REPRODUCTIVE PATTERNS OF COLD-SEEP MUSSELS IN THE GULF OF MEXICO AND NORTHWESTERN ATLANTIC

by

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THESIS ABSTRACT

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Continuous or semi-continuous reproduction is the norm in deep-sea animals, with exceptions explained by seasonal pulses of surface-derived phytodetritus. Chemosynthesis-based ecosystems such as cold seeps have an independent nutritional supply and are often thought of as decoupled from surface productivity. This thesis explores reproductive patterns of four bathymodiolin mussel species from 14 cold seeps (320 to 3300 m depth) in the Gulf of Mexico (2014) and the northwestern Atlantic (2015). Using paraffin histology, I determined maturity stages for male and oocyte sizes for female mussels. All species at all sites reproduced periodically and synchronously, with geographic synchrony among sites. This suggests that mussels rely on a site-independent cue such as seasonal phytodetrital flux to synchronize reproduction, providing evidence for a stronger coupling between surface productivity and chemosynthesis-based fauna than previously expected. Mature oocytes were of similar size for all species at all depths, suggesting that egg size is phylogenetically constrained.

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For my mom, Catherine Plowman,

who taught me to follow my dreams

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CHAPTER I

GENERAL INTRODUCTION

Chemosynthesis-based ecosystems, areas where primary production is driven by microbial oxidation of inorganic compounds, were first discovered in the deep sea around 40 years ago. Diffuse hydrothermal vents were discovered in 1977 on the Galapagos Spreading Center (Corliss & Ballard, 1977; Corliss *et al.*, 1979), and black smoker vents were found on the East Pacific Rise in 1979 (Spiess *et al*., 1980). Both of these hydrothermal vent habitats are characterized by the expulsion of chemical-, metal-, and salt-rich fluids much hotter than the surrounding seawater (up to 407^{o} C compared to 2-4^{o} C ambient temperature) (Haase *et al*., 2009). In contrast, cold seeps are areas characterized by continual seepage of hydrocarbons, typically methane and/or oil, at temperatures near that of ambient seawater (Gage & Tyler, 1991). The first biological community living at a cold seep was discovered on the Florida escarpment in the Gulf of Mexico in 1984 (Paull *et al*., 1984; Hecker, 1985), though the existence of marine hydrocarbon seeps were known long before this.

Both hydrothermal vent and cold-seep habitats are home to a unique set of endemic megafauna (Gage & Tyler, 1991). Chemosynthetic bacterial communities flourish year-round in these habitats by oxidizing methane (methanotrophy) or hydrogen sulfide (thiotrophy) to create organic carbon molecules needed for growth and survival (MacAvoy *et al*., 2002). Dense communities of tubeworms, clams, and mussels harbor these chemosynthetic bacteria in their gills and feed off the bacterial metabolites as a continual food source (Kennicutt *et al*., 1985; Gage & Tyler, 1991). Chemosynthetic

communities in turn provide food and habitat for a diversity of consumers including gastropods, polynoid worms, alvinocarid shrimps, and galatheid crabs, as well as large, mobile predators such as fish and sharks (MacAvoy *et al.*, 2002).

Mytilid mussels are represented at deep-sea chemosynthetic communities by the subfamily Bathymodiolinae (Gustafson *et al*., 1998) that also includes deep-sea genera that live on wood falls. Within this subfamily, the genus *Bathymodiolus* Kenk and Wilson, 1985 consists entirely of mussels that live at hydrothermal vents and cold seeps (Distel, 2000). Species of *Bathymodiolus* obtain the majority of their energy through thiotrophic and/or methanotrophic bacteria that live in their gills (Fiala-Medioni *et al.*, 1986), though some species have also been observed filter-feeding on bacteria and phytodetritus in the water column (Page *et al.*, 1990, 1991; Pile & Young, 1999).

Bathymodiolus mussels are often among the dominant members of the communities at hydrothermal vents and cold seeps (MacDonald *et al*., 1990), making knowledge of their biology and ecology important to understanding the ecosystem as a whole. While research has been dedicated to how bathymodiolin mussels obtain energy, relatively little is known about their reproduction or larval ecology. Most shallow-water mytilids have a seasonal gametogenic cycle followed by broadcast spawning and external fertilization, resulting in pelagic planktotrophic veliger larvae that eventually settle onto benthic substrata (Mackie & Wilbur, 1984). The cold-seep mussel *Bathymodiolus childressi* is known to have a similar synchronous periodic gametogenic cycle (Tyler *et al.*, 2006), as well as embryonic development that closely follows that of shallow-water mytilids (Arellano & Young, 2009) and planktotrophic veliger larvae that have been found in surface waters (Arellano *et al*., 2014). Seasonal gametogenic cycles are also

known for the hydrothermal vent species, *Bathymodiolus azoricus* (Comtet and Desbruyères, 1998; Colaço *et al*., 2006) and *Bathymodiolus puteoserpentis* (Hessler *et al*., 1988).

Embryonic and larval development have not been studied in bathymodiolin mussels other than those species mentioned above, but the developmental mode can be inferred from egg and larval shell sizes. Typically, larvae of species with small eggs are planktotrophic, while large eggs yield lecithotrophic larvae (non-feeding, due to a large yolk reserve) (Thorson, 1950). The larval shell in mytilid mussels contains two parts, the prodissoconch I and II. Prodissoconch I is produced from energy reserves in the egg; if it were larger than prodissoconch II, the larva is likely lecithotrophic (Ockelmann, 1965). If prodissoconch II, produced during larval feeding, were larger, this is indicative of planktotrophy (Ockelmann, 1965). The hydrothermal vent species *Bathymodiolus azoricus* (Colaço *et al*., 2006), *Bathymodiolus puteoserpentis* (Hessler *et al*., 1988), *Bathymodiolus elongata* (Le Pennec & Beninger, 1997) and *Bathymodiolus thermophilus* (Berg, 1985) are all thought to have planktotrophic larvae, based on their small egg sizes and comparatively larger prodissoconch II shells. The cold-seep species *Bathymodiolus heckerae* is also thought to have planktotrophic larvae because its prodissoconch II is larger than prodissoconch I (Turner & Lutz, 1984; Gustafson & Lutz, 1994).

Although the reproductive timing and larval mode are known for a few species and can be inferred for a couple more, we still don't know much about the reproduction of bathymodiolin mussels from cold seeps or how depth affects reproductive patterns. The aim of this study is to fill a significant gap in knowledge for an ecologically important taxon, bathymodiolin mussels. I describe the periodicity of gametogenesis and

other reproductive patterns of bathymodiolin mussels from cold seeps in the Gulf of Mexico and northwestern Atlantic Ocean.

Framework

Studies of gametogenic periodicity in deep-sea animals are typically conducted by collecting samples periodically throughout the year. This collection method allows for the observation of the timing of each gametogenic stage. There has long been an assumption that observing a single reproductive event such as spawning is not sufficient to determine gametogeneic periodicity or lack thereof (Giese, 1959). However, the need for samples across multiple time points is likely one of the main reasons why, with a few notable exceptions (e.g. the Rockall Trough off Scotland), little is known about reproductive periodicity in most parts of the deep sea (reviewed by Young, 2003). There are both financial and logistical constraints to regular deep-sea sampling. Samples for this study were collected via submersible as part of a larger project examining larval connectivity among cold-seeps (SEEP-C). Sampling times were thus constrained by the goals and field program of the SEEP-C project, and all samples were collected in summer months in 2014 (Gulf of Mexico) and 2015 (Western Atlantic Margin). Nevertheless, important information can be garnered from opportunistic deep-sea samples.

There is a precedent in the literature for "snap-shot" reproductive studies such as this one. Paul Tyler, in his 1982 study on deep-sea echinoids (Tyler *et al*., 1982) used periodic samples from the Scottish Marine Biological Association's permanent stations in the Rockall Trough to outline three criteria for documenting synchronous, periodic reproduction. These were more concisely summarized and used in a later paper on the gametogenic periodicity of *Bathymodiolus childressi* (Tyler *et al*., 2006): (1) narrow

standard error for sample mean oocyte diameter, (2) minimal variation in oocyte sizes within individuals, and (3) significant differences in oocyte sizes among different seasons. The first two points can be determined for each time-point sample, or for a single "snap-shot" sample. The third criterion concerns differences among seasons. It follows, therefore, that samples obtained in the same season should demonstrate no significant differences in oocyte sizes among samples in a synchronous, periodically reproducing population.

I have created a diagram (using hypothetical data) to show the predicted patterns of oocyte sizes for each reproductive mode (Figure 1). This diagram was based on the aforementioned criteria and published examples of periodic synchrony in a bathymodiolin mussel (Tyler *et al*., 1982, 2006), continuous reproduction in a deep-sea holothurian(Tyler *et al*., 1992) and semi-continuous reproduction in bivalves (Järnegren *et al*., 2006). Oocyte size-frequency analysis is a more powerful means of detecting seasonal gametogenic cycles than gonad or maturity indices (Grant & Tyler, 1983).

Continuous reproduction is often asynchronous within a population but may take different forms. Individuals may undergo continuous gametogenesis and spawn frequently (Rokop, 1974). Continuous reproduction is characterized by a broad distribution of oocyte sizes within each individual and frequency peaks that may or may not line up across individuals (Figure 1A). Within a population, there are also oocytes in all size classes present at a single time, no or multiple frequency peaks, and narrow standard errors (Figure 1B). However, reproduction may also be asynchronous within a population but not continuous at the level of individuals. This pattern occurs when individual gametogenic cycles are staggered, leading to a continuous production of

gametes on a population level, even if each individual only spawns once a year (Rokop, 1974).

Graphically, population asynchronicity is observed as a single cohort of oocytes within each individual, with a frequency peak centered on one or a few size classes, but the peaks do not match up among individuals in the same population (Figure 1C). Within a population, there are oocytes present in every size class, with no distinct frequency peak and very large standard errors (Figure 1D).

Some deep-sea organisms also exhibit semi-continuous reproduction, characterized by the initiation of one cohort of oocytes before the previous cohort has spawned or finished developing (Järnegren *et al*., 2006). In this case, each individual may have oocytes present in all or most size classes with two distinct peaks, one representing each cohort of oocytes (Figure 1E). Cohorts could be asynchronous across individuals, leading to population size-frequency distributions that look similar to continuous reproduction (Figures 1B or 1D), or the cohorts could be synchronous, leading to a population with two distinct frequency peaks and narrow standard errors (Figure 1F).

Seasonal, or periodic, reproduction is initiated by synchronous gametogenic cycles between individuals in a population and is controlled by specific cues for the initiation of gametogenesis and/or spawning (Grant & Tyler, 1983). This synchronicity means individuals are producing only one cohort of oocytes at a given time and have size-frequency distributions that are centered on one frequency peak (Figure 1G). Individuals within a population are all developing together, so their oocyte size-frequency peaks match. The population frequency distribution looks very similar to a single individual and has narrow standard errors (Figure 1H).

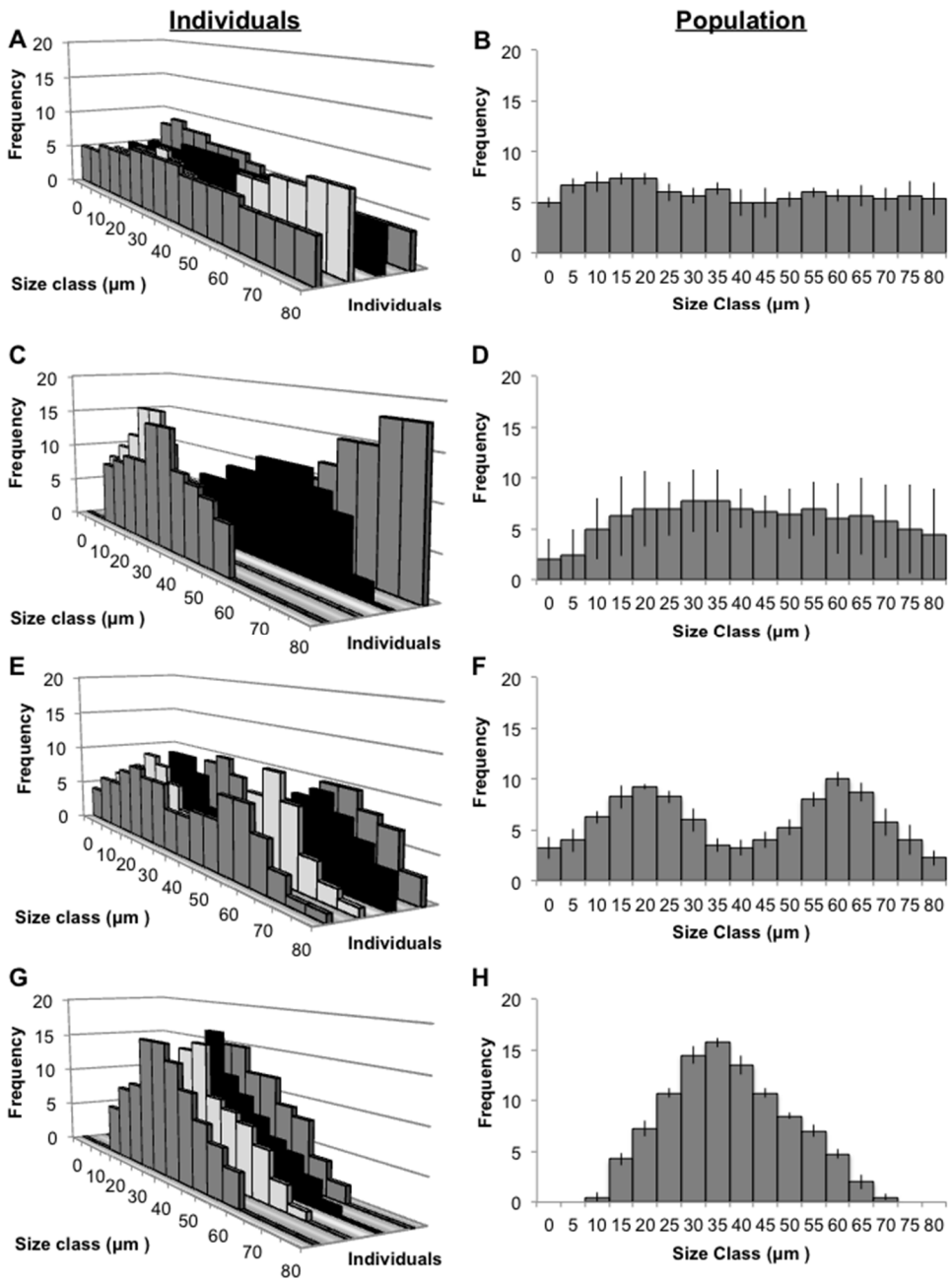

Figure 1. Schematic representation of oocyte size-frequency distributions of different reproductive modes, based on published accounts, but created with hypothetical data. A, Oocyte sizes of four hypothetical individuals with continuous reproduction and asynchronous spawning within the population. B, Average size-frequency distribution for the population in A. C, Oocyte sizes of four hypothetical individuals that each spawn in a single event, but are asynchronous within the population. D, Average size-frequency distribution for the population in C. E, Oocyte sizes of four hypothetical individuals undergoing semi-continuous reproduction. F, Average size-frequency distribution for the population in E. G. Oocyte sizes of four hypothetical individuals with synchronous, periodic/seasonal reproductive mode. H, Average size-frequency distribution for the population in G. Error bars represent ± 1 standard error.

Given the aforementioned framework, I used this study to (i) compare the reproductive patterns of *Bathymodiolus childressi* across a bathyal depth gradient (320 m to 2200 m), (ii) investigate the reproductive patterns of three other cold seep bathymodiolin mussels: *B. brooksi*, *B. heckerae* and an undescribed species, *B. sp. nov.*, and finally, (iii) compare the patterns among species.

CHAPTER II

REPRODUCTIVE PATTERNS OF *BATHYMODIOLUS CHIDRESSI* IN THE NORTHWESTERN ATLANTIC AND THE GULF OF MEXICO

Introduction

All mussels inhabiting oceanic cold seeps or hydrothermal vents belong to the family Mytilidae, subfamily Bathymodiolinae. Within this subfamily, the most speciose genus is *Bathymodiolus*, described from specimens collected from the Galapagos Rift. Species of *Bathymodiolus* are often among the dominant members of chemosynthetic communities (MacDonald *et al*., 1990). All species in the genus have chemosymbionts, either thiotrophic or methanotrophic bacteria, in their gills (Fiala-Medioni *et al*., 1986; Fisher *et al*., 1987). At least two mussel species are also able to filter-feed on particles in the water column. i) Ship-based experiments with *B. childressi* from the Brine Pool NR1 seep in the Gulf of Mexico showed significant decreases in cyanobacteria, bacteria, and protozoans in water collected from the mussels' excurrent siphons in comparison to the natural ocean water used in the experiment (Page *et al*., 1990; Pile & Young, 1999). ii) *B. thermophilus*, has been observed filter feeding in laboratory experiments (Page *et al*., 1991). Histological analysis of a third species, *B. azoricus,* showed particulate feeding levels (based on the appearance of the digestive gland) increased significantly in response to the seasonal increase in surface productivity (Dixon *et al*., 2006).

The reproduction of some *Bathymodiolus* species from hydrothermal vents has been studied: *B. azoricus* (Colaço *et al*., 2006; Dixon *et al*., 2006), *B. puteoserpentis* (Hessler *et al*., 1988), *B. elongates* (Le Pennec & Beninger, 1997), and *B. thermophilus* (Berg, 1985). All have egg and larval shell sizes indicative of planktotrophy (Arellano &

Young, 2009; Chapter I). *Bathymodiolus childressi* is the best-studied cold-seep bathymodiolin mussel and it has been confirmed to have planktotrophic larvae (Arellano & Young, 2009). Populations of this species from the Brine Pool NR1 cold seep in the Gulf of Mexico have synchronous, periodic reproduction and spawn in the fall and winter (Tyler *et al*., 2006). The presence of distinct size classes in adult *B. childressi* also indicates seasonal recruitment (MacDonald *et al*., 1990; Smith *et al*., 2000; Arellano & Young, 2010). The ultrastructure of gametogenesis for *B. childressi* from the Brine Pool has been described and revealed that the species is gonochoric (dioecious) and that gametogenesis closely resembles that of seasonal, shallow-water mytilid mussels (Eckelbarger & Young, 1999).

Even though some aspects of reproduction have been fairly well-studied in *B. childressi*, most samples were from only a single location, the Brine Pool NR1 (Gulf of Mexico) at a depth of 650 m, on the shallow end of *B. childressi*'s bathymetric range (527–2222 m from Carney *et al*., 2006). While it is unlikely that deeper populations would have different reproductive modes, it is possible that differences in the duration of the reproductive season, periodicity of reproduction, or onset of reproduction might shift as a function of depth. Indeed, such changes might be expected if reproduction were dependent on detrital food sources from surface waters since less detritus would make it to deeper sites. In this chapter, I elucidate the reproductive patterns of *B. childressi* in the deeper parts of its bathymetric range and compare reproductive patterns across the whole sampling range (320 m to 2200 m).

Bathymodiolin mussels live below the photic zone (0 to roughly 200 m) at great enough depth to experience relatively constant temperatures. However, the Brine Pool

NR1 seep in the Gulf of Mexico has seasonal temperature variations of about 1 ºC (Arellano & Young, 2011) so shallower seeps may experience greater temperature fluctuations. Temperature is known to control reproduction in many shallow-water invertebrates (Orton, 1920; Giese & Pearse, 1974), so differences in water temperature may lead to different reproductive patterns in *B. childressi* reproduction across its depth range. Seasonal detrital falls have been hypothesized to control reproductive seasonality in deep-sea animals (Lightfoot *et al*., 1979; Tyler & Young, 1992; Tyler *et al*., 1994; reviewed by Mercier & Hamel, 2009). Because bathymodiolin mussels have a continuous food supply from their symbiotic bacteria, the role of detrital seasonality is not clear. It is likely that they need a source of nitrogen (Lee & Childress, 1996; Pile & Young, 1999) and essential amino acids or fatty acids not available from chemosynthesis in order to reproduce (Page *et al*., 1990; Pile & Young, 1999). Indeed, the spawning of *B. childressi* from the Brine Pool NR1 site in October through February (Tyler *et al*., 2006) does appear to align with surface phytoplankton peaks in December through February in the northwestern Gulf of Mexico (Müller-Karger *et al*., 1991). Since *B. childressi* is known to have planktotrophic larvae, this seasonality could also be an example of Crisp's Rule, which says marine organisms with planktotrophic larvae time their reproduction so that the larvae are in the water column when planktonic food is highest (Quasim, 1956).

Deeper populations of *B. childressi* experience less fluctuation in temperature than shallower populations, and are further removed from photosynthetic food sources. If nitrogen or essential nutrients are required for initiating gametogenesis, as has been suggested, individuals in these deeper populations might acquire the substances later in the year, or accumulate them more slowly than shallower populations. I, therefore,

hypothesized that deeper populations of *B. childressi* will show an increased tendency toward continuous or asynchronous reproduction than the shallower populations do and that they also might reproduce later in the year.

Materials and Methods

Sample Collection and Preservation

A total of 252 individuals of *Bathymodiolus childressi* were collected from known methane seeps in the Gulf of Mexico (79 mussels) during May/June 2014 and along the Western Atlantic Margin (173 mussels) in July 2015 (Figure 2, Table 1). Clumps of mussels were collected using the manipulator arm of DSV *Alvin* and brought to the surface in a closed Plexiglas box that kept mussels near bottom temperature during transport to the surface. Once on board the support vessel R/V *Atlantis*, mussels were identified morphologically and removed from their shells (identifications were later verified with molecular techniques; C.L. van Dover and B. Ball personal communication). Mussel soft tissues were preserved in 10% buffered formalin and shipped to the Oregon Institute of Marine Biology (OIMB) in Charleston, OR. After 8 – 10 months, samples were rinsed and transferred to 70% ethanol.

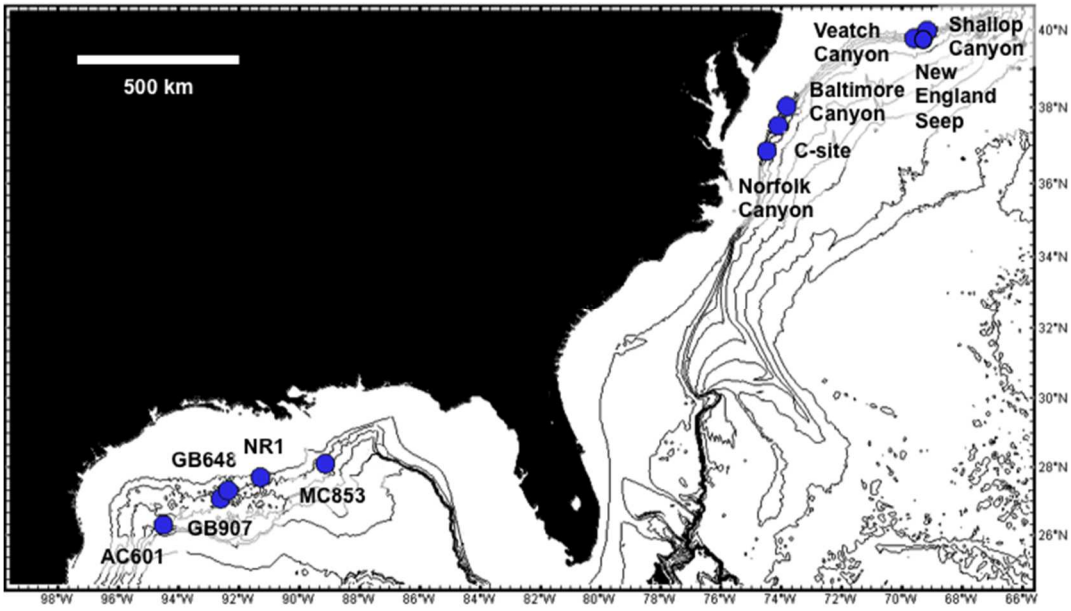

Figure 2. Map of collection sites for *Bathymodiolus childressi* in the Gulf of Mexico (summer 2014) and along the Western Atlantic Margin (summer 2015). Longitude is shown along the bottom axis and latitude along the right axis. Depth contours represent 500 m.

Table 1. Bathymodiolin mussels collected from the Gulf of Mexico (GoM) and the Western Atlantic Margin (WAM). "Indeterminate" denotes individuals lacking gonad tissues.

Depth (m)	Site	Basin	Latitude (° N)	Longitude (° W)	Temp (° C)	Date Sampled	N	% Male	% Female	% Indeterminate
320	Shallop Canyon	WAM	39.9961	69.1260	8.6	July 24, 2015	11	27.3	63.6	9.1
400	Baltimore Canyon	WAM	38.0486	73.8214	8.5	July 20-21, 2015	38	55.3	36.8	7.9
650	NR1	GoM	27.7237	91.2877	9.4	May 27, 2014	23	65.2	30.4	4.3
970	GB648	GoM	27.3389	92.3607	4.9	June 3, 2014	13	30.8	69.2	0.0
1020	C-site	WAM	37.5423	74.1019	4.4	July 17 & 19, 2015	30	70.0	30.0	0.0
1070	MC853	GoM	28.1242	89.1414	4.3	May 22, 2014	43	76.7	16.3	7.0
1260	GB907	GoM	27.0919	92.6190	4.3	June 2, 2014	19	21.1	57.9	21.1
1420	Veatch Canyon	WAM	39.8050	69.5930	4.3	July 23 & 25, 2015	36	41.7	52.8	5.6
1420	New England Seep	WAM	39.8693	69.2855	4.3	July 27, 2015	30	46.7	46.7	6.7
1460	Norfolk Canyon E	WAM	36.8712	74.4742	4.0	July 15, 2015	24	70.8	29.2	0.0
1600	Norfolk Canyon W	WAM	36.8682	74.4881	4.1	July 16, 2015	13	46.2	53.8	0.0
2200	AC601	GoM	26.3197	94.5150	4.3	May 30, 2014	18	33.3	55.6	11.1

Histological Analysis

To determine gametogenic stages and oocyte diameters, a ~1 cm^3 cube containing gonadal tissue was dissected from the middle of the visceral mass of each mussel for histological analysis. Dissected tissue was soaked in 95% ethanol for 18-24 h then soaked three times for 2 h in 100% ethanol to ensure complete sample dehydration. Tissues were then soaked in 99.9% toluene > 24 h for clearing. Cleared tissues were infiltrated with molten paraffin wax for 24 h and then embedded in paraffin blocks. Tissue sections (7 μm thick) were cut using a rotary microtome, allowed to expand in a warm water bath, then mounted on slides and left to dry and adhere overnight. Slides were stained with the acidophilic nuclear stain hematoxylin and the basophilic cytoplasmic stain eosin to enable visualization of the gonad tissues (Galigher & Kozloff, 1971). These stains enable gender differentiation by causing nuclei (including sperm heads) to appear dark blue and egg cytoplasm to appear light pink. Cover slips were mounted using Permount® mounting medium. Two slides from each specimen, containing about 5–7 tissue sections, were examined using an Olympus BX50 compound light microscope at 200× magnification.

Body Length

The length of each mussel (anterior edge of mantle to posterior edge), without shell, was measured to the nearest millimeter, prior to dissection of the gonad. Lengths were then compared between sexes: male, female, hermaphrodite, and indeterminate.

Maturity Stages

To assess male development, maturity stages were assigned based on those described for *Bathymodiolus childressi* by Tyler *et al*. (2006). Females were not assigned maturity stages since their gametogenic patterns are well characterized by oocyte sizes. The 6 stages of males were as follows (Figure 3):

Stage 1: Onset of spermatogenesis. Proliferation of spermatogonia (early cell type that will give rise to 4 spermatids) and spermatocytes (intermediate cell type that will give rise to 2 spermatids) begins around the edge of the acinus (small, sack-like cavity inside the gonad where gametes are produced) (Figure 3A).

Stage 2: Appearance of spermatids. Spermatids, or developing sperm, start appearing in the lumen of the acinus (Figure 3B).

Stage 3: Proliferation of spermatids. Lumen of the acinus begins to fill with spermatids and fully developed sperm, or spermatozoa (Figure 3C).

Stage 4: Whorls form. Lumen of the acinus becomes so full of spermatozoa that whorls begin to form (Figure 3D).

Stage 5: Ripe. Whorls of spermatozoa fill the acinus lumen and the individual is ready to spawn (Figure 3E).

Stage 0: Post-spawning. Deflated acinus is surrounded by a large number of somatic cells and contains only a few peripheral spermatogonia (Figure 3F).

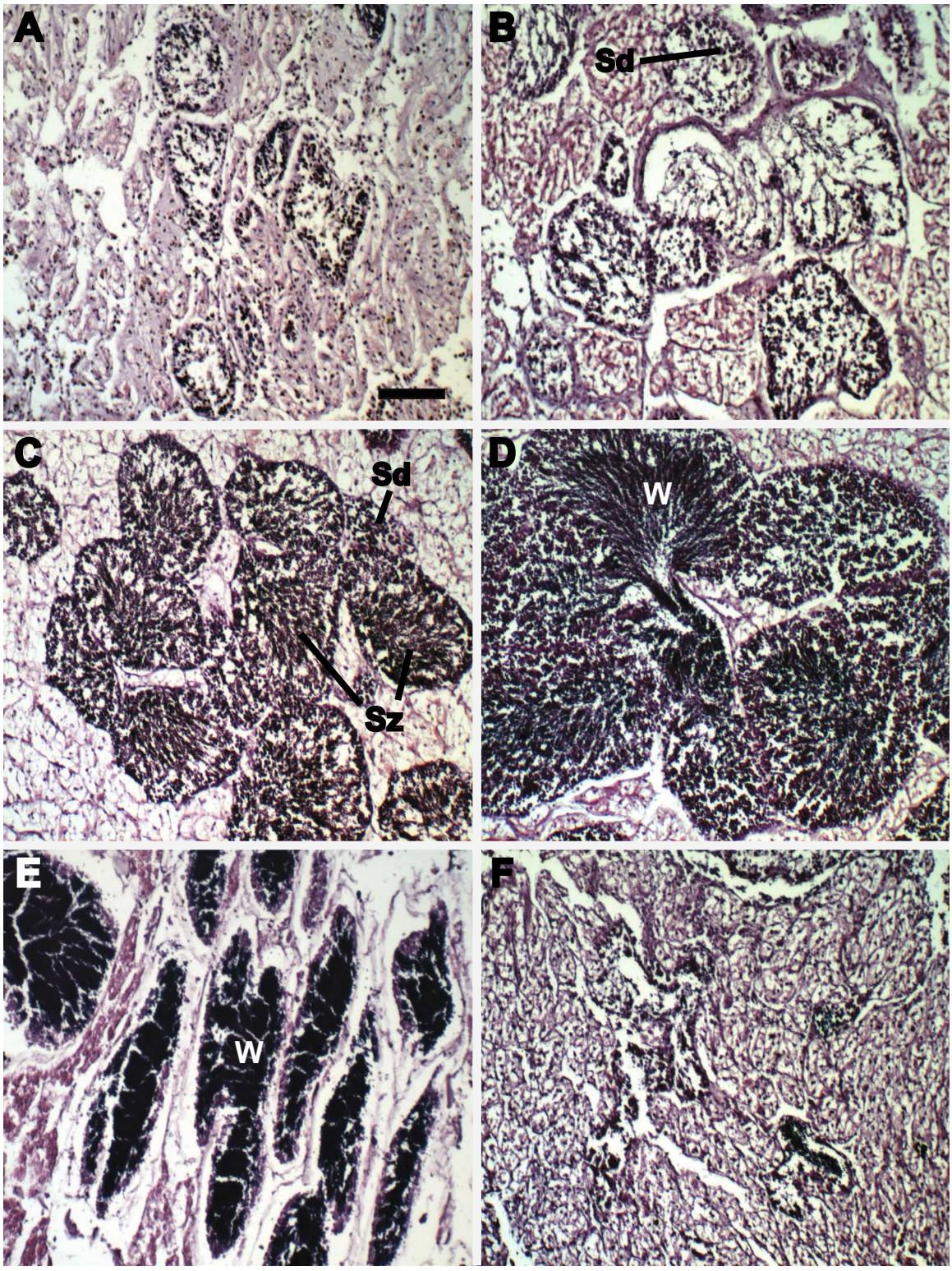

Figure 3. *Bathymodiolus childressi* testis development and male maturity stages. A, Stage 1: Onset of spermatogenesis. Scale bar is 100 μm and applies to all images. B, Stage 2: Appearance of spermatids (Sd). C, Stage 3: Proliferation of spermatids and appearance of spermatozoa (Sz). D, Stage 4: Whorls of spermatozoa form. E, Stage 5: Ripe and ready to spawn. F, Stage 0: Post-spawning.

Oocyte Diameters

Histological sections of each female were photographed using a Canon PowerShot S3 IS (6.0 megapixels) camera attached to a compound microscope, and oocytes were measured using ImageJ version 64 (Rasband, 2016). A stage micrometer was photographed at the same magnification as the slides and used for size calibration. The areas of 100 haphazardly selected oocytes from each female were measured by tracing of the outline of each cell using the freehand trace tool in ImageJ. The software reported the area of each cell and calculated the feret diameter using the standard equation for the area of a circle. Feret diameter is often used to measure oocytes because it smooths out any irregularities in shape (e.g. Tyler *et al*., 2006). Only those oocytes with clear nucleoli were measured to avoid measuring portions of the same oocyte from different sections and to standardize, as much as possible, the cross-section of the oocyte (Grant & Tyler, 1983). Diameters were measured for 100 oocytes following Tyler *et al*. (2006) and to ensure adequate sampling of each individual. All measurements were examined to find a minimum, median, mean, and maximum oocyte size for each site. Then, the 100 measurements for each individual were averaged to obtain a mean oocyte size per individual.

Statistical Analysis

Oocyte size data did not meet the assumptions of parametric statistical analyses (homogeneity of variances and normality). Therefore, non-parametric statistics were used. A Kruskal-Wallis test, with a Dunn's multiple comparisons post-hoc test, was used to test oocyte size differences among sites. A chi-squared contingency table analysis was used to test for uneven distributions in male maturity stages among sites. A Kolmogrov-

Smirnov test was used to test the distribution of body lengths between males and females. Body length data did meet assumptions of parametric statistical analyses, so a two-way ANOVA was used to test the effect of sex, site, and their interaction on mussel body length. These tests were all performed in the statistical software package RStudio version 1.0.136 (R Core Team, 2016).

Results

Bathymodiolus childressi is gonochoric with a maximum oocyte diameter around 80 μm (Table 2). One mussel (out of 252 total) was a hermaphrodite. This individual, collected at MC853 (1070 m) in the Gulf of Mexico, had a body length (without shell) of 56 mm. Its gonads contained developed spermatids and spermatozoa along with pre-vitellogenic and some newly vitellogenic oocytes in the same acinus (Figure 4).

Table 2. Minimum, median, mean, maximum, and standard error of oocyte sizes for each site. N = number of individuals, n = number of oocytes.

					Oocyte sizes (μm)				
Site	Depth (m)	Region	N	n	Min	Median	Mean	Max	SE
Shallop Canyon	320	WAM	7	700	6.21	17.41	22.17	67.57	0.53
Baltimore Canyon	400	WAM	13	1300	10.01	32.62	33.08	67.47	0.35
NR1	650	GoM	7	700	7.75	26.07	26.59	77.48	0.41
GB648	970	GoM	9	900	6.87	27.74	28.28	77.99	0.39
C-site	1020	WAM	9	900	10.87	35.92	37.82	72.27	0.47
MC853	1070	GoM	8	800	5.11	21.38	22.94	71.55	0.38
GB907	1260	GoM	11	1100	5.21	18.01	20.45	70.77	0.31
Veatch Canyon	1420	WAM	15	1500	6.10	11.63	13.91	52.01	0.19
New England Seep	1420	WAM	14	1400	5.35	11.69	15.88	67.46	0.30
Norfolk Canyon E	1460	WAM	7	700	11.53	32.95	35.28	76.22	0.52
Norfolk Canyon W	1600	WAM	6	600	10.26	29.76	33.69	71.04	0.56
AC601	2200	GoM	10	1000	4.85	16.50	20.62	72.16	0.41

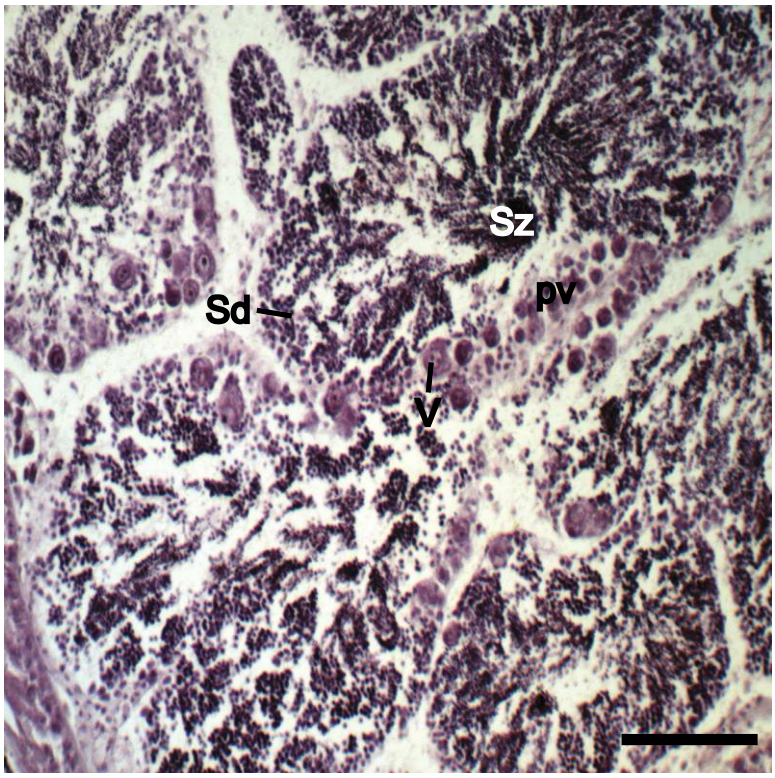

Figure 4. Hermaphroditic individual of *Bathymodiolus childressi* collected from MC853 in the Gulf of Mexico, May 2014. Within the same acinus, it has developing spermatids (Sd) and spermatozoa (Sz) indicative of a stage 3 male, along with previtellogenic oocytes (pv) and oocytes just starting vitellogenesis (V). Scale bar is 100 μm.

Reproductive Periodicity

Individuals and populations of *Bathymodiolus childressi* at all depths (320 to 2200 m) were found to be reproducing synchronously and periodically, based on individual size-frequency distributions of oocyte sizes (Figures 5–6), and average size-frequency histograms for each site (Figures 7–8).

Mussels at a few sites (C-site and Norfolk Canyon) had a wide range of oocyte sizes within individuals but mussels at all other sites had a narrow distribution of sizes centered around one frequency peak (Figures 5 and 6). While there was individual variation within sites, the shape of size-frequency oocyte distributions was similar within each population (site), and the frequency peaks occurred in approximately the same size

class (Figures 7 and 8). These results are indicative of reproductive synchrony within each population.

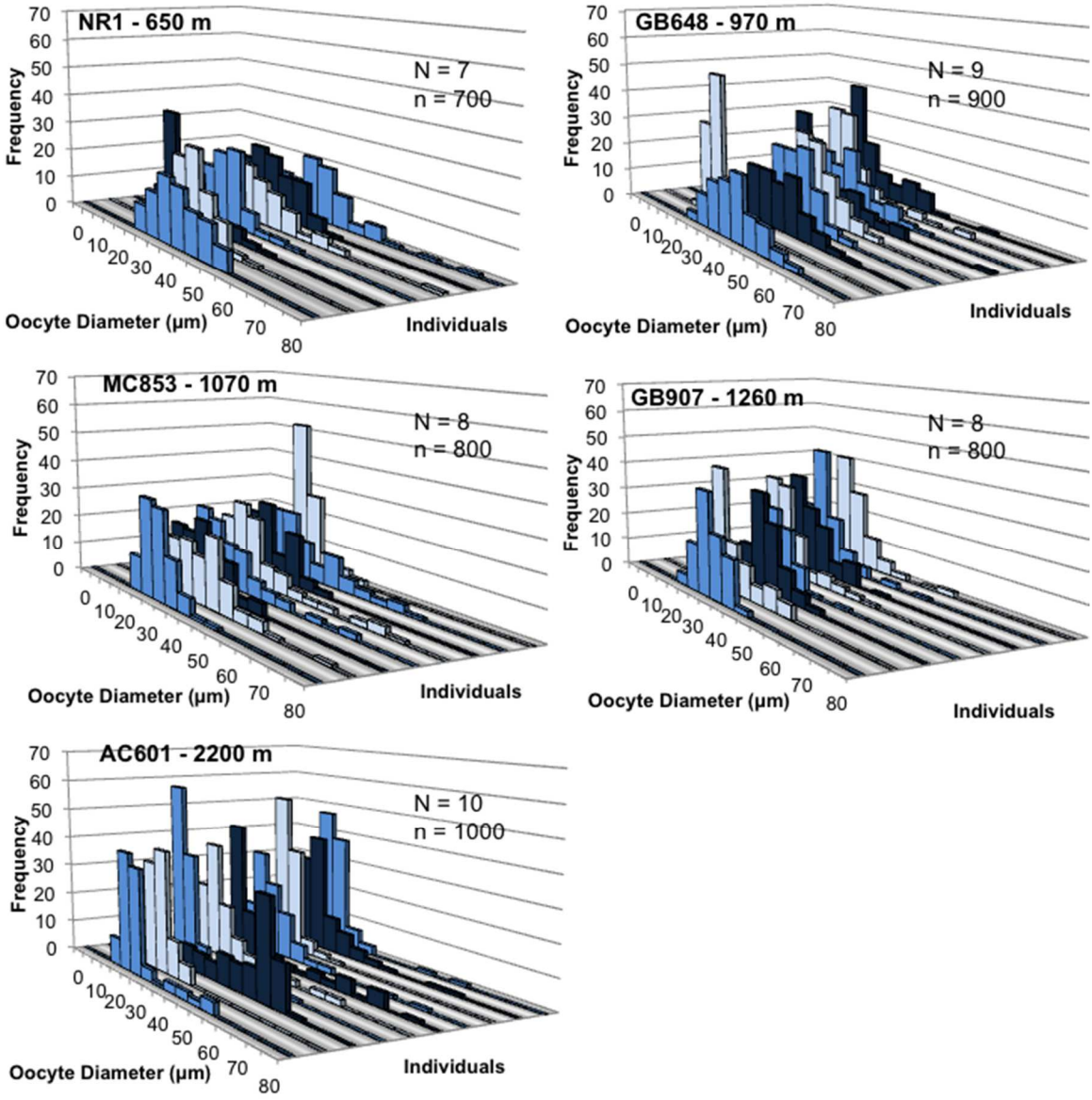

Figure 5. Individual oocyte size-frequency distributions, grouped by site, for *Bathymodiolus childressi* collected in the Gulf of Mexico in the summer of 2014. N = number of individuals, n = number of oocytes measured. Different shades are used to aid in distinguishing among individual mussels.

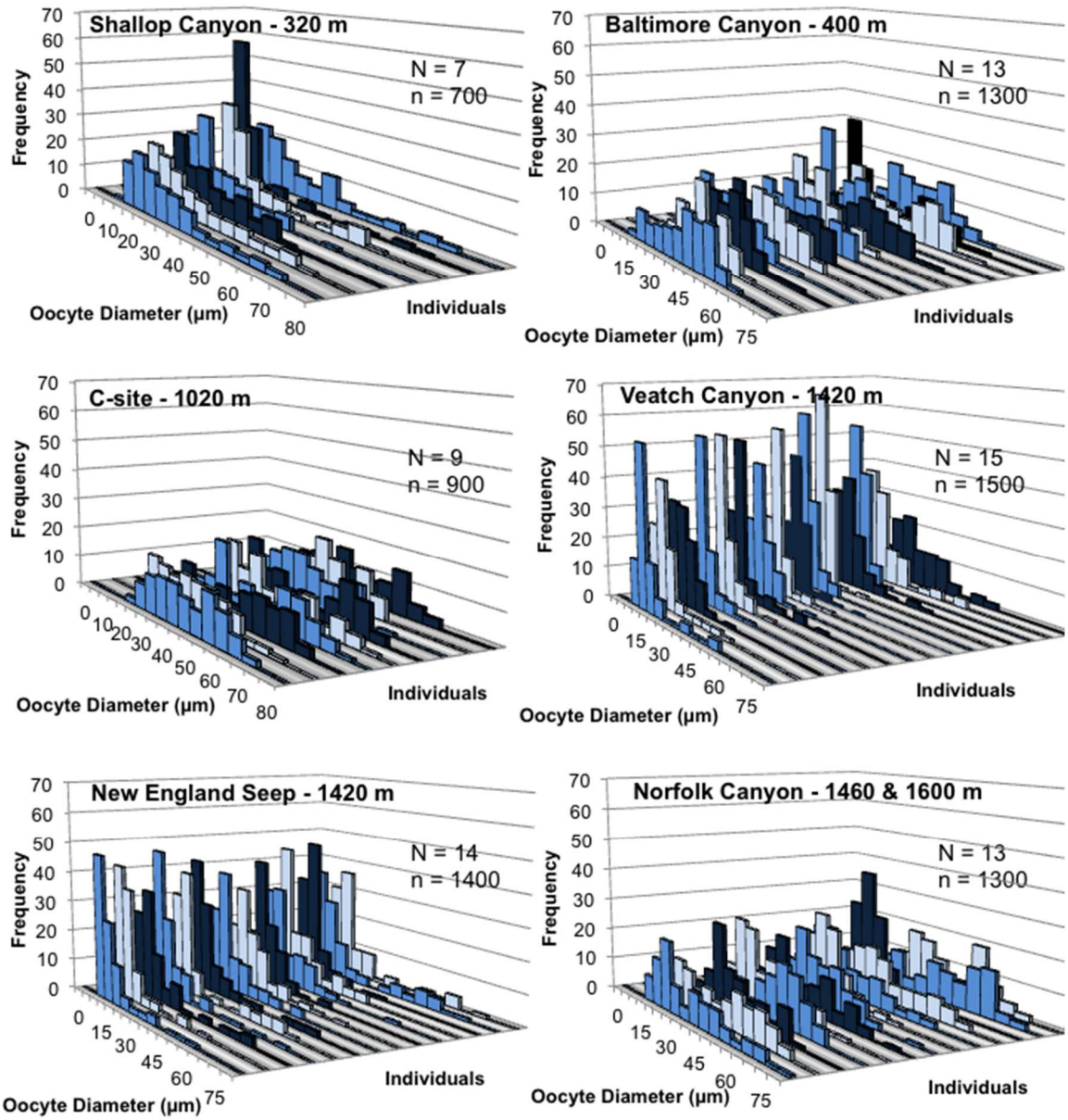

Figure 6. Individual oocyte size-frequency distributions, grouped by site, for *Bathymodiolus childressi* collected along the Western Atlantic Margin in the summer of 2015. N = number of individuals, n = number of oocytes measured. Different shades are used to aid in distinguishing among individual mussels.

Average oocyte size-frequency distributions for each site (Figures 7 and 8) show synchronicity among individuals and sites, indicating geographic synchrony. For example, sites NR1, GB648 and GB907 (Figure 7) are all near (37–150 km) each other in the Gulf of Mexico and the mussels have a range of oocyte diameters between 10 and 50–

60 µm with a frequency peak around 25–30 µm. New England Seep and Veatch Canyon in the Atlantic (Figure 8) are at the same depth (1420 m) and very close (27 km) together geographically. They both have oocyte sizes ranging from 10 to 60 µm with a frequency peak around 10–15 µm, which is dramatically different from other sites on the Western Atlantic Margin.

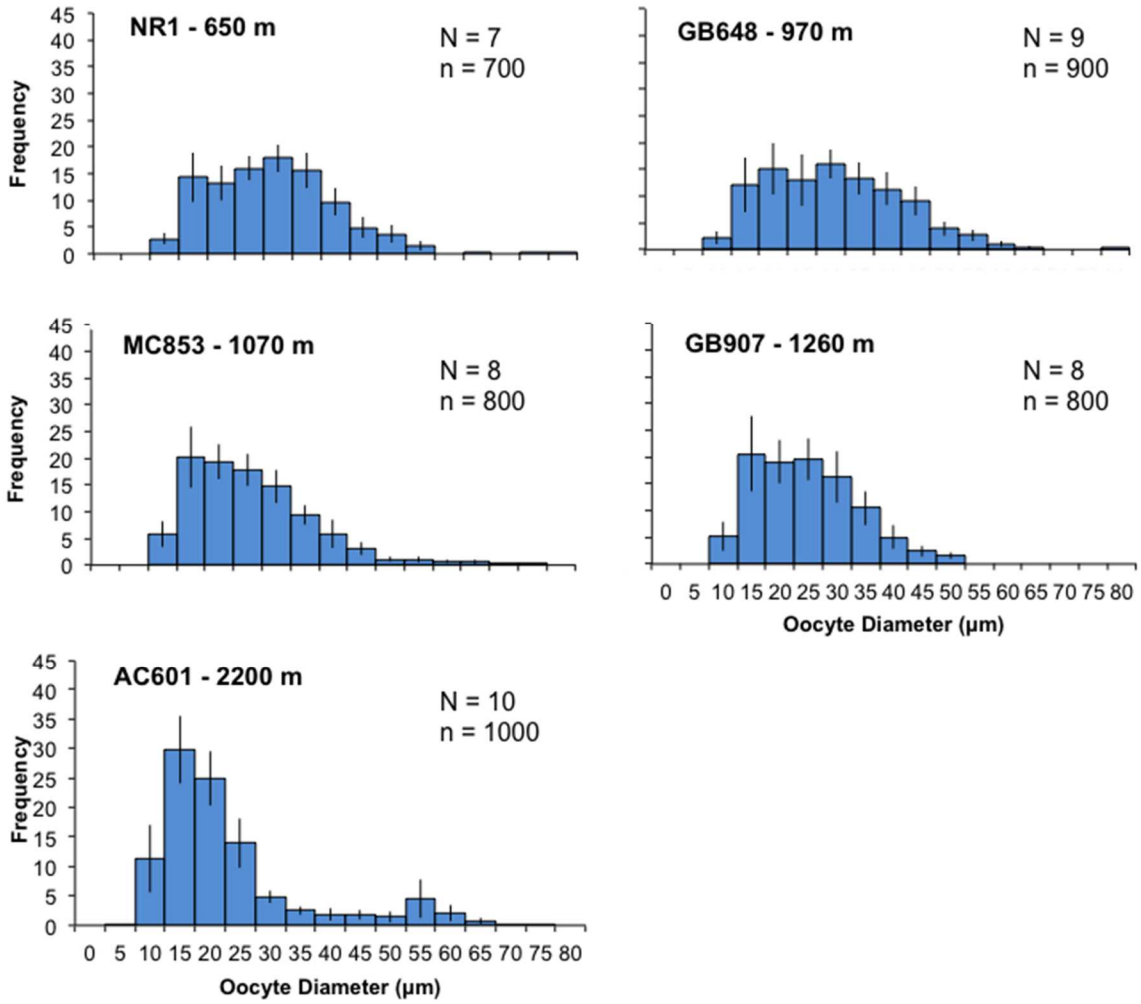

Figure 7. Average oocyte size-frequency distributions for *Bathymodiolus childressi* populations at each site in the Gulf of Mexico in summer 2014. N = number of mussels, n = number of oocytes. Error bars represent ± 1 standard error.

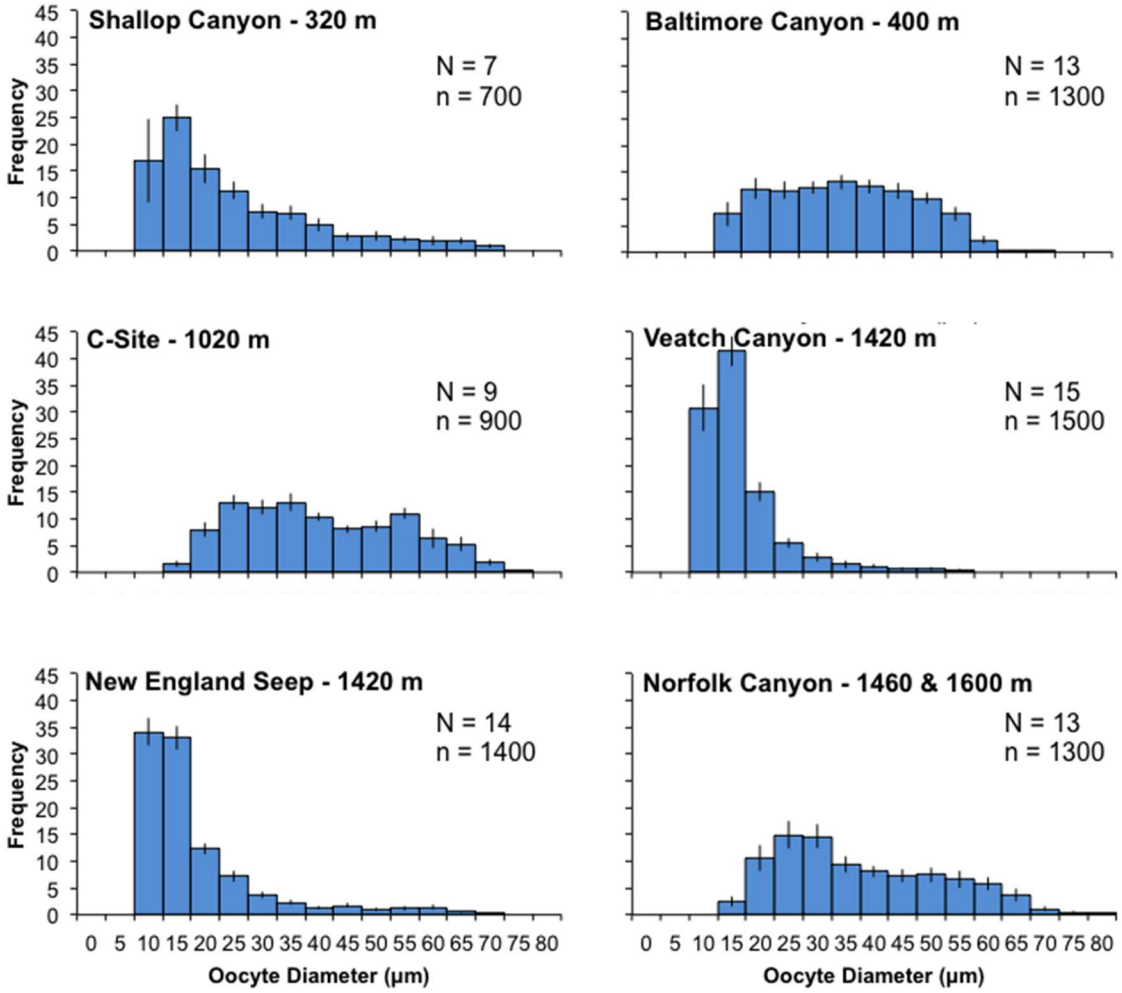

Figure 8. Average oocyte size-frequency distributions for *Bathymodiolus childressi* populations at each site on the Western Atlantic Margin in summer 2015. N = number of mussels, n = number of oocytes. Error bars represent ± 1 standard error.

Oocyte Sizes

Oocyte feret diameter differed significantly among sites (Kruskal-Wallis, $H = 81.97$, $df = 11$, $p < 0.001$; Figure 9)

A Dunn's post-hoc test with a Bonferroni correction showed four groups of sites, with oocyte diameters being significantly different among but not within groups of sites. These groups do not appear to align with depth or region (GoM vs. WAM). Veatch Canyon (WAM, 1420 m) had the smallest mean oocyte diameter at 13.91 ± 0.71 µm. C-

site (WAM, 400 m) had the largest diameter at 37.82 ± 1.11 µm; all other sites had mussels with mean oocyte diameters between 15 and 36 µm.

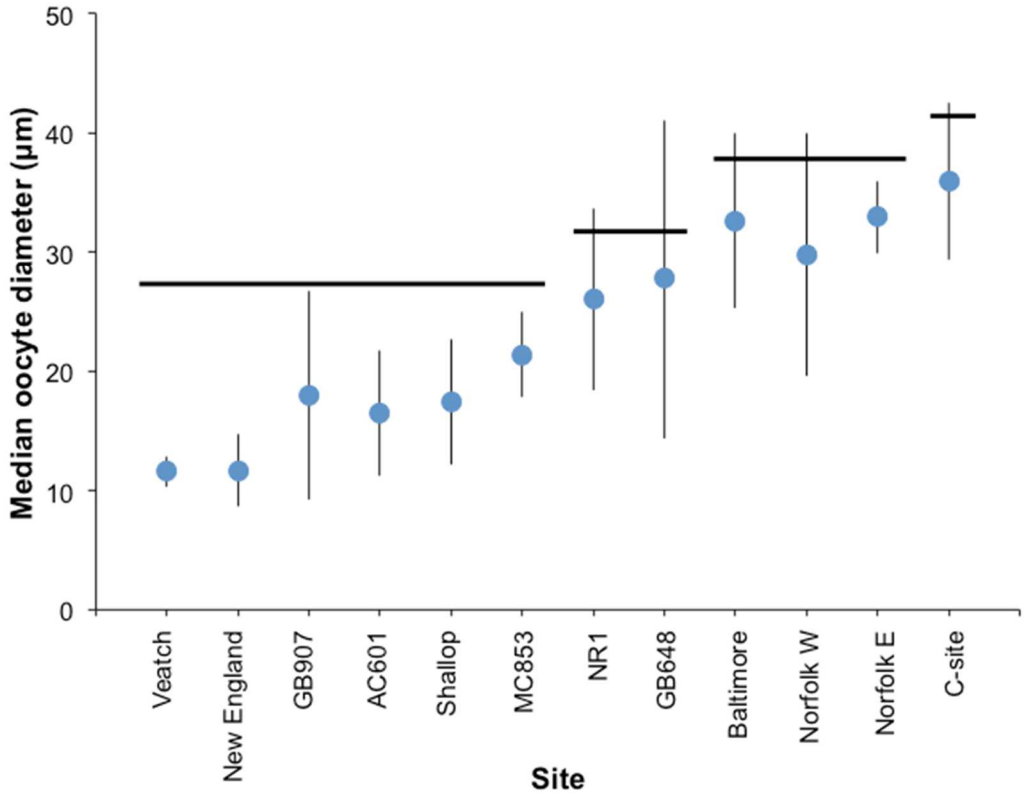

Figure 9. Median oocyte feret diameters of *Bathymodiolus childressi* collected from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015). Error bars represent the inter-quartile range. Horizontal lines denote groups of statistically similar sites.

Maturity

Individuals at almost every maturity stage were found in both the Gulf of Mexico and the Western Atlantic Margin (Table 3).

The relative distribution of male maturity stages revealed significant differences among sites (chi-squared contingency table, $\chi^2 = 49.45$, df = 33, p = 0.03). None of the z-scores in this test were significant so the location of the differences cannot be ascertained. Baltimore Canyon (400 m) was the only site with a ripe (stage 5) male individual and GB907 (1260 m) was the only site with a post-spawning (stage 0) male. The population

at New England Seep (1420 m) had more males at later maturity stages than the population at Veatch Canyon (1420 m), despite being at the same depth. The shallower Norfolk Canyon E population (1460 m) had more males at later maturity stages than Norfolk Canyon W (1600 m) even though they are from sites extremely close (1.3 km) together.

Table 3. Number of male *Bathymodiolus childressi* individuals at each maturity stage for each collection site from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015). N = number of individual mussels.

			Testis development stage						
Site	Region	Depth (m)	1	2	3	4	5	0	N
Shallop Canyon	WAM	320	0	1	1	1	0	0	3
Baltimore Canyon	WAM	400	0	2	11	7	1	0	21
NR1	GoM	650	2	2	7	4	0	0	15
GB648	GoM	970	1	1	2	0	0	0	4
C-Site	WAM	1020	0	10	9	2	0	0	21
MC853	GoM	1070	2	14	14	3	0	0	33
GB907	GoM	1260	1	0	2	0	0	1	4
Veatch Canyon	WAM	1420	3	7	3	0	0	0	13
New England Seep	WAM	1420	2	5	5	1	0	0	13
Norfolk Canyon E	WAM	1460	1	2	9	5	0	0	17
Norfolk Canyon W	WAM	1600	2	1	3	0	0	0	6
AC601	GoM	2200	1	0	2	2	0	0	5
		Totals:	15	45	68	25	1	1	155

Females were not assigned maturity stages, but it is important to note that the ovary developed in a similar fashion as the testes (Figure 10): onset of oogenesis (Figure 10A) followed by the initiation (Figure 10B) and continuation of vitellogenesis (process of adding yolk to oocytes; Figure 10C), then the proliferation of free oocytes that have completed vitellogenesis (Figure 10D) and fill the lumen of the acinus until the individual is ripe and ready to spawn (not shown since no ripe females were collected in this study). After spawning, the acinus deflates and may contain a few un-spawned oocytes (Figure 10E).

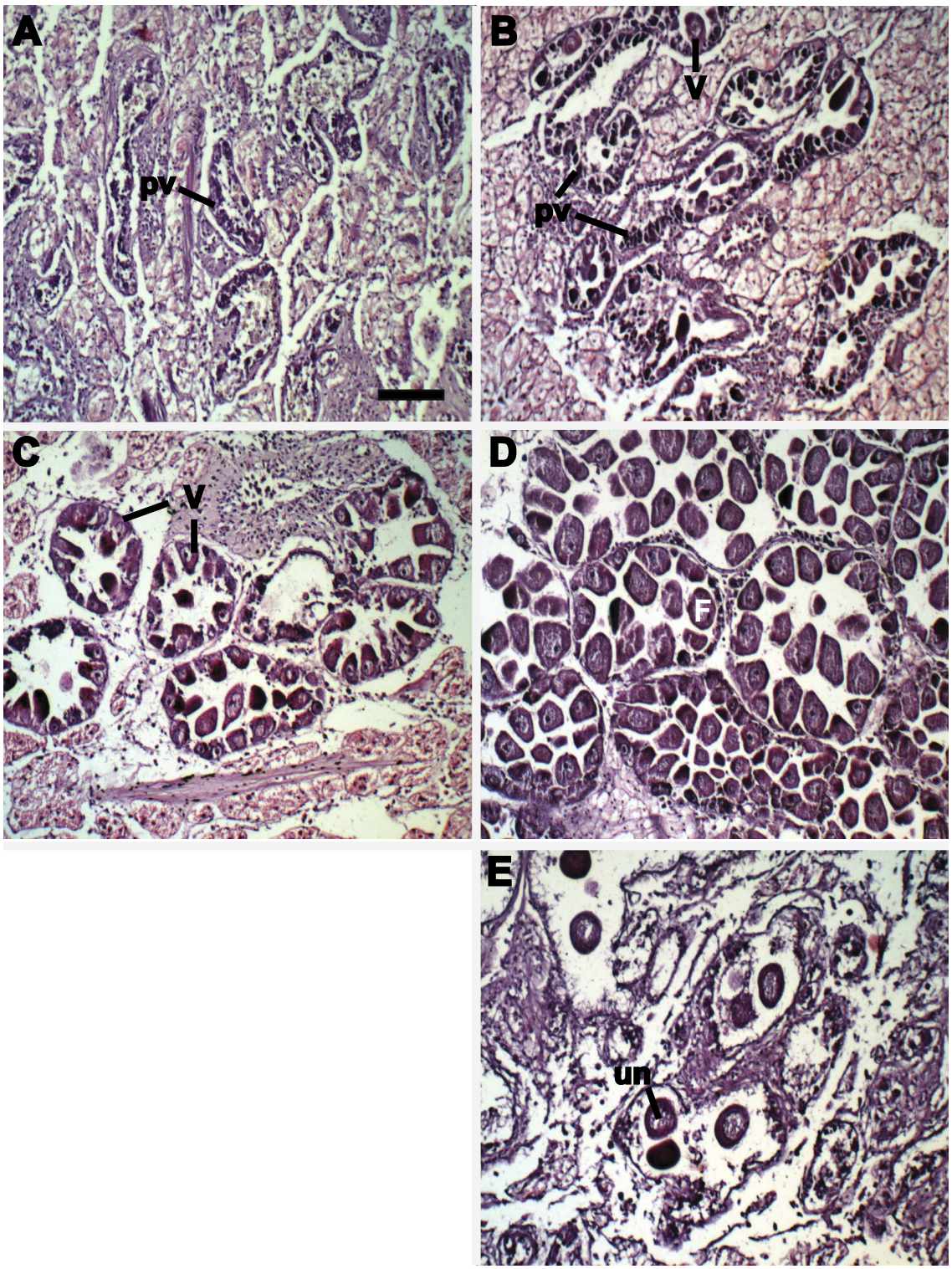

Figure 10. *Bathymodiolus childressi* ovary development. A, Onset of oocgenesis. Scale bar is 100 μm and applies to all images. B, Previtellogeneic oocytes (pv) start to undergo vitellogenesis. C, Proliferation of vitellogenic oocytes (V). D, Free oocytes (F) start to fill the lumen of the acinus in preparation for spawning. No ripe individuals were found during this study. E, Post-spawning deflated acinus with a few un-spawned oocytes (un) left over.

Site Comparisons

Significant differences among sites were found using both oocyte feret diameter (Figure 9) and male maturity stages (Table 3). In neither case do these differences appear to be directly related to depth or region (GoM vs. WAM) alone.

C-Site (WAM, 1020 m) is at an intermediate depth and had mussels with the largest oocyte diameter, along with males in intermediate developmental stages. Veatch Canyon (WAM, 1420 m), also at an intermediate depth, had mussels with the smallest mean oocyte diameter and the highest number of males found in Stage 1. Mussels at Veatch Canyon did have a mean oocyte diameter and male maturity stage distribution similar to New England Seep (WAM, 1420 m) and Shallop Canyon. Both are very close (<46 km) to Veatch Canyon geographically.

Geographic location seems to relate to some of the reproductive patterns described here, but it also does not fully explain the differences among sites. The statistical groups based on mean oocyte feret diameters (Figure 9) did not form groups within each region (GoM vs. WAM), but instead all groups include sites from both regions. The two sites from the Garden Banks area in the Gulf of Mexico, GB648 (970 m) and GB907 (1260 m), have mussels with similar distributions of male maturity stages and similar mean oocyte feret diameters, but mussels at GB648 have a mean oocyte diameter closest to that of NR1 (GoM, 650 m). NR1 is 76 km further away from GB648 than the other Garden Banks site is, but is NR1 closer to GB648 in depth.

Geographic trends are further confounded by the sites near the mouth of the Chesapeake Bay: Norfolk Canyon E (1460 m), Norfolk Canyon W (1600 m), C-Site, and Baltimore Canyon. C-site lies in between Norfolk Canyon and Baltimore Canyon

geographically and in depth, yet the mussels there have the highest mean oocyte feret diameter of the three sites. Baltimore Canyon has the most mature male individuals, while C-Site and the two Norfolk Canyon sites have very similar distributions of male maturity stages. The two Norfolk Canyon sites have statistically the same mean oocyte diameter, yet the shallower East site has more mature males than the deeper West site.

Body Lengths

Reproductive *Bathymodiolus childressi* males had statistically smaller body sizes than reproductive females (K-S test, $D = 0.35$, $p < 0.001$) with some overlap (Figure 11). Results of a two-way ANOVA showed sex and site both had a significant effect on body length, but their interaction does not (Table 4). Indeterminate individuals were found at a range of body sizes and the single hermaphroditic individual was at a median body size.

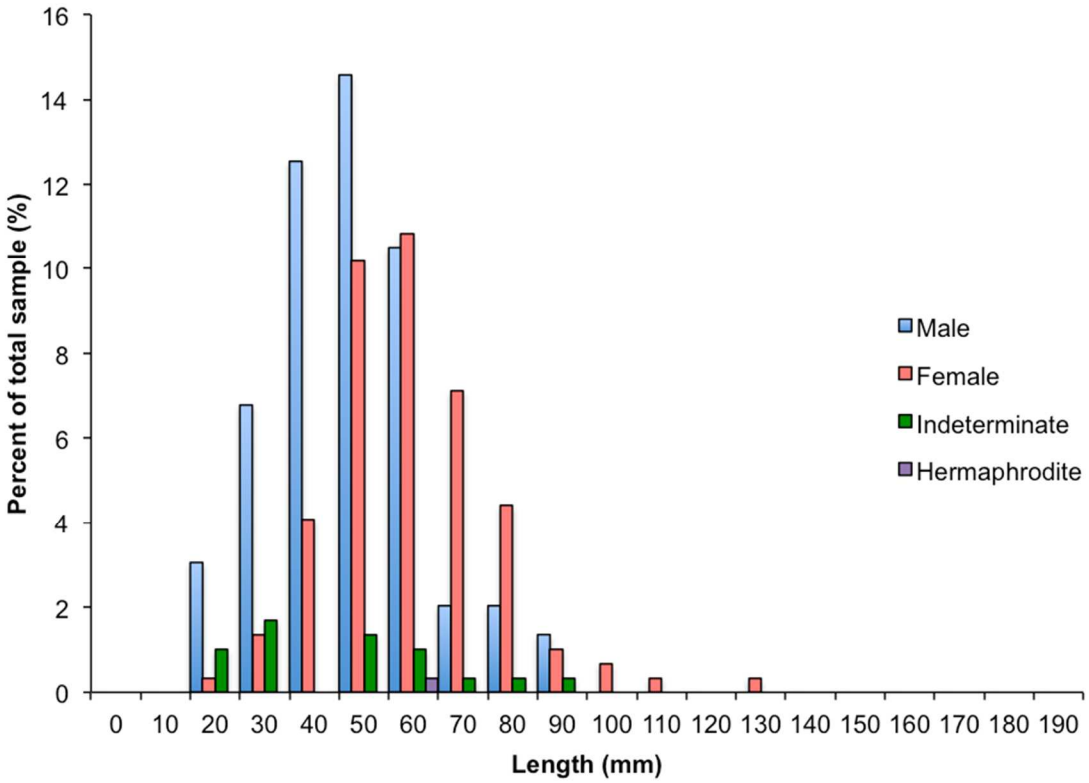

Figure 11. Percentage of the total sample of *Bathymodiolus childressi* mussels in each body length size class for males, females, indeterminate, and hermaphroditic individuals collected from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015).

Table 4. Results of a two-way ANOVA on *Bathymodiolus childressi* body length for samples collected from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015).

Response: Length						
	df	SS	MS	F value	p-value	Partial Eta Sq
Sex	2	10641	5320.6	27.052	< **0.001**	0.177
Site	11	17010	1546.3	7.862	< **0.001**	0.256
Sex:Site	10	1831	183.1	0.931	0.505	0.036
Residuals	252	49563	196.7			

Discussion

Reproductive Periodicity and Phytoplankton Connection

Bathymodiolus childressi is gonochoric and has synchronous periodic reproduction, confirming previously published results by Tyler *et al*. (2006). Most previous studies (e.g. Eckelbarger & Young, 1999; Tyler *et al*., 2006; Arellano &Young, 2009) on the reproduction of *B. childressi* used specimens from the Brine Pool NR1 site and/or sites in the Garden Banks and Bush Hill region of the Gulf of Mexico (all on the western side), but I found gonochorism and synchronous periodic reproduction at all 5 sites in the Gulf of Mexico and 6 sites along the Western Atlantic Margin, spanning a depth range of 320 m to 2200 m. Thus, the hypothesis that deeper populations of *B. childressi* would tend toward continuous or asynchronous reproduction was not supported. A seasonal or periodic reproductive pattern may be counter-intuitive for organisms living in a relatively stable environment with a constant chemosynthetic food source (Tyler & Young, 1992); however, it may be adaptive for species with planktotrophic larvae, as phytoplankton blooms are seasonal. In fact, the widely accepted Crisp's rule states that the breeding periodicity of marine organisms with planktotrophic larvae is likely timed so that the larvae hatch during the season with the most planktonic food (Qasim, 1956).

Regional geographic synchronicity occurs among sites (Figures 7 and 8). This geographic synchrony indicates the mussels rely on a site-independent cue to synchronize their reproduction, i.e. a cue that is present at all sites and not specifically related to any methane seep. One potential example is a seasonal phytoplankton bloom in the surface waters over the general extended study area. Tyler *et al*. (2006), in their initial description of gametogenesis in *B. childressi*, reported a definitive relationship between the reproductive seasonality of the Brine Pool population and phytodetrital production. The authors postulated that nitrogen, available from surface-derived detritus (but not from methane), could be the limiting factor for the mussels' production of gametes. Dixon *et al*. (2006) also found evidence of seasonal reproduction in the hydrothermal vent species *B. azoricus* that statistically correlated with a local phytoplankton bloom.

My data also support the role of a temporal cue, possibly related to surface production, in synchronizing *Bathymodiolus childressi* reproduction at methane seeps. All mussel populations sampled in this study had periodic, synchronous reproduction, yet there are some differences in reproductive patterns among sites that may be controlled by a combination of depth and geographic location, though the observed trends are neither clear nor obvious. Lightfoot *et al*. (1979) proposed the hypothesis that surface-derived phytodetritus could act as a reproductive cue for bathyal species. Deeper sites may experience a phytodetritus pulse later in the season than shallower sites. Phytodetrital aggregates can sink at a rate of about 125 m per day (Gooday & Turley, 1990), meaning the shallowest sites would have access to this food source and possible reproductive cue mere days after the surface phytoplankton bloom starts to die, while the deeper sites would not receive the food pulse until about a month later.

Mussels at the northernmost sites had smaller mean oocyte sizes (Figure 9) and few individuals in later maturity stages (Tables 3) then more southern sites. The northwestern Atlantic Ocean experiences its largest phytoplankton bloom in September – January (Yoder *et al*., 2000), while the Gulf of Mexico experiences its largest bloom in December through February (Müller-Karger *et al*., 1991) (Figure 12). It is possible the earlier phytoplankton bloom causes northern Atlantic mussels to spawn earlier in the year.

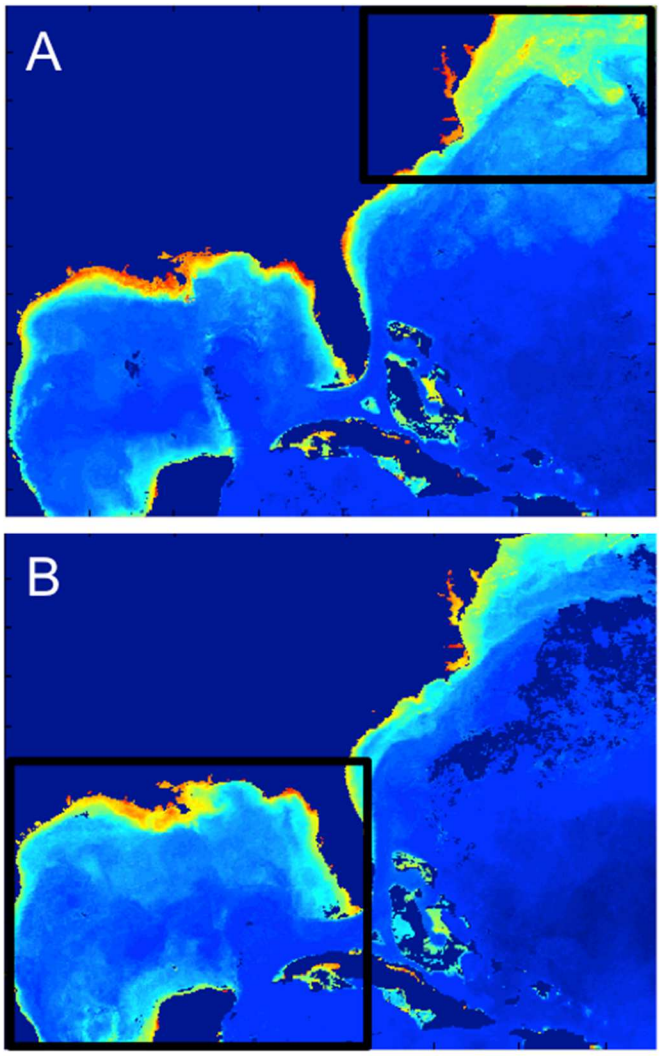

Figure 12. Chlorophyll a concentration maps for the study area in 2014. Warmer colors represent higher concentrations than cooler colors. A, October. B, January. Black boxes highlight the region with the largest bloom. Data were obtained from the NASA Ocean Color website

The results of this study lend support to the idea that *Bathymodiolus* mussels in chemosynthetic systems rely on an increase in phytodetrital food as a reproductive cue. Many studies have shown nitrogen and fatty acids to be essential for gametogenesis (e.g. Rodegker, 1964; Kluytmans *et al*., 1985; Page *et al*., 1991). Food availability for planktotrophic larvae has also been shown to control recruitment success (reviewed by Olson & Olson, 1989), lending further support to the use of phytodetritus as a spawning cue for deep-sea chemosymbiotic mussels.

Body Length

Bathymodiolus childressi males reach sexual maturity at a smaller body size and likely younger age than females. Females also appear to reach larger maximum body sizes than males (Figure 11). This type of sexual dimorphism is rare in bivalve molluscs (Mackie & Wilbur, 1984). The one hermaphrodite individual found was in an intermediate size class that also contained male and female individuals. A few hermaphroditic individuals have been seen before in *B. childressi* (Tyler *et al*., 2006) and it is not uncommon for gonochoric molluscan species to have a few putative hermaphroditic individuals (Mackie & Wilbur, 1984).

Indeterminate individuals lacked gonad tissue when dissected, possibly due to sexual immaturity, but their presence across most body length size classes points toward other possible causes. Disease or parasites could lead to a lack or reduction of gonad tissue. A *Bucephalus*-like trematode parasite and a *Chlamydia*-like bacterial organism have both been reported to parasitize the gonads of *B. childressi* (Tyler *et al*., 2006),

though in both cases the parasites were visible in their histological preparations and no parasites were seen in this study.

At the Brine Pool NR1 site, *B. childressi* is known to live in groups of distinct size classes (MacDonald *et al*., 1990; Smith *et al*., 2000; Arellano & Young, 2010) and this could be true for other sites as well. The statistically significant effect of site on body length is likely an artificial result of the sampling method since only a single group of mussels was collected from each site. Alternatively, there could be variations in the relative ages of the mussel communities at each seep site. Since mussels increase in size with age, older communities may have more large mussels than younger communities, resulting in size differences between sites.

Conclusions

Bathymodiolus childressi is a gonochoric species with uncommon examples of hermaphroditism. This species has a seasonal and synchronous reproductive cycle at populations throughout its full bathymetric range (320 to 2200 m). Most of the mussels in this study were in a period of gametic growth and proliferation (Stages 2 – 4) and were collected during the summer. These results are consistent with the reproductive season described in Tyler *et al*. (2006). Seasonality of reproduction in *B. childressi* could be tied to surface productivity, and it may be affected by both depth (temperature and pressure) and geographic location (latitude and food availability). Future studies should focus on determining the exact nature of the reproductive cue.

CHAPTER III

REPRODUCTIVE PATTERNS IN FOUR CONGENER COLD-SEEP MUSSELS

Introduction

Bathymodiolin mussels (family Mytilidae, subfamily Bathymodiolinae, genus *Bathymodiolus*) are dominant members of megafaunal communities at hydrothermal vents and cold seeps (MacDonald *et al*., 1990). All species have chemosymbiotic bacteria that provide nutrients to the mussels by reducing the methane and/or hydrogen sulfide produced and seeps and vents (Fiala-Medioni *et al*., 1986; Fisher *et al*., 1987). Some species are also known to filter feed on photosynthetically produced organic matter (Page *et al*., 1990, 1991; Pile & Young, 1999; Dixon *et al*., 2006).

While the chemosymbiosis and feeding of these mussels have been studied, along with their spatial distributions (see MacDonald *et al*., 1990; Smith *et al*., 2000; Bergquist *et al*., 2005), little is known about their reproduction. Hydrothermal vent species *Bathymodiolus azoricus* (Colaço *et al*., 2006; Dixon *et al*., 2006), *B. puteoserpentis* (Hessler *et al*., 1988), *B. elongates* (Le Pennec & Beninger, 1997), and *B. thermophilus* (Berg, 1985) all have egg and larval shell sizes indicative of planktotrophy. Two of these species, *B. azoricus* (Comtet & Desbruyères, 1998; Colaço *et al*., 2006) and *B. puteoserpentis* (Hessler *et al*., 1988) are also known to have seasonal gametogenic cycles.

Reproduction of the cold-seep species *Bathymodiolus childressi* is well known. Gametogenesis in this species resembles that of its shallow-water mytilid relatives (Eckelbarger & Young, 1999), and its larvae have been confirmed as planktotrophic by shell growth in wild populations, though larvae have never been observed to feed in laboratory culture (Arellano & Young, 2009). *Bathymodiolus childressi* has a seasonal

gametogenic cycle and spawns in the winter (Tyler *et al*., 2006). Comparisons of populations from its full bathymetric range of 320 to 2200 m showed similar seasonal reproduction at all depths, though there appeared to be some geographic differences in timing (Chapter II).

The present study aims to (i) elucidate the reproductive patterns of three other bathymodiolin mussels species from the Gulf of Mexico and the northern Western Atlantic Margin: *Bathymodiolus brooksi, B. heckerae* (Gustafson *et al*., 1998) and an undescribed species *B. sp. nov.*, and (ii) compare these species to *B. childressi*.

Nothing is known about the reproduction of *B. brooksi*, *B. heckerae*, or *B. sp. nov.*, though *B. heckerae* is thought to have planktotrophic larvae based on larval shell sizes (Turner & Lutz, 1984; Gustafson & Lutz, 1994). Of the three described species, *B. childressi* has the shallowest distribution and the broadest bathymetric range (320 – 2267 m) (Chapter II; Gustafson *et al*., 1998). *Bathymodiolus brooksi* is found deeper (2222 – 3313 m), and *B. heckerae* occurs only at deep seeps (3313 m) (Gustafson *et al*., 1998). *Bathymodiolus childressi* and *B. brooksi* occur at water temperatures of 4.25 – 6.3^{o} C. No temperature range for *B. heckerae* has been reported, but it has been previously found at the Florida Escarpment (Gustafson *et al*., 1998) and Blake Ridge (Van Dover *et al*., 2003) seep sites, which have temperatures of 4.4 and 3.0 ^{o}C respectively (present study). *Bathymodiolus sp. nov.* was discovered only recently at the DC-583 site in the Gulf of Mexico (C. Fisher, Penn. State Univ., unpublished data). Since *B. childressi* reproduces seasonally at all depths and there is some evidence that vent bathymodiolin mussels do as well, I hypothesized that *B. brooksi*, *B. heckerae*, and *B. sp. nov.* also reproduce seasonally with a pattern similar to that of *B. childressi*.

Bathymodiolus childressi larvae are planktotrophic, and some have been found in surface plankton tows (Arellano *et al*., 2014). Larvae from eggs spawned at deeper sites would have farther to travel to the surface, where planktonic food is more abundant (Young *et al*., 1996). Therefore, I also hypothesized that deeper living species will have larger egg sizes (to provide enough energy for their larvae to migrate to a depth with sufficient food) than their shallower conspecifics.

Materials and Methods

Sample Collection and Preservation

A total of 157 mussels were collected from known methane seeps in the Gulf of Mexico (32 *Bathymodiolus heckerae*, 87 *B. brooksi*, and 10 *B. sp. nov.*) during May/June 2014 and along the Western Atlantic Margin (28 *B. heckerae*) in July 2015 (Table 5, Figure 13). Clumps of mussels were collected using the manipulator arm of DSV *Alvin* and brought to the surface in a closed Plexiglas box. Once on board the support vessel R/V *Atlantis*, mussels were identified morphologically and removed from their shells (identifications were later verified by colleagues using molecular techniques). The soft tissue was preserved in 10% buffered formalin and shipped to the Oregon Institute of Marine Biology (OIMB) in Charleston, OR. After 8 – 10 months, samples were rinsed with RO water and transferred to 70% ethanol.

Histological Analysis

Samples were processed for histology using the same methods as Chapter II and stained with hematoxylin and eosin to enable visualization of eggs and sperm (Galigher & Kozloff, 1971; see Chapter II).

Table 5. Bathymodiolin mussels collected from the Gulf of Mexico (GoM) and the Western Atlantic Margin (WAM). "Indeterminate" refers to individuals lacking gonad tissue.

Depth (m)	Site	Basin	Latitude (ºN)	Longitude (ºW)	Temp (ºC)	Species Present	Date Sampled	N	% Male	% Female	% Indeterminate
1070	MC853	GoM	28.1242	89.1414	4.3	*B. brooksi*	May 23, 2014	17	35.3	58.8	5.9
2160	Blake Ridge	WAM	32.4950	76.1910	3.0	*B. heckerae*	July 9-12, 2015	26	38.5	53.8	7.7
2200	AC601	GoM	26.3197	94.5150	4.3	*B. brooksi*	May 30, 2014	20	65.0	25.0	10.0
2500	DC583	GoM	28.3915	87.3889	4.3	*B. sp. nov.*	June 6-7, 2014	10	80.0	20.0	0.0
3300	Florida Escarpment	GoM	26.0289	84.9113	4.4	*B. brooksi*	June 10-11, 2014	5	40.0	60.0	0.0
						B. heckerae	June 10-11, 2014	33	51.5	27.3	21.2

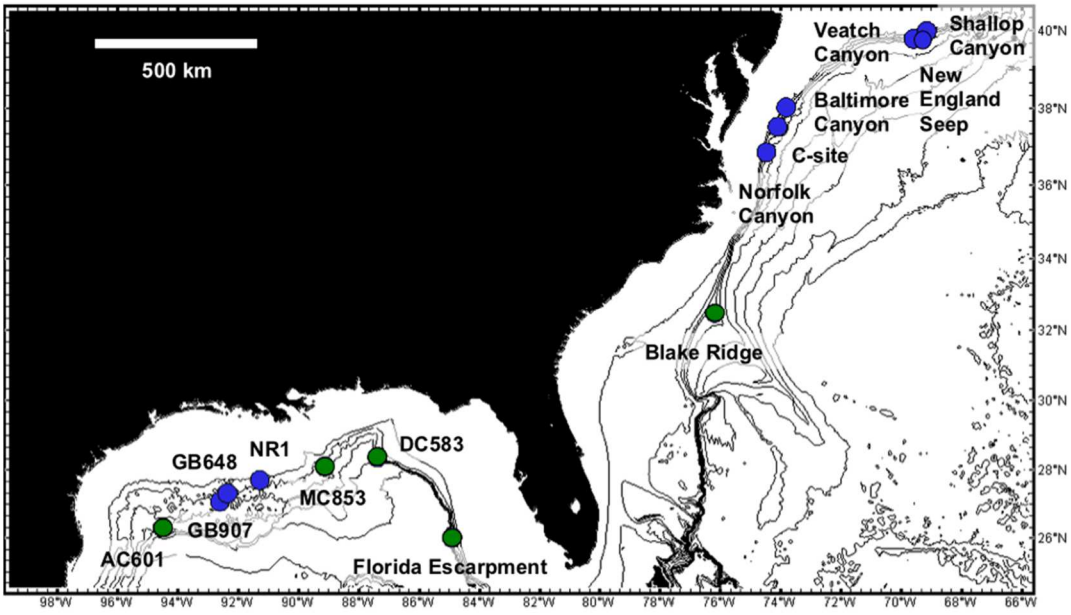

Figure 13. Collection sites for bathymodiolin mussels in the Gulf of Mexico (summer 2014) and along the Western Atlantic Margin (summer 2015). Green circles indicate sites used in this study and blue circles indicate sites from Chapter II and are displayed for comparative purposes. Longitude is shown along the bottom axis and latitude along the right axis. Depth contours represent 500 m.

Maturity Stages

Maturity stages were assigned for male mussels in this study based on those used for *B. childressi* in Chapter II (Tyler *et al*., 2006). See Chapter II for full definitions of stages and for pictorial examples.

Stage 1: Initiation of gametogenesis

Stages 2–4: Proliferation and growth of gametes

Stage 5: Ripe individual, ready to spawn

Stage 0: Post-spawn.

Oocyte Diameters

Histological sections of each female were photographed and oocyte feret diameters were measured using the same methods as in Chapter II.

Statistical Analysis

Oocyte size and maturity data from *Bathymodiolus childressi* (Chapter II) were included in all statistical analyses in this chapter for comparisons among species. Oocyte size data violated the assumptions of normality (Shapiro-Wilk test) and homogenous variances (Levene's test), so non-parametric statistics were used. A Scheirer-Ray-Hare test (non-parametric equivalent of a 2-way ANOVA) was performed to determine if oocyte diameters differed significantly by site, species, or their interaction. For sites that had two co-occurring species, Student's t-tests or Mann-Whitney U tests (in cases of heterogeneous variances) were used to determine if the mean or median oocyte sizes for the two species differed significantly. These tests were all performed in the statistical software package RStudio version 1.0.136 (R Core Team, 2016).

Primer version 6 (Clarke & Gorley, 2006) was used to compare oocyte diameters among regions and with environmental factors of temperature, latitude, longitude, and depth. A fourth-root transformation was used to balance the data among sites, and then used to create a Bray-Curtis similarity matrix. A 2-way ANOSIM of depth nested within region was performed to test the differences in oocyte sizes among sites and a non-metric multi-dimensional scaling plot (nMDS) was created to show the relationship between sites based on their depth and region. A BEST (Bio-Env) routine was run with normalized physical factors (temperature, latitude, longitude, and depth) to test whether these factors correlate with the oocyte sizes.

A chi-squared contingency table analysis was also performed in RStudio to test the differences in the relative distribution of male maturity stages among sites.

Results

All species used in this study, *Bathymodiolus brooksi, B. heckerae* and *B. sp. nov.*, were found to be gonochoric. No hermaphrodite individuals were found in any of these species. I also found evidence for synchronous, periodic reproduction in all three species that aligns with the seasonal patterns described for *B. childressi* as described by Tyler *et al*. (2006) and in Chapter II.

Differences in Diameter

Oocyte ferret diameters differed significantly among sites (Table 6; Scheirer-Ray-Hare, $H = 15.367$, $p < 0.001$, $N = 149$), but not among species ($H = 1.012$, $p = 0.314$, $N = 149$). The interaction of site and species was also not significant ($H = 0.003$, $p = 0.953$, $N = 149$). Since sites varied in physical location as well as depth and their associated factors (temperature and pressure), a nMDS plot was created to show the relationship between sites based on their depth and region (accounts for both longitude and latitude), using oocyte size-frequency data (Figure 14). The plot has a fairly low stress value (0.09) meaning it adequately shows the underlying pattern, but there were no clear groupings based on depth or region. The results of a 2-way ANOSIM of depth nested within region showed region to be marginally significant ($R = 0.212$, $p = 0.057$). The best correlation of environmental factors to oocyte sizes (BEST, BIO-ENV) was a combination of depth, latitude, and longitude, but only had a Spearmen correlation value of $\rho = 0.199$.

Table 6. Results of a Scheirer-Ray-Hare test for the effects of site, species, and their interaction on oocyte sizes of bathymodiolin mussels collected from the Gulf of Mexico (2014) and from the northern Western Atlantic Margin (2015).

	SS	H	df	p-value
Site	28200	15.370	1	**<0.001**
Species	1860	1.010	1	0.314
Interaction	6.520	0.003	1	0.953

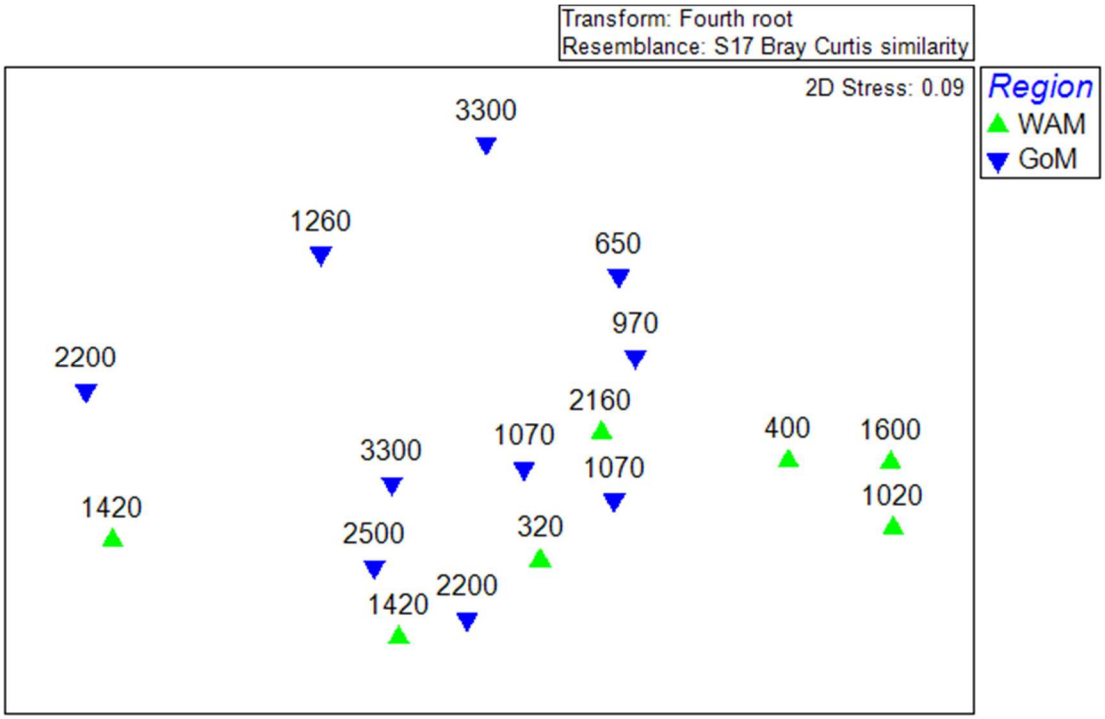

Figure 14. nMDS plot showing the relationships among sites based on oocyte size-frequency data from bathymodiolin mussels collected from the Gulf of Mexico (GoM) in 2014 and the northern Western Atlantic Margin (WAM) in 2015.

Co-occurring Species

Three of the 5 locations sampled contained two different species (Figure 15), but no site had more than two. Both *Bathymodiolus childressi* and *B. brooksi* were collected at MC853 in the Gulf of Mexico (1070 m depth). There was no significant difference

between oocyte diameters in *B. childressi* ($\bar{x}$ = 22.94 µm ± 2.02 SE, N = 8) and *B. brooksi* ($\bar{x}$ = 25.76 µm ± 3.41 SE, N = 10) at this site (Student's t-test, t = -0.67, df = 16, p = 0.514, N = 18). These two species also co-occurred at AC601 in the Gulf of Mexico (2200 m depth) but again had no significant difference in oocyte diameter (*B. childressi* $\bar{x}$ =20.49 µm ± 3.29 SE, N = 9; *B. brooksi* $\bar{x}$ = 16.89 µm ± 0.81 SE, N = 5; Mann-Whitney U = 34, p = 0.309, N = 14). At the Florida Escarpment in the Gulf of Mexico (3300 m depth), *B. brooksi* and *B. heckerae* co-occurred, and there was a significant difference between *B. brooksi* ($\bar{x}$ = 22.95 µm ± 1.65 SE, N = 3) and *B. heckerae* ($\bar{x}$ = 19.10 µm ± 0.78 SE, N = 9) oocyte diameters at this site (t-test, t = -2.35, df = 10, p = 0.041, N = 12).

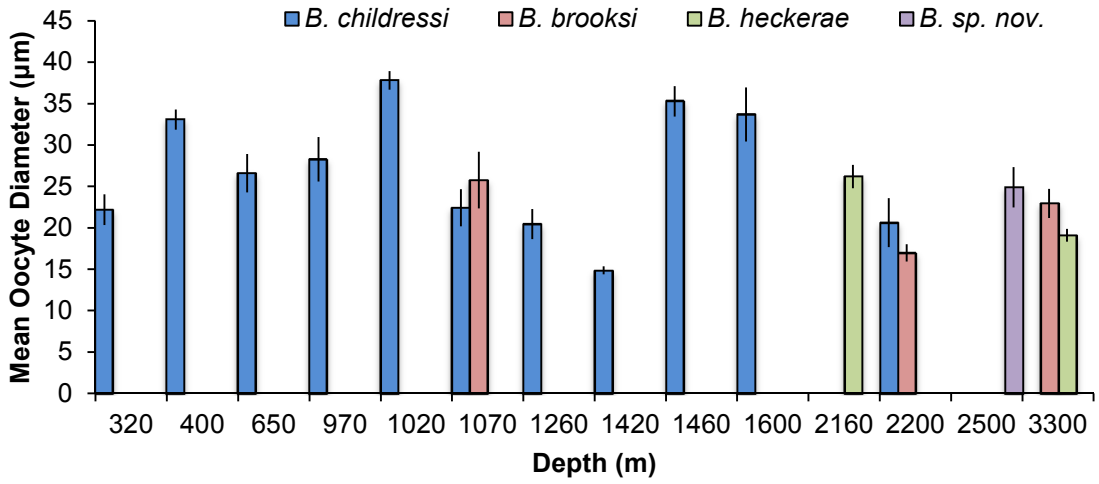

Figure 15. Mean oocyte feret diameters for each depth, color-coded by species. Error bars represent ± 1 standard error.

Reproductive Periodicity

Using the framework discussed in Chapter I, Figure 1, I found that populations of *Bathymodiolus brooksi, B. heckerae,* and *B. sp. nov.* all had synchronous and apparently seasonal gametogenic cycles, based on mean oocyte sizes for each depth (Figure 15), individual size-frequency distributions of oocyte sizes (Figure 16), and average size-

frequency histograms for each site (Figure 17). For sites where *B. childressi* was also present, those data are included on graphs for comparative purposes.

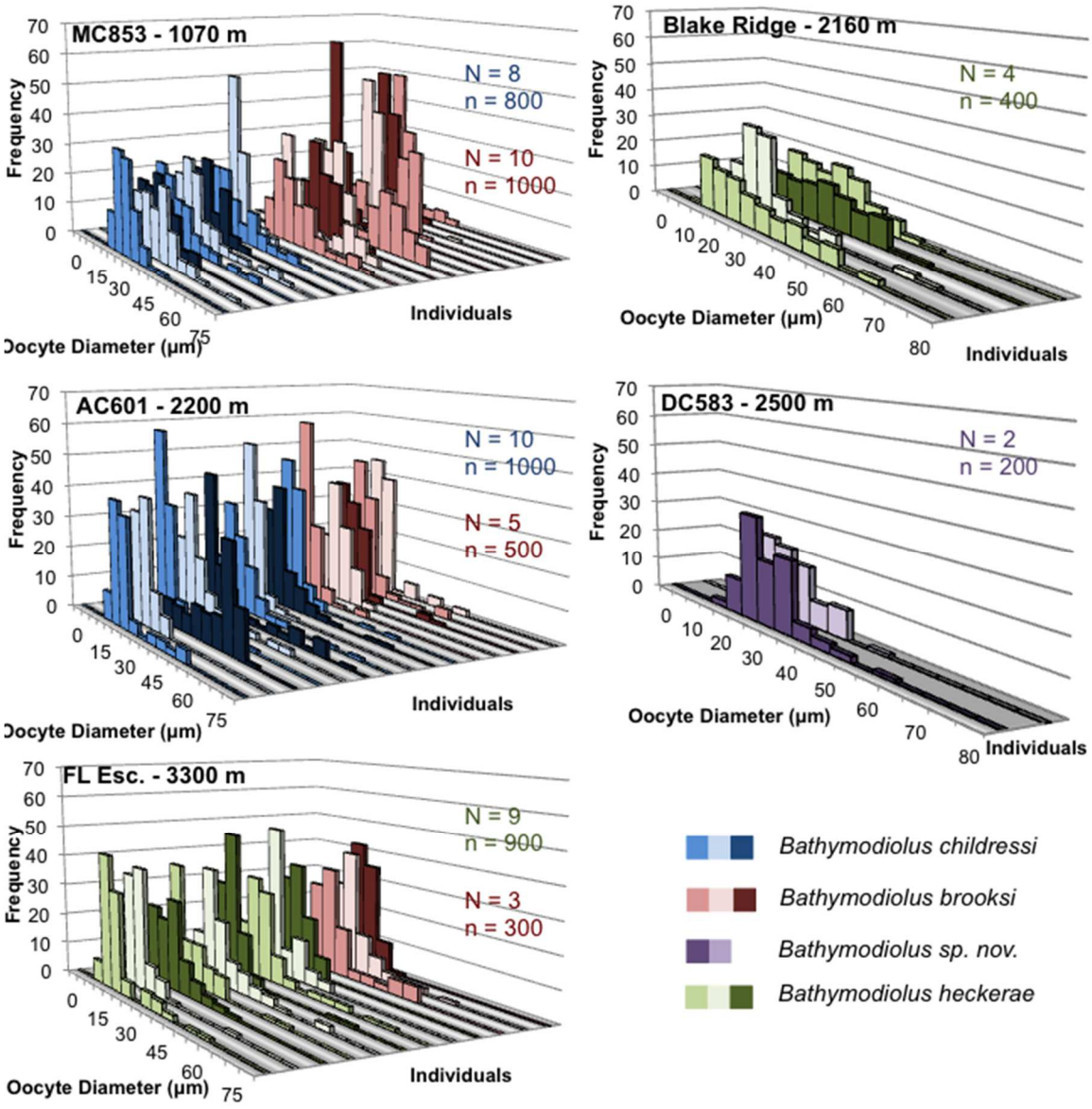

Figure 16. Individual oocyte size-frequency distributions, grouped by site, for bathymodiolin mussels collected from the Gulf of Mexico (2014) and from the northern Western Atlantic Margin (2015). Different shades are used to help distinguish among individual mussels. N = number of individuals sampled, n = number of oocytes measured.

Mean oocyte diameter varied across depths, but all sites had narrow standard errors, ranging from 0.47 (*B. childressi*, 1420 m) to 3.41 (*B. brooksi*, 1070 m). Means ranged from 14.86 μm (*B. childressi*, 1420 m) to 37.82 μm (*B. childressi*, 1020 m), and the overall maximum oocyte diameter was ~80 μm.

Every mussel individual had a size-frequency distribution of oocyte feret diameter indicative of periodic reproduction (Figure 16). A few individuals at Blake Ridge (WAM, 2160 m), AC601 (GoM, 2200 m), and Florida Escarpment (GoM, 3300 m) had a fairly wide distribution of oocyte sizes, but most individuals had a narrow unimodal distribution. While there was some variation among individuals within sites, the shape of each individual distribution was similar, and the frequency peaks occurred in approximately the same size class (Figure 16). These results indicate reproductive synchronicity within each population. Interestingly, *Bathymodiolus heckerae* at Blake Ridge (WAM, 2160 m) and the Florida Escarpment (GoM, 3300 m) had different size-frequency distributions. At sites where *B. childressi* co-occurred with *B. brooksi* (MC853 and AC601, both in GoM), individuals of both species had very similar size-frequency distributions (Figure 16). This was also true for the Florida Escarpment, where *B. heckerae* co-occurred with *B. brooksi,* indicating synchronicity between species.

Average oocyte size-frequency distributions for each site indicate synchronous and periodic reproduction while also showing some geographic synchronicity (Figure 17). For example, MC853 (GoM, 1070 m) and AC601 (GoM, 2200 m) are geographically close to each other, and both have communities of *Bathymodiolus brooksi* co-occurring with communities of *B. childressi*. Both species had very similar oocyte size-frequency distributions with a peak around 15-20 μm. While the individual size-

frequency distributions for *B. heckerae* were slightly different between Blake Ridge and the Florida Escarpment, the population size-frequencies (Figure 17) were very similar, with oocyte sizes ranging from 10 µm to ~70 µm and a peak around 20 µm.

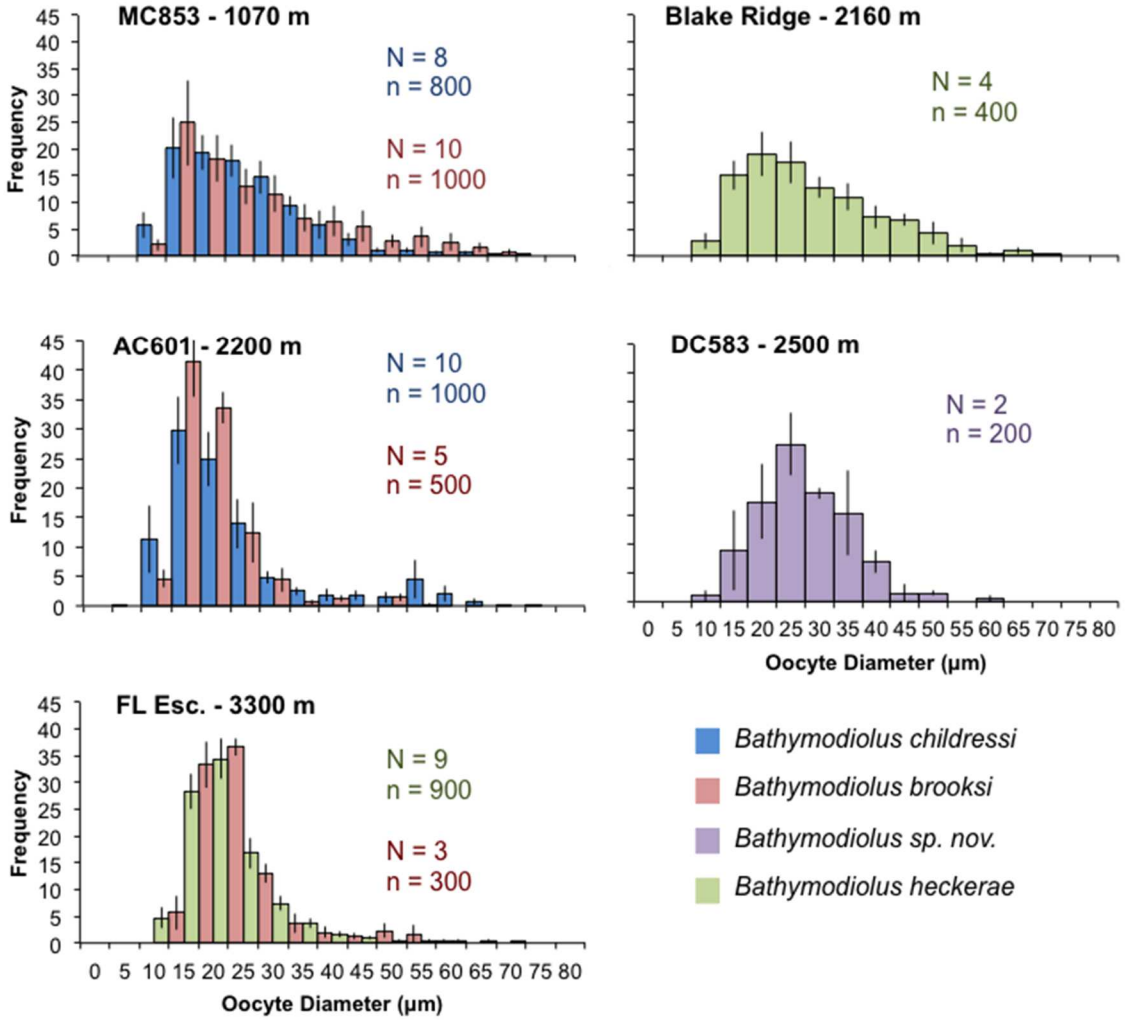

Figure 17. Average oocyte size frequency distributions of bathymodiolin mussels collected from the Gulf of Mexico (2014) and the northern Western Atlantic Margin (2015). Error bars represent standard error. N = number of individuals sampled, n = number of oocytes measured.

Maturity

Maturity stages are reported only for males because female reproductive patterns have been well-described using oocyte diameters. Individuals at almost every maturity

stage were found in all three species (Table 7). Since females showed no significant within-species variability in oocyte feret diameters, male maturity stage frequencies were summed and plotted for each species (Figure 18). A chi-square contingency table analysis showed significant differences in the relative maturity stage distributions among species ($\chi^2 = 208.93$, df = 15, $p < 0.010$). Both the graph and the chi-square contingency table (residual z-scores in Table 8) showed that the deeper-living species, *Bathymodiolus brooksi* and *B. heckerae*, had more early (stage 1 – initiation of gametogenesis) and late (stage 0 – post spawning) stages than the shallower *B. childressi* and *B. sp. nov.*

The contingency table also shows that *B. brooksi* had fewer individuals in stages 3 and 4 than expected, while *B. childressi* had more individuals in stage 4 than expected and *B. sp. nov.* had more individuals in stage 3, but fewer in stage 4 than expected.

Table 7. Number of male bathymodiolin mussel individuals in each maturity stage for each collection site from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015).

				Testis development stage						
Species	Site	Region	Depth (m)	1	2	3	4	5	0	N
Bathymodiolus brooksi	MC853	GoM	1070	2	1	1	0	0	0	4
	AC601	GoM	2200	6	1	1	0	0	4	12
	FL Esc.	GoM	3300	1	1	0	0	0	0	2
			Totals:	9	3	2	0	0	4	18
Bathymodiolus heckerae	Blake Ridge	WAM	2160	1	5	1	0	0	3	10
	FL Esc.	GoM	3300	9	1	6	1	0	0	17
			Totals:	10	6	7	1	0	3	27
Bathymodiolus sp. nov.	DC583	GoM	2500	0	2	6	0	0	0	8
			Totals:	0	2	6	0	0	0	8

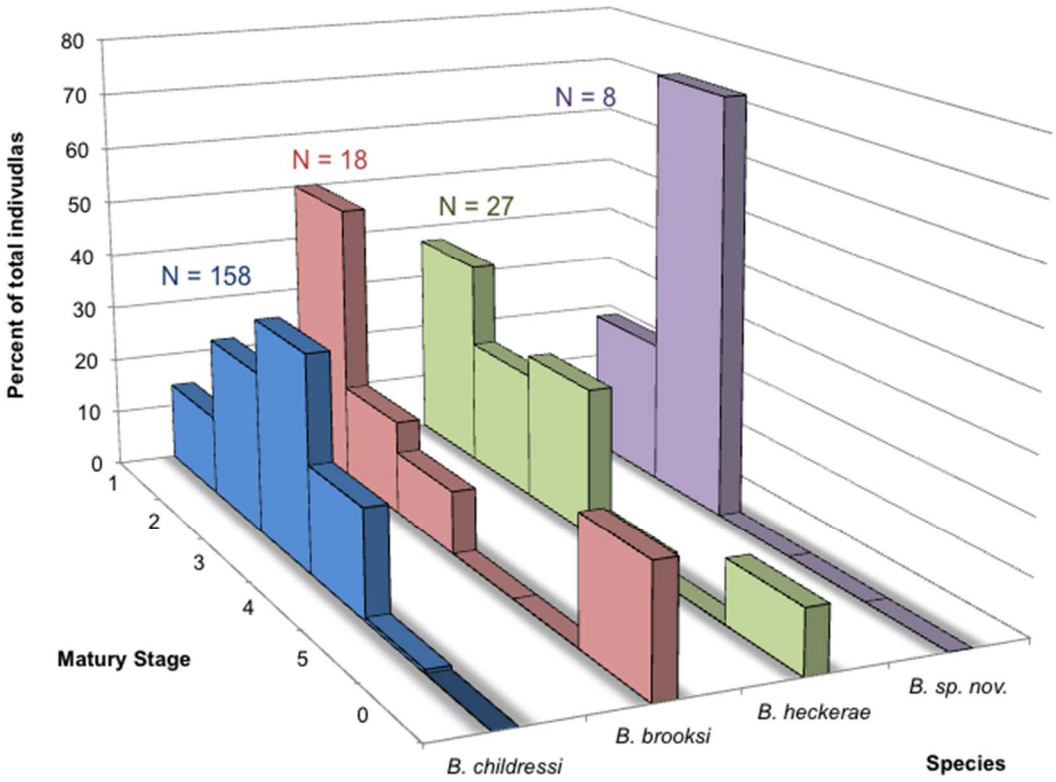

Figure 18. Frequency distributions of male maturity stages for each species of bathymodiolin mussels collected from the Gulf of Mexico (2014) and the Western Atlantic Margin (2015). N = number of individuals sampled.

Table 8. Z-score residuals from a chi-square contingency test on the relative distributions of male maturity stages for each species of bathymodiolin mussel collected from the Gulf of Mexico (2014) and the northern Western Atlantic Margin (2015). Bold-faced values are statistically significant and ** means $p < 0.01$.

Species	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 0
Bathymodiolus childressi	**-2.25****	1.12	0.08	**5.59****	1.19	**-2.89****
Bathymodiolus brooksi	**4.93****	-1.34	**-4.31****	**-2.38****	-0.40	**4.81****
Bathymodiolus heckerae	**2.35****	-0.18	-1.89	-0.83	-0.40	0.96
Bathymodiolus sp. nov.	**-5.02****	0.40	**6.12****	**-2.38****	-0.40	**-2.89****

Oocyte Sizes

Few of the females sampled were mature, making it impossible to calculate a mean mature oocyte size. Instead the range of maximum oocyte sizes seen at each site was compared with values for *Bathymodiolus childressi* and other bathymodiolin and

mytilid species (Table 9). Maximum oocyte sizes were very similar for all *Bathymodiolus* species at all sites in this study. Oocyte diameters were also similar among bathymodiolin species from vents and seeps and in comparison to shallow-water *Mytilus edulis*.

Table 9. Ranges of maximum oocyte feret diameters for the three *Bathymodiolus* species in this study, compared with those for *B. childressi* from Chapter II and published values for various bathymodiolin mussels and *Mytilus edulis*.

Species	Habitat	Reproduction	Depth (m)	Max diameter (μm)	Source
Bathymodiolus brooksi	Cold Seeps	Gonochoric, seasonal reproduction	1070	45 - 54	Present Study
			2200	48 - 50	
			3300	54 - 55	
Bathymodiolus heckerae	Cold Seeps	Gonochoric, seasonal reproduction	2160	50 - 65	
			3300	46 - 67	
Bathymodiolus sp. nov.	Cold seeps	Gonochoric, seasonal reproduction	2500	45 - 57	
Bathymodiolus childressi	Cold Seeps	Gonochoric, seasonal reproduction	320	53 - 67	Chapter II
			400	46 - 67	
			650	41 - 77	
			970	51 - 78	
			1020	54 - 72	
			1070	43 - 71	
			1260	40 - 70	
			1420	40 - 67	
			1460	60 - 76	
			2200	52 - 68	
B. childressi	Cold Seeps	Gonochoric, seasonal	650	50 - 80	Tyler et al., 2006
B. azoricus	Hydrothermal vents	Seasonal?	850	70 - 80	Colaço et al., 2006
B. puteoserpentis	Hydrothermal vents	Unknown	3450	50 - 60	Hessler et al., 1988
B. elongates	Hydrothermal vents	Unknown	~1900	50 - 60	Le Pennec and Beninger, 1997
B. thermophilus	Hydrothermal vents	Unknown		~50	Berg, 1985
Mytilus edulis	Intertidal	Gonochoric, seasonal	0	60 - 90	Lutz & Kennish, 1992

Discussion

Reproductive Periodicity

The results of this study show that three species of bathymodiolin mussels from cold seeps, *Bathymodiolus brooksi, B. heckerae., and B. sp. nov.*, are all gonochoric and have seasonal, periodic reproductive cycles. A seasonal or periodic reproductive pattern is counter-intuitive for organisms living in a relatively stable environment with a constant chemosynthetic food source (Tyler & Young, 1992); however, it may be adaptive for species with planktotrophic larvae, as phytoplankton blooms are seasonal.

The geographic synchronicity seen among sites indicate the mussels are relying on a site-independent cue to synchronize their reproduction, i.e. a cue that is present at all sites and not specifically related to any methane seep, such as a seasonal phytoplankton bloom in the surface waters over the study area. It has previously been hypothesized that surface productivity could control seasonal reproduction in *Bathymodiolus childressi* (Tyler *et al*., 2006; see Chapter II) and the vent species *B. azoricus* (Dixon *et al*., 2006). The results of the present study support the role of a temporal cue, possibly related to surface production, in synchronizing *Bathymodiolus* reproduction at methane seeps.

Environmental Factors

The lack of strong correlation between oocyte size data and the environmental factors of depth, temperature, longitude, latitude, and region, point to a different environmental cue influencing the synchrony of gametogenesis. A combination of depth and location provided the strongest explanation for variation in the oocyte size data, and this result could lend further support to the hypothesis that phytodetrital flux is acting as a reproductive cue for bathymodiolin mussels. The Western Atlantic Margin experiences a

surface phytoplankton bloom slightly before the Gulf of Mexico (Friedland *et al*., 2015) and has shallower sites that would likely receive sinking phytodetritus earlier than the deeper Gulf of Mexico sites, though this would depend on current speeds.

Oocyte Sizes

Even though not all of the mussels sampled were mature, their maximum oocyte feret diameters of free oocytes give a good indication of what their egg size would be since these oocytes are ready to be spawned (Tyler *et al*., 2006; Arellano & Young 2009). All four species of bathymodiolin mussels found in the Gulf of Mexico (two are also found along the northern part of the Western Atlantic Margin) had similar maximum oocyte sizes with no trend across depth. These oocyte sizes are indicative of planktotrophic larvae (Thorson, 1950), as in all previously studied *Bathymodiolus* species (Chapter I). Maximum oocyte diameters are also similar among other *Bathymodiolus* species from hydrothermal vents and even the intertidal *Mytilus edulis,* suggesting that egg size is phylogenetically constrained within the genus *Bathymodiolus* and possibly across mytilids. Eckelbarger and Watling (1995) showed that certain aspects of reproduction, such as egg size, are often phylogenetically constrained as a result of the metabolic processes creating the egg, for many deep-sea invertebrates. Gustafson and Lutz (1994) and Bouchet and Warén (1994) also showed that developmental strategies of deep-sea molluscs are phylogenetically constrained.

Conclusions

Bathymodiolus brooksi, B. heckerae, and the undescribed *B. sp. nov.* are all gonochoric and have a synchronous seasonal reproductive cycle. This seasonal pattern aligns with that seen in *B. childressi* from seeps in the same areas and suggests the

influence of surface phytoplankton production. However, more research is needed to elucidate exactly how these mussels may be using phytodetritus as a reproductive cue.

Egg size may be phylogenetically constrained within the genus *Bathymodiolus* and possibly within the larger mytilid family. An investigation into the ultrastructure of gametogenesis for each of these species would be needed to determine if the metabolic processes responsible for the production of gametes are similar.

CHAPTER IV

GENERAL CONCLUSIONS

"Snap-shot" Framework

Most deep-sea reproductive studies looking at periodicity are conducted with seasonal sampling, and while that method would have provided more information about the timing of the gametogenic cycle as well as spawning times, the "snap-shot" sampling method used here still produced valuable new information. Based on previously published reproductive studies of deep-sea animals, there are distinct patterns in oocyte size-frequency distributions indicative of each reproductive mode (continuous vs. periodic, and asynchronous vs. synchronous) as explained in the Framework section of Chapter I. These previously established patterns have allowed me to infer reproductive mode (synchronous periodicity) from single time-point samples. Since deep-sea samples are logistically and financially difficult to obtain, more single time-point studies of this nature could be used to gain a basic insight into an organism's reproductive biology and then used to develop more targeted sampling plans.

Study Goals

There were three main objectives for this thesis: i) compare the reproductive patterns of *Bathymodiolus childressi* across a depth gradient of 320 m to 2200 m, ii) investigate the reproductive patterns of three other cold seep bathymodiolin mussels: *B. brooksi*, *B. heckerae* and an undescribed species, *B. sp. nov.*, and iii) compare the patterns among species.

My original hypotheses were that deeper populations of bathymodiolin mussels would show increased tendency toward continuous or asynchronous reproduction than

the shallower populations. I also hypothesized that species found at deeper sites would have larger egg sizes to provide more of a nutrient reserve for their larvae to use on their longer trek to food-abundant surface waters.

Major Results

This was the first ever reproductive study for three of the species: *Bathymodiolus brooksi, B. heckerae,* and the undescribed species *B. sp. nov.* Populations of those species, as well as *B. childressi* were found to be synchronously and periodically reproducing at all depths. There was some individual and population-level variation, but no apparent between-species differences in the gametogenic cycle or timing. Populations also showed geographic synchrony, indicating that some factor acting on a much larger scale than a single cold seep is controlling the reproductive periodicity.

All mussel species, at all depths, had similar maximum oocyte feret diameters. The maximum diameters for my samples are slightly lower than the previously published value for *B. childressi* (50 - 80 μm; Tyler *et al*., 2006). This discrepancy may be because few mussels in this study had mature oocytes and none of them were ripe. However, the maximum oocyte sizes from this study still fall within that range, the same range reported for bathymodiolin mussels from hydrothermal vents and even shallow-water mytilids. Similar maximum oocyte sizes for all *Bathymodiolus* species that have been studied indicate that egg size is likely phylogenetically constrained within bathymodiolin mussels. Since the egg sizes for bathymodiolin mussels are also similar to those of shallow water mytilids, it is possible that egg size is phylogenetically constrained within the whole Mytilidae family.

Phytoplankton Connection

It has previously been hypothesized that the seasonality of *B. childressi* (Tyler *et al*., 2006) and the vent species *B. azoricus* (Colaço *et al*., 2006) is controlled by seasonal blooms of phytoplankton. While this is unexpected for a chemosynthesis-based ecosystem, it is expected for organisms that have planktotrophic larvae (Qasim, 1956). The geographic synchrony shown in this study also supports the hypothesis that the reproductive seasonality in cold-seep mussels is being driven by surface productivity.

The connection between surface productivity and reproduction in the deep sea was hypothesized almost 40 years ago (Lightfoot *et al*., 1979), but its application to chemosynthesis-based ecosystems started to become apparent just over a decade ago (Dixon *et al*., 2006; Tyler *et al*., 2006). This could have important implications for global nutrient cycling as well as conservation of communities at cold seeps and hydrothermal vents.

Even though these communities are far from human interaction, they do face threats from anthropogenic sources. Resource extraction such as offshore oil drilling (Fisher *et al*., 2014) and deep-sea mining of minerals and metals (Van Dover *et al*., 2012) can poison and damage organisms living at vents and seeps. Global climate change is extending areas of hypoxic and anoxic water, which could profoundly alter these communities due to the high dissolved oxygen demand of chemosynthesis (Childress & Girguis, 2011). Along with these threats that have already been identified, an increase in pollution or acidification, or change in ocean circulation, has the potential to decouple these ecosystems from their expected seasonal phytodetrital pulse. If phytodetritus is indeed a spawning cue in bathymodiolin mussels as well as necessary food for their

planktotrophic larvae, a drastic change in the time or abundance of phytodetritus could lead to inefficient spawning, resulting in fewer larvae. These larvae in turn would have less food needed to grow to settlement size, resulting in less recruitment.

Areas for Future Study

Future studies should directly test the role of phytoplankton as a reproductive cue in bathymodiolin mussels. Is it truly a spawning cue? If so, is it the only one? What would happen if the mussels did not receive a phytodetrital cue? More work could also be done on the role of phytodetritus on bathymodiolin larvae. How long can a larva survive without feeding? Does the abundance of phytodetritus effect their vertical migration and thus their current-driven dispersal trajectories?

The likely phylogenetic constraint on egg size is also interesting and deserves further study. Examining egg sizes for more species than were included in this study could help strengthen this hypothesis. It could likely be tested with ultrastructural analyses of gametogenesis in more bathymodiolin species as well as other mytilid mussels. Ultrastructural work could determine if the metabolic processes for creating gametes (i.e. vitellogenesis) as well as gamete morphology was the same across the subfamily Bathymodiolinae or the family Mytilidae.

This thesis presents new knowledge on the reproductive biology of bathymodiolin mussels that will hopefully aid future studies. Chapter I also outlines and illustrates a framework for elucidating reproductive mode using samples from one or a few time periods. This framework should be widely applicable and will hopefully increase the number of reproductive observations across taxa.

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