotic species, 15 of which were present in the candidate gene list for maintenance of Symbiodinium symbiosis (Gonzalez-Pech et al. 2017) but 2 of those overlapped with the nonsymbiotic species (Table 1.3). Eleven additional GO terms were also found in common with the nonsymbiotic species (Table S1.1, S1.2). The nonsymbiotic species group enrichment produced a total of 35 significant (p-value < 0.05) GO terms (Table S1.2), 4 of which were on the candidate gene list (Table 1.3).

Orthofinder found M. papua to have the largest number of genes (33,845) and species specific orthogroups (1,219) compared to the other included genomes, with 92% of genes assigned to orthogroups (Fig. 1.2). The symbiotic species all had over 90% of genes assigned to orthogroups with non-symbiotic species all close to 85%. The species tree produced by Orthofinder and Orthovenn3 agreed with previously published relationships (Daglio & Dawson 2017) (Fig. 1.3).

The symbiotic group of species shared 989 orthologues (Fig. 1.4), with GO terms including interspecies interactions, multiorganism behavior, and multiorganism process, of particular interest with regard to host-symbiont interactions (Table 1.3, S1). The non-symbiotic group had 160 unique orthologues but also included multiorganism process (Fig. 1.4, Table S1.2).

1.5 Discussion

1.5.1 Mastigias papua

Longer read PacBio Sequencing has provided an improved methodology for sequencing difficult organisms in contrast to second generation sequencing (Rhoads & Au 2015). The PacBio *M. papua* genome improved from a highly fragmented assembly with over 12k scaffolds, to 1,213 scaffolds leaving room for improvement considering the chromosome prediction of 21 in *R. esculentum* (Li et al. 2020). BUSCOv5 quality scores improved, and genome size increased from Illumina to PacBio methodology. PacBio sequencing technology produced an improved genome predicting 33k genes, this estimate is more than the other scyphozoans here *C. xamachana*, *R. esculentum, A. aurita, S. malayensis,* and *C quinquecirrha* and other cnidarians, 23,677 in *Acropora digitifera*, and 27,273 in *Nematostella vectensis* for example (Kitchen et al. 2015). With the M2 and the PacBio genome sequenced from the same individual, the variation in gene content is likely an artifact of the difference in sequencing quality.

1.5.2 Symbiotic vs. Non-symbiotic Species

Differences between quality and ortholog content were present across all genomes (*Mastigias papua, Cassiopea xamachana, Rhopilema esculentum, Aurelia aurita, Sanderia malayensis,* and *Chrysaora quinquecirrha)* and could not necessarily be attributed to symbiotic or non-symbiotic characteristics. *S. malayensis* had the highest completeness percentage as estimated by BUSCOv5, followed by *R. esculentum*, *M. papua* and so forth (Table 1.2). 5,033 orthologs were shared by all species. Most of *S. malayensis* genes were in species-specific orthogroups, perhaps related to it being the only monodisc strobilator outside Kolopophorae. *M. papua* had the highest number of orthologs specific to its species, genes overall, and a high percentage of its genes in orthogroups, followed by *C. xamachana* (Fig. 1.2). This is unlikely an artifact of quality and overall completeness as *R. esculentum* had a higher completeness and a chromosome level genome (Li et al. 2020). A potential explanation may be related to

symbiosis requiring increased gene content due to additional genes needed for maintenance.

1.5.3 Concluding remarks

Prior to publication, the *M. papua* genome has contributed to studies of targeted sequencing assessing coevolution between scyphozoan hosts and zooxanthellae, and intraspecific population genomics including symbiotic and aposymbiotic population subgroups (Ch.'s 2 & 4). The potential for utilizing the *M. papua* genome to improve understanding of symbiosis and cnidarian ecology in changing environments has been supported here with the identification of genes related to symbiotic relationship maintenance in Scyphozoa. This result is a crucial starting point for identification of genes related to the breakdown of photosymbiosis. The results of my work coupled with improvements in quality of more scyphozoan genomes will allow for advanced studies in identifying genomic mechanisms of photosymbiosis.

The next step for this study would be to rerun analyses with the newly developed *M. papua* genome PacBio sequenced at 269x, adding >200 Mbp of data from the Wellcome Sanger Institute's Aquatic Symbiosis Genomics (ASG) project (McKenna et al. 2021). The increase in data would add to this study and potentially help find supplemental commonalities with *C. xamachana* but would not negate the current findings. ASG is sequencing additional scyphozoans that can be added to this comparison. The additional symbiotic species will be of specific interest due to their current lack of availability. A larger dataset will allow for higher confidence conclusions of genomic patterns contrasted between presence and absence of photosymbiosis. It would then be worthwhile to assess a broader list of candidate symbiosis genes found important to Anthozoa-zooxanthellae symbiosis (Meyer & Weis 2012). Transcriptomic comparisons between symbiotic and nonsymbiotic scyphozoans, with an initial focus on regulation of the significant candidate genes found above, would then be the next level of support for identifying the genomic underpinnings of symbiosis in Cnidaria.

1.6 Acknowledgements

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1.7 References

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1.8 Tables

Table 1.1: Intraspecific sequencing statistics between *Mastigias papua* M1, M2, and final PacBio Genome.

	M1	M2	PacBio
Sequencing Technology	Illumina	Illumina	PacBio
Total Length (Mb)	237.82	238.59	409.39
Coverage	0.38x	40.88x	19.04x
Longest Scaffold (bp)	160,870	4,196,601	3,287,832
Number Scaffolds > 1kb	24,876	4,613	1,988
Contig N50 (kb)	8.38	13.63	513.61
Number Gaps	32,458	40,227	255
% Genome Gaps	1.5	1.82	0.01

Table 1.2: Statistics on genome coverage and quality.

Annotated Genome	Size (Mb)	% Missing	% Partial	% Complete
Mastigias papua	409.39	7.55	8.28	84.17
Cassiopea xamachana	410.2	8.91	11.53	79.56
Rhopilema esculentum	275.42	6.81	7.76	85.43
Aurelia aurita	381.9	8.18	10.9	80.92
Chrysaora quinquecirrha	341.7	13.1	10.17	76.73
Sanderia malayensis	186.8	6.5	7.76	85.74

Table 1.3: Significant GO Terms for Symbiodiniaceae maintenance in symbiotic vs. nonsymbiotic scyphozoan genomes.

GO Term ID	Count	Function	GO Term Category	p-value
Symbiotic Species (Mastigias papua & Cassiopea xamachana)				
GO:0045944	3	Regulation of transcription from RNA pol. II promoter	Biological process	1.68E-19
GO:0006979	2	Response to oxidative stress	Biological process	6.57E-14
GO:0045893	3	Positive regulation of transcription, DNA-templated	Biological process	7.61E-14
GO:0006814	2	Sodium ion transport	Biological process	3.60E-13
GO:0051301	2	Cell division	Biological process	5.20E-09
GO:0019233	3	Sensory perception of pain	Biological process	1.05E-06
GO:0055114	2	Oxidation-reduction process	Biological process	3.10E-06
GO:0016485	2	Protein processing	Biological process	3.76E-06
GO:0014883	22	Transition between fast and slow fiber	Biological process	1.72E-05
GO:0007584	3	Response to nutrient	Biological process	0.00135365
GO:0008360	3	Regulation of cell shape	Biological process	0.00909366
GO:0034220	5	Ion transmembrane transport	Biological process	0.01074489
GO:0031667	2	Response to nutrient levels	Biological process	0.02162155
Non-Symbiotic Species (Rhopilema esculentum, Aurelia aurita, Chrysaora quinquecirrha, & Sanderia malayensis)				
GO:0006357	5	Regulation of transcription from RNA pol. II promoter	Biological process	4.20E-16
GO:0055085	8	Transmembrane transport	Biological process	2.60E-08
Shared Between Symbiotic and Non-Symbiotic Species				
GO:0016032	3	Viral process	Biological process	2.73E-36
GO:0042060	5	Wound healing	Biological process	2.58E-18

1.9 Figures

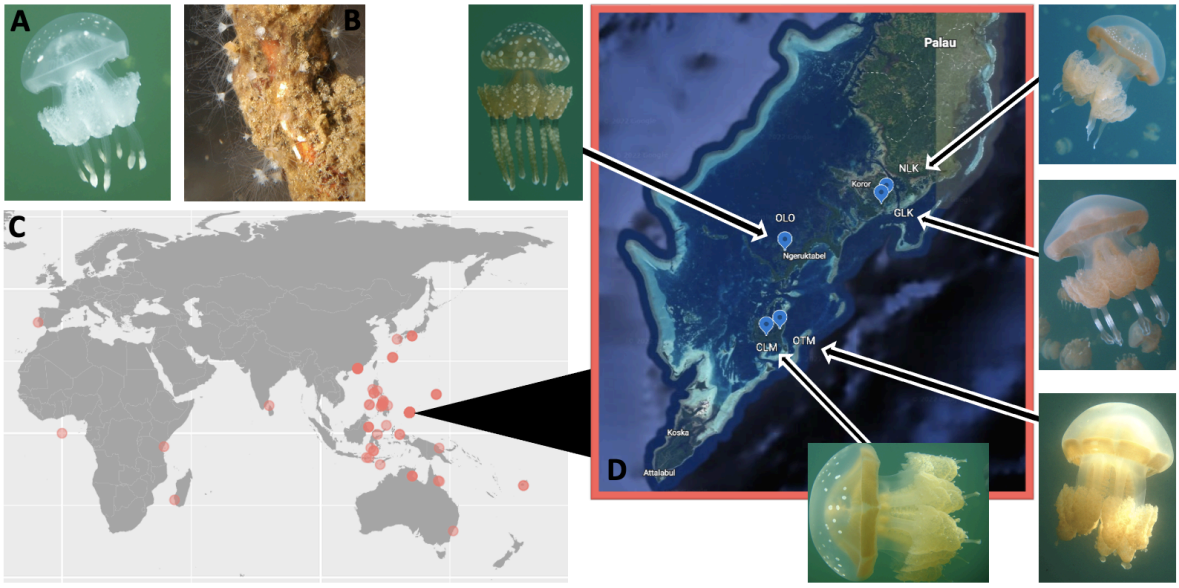

Figure 1.1: Occurrence map and Palauan subspecies phenotypes of *Mastigias papua*. A) Aposymbiotic individual in Clear Lake (CLM) following El Niño Southern Oscillation. **B**) Polyp colony of *Mastigias papua.* **C**) World map limited to the Eastern Hemisphere with GBIF species occurrence points for *Mastigias papua* in red. **D**) Photographs and lake sites of *M. papua* subspecies specific to Palauan marine lakes: Clear Lake (CLM), Uet era Ongael (OLO), Ngermeuangel (NLK), Goby Lake (GLK), and Ongeim'l Tketau (OTM).

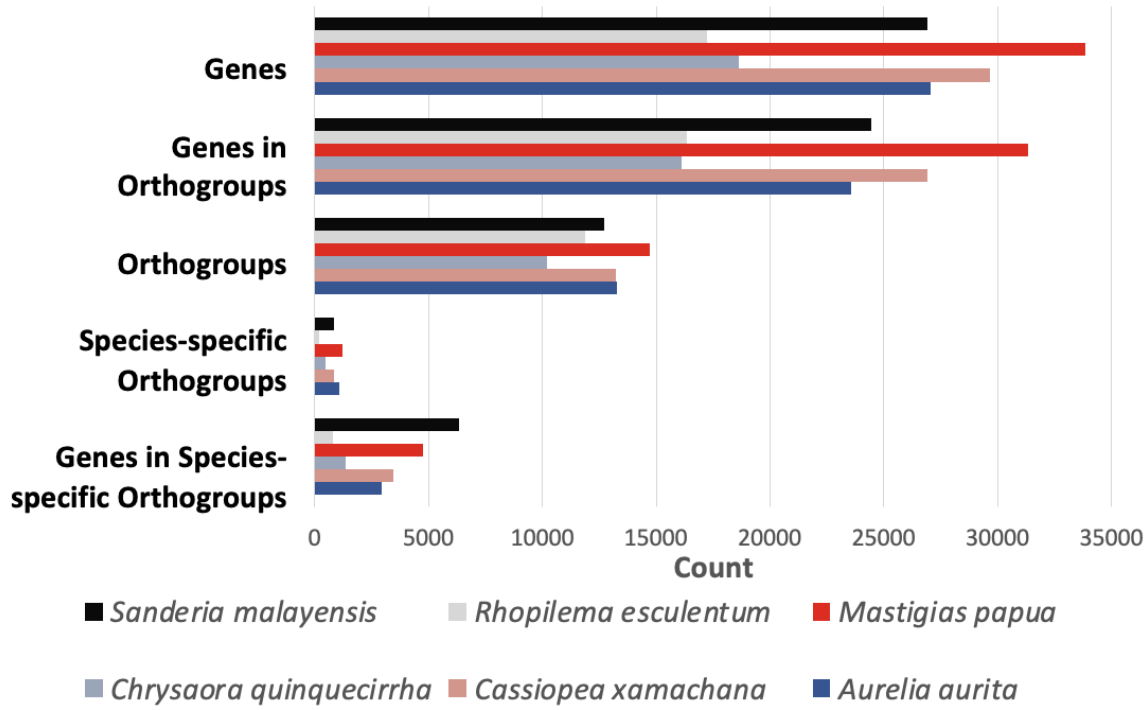

Figure 1.2: Orthogroup and total gene counts for each species as determined by Orthofinder. Symbiotic species, *Mastigias papua* and *Cassiopea xamachana*, are represented as variations of red bars. All other non-symbiotic species are variations of blue, gray, and black.

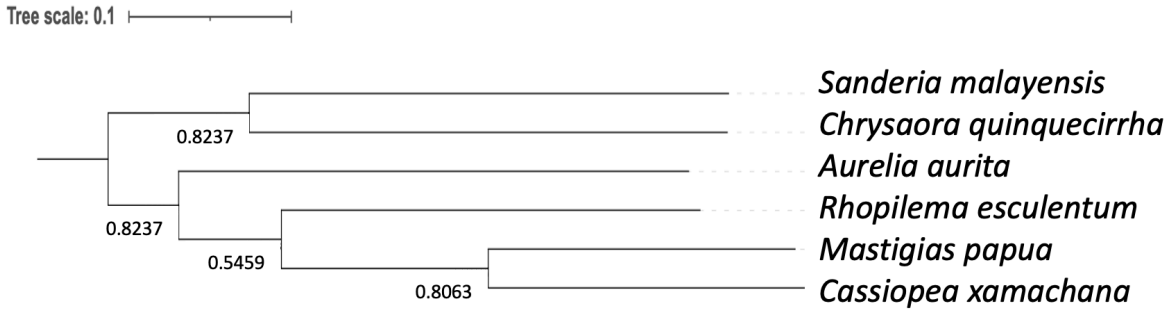

Figure 1.3: Maximum likelihood Scyphozoa species tree from OrthoFinder. Tree represents relationships of orthogroups. .

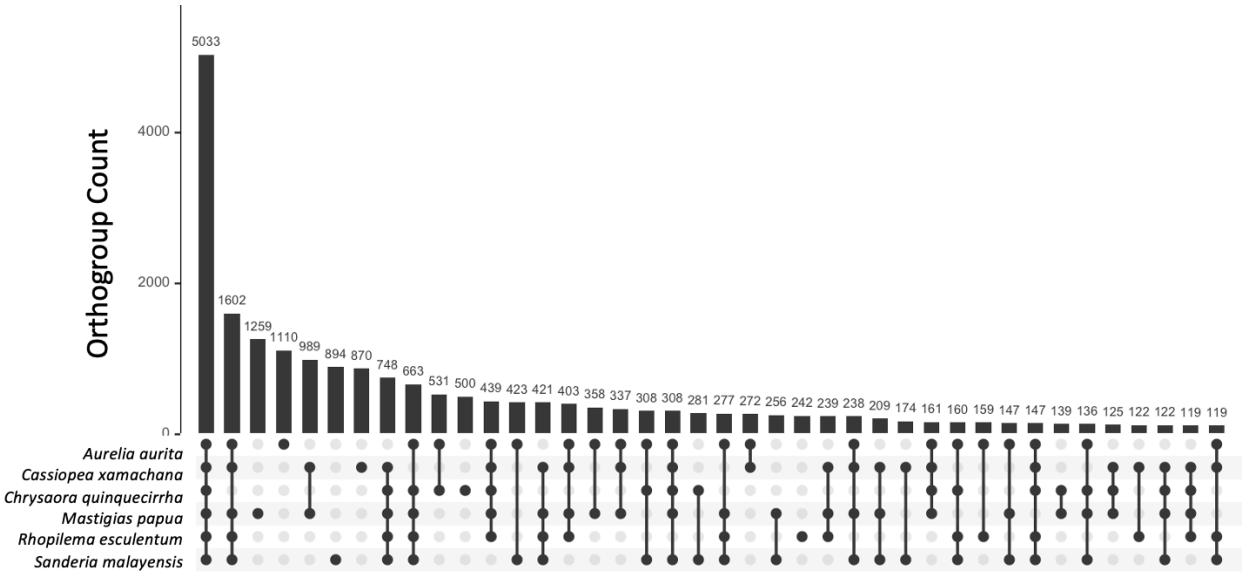

Figure 1.4: Overlap of orthogroup clusters shared between species. The bar cluster count is representative of the species signified underneath the bar by a filled in dot. The first bar, for example, is the number of orthogroups all species have in common. The symbiotic group is present in the fifth row. The non-symbiotic group is present in the thirtieth bar.

Ch. 2 Coevolution of Symbiodiniaceae and Scyphozoa

2.1 Abstract

Symbiotic species belonging to Cnidaria—such as corals and jellyfish—play significant roles in communities across the globe that are being impacted by changing climates. The adaptive potential of cnidarians can be attributed, at least in part, to the presence or absence of their photosynthetic endosymbionts belonging to Symbiodiniaceae. There are few well-defined characteristics of symbiotic cnidarian ecological relationships and even less is known about their coevolutionary potential. To address the degree of coevolution between Cnidaria and Symbiodiniaceae, I designed two BAIT sets for capturing ultraconserved elements (UCEs) from jellyfish host and Symbiodiniaceae separately, though utilizing the same tissue. With these data I constructed separate maximum likelihood phylogenies of host and endosymbiont using RAXML then performed a cross comparison. The host UCE phylogeny supported family level relationships from other markers but contained mismatched species from Rhizostomatidae, Cyanedidae, Catostylidae, and Lychnorhizidae. The Symbiodinaceae UCE phylogeny did not directly mirror that of the host phylogeny, nor did goodness of fit statistics reflect significant phylogenetic parallelization. Phylosymbiosis was not supported by these data, but exploring additional aspects of the relationship may provide support for coevolution.

2.2 Introduction

Piecing together the past with clues from the present is a challenge in the evolutionary study of any species. The complexities continue when considering species interactions and coevolution. Species interactions can include those between a species and closely related clades, species in the same community, and holobionts: a host and its symbiont(s) (Langerhans 2008). Holobiont coevolution can be identified through cospeciation, gene by gene alterations, coadaptation, species diversification patterns mirrored between two species (Thompson 1989) and phylosymbiosis: cospeciation seen between microbes and their hosts as mirrored by their phylogenies (Gonzalez-Pech et al. 2024). Study of holobiont coevolution is an established field (Ehrlich & Raven 1964) and has seen rapid development with recognition of the prevalence of symbioses. This is in part due to next generation sequencing, e.g. through the Human Microbiome Project (McGuire et al. 2008), and Aquatic Symbiosis Genomics (McKenna et al.

2021). Testing of hypotheses predicting coevolutionary patterns from the macroevolutionary to community scale have also increased (Agrawal & Zhang 2021). Hypotheses of coevolution between hosts and their symbionts have been tested in marine invertebrates (O'Brien et al. 2019). Noteworthy examples are seen in Cnidaria as both parasitic symbionts of other invertebrates and fish (Holzer et al. 2018), and mutualistic hosts of Symbiodiniaceae (Baumgarten et al. 2015).

All cnidarian classes include genera that can be symbiotic with Symbiodiniaceae a family of dinoflagellates, also referred to as zooxanthellae. Symbiodiniaceae can exist as free-living organisms but also endosymbiotic, photosynthetic dinoflagellates that associate with cnidarians in mutualistic metabolic exchange (Djeghri et al. 2019). Taxonomic diversity of Symbiodiniaceae has been redefined through time with new classifications of genera and clades (Price & Bhattacharya 2017, Liu et al. 2018, LaJeunesse et al. 2018). Symbiodiniaceae can bleach in response to environmental perturbation as they reduce contribution to the mutualism in favor of restoring their population. Bleaching is widely known in coral reefs (Allen-Waller & Barott 2023) but is also possible in Medusozoa which appear to be less dependent on the symbiont for short-term survival (Ch. 4). Many questions remain in the Medusozoa-Symbiodiniaceae symbiosis such as: the amount of symbiont necessity for host life, mechanistic drivers of the interaction, and which partner drives bleaching?

Scyphozoan jellyfish present an opportunity to explore coevolution in motile organisms across all major seas of the world. The photosymbiotic mutualistic association between Scyphozoa and Symbiodiniaceae is particularly concentrated within the Kolopophorae clade of Discomedusae in Scyphozoa (Djeghri et al. 2019). It is also present in Linuchidae and Nausithoidae, two families of coronates sister to Discomedusae (Djeghri et al. 2019). Despite the supported presence of Symbiodiniaceae in these clades, population level variation can occur, impacting the degree of individual symbiosis. Scyphozoa acquire their zooxanthellae primarily via horizontal transmission from the environment as polyps, a common attribute of mutualistic symbiosis. Vertical transmission from parent to offspring can also occur, a characteristic more commonly seen in parasites (Sachs & Wilcox 2006). Acquisition of symbionts can affect the nature of the relationship and cospeciation potential (Sachs & Wilcox 2006). Many scyphozoan hosts are selective toward certain symbiont species and will filter out unwanted taxa (e.g. Grajales & Sanchez 2016). Despite an understanding of the general relationship, ecological dynamics of the

Scyphozoa-Symbiodiniaceae symbiosis remain vague, and coevolutionary potential even more so.

Advancing genomic and ecological tools have made it possible to increase the depth of study and hypotheses that can be tested in marine holobiont coevolution. Phylogenomics of Scyphozoa and Symbiodiniaceae separately, have improved through time with new markers and methodology, but gaps and low support remain (Gomez-Daglio & Dawson 2017, Bayha et al. 2010, LaJeunesse et al. 2018). Ultraconserved elements (UCEs) combine conserved regions flanked by highly variable sites to resolve order to population level taxonomic relationships. UCEs have proven useful in resolving many taxonomic clades (e.g. McCormack et al. 2012, Crawford et al. 2012, McCormack et al. 2013) including within the cnidarian class Anthozoa (Quattrini et al. 2014) but have yet to be explored in Scyphozoa. Herein, I utilize targeted sequencing of UCEs and their flanking regions in host Scyphozoa and symbiont Symbiodiniaceae to resolve the host phylogeny and assess phylosymbiosis of the holobionts. I hypothesize that 1. host UCE signatures are evolutionarily determined, and 2. there is a phylosymbiotic pattern of coevolution between the host and Symbiodiniaceae.

2.3 Methods

2.3.1 DNA Extractions

We extracted total DNA, using CTAB-Phenol Chloroform extraction (Schiebelhut et al. 2016), from 170 archived specimens that included genera from each family of Scyphozoa, except Atorellidae and Drymonematidae which were not present in our archive. I aimed for 3 individuals from each species where available. Following extraction, DNA was quantified with the Qubit dsDNA broad range kit, then sent to Daicel Arbor Biosciences (DAB) for probe capture and sequencing.

2.3.2 Probe creation

Phyluce (Faircloth et al. 2012, Faircloth 2016) was used to create our host and zooxanthellae UCE probe set with the following steps. The Scyphozoa host genome set was developed with *Mastigias papua* (Ch. 1) as the base genome and reads were simulated from available scyphozoan genomes using *ART* (Huang et al. 2012) to avoid sequencing errors. The scyphozoan genomes included: *Cassiopea xamachana* (Ohdera et al. 2019), *Rhopilema esculentum* (Li et al. 2020), *Aurelia aurita* (Gold et al. 2019), *Lychnorhiza* (Maronna et al. in prep), *Sanderia* (Maronna et al. in prep), *Stomolophus* (Maronna et al. in prep), and *Nemopilema nomurai* (Kim et al. 2019). The simulated reads were then mapped to *Mastigias papua* using Bowtie 2 version 2.3.4.3 (Langmead

& Salzberg 2012) with alignment rates from 0.4-2%. An initial test set was created using the fully developed *Aurelia aurita* genome as the base genome but produced a lower number of probes representing fewer UCE loci so *M. papua* was selected. Alignment files were sorted and filtered to remove repetitive intervals. Alignments revealed 56,825 loci shared between *M. papua* and at least one other taxon, as more taxa were considered the shared loci declined to only 23 shared across all seven taxa. As such, I chose to proceed with the set of loci shared between *M. papua* and at least 2 other taxa to conserve a reasonable minimum number of loci: 9,116. These loci included the following counts by species: *A. aurita*: 5360, *C. xamachana*: 4953, *R. esculentum* : 4134, *Stomolophus*: 3787, *N. nomurai*: 2162, *Lychnorhiza*: 1620, *Sanderia*: 1450. This set of loci was extracted from the base genome at 160bp intervals to allow for 40bp overlap between two 120bp probes. This includes a central UCE and its flanking region. Loci were masked leaving 7,714 loci that produced 14,994 probes. Duplicate loci were removed and the bait set was aligned to the exemplar Scyphozoa genomes and three additional outgroup genomes: Hydrozoa *Hydra vulgaris* (Chapman et al. 2010), Cubozoa *Morbakka virulenta*(Khalturin et al. 2019) and Staurozoa *Calvadosia cruxmelitensis* (Ohdera et al. 2019). This step increases the applicability of the probe set at higher taxonomic levels. The loci were extracted from each genome at 180 bp and then queried for consistency allowing us again to see the number of loci shared with *M. papua* and one other taxon: 775, to all ten taxa: 11. Here I proceeded the loci shared between *M. papua* and 2 other taxa: 518 loci. Across species shared loci counts included *A. aurita*: 218, *C. xamachana*: 368, *R. esculentum* : 378, *Stomolophus*: 378, *N. nomurai*: 358, *Lychnorhiza*: 413, *Sanderia*: 246, *C. cruxmelitensis*: 73, *M. virulenta*: 64, *H. vulgaris*: 53. This loci set was used to generate the final bait set of 4,928 probes that were filtered down to 4,743 probes after removing duplicates. An insilico test of the bait set was performed by aligning the genomes to *M. papua*, matching the resulting contigs to the bait set, then aligning and trimming those conserved loci for a final insilico UCE set. I generated a phylogenetic tree to view these UCE insilico relationships using *RAXML* v8.2.12 (Stamatakis 2014) on CIPRES v.3.3 (Miller et al. 2010).

The above process was repeated for the Symbiodiniaceae probe set using *Symbiodinium goureaii* (reefgenomics http://symbs.reefgenomics.org), now known as *Cladocopium goureaii*, as the base taxon and *Symbiodinium*,*Symbiodinium microadriacticum (*GCA_001939145.1), *Symbiodinium kawagutii (*reefgenomics http://symbs.reefgenomics.org), now known as *Fugacium kawagutii*, and *Breviolum minutum (*GCA_000507305.1) as exemplar genomes to represent Clades C, A, F, and B, respectively, with genomes unavailable for Clades D, E, G, H, and I. Outgroup species included: *Trichodesmium erythraeum* (GCF_000014265.1), *Crocosphaera watsonii*(GCF_000235665.1), *Moorea producens* (GCF_000211815.1), *Symbiodinium pilosum* (GCA_905231905.1),*Prorocentrum minimum* (GCA_001652855.1), and *Polarella glacialis* (GCA_905237085.1). Initial alignment rates ranged from 0.33-1.09%. The first query produced 36,749 loci shared between *S. goureaii* and at least one other taxon to 1,007 loci shared across all 5 taxa. I proceeded with the loci shared between *S.*

goureaii and at least two other taxa: 11,650 loci, across species this was *S. kawagutii*: 9581, *S. microadriacticum*: 7503, *B. minutum*: 7131, *Symbiodinium*: 3943 loci. After masking, this was reduced to 8,362 loci represented with 16,026 probes, and 2,225 probes, targeting 318 loci after removing duplicates. The final BAIT sets contained 4,743 probes targeting 518 UCE Scyphozoa loci and a set of 2,225 probes targeting 318 UCE Symbiodiniaceae loci.

2.3.3 UCE Capture and Sequencing

Fifty microliters of extracted DNA for each sample were shipped in liquid format in individual 1.5-mL tubes to Daicel Arbor Biosciences. The gDNA was transferred to a plate, fully dried, and resuspended to 60µL per sample with EBT (10 mM Tris-Cl, 0.05% Tween-20). The total DNA was quantified spectrofluorimetrically. Samples were purified via SPRI beads at a 1.6X ratio and were quantified again via spectrofluorometry. Up to 415ng of purified gDNA was taken through an Illumina TruSeq-style library preparation method optimized for targeted capture. A fragmentation and size selection protocol designed to produce an average insert length of approximately 500nt was followed. Unique dual-index combinations were added to each sample via 6–14 cycles of PCR amplification. The indexed libraries were quantified with both a spectrofluorimetric assay and quantitative PCR.

To prepare for capture, libraries were pooled in equimolar ratios in 6-to-14-plex reactions and dried down to 7µL by vacuum centrifugation. Captures targeting the host DNA were performed using DAB's custom myBaits 1-20K kit (design ID D10274MSTGS) following the myBaits v5 protocol with an overnight hybridization. The supernatant from the overnight hybridization was saved. Hybridization and washes were performed at 65°C. For capture of a subset of the pools with a second custom myBaits 1-20K kit targeting zooxanthellae DNA (design ID D10273JFSym), 80% of the saved supernatant volume was concentrated to 25µL and used as input for capture following Klunk et al. 2019 and the myBaits v5 protocol as above. For each capture reaction, half of the volume of beads in the elution buffer were amplified for 10 cycles. For the symbiont captures, the second half of the bead volume was amplified for 10 cycles and combined with the product of the first half. Final capture pools were quantified again with both a spectrofluorimetric assay and a quantitative PCR assay and were also visualized on an Agilent Tapestation 4200. Captures were pooled at equimolar ratios for sequencing with separate pools for the host and symbiont captures. The pools were sequenced on the Illumina NovaSeq 6000 platform on partial S4 PE150 lanes using v1.5 chemistry.

2.3.4 Phylogenomics

CCmetagen (Marcelino et al. 2020) was run on the full probe capture raw reads to assess microbial gene content and contamination for Symbiodiniaceae and Scyphozoa separately. Raw reads of scyphozoan hosts with available genomes and the full set of Symbiodiniaceae were also aligned with *bbmap* (Bushnell 2024) to all available host and

Symbiodineacae genomes used during BAIT design to confirm assigned scyphozoan identity and determine Symbiodiniaceae species identifications. Since BAIT design, additional Symbiodiniaceae genomes have become available: *Effrenium voratum* (GCA_963377175.1), *Symbiodinium necroappetens* (GCA_905231915.1), *Symbiodinium natans* (GCA_905221605.1), and *Symbiodinium pilosum* (GCA_905231905.1) and were added to the *bbmap* run. Clades A, B, C, and F were represented in this run based on available genomes. The species chosen for individual identity was based on the highest percentage of mapped unambiguous reads.

Demultiplexed Scyphozoa and Symbiodiniaceae sequences were analyzed separately with the Phyluce environment (Faircloth et al. 2012, Faircloth 2016). Raw reads were cleaned and assembled, then searched for UCE loci. These loci were extracted and aligned across individuals. Alignments were compared between the full set of individuals, a modified set of individuals that removed samples contributing high quantities of gaps to the alignment, and at different percentages of loci inclusion across species. *RAXML* v8.2.12 (Stamatakis 2014) on CIPRES v.3.3 (Miller et al. 2010) was used to generate the final maximum likelihood phylogeny for Scyphozoa ceasing after 200 bootstraps, and Symbiodiniaceae ceasing after 550 bootstraps with the GTRCAT approximation and GTRGAMMA model. The final trees were midpoint rooted and visualized in Tree of Life (Letunic & Bork 2006).

2.4 Results

2.4.1 Scyphozoa

Only individuals that were assigned to a species with a currently available genome were able to be assessed for correct species assignment based on morphology. An average of 345,707 contigs across an average of 92,504,655bp were assembled per individual with an average length of 288bp. These contigs represent repeated returns of a total of 422 unique UCE loci that were assembled and aligned between individuals. The initial alignment of all individuals had 21.72% gaps and the chosen alignment for the final tree had 19.26% gaps with 39.09% invariant sites. Low UCE counts were returned in some individuals of *Cyanea capillata, Cassiopea andromeda, Rhopilema verilli, Atolla wyviellei, Atolla parva, Poralia rufescens, Chrysaora fuscecens, Phyllorhiza peronciperui, Crambione mastigiopore, Crambionella Orsini, and unidentified species of Chrysaora, Phacellophora, Paraphyllina, Periphylla, Linuche, and Stephanoscyphus* (Table S2.1).

The maximum likelihood scyphozoan UCE tree presents Coronatae sister to Discomedusae (Fig. 2.1). Within the Discomedusae, a clade of two semaeostomes — *Pelagiidae* and *Cyaneidae* — is sister to a clade containing the semaeostome *Ulmaridae* and the Rhizostomeae. Within the Rhizostomeae, the scapulates *Stomolophidae* and *Rhizostomatidae* are sister taxa, and the suborder Kolopophorae contains all expected families (*Cepheidae, Mastigiidae, Thysanostomatidae, Versurigidae*,and *Cassiopeidae*).

There are a few individuals in unexpected placements including: *Crambione mastigiophora*, and *Rhizostoma pulmo* within Ulmaridae and *Desmonema dalmatinum* within Dactyliophorae.

2.4.2 Symbiodiniaceae

Species identifications mapped a range of 0.001-48.75% per individual reads to available reference genomes with the most highly mapped being chosen as species identity. Low UCEs were returned for all control nonsymbiotic species. A total of 260 UCE loci were identified and aligned between individuals.

An average of 491,818 contigs across an average of 93,075,774 bp were assembled per individual with an average length of 193bp. These contigs represent repeated returns of 314 total UCE loci that were then assembled and aligned between individuals. The alignment had 23.20% gaps and 61.37% invariant sites. As with the host reads, some samples returned low levels of UCEs causing those samples to be excluded from the final phylogeny including *Cephea, Catostylus, Versuriga, Nausithoe, Phyllorhiza* species along with *Pseudorhiza haeckeli*, *Rhizostoma*,*Stomolophus meleagris*, and *Aurelia labiata* that were included as non-symbiotic species controls.

The Symbiodiniaceae maximum likelihood phylogeny represents Clades across the Symbiodiniaceae family including A (*S. microadriaticum*), B (*Breviolum minutum*), and C (*C. goreau*i) (Fig. 2.2). Symbiont UCE profiles that cluster together include those from the *Cotylorhiza*, *Linuche, Phyllorhiza, Mastigias* and *Cassiopea* hosts (Fig. 2.2). *Thyanostoma* and *Nausithoe* have individuals spread out rather than clustered, though bootstrap supports were low with these distinctions.

2.4.3 Host-Symbiont relationships & correlates

Phylosymbiosis

The coevolutionary comparative tree has parallel relationships between host:symbiont pairs of *Linuche*, *Phyllorhiza peroncispeuri*, *Cotylorhiza*, and *Cassiopea ornata* (Fig. 2.3). Symbiodiniaceae hosted by *Mastigias papua* form a clade that contains an individual from *Versuriga* and *Thyanostoma* (Fig. 2.3). Procrustes analysis shows high clustering between *Mastigias*, *Cotylorhiza*, *Phyllorhiza*, *Thyanostoma*, *Nausithoe*, and *Versuriga* species; *Linuche* and *Cassiopea* host species branch outward into their own clusters despite their symbionts remaining close to the main cluster (Fig. 2.4). The goodness of fit test comparing these trees returned an insignificant match, suggesting not enough support for phylosymbiosis.

2.5 Discussion

Our hypothesis of host scyphozoan UCE signatures representing evolutionary phylogenetic patterns of other markers was supported for some species and unrobust in

others. I also hypothesized that there would be a phylosymbiotic pattern of coevolution when comparing host and symbiont phylogenies. Again, there were phylosymbiotic patterns for some species associations and not others. Overall, this suggests the need for more individuals per species to rule out the role of sample quality or unique instances of nonconformity to the general patterns. I assess the outcomes of this study from the phylogenetic, spatial, and coevolutionary perspective for Scyphozoa and Symbiodiniaceae.

2.5.1 Scyphozoa phylogeny

The resulting phylogeny generally matches the accepted family structure of Scyphozoa with some caveats (Bayha et al. 2010, Gomez-Daglio & Dawson 2017, summarized in Djeghri et al. 2019). Caveats included *Desmonema dalmatinum* which should be in Semaeostomeae Cyaneidae, not Rhizostomeae. The Rhizostomeae *Rhizostoma pulmo*, and *Crambione mastigiphora* are incorrectly placed within Ulmaridae of Semaeostomeae. These unexpected individual placements could be tied to misidentification at sample collection, though investigation is still in progress. The more likely cause is related to variation during probe captures that can alter the UCE profile. Samples of the same species that were collected at the same sample site clustered together with low phylogenetic distance, supporting UCEs as a reliable marker for scyphozoan host relationships and enforcing the reliability of our bait set.

The bait set could have potentially been improved with altering the outgroups included in the final steps of design that decreased the loci represented from ~7000 to 500, though the advantage of outgroups used were chosen to provide loci representative across all species of Scyphozoa. While there was a similar decline at this step in the *phyluce* tutorial, it was not as drastic. The counts were not a concern as they were also close to the 720 UCE loci represented in the anthozoan bait set (Quattrini et al. 2018). Perhaps, though, a larger bait set would have been beneficial to resolving some of the individuals that did not follow family level groupings.

2.5.2 Symbiodiniaceae phylogeny

The Symbiodiniaceae tree has clustering of most individuals by host species and has a split between *Symbiodinium, Breviolum* and *Cladocopium* genera. While this is the first UCE bait set for Symbiodiniaceae, it could be improved upon now that more genomes are available. Compared to Scyphozoa and other bait sets, the 300 represented loci suggests room for improvement. Many loci were lost to duplication and outgroup alignment, a necessary process due to the low availability of genomes at the time of bait design and data collection. The species identifications for Symbiodiniaceae may include misidentifications as not all species' genomes are available for mapping. This could have been mitigated by running an additional ITS2 sequencing step for species identification.

I included an enhanced subset of *Mastigias papua* to compare localized sampling of host and symbionts. While *M. papua* was grouped by lake for scyphozoan UCEs, the Symbiodiniaceae UCEs are mismatched by lake site. This result is surprising as hosts acquire their symbiont from the environment, therefore, within lake similarity should be higher than between lakes. These relationships have low bootstrap support, however, and may just be artifacts of low resolution at the population level due to high homoplasy and low variation. I had attempted to combat this by using *M. papua'*s common Symbiodiniaceae species as the main reference genome during design.

2.5.3 Coevolution

Instances of reciprocal evolutionary trait changes are preferred characteristics in support of coevolution (Thompson 1989) as opposed to congruent associations between species (Janzen 1980). This would exclude the superficial assumption, for example, that coral has coevolved with Symbiodiniaceae because they are associated but would include the conclusion that populations have coevolved when observing reciprocal adaptations of calcification rates and bathymetric zonation (Pomar & Hallock 2007). Specific to our study, the species relationships here show some congruency at the basic level of phylosymbiosis but not enough to support speciation. These limitations were due to few available individuals within a species returning high quality sequencing. The results of my work remain important for initiating understanding of the interconnections between Scyphozoa and Symbiodiniaceae. Phylosymbiosis appeared present at the population level when 3+ individuals were present, e.g. *Mastigias papua*, *Cotylorhiza*, and *Cassiopea ornata*. This is likely tied to the availability of Symbiodiniacae for infection being dependent on environmental presence. I would also like to explore phylosymbiosis in more populations with phylogeography in mind. This would require intentional collection site mapping as opposed to archival sample availability.

To further develop the study, the bait set should be supplemented and redesigned with newly available host and symbiont genomes then applied to a larger sample set. Since many individuals were lost to sequencing quality, an increased sample size would benefit more robust conclusions when redefining phylogenetic relationships of Scyphozoa and Symbiodiniaceae. Tests for selection and horizontal gene transfer between the host and symbiont could be explored in the UCE dataset as alternative coevolutionary support, particularly if the host and symbiont have shared UCEs when applying the Symbiodiniaceae probes to scyphozoan controls. The longstanding association of Scyphozoa and Symbiodiniaceae, coupled with strong metabolic exchange, and evidence of behavioral interdependence (see Ch. 4) suggest we have only begun to explore the coevolutionary potential in this holobiont.

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2.7 Figures

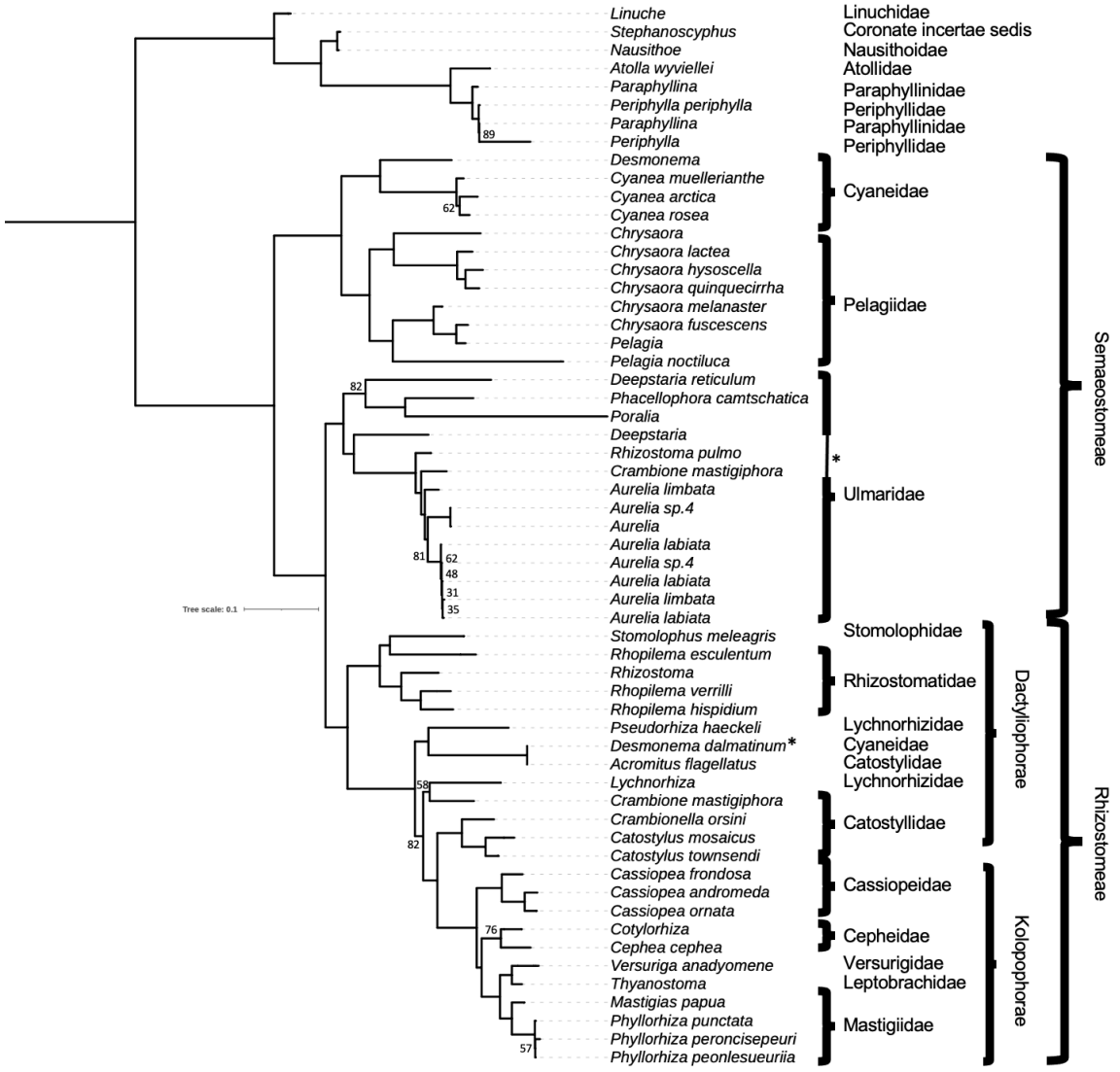

Figure 2.1: Scyphozoa host ultraconserved element maximum likelihood RAXML-ng tree. The tree is midpoint rooted, species are labeled by family with higher classifications labeled for distinction. Bootstrap support values <90% are stated, all other bootstraps were >90%. Multiple individuals of the same species sister to each other were removed for readability, the full tree with all individuals can be seen in Fig. S2.1. *Denotes unexpected topology, the top indicates a split within Ulmaridae of Rhizostomatidae and Catostylidae individuals, and the bottom highlights a problematic Cyaneidae individual within Dactyliophorae.

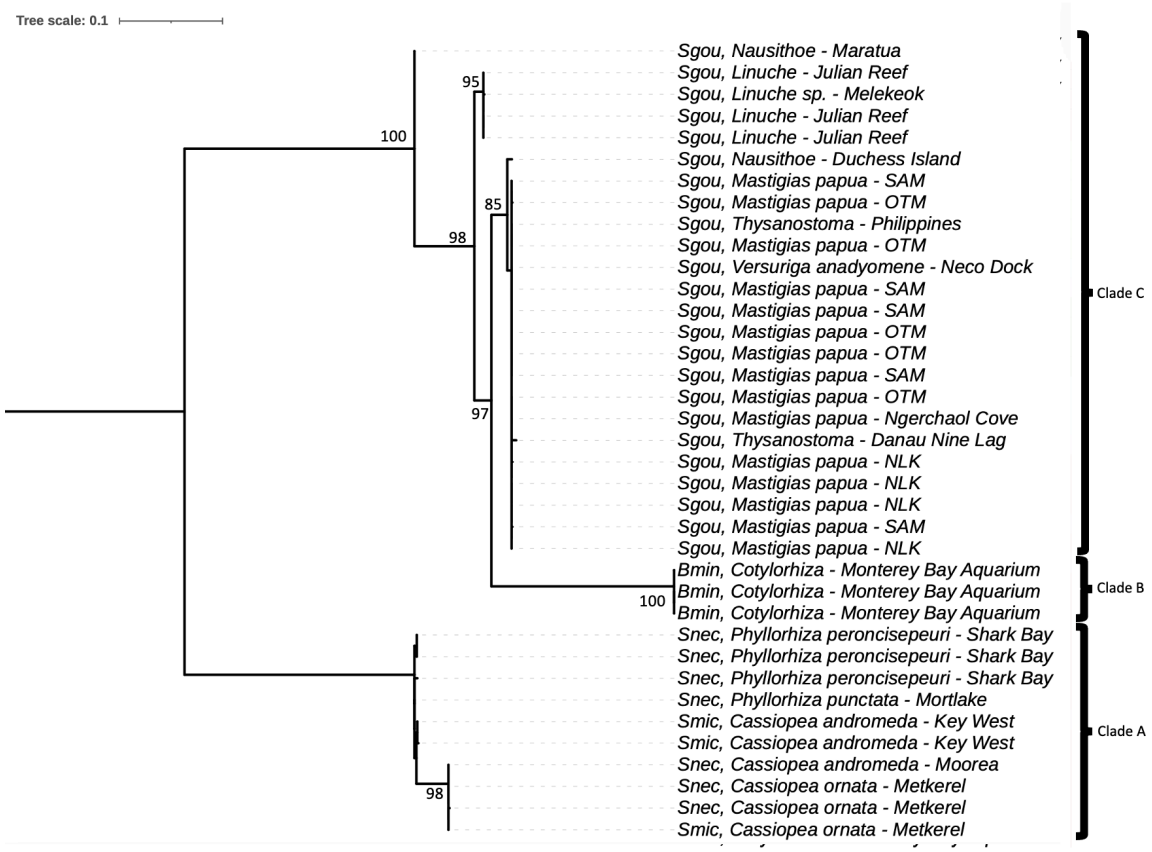

Figure 2.2. Symbiodiniaceae ultraconserved element maximum likelihood RAXML-ng tree. The tree is midpoint rooted. Naming includes the primary symbiont species (Sgou - *Symbiodinium goureaii*, Smic - *Symbiodinium microadriacticum*, Snec - *Symbiodinium necroappetens*) identified by *bbMap*, followed by host species, and collection site code. Symbiodiniaceae clades (LaJeunesse et al. 2018) are labeled in brackets on the right side. Bootstraps values greater than 75 are included.

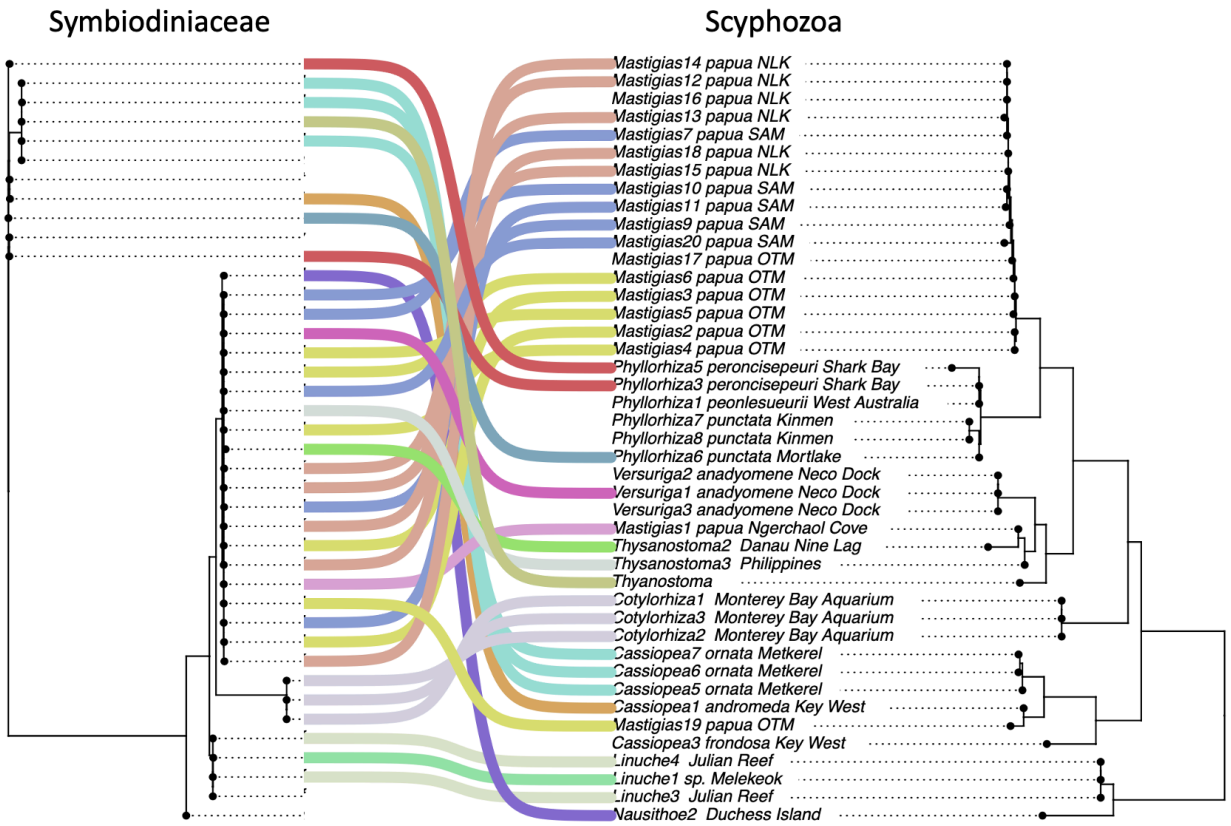

Figure 2.3: Associations of Symboidiniaceae (left) and host Scyphozoa (right) UCEs. Host tree and bootstraps are trimmed from Fig. 2.1, Symbiodiniaceae bootstraps are the same as Fig. 2.2. Tips are named by host Scyphozoa species and collection site. Branch lengths are arbitrarily set for clear viewing of relationships. The linkage is missing from species that do not have a corresponding host:symbiont sample.

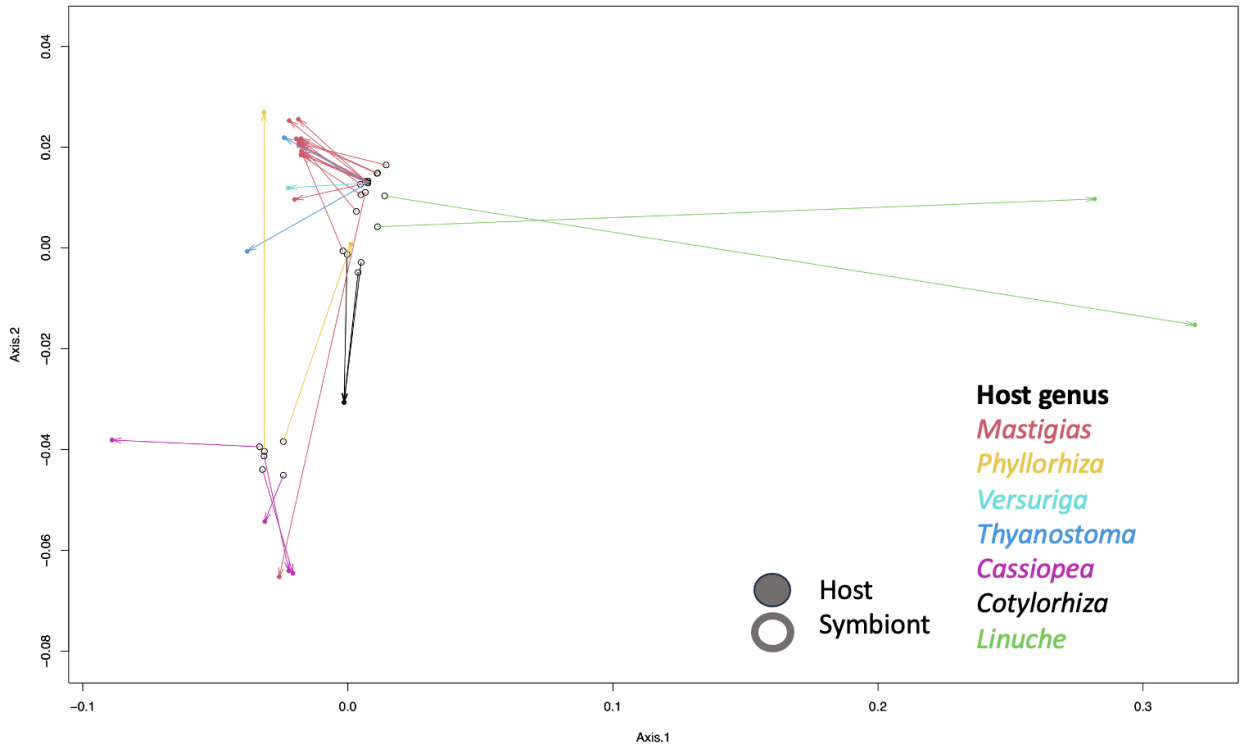

Figure 2.4: Procrustes plot of vector linkages between UCE loci of scyphozoan hosts and Symbiodiniaceae symbionts. Open circles represent Symbiodiniaceae samples and have vectors that point to closed circles of the scyphozoan host. All points are color coded by host genus.

Ch. 3 An ethological framework for deconstructing complex behaviors of Scyphozoa

3.1 Abstract

Diversity among gelatinous zooplankton has been studied primarily through a functional lens — i.e. how things work — as opposed to an ethological lens of why they work. This historical bias can be attributed in part to the technical difficulty of accessing gelatinous zooplankton for observation in their natural environments and in part to a presumed lack of complexity. Thus, the behaviors of gelatinous zooplankton remain under-explored—and ecologically consequential. On this 50th anniversary of blue-water diving, we take stock, from an evolutionary perspective, of our knowledge of jellyfish behavior from both ethological and functional perspectives, particularly of Scyphozoa. I consider individual through population to species level phenomena. I summarize behavioral categories to include movement, feeding, reproduction, and mass occurrence. I ask about their roles in ontogeny and symbioses. I deconstruct ethological observations of individuals as complexes of behavioral traits. I characterize species' differences and similarities as studies of jellyfish can highlight behavioral syndromes in early diverging taxa. I conclude I have reached the limits of the now largely conventional, phenomenological approaches, and make suggestions for structuring future trait-based behavioral studies of Scyphozoa. These conclusions can be applied in a broader context to other gelatinous zooplankton, which will be crucial to understanding responses of these ecologically influential taxa to changing oceans.

3.2 Introduction

Group behavior is among the most intriguing of biological phenomena: the elastic murmurations of starlings (King & Sumpter 2012), schooling of euphausiids (Hamner 1984), the streaming columns of ants (e.g. Burd et al. 2002). Despite the collective focus, these phenomena emerge from the actions, interactions and cooperation of individuals. Studies of ants and slime molds show group behaviors can emerge from a few basic rules (Reid & Latty 2016), and some epiphenomena may require certain numbers of participants. Base behaviors of a critical number of individuals in the group onset the emergent group properties (Giardina 2008). Jellyfishes too form large groups resulting from various factors — aggregations, blooms, and swarms — but the emphasis in the literature has been on the numbers of individuals. Specifically, the demographics of 'true blooms' (a rapid increase in population size in-place) as opposed to a redistribution, and physical factors that may accumulate largely 'planktonic' taxa are focused on.

Jellyfish behavior has been treated somewhat phenomenologically — (e.g. Graham et al. 2001; Hamner & Dawson 2009) — rarely deconstructing features that surround the behaviors. Yet, we know that differences in migrations are accompanied by population-level differences in aspects of individual behavior, such as swimming speed and direction, turning rate, pulse rate, and shadow response (Dawson & Hamner 2003). Moreover, we know that differences in behavior are allied with differences in morphology (e.g. Blough et al. 2011, Costello et al. 2021). Arguably, therefore — as for starlings, euphasiids, and ants — understanding aggregate behaviors (blooms, swarms, etc.) in jellyfishes may demand an understanding of individual behavioral traits, and furthermore understanding how these relate to morphological traits and their environments, but the literature remains deficient.

The incomplete record of jellyfish behavior can be tied to the history of scientific approaches. Early ethologists debated the inclusion of invertebrates, such as jellyfish, as model systems for interspecific behavioral variation (Beach 1960, von Frisch 1950, Schneirla 1929) but decided they were irrelevant based on beliefs that complex behavioral patterns require complex nervous systems (Hilgard 1948, Simpson 1949). In retrospect, the debate suffered from a lack of approaches for studying pelagic invertebrates, including Scyphozoa. *In situ*; researchers relied on information gained from large-scale net captures that provide little if any behavioral information (Raskoff et al. 2002). These methods improved toward the 1950s with live capture and lab holding of gelatinous zooplankton for neurological investigations (Mile 1938, Brewer 1978, Hamner et al. 1994; Raskoff et al. 2002). With Hamner and colleagues' (1975) emphasis on using SCUBA to observe gelatinous zooplankton in pelagic zones, we came to understand that gelatinous zooplankton were capable of more complex behaviors. We now know scyphozoans, and other medusozoans, perform ecologically important behaviors at individual and group scales, for example: migration, aggregation, and active prey capture (e.g. Matsumoto & Hamner 1988, Graham et al. 2001, Lewis & Long 2005, Nath et al. 2017, Ohdera et al. 2018). The nature of open water opportunistic observations can be difficult to replicate and leaves gaps in the literature. The first step to filling these gaps is by reviewing the literature to identify how we can proceed.

Approaching the literature of jellyfish behavior in an ethological framework begins by assessing the function of characters (Tinbergen 1963) from the individual to population level while considering abiotic and biotic influences. Arai's (1997) benchmark on the functional biology of scyphozoans included examples of behavioral patterns related to morphology and the resulting locomotion, feeding, reproduction, and ecology (e.g. migration and blooms). She assessed the functional biology of individual medusae, yet we can apply these same categories as a framework for assessing collective behaviors. Eibl-Eibesfeldt and Kramer (1958) developed the interconnection between structure and behavior from an ethological perspective. They emphasized the necessity of environmental context and phylogenetics with consistency of behaviors between closely

related species. Aspects of behavioral patterns can be further expanded and studied across the levels of molecule, individual, group, and population (Wcislo 1989). As with the shift toward observations of pelagic medusae (Hamner et al. 1975), a new shift is required to contextualize actions of medusae from the functional perspective toward behavioral patterns as well. Our goal here is to provide a more holistic view of behavior in jellyfishes by addressing the individual morphological and behavioral foundations of complex behaviors.

Here I incorporate ideas of Eibl-Eibesfeldt and Kramer (1958), Wcislo (1989), Tinbergen (1963), and Arai (1997) to better understand the origins and complexity of scyphozoan behaviors in the literature and their interrelations (Fig. 3.1, 3.2, 3.3). I employ a generalized definition of behavior—the actions of an organism in response to its environment—to encompass the variety of behavioral applications from functional dynamics of Scyphozoa. Where possible, I address the questions: **1.** Can we reassess functional interactions as behavior, which studies do so already? **2.** Are there distinguishable phylogenetic behavioral trends? **3.** Can we use a trait-based approach to scyphozoan behavior? As quantitatively trait-based approaches have transformed our understanding of jellyfish diversity, we believe a trait-based approach to behavior will transform our understanding of functional biology here.

3.3 Methods

3.3.1 Literature Review

A literature search was conducted on 17 May 2021 with the Web of Science using ‘medusa* + behavior’ (accounts for ‘behaviour’ as well). The results were searched for the behavioral keywords: feeding, movement, reproduction, and mass occurrence, coinciding with Arai’s (1997) chapters that include behavioral patterns of function: *Feeding*, *Locomotion*, *Reproduction*, and *Physical Ecology* (Arai 1997). On 19 May 2021 the initial search was narrowed to Scyphozoa with the key behavioral categories on Web of Science (Scyphozoa and behav* or feed* or reproduc* or migrat* or aggrega*) with unrelated categories excluded (Table S3.1, S3.2 for complete list). Papers were included in further analyses if they passed a relevancy test from each level of titles, then abstracts, then full publications. A paper was deemed relevant if the sole focus of the paper was specific to scyphozoans and their behavior in an attempt to attain papers most closely following an ethological framework specific to scyphozoans. Google Scholar was used to search for additional papers in each category (e.g. “Scyphozoa feed*”, “Scyphozoa reproduc*”,“Scyphozoa migrat*”, “Scyphozoa aggregat*”), and references from the initial key studies that appeared relevant but were missed in the Web of Science search. Behaviors from species outside of Scyphozoa are included if they add context to scyphozoan behaviors.

3.4 Results

3.4.1 Literature Review

The initial 2021 searches returned 1,296 studies related to medusae behavior across Scyphozoa, Hydrozoa and Cubozoa. When narrowed to Scyphozoa, 360 total studies were returned (Table S3.2) and narrowed to 54 studies deemed relevant, 9 of which related to mass occurrence, 27 on movement, 8 in feeding, and 9 in reproduction..

3.5 Discussion

Observations of behavioral phenomena have utility for recognizing the breadth of potential behaviors but few studies have fully explored an organism's behavior through the mechanistic, functional, developmental, and phylogenetic lens to encompass a complete ethological framework (Tinbergen 1963, Bateson & Laland 2013). Behavioral literature on Scyphozoa has not escaped this trend, integrative literature (e.g. Schwab 1977) that includes all, or at least multiple, components of the behavioral framework remain sparse.

This is surprising considering the wide knowledge of scyphozoan biology from neural networks (e.g. Passano 2004, Pallasdies et al. 2019), to anatomy (e.g. Berking & Hermann 2007), ontogeny (Helm 2018), and evolution (Hamner & Dawson 2009).

3.5.1 Behaviors

Reviewing the literature led to four overarching behavioral categories: movement, feeding, reproduction, and mass occurrence. These behaviors can be assessed from the individual to group level of function (Fig. 3.1) and across ontogenetic stages. For example, Scyphozoa movement includes planulae settlement, and medusae swimming behavior, obstacle avoidance, and sleep. We can consider these individual movements mingling into group level migrations. The baseline behaviors of movement can be translated into feeding at the individual level with prey capture techniques and prey preferences. Feeding can also differ across ontogenetic stages with filter feeding at the polyp stage and active feeding in later forms. Reproductive behaviors of fertilization can be thought of at the individual level of movement, such as spermcasting, or the population level with increased density of individuals in aggregations during reproductive seasons. Mass occurrences have other population level forms as well, including swarms and blooms. These can be broken down into functions at the individual level of movement or feeding, and considered at group level for reproduction or resulting from migrations.

Movement

From flying through the air, walking terrestrial terrains, and swimming in aquatic spaces, the fitness of motile organisms relies on adaptation to the demands of their physical

environments (Schaeffer & Lindstedt 2013). Movement at the individual level can be impacted by the organism's traits, conspecifics, interacting species, and abiotic factors, leading to variation in individual movement patterns while causing downstream effects on populations, communities, and ecosystems (Shaw 2020). The ethology of movement can be narrowed to an initial focus on the functional biomechanical attributes. Movement behaviors can be studied quantitatively with tools from the physical sciences (Brown & de Bivort 2018), particularly when exploring hydrodynamics of swimming behavior (Costello et al. 2021). Medusozoans have muscular systems similar to those of pre-Cambrian ancestors that are central to studying evolution of aquatic motility with applications to invertebrates and vertebrates (Wang et al. 2022, Costello et al. 2021, Gold 2018). The same importance can be applied throughout the life cycle and from individual to population levels of their behavior when looking at planula settlement, swimming behavior, obstacle avoidance, migration, and sleep.

Planulae Settlement

An ethological approach to Scyphozoa planula settlement offers a link between life history and environmental factors. The settlement of planulae is suggested to be preferential for areas based on decreased predation, decreased solar exposure, gravity (i.e. abiotic factors), and photoreception (i.e. traits) (Ceh & Riascos 2017). *Cassiopea xamachana* planulae select shaded undersides of mangrove leaves, this settlement behavior is not found to be dependent on conspecific density (Fleck & Fitt 1999). Preferential selection toward artificial substrates was found in five scyphozoan species, *A. aurita, Cyanea capillata, Cyanea lamarckii, Chrysaora hysoscella*, and *Rhizostoma octopus* (Holst & Jarms 2007). Artificial substrates are increasing in marine environments due to pollution, thus increasing habitats for planulae to settle and continue their life cycle, potentially contributing to blooms (Holst & Jarms 2007). Settlement assessments of *Chrysaora plocamia* in the lab found preference for upside-down settlement on horizontal, sheltered, green and red substrates, suggesting a role for photoreception in substrate selection (Ceh & Riascos 2017). Following the pelagic to sessile transition of planula to polyp is the motile stage of medusa that applies active movement.

Swimming behavior

Medusozoans have previously been thought of as solely passive dispersers on ocean currents, but this generality has been questioned with expanding studies demonstrating swimming capabilities and responsiveness to objects (e.g. Hamner 1995, Hamner et al. 1995, Garm et al. 2007, Neil and Askew 2018). Scyphozoans are thought to primarily use rowing propulsion–a paddlelike partial bell contraction–but medusa size and aspect ratio of the bell is a more reliable indicator as small medusae commonly use jet propulsion–full expansion and contraction of the bell to divert water underneath it then expel it outward (Gemmel et al. 2021). The swimming capabilities of medusae shift through ontogeny impacting the available potential for certain behaviors. Dominant, outside viscous forces on juvenile/small individuals shift to inertial forces on mature/large

individuals when considering Reynold's number (Re). While size can affect swimming type, contraction frequencies were found to be species-specific and independent of water flow speed in a study of six scyphozoan species (Yang et al. 2018). Rhizostomeae are efficient swimmers, utilizing fluid expulsion during bell contraction and relaxation that creates a vortex for propulsion (Neil and Askew 2018). *Aurelia labiata* have been observed to alter their swimming direction in response to touch and salinity (Albert 2012, 2014), a reaction to biotic and abiotic elements of the immediate environment that can be presented as a behavior.

Obstacle avoidance

Obstacle avoidance, as a motivation for movement or stimulus response, can be driven by real-time cues and provide behavioral adaptations to habitats. As seen above in *A. labiata*, for example, alter swimming direction in response to touch (Albert 2014). In marine lakes *M. papua* displays shadow avoidance, a weighted trade-off response between maximizing predator avoidance and maintaining photosynthetic endosymbionts in sunlight (Hamner 1995, Dawson & Hamner 2003). This behavior requires the medusae to sense the difference in light intensity and then perform the movement away from the shadow. A potential explanation for the light response mechanism is seen in visually guided obstacle avoidance by the cubomedusae *Tripedalia cystophora* and *Chiropsella bronzie* (Garm et al. 2007). *T. cystophora* were determined to be more sensitive to obstacles resulting from behavioral adaptation to their habitats (Garm et al. 2007). In a laboratory setting, *Chironex fleckeri* avoided dark objects as a behavioral adaptation to sea turtle predation in natural habitats (Hamner et al. 1995). Scyphozoa also have eye spots, rhopalia, though they are less complex structures in comparison to the eye forms in box jellyfish. We would benefit from further study of the role of scyphozoan rhopalia in obstacle and predator avoidance.

Migration

Diel vertical and horizontal migration are patterns of a top to bottom or left to right, or vice versa, movement in respect to the water column that occurs within a 24 hour period. Vertical and horizontal migration has been documented in some medusae species, most often dependent on nutrient acquisition, or responses to shadows, predators, or light (Arai 1997). Responses from anatomical receptors, such as rhopalia, to environmental cues, such as (sun/twi)light, pressure, temperature, and salinity, working singularly or in combination are key components of medusae migratory patterns (Graham et al. 2001). *Aurelia*, for example, was observed to promote reproductive success in aggregations with sun-compass horizontal migration in two bays of British Columbia (Hamner et al. 1994). *Aurelia* are also seen to combat tidal dispersal in Roscoe Bay, Canada with vertical migrations (Albert 2007). Medusae can also alter their migrations to match prey capture. *Aurelia aurita* in the marine lakes of Palau, for example, vertically migrates to the surface at night and then downward during the day, potentially following prey (Hamner et al. 1982).

Publications on horizontal migrations of medusae were considered rare twenty years ago (Graham et al. 2001) and that continues to be the case as our literature search returned ten studies, most of which were published before 2012. *Mastigias papua* in the marine lakes of Palau occupy a majority of this niche as they present a unique opportunity for understanding migration from a natural laboratory. Each population has adaptive morphological (Dawson 2005a, 2005b) and behavioral (Dawson & Hamner 2003, Hamner & Hauri 1981) phenotypes specific to the lake limnology (Hamner & Hamner 1998) despite occupying a single small archipelago. Past work has suggested the behavioral phenotypic variation between lake and lagoon populations is driven by sun compass, light, and basin morphometry (Hamner and Hauri 1981), as well as relaxed selection, morphological trade-offs, and predator-mediated selection (Dawson & Hamner 2003). Novel observations suggest a role of their photosynthetic endosymbiont Symbiodiniaceae (in prep). *Mastigias papua* jellyfish migrate vertically in response to ammonium availability as the uptake benefits the symbiont, maintaining 70% of their population in the upper layer of the water column during the day (Graham et al. 2001). This migration directly benefits the symbiont and is metabolically taxing on the host with a downstream nutritional benefit from nutrient transfer to the host following photosynthesis, rather than an immediate one such as consuming prey.

Sleep

Sleep, or the regulated lack of movement and responsiveness, is relatively novel in the study of Scyphozoa. The most developed sleep study was on *Cassiopea* spp. that showed scheduled periods of nightly quiescence paired with delayed stimulation response (Nath et al. 2017). These are symbiotic medusae and can use their sleep-like state to save energy during low light levels when Symbiodiniaceae are inactive. There are examples of sleep-like states in Cnidaria outside of Scyphozoa, for example *Hydra vulgaris* (Kanaya et al. 2020). *Chironex fleckerii*, have also exhibited 15-hour sleep periods attributed to their high neural processing (Kavanau 2006). The lack of nighttime activity in *Carukia barnesi* could suggest a state of energy-saving quiescence similar to sleep seen in other medusae but has not been as deeply supported by research (Courtney et al. 2015). In *Carybdea marsupialia*, rhopalia exposure to light stimulates swimming activity whereas lack of light causes temporary inhibition of swimming (Satterlie 2014). Sleep as a behavior of medusae is in early development with speculation, not necessarily support, in other medusae species based on decreased activity (Seymour et al. 2004, Gram et al. 2012) further study of Scyphozoa is needed.

Feeding

Publications on Medusozoa feeding behaviors date back to the 1849 American Academy of Science Memoirs including observations and functional descriptions of Staurozoa, then ‘Staurophora’, medusae mouths and feeding on smaller medusae (Agassiz 1850). Since then, feeding behavior has been functionally classified with use of appendages in prey capture such as cnidocytes–stinging cells that immobilize prey–and tentacles that

transfer prey to the mouth (Arai 1997). The life cycle of Scyphozoa includes metamorphosis, altering morphology and therefore physical capabilities. In other words, jellyfish feeding behaviors change with ontogeny (Browne 1995), a characteristic of plankton that emphasizes a difference in terrestrial and marine systems. Scpyhozoa generally feed on zooplankton. Size and species of zooplankton depend on environment availability, and predator life stage and size (Purcell 1997, McCloskey et al. 1994). Some species can also take up dissolved organic matter (Skikne et al. 2009) and form mutualisms with zooxanthellae, family Symbiodiniaceae, based on metabolic exchange (McCloskey et al. 1994, Muscatine et al. 1986). Medusa hosts provide the vector for sun exposure and other nutrient acquisition, and Symbiodiniaceae contribute metabolites (e.g. Verde & McCloskey 1998). When exploring the feeding behavior of medusae, the presence/absence of zooxanthellae must be considered to understand the degree of nutritional requirement fulfillment.

We can reframe the functional approach to feeding behavior with ethological studies of prey types, frequency of consumption, and prey capture techniques (Barton et al. 2013) utilizing gut content analysis, stable isotopes, and field/lab observations. *Stygiomedusa gigantea*, for example, use their oral lobes to trap prey (Benfield & Graham 2010); can we look at this functional use of the oral lobe and ask if there is selective feeding via mechanical or behavioral mechanisms? Is there mediation or a pattern to when they feed? Do they feed the same at all life stages?

Prey capture

Immature Scyphozoa have prey types and capture techniques that differ between species, with life stage, and with environmental availability. Ostman (1997) observed scyphopolyp prey to include abundant plankton such as copepods and cladocerans. *Aurelia coerulea* polyps in Jiaozhou Bay, China increased digestion rates of copepods in response to environmental availability and temperature (Pengpeng et al. 2021). *Aurelia aurita* ephyrae to larval stage use active prey capture to feed on phytoplankton and copepods (Bamstedt et al. 2001). *Aurelia coerulea* ephyrae feed on ciliates in lab settings, with their continued development dependent on prey type (Kamiyama 2018). *Chrysaora quinquechirrha* ephyrae have shown preference in the lab for rotifers, copepod nauplii and ctenophore larvae while avoiding Symbiodinium (Oleson et al. 1996). These results appear selective but may be an artifact of the preys' lack of escape behaviors. Whether this has elicited a co-occuring selective response in *C. quinquechirrha*, or if predation is opportunistic, remains unknown.

Habitat availability of plankton has a direct influence on medusae prey types but is not the only determinant. *Aurelia aurita*, for example, is suggested to use chemical induction in recognizing prey types (Arai 1991). *A. aurita* preys on micro- and mesozooplankton, particularly copepods, in the Inland Sea of Japan (Uye & Shimauchi 2005). *Chrysaora fuscescens*, *Aurelia labiata* and *Phacellophora camtschatica*, in contrast, were observed to feed on euphausiids, gelatinous plankton and cladocerans while avoiding copepods in

the Northern California Current (Suchman et al. 2008). *Cyanea capillata*, *Aurelia labiata* and *Aequorea aequorea* medusae in Alaska feed on copepods, larvaceans and cladocerans (Purcell 2003). *Catostylus mosaicus* in Australia feed on zooplankton as well, but are observed to feed on smaller numbers of large taxa such as *Lucifer sp.* and shrimp (Pitt et al. 2008). *Aurelia sp.* of Mljet Island feed on copepods, nauplii, and ciliates (Turk et al. 2008).

Convergence of morphological traits can be assessed in species that behave similarly. Three medusozoan species with similar foraging patterns were compared. It was found that upstream prey capture occurs in medusae with leading tentacles and downstream capture occurs in medusae with trailing tentacles (Colin et al. 2006). The cubozoan, *Carukia barnesi*, twitches their tentacles more frequently to attract larger prey. Courtney et al. (2015) classify the behavior as sophisticated prey capture in contrast to grazing. This is not to suggest a lack of complexity in grazing behavior, however, as *Aurelia aurita* selectively graze on larger microplankton (Stoecker 1987). *Pelagia* is sometimes classified as an opportunistic feeder rather than selective, contributing to its dominant presence in the Central Mediterranean Sea during winter (Rosa et al. 2013). *P. noctiluca* prey-capture includes using its tentacles to paralyze, then feed the prey to a nearby oral arm, they also release prey occasionally suggesting prey selection (Sandrini & Avian 1989, Bosch-Belmar et al. 2016).

Common modes of medusae feeding are documented in *Cassiopea xamachana* that manipulate water currents with bell contractions and direction change while swimming (Ohdera 2018). The manipulation of water causes nutrients to flow over oral arms where prey are consumed (Ohdera 2018). *Aurelia labiata* has been observed to catch prey on its bell margin and transport it to a gastric pouch (Albert 2014). Similarly, *Lychnorhiza lucerna* use bell contractions to transfer prey to their oral arms, the contraction currents allow them to catch prey, such as fast-swimming copepods, that ride the currents (Nagata et al. 2016). This behavior is likely present in many other species as well since contracting the bell is a common ability across Scyphozoa.

There have been observations of predator-prey interactions between species of medusae including *Cyanea capillata* (predator) and *Aurelia aurita* (prey) (Hansson 1997). *Drymonema larsoni* prey on *Aurelia sp.* in the Gulf of Mexico (Bayha et al. 2012). Scyphomedusa on the West Kamchatka shelf prey on smaller medusae (Gorbatenko et al. 2009). There are many reported observations of Scyphozoa preying on ephyrae and adult gelatinous zooplankton of the same and other species, sometimes contributing over 80% of the predator's diet (Purcell 1991).

We can look to cubozoans for additional unique feeding behaviors that have yet to be explored in scyphozoans. Habitat awareness during feeding is suggested when observing the cubozoan *Chironex fleckeri* in common aquaria. They will often not feed naturally, requiring prey be placed on the manubrium (Hamner & Hamner 1995). In

contrast, when housed in planktonkreisels, circular aquatic tanks without corners, *C. fleckeri* extend tentacles to capture and ingest prey (Hamner & Hamner 1995). *C. fleckeri* also have a unique feeding behavior when in blue light: they swim in figure-eight patterns, with slow pulsation rate and spread out tentacles (Gershwin & Dawes 2008). *C. barnesi* is observed to extend and twitch tentacles and cast nematocyst clusters for prey capture only when in light conditions (Courtney et al. 2015). Feeding can also be temporally dependent. Using field observations, Stewart (1995) found *Tripedalia cystophoa* do not feed during active breeding. When not breeding they feed actively on copepods at light shafts in the mangrove (Stewart 1995). These feeding behaviors tie into the other behavioral categories of obstacle avoidance, movement, and reproduction. Habitat awareness and feeding in relation to reproductive cycles could all be further studied in Scyphozoa.

Reproduction

Fertilization can be categorized as active copulation or passive spermcasting. Mature adult *Periphylla periphylla* in Lurefjord were observed to enhance their reproduction by migrating to the surface at night forming aggregations, increasing abundance, and decreasing distance between individuals (Bamstedt et al. 2020). This also increased the opportunity for entangling tentacles, suggesting courtship (Tiemann 2008). Aggregations (see below) are suggested to improve reproductive success in medusae by reducing sperm dilution with passive spermcasting (Larson 1992). This can lead to inbreeding among siblings that swarm together as suggested by genomic data from *Pelagia noctiluca* (Aglieri et al. 2014).

For more examples of reproductive behavior potentially still unobserved in Scyphozoa we can look to other classes. Cubomedusae can fertilize internally or externally both actively and passively (Garcia-Rodriguez et al. 2020). A more specific courtship behavior is observed in *Copula sivickisi*: females accept spermatophores from multiple males and then insert the gamete into the manubrium ultimately producing a single embryo (Lewis & Long 2005). This gamete transfer includes cnidocytes that serve to protect the embryo (Garm et al. 2015).

Mass Occurrence

Interest in mass occurrences of jellyfish has increased in recent years due to the increased frequency of blooms as anthropogenically forced climate and ecological dynamics change. These blooms are often harmful to near-term human endeavors due to the impact on fishing, drainage systems, and sting threats (Dybas 2002). Following Graham et al. (2001), Hamner and Dawson (2009) clarified and refined the categories of medusozoan mass occurrence as aggregations (accumulation of interacting individuals), swarms (aggregations that result from behavioral accumulation), apparent blooms (aggregations resulting from chemical or physical phenomena), and true blooms (a

seasonal increase in population size due to reproductive influx). Taxonomically, aggregations are suggested to have an early evolutionary presence as all medusozoan classes have species that can aggregate, but true blooms and swarms are specific to Cubozoa, Scyphozoa-Discomedusae (Hamner & Dawson 2009), and some larger Hydrozoa (Gul & Gravili 2013). A set of characteristics that encourage medusozoan occurrence en masse include strobilation ability, podocyst formation, oral arm presence, large body size and shallow water habitats (Hamner & Dawson 2009). Behaviors that lead to mass occurrence can be considered in terms of swarms, aggregations and apparent blooms but not true blooms as they are abiotic rather than biotically driven. Nonetheless, as the below examples illustrate, *en masse* behavior is the result of individually determined group organization.

Swarms

Swarms are behaviorally driven aggregations and can lead to negative outcomes for food webs and anthropogenic activities like all mass occurrence. *Periphylla periphylla*, are aposymbiotic and normally solitary deep sea medusae, but have been observed to actively seek out conspecifics forming small swarms for prey capture (Kaartvedt et al. 2015). There have been recent reports of the first accounts of swarms near India for *Acromitus flagellatus* and *Cyanea nozakii*. *A. flagellatus* swarms are attributed to opportunistic feeding behavior in Sundarbans (Siddique et al. 2022a). *C. nozakii* in the Indian Ocean (Siddique et al. 2023) have temporally increased swarming across the last nine years (Siddique et al. 2022b). The concerns of eutrophication, hypoxia, acidification, decreased tourism, and decreased fishing resulting from these swarms in India can be projected on other oceans as changing climates are promoting swarming conditions (Siddique et al. 2022). The Mediterranean Sea hosts swarms of *Rhopilema nomadica* (Siokou-Frangou 2006) and *Pelagia noctiluca* (Goy et al. 1989). *P. noctiluca* is modeled to swarm every twelve years depending on environmental conditions (Goy et al. 1989). It would be notable to assess whether this pattern has been maintained in the changing climate. The *P. noctiluca* swarms were also found to promote inbreeding amongst siblings that tended to swarm together, as noted above in Reproduction (Agileri et al. 2014).

Aggregations

Aggregations are accumulations of individuals, resulting from environmental changes, that interact promoting various outcomes such as reproduction or prey capture. Swimming behavior in aggregations can vary. As density of individuals is high, movement becomes convoluted and might be thought of as medusae passively bumping into one another maintaining the aggregation due to inability to escape. *Linuche unguiculata* patches in the Caribbean Sea and Indo-Pacific Ocean are maintained by passive swimming in Langmuir circulations that upwell nutrients, but the medusae use active swimming in horizontal, circular patterns to maintain their presence in Langmuir cell orientations when the wind is too slow to do so (Larson 1992). Langmuir cells promote aggregations of medusae providing access to planktonic prey but also make

medusae available as a prey source to avian predators (Hamner & Schneider 1986). Hydromedusae and scyphomedusae have been observed aggregating in Langmuir circulation cells in the Bering Sea (Hamner & Schneider 1986) and Caribbean Sea (Larson 1992). Aggregated swimming behavior can also include migrations. *Aurelia labiata* in Prince William Sound, Alaska is observed to swim in the same direction vertically in dense aggregations due to fluid convergences or collision avoidance (Purcell et al. 2000).

Apparent Blooms

Abiotic factors can promote apparent blooms, as opposed to true blooms resulting from seasonal reproductive cycling. Temperature and salinity gradients at haloclines can encourage accumulation of individuals into apparent blooms. Small hydromedusae respond behaviorally by slowing swimming speed or turning around to avoid the physiological stress of diffusion required to meet varying seawater conditions (Graham et al. 2001). The same response in larger scyphomedusae is more likely due to an active behavioral adaptation, rather than physiologically motivated, as their tissues adjust more rapidly to haloclines (Graham et al. 2001). *Nemopilema nomurai*, for example, was found to swim toward infrared wavelengths, or warmer waters (Ohtsu & Uye 2011). Increased water temperature, eutrophication, and coastal modification are additional abiotic contributors to blooms, such as the recent increase of *Nemopilema nomuraii* in the East Asian Marginal (Uye 2008).

M. papua of Jellyfish Lake, Palau are an apparent bloom of millions of symbiotic medusae sequestered in a landlocked marine lake. These medusae are estimated to contribute 16% of the total primary productivity in the lake and take up a large portion of biovolume (McCloskey et al. 1994). *M. papua* is an interesting case in the sense that it is symbiotic, which is thought to be inconsistent with blooms. Blooms are linked to an increase in planktonic food supply, therefore, aposymbiotic Medusozoa are thought to be more capable of blooming than holobionts that are limited to the number of present zooxanthellae (Hamner & Dawson 2009). This suggests dense accumulations of zooxanthellae in Jellyfish Lake, a characteristic lacking for habitats of other holobiont species that do not bloom, and other *M. papua* in Palauan lakes with smaller populations of medusae.

3.6 Conclusions and Future Directions

3.6.1 Concluding Remarks

Considering behaviors from the individual to population level (Fig. 3.1) and the interconnections between categories led to deliberation in sorting studies. Migration, for example, could be considered as movement or mass occurrence. Ultimately, I determined it to be a focus of the direct action of movement that led to the resulting migratory behavior rather than a focus of the population level outcome. Movement had

the largest number of studies in the returned search, supporting movement at the basis of all other behaviors, highlighting the diversity of behaviors available to motile organisms. This should not discount behavior in more sessile forms, such as the polyp, or the lack of movement, i.e. sleep. The presence of sleep in early diverging metazoans, which lack centralized nervous systems, is a prime example of the applicability of invertebrates to behaviors in further diverged taxa.

Unfortunately, there remain areas I cannot yet address. Once we are able to identify phylogenetic trends of behavior in Scyphozoa (Fig. 3.2), and other Medusozoa, we can use these taxa to their fullest potential for informing patterns of these behaviors in other species, conservational efforts, and predicted responses of medusae to changing environments. This ability would be of particular importance for predicting and responding to blooms (Hamner & Dawson 2009). Attempting to make interspecific conclusions of behavioral patterns has made apparent the lack of comprehensive ethological studies in Scyphozoa (Fig. 3.2). Without contemporary technology many pelagic species were difficult to observe and measure. When observed, the studies lack replication leaving us to question how typical these behaviors are naturally. This trend has created many gaps in the literature of Scyphozoa behavior related to behavioral genetics, ontogeny, symbiosis, and the ability to confidently complete an ethological assessment of phylogenetic patterns of behavior for accurate predictions. Among many questions, we are left to wonder about the behavior of the ephyrae, for example, nor can we accurately determine the extent of polyp behavior as most studies focus on medusae.

3.6.2 Holoethology?

In defining scyphozoan behavior, the holobiont state of the medusae should be considered when applicable. With observations of contrasting behavior between aposymbiotic and holobiotic organisms (e.g. Ch. 4) the Symbiodiniaceae should be considered when studying any aspect of the holobiont (Djeghri et al. 2019). Unfortunately, the role of the dinoflagellate has not often been considered but is mentioned above when available. The role of symbionts in host behavior has more often been studied in parasitic interactions such as trematode (*Leucochloridium* spp.) manipulation of snail (*Succinea putris*) behavior, causing it to be less cautious and more easily eaten by birds to finalize the parasitic life cycle (Wesolowska & Wesolowski 2014). The same interaction is seen with the modification of behavior in parasitized killifish, making them more easily preyed on by the final host bird (Lafferty & Morris 1996). These show the manipulation of host behavior for the symbiont's benefit but are specific to horizontally transmitted parasites that are not tied to host reproduction. Parasitic interactions are often associated with horizontal transmission (Lesser et al. 2013); the mutualistic symbiosis between Scyphozoa and Symbiodiniaceae includes horizontal transmission during ingestion from the environment by polyps, ephyrae and medusae (Sachs & Wilcox 2006) presenting a caveat to the parasitic transmission pattern. Experimental manipulation of transmission types in *Cassiopea xamachana* reveals lower

fitness values with horizontal transmission in comparison to vertical transmission (Sachs & Wilcox 2006).

3.6.3 Recommendations

It will be interesting to update the search in 2024 to assess optimism for the field with the number of scyphozoan behavioral studies published since 2021, and note any improvements to methodologies. One exemplar ethological study of twenty-six hydrozoan species' methodically reports in-lab behavioral patterns of feeding and movement in polyps and medusae using microscopy and recordings (Miglietta et al. 2000). When designing ethological studies of Medusozoa we should consider Tinbergen (1963) and Beach's (1960) questions: "What is its purpose?", "How did the behavior get to be what it is? What are the external causes of the behavior? What internal mechanisms mediate it?" (e.g. Fig. 3.3). If we hope to achieve medusae as model systems for behavior due to their early divergence in Metazoa, we must answer the above questions. Beyond the scope of this paper, the role of behaviors can extend to the impact on communities and ecosystems (Shaw 2020), an important consideration for Scyphozoa moving forward with changing climates increasing potential for blooms in some species and decimating populations of other species.

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3.8 Figures

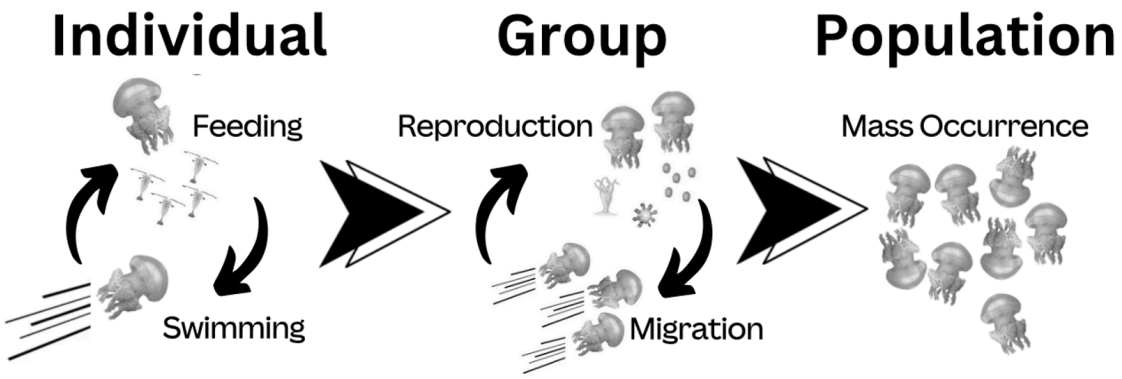

Figure 3.1: Proposed interconnections of behavior categories from individual to population level. Feeding behaviors including opportunistic foraging and active prey capture, often involve movement such as swimming and obstacle avoidance, and vise versa, these are considered at the individual level as they are performed by singular organisms. The result of these behaviors can then contribute to reproduction, including settlement and fertilization, and migration at the group level which require multiple individuals displaying the pattern to be recognized. Migration can in turn be driven by reproductive seasons. For example, mass occurrence of medusae can result from population aggregation, swarming or blooming as a result of reproduction. These relationships can also occur from individual to population level and vice versa. For example, mass occurrence is influenced by and can influence reactions at the individual level of feeding and swimming.

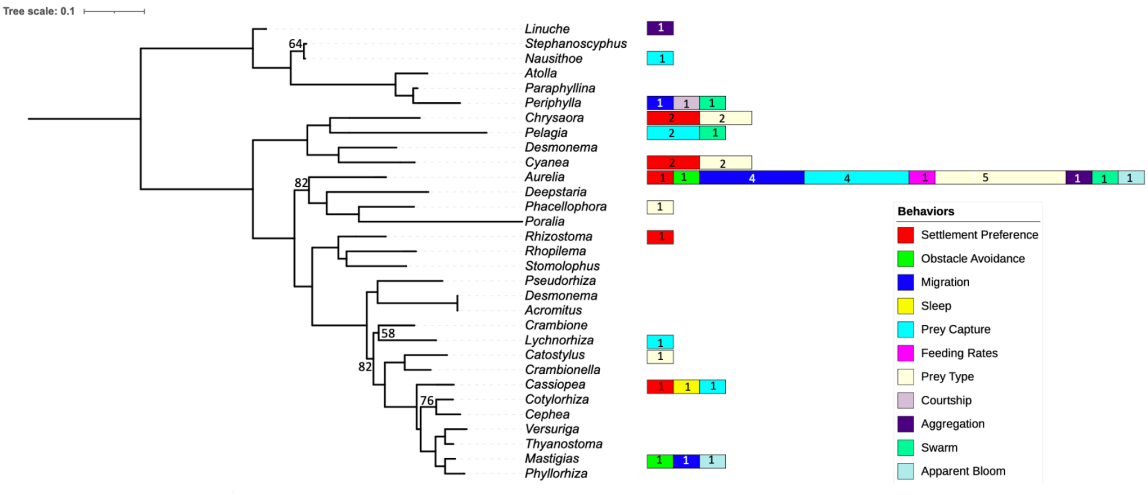

Figure 3.2: UCE phylogeny of Scyphozoa species with multibar chart of behavioral studies available in the literature. The tree is adapted from Ch. 3, trimmed to genus level, with bootstrap values <90 shown. The bar size represents counts of studies from each behavioral category ranging from 1-min to 5-max studies.

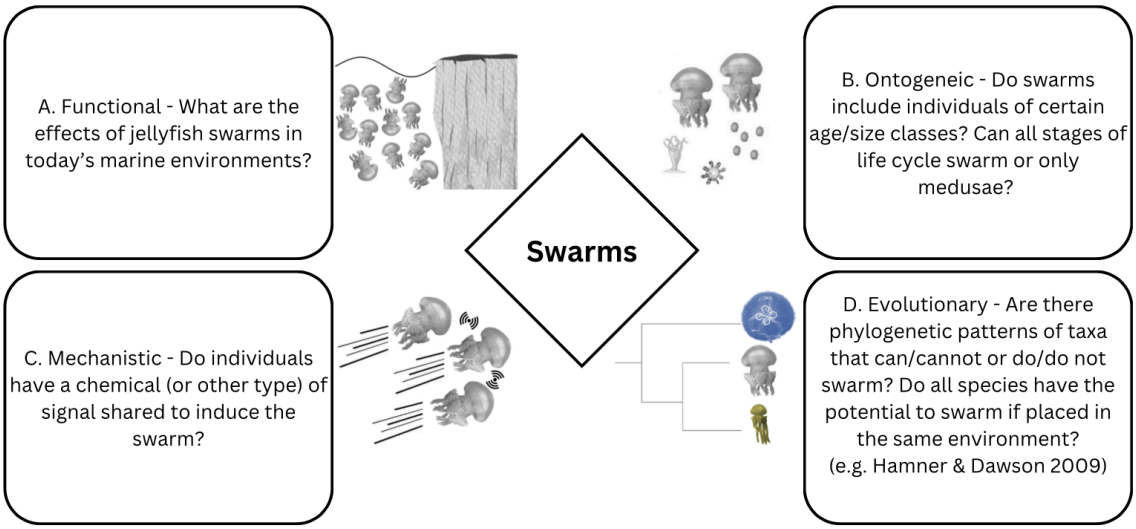

Figure 3.3: Tinbergen's Ethological Framework as applied to the example of swarms in Scyphozoa. Tinbergen's (1963) four questions are asked of swarming behavior: A) Function - What is it for?, B) Ontogeny - How did it develop?, C) Mechanistic - How does it work?, and D) Evolutionary - How did it evolve? Where available, examples from the literature are provided that ask and answer related questions.

Ch. 4 Symbiont presence alters host jellyfish migratory behavior

4.1 Abstract

The role of endosymbionts in host behavior manipulation remains unknown in many systems, particularly for mutualistic relationships. Marine lakes provide natural laboratories for observing behaviors under relatively controlled conditions for applications to pelagic oceanic environments. I observed migratory behavior of the mutualistic cnidarian *Mastigias papua*-Symbiodiniaceae during a rare occurrence of syntopic subgroups of photosymbiotic and aposymbiotic individuals. I obtained whole genome sequences from individuals of both subgroups and past populations to ensure genetic homogeneity. I mapped migration along transects, instantaneous swimming direction, and population distributions for *M. papua*. I found a significant migratory pattern for symbiotic jellies swimming into the eastern lake basin in the morning and western lake basin in the afternoon tracking sun orientation. Aposymbiotic jellies, in contrast, had random swimming orientations and no significant migratory pattern. This study reveals the role of symbionts in mediating host migratory behavior, further suggesting a symbiont's capabilities to manipulate pelagic host behavior.

4.2 Introduction

In marine systems, corals have long been the model symbiotic cnidarian, but this model presents taxonomic gaps, e.g. jellies. Coral reefs are keystone holobionts that display the impacts of changing climates on symbiotic relationships between hosts and Symbiodiniaceae, also known as zooxanthellae. Additionally, behavioral cues of holobionts are often emphasized in parasitic relationships but ignored in commensal and mutualistic holobionts (Hosokawa & Fukatsu 2020). Partial exceptions include work done in mutualistic anemones that support photo-movement (Foo et al. 2020), and endosymbiont manipulation of host circadian clocks (Sorek et al. 2018). Again, these are primarily sessile holobiont behaviors. Pelagic holobionts, particularly those that are photosymbiotic, offer a solution as they exhibit complex behaviors in response to environmental cues (e.g. Dawson 2005). Pelagic holobiont associations can exist as mutualistic or parasitic (Not et al. 2016), and along a continuum (Lesser et al. 2013). The emerging model of *Cassiopea* fills the gap to an extent but is limited for applications of the roles of behavior due to its primarily sessile benthic state. Despite the vastly informative work completed with pelagic symbioses, gaps remain in understanding the breadth of endosymbiont's influence on host phenotypes.

Mastigias papua-Cladocopium is a mutualistic cnidarian with well-documented behavioral patterns and variation among populations (Dawson & Hamner 2003). *M. papua* inhabits isolated marine lakes, lagoons, and pelagic environments providing a variety of populations to compare behavioral interactions. Acquisition of symbionts can be horizontal, from the environment, or vertical, through generations, altering the nature of the relationship and cospeciation potential (Sachs & Wilcox 2006). The photosynthetic zooxanthellae, *Cladocopium* sp. (LaJeunesse et al 2018), is primarily incorporated into *M. papua's* symbiome during the polyp life stage in which they are required for strobilation, i.e. asexual reproduction or the budding off of ephyrae from the polyp (Sugiura 1964), though exceptions have been documented (Rahat & Adar 1980, Dawson et al. 2001). Zooxanthellae can also be incorporated into the *M. papua* symbiome with consumption at the ephyrae and medusae stages (Sigura 1964). The zooxanthellae are found in all medusae host tissue types, except gonadal tissue, with common quantification of 30% in the bell and the other 70% distributed in oral arms, terminal clubs, etc. (Muscatine & Marian 1982). The zooxanthellae can contribute 100% of host carbon requirements (McCloskey et al. 1994), but medusae often continue to consume plankton. Consumption of plankton can be the differentiating factor in maintaining survival when zooxanthellae bleach during climatic perturbations. *M. papua* inhabits a variety of marine environments, presenting the opportunity to study symbioses in response to environmental perturbations and broader application possibilities such as migratory response to changing climate.

M. papua behavioral differences have been primarily observed in timing and directionality of horizontal migrations (Hamner & Hauri 1981) and timing of depth distribution in vertical migrations (Dawson & Hamner 2003). There are three hypotheses for these differences: relaxed selection, shadow (i.e. predator) avoidance, and sustaining zooxanthellae (Hamner & Hauri 1981, Dawson & Hamner 2003). Marine lake medusa swimming speed is significantly slower than in lagoon populations, possibly resulting from relaxed selection (Dawson & Hamner 2003). Slower swimming speed may have contributed to development of shadow avoidance behavior for adaptive avoidance of predatory anemones in the shadowed lake edges. Additionally, the horizontal migratory patterns lead to shadow avoidance, maintaining the populations in sunlight throughout the day (Hamner & Hauri 1981). Horizontal migrations have also been attributed to zooxanthellae maintenance along with nightly vertical migrations to nitrogen-rich depths fertilizing zooxanthellae for daily photosynthesis (Dawson & Hamner 2003, Muscatine & Marian 1982). The literature is lacking in further investigation of the role and mediation of zooxanthellae in invertebrate host behavior. Bleached cnidarian behavior, in particular, has yet to be studied. Herein I present an exploratory, comparative study of cooccurring symbiotic (golden) and aposymbiotic (white) individuals (Fig. 4.1). Answering the question, do golden and white populations show the same horizontal migration pattern?, will reveal whether symbionts play a role in determining host behavior.

4.3 Methods

4.3.1 Location and Climatic Context

Ongeim'l Tketau (OTM), also commonly known as "Jellyfish Lake", is a meromictic marine lake in Palau, on Mercherchar island (Fig. 4.2). The western section of the lake maximizes depth at 30m. The lake is approximately 420m from west to east and 100-150m from north to south. The lake is surrounded by vegetative karst with an outer edge that is primarily mangrove and shelf habitat (Patris et al. 2019). The biodiversity consists of fishes, algae, anemone, mussels and sponges (Hanzawa et al. 2012, Patris et al. 2019). OTM usually houses a mass occurrence of over one million *M. papua* medusae in less than 60,000m^2 surface area. The 1998 El Niño Southern Oscillation (ENSO) increased marine lake temperatures upwards of 3°C associated with mass mortality of the population (Dawson et al. 2001). Evidence of white, likely bleached, medusae in Clear Lake were documented (Dawson et al. 2001). Following ENSO in 2016, *M. papua etpisoni* of OTM were again 100% decimated and remained absent until 2017, the population recovered to near average historical size in 2019 (Patris et al. in prep).

4.3.2 Field observations

During fieldwork on 24 and 27 June 2018, we observed the population of *Mastigias* in OTM had recovered slightly. Additionally, a novel occurrence of two types of medusae were present: those appearing white, presumably either lacking or greatly reduced zooxanthellae, and those appearing golden, i.e. holobionts with zooxanthellae. During data collection on the 24th, the weather was clear and sunny all day. On the 27th, the weather was clear and sunny until mid-afternoon when clouds would shade portions of the lake for roughly 5-10 minutes at a time.

Horizontal distributions

Transects

We swam three parallel east-west transects spaced approximately 15m apart. Following Dawson & Hamner (2003), each transect was divided into five sections: west [W], center-west ['C], central [C], center-east [C'], east [E] (Fig. 4.2D). On Day 1, 24 June 2018, we swam west to east in the morning (0930-1050 local time) and East to West in the early afternoon (1200-1300). On the second day, 27 June 2018, the same transects were swum during three intervals (0646-0803, 1016-1118, 1521-1610) to survey the full daily migration. Each swimmer noted the time entering the section (W, 'C, C, C', E) of the lake, then every medusa within the swimmer's transect line was identified by color (golden/white), and bell diameter was estimated into a small, medium, large or very large size category (size categories: small (S) > 70mm, medium (M) 70-140, large (L) 140-180, very large (V) >180mm). It should be noted, in the largest congregation of migrating medusae, there was likely an underestimation of the high density of individuals.

Instantaneous swimming direction
Instantaneous swimming direction (ISD) was measured by a snorkeler aligning a compass with the oral-aboral axis of medusae swimming past a central location in the lake, and taking the bearing, following Dawson & Hamner (2003). For each medusa ISD, time of measurement, medusa color (golden or white), and estimated bell size were collected.

4.3.3 Data analysis

Horizontal distributions

Transects
Data from each swimmer were concatenated by lake sector, collection time, medusa color, and bell diameter. Bell diameter data were binned by 10mm increments and analyzed using R's v3.5.1 (R Core Team 2018) chisq.test with null hypothesis (H_0) calculated as H_0 = n (sample size) / k (number of bins = 20). Calculations were done separately for golden and white medusae in R v3.5.1 (R Core Team 2018).

Instantaneous Swimming Direction
Bearings were binned into 30-degree sectors and analyzed using R's chisq.test with null hypothesis (H_0) pertaining to all medusae oriented equally in all directions and calculated as H_0= n (sample size) / k (number of bins = 12). Calculations were done separately for golden and white medusae in R v3.5.1 (R Core Team 2018).

Genetic variation

To test whether behavioral differentiation could be due to population differentiation, tissues from twelve white and twelve golden medusae from OTM 2018 collections, and seven golden samples from OTM 2013 collections were CTAB extracted (Schiebelhut et al. 2016). The extractions were whole genome sequenced on an Illumina NovaSeq at the DNA Technologies & Expression Analysis Core, University of California, Davis. BBDuk (Bushnell 2023) was used to remove adapters and bwa-mem (Li & Durbin 2009) was used to align sequences to our *Mastigias papua* reference genome (Ch. 1). Picard (http://broadinstitute.github.io/picard) was used to create a sorted bam file, gatk MarkDuplicates was used to mark and remove duplicates then gatk HaplotypeCaller was used to identify haplotypes, SelectVariants and VariantFiltration were used to pull and filter SNPs (McKenna et al. 2010). The SNPs were used to create a phylogeny in raxml-ng v.1.1.0 (Stamatakis 2014) on CIPRES (Miller et al. 2010), and Weir Cockerham F_{st} estimates with PCA in Rstudio v.4.2.2. (R Core Team 2018).

4.4 Results

4.4.1 Horizontal distributions

Transects
Transect surveys showed golden population densities had an overall gradual increase in density from 3% west to 37% east in the morning (Fig. 4.3A golden circles) and from east to west in the late afternoon (Fig. 4.3C golden circles). In contrast, the white densities showed random variation in population density distribution across the lake sectors (Fig. 4.3A, 4.3C white circles).

Morning (Fig. 4.3A) and late afternoon (Fig. 4.3C) transects showed the size distributions from small to very large. During the morning migration, sizes of golden medusae were evenly distributed across lake sectors compared to primarily small-sized medusae observed in the west and larger medusae remained eastward in the afternoon. White populations primarily consisted of large medusae with similar distribution across the lake in the morning and afternoon. White medusae were oriented randomly.

Instantaneous Swimming Direction
Golden medusae showed a significantly non-random bearing eastward in the morning (Fig. 4.3B, p-value=4.61e-15) and west in the afternoon (Fig. 4.3 D, p-value=2.47e-13). White medusae were found to have an insignificant, random bearing in the morning (Fig. 4.3B, p-value=0.37) and in the afternoon (Fig. 4.3D, p-value=0.95). White medusae populations consisted largely of medium sized bell diameters, while golden medusae were spread across size classes (Fig. 4.4).

4.4.2 Genetic variation
Whole genome sequencing of white and golden medusae sampled in 2018, and golden medusae sampled in 2013, have no population variation temporally. There was some variation between white and golden individuals of 2018, though most clustered in the PCA (Fig. 4.5). Low F_{st} estimates (<0.05) support overall minimal population differentiation (Table 4.1).

4.5 Discussion
Benthic organisms, particularly corals, dominate marine symbiosis literature, limiting opportunities to informatively explore interactions of pelagic organisms. Organisms in pelagic habitats require various adaptations to combat exposure to diverse stimuli during vertical and horizontal movement. This complexity leads to many unanswered questions on the biology, ecology, and (co)evolution of pelagic symbiosis. The rare occurrence of white medusae in the marine lake Ongeim'l Tketau, Palau, provided an unparalleled natural opportunity to examine the behavioral impacts of symbioses in a pelagic cnidarian, the medusoid phase of *Mastigias papua*. Our analyses provide two primary insights. **1. *Symbiodiniaceae play a role in host behavior.*** Azooxanthellate and zooxanthellate medusae living syntopically differ behaviorally but are genetically indistinguishable. It can thus be concluded that the individuals are from the same population, and their alternate phenotypes are the result of presence/absence of

zooxanthellae. **2. M. papua *have modified, rather than complete, dependence on Symbiodiniaceae***. The white *Mastigias* subgroup included individuals at all visible life stages (size classes) suggesting metabolic/physiological self-sufficiency to maintain growth despite previous assumptions about *M. papua* reliance on zooxanthellae. There was variability in the degree of zooxanthellae presence in white individuals as signified by golden patches and different degrees of overall transparency.

4.5.1 Role of Endosymbiont in Host Behavior

For over 40 years, golden medusae in OTM have been observed performing diel horizontal migration (DHM) in response to the intensity and direction of sunlight (Hamner & Hauri 1981, Dawson & Hamner 2003, Cimino et al. 2018). Weak migration has been noted during periods of overcast weather (Dawson & Hamner 2005). Our new observations during sunny conditions of weak DHM in white medusae support breakdown of the cue for migration. Golden medusae showed a significantly non-random bearing eastward in the morning and west in the afternoon, supporting horizontal migration consistent with Dawson & Hamner (2003) and Cimino et al. (2018). White medusae had random swimming directions. These observations provide the necessary preliminary data to indicate a role of zooxanthellae in determining host behavior, altering at a minimum where the medusae are going. By including genetic data and multiple size classes we can confidently attribute the results to presence/absence of zooxanthellae. Our observational work presents an exciting area for further study in *M. papua* populations that are rapidly evolving in isolated landlocked marine lakes of Palau and ocean populations (Dawson & Hamner 2005). The symbionts' role will become equally intriguing as we contemplate the potential for obligate symbiosis, cospeciation (Shropshire & Bordenstein 2016), and behavioral evolution (Foster 1999).

The interpretation of holobiont behavior must consider host adaptation and directly mediated symbiont manipulation as both may occur simultaneously (Hosokawa & Fukatsu 2020). *C. xamachana* presents the possibility to explore both in their behavior of settling upside down in the benthos. This settling behavior provides minimized energy expenditure while constantly exposing their zooxanthellate oral arm tissues for photosynthesis. This settling behavior is a seemingly opposite technique for zooxanthellae photosymbiosis exposure, compared to the motile manipulation in *M. papua*. As a possible morphological adaptation in response, *C. xamachana* oral arm tissues also contain blue pigment thought to act as protection from solar damage (Blanquet & Phelan 1987). Pigments have also differed between lagoon, oceanic and marine lake populations of *M. papua* (personal observations). Additional, behavioral adaptation includes *C. xamachana* nighttime circadian sleep-like states (Nath et al. 2017). The role of zooxanthellae in mediating this behavior is unclear but possible considering the decreased availability of energy to support daytime pulsing activities due to lack of photosynthesis at night. It is undoubted that *M. papu*a benefit metabolically from the presence of the zooxanthellae under 'usual' circumstances (e.g. Verde & McCloskey 1998), but when the energy is being expended to acquire more resources for

the zooxanthellae, is the medusae really benefiting? When zooxanthellae are not present, *M. papua etpisoni* do not follow a significant migratory pattern and are suspected to rely on planktonic prey. What are the signals that cue the medusae nerve net in on presence/absence of zooxanthellae, consequently altering behavior from migration to prey? Is it the presence/absence of the endosymbiont or nutrition that alters their behavior? Similar questions have recently been asked of commensal and mutualistic insect symbioses with microbes, and whether it is symbiont or host benefit that drives host behavior but are more commonly addressed in parasitic interactions (Hosokawa & Fukatsu 2020).

Consideration for the role of symbionts in holobiont behavior has primarily been studied in parasitic interactions that depend on host manipulation for continuation of the parasite life cycle. This dependence is often based on parasitic transmission occurring vertically through host life stages, rather than horizontally through the environment (Sachs & Wilcox 2006). Horizontal transmission in mutualistic Scyphozoa present a caveat to this idea. When compared in the lab, alteration of transmission type in *Cassiopea xamachana* revealed lower host fitness estimates for zooxanthellae transmitted horizontally v. vertically (Sachs & Wilcox 2006). When speculating the possibility of behavioral ‘mind control’ in horizontally infected *M. papua*, does it become an intermediate between parasitic-mutualistic associations? Fitness comparisons are needed to assess whether aposymbiotic medusae are better off. Hosokawa & Fukatsu (2020), consider the role of host insects and microbial symbionts in influencing behaviors sparking transmission. In *M. papua etpisoni*, the possibility of transmission manipulation is unclear considering the primarily horizontal transmission and possible requirement of a symbiont during strobilation. Such becomes once more convoluted when considering the observation of multiple life stage azooxanthellate medusae.

I attempted a differential gene expression (DGE) study to answer the questions regarding the mechanism behind host migration. The design would have included comparing gene expression between the white and golden subgroups. RNA sequencing produced low quality reads for tissues collected in the field and preserved in RNALater so I did not proceed. This study would, therefore, require alternate preservation methods such as flash freezing with liquid nitrogen or sequencing with portable devices in the field. These were not possible due to lack of resources and the remote location of the marine lakes. Lab based experiments would be unrealistic as the behavior may be tied to the environmental conditions and location of the sun. Transinfection experiments could be performed in the lab to test if behavioral manipulation varies based on lake-specific zooxanthellae ecotypes since behavioral variation is documented between lake populations (Dawson & Hamner 2003). For example, infecting an OTM *M. papua* with zooxanthellae from another lake and observing whether it practices the OTM or new lake migratory pattern.

4.5.2 Aposymbiotic strobilation in M. papua

It was suggested that the Clear Lake, Palau (CLM) medusae from 1998 were strobilated aposymbiotically, budded ephyrae without the zooxanthellae present, because they did not contain any discernible remnant of any Symbiodiniaceae, which if present would suggest post-strobilation bleaching (Dawson et al. 2001). This was again the case for white jellies in OTM. Despite having seen white *M. papua*, we do not know how long these individuals survived without the zooxanthellae. The possibility of aposymbiotic strobilation challenges prior research results that have suggested zooxanthellae are required for strobilation (Sugiura 1964). More work is needed to fully determine the point and viability of symbiont loss in *M. papua*.

4.5.3 Marine lakes and beyond

Encompassing greater than 70% of the Earth's surface, with only 11% of extant species described, marine systems require interdisciplinary approaches to study as it is (Luyapert et al. 2019). Moving forward, studying biology in marine systems will require consideration of organisms as holobionts, the host and symbiont combined rather than individually, as we now know the prevalence of symbiosis traverses scales from microbiomes to coral reefs (Gonzalez-Pech et al. 2023, Dittami et al. 2021). Climate change adds a whole suite of complexities to accompany already difficult questions in ethology, ecology, and evolution. Despite this, scientists have made headway on identifying some consistencies, such as horizontal gene transfer and metabolic exchange, among marine holobionts (Gonzalez-Pech et al. 2023).

Coevolution of Medusozoa and zooxanthellae is still not documented, but behavioral (represented here with horizontal migration) and morphological (represented here with size differentiation) adaptations in presence of the symbiont are supported. Advancements in genomic technologies have contributed to the possibility of exploring coevolution in symbiotic interactions. Ip et al. (2021) found evidence for coevolution of a deep-sea clam and its resultant endosymbiont's reduced genome primarily maintaining host specific metabolic pathways. This could be possible in *M. papua* as the main co-functional link with *Cladocopium* is metabolic. Additionally, there was evidence of host horizontal gene transfer from symbiotic bacteria in the deep-sea clam (Ip et al. 2021). In contrast, coevolution between sea anemones and their endosymbionts, *Symbiodinium*, was generally unsupported but possible at species/genera levels and linked to biogeography (Grajales et al. 2015). With the rapid evolution occurring in the *M. papua* of Palau's landlocked marine lakes, phylogeographic coevolution is a possible avenue for future work. Environment has been found as an important indicator of endosymbiont species in a sponge host as well (van der Windt 2020).

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4.8 Tables

Table 4.1: Comparing F_{st} estimates of OTM *Mastigias papua* 2018 subgroups and 2013 population using single nucleotide polymorphisms.

Population	Weir Cockerham Mean	Weir Cockerham Weighted
2018 White & Golden	0.031	0.051
2018 White, 2018 Golden & 2013 Golden	0.02	0.03
2018 Golden & 2013 Golden	-0.001	0.013

4.9 Figures

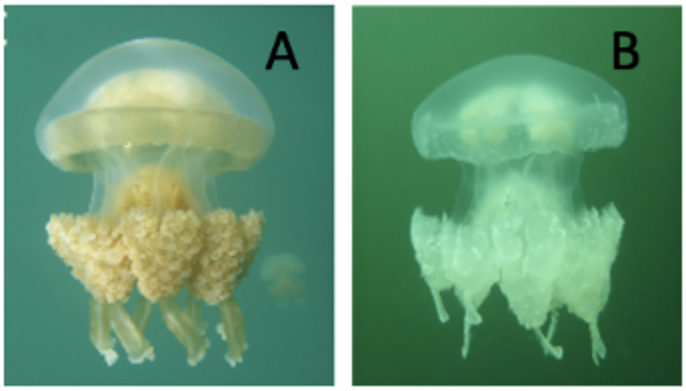

Figure 4.1: Phenotypic variation in novel subgroups of *Mastigias papua etpisoni* from Ongeim'l Tketau, Palau 2018. A. The commonly observed, zooxanthellate, 'golden' *M. papua*. B. White, putatively azooxanthellate or dezooxanthellate, *M. papua*.

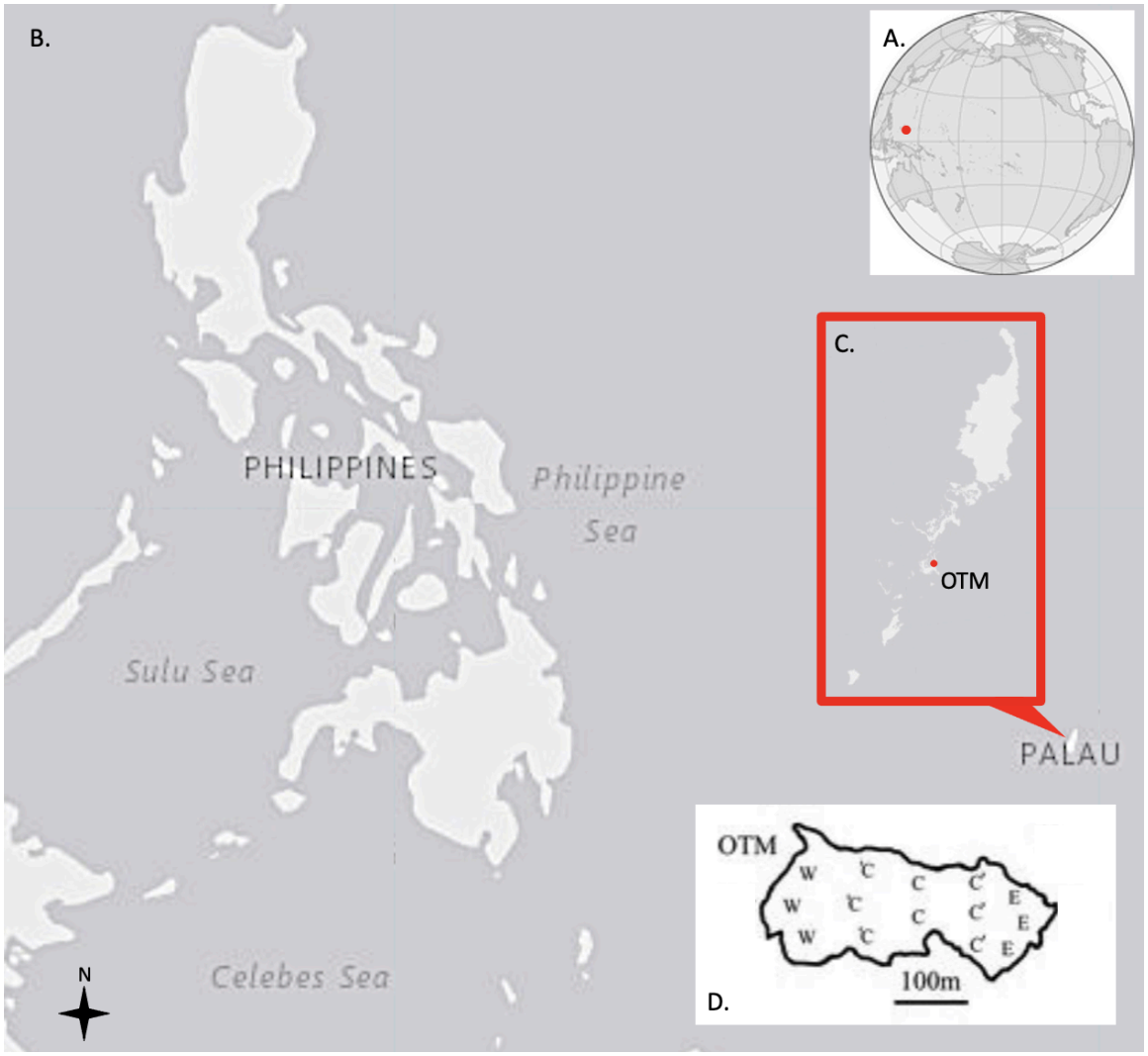

Figure 4.2: Location of Ongeim'l Tketau (OTM), Palau. A) Orientation of Palau (red dot) within the North Pacific Ocean. **B)** Orientation of Palau with respect to the Philippines. Compass orientation is signified in the bottom left. **C)** Map of Palau with OTM pinpointed (red dot). **D)** Schematic of OTM lake shape and sectors used in behavioral observation transect lines from west to east (west [W], center-west ['C], central [C], center-east [C'], east [E]). (Adapted from Dawson & Hamner 2003)

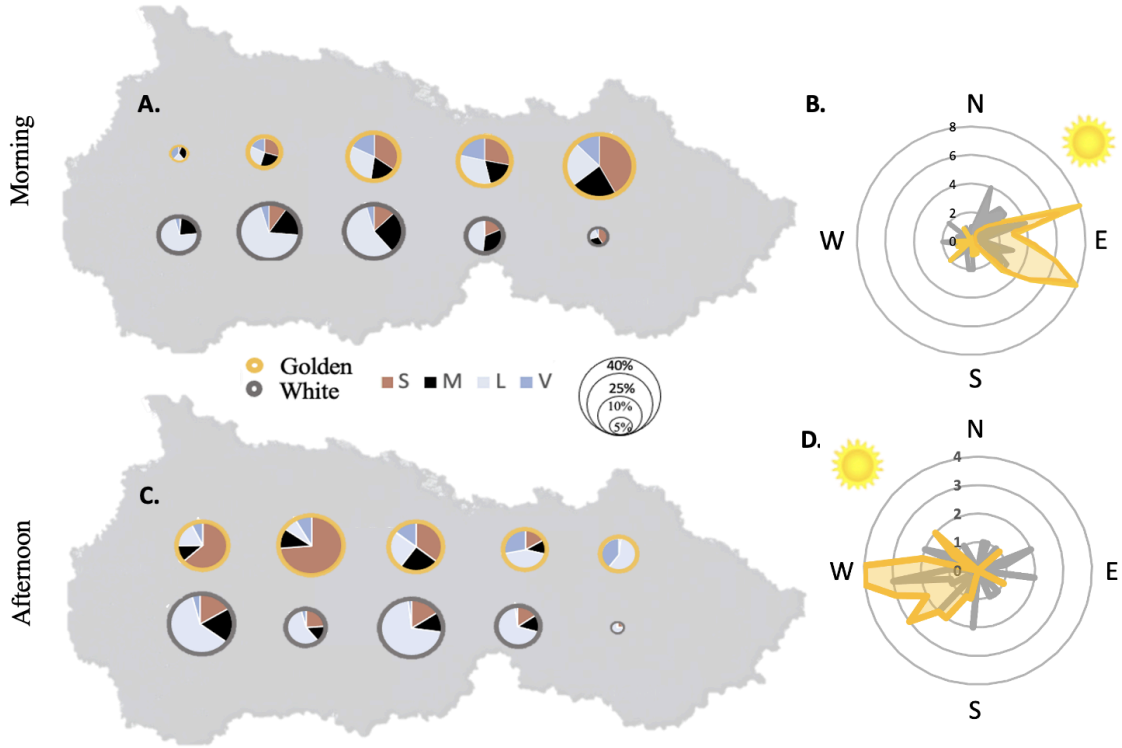

Figure 4.3: Morning and afternoon horizontal migration distributions of *Mastigias papua etpisoni* in OTM, Palau. A) Morning transect size distributions based on bell diameter (S < 70mm, M 70-140, L 140-180, V >180mm) of *M. papua etpisoni* within each lake sector from West (left) to East (right). Size of pie charts represent the within subgroup population proportions of white and golden medusae separately. Zooxanthellate (golden circles) from West to East: 3%, 9%, 27%, 24%, 37% and Azooxanthellate (gray circles) from West to East: 13%, 37%, 29%, 17%, 3%. **B)** Morning instantaneous swimming direction bearings represented by directional plot point of *M. papua etpisoni* of zooxanthellate (golden line) and azooxanthellate (gray line) individuals. Number of individuals is represented by the diameter of the circle the point resides within. Sun orientation is represented by the location of the sun icon. P-values - azooxanthellate 0.37, zooxanthellate 4.61E-15 **C)** Afternoon transect results of population distributions across the lake (circle size) and bell diameter size (pie chart divisions) of Zooxanthellate (golden circles) from West to East: 18%, 30%, 22%, 17%, 13% and Azooxanthellate (gray circles) from left to right: 30%, 14%, 36%, 18%, 1%. **D)** Afternoon instantaneous swimming direction bearings represented by directional plot point of *M. papua etpisoni* of zooxanthellate (golden lines) and azooxanthellate (gray lines) individuals. Number of individuals is represented by the diameter of the circle the point resides within. Sun orientation is represented by the location of the sun icon. P-values - azooxanthellate 0.95, zooxanthellate 2.47E-13.

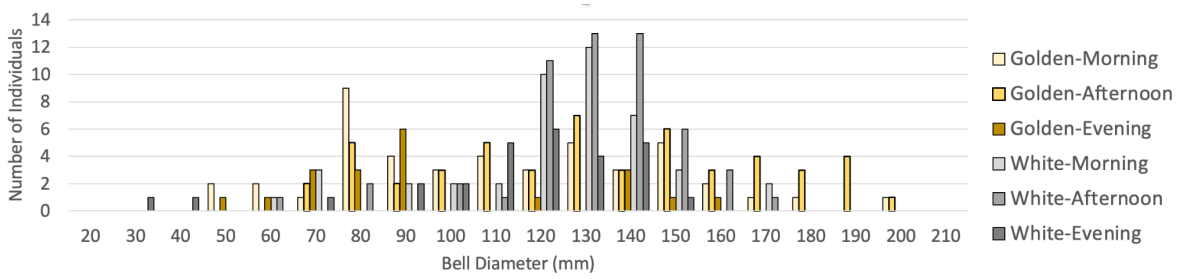

Figure 4.4: Bell diameter size (mm) variation for golden and white *M. papua etpisoni* measured during ISD surveys. The bar graph shows the number of individuals binned by bell diameter in mm, by color, and time of day. Golden bars represent symbiotic medusae from morning (light gold) to evening (dark gold), and gray bars signify non-symbiotic medusae from morning (light gray) to evening (dark gray).

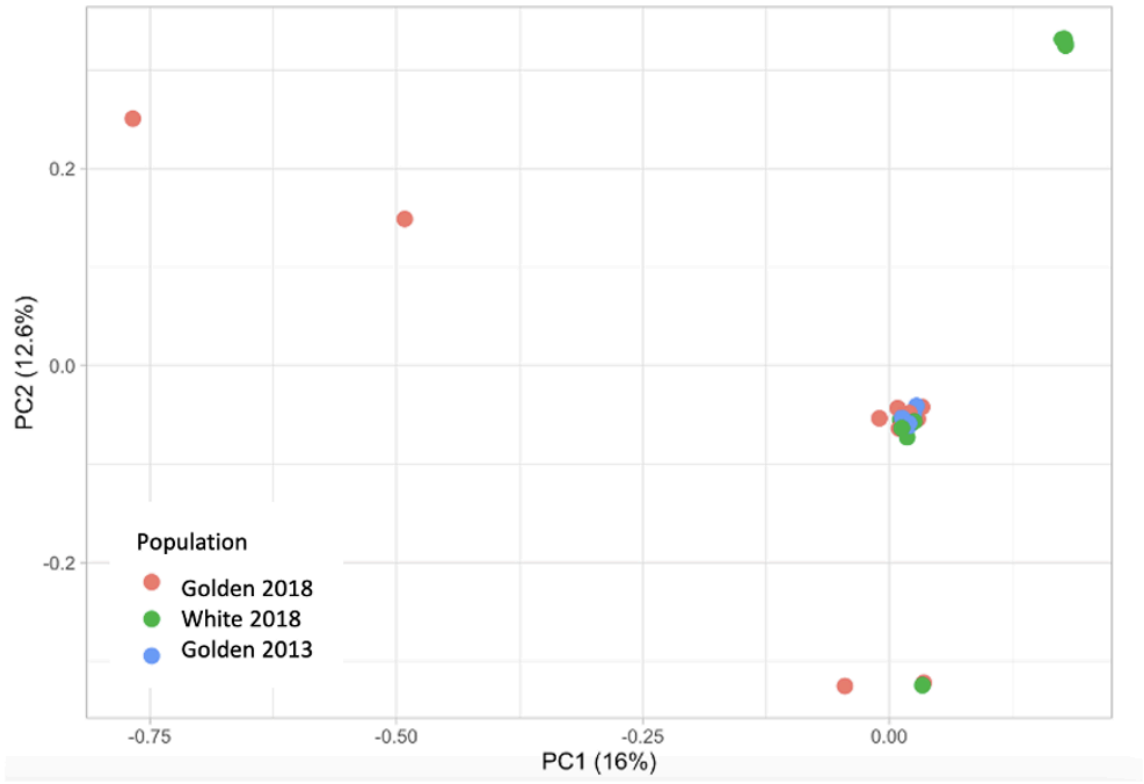

Figure 4.5: PCA of whole genome sequencing single nucleotide polymorphisms for OTM 2018 subgroups and 2013 population. Red points represent Golden 2018 individuals, Green dots represent White 2018 individuals, and Blue dots represent Golden 2013 individuals.