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Distribution and Dynamics of a Defensive Symbiosis in the Bugula neritina (Bryozoa) Sibling Species Complex

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DISTRIBUTION AND DYNAMICS OF A DEFENSIVE SYMBIOSIS IN THE *BUGULA NERITINA* (BRYOZOA) SIBLING SPECIES COMPLEX

by

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Under the Direction of Nicole B. Lopanik, PhD

ABSTRACT

Geographical differences in selective pressure may shift the relationship outcome in mutualistic symbioses from positive to negative. The marine bryozoan *Bugula neritina* is a colonial invertebrate common in temperate waters worldwide. Evidence suggests that an uncultured vertically transmitted symbiont, "*Candidatus* Endobugula sertula," hosted by *B. neritina* produces the polyketide bryostatins, which protect vulnerable larvae from predation. Studies of *B. neritina* along the Western Atlantic coast revealed a complex of two morphologically similar sibling species separated by an apparent biogeographic barrier: the Type S sibling species was found south of Cape Hatteras, North Carolina, while Type N was found to the north. Interestingly, the Type N colonies lacked *E. sertula* and defensive bryostatins; their

documented distribution was consistent with traditional biogeographical paradigms of latitudinal variation in predation pressure. Upon further sampling of *B. neritina* populations, we found that both host types occur in wider distribution, with Type N colonies living south of Cape Hatteras, and Type S to the north. Distribution of the symbiont, however, was not restricted to Type S hosts. Genetic and microscopic evidence demonstrates the presence of the symbiont in some Type N colonies and larvae, and they are apparently endowed with defensive bryostatins. Molecular analysis of the symbiont from Type N colonies suggests an evolutionarily recent acquisition, which is remarkable for a symbiont thought to be transmitted vertically only. Furthermore, most Type S colonies found at higher latitudes lack the symbiont, indicating that this relationship may be more flexible than previously thought. Transplant and common-garden experiments further suggest that the endosymbiont's geographical range is mediated by a combination of environmental impact on symbiont growth and a fitness cost on the host imposed by association with *E. sertula*. These results provide insight into possible mechanisms of regulation of context-dependent mutualisms.

INDEX WORDS: Biogeography, Bryostatin, Bryozoa, *Bugula neritina*, Marine ecology, Symbiosis

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DEDICATION

To Laura, and for all of the animals who helped us learn more about their world.

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1 INTRODUCTION

1.1 Biogeography

1.1.1 Overview

Biogeography, defined as the inference of biological phenomena from the distributive patterns of organisms and their behaviors, is often a key component in understanding the dynamics of ecological interactions. The application of biogeography might be observed most dogmatically with regard to feeding behaviors. Activities of consumption, including both herbivory and predation, often demonstrate clear patterns of latitudinal variation, in which feeding is increased in areas nearer to the equator (Vermeij, 1978). This has been shown not only in consumption rates of terrestrial plants such as broad-leaved forest trees and in the herbivore damage of salt marsh grasses, but also in predation of marine animals such as crustaceans and gastropods [e.g., (Heck and Wilson, 1987; Irie and Iwasa, 2003, b; Pennings and Silliman, 2005; Schemske et al., 2009)].

Other ecological phenomena have more recently joined herbivory and predation as standard subjects of biogeographical research. Of special relevance to the research discussed here are the production of natural products and the interaction of organisms known as symbiosis. In almost all cases, it is possible to point to exceptions to accepted biogeographic patterns; for example, some temperate brown algae produce larger amounts of herbivore-deterrent phlorotannins than their tropical counterparts (Targett et al., 1995). Indeed, there has been recent concern that biogeographical interpretations of organismal interactions such as predation and herbivory may be overemphasized due to researcher bias (Moles and Ollerton, 2016). However, there is much work to do in resolving this potential conflict, as interpretations may be confounded by variation in factors such as predation within a single region (Heck and Wilson,

1987) or by viewing the interaction of consumer and prey from the opposite side of the "arms race," such as observations that amphipod populations do not demonstrate a correlation between latitude and tolerance for some chemically rich seaweeds (Sotka et al., 2008). For the purposes of this dissertation, work has been carried out with the understanding that the majority of evidence points to a general rule of latitudinal variation in these factors, with the most important being the biogeographical dogma of feeding behaviors. Until further resolution of current dissent, exceptions must be viewed as requiring more in-depth examination to uncover variables allowing for opposition to the trend.

1.1.2 Roles and distribution of natural products

Natural products have proven to be a fertile topic for research over the past several decades, primarily due to the staggering spectrum of pharmacological activities demonstrated by naturally-occurring metabolites, from anti-inflammation to protection against pathogens ranging from viruses to protozoans (Mayer et al., 2007). This diversity in bioactivity is reflected in the variety of ecological roles assumed by natural products. In nature, these compounds may serve as behavioral cues (Hay, 2009), as protection against microbial fouling (Haber et al., 2011a, b), or as agents inhibiting the settlement of competing organisms (Piazza et al., 2011a, b). Simply examining the role of a single class of chemicals, as found within a single class of animals, can reveal diversity in the ecological value of natural products. In insects, for example, polyketide metabolites may aid the animal in both communication and chemical defense against predation and infection. This is especially evident in cockchafers, European beetles in which the polyketide benzoquinones serve as both sex pheromones for adults and antimicrobial defenses

for larvae in soil. Benzoquinones, meanwhile, have also been credited for defense against predation in earwigs and termites [reviewed in (Pankewitz and Hilker, 2008)].

The use of bioactive metabolites as chemical defenses against feeding behaviors may be seen in many natural systems. This has been demonstrated in herbivory on terrestrial plants such as milkweeds, which harbor the toxic cardenolides (Rasmann et al., 2009), as well as in marine organisms like brown algae, which produce the feeding-deterrent, phenolic phlorotannins (Haavisto et al., 2010). In addition, many slow or sessile invertebrates which, like plants, are often dependent upon non-locomotive means to avoid feeding behaviors, have been sources of predator-deterrent natural products. This is especially true in marine environments, where chemical defenses have been characterized in animals such as tunicates (Nuñez-Pons et al., 2010a, b), sea cucumbers (Van Dyck et al., 2010), nudibranchs (Cimino and Ghiselin, 1999), sponges (Haber et al., 2011a), corals (Fleury et al., 2008a, b), and infaunal worms (Kicklighter et al., 2003).

Perhaps due to the often protective capacity of natural products, and in a pattern mirroring the distribution of feeding behaviors by latitude, natural products of ecological interest appear to be more common in tropical species than in similar temperate organisms. Much of the evidence for this trend has been collected indirectly through consumer feeding assays, tying this biogeographic observation to the predation trends discussed above. Feeding behaviors and defensive natural product levels may thus serve as proxies for one another. Kicklighter & Hay (2006), for example, examined 81 species of marine worms and found that those from temperate environments were more likely to be palatable to predators, while Bolser & Hay (1996) similarly showed that palatability of seaweeds to sea urchins rises with increasing latitude. More direct evidence is found in the observation that tropical and subtropical seaweed populations are the

sources for a majority of seaweed secondary metabolites studied thus far [reviewed in (Hay, 1996)], and in the correlation in salt marsh plants between latitude and palatability of polar extracts by Siska *et al.* (Siska et al., 2002).

1.1.3 Symbiotic relationships and their distribution

An increasingly recognized trend in natural product investigations is the discovery that ecologically relevant metabolites may be synthesized not by the organism in which they are discovered, but by microbial symbionts living in association with the host (Crawford and Clardy, 2011; Piel, 2009). For example, pathogenic fungi of the genus *Rhizopus* cause the disease rice seedling blight via the antimitotic phytotoxin rhizoxin. Investigations into the biosynthesis of this metabolite revealed its source to be an endosymbiotic bacterium of the genus *Burkholderia* (Partida-Martinez and Hertweck, 2005). Similarly, the tunicate-derived cyclic peptides known as patellamides have been shown to originate not from the animals themselves, but from cyanobacterial symbionts living within the tunicates (Schmidt et al., 2005). Such associations allow eukaryotes to benefit from the greater metabolic and biochemical diversity found in the prokaryotic domains (Haygood et al., 1999).

Importantly, mutualistic associations among organisms are often demonstrated to occur more frequently at lower latitudes. In terrestrial systems, examples include pollination and seed dispersal by animals, ant-plant symbioses, and the occurrence of fungal endophytes [reviewed in (Schemske et al., 2009)]. In marine environments, differences along latitudinal lines can be even more striking, especially when comparing temperate environments with the coral reef communities of the tropics. Not only are cleaning symbioses more likely to be found among metazoans, but mutualistic associations between animals and microbial endosymbionts may be

much more common, as exemplified in Yonge's (1957) observation that while symbioses between coelenterates and photosynthetic zooxanthellae occur infrequently in temperate waters, they are quite common among such animals in coral reefs [reviewed in (Schemske et al., 2009)].

1.1.4 Biogeography of the holobiont

Interestingly, while the biogeographical clines and boundaries that define the geographical distribution of an organism (and the ecological underpinnings of these limits) have been studied for many metazoans (Diaz-Ferguson et al., 2010; Pennings and Silliman, 2005; Wares, 2002), the importance of these limits to the distribution of symbionts of those metazoans and the partners together (the holobiont) has rarely been thoroughly explored. While many symbiotic relationships are considered mutualisms, in which both partners benefit, there can be physiological costs associated with hosting a symbiont (Vorburger and Gouskov, 2011). For some of the most well-studied examples of metazoan-prokaryotic symbioses, this physiological cost is rendered moot by the absolute necessity of the association. For example, the intracellular bacteria *Buchnera* aid aphids nutritionally by providing essential amino acids to the insects. As *Buchnera* are reciprocally reliant upon aphids for their living environment, the symbiosis represents a truly obligate partnership (Douglas, 1998).

Beneficial defensive symbionts, meanwhile, facilitate the survival of the host against enemies such as pathogens, competitors, or predators (Lopanik, 2014). The nature of defensive symbiosis means that the ecological challenges they help to overcome are often ephemeral and may lead to drastically varying importance among populations. For example, the relationship between cleaning gobies and longfin damselfish is beneficial in high densities of damselfish ectoparasites, but shifts to a more parasitic interaction at low ectoparasite densities (Cheney and

Cote, 2005). In the absence of the selective pressure, the costs of hosting a symbiont may eclipse the potential benefits of a partnership, resulting in an unpartnered host with greater fitness than a partnered host (Oliver et al., 2008; Polin et al., 2014; Thomas et al., 2013). Selection over time would then ultimately result in symbiont loss. Defensive symbionts that are vertically transmitted are thought to represent a significant parental investment. This implies both that the symbionts are beneficial to the host, and that the host and symbiont have developed mechanisms to prevent loss of the association; therefore, symbiont loss represents an irreversible evolutionary milestone. The increase in aposymbiotic host frequency in the absence of the enemy would then suggest that the symbiont imposes a cost on its host [reviewed in (Clay, 2014)]. The interplay of symbiont cost-benefit and biogeography is likely important to host/symbiont partners with widespread distribution, but not well understood.

1.2 The bryozoan *Bugula neritina*

1.2.1 Symbiosis

The group of arborescent marine bryozoans including the genus *Bugula* provides an interesting platform for the exploration of the importance of geographic variation to symbiotic interactions. Among commonly studied *Bugula* and closely related species in the genera *Bugulina*, *Crisularia*, and *Viridentula* [updated nomenclature found in (Fehlauer-Ale et al., 2015)], at least four are known to harbor bacterial symbionts seemingly absent from a number of related species within the clade (Davidson and Haygood, 1999; Lim and Haygood, 2004; Lim-Fong et al., 2008; Woollacott, 1981). Vertical transmission of these endosymbionts is suspected due to their presence in the larvae and larval brood chambers, or ovicells (Fig 1.1), of colonies (Sharp et al., 2007).

Figure 1.1 Close-up view of branches from a B. neritina *colony showing ovicells (as indicated by arrow) where larvae are brooded. Associated endosymbionts are concentrated in these chambers. Photo by Meril Mathew*

This suspected vertical transmission has also led to a high degree of congruence between the phylogenetic topologies of host and symbiont, with just a single host-switching event indicated in genetic comparison across the clade (Lim-Fong et al., 2008). Within the group, the cryptic species complex of the cosmopolitan bryozoan *Bugula neritina* (Fig. 1.1) allows for even more direct exploration of the ties between symbiosis and host ecology. *Bugula neritina* is known to harbor a γ-Proteobacterial symbiont that is, to date, uncultivated (Haygood and Davidson, 1997; Woollacott, 1981). "*Candidatus* Endobugula sertula" is found in all life stages of the host: in larvae, it is found in a circular groove located along the aboral pole called the

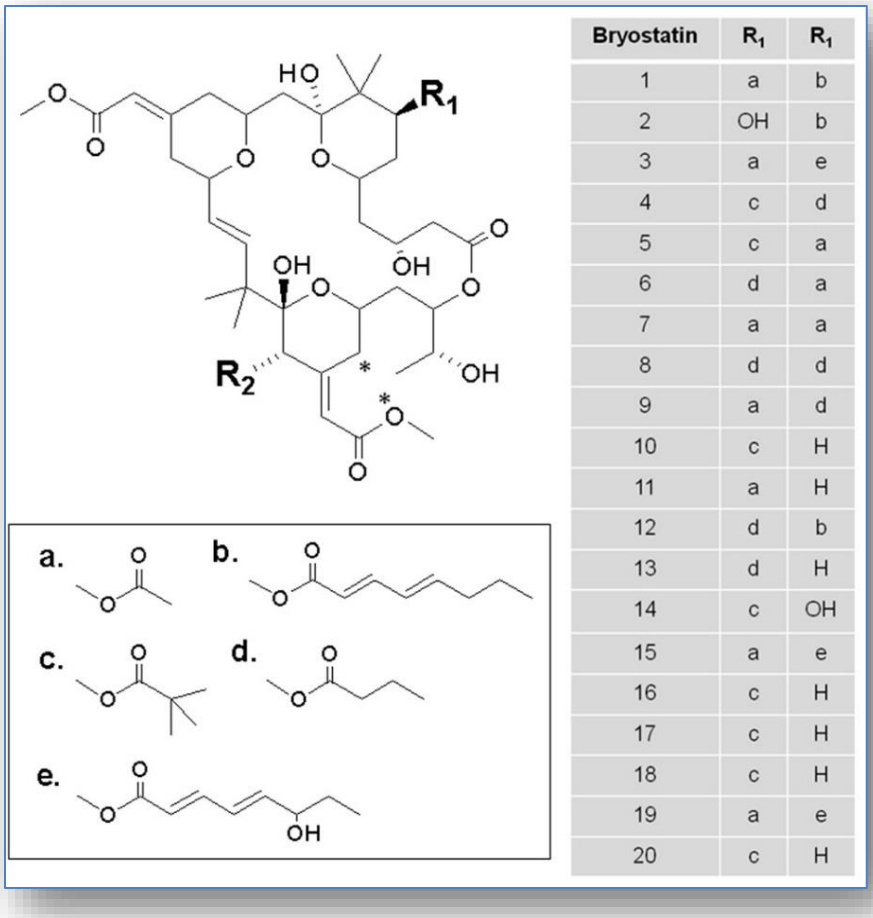
pallial sinus (Haygood and Davidson, 1997; Woollacott, 1981); in adult colonies, it is found in funicular cords, which serve as a vascular system, transporting nutrients and wastes throughout the colony (Woollacott and Zimmer, 1975) and to a developing embryo in an ovicell (Sharp et al., 2007).

Figure 1.2 B. neritina *colony pulled from a floating dock in North Carolina, composed of hundreds of genetically identical individuals. Photo by Michelle Ventura*

1.2.2 Role of E. sertula

Evidence from antibiotic-curing experiments suggests that *E. sertula* is the source of the bryostatins (Fig. 1.3) (Davidson et al., 2001; Lopanik et al., 2004), complex bioactive polyketides with medical potential [reviewed in (Trindade-Silva et al., 2010)]. Feeding assays with extracts of *B. neritina* and purified bryostatins revealed that these symbiotically produced

compounds are unpalatable and may serve to protect the vulnerable host larvae from predators, suggesting an association in which bryostatin presence may drive maintenance of symbiosis over generations. Both *B. neritina* larvae and larval extracts are unpalatable to co-occurring invertebrate and vertebrate predators including oysters (Tamburri and Zimmer-Faust, 1996), corals, sea anemones, filefish, and the pinfish, *Lagodon rhomboides* (Lindquist, 1996; Lindquist and Hay, 1996; Lopanik et al., 2004; Lopanik et al., 2006). Larvae have also shown a marked ability to survive and metamorphose after rejection by predators, with >90% successful after rejection by the coral *Oculina arbuscula* (Lindquist, 1996) and 100% metamorphosing after attacks by *L. rhomboides* and the filefish *Monocanthus ciliatus* (Lindquist and Hay, 1996).



Bryostatin	R_1	R_1
1	a	b
2	OH	b
3	a	e
4	c	d
5	c	a
6	d	a
7	a	a
8	d	d
9	a	d
10	c	H
11	a	H
12	d	b
13	d	H
14	c	OH
15	a	e
16	c	H
17	c	H
18	c	H
19	a	e
20	c	H

Figure 1.3 Structure of the first 20 bryostatins, with substituent groups at R_1 and R_2 indicated in table. Bryostatins 3, 19, and 20 also feature cyclization at points indicated with asterisks (*). (Adapted from Davidson & Haygood, 1999.)

Importantly, the defensive bryostatins appear to be most concentrated in the larval stage of the *B. neritina* life cycle (Lindquist and Hay, 1996; Lopanik et al., 2004; Lopanik et al., 2006), suggesting that alternative protective means such as structural defenses may be more crucial to adult colonies. The *B. neritina-E. sertula* association is one of the most well-characterized examples of a marine system involving defensive natural products that are produced by a microbial symbiont (Lopanik, 2014). At least one terrestrial analog has been identified, in which

the polyketide pederin, produced by a bacterial endosymbiont, prevents predation on the larval stage of the rove beetle genus *Paederus* (Kellner, 2002; Kellner and Dettner, 1996).

1.2.3 Traditional view of B. neritina-E. sertula *biogeography*

While the presence of defensive metabolites suggested that the association with *E. sertula* may provide a distinct advantage for larval survival, some populations of *B. neritina* were shown to lack these endosymbionts and, consequently, the suite of bryostatins associated with them (Lopanik et al., 2004; McGovern and Hellberg, 2003). Further genetic characterization over the past several years has identified three cryptic sibling species of *B. neritina* based upon the sequence of the mitochondrial cytochrome *c* oxidase I (COI) gene: (1) Type S ("Shallow"/"Southern") found in temperate environments worldwide, (2) a Type D ("Deep") variety found only on the Eastern Pacific coast, and so named due to its occurring at depths 9 m or greater in southern California, and (3) Type N ("Northern") found only on the Western Atlantic coast, initially in Delaware and Connecticut (Davidson and Haygood, 1999; Mackie et al., 2006; McGovern and Hellberg, 2003). These so-called "sibling species" have recently been proposed as distinct biological species (Fehlauer-Ale et al., 2014).

Types S and D *B. neritina* from California house closely related strains of *E. sertula*, and bryostatin composition has been shown to vary between the host sibling species, which could be attributed to either host or symbiont (Davidson and Haygood, 1999). The *bry* gene cluster, which prescribes bryostatin biosynthesis, shows minimal variation between the sibling species, demonstrating 98% identity and differing most significantly in the genomic location of several accessory genes, with the entire cluster found on a single locus for Type S but on two for Type D (Sudek et al., 2007). Perhaps most notably, Type N populations initially gave no evidence of

endosymbionts or of bryostatin production. Although extracts of Type N *B. neritina* were shown to be significantly unpalatable to a predator from North Carolina (NC), decreasing palatability by nearly 40%, these Type N samples were not as deterrent as extracts with bryostatins, which reduced feeding by 80% (Lopanik et al., 2004). Because of this pattern of palatability and of sibling species location, researchers proposed a biogeographic division on the North American east coast between the ranges of Type N and S *B. neritina*, with the traditional boundary Cape Hatteras (Briggs, 1974; Briggs and Bowen, 2012; McCartney et al., 2013) initially speculated as a point of transition.

1.2.4 Impetus for this study

Work continued on this system with little to refute these initial estimations of the range of Type N *B. neritina* or the necessity of bryostatins for survival in the higher predation risk of the southern Atlantic coast. For 13 years, Lopanik and co-workers collected and analyzed the genetics and bryostatin composition of colonies in Morehead City, NC, and Indian River Inlet, DE, and never noticed a Type N individual inhabiting North Carolina, nor a Type S individual in Delaware, despite the potential for widespread dispersal via shipping (Mackie et al., 2006). In 2010, we began assessing the genetic and chemical composition of *B. neritina* populations along the United States' east coast with the goal of uncovering differences in local bryostatin composition and symbiont identity. Initial investigations of *B. neritina-E. sertula* populations from along the east coast demonstrated that the *B. neritina* population from a site near Beaufort, SC, contained a mix of Type N and Type S individuals. This result was also obtained for a previously collected (2008) pooled larval sample from a nearby location.

Further experimentation revealed the likely presence of the Type N species in *B. neritina* samples from the North Carolina coast dating back as far as 2008. Meanwhile, exploration of southern locations in search of more Type N samples, and subsequent polymerase chain reaction (PCR) targeting symbiont DNA, showed the possibility of the vertically transmitted *E. sertula*'s having an association with Type N animals. These seemingly novel findings, coupled with colleagues' discovery of Type S colonies as far north as Maryland, led us to examine *B. neritina* populations along the Atlantic coast more closely in order to correlate varying symbiotic and bryostatin status with the distribution of both sibling species.

1.3 Goals of this study

The objectives of this study were built upon the questions raised by these challenges to accepted *B. neritina-E. sertula* biogeography. Broadly, the research goals fall into three realms:

1.3.1 Distribution of B. neritina *sibling species and* E. sertula

The first objective was to gain a greater understanding of the ranges of Types N and S *B. neritina* in order to determine if the exceptional results prompting this research were unique or part of a larger pattern. Alongside this exploration, testing of *B. neritina* colonies for symbiont presence would reveal any correlative biogeographic tendencies. Specifically, the following hypotheses were addressed:

1. *Types N and S* B. neritina *are readily found outside their initially proposed ranges which include a sharp divide at the traditional biogeographic boundary of Cape Hatteras.*

2. *The endosymbiont* E. sertula *can be found in many of the Type N animals inhabiting southern waters; conversely, Type S* B. neritina *found north of Cape Hatteras may lack the association with* E. sertula.

1.3.2 Characterization of E. sertula *associated with Type N* B. neritina

Secondly, I addressed questions of identity posed by the apparent association of the symbiont *E. sertula* with Type N *B. neritina*. This included both phylogenetic placement, for indications of the Type N animals' acquisition of the symbiont (e.g. supporting coevolution of all three *B. neritina* sibling species and their symbionts), and characterization of the protective capacity of the Type N-associated *E. sertula*. Specifically, we addressed these hypotheses:

1. *Supporting its suspected vertical transmission,* E. sertula *associated with Type N* B. neritina *in nature represent a third lineage distinct from that found in Types D and S* B. neritina.

2. E. sertula *associated with Type N* B. neritina *play a role equivalent to that in* Type S *animals, protecting vulnerable larvae from predators, and will consequently have similar deterrent properties and relatively effective biochemical profiles.*

1.3.3 Dynamics of the B. neritina-E. sertula *association*

Finally, the discovery of colonies of *B. neritina* that share a sibling species identity (Type S) but differ in symbiotic status raised questions of the mediation of this association. The capability of individuals to gain or lose the endosymbiont in nature is largely unexplored. I

chose to approach the issue from two perspectives, that of the system's reaction to a changing environment, and the balance of positive and negative fitness effects resulting from the bryozoan's harboring of the symbiont. I was guided by these hypotheses:

1. *There is a demonstrable cost to maintaining the association with* E. sertula *that may serve as a selective agent in populations of* B. neritina.

2. *The cost-benefit ratio of harboring* E. sertula *modulates symbiosis in concert with differing tendencies for the organism to maintain association in direct reponse to differing environments.*

2 DISTRIBUTION OF *B. NERITINA* SIBLING SPECIES AND *E. SERTULA*Introduction

2.1.1 Biogeography of B. neritina *and* E. sertula

For the bryozoan *B. neritina*, while the presence of defensive metabolites suggested that the association with *E. sertula* may provide a distinct advantage for larval survival, some populations of *B. neritina* were shown to lack these endosymbionts and, consequently, the suite of bryostatins associated with them (Lopanik et al., 2004; McGovern and Hellberg, 2003). While Types S and D *B. neritina* from California house closely related strains of *E. sertula*, Type N populations initially gave no evidence of endosymbionts or of bryostatin production and proved to be more palatable to predators than populations associated with *E. sertula* (Lopanik et al., 2004).

Biogeographic patterns of palatability and of sibling species location led researchers to propose a division on the North American east coast between the ranges of Type N and S *B. neritina*, with the traditional boundary Cape Hatteras (Briggs, 1974; Briggs and Bowen, 2012; McCartney et al., 2013) thought to be a point of transition. In this separation of sibling species along the Western Atlantic coast, survival of Type S *B. neritina* in lower-latitude waters would be enabled by maintenance of the symbiosis and associated defensive metabolites in the presence of higher predation pressure (Vermeij, 1978). Its spread northward, meanwhile, would be limited by environmental tolerance limits of the Type S bryozoan or its symbiont (Lopanik et al., 2006; McGovern and Hellberg, 2003).

2.1.2 Exceptions to the initial **B. neritina** *biogeographical paradigm*

In 2010, we began assessing the genetic and chemical composition of *B. neritina* populations along the United States' east coast with the goal of uncovering differences in local bryostatin composition and symbiont identity. Initial investigations of *B. neritina-E. sertula* populations from along the east coast revealed a conflicting bryozoan sibling species profile from populations collected in some southern locations. Restriction fragment length polymorphism (RFLP) analysis of COI amplicons obtained from pooled larval DNA demonstrated that the *B. neritina* population from a site near Beaufort, SC, contained a mix of Type N and Type S individuals. This result was also obtained for a previously collected (2008) pooled larval sample from a nearby location.

Further experimentation with primers designed to differentiate between Type N and S COI sequences also revealed the likely presence of the Type N species in *B. neritina* samples from the North Carolina coast dating back as far as 2008. Perhaps most intriguingly, early exploration of southern locations in search of more Type N samples, and subsequent polymerase chain reaction (PCR) targeting symbiont DNA, showed the possibility of the vertically transmitted *E. sertula*'s having an association with Type N animals. Coupled with colleagues' discovery of Type S colonies as far north as Maryland, these inconsistencies with previous reports led us to examine *B. neritina* populations along the Atlantic coast more closely in order to better understand the geographic and phylogenetic relationship of Type N and Type S, as well as to correlate varying symbiotic and bryostatin status with the distribution of both sibling species.

2.2 Methods and materials

2.2.1 Sampling of adult B. neritina

Adult *B. neritina* colonies were collected from floating docks in coastal locations. No specific permits were required as the studies did not involve endangered or protected species. Floating docks included public and private property, and permissions were sought from the owners or dock masters prior to collecting. All samples were pulled from docks by hand and thus represent animals found close to the water's surface. In many cases, colonies were chosen haphazardly. In some instances, the Type N or S phylotype was targeted for specific investigation, a direction made possible by slight morphological differences observable within multiple mixed *B. neritina* populations (details below). For the coastal survey, sites ranged from Indian River, DE, to St. Augustine, FL (sites listed in Table 2.1), and samples were collected from 2010-2013.

Table 2.1 Locations and GPS coordinates of sites sampled for the coastal survey of B. neritina *and* E. sertula.

Location	Latitude	Longitude
Indian River, DE	38.612	-75.072
Ocean Pines, MD	38.386	-75.13
Chincoteague, VA	37.92	-75.405
Wachapreague, VA	37.604	-75.687
Oyster, VA	37.289	-75.923
Rudee Inlet, VA	36.832	-75.976
Beaufort, NC	34.717	-76.666
Radio Island, NC	34.714	-76.68
Morehead City, NC	34.72	-76.707
Salter Path, NC	34.69	-76.887
Murrell's Inlet, SC	33.557	-79.031
Isle of Palms, SC	32.823	-79.73
Beaufort, SC	32.466	-80.666
Hunting Island, SC	32.346	-80.469
Tybee Island, GA	31.991	-80.852
Jacksonville, FL	30.299	-81.413
St. Augustine, FL	29.946	-81.307

2.2.2 Collection of larvae

Whenever possible, adult colonies were kept in flowing seawater tables [at the University of North Carolina – Chapel Hill's Institute of Marine Sciences (UNC-IMS) in Morehead City, NC, or the University of Delaware's College of Earth, Ocean, and Environment, Hugh R. Sharp Campus in Lewes, DE] to support several days' collection of larvae. When circumstances did not allow this, large plastic coolers were placed on floating docks in field locations, with pumps suspended in the water to allow constant flow through the cooler, and colonies were kept in these coolers. In all cases, colonies were covered overnight to minimize light exposure. In the

morning, colonies were placed into glass jars or dishes in order to stimulate the release of larvae through exposure to sunlight. Larvae were then collected manually by pipet.

2.2.3 DNA extraction

For *B. neritina* samples subjected to DNA analysis, care was taken after collection to ensure that the selected zooids represent a single colony rather than multiple associated individuals. Distal zooids were cut from several branches in order to avoid ovicells in more proximal reproductive zooids. In several cases, larvae were also collected as they were released by the colonies in subsequent days (variously for both individual colonies and pooled adult samples). When colonies were not immediately subjected to DNA extraction, zooids were cut and stored in RNALater RNA Stabilization Reagent (Qiagen, Valencia, CA) or 70-100% ethanol at -20°C. Similarly, larvae not immediately processed through DNA or chemical extraction were stored in RNALater or 100% methanol for later analysis, or were fixed for fluorescence *in situ* hybridization (FISH; see below). For both adult and larval samples, DNA was extracted using either ZR Fungal/Bacterial DNA MiniPrep or MicroPrep (Zymo Research, Irvine, CA), or DNEasy Blood and Tissue Kit (Qiagen), and following the manufacturer's instructions. Quality and quantity were assessed by spectroscopy (NanoDrop 1000, Thermo Fisher Scientific, Wilmington, DE).

2.2.4 Molecular characterization of host and endosymbiont

PCR amplification of the mitochondrial COI gene was carried out using the universal invertebrate primers LCO1490 and HCO2198 (Folmer et al., 1994) or the *B. neritina*-specific primer pair BnCOIf and BnCOIr (see Table 2.2 for all primers and probes used in these studies).

Restriction fragment length polymorphism (RFLP) analysis was performed on amplicons using the restriction endonucleases *Dde*I, which only digests Type S COI products, or *Hha*I, which cuts amplicons from Type N individuals. In order to identify host phylotypes not documented amongst the individual adult colonies collected, PCR was also performed on DNA from the pooled larvae of a large number of colonies (>200) using primers targeting polymorphisms in the Type N and S COI genes.

The presence of the symbiont *E. sertula* was assessed in DNA by PCR targeting its 16S ribosomal RNA (16S rRNA) gene [primers EBn16S_254f and EBn16S_643r, Bn240f and Bn1253r (Haygood and Davidson, 1997)], or the bryostatin biosynthetic gene *bryS* (Sudek et al., 2007) (primers BryS_576f and BryS_774r). PCR amplicons and restriction digested products were visualized after agarose gel electrophoresis. For selected samples, the COI or 16S PCR amplicon was purified using the GeneJET PCR Purification Kit (Thermo Scientific, Pittsburgh, PA) and sequenced at the Georgia State University Core Facility. DNA sequences were trimmed, assembled, and compared using the Lasergene software suite (DNASTAR, Madison, WI).

Table 2.2 Primers and probes used in these studies.

Name	Sequence	Target	Source
LCO1490	GGTCAACAAATCATAAAGATATTGG	Invertebrate COI	(Folmer et al., 1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Invertebrate COI	(Folmer et al., 1994)
BnCOIf	ACAGCTCATGCATTTTTA	*B. neritina* COI	This work
BnCOIr	CATTACGATCGGTTAGTAG	*B. neritina* COI	This work
BnCOIF	TTGATACTGGGGGCTCCTGATATG	*B. neritina* COI (for Q-PCR)	(Lopanik et al., 2004)
BnCOIR	AAGCCCGATGATAAGGGAGGGTA	*B. neritina* COI (for Q-PCR)	(Lopanik et al., 2004)
Bn_COI_N_129f	CACCGGTAGAGATAAAAGTAAT	Type N *B. neritina* COI	This work
Bn_COI_N_615r	CGAATTAAGACAACCTGGTAGT	Type N *B. neritina* COI	This work
Bn_COI_S1_129f	CACTGGTAAAGATAAAAGTAAC	Type S *B. neritina* COI	This work
Bn_COI_S1_615r	AGAATTAAGACAACCAGGCAGC	Type S *B. neritina* COI	This work
Bn240f	TGCTATTTGATGAGCCCGCGTT	*E. sertula* 16S	(Haygood and Davidson, 1997)
Bn1253r	CATCGCTGCTTCGCAACCC	*E. sertula* 16S (used as primer and probe)	(Haygood and Davidson, 1997)
EBn16S_254f	TACTCGTTAACTGTGACGTTACTC	*E. sertula* 16S (includes Q-PCR)	(Mathew and Lopanik, 2014)
EBn16S_643r	ACGCCACTAAATCCTCAAGGAAC	*E. sertula* 16S (includes Q-PCR)	(Mathew and Lopanik, 2014)
1055F	ATGGCTGTCGTCAGCT	Eubacterial 16S	(Ferris et al., 1996)
1492R	TACGGYTACCTTGTTACGACTT	Eubacterial 16S	(Lane, 1990)
Eub338	GCTGCCTCCCGTAGGAGT	Eubacterial 16S (used as probe only)	(Amann et al., 1990)
EBn16S_621f	CCTTAGAGTTCCCAGCCAAAC	*E. sertula* 16S (for ITS sequencing)	This work
ITS-23S-r2	TSTGRDGCCAAGGCATCCA	Eubacterial 23S (for ITS sequencing)	After (Garcia-Martinez et al., 1999)
BryA_7987f	AAATGATGGACCCACGTCAAC	*bryA*	This work
BryA_9579r	TCTCAGACACTGCCTCTG	*bryA*	This work
BryS_576f	CATTGACAGTCAGTTCTTCATTGA	*bryS*	This work
BryS_774r	CTTTTCCAGATTGAGTTTTTAACCA	*bryS*	This work
bryP-B-5849f	TTATAGAATAATGAAGTTGCTG	*bryP-B* intergenic region	This work
bryP-B-6236r	AAAAATTCCTCAACAACGACC	*bryP-B* intergenic region	This work

2.2.5 Fluorescence in situ hybridization (FISH)

Larvae designated for FISH were fixed for 1 h in 4% paraformaldehyde in MOPS-NaCl buffer, then stored in 70% ethanol at -20° C. Hybridization of larvae followed the protocol previously described (Lim and Haygood, 2004) using a Cy3-labeled universal

eubacterial probe [Eub338 (Amann et al., 1990)] on a subset of larvae, and a Cy3-labeled "*Ca.* Endobugula sertula"-specific probe [Bn1253r (Haygood and Davidson, 1997)] for the rest of the larvae (Table 2.2). To prepare for hybridization, larvae were rinsed in phosphate buffered saline (PBS) and then transferred to hybridization buffer. Probes were added to obtain a final concentration of 5 ng/µl. After incubation at 46° C for 3-4 h, all samples were rinsed in wash buffer and incubated twice in wash buffer for 20 min at 48° C. Larvae were finally washed in PBS with 0.1% Tween-20 and stored at 4° C until observation. Samples were mounted with Vectashield (Vector Labs, Burlingame, CA) and then viewed using an Olympus FluoView FV1000 confocal microscope, with images composed of stacks of 60-80 optical sections 1 µm apart. All images were captured with identical laser intensity and gain settings.

2.2.6 Clone libraries of B. neritina*-associated bacteria*

Concurrently, colleagues generated clone libraries of 16S rRNA gene amplicons with the universal primers 1055f and 1492r for four adult *B. neritina* colonies, using the Invitrogen TOPO TA Cloning Kit for Sequencing (Life Technologies, Carlsbad, CA), with 164 clones sequenced at Virginia Commonwealth University's Nucleic Acid Research Facility. Colonies included two Type S from Oyster, VA, determined to be aposymbiotic by PCR analysis, one symbiotic Type S from Oyster, and one symbiotic Type N from Salter Path, NC. Clone identities were determined by BLAST searches, and the proportions of "*Ca.* Endobugula sertula" clones obtained from each sample were analyzed using Fisher's Exact Test.

2.3 Results

2.3.1 Ranges of Types N and S B. neritina

Fourteen locations were sampled in the 2010-2013 survey of coastal *B. neritina* populations. Sibling species were readily found outside of their initially proposed biogeographic ranges; haphazard colony collection revealed least four locations in which the atypical species were found among the populations. As expected, a majority of haphazardly collected *B. neritina* found south of Cape Hatteras were of the Type S sibling species, but sites in Beaufort, SC, and Salter Path, NC, included Type N animals. To the north of Cape Hatteras, two locations (Ocean Pines, MD, and Oyster, VA) included Type S *B. neritina*. The Oyster location was extensively sampled (n=100) and had the largest proportion of Type S animals (17%) among high-latitude locations (those north of Cape Hatteras).

	Host phylotype	
Location	N	S
Indian River, DE	31	0
Ocean Pines, MD	22	2
Chincoteague, VA	10	0
Wachapreague, VA	17	0
Oyster, VA	83	17
Rudee Inlet, VA	19	0
Beaufort, NC	0	50
Radio Island, NC	0	26
Morehead City, NC	0	26
Salter Path, NC	13	1
Beaufort, SC	3	12
Tybee Island, GA	0	14
Jacksonville, FL	0	10
St. Augustine, FL	0	10

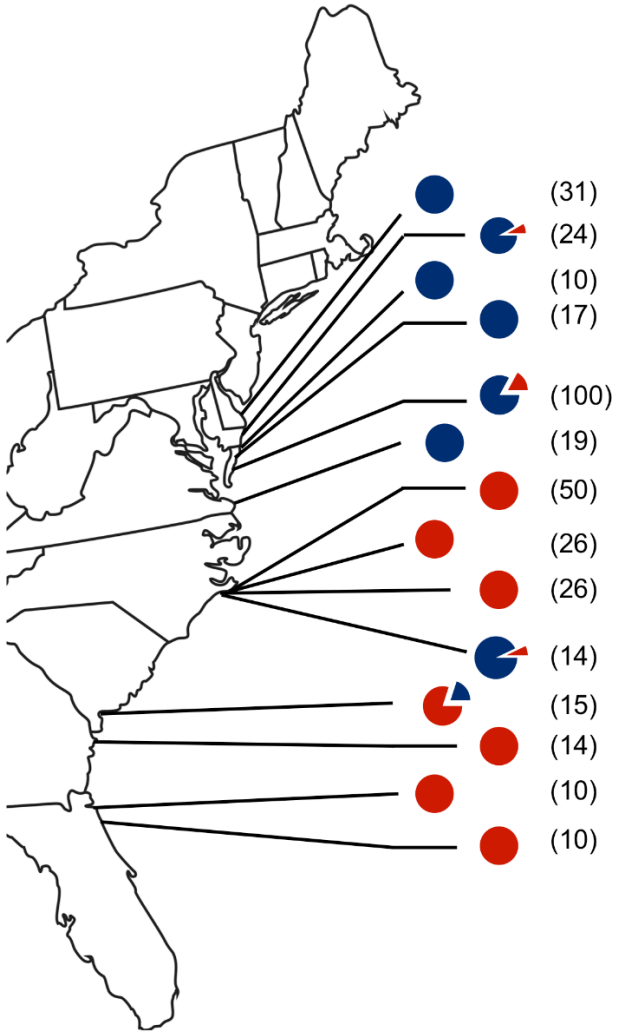


Figure 2.1 B. neritina *sibling species collected by haphazard sampling. Proportions at each site indicated by blue (Type N) and red (Type S) in charts. (From Linneman et al., 2014)*

2.3.2 Morphological differentiation of Types N and S B. neritina

One of the fundamental difficulties that emerged in exploring differences between Types N and S *B. neritina* was the inability to distinguish between the two without genetic analysis. As the coastal survey progressed, subtle morphological differences became apparent which enabled

the targeting of one sibling species in a mixed community. While morphometric differences have not been established, the consistent appearances of Type N *B. neritina* in regular, conical tufts, and Type S *B. neritina* as less ordered, more "ragged" arborescent groupings has allowed for the ability to distinguish between the two to be shared among multiple researchers working with the animals. Close examination and tactile assessment are often required for adequate differentiation. Type S colonies, for example, often feel more rigid than those of Type N. In addition, Type S may demonstrate a rustier color than its counterpart, which is often more purple in color. These subtleties can make sibling species identification especially difficult for young colonies. Examples of the sibling species' morphological comparison, with typical representatives of each, are shown in Figure 2.2.

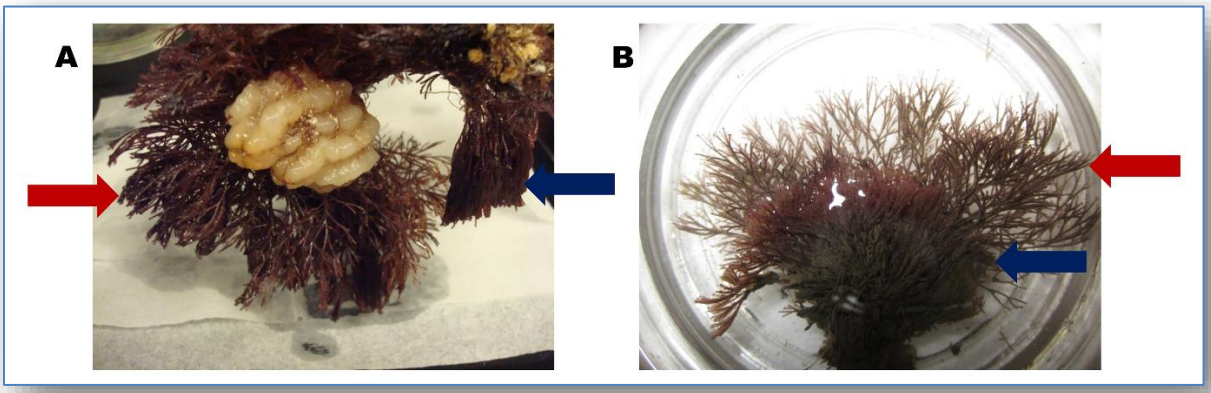


Figure 2.2 Colonies collected together in (A) Beaufort, NC, and (B) Oyster, VA. Blue arrows indicate Type N colonies; red indicate Type S. Images demonstrate both co-occurrence of sibling species and means of morphological differentiation. (From Linneman et al., 2014.)

2.3.3 Targeting atypical sibling species

The ability to target Type S or N *B. neritina* in mixed communities, enabled by recognition of subtle morphological differences, revealed even more atypical sibling species among the sites surveyed. When public docks at Oyster, VA, were targeted for Type S colonies, they were easily found among the Type N animals (see comparison of Type S animals included in Figure 2.1, haphazardly collected colonies, with those of Figure 2.4, all colonies assayed for the presence of *E. sertula*). More dramatically, collection efforts targeting Type N adult colonies revealed their presence among Type S colonies at a number of other sites: Morehead City, NC; Radio Island, NC; and Hunting Island, SC.

For a number of populations, pooled larvae a from large number of adults (>200) were used to investigate the possibility of atypical sibling species' presence not uncovered by either haphazard or targeted sampling. While these sibling-specific PCRs did not reveal any additional sites of Type S habitation in northern locations, the presence of Type N animals was indicated as far south as the coast of Florida (Figure 2.3).

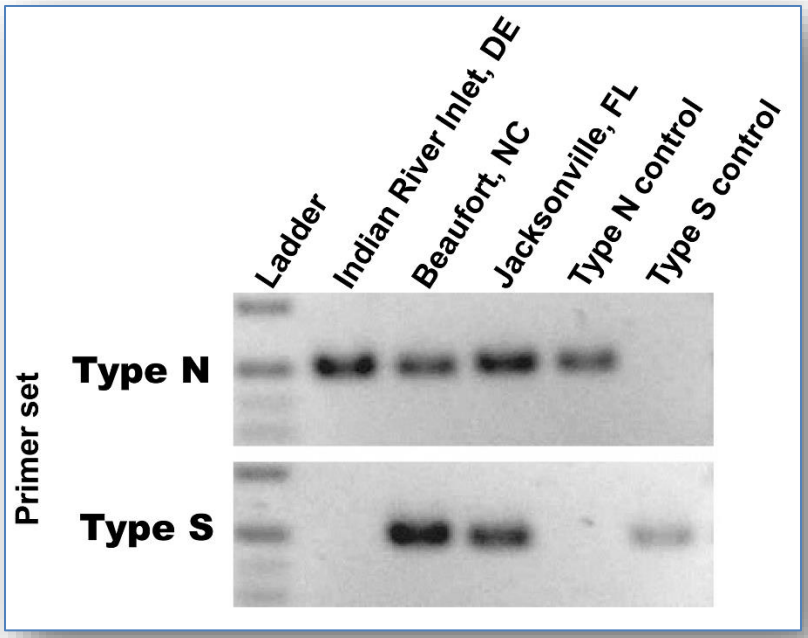


Figure 2.3 B. neritina *sibling species-specific PCR indicating Type N's presence at least as far south as Jacksonville, FL. (From Linneman et al., 2014.)*

2.3.4 Ranges of E. sertula *in Types N and S* B. neritina

A vast majority (94%) of colonies collected north of Cape Hatteras tested negative for the presence of *E. sertula* (Figure 2.4). This was especially true for Type N animals, for which no symbiotic colonies were found. In contrast, our collection revealed 95% of colonies to be symbiotic when located south of Cape Hatteras. Just four aposymbiotic Type S colonies were found south of Cape Hatteras (2%). Type N *B. neritina*, similarly, was largely symbiotic when south of the Cape (85%). It should be noted that quantities of both phylotypes in a specific location may differ between the geographic figures presented here, as symbiont occurrence was determined both for colonies collected haphazardly and in targeted sampling (see Chapter 2), and not all haphazardly selected colonies were assayed for symbiont presence.

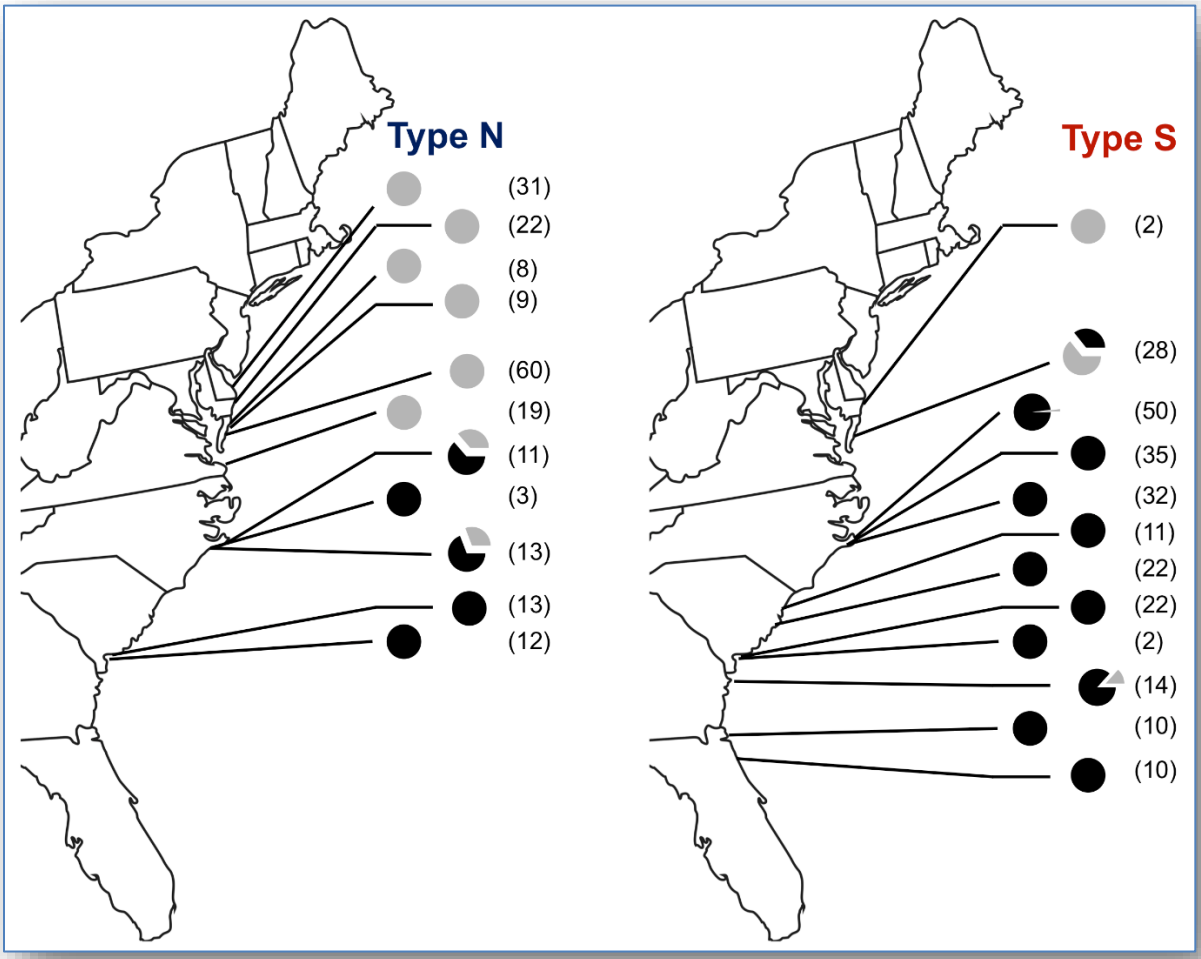


Figure 2.4 Symbiotic status of sampled colonies. Proportions at each site indicated by black (symbiotic) and gray (aposymbiotic) in charts. Results from both haphazardly collected and targeted samples are included. Number of colonies sampled shown beside each graph.

2.3.5 Sequencing of B. neritina *COI*

Type S COI sequences obtained in this study were identical to the Southern (Type S) clade of McGovern and Hellberg (McGovern and Hellberg, 2003) over the 624 overlapping bases and showed 99.8% identity to the Type S COI sequence of Davidson and Haygood

(Davidson and Haygood, 1999), differing by just 1 bp along 483 bases. Type N COI sequences were identical to that of the Northern (Type N) clade reported in McGovern and Hellberg (McGovern and Hellberg, 2003).

2.3.6 Confirmation of atypical sibling species-symbiotic status pairings

The presence of the endosymbiont in larvae collected solely from low-latitude Type N *B. neritina* was additionally demonstrated by PCR targeting the *E. sertula brySgene*. This result further supports the finding of *E. sertula* among Type N adults and more importantly confirms the transmission of symbiont from adult colonies to the next generation. Furthermore, the existence of all four pairings of sibling species identity and symbiotic status was confirmed by FISH (Figure 2.5). Molecular probes specific to *E. sertula* as well as to eubacteria demonstrate the presence of the endosymbiont in the pallial sinus of both Type N and S *B. neritina* larvae which were designated symbiotic by PCR. Similarly, FISH probes failed to reveal the endosymbiont in Type S and Type N animals determined to be aposymbiotic by PCR.

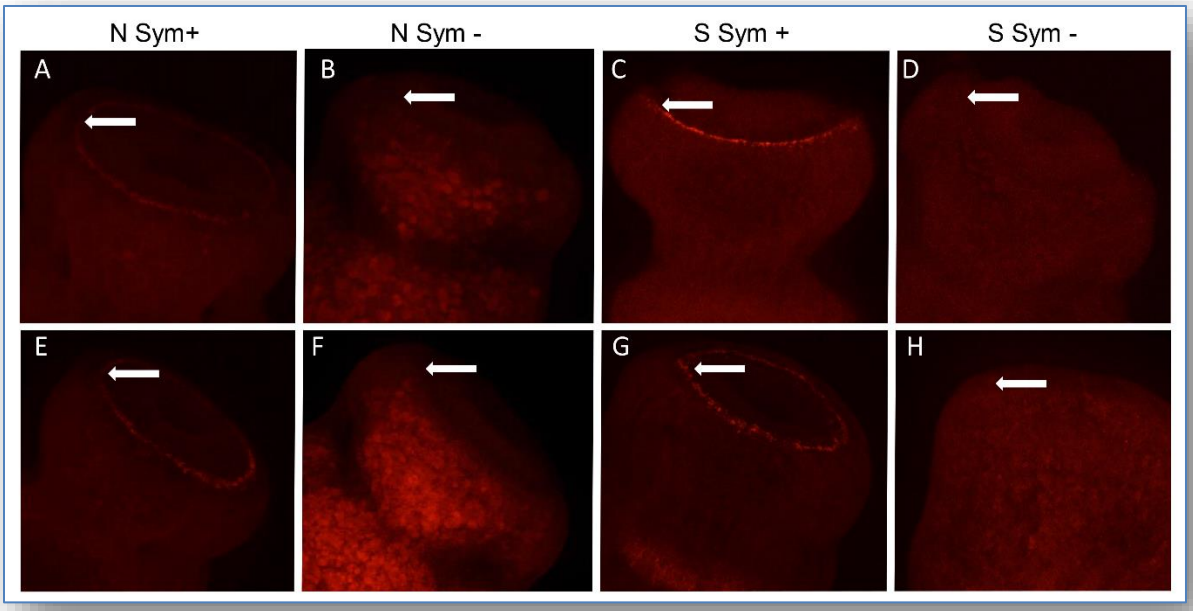


Figure 2.5 Confirmation of B. neritina *symbiotic status via fluorescence in situ hybridization (FISH). White arrows indicate the circular pallial sinus on the aboral surface of the larva. (A–D) FISH micrographs of larvae using the Eub338 (eubacterial) probe. (E–H) FISH micrographs of larvae using the symbiont-specific Bn1253r (*E. sertula*-specific) probe. Diameter of pallial sinus is approximately 150 mm. (From Linneman et al., 2014.)*

2.3.7 Analysis of B. neritina*-associated bacteria*

In experiments carried out by colleagues at Randolph-Macon College in Ashland, VA, clone libraries were generated using eubacterial universal primers and DNA extracted from four adult *B. neritina* colonies (two aposymbiotic Type S, one symbiotic Type S, one symbiotic Type N). A total of 164 clones were generated and analyzed. Sequencing revealed the presence of *E. sertula* DNA among the clones derived from symbiotic animals (8/85 clones), but none among those constructed using aposymbiotic animals (0/79) (Linneman et al., 2014). The proportion of clones matching the endosymbiont were significantly different for animals identified as symbiotic and aposymbiotic by the PCR assay (p=0.007, two-tailed Fisher's Exact Test).

2.4 Discussion

2.4.1 B. neritina *and latitude*

For Western Atlantic *B. neritina* populations, previous sampling had indicated biogeographic separation between two sibling species that varied in their symbiotic status (Davidson and Haygood, 1999; McGovern and Hellberg, 2003). It was proposed that the aposymbiotic Type N *B. neritina*, which was documented only at higher latitudes, did not possess a defensive symbiont because of lower predation pressures, and that the Type S colonies were symbiotic because they inhabited areas of higher predation pressure. During the course of our study, we discovered that the distribution of the host was much broader than previously thought, while that of the symbiont appears to be restricted by biogeography, instead of host phylotype, as previously thought.

2.4.2 Ranges of B. neritina *sibling species*

Both Types N and S *B. neritina* can be found across a much wider range of the United States' Atlantic coast than initially observed (McGovern and Hellberg, 2003). This is the second report of these sibling species co-occurring, as well as of their occurrence on both sides of Cape Hatteras (Fehlauer-Ale et al., 2014). Both symbiotic Type N and aposymbiotic Type S colonies were unexpected findings in this study. The existence of these previously undescribed types may have been a crucial factor enabling the spread of each sibling species beyond the ranges initially understood. Additionally, while Cape Hatteras is not a strict biogeographic boundary for these sibling species, there is still a marked inversion in relative prevalence occurring around this latitude. Physiological factors likely favor Type N *B. neritina* at higher latitudes and Type S at

lower, although population structure may be based both on fitness in and initial settlement of a region.

Sequencing completed thus far on conspecific colonies from opposite sides of the Cape has not revealed separate lineages within sibling species that are differentially adapted to varying latitudes. Although *B. neritina* larvae have a very short pelagic phase (0.5-2 hr), suggesting a limited dispersal (Keough, 1989; Keough and Chernoff, 1987), adult colonies are often found on the hulls of ships (Carlton and Hodder, 1995; Floerl et al., 2004; Gordon and Mawatari, 1992; Hewitt, 2002) and are thought to be dispersed anthropogenically (Mackie et al., 2006; Ryland et al., 2011). This transport should result in widespread availability of all haplotypes in many locations, such that the documented population structure may be the result of a marked selection pressure.

2.4.3 Range of the endosymbiont E. sertula

Regardless of host identity, symbiosis in sibling species of *B. neritina* is more common at lower latitudes, perhaps reflecting the higher predation pressure in regions nearer to the equator (Manyak-Davis et al., 2013; Vermeij, 1978). While biogeographic structuring has been noted in marine endosymbionts (Sanders and Palumbi, 2011), the latitudinal variation in *B. neritina-E. sertula* symbioses may have novel implications for the bryozoan host. For example, while both sibling species are capable of harboring *E. sertula*, symbiosis is more likely to be observed in Type S colonies on both sides of Cape Hatteras, possibly indicating that that they more readily associate with *E. sertula* than do Type N *B. neritina*. An easier association with a symbiont conferring fitness in the face of higher predation pressure may also serve as one of the physiological factors favoring Types N and S *B. neritina* in their traditionally recognized ranges,

and may in fact be the most important factor that indicated the separation of the two sibling species in almost all studies up to this point. Importantly, the question of identity of the endosymbiont found in Type N *B. neritina* must be addressed, so that implications for ease of association with both sibling species may be assessed.

3 CHARACTERIZATION OF *E. SERTULA* ASSOCIATED WITH TYPE N *B. NERITINA*

3.1 Introduction

The discovery of *E. sertula* in Type N *B. neritina* was surprising and immediately raised questions of the origin and purpose of this association. Strictly vertical transmission of the endosymbionts of *B. neritina* has been suspected due to the bacteria's presence in the larvae and larval brood chambers of colonies (Sharp et al., 2007). In addition, no previous work has demonstrated the incidental capability of antibiotic-cured *B. neritina* to increase its symbiont load while in the presence of fully symbiotic animals [for example, (Lopanik et al., 2004)]. While host switching may be a rare event in the past, the suspected vertical transmission has ultimately led to a high degree of congruence between the phylogenetic topologies of host and symbiont (Lim-Fong et al., 2008).

Types D and S *B. neritina* from California house closely related strains of *E. sertula* which differ at just four nucleotide sites (0.4% of the 996-bp shared sequenced region) in the small subunit ribosomal RNA gene (16S rRNA). In addition, bryostatin composition has been shown to vary between the Type S and D populations (Davidson and Haygood, 1999), and the *bry* gene cluster shows some amount of variation between the sibling species, demonstrating 98% identity and differing most significantly in the genomic location of several accessory genes (Sudek et al., 2007). This understanding led to an initial hypothesis that the endosymbiont associated with Type N *B. neritina* would represent a third *E. sertula* lineage. The lack of symbiont in Type N populations from earlier studies could thus be attributed simply to their location north of Cape Hatteras. The mechanism leading to the loss of this association could be similarly invoked for the newly-discovered aposymbiotic Type S individuals.

It is possible, of course, that the Type N endosymbiont indicates the initiation of symbiosis for at least one *B. neritina* sibling species at some point after its loss within the lineage. This left open the chance that we might see topological incongruence between host and symbiont phylogenies similar to that seen deeper within the bryozoan clade containing *B. neritina* by Lim-Fong et al. (2008), and that host-switching may be more than a one-time event for endosymbionts of the genus *Endobugula*. Additionally, more data were needed to establish whether the *E. sertula* associated with southern Type N *B. neritina*, but not those in northern waters, could produce defensive chemistry similar to those associated with Type S animals. To investigate these possibilities, I compared the Type N symbiont with those of the other two *B. neritina* sibling species.

3.2 Methods and materials

3.2.1 Collection of B. neritina *and preparation for assays*

Adult *B. neritina* were collected from floating docks in coastal locations as described in Chapter 2. Briefly, colonies were taken directly from floating docks in coastal locations from 2010-2013. When possible, adults were kept in flowing seawater tables, and in all cases were covered overnight to minimize light exposure. In the morning, they were placed into glass jars or dishes in order to stimulate the release of larvae through exposure to sunlight. Larvae were then collected manually, by pipet. DNA from both adult and larval samples was extracted using either ZR Fungal/Bacterial DNA MiniPrep or MicroPrep, or DNEasy Blood and Tissue Kit, and following the manufacturer's instructions, with animals not immediately processed similarly stored in RNALater RNA Stabilization Reagent, 70-100% ethanol, or 100% methanol for later analysis. Quality and quantity of DNA were assessed by spectroscopy.

3.2.2 Molecular characterization of host and endosymbiont

As in the survey previously described, PCR amplification of the mitochondrial COI gene was carried out using the universal invertebrate primers LCO1490 and HCO2198 (Folmer et al., 1994) or the *B. neritina*-specific primer pair BnCOIf and BnCOIr. RFLP analysis was again performed on amplicons using the restriction endonucleases *Dde*I, which only digests Type S COI products, or *Hha*I, which cuts amplicons from Type N individuals.

The presence of the symbiont *E. sertula* was assessed in DNA by PCR targeting its 16S ribosomal RNA gene [primers EBn16S_254f and EBn16S_643r, Bn240f and Bn1253r (Haygood and Davidson, 1997)], or the bryostatin biosynthetic gene *bryS* (Sudek et al., 2007) (primers BryS_576f and BryS_774r). Additional PCRs were run using primers targeting elements of the *bry* gene cluster, which prescribes synthesis of bryostatins. Type N *B. neritina* samples determined to be symbiotic were assayed for the presence of either the *bryA* component, or the intergenic region between *bryP* and *bryB* (Figure 3.1), which should be diagnostic for a gene cluster like that of *E. sertula* associated with Type S *B. neritina* (vs. Type D, Figure 3.1) (Sudek et al., 2007). PCR amplicons were visualized after agarose gel electrophoresis.

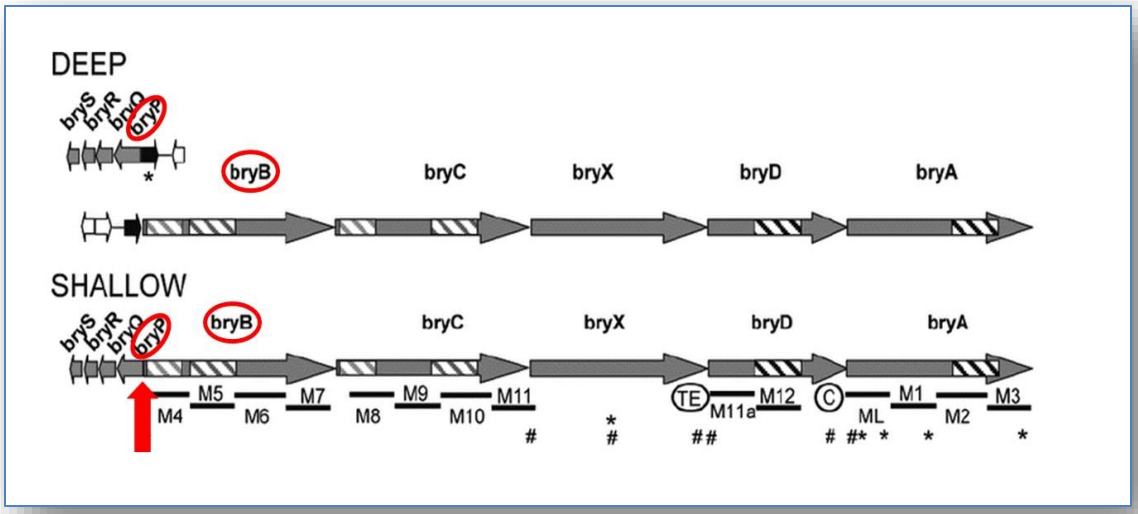


Figure 3.1 Gene cluster prescribing bryostatin biosynthesis. Arrow indicates bryP-B *intergenic region targeted by PCR, indicating a Type S-like* bry *gene cluster. [Modified from Sudek et al. (2007).]*

For selected samples, the COI or 16S PCR amplicon was purified using the GeneJET PCR Purification Kit and sequenced at the Georgia State University Core Facility. An additional PCR was performed for a number of samples using the primers EBn16S_621f and ITS-23S-r2 to amplify a contiguous fragment of the ribosomal RNA gene containing the 3' end of the 16S rRNA gene (for identity confirmation) and the complete internal transcribed spacer (ITS) region leading to the 5' end of the 23S rRNA gene. These products were purified with the GeneJET kit and sequenced at Yale University's DNA Analysis Facility (New Haven, CT).

3.2.3 Chemical extraction and analysis of B. neritina *larvae*

Crude extracts were obtained from larvae stored in methanol using a six-step process of exhaustive extraction, with the first and last steps using 100% methanol, and the remaining four using an approximately 1:1 mixture of methanol and dichloromethane as in

(Lopanik et al., 2004). Solvents were removed by rotary evaporation and extracts were dissolved in a 6:3:1 mixture of methanol:dichlormethane:water according to the number of larvae collected, based upon counts conducted at the time of collection or immediately after extraction. Extracts were analyzed via high-performance liquid chromatography (HPLC, with photodiode array detection, Shimadzu, Columbia, MD) eluted on a C18 analytical column (250x4.6 mm Gemini 5 µm, Phenomenex, Torrance, CA) with a gradient time program using water and acetonitrile as solvents. Chromatogram peaks were examined for maximum absorbance at or near 229 nm, a wavelength previously demonstrated to be diagnostic for bryostatins found along the Atlantic coast of the United States (Lopanik et al., 2004).

3.2.4 Feeding assays

The difficulty in finding sufficient numbers of symbiotic Type N *B. neritina* larvae prohibited feeding assays for much of the time covered by this work. In 2015, however, attempts began in earnest to carry out assays that would demonstrate deterrence of predators due to association of Type N *B. neritina* with *E. sertula*. Four attempts were made in total. These began in March 2015, when larvae were collected from six colonies gathered from floating docks near Beaufort, SC, and settled in six-well polystyrene plates for field growth in North Carolina. The goal was to begin this experiment early in the spring growth period for *B. neritina*, allowing for harvesting of next-generation larvae and their subsequent use in feeding assays later in the season.

Larvae were divided for settlement between control plates containing filtered seawater and antibiotic plates containing filtered seawater with 100 µg/ml gentamycin. This treatment continued for 2-3 days in order to eliminate *E. sertula* from juvenile colonies; water was

regularly changed in wells to ensure antibiotic efficacy. A small amount of tissue (~10-20 zooids) was cut from each colony and ground in 20 µl phosphate-buffered saline. An aliquot of 2 µl of this mixture was then used as template in PCR in order to determine the phylotype and symbiotic status of each colony, as described in Chapter 2. All juvenile colonies were mapped within wells, and plates were suspended from a dock in the waters of Morehead City, NC (in Bogue Sound).

When the first experiment was followed up in May 2015, a second group of plates was similarly generated for transplant to the University of Virginia's Anheuser-Busch Coastal Research Center, in Cape Charles, VA. Parent colonies came from Salter Path, NC, and Russ Point Boat Landing on Hunting Island, SC. A total of 20 colonies were used in preparing five control and five gentamycin-treated plates. Juvenile colonies were again mapped, and parental phylotype and symbiotic status was determined as above, with some samples' requiring later extraction and analysis of DNA from zooids stored in RNAlater.

In addition, feeding assays were attempted using whole larvae collected from six symbiotic Type N colonies gathered on Hunting Island and two aposymbiotic Type S colonies gathered at the Morehead City Yacht Basin in Morehead City, NC. Predators included juvenile pinfish (*L. rhomboides*) caught at a public dock in Morehead City and polyps of the sea anemone *Aiptasia pallida* collected from a filtration pond at the UNC-IMS. Krill eyes cut into quarters were used as additional control foods.

The second group of settled plates was recovered from Virginia approximately two months later, in July 2015. After clearing new recruits and fouling organisms from the plates as much as possible, adult colonies were set out for collection of larvae for use in feeding assays as described in Chapter 2. In addition, the adult, colonial offspring of four individuals collected in

May 2015 were used for chemical extraction. Ovicell-bearing portions of the colonies were selected, as Lopanik *et al.* (2006) demonstrated that extracts of ovicell-bearing adult *B. neritina* tissue significantly reduced feeding by pinfish, while ovicell-free zooids did not. These portions were cut and allowed to dry for >2 h on paper towels. The volume of each portion was measured by placing the animals in a graduated cylinder containing filtered seawater, and chemical extraction was performed on the zooids as described above. Dried extracts were redissolved in methanol, then spread onto a glass dish and allowed to dry. Squid paste with added sodium alginate was spread onto the dish, then stirred and kneaded liberally to allow the mixture of extract throughout. Squid paste volume was either equal to the volume of *B. neritina* extracted or 1/3 colony volume, depending on the assay. Control food was made using the above procedure, but without dissolving anything in the methanol used. After adequate mixing, the squid paste was divided into ~5 µl pellets and sprayed with calcium chloride to bring the paste to a pasta-like consistency. These pellets were then rinsed and used in feeding assays with pinfish and sea anemones, which were collected as before. Standard procedure was to offer a control pellet to ensure that the predator was hungry, then offer a pellet with extract if the control pellet was eaten. If the treatment pellet was rejected, a second control pellet was offered to ensure that the rejection was not due simply to satiation.

Finally, in November 2015, I attempted to simply locate sufficiently gravid symbiotic Type N *B. neritina* so that feeding assays could be performed using extracts from larvae of natural populations. *A. pallida* were again collected from the UNC-IMS filtration pond, but pinfish, which were more difficult to catch at their autumn age and size, were acquired from another UNC researcher. Adult *B. neritina* colonies were gathered from floating docks in Beaufort, NC, and from Mariner's Point and Homer's Point Marina in Salter Path, NC (with the

latter two sites known to host reasonable numbers of Type N *B. neritina*). Colonies from individual locations were pooled for collection of larvae, and tissue was combined for assessment of phylotype and symbiotic status (as described above) to ensure that the groups could be considered collectively as a single phylotype and status. Larvae were extracted using methanol and dichloromethane, as described above, and feeding assays were carried out as in July 2015, with squid paste volume equal to the volume of *B. neritina* extracted or 1/2 larval colony volume, depending on the assay. (The method was altered after initial concerns that the fish, due to their size or time in captivity, were not reacting as in previous feeding assays to extract from symbiotic animals, including those of Type S.) Additional aposymbiotic Type N control larvae were also used in these procedures from previously collected samples originating in the Indian River Inlet, DE, and public docks in Oyster, VA. All statistical analyses for feeding assays were performed using VassarStats software.

3.3 Results

3.3.1 Phylogeny of Type N-associated E. sertula

Amplicons of the 16S rRNA gene from Type N symbionts collected from Radio Island, NC, Beaufort, SC, and Hunting Island, SC, had sequences identical to those of Type S symbionts from Hunting Island, SC, and Beaufort, SC. These sequences also matched the 16S rRNA gene sequence reported by both Davidson and Haygood (Davidson and Haygood, 1999) and McGovern and Hellberg (McGovern and Hellberg, 2003) for Type S *E. sertula*. Sequences obtained using primers starting at position 621 in the 16S rRNA gene and ending in the 5' region of the 23S rRNA gene also matched previous 16S sequences. An ITS region of ~850 bp was amplified and demonstrated to be identical between the Type N and Type S symbionts examined.

After verification of contiguity from the *E. sertula* 16S sequence to its newly characterized ITS sequence, further sequencing was performed from the 3' end of the amplicon (starting in the 23S region). All six samples (3 Type N, 3 Type S) were identical across 932 bp of shared sequencing.

Significantly, this identity is in stark contrast to Type D ITS sequences obtained from California colonies. Restriction analysis of host COI amplicons with *Msp*I indicated that all California samples from Catalina Island (n=7) were of the Type D sibling species, with further digestion of 16S rRNA gene amplicons using *Nhe*I (Davidson and Haygood, 1999) revealing the endosymbiont to also be of Type D. Sequences from two Type D ITS amplicons differed from both the Type N and Type S ITS by 4.0% and 4.3% (Table 4.1) depending upon the length of quality read. Among *B. neritina* samples taken from Bodega Bay, one proved to be of sufficient quality for DNA characterization. This colony was confirmed to be Type S by COI restriction digestion with *Dde*I and *Msp*I. The ITS sequence amplified from this sample was identical to those of Type S and N *E. sertula* from the Atlantic coast, reducing concerns that the differences between the Type D symbiont ITS and that of Types N and S are a consequence of location. Instead, endosymbiont identity appears to consistently relate to host phylotype.

3.3.2 Bryostatins and bryostatin genes associated with Type N E. sertula

PCRs indicated the presence of the *bryA* gene in *E. sertula* associated with Type N *B. neritina*, as well as a short (~200 bp) intergenic region between the genes *bryP* and *bryB*, demonstrating similarity to the layout of the Type S *bry* gene cluster. Analysis of bryostatin composition in Type N symbiotic larval extracts by HPLC at 229 nm illustrates a chemical profile that is also very similar to that of pooled larvae from largely Type S populations (Figure

3.3). Interestingly, larvae from aposymbiotic Type N *B. neritina* found alongside symbiont-protected Type S animals in North Carolina showed few peaks that appear to be bryostatins when analyzed in this manner. While the co-occurrence of these protected and unprotected colonies is somewhat surprising, the stark chromatographic contrast between symbiotic and aposymbiotic Type N animals highlights the relative similarity of symbiotic Type N larvae to Type S larvae from both Beaufort and Morehead City, NC.

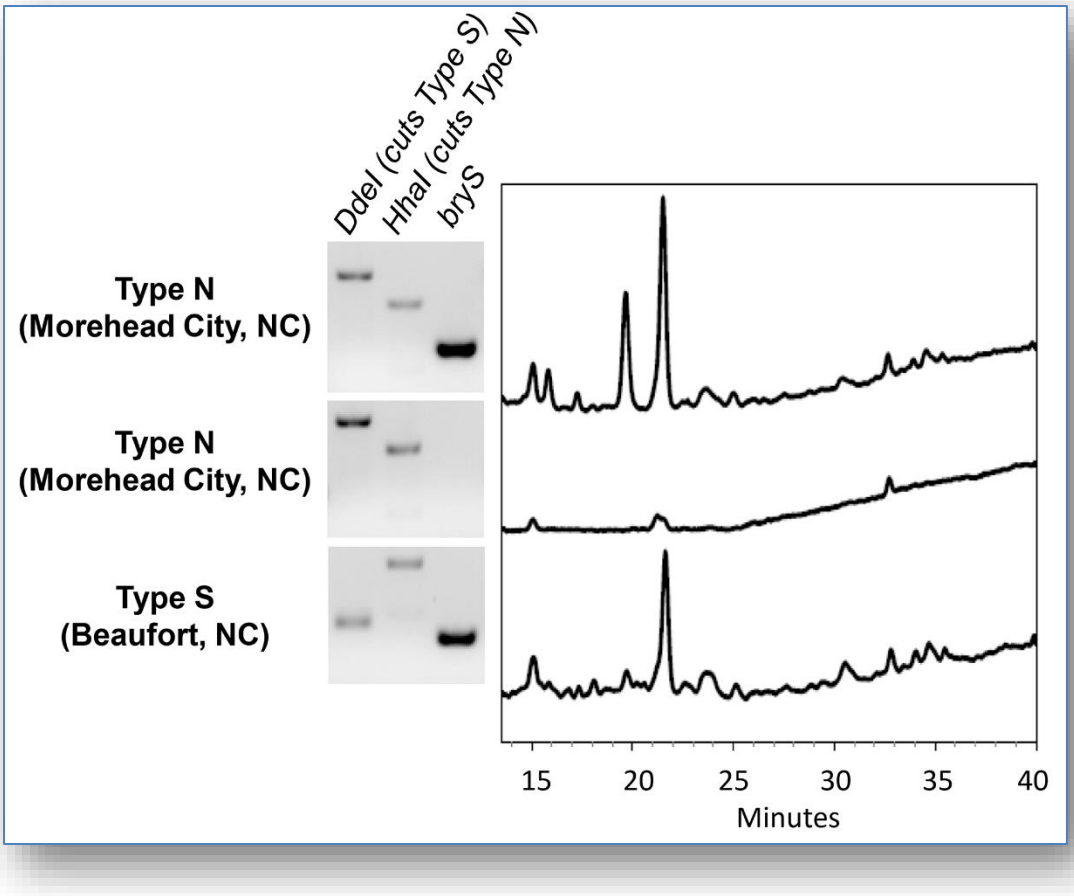


Figure 3.2 Molecular characterization of B. neritina *and HPLC analysis of crude larval extract – Agarose gel showing* Dde*I and* Hha*I restriction digestion of* B. neritina *adult or pooled larval cytochrome* c *oxidase I and amplification of* bryS *to demonstrate presence of symbiont; and absorbance at 229 nm (which is diagnostic of bryostatins) of extract collected from larvae represented by this gDNA. (From Linneman et al., 2014.)*

3.3.3 Feeding assays

All of the feeding assay attempts were met with difficulties in completion or inconclusive results. The first settled plates, from March 2015, were too overgrown by the time of planned assays in May 2015 to allow for separation of experimental colonies from new recruits. May 2015 assays using whole larvae could not be completed due to predators' inability or unwillingness to give attention to the larvae. In July 2015, feeding assays were completed with

extracts from adult *B. neritina* colonies. Parental colonies were all found to be Type N, symbiotic animals. In the first two assays, in which extract concentration was equal to that found in the colonies, all pellets were eaten by *L. rhomboides* (23/23 from gentamycin-treated colonies, 19/19 from untreated colonies) and sea anemones (5/5 from untreated colonies) with no appearance of rejection. For the remaining two assays performed, the protocol was modified to use extracts at 3x the concentration found in the animals, in order to see if rejection could be elicited by increasing the amount of deterrent metabolite. However, once again, all pellets were eaten by both pinfish (9/9 from gentamycin-treated colonies, 13/13 from untreated colonies) and anemones (8/8 from gentamycin-treated colonies, 8/8 from untreated colonies). Two fish did initially reject part of the experimental pellets, but both ingested at least a portion, and one even returned to the initially rejected fragment to eat it.

In November 2015, the assays began with an unexpected complication. Squid paste pellets containing extracts from symbiotic Type S *B. neritina*, demonstrated in previous experiments (Lopanik et al., 2004; Lopanik et al., 2006) to be readily rejected by pinfish, showed very little difference in palatability as compared to control pellets (6/7 were eaten by fish who accepted the initial control pellet; p=0.500, McNemar's test). Similarly, 7/7 pellets containing Type N extract were eaten. Because extracts were in very short supply, and there were questions about the suitability of the pinfish for the experiment, it was decided to double the concentration within the pellets for further assays. This still resulted in acceptance of the food pellet by 5/7 fish for Type S *B. neritina* extract (p=0.250) and 7/8 for Type N (p=0.500). All pellets (4/4) containing the limited available extract from aposymbiotic Type N larvae were also eaten by pinfish, showing no significant difference from symbiotic larvae of either phylotype (vs. Type S, p=0.382; vs. Type N, p=0.667; one-tailed Fisher's exact tests).

However, a somewhat informative result could be found in observation of sea anemones used in the study. While all anemones willing to ingest the first control pellet also ate the experimental pellet (n=16 in total), and animals were observed for >1 hour to ensure the pellets were kept down, a check of the anemones 5 hours after the day's first assay revealed that a number of the *A. pallida* had regurgitated the food. Two of these anemones were involved, about 3 hours apart, in experiments with both Type N and Type S extract, so they are excluded from analysis here. For anemones only fed control pellets and those containing extract from symbiotic Type N *B. neritina* larvae, 4/12 later regurgitated the pellets. This approaches significance via McNemar's test for significance of changes (p=0.063), although issues may certainly be raised, not only with the test's failure to reach 95% confidence, but also with a methodology that fails to discriminate between the effects of the control and the experimental food pellets.

3.4 Discussion

The similar chemistry associated with the symbionts of both Types N and S *B. neritina* suggest that their role in defending bryozoan larvae applies to both sibling species. While feeding assays could be described at best as inconclusive, HPLC analysis revealed a high degree of similarity between extracts from symbiotic larvae of both phylotypes. In addition, the Type N *bry* biosynthetic gene cluster appears to have retained the topology associated with Type S symbionts, with accessory genes immediately upstream of the *bryB* gene, as opposed to Type D *E. sertula*, in which accessory genes have not been found within ~30 kb upstream of *bryB* (Sudek et al., 2007). More importantly, analyses using 16S rRNA gene and ITS sequences give no indication that the symbiont associated with Type N *B. neritina* differs from that of Type S

colonies. This is surprising if *E. sertula* is subject to strict vertical transmission. Endosymbionts associated with Type S and D *B. neritina* have demonstrated differences of up to 0.5% even in the slowly evolving 16S rRNA gene sequence, with host COI sequences varying by 8.2% (Davidson and Haygood, 1999; McGovern and Hellberg, 2003). In comparison, Type N and Type S COI sequences differ by 11.5%. One would expect this difference to correlate with an equal or greater likelihood of variation in the symbionts' 16S rRNA gene identity, but none is observed. Indeed, multilocus phylogenetic analysis of the *B. neritina* complex indicates that Type N animals form a sister group to the clade containing Types S and D *B. neritina* (Fehlauer-Ale et al., 2014), further suggesting that cospeciation of host and symbiont would lead to greater divergence between Type S and Type N symbionts.

The slow evolution of the 16S rRNA gene, however, makes in-depth comparison difficult. In fact, while the "*Ca.* Endobugula sertula" sequences obtained by McGovern and Hellberg (McGovern and Hellberg, 2003) from Type S *B. neritina* differed from those of Haygood and Davidson's (Haygood and Davidson, 1997) Type S sequences by just 2 bp (0.2%) and Type D sequences by 4-5 bp (0.5%), there were an additional 2 bp that were unique to the later Type S sequences and earlier Type D sequences, while not being shared by the first characterized Type S symbionts. The 16S rRNA gene alone may simply be unreliable for elucidating "*Ca.* Endobugula sertula" phylogeny. In contrast, while 16S rRNA gene sequences may not provide a fast enough clock for contrast, differences might still be seen in ITS sequences (Table 1), as they have been demonstrated to vary up to 9% among clades even within a single bacterial species (Erwin and Thacker, 2006). Cytochrome oxidase I variation among *B. neritina* sibling species is well within the expected range for congeneric species of invertebrate phyla, which average 11.3% (Hebert et al., 2003).

Table 3.1 Percent identity of marker sequences among B. neritina sibling species. Types D and S show the greatest COI identity, but Type D differs from both Types S and N in symbiont markers.

Marker	Type	N	S
COI	D	89.6	91.8
	N		88.5
16S	D	99.6	99.6
	N		100
ITS	D	95.7	95.7
	N		100

Coevolution of host and symbiont would lead to the expectation of associated ITS variation. While whole-genome sequencing may reveal subtle differences, the identities indicated by the selected genes leave no room for ambiguity (see 100% identity between Types N and S in Table 3.1). That genetic differences have not been observed between Type N and Type S *E. sertula* leaves open the possibility of horizontal transmission of the symbiont between these two sibling species. While potentially not the primary mode of symbiont transfer, such events have been observed in other symbionts demonstrating vertical transmission with a high degree of fidelity (Jaenike et al., 2010), and I note that co-analysis of *Bugula* host and symbiont phylogenetic topologies reveals the probability of at least one host-switching event in the lineage's past (Lim-Fong et al., 2008). The observed similarity between Type N and S

symbionts, both genetically and chemically, may be the result of a very recent horizontal transmission event, potentially enabled by uptake into the colony's funicular system via the gut. Further investigation of the sibling species' invasion patterns and experimentation with horizontal acquisition of their symbiont may shed more light on the history of this mutualism.

4 INVESTIGATION OF *BUGULA NERITINA-ENDOBUGULA SERTULA* DYNAMICS

4.1 Introduction

The identification of both Type N and Type S *B. neritina* colonies that differ in symbiotic status raised questions regarding the mediation of this association. While it is believed that *B. neritina* does not readily gain the endosymbiont in nature, the capability of individuals to abandon the association is largely unexplored. Environmental factors may limit the survival of the endosymbiont in high-latitude waters, with unprotected aposymbiotic animals found only transiently at low latitudes due to anthropogenic dispersal of adult *B. neritina*.

Perhaps more importantly, the varying association of both *B. neritina* sibling species according to latitude may be the result of a balance of positive and negative fitness effects driven by the bryozoan's harboring of the symbiont. As noted previously, beneficial defensive symbionts may protect host organisms from pathogens, competitors, or predators (Lopanik, 2014). These ecological dangers are necessarily context-dependent. An example can be seen in the relationship between cleaning gobies and longfin damselfish, which is beneficial in high densities of damselfish ectoparasites, but shifts to a more parasitic interaction at low ectoparasite densities (Cheney and Cote, 2005). It is likely that many ecologically relevant scenarios, such as low predation pressure, will cause the cost of hosting a symbiont to eclipse the potential benefits of a partnership. Thus, unpartnered hosts should have greater fitness than those maintaining the symbiont. This has been shown in other host/symbiont interactions, including those between the pea aphid and its protective symbiont, *Hamiltonella defensa*, and the cleaning symbiosis of crayfish and branchiobdellidan worms (Oliver et al., 2008; Polin et al., 2014; Thomas et al., 2013).

In this circumstance, selection over time would then ultimately result in symbiont loss. This may be especially true for host-dependent, vertically transmitted symbionts, which may require a significant energetic investment from the host in order to maintain the association. An increase in aposymbiotic host frequency in environments with few targets for the symbiont's defensive capabilities suggest that the symbiont imposes a cost on its host [reviewed in (Clay, 2014)]. There may, in fact, be an interplay of host cost-benefit comparison and biogeographical tendencies of symbiont survival that serves to mediate such relationships across wide distributions. With this in mind, experiments were undertaken to determine whether or not a cost could be determined for the bryozoan *B. neritina*'s association with *E. sertula*, and to see if different environments directly resulted in differing symbiont levels.

4.2 Methods and materials

4.2.1 Exploratory transplant

In late October 2012, six colonies each from Radio Island Marina and Morehead City Yacht Basin, along with five from Oyster, VA, were selected from sampled animals, with larvae collected as described in Chapter 2. Larvae were allowed to settle in filtered seawater placed in 6-well polystyrene plates. Each well contained larvae from a single parent, and each parent's larvae were split between two plates, for separate placement in North Carolina and Virginia. After settlement, juvenile colonies were mapped so that new recruits could be identified after placement. Tissue samples were cut from each colony for DNA extraction and subsequent phylotyping and assay for the presence of symbiont.

Plates were divided between two locations for placement, a flowing seawater table at the UNC-IMS in Morehead City, NC, and a floating dock at the University of Virginia's Anheuser-

Busch Coastal Research Center, in Cape Charles, VA. All plates were checked approximately one month later to ensure colony growth. In January 2013, up to three colonies were selected haphazardly from each well, and counts were made of the zooid number as a measure of each colony's size. (In this and all further growth experiments, a general window of 1-3 months after settlement was targeted for measurement, as this period would ideally provide adequate growth time and allow for appropriate scheduling of travel while avoiding overgrowth that would hinder or prevent data collection.) In March and May 2013, zooid counts were performed for all remaining colonies. In addition, colonies were sacrificed in March 2013 and stored in RNAlater for subsequent PCR analysis. DNA was later extracted using the ZR Fungal/Bacterial DNA MiniPrep, as previously described. After checking sample quality and quantity by spectroscopy, useable templates were diluted in a 1:5 series to concentrations of 10 ng/µl, 2 ng/µl, 0.4 ng/µl, and 0.08 ng/µl. PCR was run using the cytochrome *c* oxidase I primers BnCOIf and BnCOIr to assess the presence of host DNA, and the *brySP* primers BryS_576f and BryS_774r to assess the presence of symbiont DNA. Results of this semi-quantitative PCR were visualized via agarose gel electrophoresis.

4.2.2 Expanded transplant

From colonies collected in late October 2013, larvae were collected and settled from six South Carolina colonies and five Virginia colonies. For settlement, larvae were divided into control plates containing filtered seawater and antibiotic plates containing filtered seawater with 100 µg/ml gentamycin, as described in Chapter 3. This treatment continued for 3 days in order to eliminate *E. sertula* from juvenile colonies; water was regularly changed in wells to ensure antibiotic efficacy. All juvenile colonies were mapped within wells, and plates for this

experiment were placed beneath docks in Cape Charles, VA, and Bogue Sound in North Carolina. Tissue was cut from each parent colony for phylotyping and symbiont assays.

In mid-December, transplanted plates were cleared of new recruits, and colony sizes were determined by counting the total number of zooids. At least three prominent and accessible colonies, if available, were selected to represent each well. For wells containing 3+ colonies, one was sacrificed and placed in RNAlater for subsequent DNA extraction and quantitative real-time PCR (Q-PCR) analysis. The time of the counts and sacrifices represented a span of 40-43 days of growth, dependent upon the day of settlement and travel to locations for data collection.

4.2.3 Common garden experiments

In May and November 2014, *B. neritina* colonies from floating docks in Beaufort, NC, and the Morehead City Yacht Basin, respectively, were used in collection of larvae, which were settled and subjected to gentamycin treatment as described above. In November 2014, an additional experimental group was also subjected to antibiotic treatment with 100 µg/ml streptomycin in order to control for secondary effects of antibiotic treatment. Plates were transported to aquaria at Georgia State University, Atlanta, GA, and grown at room temperature under 12 hour/12 hour light/dark cycles. Colonies were fed at least three times per week, typically ~1.2 liters (per aquarium of *B. neritina*) of *Rhodomonas* CCMP768 (stocks acquired from the Provasoli-Guillard National Center for Marine Algae and Microbiota) that had been grown to near-maximum density and spun down in a benchtop centrifuge.

After 24 days in a shared aquarium, the May 2014 plates were divided into three temperature conditions (Cold, Room, Warm). These aquaria were all initially ~21-22°C, similar to the shared tank, and the Cold and Warm aquaria were gradually altered over the course of 11

days to ~14°C and ~26°C, respectively. Throughout the experiment, the Cold aquarium was the most difficult to keep stable, varying from 11.6°C to 14.8°C. All aquaria were monitored and adjusted to ensure the most stable temperatures possible. After 30 days, colonies from each plate were sacrificed and placed into RNAlater for subsequent DNA extraction and Q-PCR analysis. To support the recovery of adequate DNA concentrations, all colonies within a single well were treated as a single sample.

For the plates settled in November 2014, zooid counts were taken after 15 and 31 days. For the first count, all colonies were considered (n=171); in the second, half of each of the gentamycin- and streptomycin-treated plates were haphazardly selected for counting (total n=109).

4.2.4 Quantitative real-time PCR

For DNA samples from the May 2014 common garden experiment and the December 2014 transplants, relative quantification of symbiont levels was carried out using the method of Mathew and Lopanik (2014). Q-PCR was performed using the primer sets BnCOIF/BnCOIR, targeting the host COI gene (Lopanik et al., 2004), and EBn16S_254f/EBn16S_643r targeting the endosymbiont's ribosomal RNA gene (Mathew and Lopanik, 2014). The DyNAmo HS SYBR Green qPCR kit (Finnzymes) was used, including SYBR Green I flurorescent dye and ROX passive reference dye. Reactions were carried out in duplicate on an Applied Biosystems 7500 Fast Real-Time PCR system, with an initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with subsequent melt-curve analysis to indicate nonspecific products. Each sample's mean threshold cycle (C_T) values for 16S and COI were then used to calculate a ratio indicative of symbiont

level within the sample, with higher values' indicating less symbiont DNA due to the use of C_T values in the numerator.

4.2.5 Data analysis

Data from transplant and common garden experiments were analyzed via Student's *t*-test and one-way and multi-factor analyses of variance (ANOVAs), using PASW Statistics software (SPSS Inc., Chicago, IL).

4.3 Results

4.3.1 Impact of symbiont on B. neritina *growth in transplant experiments*

For the initial transplant carried out in 2012, with data collected at three points in early 2013, all colonies originating in North Carolina were symbiotic Type S *B. neritina*, while all colonies of Virginia origin represented aposymbiotic Type N. The largest colony counted within each well was considered. These colonies represented the largest remaining colony from each parent colony as found in both transplant locations. Using locations of origin and of transplant placement as independent variables, all data were normal by Shapiro-Wilk and Kolmogorov-Smirnov tests, and homoscedastic via Levene's test for equality of variances. Colonies grew significantly better in Virginia during the first three months [47.2 zooids/colony in VA, 19.2 zooids/colony in NC; $F(1,17)=48.310$, $p<0.001$ by ANOVA]. The possibility of this significance was anticipated, as the resource availability in the Virginia location was thought to be potentially stronger than that in the North Carolina water table. This difference faded to non-significance in the counts two and four months later [$F(1,22)=1.800$, $p=0.193$; and $F(1,11)=0.375$, $p=0.553$, respectively]. No significant interaction ever emerged between the variables of origin and

placement locations [$F(1,17)=0.695$, $p=0.416$; $F(1,22)=1.774$, $p=0.197$; $F(1,11)=0.119$, $p=0.737$, chronologically].

Interestingly, for all three points of data collection, colonies originating in Virginia, which were aposymbiotic, were significantly larger than those of North Carolina origin (all symbiotic; see Figure 4.1). This difference became more pronounced over the course of the experiment, with the margin ranging from 12.8 zooids in January 2013 to 195.8 zooids in May 2013. It must be noted that due to the results of phylotyping and symbiont assays, location of origin accounts for two important variables in the *B. neritina* colonies – sibling species identity and symbiotic status. Thus, symbiotic status was manipulated in the next experiment to ensure otherwise equivalent groups.

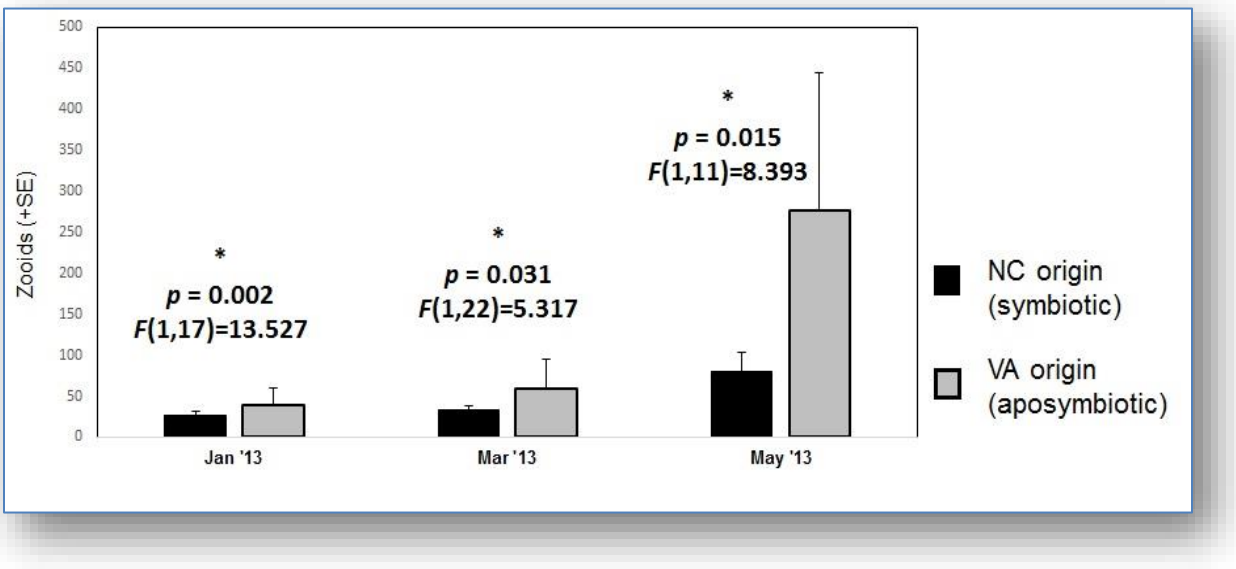


Figure 4.1 Growth of transplanted B. neritina *colonies, as measured by number of zooids per colony. Largest surviving colony that could be found for each parent in both locations considered. n=21, n=26, n=15, chronologically.*

For the second transplant experiment, begun in November 2013, the offspring of six South Carolina and six Virginia colonies were measured. All Virginia colonies were Type S, while South Carolina colonies were split evenly between Types N and S. All but one parent colony, a representative from Virginia, tested positive for the presence of *E. sertula*. This initially aposymbiotic colony's offspring were thus removed from further analysis. Gentamycin-treated colonies were determined to be significantly reduced in levels of *E. sertula* by calculation of 16S/COI Q-PCR C_T ratios. Data were normal by the Shapiro-Wilk test, with gentamycin-treated colonies' having a significantly higher mean ratio of 1.60 vs. the control colonies' 1.34 [$F(1,11)=46.175$, $p<0.001$, n=15] by factorial ANOVA using placement location and antibiotic treatment as independent variables.

Growth data were divided by location for further analysis. Due to the timing of the experiment much nearer to optimal growth conditions in North Carolina than in Virginia, *B. neritina* colonies grew significantly better in the southern location by a rather wide margin [47.1 zooids/colony in NC, 15.0 zooids/colony in VA; $F(1,142)=38.685$, $p<0.001$ by ANOVA when all variables were considered (n=154)]. With a possible additional confound due to differing morphology between Type N and S colonies, data were thus analyzed within the groups of growth location and sibling species, with site of origin serving as the sole potential interacting variable.

Two-way ANOVA revealed no interaction between site of origin and gentamycin treatment for Type S colonies in either growth location, nor did site of origin have a significant effect on colony growth. However, treated colonies significantly outgrew untreated colonies in Virginia [$F(1,55)=9.708$, $p=0.003$], while treatment had no effect on colonies grown in North Carolina (Figure 4.2).

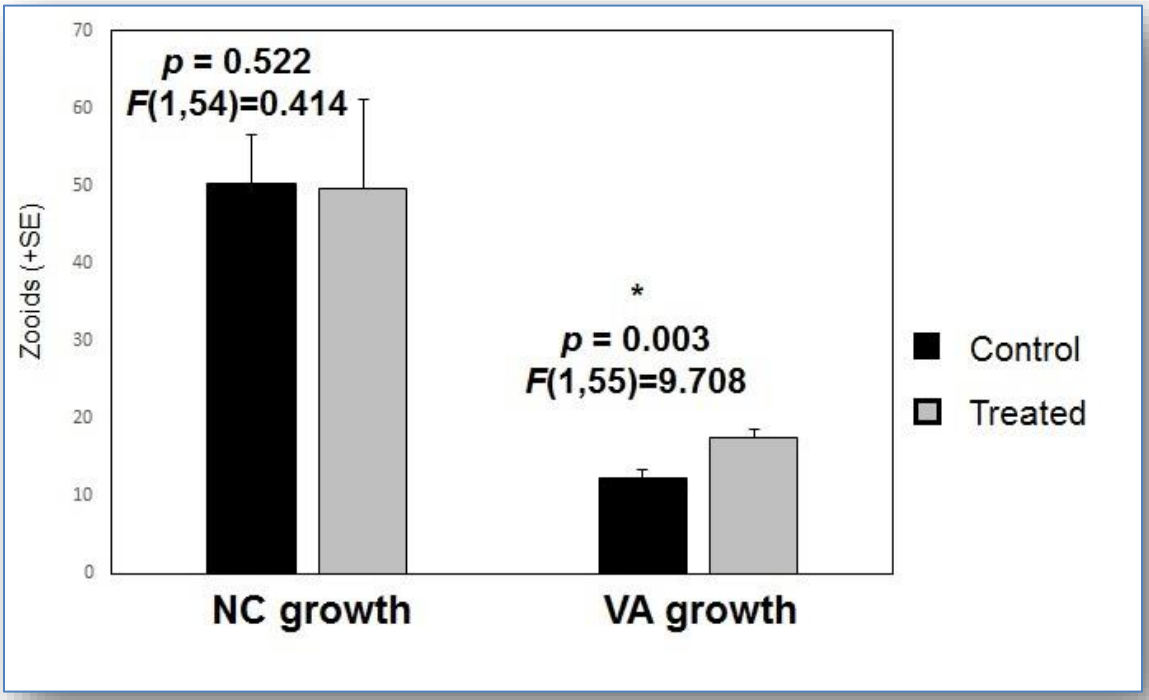


Figure 4.2 Growth of transplanted Type S B. neritina *colonies, as measured by number of zooids per colony. Up to three colonies from each parent, in both locations, considered after approximately 40 days' growth.*

Interestingly, growth location seemingly impacted Type N colonies in an opposite manner. Because all Type N colonies in this study originated in South Carolina, site of origin did not need to be factored into the analysis, and comparisons were made via Mann-Whitney *U* test after determination of unequal variances by Levene's test. While aposymbiotic Type S animals had an apparent growth advantage over those maintaining the symbiont in Virginia (but not in North Carolina), Type N colonies were larger in North Carolina when aposymbiotic ($p<0.001$) but were not impacted by symbiosis when grown in Virginia ($p=0.082$, Figure 4.3). These results represent a continuation of the trend of aposymbiotic animals' having higher

growth rates, as demonstrated in the previous experiment. Additionally, they may indicate a deeper interaction among symbiotic status, growth environment, and sibling species identity.

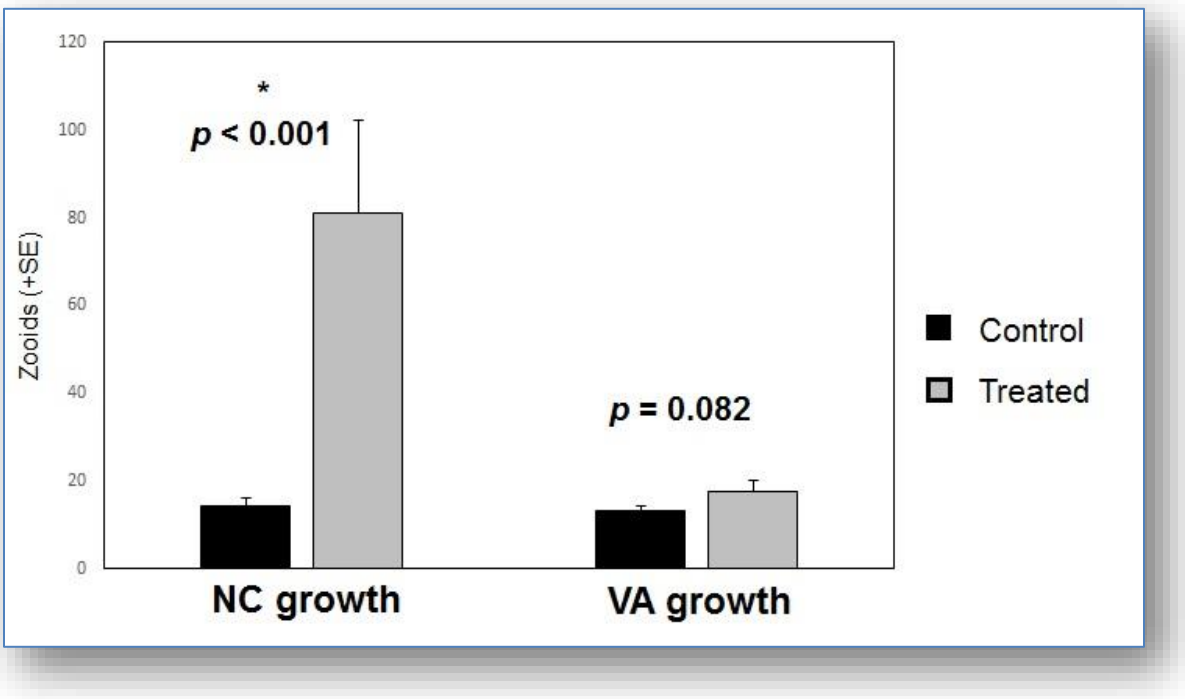


Figure 4.3 Figure 4.4 Growth of transplanted Type N B. neritina *colonies, as measured by number of zooids per colony. Up to three colonies from each parent, in both locations, considered after approximately 40 days' growth.*

4.3.2 Impact of symbiont on B. neritina *growth in common garden experiment*

For animals grown in artificial conditions at Georgia State University, all antibiotic-treated colonies were considered together in comparison to controls. These simple comparisons could be performed via one-tailed *t*-tests due to the fact that I anticipated, based upon the above work, that antibiotic-cured colonies would grow faster than those left untreated. After 15 days, mean colony sizes were nearly identical (3.85 zooids/control colony, 3.79 zooids/antibiotic-treated colony; p=0.257). At 31 days' growth, gentamycin-treated colonies had outgrown

control colonies by one zooid (11.6 vs. 10.6, *p*=0.04; Figure 4.5). Temperature was not manipulated in this experiment.

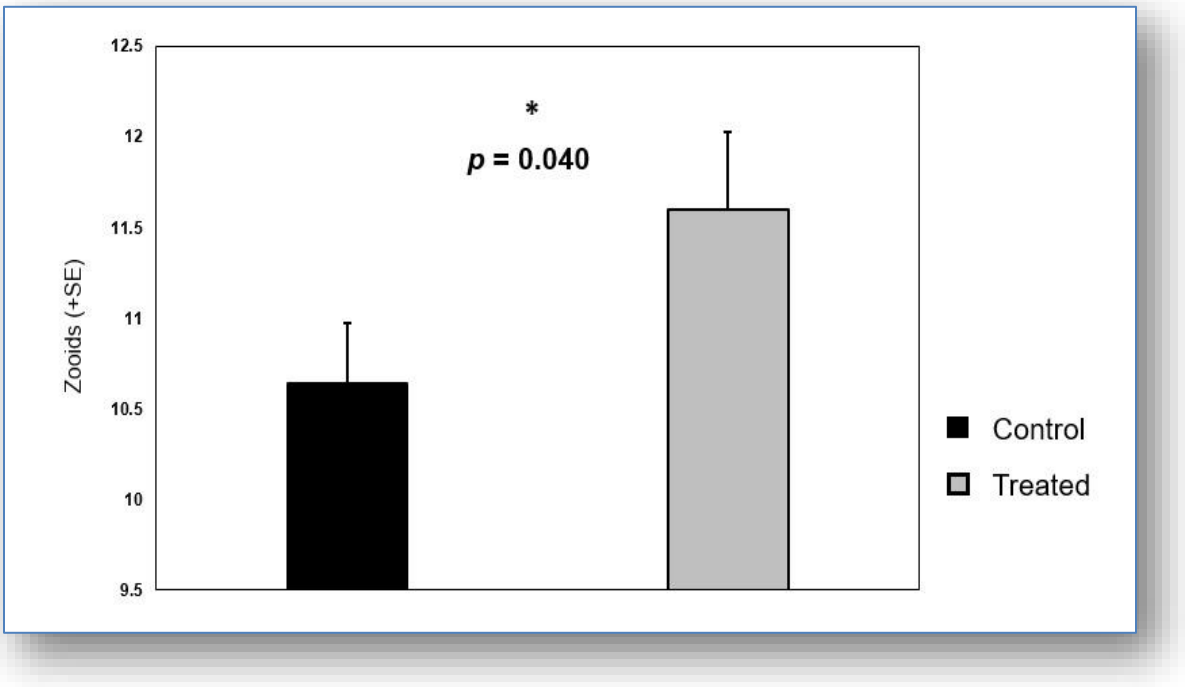


Figure 4.5 Growth of common garden B. neritina *colonies. Zooids counted after 31 days' growth (n=109, analyzed via one-tailed* t*-test).*

4.3.3 Impact of environment on symbiont maintenance

Semi-quantitative PCR using DNA templates derived from transplanted *B. neritina* colonies after approximately 4 months' growth in both North Carolina and Virginia revealed an apparently lower symbiont titer for colonies in northern waters (Figure 4.6). Template dilution to 0.8 ng/µl led to the near or complete extinction of *B. neritina* DNA sensitivity for all samples. At the concentrations examined, however, the symbiont (*brySD*) gene was only detectable for

colonies grown in North Carolina. Those grown in Virginia showed no evidence of remaining *E. sertula* DNA.

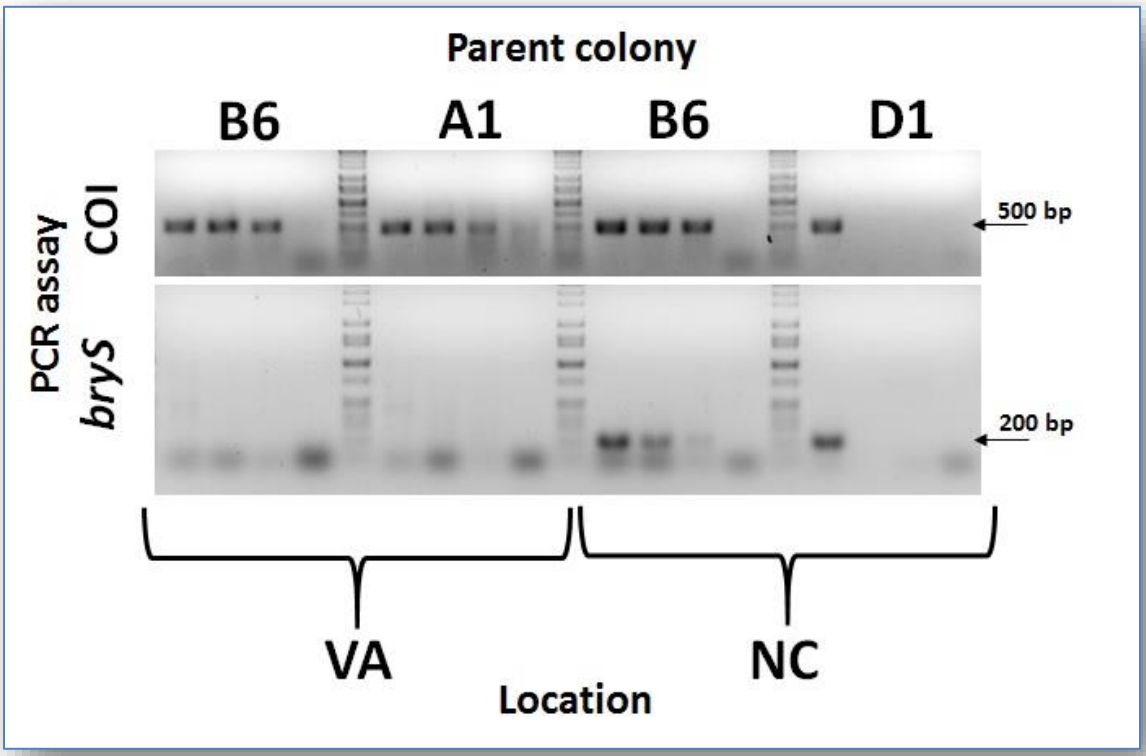


*Figure 4.6 Semi-quantitative PCR of transplanted colonies' DNA – PCR targeted host COI (top) and endosymbiont's bryostatin biosynthetic (*bryS*, bottom) genes. For each sample, PCR was run using (L-R) 10 ng, 2 ng, 0.4 ng, and 0.08 ng of template DNA. Parent colony shown at top; location of colony growth indicated at bottom.*

Interestingly, while location itself did not have a significant main effect in Q-PCR data from the transplant experiment, an interaction between location of growth and antibiotic treatment status was indicated [$F(1,11)=27.342$, $p<0.001$]. Further investigation reveals that if only the untreated control colonies are compared, the 16S/COI C_T ratio is greater for those reared in Virginia than those in North Carolina, indicating a lower symbiont level in Virginia (1.46 vs.

1.24, *p*=0.004 by two-tailed Student's *t*-test, n=9; see Figure 4.7). Strangely, for colonies that were treated with gentamycin, those grown in Virginia actually had a significantly lower ratio (and thus a higher symbiont load) than those in North Carolina (1.45 vs. 1.91, *p*=0.040 by two-tailed Student's *t*-test, n=6), although this test only included two samples from the North Carolina cohort.

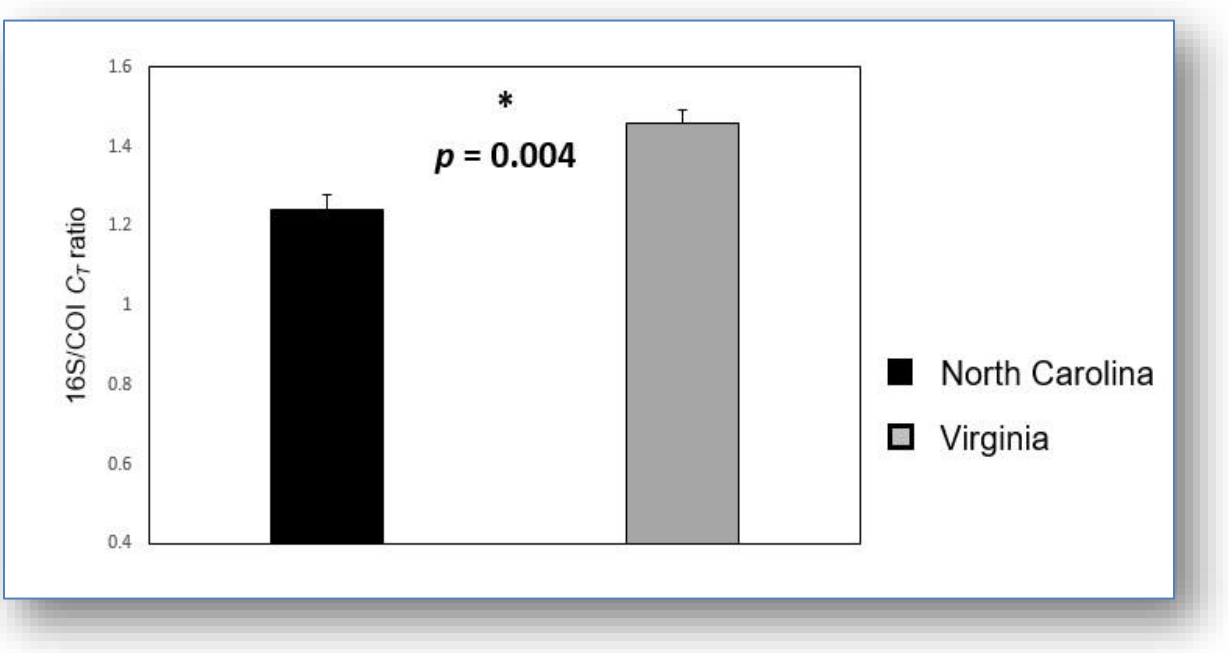


Figure 4.7 Relative abundance of E. sertula *in* B. neritina *colonies grown in North Carolina and Virginia.* C_T *ratios use results of Q-PCR targeting the* E. sertula *16S ribosomal RNA gene and the* B. neritina *mitochondrial cytochrome* c *oxidase I gene. Untreated (control) colonies are compared; higher 16S/COI* C_T *ratio indicates lower endosymbiont level. [Method of comparison from Mathew and Lopanik (2014).]*

Finally, among colonies grown in GSU aquaria under differing temperature regimes, untreated colonies exposed to cold water did have a higher ratio (1.16) than those from either the room temperature (1.00) or warm (1.07) conditions. However, this effect was not statistically significant by factorial ANOVA among all colonies considering the variables of temperature and

gentamycin treatment [$F(2,10)=2.022$, $p=0.183$, n=16], nor for untreated colonies by one-way ANOVA among the three temperatures [$F(2,8)=0.917$, $p=0.438$, n=11] or *t*-test comparing cold-water colonies to those grown in both of the warmer conditions ($p=0.257$).

4.4 Discussion

It is possible that simple environmental differences may regulate the tendency of *B. neritina* to maintain its association with the endosymbiont *E. sertula*. The data shown here suggest that more northern waters (those north of Cape Hatteras) may include some element that ultimately hinders the growth of the bacteria. While temperature is perhaps the most obvious direction to look in considering possibilities for such an environmental factor, there are certainly innumerable variables that may be critical. Perhaps more importantly, whether this is due to direct impacts on bacterial survival or to some form of sanctioning on the part of the bryozoan would require much more in-depth investigation. In either case, such a mechanism would be sufficient to explain a lack of symbiosis among northern *B. neritina* colonies – an abandonment conveniently confined to regions where the endosymbiont's benefit is minimized.

The investigations on *B. neritina* growth in the natural absence of *E. sertula* and after antibiotic treatment, however, point to a likelihood of a cost associated with the symbiosis. This cost and its associated selective effects may work in concert with any direct environmental mechanisms in determining the locations in which we are most likely to find *E. sertula* harbored by the bryozoan *B. neritina*. All three experiments described above indicated a significant cost of symbiosis on bryozoan growth in at least one group, with no groups following the opposite pattern.

The possible interplay of gentamycin treatment with growth environment and sibling species identity may be a first indicator of a deeper interaction at work. It is interesting that both sibling species grew better when aposymbiotic only in the environment that was more "foreign" to their typical site of origin (according to the traditional *B. neritina* biogeographic paradigm). Perhaps the endosymbiont burden becomes more pronounced when environmental stresses are already amplified by such a transplant. It should also be acknowledged that any apparent interactions among treatment, growth location, and sibling species identity may be influenced by an overall trend toward reduction in symbiont levels among colonies grown above Cape Hatteras. While gentamycin and growth location did not directly interact in any of the analyses, the data are bound to be skewed by a natural tempering of *E. sertula* maintenance.

Taken as a whole, the results indicate that the biogeographical distribution of *B. neritina*-*E. sertula* symbiosis, as well as the occasional sympatric association of symbiotic and aposymbiotic colonies, is likely to reflect on the symbiont's impact on host survivorship. Co-occurrence of colonies with both symbiotic statuses may be briefly observed with the presence of a small number of short-lived colonies, before selection is able to eliminate poorly adapted lineages. After all, the value of any facultative symbiosis is necessarily context-dependent (Cockburn et al., 2013).

Finally, while these experiments demonstrate just one possible hardship imposed by symbiosis, evaluation of cost within the dynamics of the lifestyle of one or more generations of participants may require observation of a host across its lifetime (Oliver et al., 2008), and may be further complicated by genetic variation within the host, as in the association of the protective symbiont *H. defensa* with the black bean aphid (Vorburger and Gouskov, 2011). The cost of symbiosis may ultimately be found to reflect not only a differing association of Type N and Type

S *B. neritina* with their bacterial symbiont, but also slightly differing lifestyles between the sibling species, or even variations in tendency for lineages within each sibling species to harbor *E. sertula*.

5 CONCLUSIONSSummary

In these studies, I explored the distribution of two sibling species of the temperate marine bryozoan *B. neritina*, and their interaction with the defensive endosymbiont *E. sertula*, the source of the bryostatins. In the traditional biogeographic paradigm of Western Atlantic *B. neritina* populations, as indicated by early surveys, North Carolina's Cape Hatteras served as a sharp boundary between symbiotic Type S animals in southern waters, and aposymbiotic Type N colonies to the north. Bryostatins produced by *E. sertula* associated with Type S *B. neritina* protect vulnerable bryozoan larvae in low-latitude, high-predation waters, while being unnecessary to Type N animals in the relatively safer waters north of Cape Hatteras.

The survey presented here alters this view, showing a much broader distribution for both sibling species. Importantly, Type N *B. neritina* colonies and larvae were also shown to harbor *E. sertula*, and it is likely that the endosymbiont serves in a similar protective capacity in both groups. Rather than sibling species identity, association with the endosymbiont may be the most important factor affecting the distribution of populations within both Types N and S *B. neritina*. Genetic comparisons of *E. sertula* associated with both sibling species showed no difference between the two, indicating a probability of horizontal transmission of the endosymbiont. This is surprising, as the endosymbiont has been thought to be solely vertically transmitted.

The dynamics of the bryozoan-endosymbiont interaction appear to be influenced by a fitness cost imposed by harboring *E. sertula*. This cost may encourage the relative success of aposymbiotic colonies in environments where predator deterrence is not a crucial factor in the survival of *B. neritina* larvae. In addition, endosymbiont maintenance is influenced by the bryozoan's environment, and may, in fact, be impaired in certain environmental regimes.

5.2 Potential hybridization issues

A concern that may be raised with regard to these results is the possibility of widespread hybridization between Types N and S *B. neritina*. As details of this bryozoan's reproduction are not well understood, it would be difficult to assert that the chance of hybridization between these sibling species has been adequately addressed. This could affect interpretations of our data in a number of ways.

If hybrids exist in the environment, one would expect the sibling species' ranges to be obscured, as some individuals may benefit from adaptations originating within the sibling species different from that of the individual's COI gene. For example, apparently Type N animals may persist at lower latitudes due to genes from the Type S lineage, and vice versa. In addition, while Type S *B. neritina* appears to associate more readily with the endosymbiont *E. sertula*, hybridization may actually obscure this tendency, as hybrid animals could be falsely identified as strictly one or the other sibling species. A greater disparity in sibling species' predisposition to symbiosis may become apparent with deeper genetic exploration. Finally, as speculation presented here regarding horizontal transmission of *E. sertula* rests upon mitochondrial genetic characterization, hybridization may confound interpretations that could be made more precise by nuclear genome sequencing.

However, it should be noted that if *E. sertula* is almost exclusively vertically transmitted, it is likely that its inheritance would correlate with mitochondrial transmission. Heritable bacterial symbionts are typically inherited maternally (Cary and Giovannoni, 1993; Wilkinson et al., 2003), though exceptions exist [see (Moran and Dunbar, 2006)]. In addition, other researchers have characterized both nuclear and mitochondrial genes from the *B. neritina* species complex worldwide, finding no evidence of hybridization, and suggested that these sibling

species be regarded as distinct biological species (Fehlauer-Ale et al., 2014). Thus, it is unlikely that hybridization between Types N and S *B. neritina* is complicating interpretation of the data regarding horizontal transmission of *E. sertula* or the distribution of sibling species.

5.3 Implications of this work

At its most practical level, the work presented here will impact anyone who targets *B. neritina* in nature for the harvesting of bioactive bryostatins. It is clear that sibling species identity is not sufficient for an assumption of symbiosis. Furthermore, Type N *B. neritina* may present the same chemical capabilities as Type S if found in low-latitude regions.

This work also raises questions regarding the initiation of symbiosis within Type N or S *B. neritina*. The most likely explanation for the observed pattern is the maintenance of symbiosis over a long period of evolutionary time within just one of the sibling species, and the eventual transmission of the endosymbiont to the divergent group. This flexibility in transmission would have parallels in other facultative symbioses between invertebrates and prokaryotes. The pea aphid symbiont *H. defensa*, for example, provides protection from parasitism in a primarily vertical association, but has also been shown to be capable of sexual transmission to new matrilines (Moran and Dunbar, 2006; Oliver et al., 2003). In a system similar to that of *B. neritina* and *E. sertula*, rove beetles with aposymbiotic parentage may acquire pederin-producing endosymbionts by feeding on symbiotic eggs while in the larval stage (Kellner, 2001, 2003). While the bryozoan clade including *Bugula* has shown evidence of host-switching at least once in the past, it is unknown if this phenomenon is common among *B. neritina*, or if the sharing of the endosymbiont is the result of a one-time historical event.

The relative tendency of each sibling species to harbor *E. sertula* may point to an origin with the Type S lineage. However, it is alternatively possible that Type S colonies' stronger association with the endosymbiont is merely a function of greater adaptation to lower latitudes, at which the *E. sertula* is thought to be more beneficial. It is noteworthy that another bryozoan genus, *Watersipora*, also demonstrates both latitudinal variation in genotype (Mackie et al., 2012) and association with putatively defensive symbiotic bacteria (Anderson and Haygood, 2007), but it is unknown if these factors are interrelated as they seem to be for *B. neritina*.

Finally, an apparent cost to harboring *E. sertula* indicates that in addition to defining the range of the symbiosis, the cost-benefit variation of the *B. neritina- E. sertula* relationship across space may, in fact, be a force behind the bryozoan's abandonment of association with the bacteria. Mutualism breakdown may be a common result of association across a variety of symbioses (Sachs and Simms, 2006). While the work presented here suggests that environment may lead to lower symbiont levels, a reliable mode of symbiont loss has yet to be demonstrated in this system in nature, leaving open the possibility of primary mediation by simple selective pressure, perhaps coupled with imperfect inheritance of the bacteria (Yule et al., 2013). Taken together, an interplay of cost-benefit balance and direct environmental effects may prove to be viable in a number of symbiotic systems.

5.4 Future studies

There are three primary lines of research that extend from the work presented here. The first would be a more detailed exploration of potential mechanics of horizontal *E. sertula* transmission. There are significant obstacles inherent in the design of such experiments. For example, differentiating between completely aposymbiotic colonies and those which are nearly

devoid of symbiont can be difficult, threatening to confound any attempts to demonstrate horizontal transfer. However, as the safest assumption is likely that inheritance is the sole expected mode of transmission, any experiment showing the possibility of horizontal transfer would drastically change approaches to the ecology of *B. neritina*. Considering the breadth of the question and the difficulties involved, the most prudent direction may be to experiment with artificial methods of introducing *E. sertula* to aposymbiotic colonies in order to establish "proof of concept." Specific environmental experiments may then be designed to explore the natural mechanics of the process.

Secondly, there is much to do in order to untangle the interactions surrounding the cost of symbiosis to *B. neritina*. The experiments shown here merely demonstrate that such a cost is observable; how the cost is impacted by varying environments and varying sibling species identity within those environments may prove to be much more complex. As the cost-benefit ratio is likely environmentally dependent, experimentation within a variety of environmental regimes will help develop the overall equation for *B. neritina*, piece by piece.

Finally, if one accepts that both selection and environmental effects on symbiont growth, whether direct or host-mediated, are involved in determining the symbiotic status of a population, the greatest question becomes the relative importance of each. Observability in experimental situations does not necessarily translate to importance in nature. In other words, while there may be a cost to harboring *E. sertula*, the actual impact of that cost in structuring natural populations may be marginal. Laboratory experiments, mathematical modeling, and *in situ* manipulation of populations may be necessary to adequately resolve this. In the meantime, the results presented here highlight the flexibility and context-dependence of organismal

interactions and the importance of investigating these relationships across scales where abiotic and biotic factors may vary.

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