12-15-2016

Symbiont Dependent Host Reproduction In The Marine Bryozoan, Bugula neritina

Meril Mathew

SYMBIONT DEPENDENT HOST REPRODUCTION IN THE MARINE BRYOZOAN, *BUGULA NERITINA*

by

MERIL MATHEW

Under the Direction of Nicole B. Lopanik, Ph.D.

ABSTRACT

Larvae of the marine bryozoan, *Bugula neritina*, are defended from predation by the bryostatins, polyketides synthesized by its uncultured endosymbiont, "*Candidatus* Endobugula sertula." Bryostatins are potent modulators of the eukaryotic signaling protein, protein kinase C (PKC) that is involved in many eukaryotic cellular processes. The close association of the host and symbiont raises the possibility of an interaction between the symbiont-produced bryostatins and host PKCs. Such interaction could impact PKC regulated host cellular processes, which could result in altered host physiology. In this study, I investigated the response of the bryozoan host in the absence of the symbiont and symbiont-produced bryostatins. Western blot analysis of protein extracts from symbiotic and symbiont-reduced *B. neritina* colonies revealed a difference

in bryostatin-activated conventional PKCs, but none for bryostatin-independent PKCs. Similar results were observed for PKCs in the model invertebrate, *Caenorhabditis elegans*, exposed to bryostatin, suggesting that the symbiont-produced bryostatins potentially modulate PKC activity and therefore PKC-mediated cellular processes in symbiotic *B. neritina* and bryostatin-exposed *C. elegans*. The number of ovicell-bearing female zooids in symbiont-reduced colonies was significantly decreased, suggesting a role of symbiont in the host reproduction. Interestingly, the female zooids in both the colony types were healthy and no anatomical or molecular differences were found except that fewer female zooids occur in symbiont-depleted colonies. The lack of difference in female zooids indicate that the symbiont does not affect the female structures and functions in the zooid, but potentially influences the early stage differentiation of the female zooid in the colony. I hypothesize that symbiont-produced bryostatins via PKC activation signal early stage differentiation of the female zooids in the colony. Additionally, microscopic investigation revealed the presence of previously undescribed 'funicular bodies' containing bacteria in the symbiotic colonies. However, the bacteria associated with the 'funicular bodies' and funicular strands in the symbiotic colonies were morphologically different and are potentially an adaptation for successful mutualistic association with the bryozoan host.

INDEX WORDS: Symbiosis, Bryostatins, Protein kinase C, Host reproduction, Differential gene expression

SYMBIONT DEPENDENT HOST REPRODUCTION IN THE MARINE BRYOZOAN, *BUGULA NERITINA*

by

MERIL MATHEW

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

SYMBIONT DEPENDENT HOST REPRODUCTION IN THE MARINE BRYOZOAN,

BUGULA NERITINA

by

MERIL MATHEW

Committee Chair: Nicole B. Lopanik

Committee: George E. Pierce

Sidney A. Crow

Eric S. Gilbert

Andrew N. Ostrovsky

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2016

DEDICATION

Dedicated to my mother, Mary Mathew, and my wife, Alphonsa Meril, for their support and prayers.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to my mentor, Dr. Nicole B. Lopanik for her guidance and supervision at every stage of my dissertation research. I am grateful to my committee members, Dr. George E. Pierce (Interim Advisor), Dr. Sidney A. Crow, Dr. Eric S. Gilbert, and Dr. Andrew N. Ostrovsky for their support and suggestions through the process.

I would like to thank Dr. Niels Lindquist for allowing me the generous use of both wet and dry lab facilities at the Institute of Marine Sciences – UNC-CH during field work. I also appreciate the help provided by the administrative and technical staff at Institute of Marine Sciences – UNC-CH during the field studies. I am thankful to Dr. Grace Lim-Fong for assistance in the collection of samples from Virginia and Delaware coast.

For my dissertation research, I had the opportunity to collaborate with various researchers. I am extremely thankful to Dr. Andrew Ostrovsky and Dr. Thomas Schwaha for their assistance with the microscopic investigation. I also would like to thank Dr. Robert Simmons for training me on microscopy techniques. I would like to credit the collaboration with Dr. Lesly-Ann Hawthorn and Dr. Sam Cheng (Integrated Genomics Facility, Georgia Regents University Cancer Center, Augusta, Georgia, USA) for RNA sequencing. I greatly appreciate the help obtained from Dr. Alexander Zelikovsky, Dr. Ion Mandoiu, Dr. Sahar Al-Seesi and their graduate students for transcriptomic analyses. I am thankful to Dr. Casonya Johnson for the guidance provided for *Caenorhabditis elegans* experiments. I would like to thank Dr. Malcolm Zellars for help with Western blot experiments. I also thank the staff at the GSU biology core facilities for training and providing access to equipment for research.

I am greatly thankful to the Department of Biology for funding this research and granting me graduate assistantship for my graduate study. I immensely appreciate my friends, especially Dr. Jonathan Linneman, Kayla Bean, Michelle Ventura, Kim Beeson, and several members of the lab for their help during field work and in the lab. Last but not the least, I am grateful for my family for their continued love, support, and prayers during my graduate study in the US.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 BACKGROUND

1.1 Host-symbiont interactions

Mutualism is a symbiotic interaction between at least two species in which both partners benefit. Many of these relationships involve eukaryotic multicellular organisms and microorganisms (Moran 2006). Microbial symbionts play an essential role in the survival and evolution of their eukaryotic hosts by either satisfying nutritional requirements or contributing to defense (Moran 2006; McFall-Ngai et al. 2013). Host-symbiont interactions resulting in the establishment and maintenance of these partnerships can be complex (Ruby 2008; Schmidt 2008; Chaston and Goodrich-Blair 2010; McFall-Ngai et al. 2013). Mutualisms can be established through vertical transmission of the symbiont from parent to offspring during host reproduction, or by horizontal acquisition of the symbiont from the environment after host reproduction (Moran 2006).

The defensive symbiotic association between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* is a well-studied model for understanding the role of host-symbiont interactions in the establishment of a horizontally transmitted symbiosis, identification of the host and symbiont, molecular signaling between the partners, symbiont induction of host morphological changes, and maintenance of the association (Rader and Nyholm 2012; McFall-Ngai 2014). Immediately upon hatching, bacterial cells including *V. fischeri* in the surrounding sea water aggregate in mucus secreted by ciliated surface epithelium of the nascent light organ of the squid (Nyholm et al. 2000). However, *V. fischeri* cells outcompete to become dominant population by attachment to the host cilia (Altura et al. 2013) and aggregation in symbiont-produced exopolysaccharide (Visick 2009). The aggregated *V. fischeri* cells alter a variety of host cellular features to increase hemocyte trafficking in the

host blood sinus (Koropatnick et al. 2002) and degradation of chitin present in the hemocytes to enter the light organ by chemotaxis (Mandel et al. 2012). During their journey through the ducts and crypt spaces of the light organ, the symbiont cells also overcome various biochemical and biophysical challenges, which include maneuvering through dense cilia lining the ducts and resistance to host-derived oxidative stress of nitric oxide and halide peroxidase [reviewed in (Nyholm and McFall-Ngai 2004)]. Furthermore, light production by the symbiont cells by bioluminescence, which provides the presumed protection to the squid from predation, is also important to maintain the symbiotic association. Mutant *V. fischeri* cells incapable of light production are unable to persist in the light organ (Visick et al. 2000). Following colonization, the *V. fischeri* cells induce development of the light organ to become functional and also mediate morphological changes in the ducts and ciliated epithelium to prevent subsequent colonization by environmental symbionts [reviewed in (Nyholm and McFall-Ngai 2004)].

Similarly, the obligate nutritional symbiotic association between the plant sap-feeding aphid *Acyrthosiphon pisum* and its primary symbiont, *Buchnera aphidicola*, demonstrates host-symbiont interactions characteristic of vertically transmitted mutualisms. *Buchnera* cells are located within specialized aphid cells called bacteriocytes (Braendle et al. 2003; Baumann 2005). The symbiont transmission occurs at the bacteriocyte-blastula interface, where *Buchnera*-specific exocytosis transiently releases *Buchnera* cells from the host bacteriocyte into the extracellular space, which are subsequently endocytosed by cytoplasm of syncytial blastula at the posterior pole (Koga et al. 2012). Metabolic complementation of nutritional requirements of both the partners makes this symbiotic association essential for survival of both partners and ensures maintenance of the relationship. Genomic and physiological analysis revealed that *Buchnera* synthesizes essential amino acids for the host and encodes genes for most of the enzymes

required for biosynthesis of essential amino acids. However, it lacks genes for production of non-essential amino acids. Interestingly, the genome of the aphid contains the missing genes of enzymes involved in essential amino acid pathway and provides non-essential amino acids for the symbiont (Shigenobu et al. 2000; Wilson et al. 2010; Hansen and Moran 2011). Another characteristic feature of obligate heritable symbionts such as *Buchnera* is genome reduction [reviewed in (Moran et al. 2008; McCutcheon and Moran 2012)]. The genome of *Buchnera* lacks the full complement of genes for cell envelope formation, transporter systems, regulatory systems, and DNA repair and recombination (Shigenobu et al. 2000). Many of these missing functions in the symbiont have been reported to be compensated by the host (Hansen and Moran 2011), making mutualism essential for *Buchnera's* survival. These studies illustrate the complexity of host-symbiont interactions in mutualistic relationships.

1.2 Symbiosis and natural products

Many ecologically relevant metabolites isolated from plants and animals have been shown to be synthesized by mutualistic microorganisms living in association with the host [reviewed in (Piel 2009; Crawford and Clardy 2011)]. These symbiont-produced natural products play an important role in the survival of the host by defending the host against pathogens, parasites, and predators (Haine 2008; Lopanik 2014; Florez et al. 2015). For instance, the anti-fouling compound ubiquinone-8 produced by a strain of *Alteromonas* sp. associated with marine sponge, *Halichondria okadai* inhibits settlement of barnacle cyprids (Konya et al. 1995). The symbiont-derived compound, pederin, confers protection to rove beetle larvae from predatory wolf spiders (Piel 2002). Similarly, cyanobacterial symbionts of the genus *Synechocystis* associate with the tunicate *Trididemnun solidum*, and are the source of the secondary metabolite, didemnin B (Rinehart et al. 1981), a feeding deterrent to coral reef fishes

(Lindquist et al. 1992). Rhizoxin isolated from the phytopathogenic fungus *Rhizopus* sp. was identified as the virulence factor for rice seedling blight disease (Iwasaki et al. 1984). Rhizoxin was later revealed to be biosynthesized by a bacterial symbiont, *Burkholderia rhizoxinica*, associated with the fungus (Partida-Martinez and Hertweck 2005). These studies demonstrate the importance of symbiont-produced metabolites to host fitness.

1.3 *Bugula neritina*

Bugula neritina (Linnaeus 1758) is a marine bryozoan (Class Gymnolamata; Order Cheilostomata; Suborder Flustrina; Family Bugulidae). It is a sessile colonial invertebrate consisting of dichotomously dividing branches of biserially arranged individuals called zooids (Fig. 1.1A). The zooids within a bryozoan colony have diverse morphology and functions, including feeding, reproduction, or anchorage to the substrate [reviewed in (Silén 1977)]. Feeding zooids are called autozooids and have a feeding polypide structure surrounded by a lighly calcified box-like cystid (Fig. 1.1B). The polypide consists of a retractable crown of ciliated tentacles around the mouth called the lophophore, a loop-shaped digestive tract, and associated muscles (Ryland 1970). Non-feeding zooids in the colony are called heterozooids, such as rhizoids for anchorage, kenozooids to strength and support the colony, vibracula for cleaning, etc. All the zooids within a colony are interconnected via a network of funicular strands, which are involved in transport of nutrients within the colony and hypothesized as homologue of blood vessels (Woollacott and Zimmer 1975; Carle and Ruppert 1983). Bryozoans reproduce both asexually and sexually. Colony growth initially occurs by budding of the first zooid, the ancestrula, which is formed by metamorphosis of a sexually produced larva, being further continued by subsequent generations of zooids produced by asexual budding.

Colonies of *B. neritina* are zooidal hermaphrodites with both the male and female gonads developing within the same zooids (Mawatari 1951). The zooids at the base of the colony are sterile, continued by simultaneous hermaphrodite fertile zooids. In the fertile zooids, spermatogenic tissue stops its activity and sperm is released early in the life cycle, while ovaries continue the oocytic production. Thus, hermaphroditic zooids eventually become females. Autozooids with a functioning ovary in a colony can be identified by the presence of specialized calcified structure called an ovicell that brood embryos (Woollacott and Zimmer 1972). Formation and development of gonads and gametes in fertile zooids depend on various factors including: cycles of polypide degeneration and regeneration (Dyrynda and Ryland 1982; Dyrynda and King 1983), age and size of the colony, and environmental conditions such as sea temperature, day-length, density and composition of neighboring communities, and water flow rates [reviewed in (Reed 1991)]. Based on structural organization of the ovary, patterns of oogenesis, site and time of fertilization and brooding of embryo, *Bugula* spp. belong to reproductive pattern III [reviewed in (Reed 1991; Ostrovsky 2013a)]. This reproductive pattern is characterized by successive maturation of few small oligolecithal oocytes in the ovary. The ovary generally forms associated with the funicular strands and the developing polypide. It is comprised of oogonia and oocytes surrounded partially by follicle cells. An incomplete cytokinesis during the division of oogonium gives rise to a primary oocyte that is connected by a cytoplasmic bridge to its nurse cell (Dyrynda and Ryland 1982; Dyrynda and King 1983; Temkin 1996; Ostrovsky 2013a). The primary oocyte accumulates yolk reserves from the maternal zooid during vitellogenesis and develops into a mature egg. Although the colonies are hermaphrodites, self-fertilization rarely occurs (Silén 1966; Johnson 2010). Sperm released by the donor colony is thought to enter the body cavity of an egg-producing zooid in a recipient colony via genital

pores, intertentacular organ (ITO) or supraneural coelomopore (SNP) (Ostrovsky and Porter 2011). Fertilization is intraovarian and precocious, occurring at the early stage of the oocyte development before the start of the vitellogenic period (Temkin 1996). However, karyogamy (fusion of male and female pronuclei) is delayed until the mature egg is ovulated into the ovicell and results in formation of an embryo (Temkin 1996). The embryo is brooded one at a time within the ovicell and receives extraembryonic nutrition from the maternal zooid via a placental analogue and the associated funicular system to develop into a lecithotrophic larva (Woollacott and Zimmer 1975; Ostrovsky 2013a). The mature larvae are released into the water column (Woollacott and Zimmer 1972). The larvae are free-swimming and settle on a substrate within 2 to 12 hours after release (Keough 1989a). Following the settlement, the larva undergoes rapid metamorphosis to form the first feeding zooid, ancestrula (Woollacott and Zimmer 1971), from which the subsequent generations of zooids asexually reproduce by budding to form the colony.

1.4 *Bugula neritina*, symbiosis, and bryostatins

The marine bryozoan, *Bugula neritina* (Linnaeus 1758), forms a symbiotic association with an uncultured γ-proteobacterium, "*Candidatus* Endobugula sertula" (Haygood and Davidson 1997). Several lines of evidence indicate that the symbiont is transmitted vertically from maternal zooid to larva, and not acquired from the surrounding seawater. First, symbiont cells are located in the maternal funicular cords connected with the placental analogue of the ovicell containing the growing embryo (Woollacott and Zimmer 1975; Sharp et al. 2007). In addition, attempts to identify symbiont cells in seawater surrounding *B. neritina* colonies have failed (Haygood et al. 1999), indicating that the symbiont is not likely acquired environmentally. In the adult colony, the symbiont cells are found within channels of funicular cords (Woollacott and Zimmer 1975; Sharp et al. 2007), involved in the transport of nutrients and waste within the

colony and to the embryo in the brood chamber, termed the ovicell (Woollacott and Zimmer 1975; Carle and Ruppert 1983). In the larvae, symbiotic bacteria reside within a circular surface invagination called the pallial sinus located on the aboral side (Woollacott 1981; Haygood and Davidson 1997). Evidence suggests that "*Ca.* Endobugula sertula" produces the bryostatins (Pettit 1996), distasteful, polyketide metabolites (Fig. 1.2) that defend vulnerable *B. neritina* larvae from predators (Lindquist 1996; Lindquist and Hay 1996; Tamburri and Zimmer-Faust 1996; Lopanik et al. 2004b). First, reduction in the titers of the symbiont cells in antibiotic treated colonies resulted in reduced levels of bryostatins (Davidson et al. 2001; Lopanik et al. 2004b). Moreover, next generation larvae from the antibiotic cured adults have a significantly lower concentration of bryostatins and are more palatable to a predator than control larvae (Lopanik et al. 2004b), illustrating the role of symbiont-produced bryostatins as predator deterrents and their contribution to host survival.

Interestingly, the association with the symbiont and presence of bryostatins in *B. neritina* is complex. Based on the *B. neritina* mitochondrial cytochrome C oxidase I (*COI*) sequences and "*Ca.* Endobugula sertula" 16S rRNA gene sequences, *B. neritina* populations form a complex of three sibling species (Davidson and Haygood 1999; McGovern and Hellberg 2003; Fehlauer-Ale et al. 2014). In the United States, two of the sibling species are found in southern California (CA): Type S, occurring above the depth of 9 m, and Type D collected at a depth below 9 m. Both the sibling species display an 8.1% difference in a region of the *COI* gene and 0.4% difference in symbiont 16S rRNA genes (Davidson and Haygood 1999). A sample from Beaufort, North Carolina (NC), was identified as Type S in genotype (Davidson and Haygood 1999). Further genetic characterization of populations from the Atlantic coast south of Cape Hatteras and the Gulf of Mexico revealed that they were part of the Type S genotype (McGovern

and Hellberg 2003). The third sibling species, Type N, was identified from populations found north of Cape Hatteras in Delaware and Connecticut and diverged 11.5% from the Type S *COI* sequences (McGovern and Hellberg 2003). Remarkably, the Type N sibling species lack any endosymbiont (McGovern and Hellberg 2003; Lopanik et al. 2004b), and no bryostatins were detected in larval extracts based on HPLC analysis (Lopanik, unpub. data). It has been hypothesized that this pattern is due to general biogeographic patterns of predation (i.e., lower predation rates at higher latitudes) (Vermeij 1978; Bertness et al. 1981; Menge and Lubchenco 1981) allowing for the selection of non-defended aposymbiotic colonies. However, recent discovery of defended (symbiotic with bryostatins) and undefended (aposymbiotic without bryostatins) Type S and N sibling species co-occurring at some sites along the East coast of the US (Linneman et al. 2014), indicate that the bryostatins may not be as important for defense of the host as previously thought.

1.5 Bryostatins and protein kinase C

The symbiont-produced bryostatins isolated from *B. neritina* have long been a target of pharmaceutical research and drug development. In 1970, crude extracts from *B. neritina* were first reported to have potent anticancer activity (Pettit et al. 1970), but the structure of bryostatin 1 was not published until 1982 (Pettit et al. 1982), due to very low concentrations within the bryozoan. To date, 20 bryostatins have been characterized from different populations of *B. neritina* (Pettit 1996; Davidson and Haygood 1999; Lopanik et al. 2004a) and are being tested as potential pharmaceuticals for the treatment of cancer, Alzheimer's disease, and HIV [reviewed in (Trindade-Silva et al. 2010)]. Bryostatin 1 binds with high affinity to the C1b region of the diacyl glycerol (DAG) binding regulatory region of human, rat, and mouse protein kinase C (PKC), a serine-threonine kinase (Kraft et al. 1986; De Vries et al. 1988; Kraft et al. 1988;

Wender et al. 1988) that is involved in the signaling cascades of many regulatory processes in eukaryotic cells (Newton 2001; Battaini and Mochly-Rosen 2007; Newton 2010). Of the ten different isozymes of PKC, bryostatin binds to the conventional (cPKC, α, β_I, β_{II}, γ) and the novel (nPKC, δ, ε, η, θ) isoforms, but not to the atypical forms (aPKC, ξ, λ/ι), as they lack the C1b binding domain (Mutter and Wills 2000). Inactive PKC is found in the cytosol with the pseudosubstrate blocking the active site (Newton 2001). Differences in the regulatory domains of the three isoforms dictate the essential cofactors necessary for activation. For c- and nPKCs, DAG released by phospholipases binds to PKC, which is allosterically activated. Its affinity for phosphatidyl serine increases and the affinity for Ca^{2+} shifts to the physiological range, resulting in release of the pseudosubstrate by a conformational change and migration to the cell membrane, the location of its substrates and regulators. After binding to bryostatin, c- and nPKC is activated briefly, autophosphorylated, translocated to the cell membrane where it phosphorylates its protein substrates, such as myristoylated alanine-rich C-kinase substrate (MARCKS) (Graff et al. 1989; Hartwig et al. 1992), rapidly accelerated fibrisarcoma (RAF) kinase (Kolch et al. 1993; Carroll and May 1994), and is then downregulated by ubiquitination and subsequently degraded by proteasomes (Clamp and Jayson 2002). Bryostatins with slight structural variations display a dramatic difference in activating PKC isoenzymes (Wender et al. 2011). For instance, bryostatin 1 induces rapid translocation of PKCβ conjugated to green fluorescent protein (GFP) to the membrane of Chinese hamster ovary (CHO) cells, whereas bryostatin 2, which only differs from bryostatin 1 by an acetate group on C7, has no effect on PKCβ. Further, there is a significant difference in the amount of PKCα, PKCδ, and PKCε remaining in the cytosol after 24-h exposure to bryostatin 1, indicating that the isoforms are

activated at variable levels by the same bryostatin. This selectivity of the bryostatins for different PKC isoforms suggests that these compounds could be used as regulators of PKC.

In a previous study to identify *B. neritina* genes differentially expressed in the symbiotic and symbiont-reduced (antibiotic-treated) Type S colonies (Mathew and Lopanik 2014), I observed very few ovicell-bearing (reproductive) zooids in the symbiont-reduced colonies. Furthermore, recent discovery of symbiotic and aposymbiotic colonies of *B. neritina* co-occurring at some sites along the East coast of the US (Linneman et al. 2014), indicate that the symbiotic association does not just have defensive role. These findings suggest an additional role of the symbiont, which is symbiont-dependent host reproduction. The goal of this study is to investigate the hypothesized role of the symbiont in host fecundity and to identify host-symbiont interactions that lead to symbiont-regulated host reproduction.

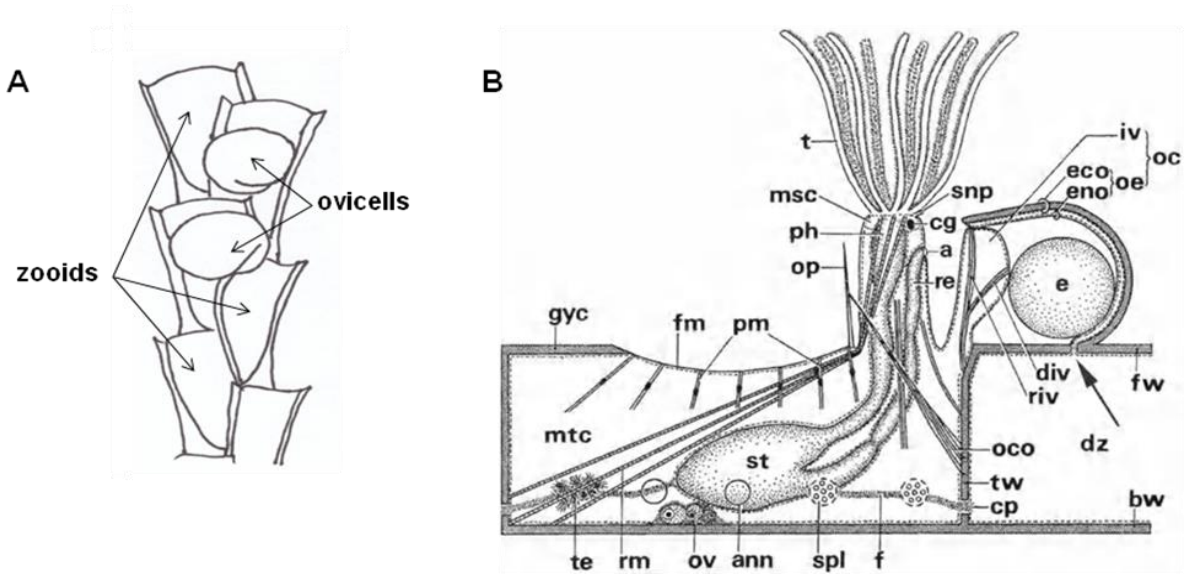

Figure 1.1 *Bugula neritina* colony morphology and zooid anatomy.
(A) Schematic representation of morphology of a branch of *B. neritina* colony with zooids and ovicells. **(B)** Zooid structure in Cheilostomata. The ooecial communication pore is arrowed. Abbreviations: a anus, ann annulus of mural pore chamber, bw basal wall, cg cerebral ganglion, cp communication pore, div depressor muscle of inner (ooecial) vesicle, dz distal zooid, e embryo, eco ectooecium, eno entooecium, f funiculus, fm frontal membranous wall, fw frontal wall, gyc gymnocyst, iv inner vesicle, msc mesocoel (ring coelom), mtc metacoel (visceral coelom), oc ovicell, oco opercular muscle, oe , ooecium, op operculum, ov ovary, ph pharynx, pm parietal muscles, re rectum, riv retractor muscle of inner (ooecial) vesicle, rm retractor muscle of polypide, snp supraneural pore, spl pore plate (septulum) in lateral wall, st stomach, t tentacle, te testis, tw transverse wall [From (Ryland 1970), with modifications by (Ostrovsky 2013a)]

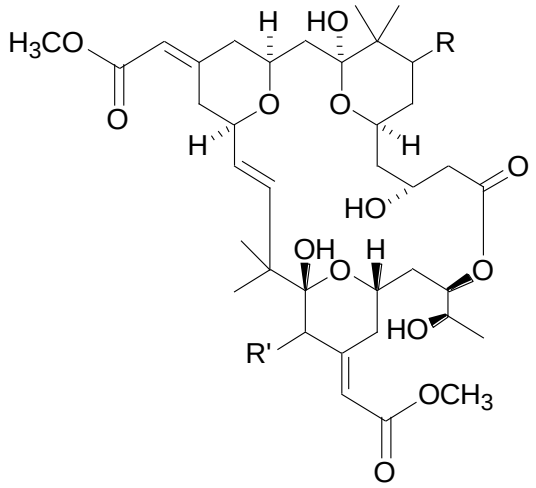

Figure 1.2 General structure of bryostatins.

2 INFLUENCE OF SYMBIONT-PRODUCED BIOACTIVE NATURAL PRODUCTS ON HOLOBIONT FITNESS IN THE MARINE BRYOZOAN, *BUGULA NERITINA* VIA PROTEIN KINASE C (PKC)

2.1 Introduction

Microbial symbionts of eukaryotic hosts are a major source of complex natural metabolites that have potent activity against pathogens, parasites, competitors, and predators of their host (Haine 2008; Piel 2009; Crawford and Clardy 2011; Lopanik 2014). These symbiont-produced compounds can have potent activity in eukaryotic cells and have therefore been investigated for a variety of therapeutic applications (Newman and Cragg 2007; Piel 2009; Gerwick and Moore 2012; Cragg and Newman 2013). For instance, the symbiont-derived anti-predatory compound, pederin, isolated from the rove beetle (Piel 2002), and pederin-like compounds, onnamides and theopederins, from a marine sponge (Piel et al. 2004), have been reported to inhibit protein biosynthesis and cell division (Narquizian and Kocienski 2000; Witczak et al. 2012). The bacterial symbiont of the fungus *Rhizopus* sp. produces rhizoxin (Partida-Martinez and Hertweck 2005), which exhibits antimitotic activity by binding to tubulin (Tsuruo et al. 1986; McLeod et al. 1996; Scherlach et al. 2006). Ecteinascidin 743, produced by a microbial symbiont of the marine tunicate *Ecteinascidia turbinata* (Rath et al. 2011), impedes DNA repair processes by several mechanisms including interfering with DNA transcription factors and binding proteins (van Kesteren et al. 2003), and is clinically approved in the European Union for treatment of soft tissue sarcomas. Despite the abundance of microbial symbiont-produced compounds and their activity in eukaryotic cellular processes, very few studies have investigated host adaptation or response to these compounds. For instance, the anti-mitotic activity of rhizoxin is tolerated by *Rhizopus microsporus* due to an amino acid

substitution in its β-tubulin gene that reduces its affinity for rhizoxins (Schmitt et al. 2008). In the mutualistic association between the insect pathogen *Photorhabdus luminescens* and entomopathogenic nematodes, the presence of the symbiont was reported to induce the growth and development of nematode juveniles into adults (Strauch and Ehlers 1998), via hydroxystilbene, an antimicrobial compound produced by the bacterial symbiont (Joyce et al. 2008). These studies illustrate the importance of symbiont-produced metabolites to host fitness, as well as host adaptation to the presence of these bioactive compounds.

The symbiont-produced bryostatins bind with high affinity to molecular regulator molecules PKCs that are essential in signaling cascades and implicated in a variety of processes including calcium signaling, lipid signaling, protein secretion, cell cycle regulation, cell reproduction, cell growth, and modification of the cytoskeleton [reviewed in (Battaini and Mochly-Rosen 2007; Akita 2008; Newton 2010; Lipp and Reither 2011; Black and Black 2013; Long and Freeley 2014; Poli et al. 2014)]. As discussed above, bryostatins have been reported to be modulators of PKC activity. Such modulation of PKCs by the bryostatins could impact the PKC mediated signaling pathways for the regulation of various cellular processes, but the potential interaction of bryostatins with the host bryozoan cells and their impact on host physiology is unknown. Host and symbiont co-evolution has been well established in many systems (Ashen and Goff 2000; Thacker and Starnes 2003; Kaltenpoth et al. 2014), especially those with vertically inherited symbionts (Moran 2006). I hypothesize that *B. neritina* hosts have adapted specifically to the presence of bryostatins produced by their microbial symbiont, most likely by a PKC-based mechanism. To investigate this, I determined host fitness in the presence and absence of the bryostatin-producing symbiont, and found that absence of the symbiont significantly affects host fecundity. In addition, some, but not all, of the host PKCs are altered in

the presence of the symbiont and symbiont-produced bryostatins. Further, the PKC-bryostatin interaction was investigated in the model organism, *Caenorhabditis elegans* to better understand their ability to affect reproduction in invertebrates, and found that while bryostatins also alter expression of some PKCs, they reduce the fecundity in the naïve, non-adapted host. Taken together, I propose that host-symbiont coevolution has resulted in the utilization of symbiont-produced natural products as cues for host reproduction in the *B. neritina*-"*Ca.* Endobugula sertula" system, which results in greater fitness of both partners.

2.2 Methods

2.2.1 Collection of Bugula neritina*, and assessment of host genotype and symbiotic status*

Adult colonies of *B. neritina* were collected by hand from floating docks either in Beaufort, NC, USA (34°42'N, 76°39'W), or in Morehead City, NC, USA (34°43'N, 76°42'W), and transported to the seawater laboratory facilities at UNC-Chapel Hill's Institute of Marine Sciences in Morehead City. The colonies were rinsed in 0.45µm filtered seawater and a few zooids were excised from individual colonies for genomic DNA extraction to determine the genotype and symbiont status of the colonies. The genomic DNA was extracted from the excised zooids using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Orange, CA, USA). The genomic DNA was used as template for PCR to amplify the *B. neritina* mitochondrial COI gene (Bn COIf and BnCOIr; Table 2.1) and the PCR amplicon was digested with restriction enzymes *Dde*I and *Hha*I to differentiate Type S and Type N colonies, respectively (Linneman et al. 2014). For symbiont-screening of the colonies, the genomic DNA was subjected to PCR amplification using symbiont-specific 16S rRNA primers (EBn16S_254f and EBn16S_643r), as well as primers that amplify a portion of the bryostatin biosynthetic gene cluster, *bryS* (Sudek et al. 2007) for confirmation (Table 2.1).

2.2.2 Fecundity assessment in antibiotic treated Bugula neritina

Type S *B. neritina* colonies were collected from Beaufort, NC, in November, 2010 and maintained overnight in the dark in flowing seawater tables. In the morning, the colonies were placed into large glass jars filled with seawater and exposed to sunlight to stimulate larval release. Pooled larvae (~100) were pipetted into six-well polystyrene plates (n = 6 replicate plates per treatment) containing filter-sterilized seawater with either the antibiotic, gentamicin (100 µg/mL; MP Biomedicals, LLC., Solon, Ohio, USA) (treatment) or seawater with a small volume of distilled water (control). The larvae in the plates were allowed to settle and metamorphose. The antibiotic-treated newly metamorphosed juveniles were exposed to gentamicin (100 µg/mL) for 10 h over 3 consecutive days, similar to previous studies (Davidson et al. 2001; Lopanik et al. 2004b; Mathew and Lopanik 2014). Each day after the 10 h gentamicin treatment, the developing juveniles were fed by placing them in an indoor artificial environment supplied with unfiltered seawater from Bogue Sound, Morehead City, NC, USA. After the 3-day treatment, the gentamicin-treated and control (symbiotic) group of juveniles were permanently placed in the indoor artificial environment with unfiltered flowing seawater for 5 months for outgrowth. For fitness assessments, thirteen mature control and gentamicin-treated colonies from each experimental group were randomly collected. Size of the colonies was measured by counting the number of branch bifurcations (Keough 1989b; Lopanik et al. 2004b), and the fecundity of the colonies was determined by counting the number of ovicells.

Small portions of randomly selected treated and control colonies (n = 6) from each replicate plate (n = 6) were dissected to determine the relative symbiont levels and assess PKC expression in the two types of colonies. The relative symbiont levels the gentamicin-treated and control *B. neritina* colonies was determined by quantitative real-time PCR (qPCR) using

symbiont-specific 16S rRNA primers (EBn16S_254f and EBn16S_643r) and *B. neritina COI* qPCR primers (BnCOIQf and BnCOIQr) (Table 2.1). The qPCR reactions were performed using a hot start version of modified *Thermus brockianus* (*Tbr*) DNA polymerase along with SYBR Green I fluorescent dye, and ROX passive reference dye (DyNAmo HS SYBR Green qPCR kit, Finnzymes, Espoo, Finland) under the following parameters: initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension of the amplified products at 72°C for 5 min. Each qPCR reaction was performed with three technical replicates and the experiment was repeated three times in a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Efficiency of the PCR reactions with each set of primers was performed with known concentrations of purified PCR amplicon standards, and was found to be within the recommended range (BnCOI: 104.9%, EBn16S: 94.6%). Melt curve analysis of the reaction products was performed after the amplification to assess the synthesis of any non-specific products. An optimum threshold cycle (C_T) value within the early exponential phase of the amplification curve was determined for each reaction using 7500 software version 2.0.1 (Applied Biosystems, Foster City, CA, USA). Relative symbiont levels in pooled colonies from each replicate plate were determined by calculating the ratio of the mean C_T value for symbiont 16S rRNA gene to the mean C_T value for the *B. neritina COI* gene as in (Mathew and Lopanik 2014). Colonies from plates that displayed the greatest level of symbiont reduction, as well as random control colonies, were selected for further protein analysis.

2.2.3 PKC expression in antibiotic treated Bugula neritina

For *B. neritina* PKC expression analysis, total protein was extracted from the dissected zooids using the Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany). The

zooids were lysed in Qproteome mammalian lysis buffer containing protease inhibitor and benzonase nuclease using a sterile 1.5 mL homogenization pestle and battery-operated hand-held pestle grinder system, and stored at -80°C. The total protein was precipitated with acetone according to the manufacturer's instructions. The precipitated protein pellet was dissolved in urea (8M) and the quantity of crude protein was assessed with the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) against a serially diluted standard of bovine serum albumin. The crude total protein extract was stored in a -80°C freezer and subjected to Western blot analysis of PKCs (see below).

2.2.4 PKC expression in naturally symbiont-reduced Bugula neritina

The host PKC expression analysis was also carried out in naturally symbiont-reduced colonies. Adult colonies were collected from several different locations: from Radio Island Marina (34°43'N, 76°41'W) and Yacht Basin Marina (34°43'N, 76°42'W), Morehead City, NC, USA in March 2012, as well as from Oyster public docks, Oyster, Virginia (VA), USA (37°17'N, 75°55'W), and Indian River Marina, Delaware (DE), USA (38°36'N, 75°4'W) in June 2012. The individual colonies were genotyped and their symbiotic status determined as described previously. The dissected zooids from individual colonies were lysed as described above, and stored at -80°C until processed further for extraction of total protein. Total protein was extracted and quantified as described above. The analysis of the host PKCs in the naturally symbiont-reduced colonies was performed by Western blot assay (see below).

2.2.5 Treatment of Caenorhabditis elegans *with PKC activator molecules*

Wild-type adult *C. elegans* (N2) worms were grown on Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 for two days at 20°C. The washed eggs were transferred to previously prepared *E. coli* OP50 seeded NGM agar (10 mL) plates

containing different concentrations of the PKC activator molecules, phorbol 12-myristate 13-acetate (PMA; 0.2 and 0.1 µg/mL) and bryostatin 1 (0.2, 0.1, 0.08, 0.05, and 0.01 µg/mL) (EMD Chemicals, Inc. San Diego, CA, USA), and control experiment plates containing 0.1% DMSO (diluent used for PKC activators). PMA was used because, like bryostatins, it is also a PKC activator that binds to C1b domain of PKC. Moreover, studies on exposure of *C. elegans* to PMA have been previously reported (Lew et al. 1982; Miwa et al. 1982; Tabuse and Miwa 1983; Tabuse et al. 1995). The eggs were incubated at 20°C and grew to the L4 larval stage. A single L4 stage nematode from each of the chemical containing NGM agar (10 mL) plates was transferred to a fresh *E. coli* OP50 seeded NGM agar (3 mL) plate containing the respective chemical in triplicate experiments, and allowed to develop into the adult stage and lay eggs. Every day the newly developed single egg-laying adult worm was transferred onto a fresh plate containing the chemicals leaving the eggs laid by the adult nematode in the old plate. The young larvae hatched from the eggs in each plate were counted and the progeny of the single nematode was recorded in triplicate experiments for each concentration of PKC activator molecules and the control experiment. This routine was continued until the adult worm stopped laying eggs.

2.2.6 Total protein extraction from adult Caenorhabditis elegans

Synchronized nematode cultures were started in *E. coli* OP50 seeded NGM agar (10 mL) plates containing PMA (0.1 µg/mL), bryostatin 1 (0.1 µg/mL), and DMSO (0.1%) in triplicate. The cultures were incubated at 20°C for 3 days to allow transformation of eggs to adult stage worms. The adult *C. elegans* cultures grown in the treated NGM agar plates were harvested for total protein extraction. The worm cultures were washed three times with ice-cold M9 buffer and harvested by centrifugation at 2,000 RPM for 1 min at 4°C. Viable adult worms were separated from denser eggs and debris by the sucrose float technique, in which the pellet was

suspended in 4 mL of sterile ice-cold sucrose solution (35%) and immediately centrifuged at 1,000 RPM for 5 min at 4°C. Live adult worms floating at the top of sucrose solution (~1 mL) were transferred to a new centrifuge tube, rinsed with ice-cold M9 buffer, and then stored in a -80°C freezer at least overnight. The frozen worm pellet was homogenized in Qproteome mammalian lysis buffer containing protease inhibitor and benzonase nuclease (Qproteome Mammalian Protein Prep Kit, Qiagen, Hilden, Germany). The worm tissue was further lysed by repeated (3X) freezing and thawing with liquid nitrogen and passing the lysate through a 26-gauge syringe needle. The lysate was processed to extract total protein as previously described. The total protein extract was quantified, and stored in a -80°C freezer until used for Western blot analysis of PKCs described below.

2.2.7 Western blot analysis of PKC proteins

Total protein extracts (from control, antibiotic-treated and naturally symbiont-reduced *B. neritina* and *C. elegans*) were subjected to Western blot analysis with polyclonal antibody for bryostatin-activated cPKC isoforms that will bind to any α, β, γ cPKC isozyme (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) and antibody for the aPKC ζ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). As bryostatins do not activate the atypical PKCs, the aPKC ζ Western blot served as a negative control. Equal quantities of denatured total protein from the samples were electrophoresed on a 4-15% Mini-PROTEAN TGX precast polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The proteins separated on the gel were transferred onto a PVDF membrane (Immun-Blot PVDF Membrane For Protein Blotting, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using either an Owl HEP-1 Semi Dry Electroblotting System (Thermo Fisher Scientific, Marietta, OH, USA) or a Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in Towbin buffer (25 mM Tris, 192 mM

glycine, and 20% methanol). The membrane was washed twice for 10 min in Tris-buffered saline (TBS; 20 mM Tris, 0.5 M NaCl, pH 7.5) prior to blocking in TBS with 5% non-fat dry milk for 1 h at room temperature with agitation. The membrane was then incubated overnight with 1 µg/mL of either PKC polyclonal antibody diluted in Antibody buffer (TBS, 0.1% Tween-20, and 0.2% non-fat dry milk) at 4°C with gentle agitation. The membrane was washed twice with TBS containing 0.1% Tween-20 (TTBS) for 10 min, and then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (diluted 1:3000, Immun-Star (GAR)-AP Intro Kit, Bio-Rad Laboratories, Hercules, CA, USA) in Antibody buffer for 2 h at room temperature with gentle shaking. The membrane was rinsed three times for 10 min in TTBS, and then incubated with Immune-Star chemiluminescent substrate for 10 min at room temperature in the dark. The chemiluminescent emission on the treated membrane was visualized in an ImageQuant LAS 4000 Mini imager (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The intensity of the protein bands detected by the Western blot analysis among the different samples was compared by densitometric analysis (ImageQuant TL 7.0).

2.2.8 Statistical analysis

The data for growth and fecundity of *B. neritina*, and progeny of *C. elegans* upon treatment with PKC activator molecules are reported as means ± SE (standard error of mean), while the data for qPCR based symbiont quantification using ratio of C_T (16S) to C_T (*COI*) is presented as means ± SD (standard deviation from the mean). The data for growth and fecundity of control and gentamicin-treated colonies did not have a normal distribution and the significance of the results was determined by the non-parametric tests, Mann-Whitney U test and Welch ANOVA. An independent samples Student's *t*-test was used to compare differences in growth and fecundity of the symbiotic and naturally symbiont-reduced colonies. Significance of

differences in the data for progeny of *C. elegans* exposed to PKC activators, density of PKC bands in *C. elegans* proteins, and the ratio of C_T (16S) to C_T (*COI*) for the symbiont quantification in symbiotic (control) and symbiont-reduced (gentamicin-treated) *B. neritina* colonies were determined by one-way Analysis of Variance (ANOVA), followed by Tukey's HSD post hoc test when possible. In all the analyses, statistical significance was accepted at $P < 0.05$.

2.3 Results

2.3.1 Bugula neritina *fecundity after symbiont reduction by antibiotic treatment*

Symbiont-reduced Type S *B. neritina* juveniles were prepared by treating larvae with gentamicin during the progression of larval metamorphosis into juveniles for 3 days. After the antibiotic treatment, the juveniles matured into adult reproductive colonies over a period of 5 months. Similar to findings in a previous study (Lopanik et al. 2004b), both control and gentamicin-treated adult *B. neritina* colonies appeared to be healthy and growth of the colonies, as measured by the number of bifurcations in the arborescent colony, was statistically similar (n = 13 control and treated colonies; Mann-Whitney U test, $P = 1.000$; Welch ANOVA, $P = 0.351$ Fig. 2.1a). However, both the number of ovicells per colony and the number of ovicells per bifurcation in gentamicin-treated colonies was significantly lower compared to control colonies (Mann-Whitney U test, $P = 0.001$; Welch ANOVA, $P = 0.001$ Fig. 2.1b and c).

The symbiont titer in sexually mature control and gentamicin-treated colonies was determined by qPCR. The amount of symbiont DNA normalized to host DNA was calculated using C_T values for the 16S rRNA gene and *COI* primed reactions in each replicate as a proxy. The gentamicin-treated *B. neritina* colonies possessing a high ratio of symbiont 16S rRNA to host *COI* C_T values (indicating less symbiont DNA per unit of host DNA as C_T value is inverse

to amount of DNA) were determined (Table 2.2). The mean ratio of 16S rRNA to *COI* C_T values was significantly higher in gentamicin-treated colonies from plates T1, T2, and T3 (one-way ANOVA, $P < 0.05$; Table 2.2), while the ratios calculated from *B. neritina* colonies in all the control plates were not significantly different (one-way ANOVA, $P > 0.05$; Table 2.2). In addition, the ratio of 16S rRNA to *COI* C_T values among the treated colonies (from plates T1, T2, and T3) and the control colonies (from plates C3, C5, and C6) used for protein extraction and Western blot analysis were significantly different (independent samples Student's *t*-test, assuming equal variance, $t(16) = 11.14$, $P < 0.001$).

2.3.2 Western blot analysis of PKC proteins in antibiotic-treated Bugula neritina

Bryostatins have been shown to activate some, but not all, PKC isozymes. Therefore, changes to the host PKCs in presence and absence of the bryostatin-producing symbionts were investigated by Western blot analysis using antibodies for bryostatin-activated cPKC isozymes and a bryostatin-independent aPKC (negative control). The presence of cPKCs (α, β, or γ) and aPKC ζ in symbiotic (control) and symbiont-reduced (gentamicin-treated) *B. neritina* colonies was determined. A lower molecular weight cPKC (α, β, or γ) protein fragment (~25 kDa) was detected in the total protein extracts from symbiotic colonies but not in extracts from symbiont-depleted colonies (Fig. 2.2a). In contrast, there was no difference in the aPKC ζ isozyme fragment sizes in either type of colony, likely due to the absence of the bryostatin binding C1b site on aPKCs (Fig. 2.2b).

2.3.3 Western blot analysis of PKC proteins in naturally symbiont-reduced Bugula neritina

Adult colonies of *B. neritina* collected from different sites along the East coast in March and June 2012 were genotyped and their symbiont status determined (Fig. 2.3a-c). PKC isoforms in the total protein extracts of individual Type S and N symbiotic and naturally

symbiont-reduced colonies were investigated by Western blot analysis against cPKCs (α, β, or γ) and aPKC ζ antibodies. Similar to the Western blot results with control and gentamicin-treated colonies, a small molecular weight cPKC isozyme (~25 kDa) was detected in the total protein extracts from symbiotic Type S and N colonies but absent in symbiont-reduced samples (Fig. 2.3d). The analysis of bryostatin-independent aPKC ζ showed same number of proteins in all the samples (Fig. 2.3e).

2.3.4 Effect of PKC activators on Caenorhabditis elegans

The effect of the PKC modulators PMA and bryostatin 1 on the fecundity of *C. elegans* was performed by exposing them to variable concentrations of the molecules. PMA was used as a positive control as it also has a high affinity for PKC and binds to the C1b domain. Worms exposed to PMA laid significantly fewer eggs than those exposed to bryostatin 1 or 0.1% dimethyl sulfoxide (DMSO, diluent control) (one-way ANOVA, $F(7, 15) = 75.29$, Tukey's HSD, $P < 0.001$; Fig. 2.4). The number of eggs laid upon exposure to 0.2 to 0.05 µg/mL bryostatin 1 was significantly less than observed in 0.1% DMSO control experiments, while the number of progeny in the 0.01 µg/mL bryostatin 1 treatment was similar to that of the DMSO control.

2.3.5 Western blot analysis of PKC proteins in Caenorhabditis elegans

Investigation of conventional and atypical isoforms of PKC in *C. elegans* exposed to PMA and bryostatin 1 (0.1 µg/mL) was performed by Western blot analysis in duplicate experiments. The analysis of bryostatin-activated cPKC (α, β, γ) in protein extracts of the treated *C. elegans* detected same number of proteins in all the samples, though the intensity of a lowest molecular weight protein (~20 kDa) was different among the samples (Fig. 2.5a). The lowest molecular weight protein band in bryostatin 1 exposed nematodes was significantly more dense than the same band detected in the PMA and DMSO exposed worms (one-way ANOVA, $F(2, 4)$

= 15.73, $P = 0.01$; Tukey's HSD: bryostatin1 > PMA = DMSO, Fig. 2.5b). Analysis of bryostatin-independent aPKC ζ also detected same number of proteins in all the samples without any significant difference in the band density of proteins detected among the samples (higher molecular weight band, one-way ANOVA, $F(2, 3) = 7.94$, P = 0.063; lower molecular weight band, one-way ANOVA, $F(2, 3) = 3.66$, P = 0.156; Fig. 2.5c). The intensity of bands for one of the replicates of the bryostatin 1 exposed samples was lower because only 7.25 µg of total protein was electrophoresed compared to 10 µg for the others samples due to low protein yields.

2.4 Discussion

Microbial symbionts are a source of diverse metabolites that have been reported to target a variety of molecular and cellular processes in eukaryotic cells, and therefore, are prime candidates for drug discovery and development (Piel 2009; Cragg and Newman 2013). The association of these metabolite-synthesizing microbial symbionts with their eukaryotic hosts raises the question of interaction between host and the symbiont-produced metabolite. Very few studies have reported the response of the host to these bioactive compounds (Joyce et al. 2008; Schmitt et al. 2008). In this study, I investigated the interaction between the marine bryozoan host, *B. neritina* with its symbiont-produced bryostatins. As discussed above, bryostatins are potent modulators of c- and nPKC activity. The close association of the host and symbiont raises the possibility of an interaction between the symbiont-produced bryostatins and host PKCs. Such interaction could impact PKC mediated signaling pathways for the regulation of various host cellular processes, which could result in altered host physiology. To test this hypothesis, various aspects of the physiology of symbiotic and artificially symbiont-reduced *B. neritina* colonies were compared. In the antibiotic curing experiment, relative quantification of symbiont titers remaining in gentamicin-treated adult colonies using molecular techniques indicate that the

symbiont cells were significantly reduced compared to control colonies, but not completely eliminated. Similar results were observed in a previous study that indicated 95% elimination of symbiont in gentamicin-treated colonies (Davidson et al. 2001). Consistent with previous research, growth and development of both the control and the gentamicin-treated adult *B. neritina* colonies was not affected due to antibiotic treatment or reduction of symbiont (Davidson et al. 2001; Lopanik et al. 2004b). Interestingly, the number of reproductive ovicell-bearing zooids per bifurcation in gentamicin-treated colonies was less than the control colonies (Fig. 2.1) suggesting that the depletion of symbiont may lead to a decrease in *B. neritina* sexual reproduction. The decreased fecundity of the symbiont-depleted (gentamicin-treated) colonies, as indicated by a reduction in the number of ovicells, is likely due to an effect on female reproductive processes. Formation of ovicells in confamiliar bryozoan species such as *Bicellariella ciliata*, has been reported to occur in concert with the development of the ovary (Reed 1991; Moosbrugger et al. 2012). It is possible that the absence of the symbiont or symbiont-produced bryostatins affects the formation and development of the ovary or the formation and maturation of oocytes in the ovary in *B. neritina*, resulting in the observed reduced fecundity phenotype. Microscopic investigation of anatomical differences during formation and development of the ovary or ovicell, or formation and maturation of oocytes during oogenesis in symbiotic and symbiont-reduced *B. neritina* could answer this question.

PKCs have been reported to be important for initiation of maturation of the primary oocytes during oogenesis in various organisms (Eckberg 1988; Colas and Dube 1998; Kalive et al. 2010). Treatment of surf clam *Spisula* spp. oocytes with phorbol esters, cPKC and nPKC activators (Nishizuka 1984) similar to bryostatins, was reported to stimulate germinal vesicle breakdown (GVBD), a key event in the initiation of the meiotic maturation of the primary

oocytes (Dube et al. 1987; Eckberg et al. 1987). Similarly, PKCs were shown to induce GVBD in the annelid *Chaetopterus* spp. (Eckberg and Carroll 1987; Eckberg et al. 1996), and in vertebrate oocytes (Aberdam and Dekel 1985; Stith and Maller 1987; Kwon and Lee 1991; Rose-Hellekant and Bavister 1996; Mondadori et al. 2008). Conventional PKC-α was highly expressed in the ovary and oocytes of the starfish *Asterina pectinifera* (Miyake et al. 2009). While there is little information regarding the molecular regulation of bryozoan reproduction, the results suggest that PKC is potentially involved in *B. neritina* reproduction, especially in oocyte maturation, similar to what has been shown in evolutionarily diverse organisms. I hypothesize that symbiont-produced bryostatins act as a signal for *B. neritina* reproduction via PKC activation. The hypothesized role of PKC-bryostatin interaction in the regulation of activity of host PKC isozymes was investigated by comparing host PKCs in presence and absence of the bryostatin-producing symbiont. I compared PKC isoforms in total protein extracts from symbiotic, naturally symbiont-reduced, and gentamicin-treated *B. neritina* colonies by Western blot analysis. There was a clear difference between the cPKC (α, β, γ) proteins that are activated by bryostatins, in symbiotic hosts, and those that are from antibiotic cured and naturally symbiont-reduced animals. The lower molecular protein band in extracts from the symbiotic colonies is a potential cPKC degradation product following bryostatin activation. In contrast, the similarity of the aPKC protein profiles among all of the types of animals is expected, as the aPKCs lack a C1b binding domain and are not affected by the presence of bryostatins. Taken together, these results suggest that symbiont-produced bryostatins affect PKC activity, which may affect some host physiological processes, including reproduction. To confirm the importance of bryostatins for host fecundity, further experimentation with bryostatins and symbiont-reduced colonies is needed.

In order to better understand the effects of bryostatin on reproduction without the confounding effects of host-symbiont coevolution or alternative symbiont effectors, I measured the effects of differing concentrations of bryostatin 1 on the PKC proteins and fecundity in a naïve invertebrate, *C. elegans*. Two independent studies have demonstrated the effects of the phorbol ester, PMA, on the morphology and physiology of *C. elegans* (Lew et al. 1982; Miwa et al. 1982). In both studies, exposure to phorbol ester (0.1 μg/mL) resulted in smaller sized animals, reduced numbers of eggs laid, and uncoordinated movement of the worms. The molecular target of PMA was identified to be *tpa-1* (Tabuse and Miwa 1983; Tabuse et al. 1995) that encodes two proteins, TPA-1A and TPA-2B, with high homology to the nPKCs δ and θ (Tabuse et al. 1989; Sano et al. 1995). Bryostatin 1 has a higher binding affinity to PKC than phorbol esters (Kraft et al. 1986; De Vries et al. 1988), but its effects on reproduction in *C. elegans* have not been studied. Exposure of *C. elegans* to different concentrations of bryostatin 1 and PMA resulted in significantly decreased fecundity at high concentrations (Fig. 2.4). Reduced fecundity in *C. elegans* with bryostatin exposure contrasts with the fecundity results of *B. neritina*. This would be expected in an organism that has not co-evolved with bryostatin or bryostatin-producing symbionts. Western blot analysis of PKC isoforms in *C. elegans* exposed to bryostatin 1 and PMA showed differences in bryostatin-activated cPKCs (α, β, γ) compared to control samples, while no difference was seen for aPKC ζ (not activated by bryostatins) (Fig. 2.5a and c). The western blot analysis of PKC isoforms in *C. elegans* exposed to bryostatins supports the bryostatin-PKC interaction in this evolutionarily diverse model organism.

Host-symbiont interactions and coevolution are important for the establishment and maintenance of diverse mutualistic partnerships (Ruby 2008; Schmidt 2008; Chaston and Goodrich-Blair 2010; McFall-Ngai et al. 2013). These relationships can be established through

vertical transmission of the symbiont from parent to offspring during host reproduction, or by horizontal acquisition of the symbiont by the host from the environment (Moran 2006). Benefits of the association ensure maintenance of the relationship in both vertically and horizontally transmitted symbiosis by enhancing the fitness of host and symbiont, and efficient transmission of symbiont to the host next generation to increase the frequency of infected hosts, in the case of vertically transmitted symbionts (Moran 2006). Symbiont-dependent host reproduction may play a key role in maintenance of the association and guarantees persistence of the symbiont in the host population. The intracellular bacterium, *Wolbachia* sp., is a well-studied reproductive parasite in arthropods that causes manipulation of host reproduction to enhance its own transmission [reviewed in (Stouthamer et al. 1999; Werren et al. 2008; Engelstaedter and Hurst 2009)] and in some cases, is essential to promote host oogenesis and oocyte maturation (Dedeine et al. 2001; Zchori-Fein et al. 2006; Pannebakker et al. 2007). The mutualistic association between the plant-pathogenic fungus *Rhizopus microsporus* and its bacterial symbiont, *Burkholderia rhizoxinica*, is maintained by strict dependence of fungal sporulation upon the endosymbiont type III secretion system (Partida-Martinez et al. 2007; Lackner et al. 2011). The symbiotic association between *B. neritina* and its uncultured symbiont "*Ca*. Endobugula sertula" is thought to be established via vertical acquisition of the symbiont cells by the growing embryo within the maternal ovicell (Sharp et al. 2007). As the symbiont-produced bryostatins participate in the chemical defense of the vulnerable host larvae (Lindquist 1996; Lindquist and Hay 1996; Tamburri and Zimmer-Faust 1996; Lopanik et al. 2004b), the host, *B. neritina*, would need to ensure presence of symbiont-mediated defense for its offspring before expending its resources into reproduction. I hypothesize that the interaction between *B. neritina* and its symbiont has evolved such that host reproduction is dependent on the symbiont, bryostatins, or both to

increase frequency of symbiont infected host larvae and survivability of host larvae in the environment.

Table 2.1 Primers used in this study.

Name	Sequence (5'→3')	Target	Product size (bp)	Purpose	Reference
BnCOIf	ACAGCTCATGCATTTTTA	*B. neritina COI* gene	469	PCR	(Linneman et al. 2014)
BnCOIr	CATTACGATCGGTTAGTAG				
Bn240f	TGCTATTTGATGAGCCCGCGTT	"*Ca.* Endobugula sertula" 16S	1013	PCR	(Haygood and Davidson 1997)
Bn1253r	CATCGCTGCTTCGCAACCC				
BryS_576f	CATTGACAGTCAGTTCTTCATTGA	*bryS*	198	PCR	(Linneman et al. 2014)
BryS_774r	CTTTTCCAGATTGAGTTTTTAACCA				
EBn16S_254f	TACTCGTTAACTGTGACGTTACTC	"*Ca.* Endobugula sertula" 16S	389	PCR and qPCR	(Mathew and Lopanik 2014)
EBn16S_643r	ACGCCACTAAATCCTCAAGGAAC				
BnCOIQf	TTGATACTGGGGGCTCCTGATATG	*B. neritina COI* gene	155	qPCR	(Lopanik et al. 2004b)
BnCOIQr	AAGCCCGATGATAAGGGAGGGTA				

Table 2.2 Symbiont quantification of *Bugula neritina* colonies.
Symbiont titer in gentamicin-treated (T) and control (C) *B. neritina* colonies was investigated by quantitative real time PCR using symbiont-specific 16S rRNA gene primers and *B. neritina* cytochrome c oxidase I (COI) primers. Since C_T value is inverse to the amount of template DNA, a higher ratio of C_T (16S) to C_T (COI) indicates less symbiont DNA per unit of host DNA. The asterisks denote statistically significant difference in the ratio of symbiont DNA normalized to host DNA. SD= standard deviation from the mean.

Plate	$\frac{C_T(16S)}{C_T(COI)}$	**SD**
C1	0.88	0.18
C2	1.15	0.07
C3	0.93	0.22
C4	1.02	0.03
C5	1.04	0.19
C6	0.95	0.02
T1	1.58*	0.12
T2	1.66*	0.03
T3	1.74*	0.09
T4	1.28	0.08
T5	1.26	0.02
T6	1.26	0.03

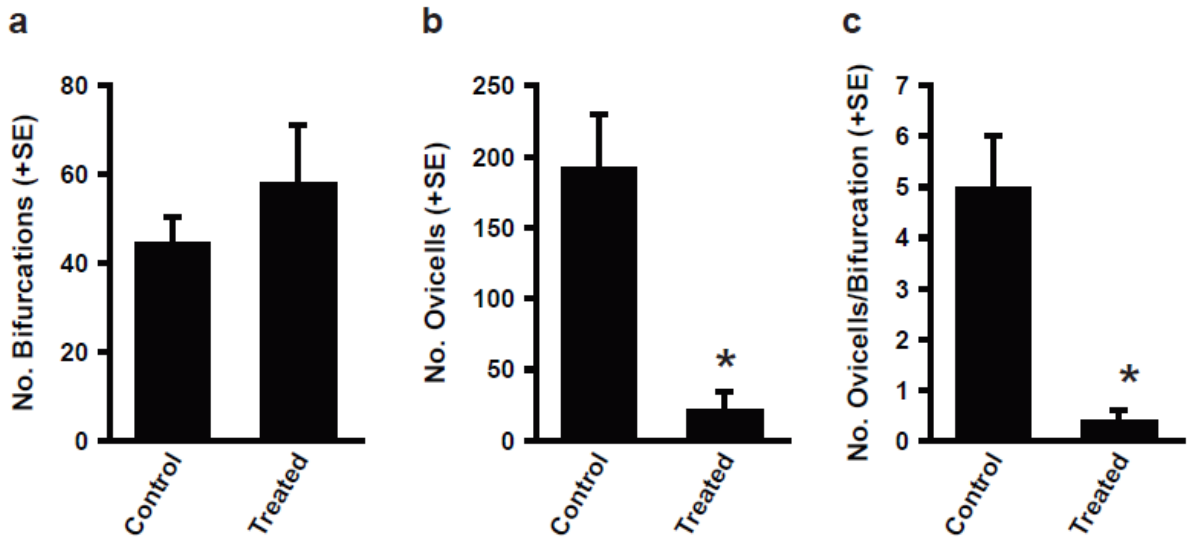

Figure 2.1 Fecundity assessment in antibiotic treated *Bugula neritina*.
a Size as measured by the number of bifurcations, **b** fecundity as measured by the number of ovicells, and **c** the fecundity normalized to colony size of control and antibiotic treated colonies. The asterisks denote significant differences (Mann-Whitney U-test, Welch ANOVA, $P < 0.05$) and SE = standard error of the mean.

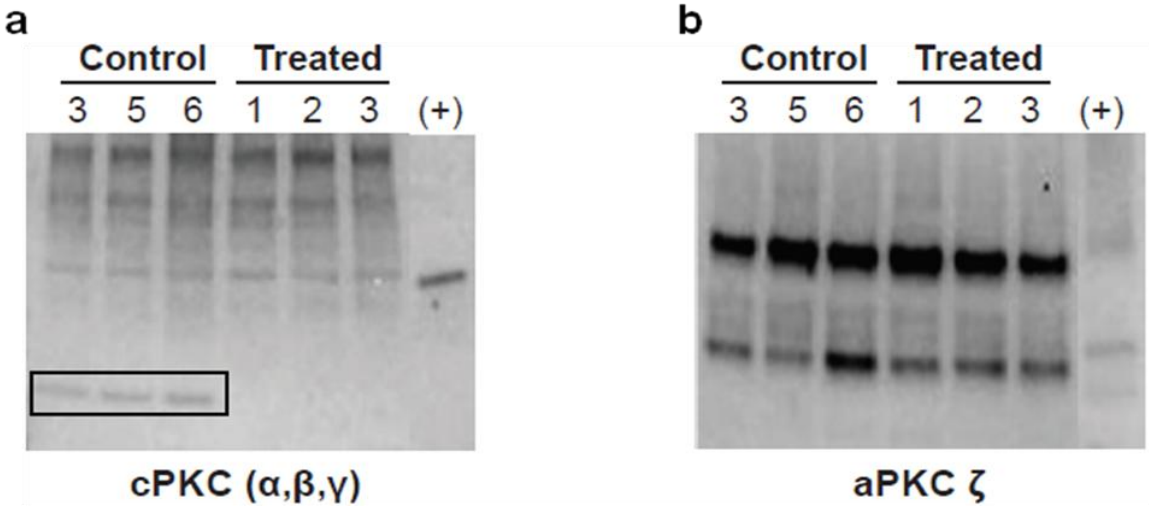

Figure 2.2 PKC expression in antibiotic treated *Bugula neritina*.
Western blot analysis of **a** conventional PKCs (α, β, or γ) and **b** atypical PKC ζ in total protein extracts of control (symbiotic) and treated (gentamicin-treated) *B. neritina* colonies. Positive control (+) = NIH/3T3 whole cell lysate. The box in **a** shows a lower molecular weight cPKC (α, β, or γ) protein fragment (25 kDa) detected only in the total protein extracts from symbiotic colonies.

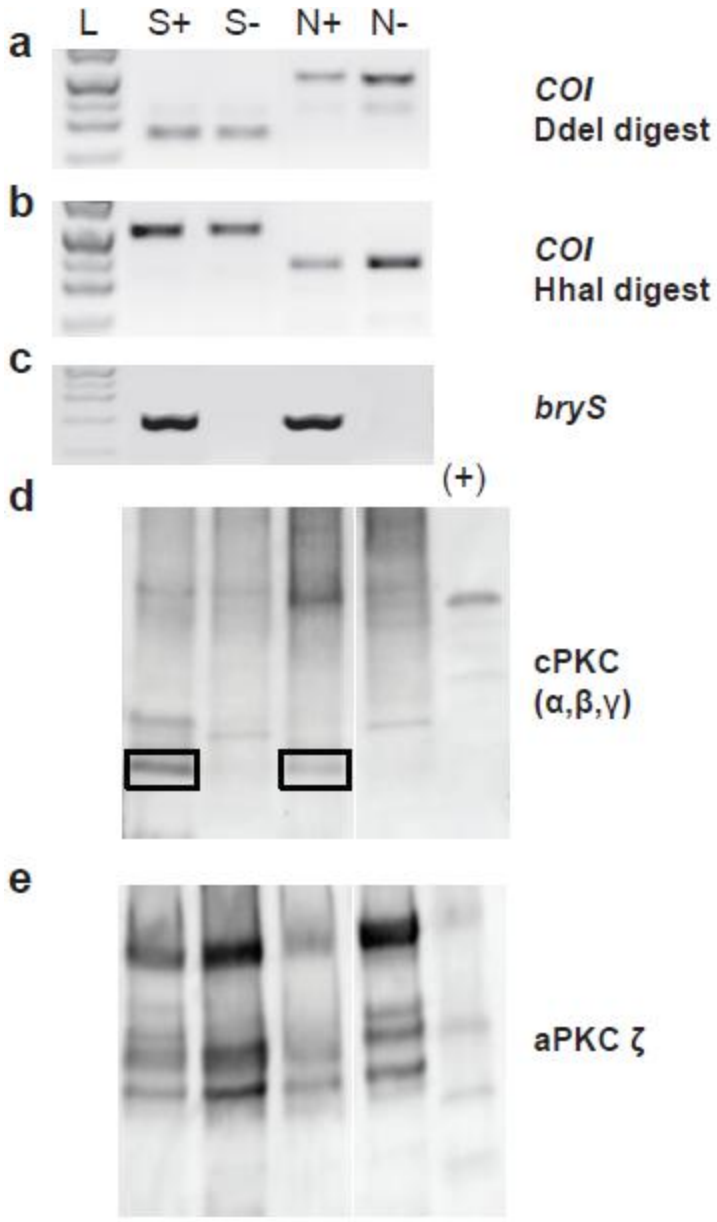

Figure 2.3 PKC expression in naturally symbiont-reduced *Bugula neritina*. Identification of *B. neritina* Type S and N genotype by restriction digestion of *B. neritina* mitochondrial COI gene amplicon with **a** *Dde*1 and **b** *Hha*1, respectively. **c** Determination the symbiotic status of the colonies by PCR detection of bryostatin biosynthetic gene cluster, bryS. L = DNA molecular weight ladder. Western blot analysis of **d** conventional PKCs (α, β, or γ) and **e** atypical PKC ζ in total protein extracts of S+ (Type S symbiotic), S- (Type S aposymbiotic), N+ (Type N symbiotic), and N- (Type N aposymbiotic) *B. neritina* colonies. Western blot positive control (+) = NIH/3T3 whole cell lysate. The box in **d** shows a lower molecular weight cPKC (α, β, or γ) protein fragment (25 kDa) detected only in the total protein extracts from symbiotic colonies.

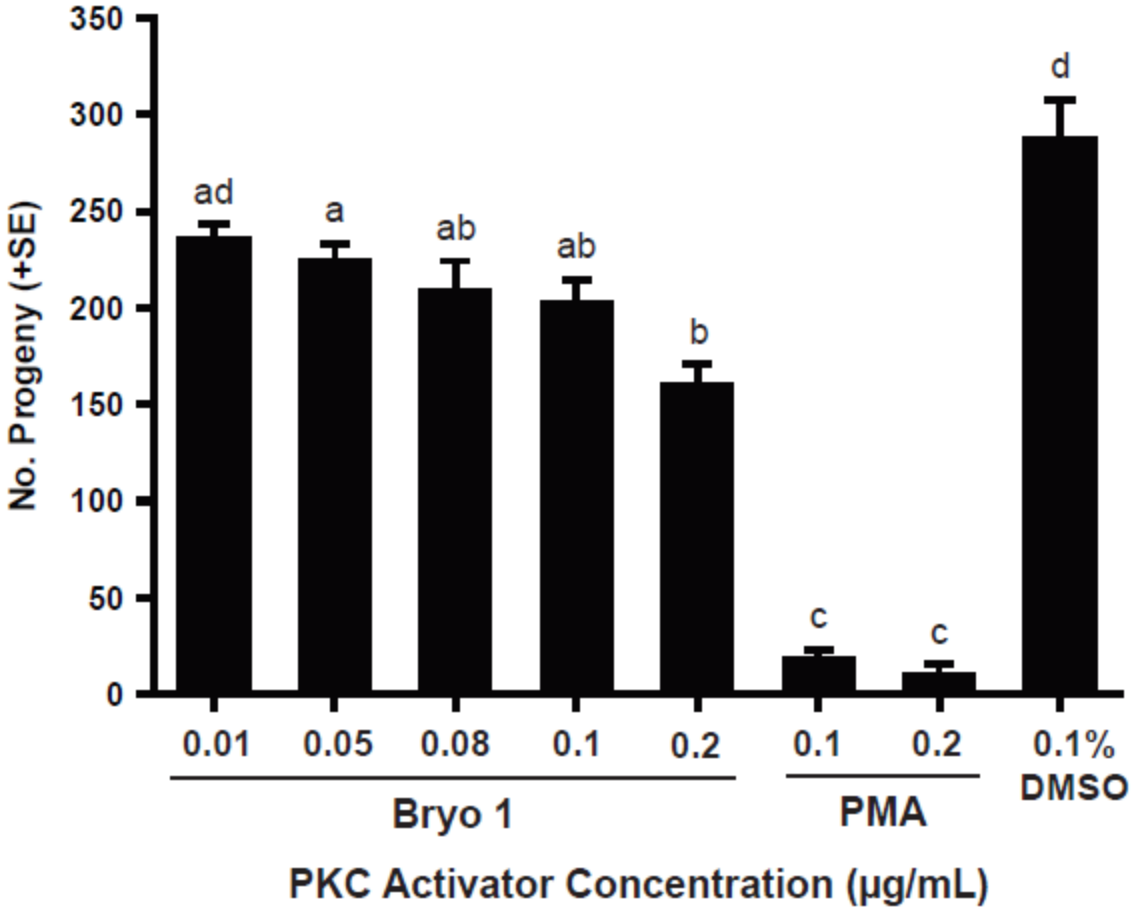

Figure 2.4 Effect of PKC activators on *Caenorhabditis elegans*.
Progeny of *C. elegans* exposed to different concentrations of PKC activator molecules, phorbol 12-myristate 13-acetate (PMA) and bryostatin 1 (Bryo 1). Dimethyl sulfoxide (0.1% DMSO) was the diluent control. The letters denote significant differences in number of progeny between treatments (one-way ANOVA, Tukey's HSD, $P < 0.05$) and SE = standard error of the mean.

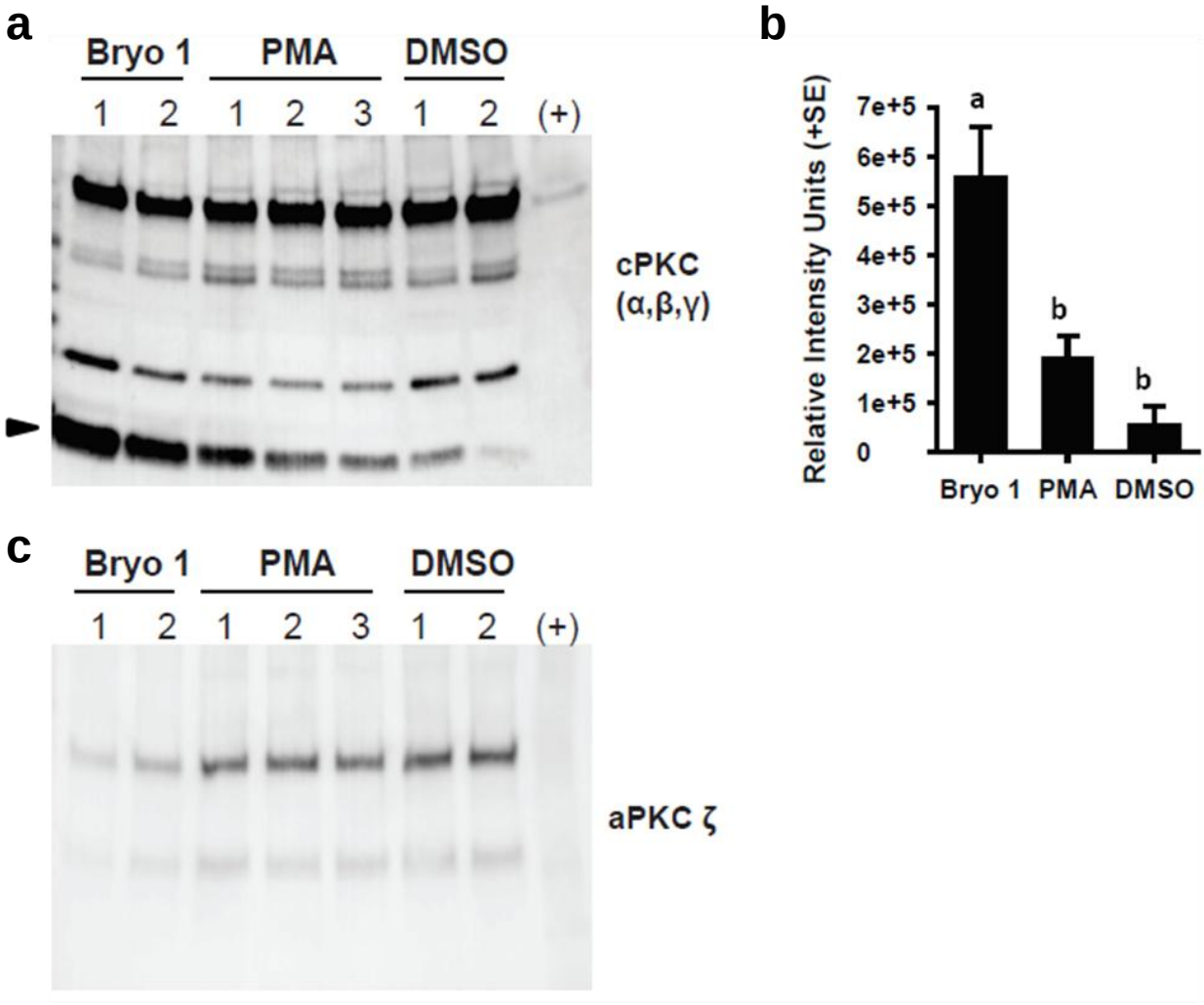

Figure 2.5 Western blot analysis of PKC proteins in *Caenorhabditis elegans* exposed to PKC activators.
a Western blot analysis of conventional PKCs (α, β, or γ) in total proteins extracts of *C. elegans* exposed to 0.1 µg/mL of PKC activators and DMSO control. **b** Relative intensity of lowest molecular weight cPKC (α, β, or γ) protein fragment (denoted by arrow in **a**) among the treatments. The letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD). **c** Western blot analysis of atypical PKC ζ in total proteins extracts of treated *C. elegans*. Western blot positive control (+) = NIH/3T3 whole cell lysate.

3 TRANSCRIPTOME ANALYSIS OF *BUGULA NERITINA*

3.1 Introduction

The transcriptome is defined as the complete set of transcripts in a cell or population of cells at a given time. The term was first used by Charles Auffray in 1996 (Piétu et al. 1999). Knowledge of the transcriptome is necessary to understand the dynamics of expression of gene transcripts and its regulation under different conditions such as differentiation, development, physiological changes, environmental influence, disease, etc. Complementary DNA microarray (Schena et al. 1995) and RNA sequencing (RNA-seq) (Mortazavi et al. 2008) are the most popular techniques used in transcriptomic studies to quantify the expression levels of genes. However, RNA-seq has gained an upper hand due to its key advantages. First, prior knowledge of gene transcripts and genomic sequence is not required for RNA-seq. Therefore, it is the method of choice for transcriptomic studies in non-model organisms, which generally lack extensive genetic information (Ekblom and Galindo 2011). Second, RNA-seq is less time consuming and efficient because transcriptome characterization (*i.e.* identification of expressed gene transcripts) and quantification of genomewide expression patterns can be achieved in a single high-throughput assay (Wang et al. 2009). A third advantage of RNA-seq over microarray is its ability to detect expression levels in novel gene transcripts (Nielsen et al. 2006). Another advantage of RNA-seq methods is very low background noise and no cross-hybridization encountered during the assay compared to fluorescently labeled hybridization probes in microarray method (Okoniewski and Miller 2006; Casneuf et al. 2007). RNA-seq approach has very high resolution to a single base, as well as has a broad dynamic range to successfully detect subtle expression differences in rare and highly expressed gene transcripts (Wang et al. 2009; Ekblom and Galindo 2011). Furthermore, technological advancements in next generation

sequencing (NGS) has allowed sample multiplexing and significant reduction in RNA-seq cost (Shishkin et al. 2015).

The above advantages of RNA-seq has revolutionized transcriptomic studies in the fields of ecology and evolution in non-model organisms and wild populations (Ekblom and Galindo 2011; Alvarez et al. 2015). The first NGS-based transcriptomic study in a non-model species was carried out on the wasp *Polistes metricus* (Toth et al. 2007). Currently, transcriptome characterization and gene expression profiling in non-model species are the most common application of NGS-based RNA-seq studies (Ekblom and Galindo 2011). Transcriptome characterization is done by *in silico* mapping of short sequence reads generated by NGS to an existing genome or *de novo* assembly to a draft transcriptome when genome information is not known, as in the case of non-model organisms (Vijay et al. 2013; Wolf 2013). Though characterization of transcriptome in non-model species is descriptive in nature, it serves as an important background for further research and ecological studies to identify the cause (Ellegren 2008; Andrew et al. 2013). In addition to identification of expressed gene transcripts, RNA-seq approach also allows quantification of transcript expression. This is done by using computational algorithms to count the number of reads mapped to a transcript and normalizing for factors such as transcript length, total number of reads, read size, and sequencing biases (Wolf 2013; Conesa et al. 2016). Furthermore, integrating RNA-seq with established targeted methods, such as qPCR and microarray, enable cost-effective large scale transcriptomic studies in to investigate interindividual and interpopulation variance in the expression of ecologically important gene transcripts (Alvarez et al. 2015; Todd et al. 2016).

Due to limited genetic information available for *B. neritina*, RNA-seq was used to characterize *B. neritina* transcriptome and to identify gene transcripts whose expression is

potentially dependent on the symbiotic association with "*Ca.* Endobugula sertula." Specifically, genes involved in the regulation of GVBD and initiation of oocyte maturation were investigated.

3.2 Methods

3.2.1 Collection of Bugula neritina *samples and RNA preparation for Illumina sequencing*

Mature colonies of *B. neritina* attached to floating docks were collected from Radio Island Marina, Radio Island, NC, USA (34°43'N, 76°41'W) in November 2011 and March 2012, as well as from Oyster public docks, Oyster, VA, USA (37°17'N, 75°55'W) in June 2012. The colonies were cleaned with 0.45µm filtered seawater and zooids were carefully dissected from individual colonies separately to avoid any cross contamination. A portion of the excised zooids were preserved in RNA*later* RNA Stabilization Solution (Ambion, Life Technologies, Carlsbad, CA, USA) at -20°C for genomic DNA extraction, while the remaining zooids were preserved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at -80°C for total RNA extraction. Genomic DNA was isolated from the preserved zooids using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Orange, CA, USA) and used for the determination of the colony genotype and symbiont status as described above. Total RNA was extracted from the TRIzol-preserved zooids of the identified colonies as per the manufacturer's instructions. The total RNA was further purified (RNeasy Mini kit, Qiagen, Valencia, CA, USA) and treated with RNase-free DNaseI to remove any contaminating DNA molecules. The integrity of the RNA was determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). The purified total RNA was processed according to standard operating procedure for preparation of adaptor-ligated cDNA library for sequencing (TruSeq RNA Sample Preparation Kit, Illumina, San Diego, CA, USA). The adapter-ligated cDNA library was hybridized to the surface of Illumina flow cell and sequenced on an Illumina sequencing platform (Illumina HiSeq 2500, San Diego,

CA, USA) at the Integrated Genomics Facility, Georgia Regents University Cancer Center, Augusta, Georgia, USA.

3.2.2 Assembly of sequenced Illumina reads

The paired-end reads were assembled *de novo* using Trinity software package (version r2013-02-25) (Grabherr et al. 2011) and the assembled contigs were annotated by performing blastx searches (Translated Query-Protein Subject BLAST 2.2.26+) against the Swiss-Prot database. The total number of reads mapped to a contig was normalized for sequencing depth and assembled contig length by computing fragments per kilobase of transcript per million mapped reads (FPKM) values using the IsoEM algorithm, which is an expectation-maximization algorithm to calculate frequency of gene isoforms in the RNA-seq data (Nicolae et al. 2011). Differential expression of annotated transcripts in the symbiotic and symbiont-reduced Type S *B. neritina* samples was determined using the IsoDE package. The IsoDE utilizes the FPKM values estimated by the IsoEM algorithm to determine fold change by bootstrapping to determine differential gene expression of the transcripts (Al Seesi et al. 2014).

3.3 Results

3.3.1 Genotyping and symbiont screening of Bugula neritina *colonies*

Randomly collected mature colonies of *B. neritina* were genotyped by restriction digestion of the of the *B. neritina* mitochondrial *COI* gene amplicon with restriction enzymes *Dde*I and *Hha*I. All the colonies collected belonged to Type S genotype as indicated by *Dde*I digested mitochondrial *COI* gene amplicons (Fig. 3.1b). The symbiotic status of the colonies was determined by PCR amplification of symbiont-specific 16S rRNA and a portion of the bryostatin biosynthetic gene cluster, *brySI* (Fig. 3.1c and d). All the colonies collected from NC were symbiotic, while the colony collected from VA lacked the symbiont. Total RNA was

purified from the above colonies and the quality of the RNA isolated was determined to have a recommended RNA Integrity Number (RIN) value greater than or equal to 8. The *B. neritina* samples used for high-throughput sequencing included: Type S symbiotic, Type S aposymbiotic, Type S symbiotic ovicell-bearing, and Type S symbiotic ovicell-free.

3.3.2 De novo assembly and differential expression of **Bugula neritina** *transcriptome*

The *de novo* assembly of the reads was performed on the union of all the reads obtained from the four *B. neritina* samples sequenced by Illumina paired-end sequencing. A total of 221,818,850 paired reads with an average length of 50 bp per read were assembled into contigs by using Trinity software. We obtained 166,951 contigs after filtering with RSEM isopct-cutoff=1.00. Out of which 76,769 were ORFs, 37,026 BLAST hits of translated ORFs against the SwissProt database, and 12,067 annotated protein hits. This indicates 59.37 % ORFs hits and 63.35 % contigs hits. Using IsoDE, 1184 transcripts were identified to be over-expressed (fold change ≥ 2) in aposymbiotic Type S *B. neritina* compared to the symbiotic colony, while 301 transcripts were under-expressed (fold change ≤ 0.5) in the aposymbiotic colony. Specifically, genes involved in the regulation of GVBD of and initiation of oocyte maturation were identified (Table 3.1). Functional annotations were assigned to the differentially expressed transcripts using the online KEGG Automatic Annotation Server (http://www.genome.jp/kegg/kaas/). The KEGG Orthology (KO) annotations were queried against closely related nematodes and flatworm in KEGG genes database. The KO annotation were performed for 1184 over-expressed (Fig. 3.2) and 301 under-expressed (Fig. 3.3) transcripts.

3.4 Discussion

Because extensive genetic information for *B. neritina* is lacking, the host bryozoan transcriptome in presence and absence of the symbiont, "*Ca.* Endobugula sertula," was

characterized by high-throughput RNA-seq method. Additionally, the expression of the identified transcripts among the bryozoan host was computationally quantified and compared using IsoEM and IsoDE programs, respectively. The genomic sequence of genes involved in the regulation of GVBD and initiation of oocyte maturation in *B. neritina* were identified and their expression among the host colonies was determined for gene expression study performed in section 4 below.

All the differentially expressed gene transcripts were assigned to functional categories by KEGG annotation. More than 120 transcripts over-expressed in the aposymbiotic colony belonged to signaling molecules and signal transduction, suggesting that presence of the symbiont potentially downregulates many of the host bryozoan's molecular signaling processes. A few of these highly expressed transcripts were closely related to protein flightless-1 (m.27690), caltractin (m.534), guanylate cyclase (m.6535), autophagy-related protein 8 (m.4463), follistatin-related protein (m.21891), histone acetyltransferase (m.17341), phototropin (m.6157), laminin (m.14155), P2X purinoceptor (m.27211), metabotropic glutamate receptor (m.2882), and gamma-aminobutyric acid receptor (m.12980). The role the above transcripts in the host-symbiont interaction are unknown and require further investigation.

The second most expressed transcripts in the aposymbiotic *B. neritina* were the ones involved in infectious diseases (83 transcripts). This is expected because in the symbiotic host the expression of these gene transcripts would be repressed to allow successful infection and maintenance of the symbiont within the host. The expression of transcripts encoding lysozyme (m.27152 and m.22675) was reduced in the symbiotic host. Lysozymes are antibacterial compounds that damage bacterial cell wall by hydrolysis of peptidoglycan and therefore function as defense against bacterial infections (Callewaert and Michiels 2010). Down-regulation of

lysozyme gene expression upon bacterial symbiont infection has been reported in other symbiotic systems. In symbiotic association between *Wolbachia* and female parasitoid wasp *Asobara tabida*, lysozyme gene is down-regulated in the symbiont-infected ovaries of the host (Kremer et al. 2012). The expression of lysozyme gene was also found to be decreased in the bacteriocytes harboring primary endosymbiont of the cereal weevil *Sitophilus oryzae* (Vigneron et al. 2012). Another transcript identified to be expressed more in aposymbiotic *B. neritina* colony is cathepsin L protease (m.28374). Cathepsins are lysosomal cysteine proteases found in metazoans (Turk et al. 2000). Cathepsin L have been proposed to play a role in controlling the bacterial symbiont population in symbiotic midgut organ of bean bug, *Riptortus pedestris* (Byeon et al. 2015) and in the symbiont-harboring bacteriocytes in pea aphid (Nishikori et al. 2009). Similarly in symbiotic *B. neritina*, the reduced expression of transcripts encoding antibacterial compounds potentially allows infection of the symbiont in the host tissues.

Additionally, forty five transcripts with a role in host immunity were down-regulated in symbiotic host colony. One of these transcripts identified is hemicentin (m.4015), which is an extracellular matrix immunoglobin protein involved in pathological processes and immune response in *C. elegans* (Vogel et al. 2006). Transcript encoding hemicentin was found to be upregulated in Caribbean coral, *Orbicella faveolata*, affected by yellow band disease (Closek et al. 2014), which is proposed to be caused by *Vibrio* spp. upon rise in seawater temperature (Cervino et al. 2004). In case of symbiotic *B. neritina*, the down-regulation of genes involved in immunity and immune response is potentially a host adaptation to reduce immune defense processes against the symbiont.

Table 3.1 Transcriptomic analysis of genes involved in germinal vesicle breakdown (GVBD) and initiation of oocyte maturation.
Fold change (FC) of gene expression in aposymbiotic Type S *B. neritina* compared to symbiotic animal.

Contig	**Annotated gene**	**FC**
23292	Cyclin dependent kinase 1(CDK1/Cdc2/p34^{cdc})	0.78
3692	Cyclin B	0.68
3823	Cyclin B1	7.58
24871	Cyclin B3	0.52
25078	Wee1 kinase	0.66
11412	Myt1 kinase	0.85
4502	Cyclin dependent kinase 7 (CDK7)	0.93
12711	Cyclin H	0.83
1582	CDK-activating kinase assembly factor Mat1	0.98
4532	Serine/threonine-protein kinase Mos	1.02
10546	Mitogen activated protein kinase (MAPK)	1.17
10547		1.22
20059		1.04
18385	Calcineurin	1.36
27705	Anaphase promoting complex/Cyclosome (APC/C)	7.75

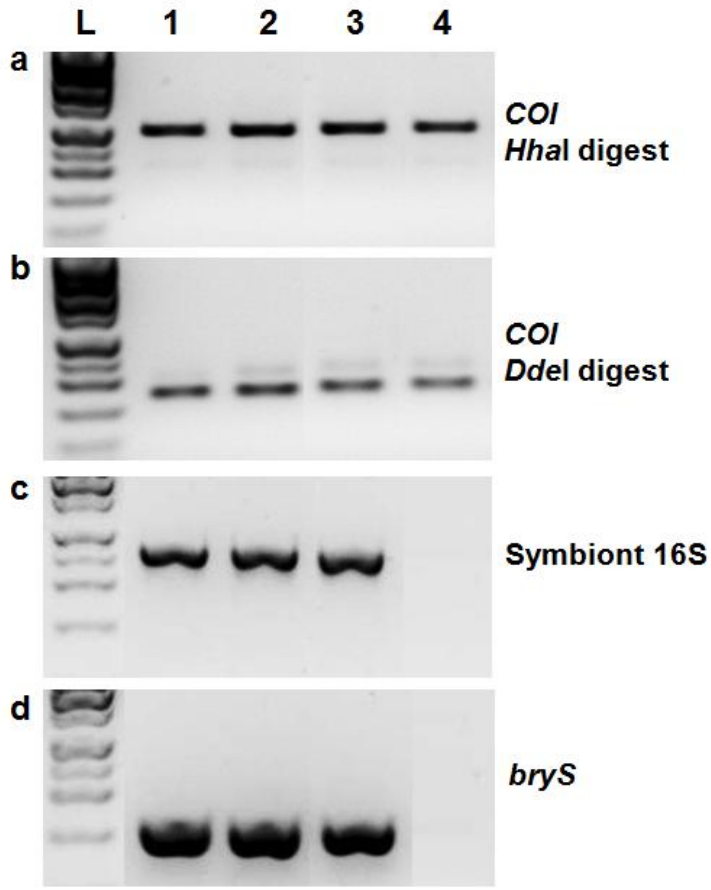

Figure 3.1 Genotyping and symbiont screening of *Bugula neritina* colonies. Identification of *B. neritina* Type N and S genotype by restriction digestion of *B. neritina* mitochondrial COI gene amplicon with **a** *Hha*1 and **b** *Dde*1, respectively. **c** Determination the symbiotic status of the colonies by PCR amplification of **c** symbiont 16S rRNA gene, and **d** bryostatin biosynthetic gene cluster, *brySS*. L = DNA molecular weight ladder, 1 = Type S symbiotic ovicell-bearing, 2 = Type S symbiotic ovicell-free, 3 = Type S symbiotic, and 4 = Type S aposymbiotic *B. neritina* colony.

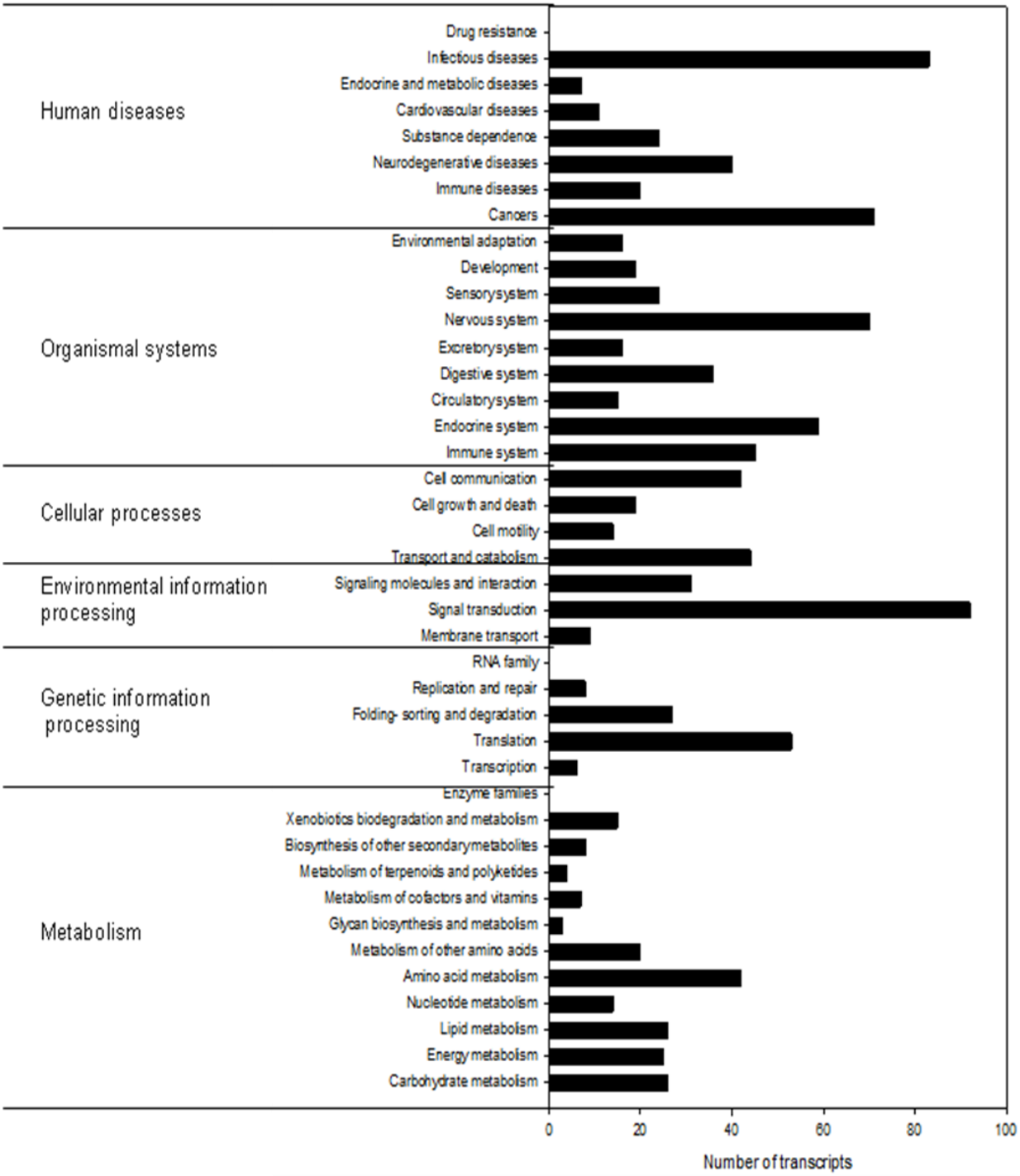

Figure 3.2 KEGG orthology annotations of over-expressed transcripts (fold change ≥ 2) in aposymbiotic Type S *Bugula neritina*.

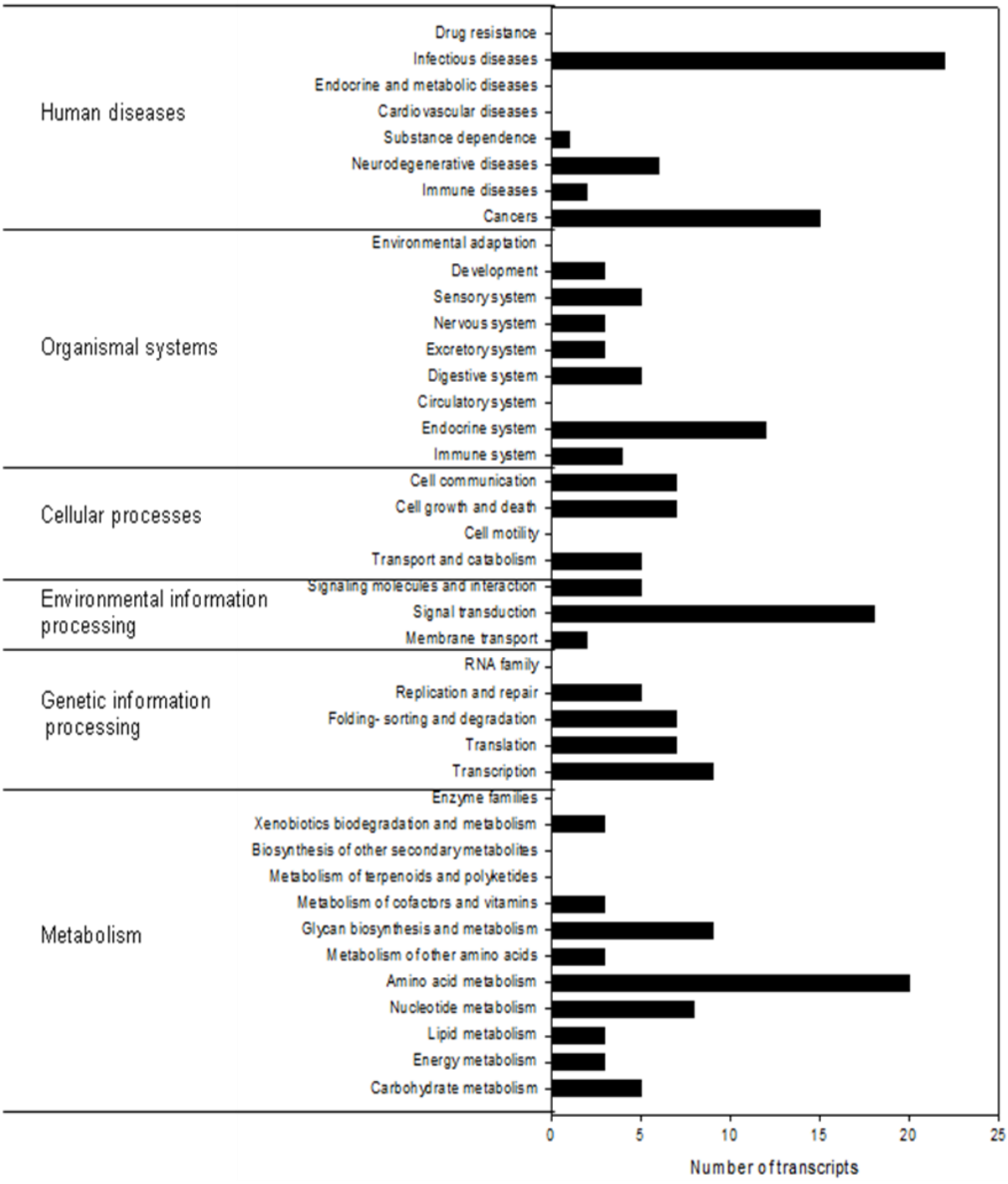

Figure 3.3 KEGG orthology annotations of under-expressed transcripts (fold change ≤ 0.5) in aposymbiotic Type S *Bugula neritina*.

4 ROLE OF SYMBIONT IN SEXUAL REPRODUCTION OF MARINE BRYOZOAN HOST, *BUGULA NERITINA*

4.1 Introduction

The nutritional and defensive benefits of mutualism provide a competitive advantage to the partners with respect to their survival and fitness. Therefore, the partners in mutualism have evolved a variety of specific mechanisms to ensure successful establishment and maintenance of the association in the succeeding generations that could be achieved by efficient transmission of symbiont in host generations increasing the frequency of infected hosts (Moran 2006). Symbiont-dependent host reproduction could guarantee the maintenance of the symbiont in the host population. The association of arthropods with bacterial parasites, *Wolbachia* sp. and *Rickettsia* sp., in some cases, has been reported to have evolved into a mutualistic interaction by providing a fecundity advantage to infected female insects (Weeks et al. 2007; Himler et al. 2011). The association of *Wolbachia* with the female parasitoid wasp, *Asobara tabida* was demonstrated to be obligatory as the bacterium is necessary for oogenesis (Dedeine et al. 2001). The presence of *Wolbachia* was shown to inhibit apoptosis of the host's nurse cells, thus allowing the oocytes to mature (Pannebakker et al. 2007). Similarly, oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, was impaired in individuals cured of the symbionts *Wolbachia* and *Rickettsia* (Zchori-Fein et al. 2006). In another example of symbiont-dependent host reproduction, the mutualistic association between the plant-pathogenic fungus *Rhizopus microsporus* and its bacterial symbiont, *Burkholderia rhizoxinica*, is maintained by strict dependence of fungal sporulation upon the bacteria (Partida-Martinez et al. 2007). In this relationship, the endosymbiont type III secretion system is necessary for sporulation of the fungal host (Lackner et al. 2011). These studies illustrate the evolution of host-symbiont

interactions that ensure the establishment and maintenance of their association by means of microbial manipulation of host reproduction. Similarly, the observed reduction in the number of fertile zooids containing an ovary (ovicell-bearing) in the gentamicin-treated (symbiont-reduced) Type S *B. neritina* colonies is likely to be influenced by the symbiont. Additionally, the interaction of symbiont-produced bryostatins and *B. neritina* PKCs, as demonstrated by Western blot analysis, suggests that the symbiont-produced compounds may affect PKC-regulated cellular processes. PKCs have been demonstrated in various studies to play a role in initiation of maturation of oocytes during oogenesis [reviewed in (Kalive et al. 2010; Deguchi et al. 2014)]. Taken together, the absence of the symbiont or symbiont-produced bryostatins potentially affects the formation and development of the ovary or the formation and maturation of oocytes in the ovary in Type S *B. neritina*, resulting in the observed reduced fecundity phenotype.

In gymnolaemate Bryozoa, the ovary is generally found on the basal wall inside the zooids, interconnected to the gut by the funicular strands. The ovary comprises of oogonia and oocytes partially surrounded by follicle cells. The female germ cell, the oogonium, undergoes an incomplete mitotic division to form an oocytic doublet consisting of a primary oocyte and its nurse cell connected by a cytoplasmic bridge (Dyrynda and Ryland 1982; Dyrynda and King 1983; Temkin 1996; Ostrovsky 2013a). The primary oocyte accumulates yolk reserves from the maternal zooid by vitellogenesis. During oocyte maturation, the primary oocyte divides meiotically to become a haploid egg. Similar to all other metazoans, the developmental progression of the primary oocyte is arrested at the prophase stage of the first meiotic division (Wourms 1987). The nucleus of the prophase-I arrested primary oocyte (called the germinal vesicle) is characterized by the presence of an intact nuclear envelope enclosing the nuclear material and preventing resumption of subsequent cycles of meiosis. In the gymnolaemate

suborder Flustrina to which *B. neritina* belongs, an intraovarian precocious sperm fusion with the arrested primary oocyte takes place (Temkin 1996; Ostrovsky 2013a). Following vitellogenesis, the disintegration of oocytic nuclear membrane, termed as germinal vesicle breakdown (GVBD), occurs just before ovulation of the vitellogenic primary oocyte from the ovary to the coelom of the maternal zooid. GVBD indicates the start of oocyte maturation by removing the prophase arrest and resumption of meiotic division of the primary oocyte. Upon ovulation of the oocyte, the nurse-cell detaches from its sibling and degenerates. The fertilized mature primary oocyte is then oviposited from the coelom of the maternal zooid to the ovicell. As soon as the oocyte appears in the brood chamber, meiosis is completed and two polar bodies are sequentially separated from the oocyte, resulting in formation of the mature ovum or an egg. The male pronucleus co-exists with the oocytic nucleus in the cytoplasm of the maturing oocyte during the entire process of oogenesis. The next step is egg activation, followed by fusion of the male and female pronuclei (karyogamy) to form a zygote that begins cleavage (Reed 1991; Temkin 1996; Ostrovsky 2013a).

Molecular mechanisms that regulate GVBD and oocyte maturation in bryozoans have not been determined. However, in metazoans, GVBD and resumption of the meiotic cycle in arrested oocytes is directly governed by the activation of the maturation promoting factor (MPF) protein complex (Masui and Markert, 1971). It comprises of a catalytic P34cdc kinase (CDK1/Cdc2) and its regulatory subunit cyclin B (Labbe et al. 1989; Dorée and Hunt 2002). Activation of MPF is caused by phosphorylation of its catalytic subunit CDK1 [reviewed in (Coleman and Dunphy 1994; Yamashita et al. 2000; Voronina and Wessel 2003; Adhikari and Liu 2014)]. The Thr161 residue of CDK1 is phosphorylated by CDK-activating kinase (CAK), which is a complex of CDK7, cyclin H, and Mat1 (Fesquet et al. 1993; Poon et al. 1993; Fisher

and Morgan 1994). Additionally, stimulation of MPF is also induced by a balance of regulation by two genes, Wee1/Myt1 kinases and Cdc25 phosphatase. Wee1/Myt1 kinases cause inhibitory phosphorylation of CDK1 at Thr14 and Tyr15 residues and subsequently inactivate MPF (Gautier et al. 1989; Mueller et al. 1995). In contrast, Cdc25 phosphatase removes the Wee1/Myt1-mediated inhibitory phosphorylation of CDK1 to turn on MPF (Rudolph 2007). The meiotic arrest of oocytes is also maintained by the anaphase promoting complex/cyclosome (APC/C)-mediated degradation of cyclin B, the regulatory component of MPF (Murray et al. 1989; Morgan 1999).

An increase in calcium ion (Ca^{2+}) concentration within the oocyte has been shown to be the signal that triggers molecular cascades for the GVBD and initiation of oocyte maturation [reviewed in (Nader et al. 2013; Sobinoff et al. 2013; Costache et al. 2014; Deguchi et al. 2014)]. The intraoocytic rise in Ca^{2+} is caused by the sperm via fertilization-induced release from the intracellular stores such as endoplasmic reticulum (Stricker 1999; Miyazaki 2006). In the eggs of marine invertebrates, the Ca^{2+}-activated phosphatase calcineurin was reported to influence APC/C-dependent cyclin B degradation (Levasseur et al. 2013). Calcium-induced activation of PKCs has been demonstrated to have a role in the GVBD and resumption of meiosis during oocyte maturation in various organisms. In marine polychaete worms, *Chaetopterus* spp., PKC has been reported to be an essential regulator of GVBD by directly activating CDK1, the catalytic subunit of MPF (Eckberg and Carroll 1987; Eckberg et al. 1996). In the surf clam *Spisula* spp., oocytes underwent GVBD upon treatment with phorbol esters, PKC activators (Nishizuka 1984) similar to bryostatins (Dube et al. 1987; Eckberg et al. 1987). Similarly, PKCs were shown to induce GVBD in vertebrate oocytes (Aberdam and Dekel 1985; Stith and Maller 1987; Kwon and Lee 1991; Rose-Hellekant and Bavister 1996; Avazeri et al. 2004; Mondadori

et al. 2008). A conceptual model for molecular regulation of GVBD and initiation of oocyte maturation is represented in Figure 4.1.

The symbiont-dependent reproduction in *B. neritina*, as indicated by a reduction in the number of ovicell-producing zooids (and, thus, ovaries) in Type S symbiont-reduced (antibiotic-treated) colonies, is likely due to an effect of the bacteria on female reproductive processes. Formation of the ovicells in brooding cheilostomes, including confamiliar species *Bicellariella ciliata*, has been reported to occur in concert with the development of the ovary (Reed 1991; Moosbrugger et al. 2012). My hypothesis is that the absence or low level of the symbiont cells or symbiont-produced metabolites (supposedly bryostatins) could potentially affect the formation and development of the ovary or the formation and maturation of oocytes in the ovary of *B. neritina*. I tested my hypothesis by comparing host fecundity in symbiotic and naturally-occurring symbiont-reduced Type S *B. neritina* colonies. Any difference in host fecundity was confirmed by investigating anatomical differences in the host female reproductive system as well as oogenesis mode in symbiotic and naturally symbiont-reduced *B. neritina* colonies collected in spring, summer, and autumn. Specifically, differences in the type, number, and size of oocytes in ovaries as well as the ovarian structure were examined in the fertile zooids of the colonies by histological sectioning and light microscopy. I also assessed difference in oocyte maturation at the molecular level. Genes involved in molecular regulation of the GVBD of primary oocyte and oocyte maturation in *B. neritina* were identified and expression of key genes involved these processes was compared in symbiotic and symbiont-reduced colonies. Interestingly, no anatomical or molecular differences was found in the ovaries among the colonies, indicating that the symbiont does not affect the female structures and functions in the zooid, but potentially decreases the proportion of female zooids in the hermaphroditic colony. However, ‘funicular

bodies' containing bacteria were present in symbiotic colonies only. Additionally, two variants of funicular strands were observed associated with morphologically different forms of bacteria.

4.2 Methods

4.2.1 Collection of Bugula neritina *colonies and fecundity assessment in naturally symbiont-reduced colonies*

Colonies of *B. neritina* were collected on the NC and VA coasts between November 2014 and December 2015 (collection sites listed in Table 4.1). Mature colonies attached to floating docks at the collection sites were randomly collected by hand. Colonies collected from the NC coast were temporarily housed in seawater laboratory facilities at UNC-Chapel Hill's Institute of Marine Sciences in Morehead City, NC, while the colonies collected from the VA coast were processed immediately at the sampling site. For genomic DNA extractions, individual colonies were rinsed in 0.45µm filtered sea water and young ovicell-free zooids at the extremities of a colony were dissected and preserved in RNA*later* RNA Stabilization Solution (Ambion, Life Technologies, Carlsbad, CA) at -20°C. Genomic DNA was extracted from the preserved colonies using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Orange, CA), and used for genotyping and symbiont-screening as mentioned above. In addition, relative symbiont levels in the colonies were determined by qPCR using symbiont-specific 16S rRNA primers and *B. neritina COI* qPCR primers as described above. The qPCR reactions were performed using a hot start version of modified *Thermus aquaticus* (*Taq*) DNA polymerase with SYBR Green I fluorescent dye, and ROX passive reference dye (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific). The reactions were performed under the following parameters: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C

for 30 s, extension at 72°C for 30 s, and a final extension of the amplified products at 72°C for 5 min.

Following the genotyping and symbiont-screening by PCR, naturally occurring symbiont-reduced Type S *B. neritina* colonies were identified. The growth and fecundity of naturally occurring symbiotic and symbiont-reduced *B. neritina* colonies (n = 8, symbiotic and n = 6, symbiont-reduced) collected from the NC coast in May and November, 2015 were determined as mentioned above.

4.2.2 Microscopic investigation of Bugula neritina

For light microscopy, fertile (ovary-bearing) zooids with ovicells (empty as well as with embryos) of the adult symbiotic and symbiont-reduced colonies of both genotypes, Type S and Type N, were excised and preserved by fixing in Bouin's fluid without acetic acid (3 parts of water saturated picric acid and 1 part of formalin) for at least 24 h at room temperature (Ostrovsky 1998). All the fixed tissue samples were dehydrated in a graded ethanol series (40-50-60-70-80-90-100%) or acidified dimethoxy-propane (DMP), and subsequently embedded in epoxy resin. Serial semi-thin sections (1 μm) were produced with a Histo Jumbo diamond knife (Diatome, Biel, Switzerland) on a Leica UC6 Ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were stained with toluidine blue and digitally photographed with an Olympus DP73 camera (Olympus, Tokyo, Japan) on an Olympus BX53 compound microscope.

4.2.3 Differential expression of Bugula neritina *genes involved in oocyte maturation*

B. neritina genes involved in the regulation of GVBD and initiation of oocyte maturation were identified by the transcriptome sequencing study discussed previously (Table 4.2). A preliminary computational analysis of differential expression of the identified genes in a symbiotic colony compared to a symbiont-reduced colony, as well as a review of literature on

previous research on oocyte maturation in metazoans, lead us to investigate differential expression of cyclin dependent kinase 1 (CDK1), cyclin B, and anaphase promoting complex/cyclosome (APC/C) genes that play an important role in GVBD and resumption of meiosis for oocyte maturation. The differential expression of the genes was examined by qPCR analysis. The expression of two housekeeping genes, 18S rRNA (Wong et al. 2010; Mathew and Lopanik 2014) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Thellin et al. 1999; Lee et al. 2002), was used to normalize the target gene expression level. Primer pairs to amplify the genes of interest were designed using Primer 3 Plus tool (Untergasser et al. 2012) (Table 4.3). The PCR amplification efficiency of the primer pair for each of the genes was assessed with known concentrations of purified PCR amplicon standards and was found to be more than 90%; CDK1: 95.82%, cyclin B: 100.37%, APC/C: 92.72%, 18S: 90.94%, and GAPDH: 96.7%.

For gene expression analysis, young zooids and ovicell-bearing zooids at the tips of branches of the symbiotic and symbiont-reduced Type S *B. neritina* colonies (n = 9 symbiotic and 8 symbiont-reduced) were separately collected and preserved in TRIzol reagent (Invitrogen, Carlsbad, California, USA). Total RNA was extracted from the samples using Direct-zol RNA MiniPrep kit (Zymo Research Corp., Irvine, California, USA) as per the manufacturer's protocol and the total RNA was treated with RNase-free DNase I to digest any contaminating DNA molecules. The RNA was cleaned and concentrated using OneStep PCR inhibitor Removal kit (Zymo Research Corp.), followed by RNA Clean and Concentrator-5 kit (Zymo Research Corp.). The concentration and purity of the RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Single-stranded cDNA was synthesized from equal amounts of total RNA from each replicate sample using SuperScript III reverse transcriptase and random hexamer primers (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen,

Carlsbad, California, USA). A control reaction lacking the reverse transcriptase was also performed to ensure that the cDNA synthesized lacks any genomic DNA contamination. An equal quantity of the single-stranded cDNA from each replicate sample was used as template for qPCR analysis using the optimized concentrations of target and reference gene primers (Integrated DNA Technologies, Coralville, Iowa, USA). The qPCR reaction for each gene of interest was performed in triplicate as described above. The relative expression level of the target genes in the symbiotic and symbiont-reduced Type S *B. neritina* samples was estimated by the comparative C_T method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen 2001).

4.3 Results

4.3.1 Genotyping and symbont-screening of Bugula neritina *colonies*

Adult *B. neritina* colonies (n = 225) were randomly collected between November 2014 to December 2015 from coastal NC and VA. All the colonies were genotyped by restriction digestion of the *B. neritina* mitochondrial *COI* gene amplicon. Twenty-four colonies belonged to Type N genotype, while the remaining 201 colonies were Type S (Table 4.1, and Fig. 4.2a and b). The colonies were also screened for the presence of the symbiont by PCR amplification of symbiont-specific 16S rRNA and a portion of the bryostatin biosynthetic gene cluster, *brySI* (Fig. 4.2c and d). Among the Type S colonies, eleven colonies were identified to be naturally occurring symbiont-reduced; 3 colonies were from the November 2014 sampling, 4 colonies from May 2015, 2 colonies from July 2015, and 2 colonies from the November 2015 collection (Table 4.1). Of the Type N colonies, 19 colonies were naturally occurring symbiont-reduced (Table 4.1). The relative symbiont titer in the colonies was also estimated by qPCR by calculating the C_T ratio (mean C_T for symbiont 16S rRNA gene to mean C_T for host *COI* gene). The C_T ratios for the naturally occurring Type S symbiont-reduced colonies was significantly

higher than the Type S symbiotic colonies (independent samples Student's t-Test, $P < 0.01$, Fig. 4.3), indicating a significant reduction of symbiont load in the colonies. Similarly, the symbiont titer in symbiont-reduced Type N colonies was significantly lower than that in symbiotic Type N colonies (independent samples Student's t-Test, $P < 0.01$).

4.3.2 **Bugula neritina** *fecundity in naturally occurring symbiont-reduced colonies*

The growth and fecundity of the naturally occurring symbiotic and symbiont-reduced *B. neritina* colonies collected in May and November, 2015 were determined. Both symbiotic (n = 8) and symbiont-depleted (n = 6) adult colonies appeared to be healthy and growth of the colonies was statistically similar (independent samples Student's *t*-test, assuming equal variance, $P = 0.346$; Fig. 4.4a). Similar to the results observed in the control and gentamicin-treated colonies, both the number of ovicells per colony and the number of ovicells per bifurcation in naturally symbiont-reduced colonies was significantly less compared to symbiotic colonies (independent samples Student's *t*-test, assuming equal variance, $P < 0.001$; Fig. 4.4b and c).

4.3.3 *Comparative anatomy of female reproductive structures in* **Bugula neritina**

The decreased fecundity of the symbiont-reduced colonies, as indicated by decrease in ovicell number, is likely due to an effect on female reproductive processes. Microscopic investigation was performed to examine anatomical differences in the female reproductive structures and oogenesis in fertile zooids containing ovaries in the symbiotic and symbiont-reduced colonies of both, Type S and Type N collected in different seasons. No visually detectable differences were observed in symbiotic and symbiont-reduced Type S *B. neritina* colonies (Fig 4.5a and c). The oligolecithal mode of oogenesis, having 1-2 oocytic doublets in the ovary, was identical in all colony types. The small ovary is positioned in the distal half of zooid on its basal wall or suspended in the zooidal coelomic cavity on funicular cords beneath

the gut. Oocytic doublets are enveloped by the flattened follicle cells. A narrow subovarian zone was detectable in some ovaries that sometimes contained oogonia (4-5 μm). Small ovulated oocytes (32-38 μm) with a light granulated cytoplasm are subsequently transported to the ovicells where very large larvae (230×190 μm) grow by aid of a placental analogue. Microscopic investigation was also performed on symbiotic and symbiont-reduced Type N *B. neritina* colonies (Fig. 4.5e and g) and the observations were similar to that in Type S colonies. Further, there were no differences in the oogenesis mode in the colonies collected in summer (July 2015) and autumn-winter (November 2014 and December 2015).

4.3.4 Presence of 'funicular bodies'

In both Type N and Type S symbiont colonies the prominent structures called 'funicular bodies' (Lutaud 1969) were found inside the zooids with and without ovaries. The round, oval or elongated (sometimes, with a central constriction) 'funicular bodies' were generally positioned on the upper surface of the blind part of the stomach (caecum) (Fig. 4.5b and f). They contained granular material of various densities that is surrounded by an envelope of flattened somatic cells of the host. These 'granules' are round or oval in shape and 1.5-2 μm in diameter, obviously representing bacterial cells staining either in light or dark blue. Each 'funicular body' (28–31× 31–65 μm) was associated with the thin funicular strands. In a few instances, 'funicular bodies' were suspended on them inside the zooidal cavity and not connected with the gut. In contrast, no such 'funicular bodies' were found in the Type S and Type N symbiont-reduced colonies.

Also, in the symbiotic and symbiont-reduced colonies of both host types, Type N and Type S, the funicular system comprised of two variants of strands: thin/narrow (3-4 μm wide) and thick/wide (11-17 μm). The thick cords looked like vessels or tubes with walls of flattened

cells and were filled with fine-granulated darkly stained material evenly or chaotically distributed within its lumen (Fig. 4.5c, e, and h). We hypothesize that these granules may represent bacteria too, although differing in the size and appearance from those in the 'funicular bodies'. The 'funicular bodies' were only seen connected with the thin strands (Fig. 4.5b and f), however, some of which also showed a stained material inside their canal. Structural and spatial relationships between thin and thick funicular strands require further study.

Finally, smaller structures (8-10 μm in diameter, also possibly of bacterial nature) were found in Type S symbiont-reduced colonies (Fig. 4.5d). They appeared as round or oval shaped dense accumulations of tiny dark granules reminiscent to those found in the thick funicular strands. These small 'bodies' were placed among the thin funicular strands inside or close to ooecial vesicle with the placental analogue. The ooecial vesicle is a retractile expansion of the zooidal wall that plugs the entrance to the ovicell. Similar small 'bodies' were also seen in one Type N symbiotic colony, but due to its insufficient preservation, the result is inconclusive. In contrast to the 'funicular bodies', a cellular envelope was not detected around the small 'bodies' that were only seen in the ovicells containing embryos.

4.3.5 Differential expression of **Bugula neritina** *genes involved in oocyte maturation*

Expression of *B. neritina* genes involved in oocyte maturation in symbiotic Type S *B. neritina* zooids was compared to that in symbiont-reduced colonies using qPCR analysis. Primer pairs designed to amplify the genes of interest were specific and the absence of any non-specific amplification was confirmed by melt curve analysis during qPCR experiments, which showed a single dissociation peak for each primer pair. The relative expression of *B. neritina*, CDK1, cyclin B, and APC/C genes normalized to expression of housekeeping genes, host 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by the comparative C_T

method ($2^{-\Delta\Delta C}{}_T$). The qPCR analysis did not show any statistically significant difference in the expression of the investigated genes between the colonies (n = 9 symbiotic and 8 symbiont-reduced colonies; independent samples Student's t-Test, P > 0.05; Fig. 4.6). The expression of the genes between symbiont and symbiont-reduced colonies was also determined to be similar in samples collected in different seasons.

4.4 Discussion

Consistent with the results observed in antibiotic-treated colonies, growth and development of the naturally occurring symbiotic and symbiont-reduced adult *B. neritina* colonies was similar. Also, the number of reproductive ovicell-bearing zooids per bifurcation in naturally occurring symbiont-reduced colonies was significantly less than the symbiotic colonies (Fig. 4.4) suggesting that the fecundity of Type S *B. neritina* is influenced by the depletion of symbiont and not due to an effect of gentamicin treatment. The decreased fecundity of the symbiont-reduced colonies seems more likely due to an effect on female reproductive processes, as any effect on male reproductive process or sperm production should have been rescued by healthy sperm from the surrounding symbiotic colonies.

The study showed that regardless of the season, colony type, and the abundance of bacteria (symbiotic vs. symbiont-reduced), the oogenesis mode is similar in all studied colonies. It is oligolechital with few small, sequentially produced oocytes that corroborate the data on *B. neritina* sexual reproduction in California (Woollacott and Zimmer 1972; Woollacott and Zimmer 1975) and the Caribbean (Ostrovsky 2013a; Ostrovsky 2013b). Also, the expression of the genes involved in GVBD that leads to initiation of oocyte maturation is similar in symbiotic and symbiont-reduced Type S colonies. The results indicate that both types of colonies form normal fertile hermaphroditic zooids that are similar with respect to the anatomy of ovary, type

and number of female cells, and molecular regulation of GVBD during oocyte maturation. However, fewer zooids in the symbiont-reduced colonies develop ovaries. This was indicated by a significant reduction in number of ovicells (Mathew et al. 2016), whose formation occurs simultaneously with the development of incipient ovary in the fertile zooid (Silén 1945; Reed 1991; Moosbrugger et al. 2012). The findings suggest that the formation of zooids with ovaria in *B. neritina* colony is influenced, but not strictly dependent on the symbiont because symbiont-reduced colonies do produce a few fertile zooids. We hypothesize that the symbiont affects host reproduction at the colony level, and not on the individual zooid level. In colonial animals such as bryozoans, individual zooids within a colony have diverse morphologies and functions, including feeding, reproduction, protection, cleaning, mechanical strength, or anchorage to the substrate [reviewed in (Silén 1977; Lidgard et al. 2012)]. Being integrated morphologically and physiologically, they still are relatively interdependent on each other in respect to their development and functioning. The depletion of the symbiont in a *B. neritina* colony does not seem to affect the reproductive function of fertile zooids but the fecundity of the colony is reduced due to their fewer numbers.

The only difference revealed is the presence and absence of the ‘funicular bodies’ in the symbiotic and symbiont-reduced colonies, respectively. While we did not study these ‘bodies’ at the ultrastructural level, their bacterial nature is very likely when comparing their structure, position, epithelial envelope, and granular content with the data from the literature, especially with that from confamiliar *Bugulina turbinata* (Lutaud 1969). Also, in fluorescent *in situ* hybridization (FISH) experiments with “*Ca.* Endobugula sertula” specific and universal eubacterial oligonucleotide probes, symbiont cells were observed in the funicular strands of the rhizoids of *B. neritina* (Sharp et al. 2007). The usage of both the probes suggests that “*Ca.*

Endobugula sertula" is the only bacterium in the funicular strands. Our findings indicate that the absence of the 'funicular bodies' that are supposedly voluminous reservoirs containing symbiont cells could explain why the symbiont-reduced colonies have fewer fertile zooids and ovicells. In bryozoans, the differentiation of germ cells to form sex cells is governed by epigenetic factors (Extavour and Akam 2003). Formation and development of gonads and gametes in bryozoan zooids depend on various factors including: cycles of polypide degeneration and regeneration (Dyrynda and Ryland 1982; Dyrynda and King 1983), age and size of the colony, and environmental conditions such as water temperature, day-length, density and composition of neighboring communities, and water flow rates [reviewed in (Reed 1991)]. We hypothesize that a low titer of the symbiont cells and, thus, reduced amount of secondary metabolites (such as bryostatins) produced by the symbiont affects an unknown molecular mechanism connected with production of female sex cells and, thus, ovaries, in symbiont-depleted colonies. In our previous study, a difference in the profile of bryostatin-activated PKC proteins among symbiotic and symbiont-reduced *B. neritina* was observed and proposed to alter a diverse range of cellular processes regulated by PKC signal transduction (Mathew et al. 2016). In the marine cheilostome bryozoan *Celleporella hyalina* (suborder: Flustrina), increased allocation to male zooids was reported to be a general response to exposure to a variety of environmental stress (Hughes et al. 2003). It was hypothesized that in hermaphroditic modular animals, stress conditions would promote male function because oogenesis and brooding of larvae warrants more energy and time than that required for spermatogenesis and sperm release. Furthermore, swimming sperm from a stressed parent colony will be dispersed farther and more likely to reach favorable environment to mate with a healthy female. Similarly, in *B. neritina*, reduction of the symbiotic or symbiont-produced compounds may induce a stress response that results in the increased production of

male gonads. The sperm released by the symbiont-reduced colony will have the opportunity to mate with a neighboring symbiotic female colony to produce symbiotic paternal offspring that is defended against predation. To confirm this hypothesis, an additional study on the spermatogenic tissue production in the symbiotic and symbiont-reduced colony should be undertaken.

Alternatively, the high titer of symbiont cells in symbiotic colonies could be associated with an increased resource allocation to the production of female gonads and would be selected because of the high survival of sexually-produced symbiotic larvae protected by symbiont-produced bryostatins (Lindquist 1996; Lindquist and Hay 1996; Tamburri and Zimmer-Faust 1996; Lopanik et al. 2004b). Such translocation of resources among specialized modules of colonial marine invertebrates has been reported in many studies (Best and Thorpe 1985; Lutaud 1985; Miles et al. 1995; Best and Thorpe 2002). Additionally, a higher proportion of fertile zooids and mass larval production in symbiotic colonies would also benefit the distribution and persistence of the symbiont cells in the next generation of the host via vertical transmission from the maternal zooid to the developing larva (Woollacott 1981; Haygood and Davidson 1997; Sharp et al. 2007). It is estimated that each larva receives an inoculum of about 2500 symbiont cells within its pallial sinus from the maternal zooid (Haygood et al. 1999). Thus, if the colony is devoid of sufficient amount of the symbiont, the larval production would be reduced to maintain the symbiont inoculum received per larva. The low level of metabolites (bryostatins) produced by the symbiont could be a signal for the host to produce fewer ovaries, *i.e.* fertile zooids [(Hillman and Goodrich-Blair 2016) and references therein]. Thus, the symbiont or the symbiont-produced bioactive compounds could influence the host genes responsible for the germ cell differentiation, thus being responsible for the reproductive plasticity of the host within

population. The existence of symbiotic and symbiont-reduced colonies belonging to one bryozoan species could also indicate an influence of some environmental constrains or an unknown cost associated with hosting the symbiont, but this requires further study.

Woollacott and Zimmer (1975) observed bacteria in the canal of the funicular strands of *B. neritina* from California on the ultrastructural level, but did not find the 'funicular bodies' that were observed in this study (Fig. 4 e and f). The close proximity of the 'funicular bodies' to the gut and the thin funicular strands, suggests that the bacteria use these reservoirs to get nutrition for active multiplication and growth. Later they are transported by an unknown mechanism via canals of the funicular system to the placental analogue of the ooecial vesicle, where they accumulate as small 'bodies'. Numerous small 'bodies' found in this area were earlier described in confamiliar *Bugulina flabellata* (Ostrovsky et al. 2009). Their bacterial nature was initially suggested based on the fact that they develop exclusively in the ovicells containing embryos (Ostrovsky 2013a), and our later ultrastructural findings of the bacterial aggregations close to placenta in *Bugulina* cf. *avicularia* from Adriatic Sea supported this (Moosbrugger, Schwaha, and Ostrovsky, unpubl. data). Further, the FISH experiments with a symbiont-specific probe demonstrated the presence of "*Ca.* Endobugula sertula" within the funicular strands in zooids as well as base of the ovicell, and in the funicular cords leading to the epithelium of the ooecial vesicle adjoining developing larva in the brood chamber (Sharp et al. 2007). Since the symbiont cells are transmitted from the ooecial vesicle to the larvae just before its release from the ovicell, it is likely that the small 'bodies' found in *B. neritina* are the aggregations of the symbiont cells gathered close to the placental analogue of the ooecial vesicle before inoculation.

The strong morphological and size difference between the granulated content of the large 'funicular bodies' and of the thick funicular strands and the small 'bodies' is enigmatic. The

question then arises if the symbiont could exist in two forms: a large stationary form that is feeding and dividing in the 'funicular bodies,' and a small motile form that is transported via thick funicular strands and assemble as small 'bodies' near the entrance of the ovicell. Bacteria are known to change their morphology during their life cycle and in response to environmental conditions [reviewed in (Yang et al. 2016)]. Many species of pathogenic bacteria, including *Escherichia, Salmonella, Campylobacter,* and *Helicobacter* have been reported to change their shape and behavior to assist infection and colonization in the host (Justice et al. 2014; Li et al. 2014; Sarem and Corti 2016). Morphological and physiological changes in *Vibrio fischeri* were observed during the initiation and establishment of the symbiotic association with Hawaiian bobtail squid, *Euprymna scolopes* (Ruby and Asato 1993). Following the initial symbiotic infection, the *V. fischeri* cells colonized in the light organ of the squid suffered a significant reduction in growth rate. Additionally, the established population of the symbiont cells in the light organ was smaller in size and non-flagellated. However, the same symbiont cells when expelled from the light organ into the environment reverted back to normal growth rate and began to synthesize functional flagella. Similarly, *Rhizobium* spp. that colonize the root nodules of leguminous plant host differentiate into nitrogen-fixing bacteroids with diverse shapes, sizes, and surface biochemistry depending on the bacterium-plant combination [reviewed in (Oke and Long 1999)]. In anaerobic protozoans, *Metopus contortus* and *Trimyema* sp., a single species of symbiotic archaebacterium belonging to *Methanocorpusculum* (methanogen) was shown to change its shape during its life cycle and interaction with the host hydrogenosome (hydrogen-evolving redox organelle) (Finlay and Fenchel 1991; Embley et al. 1992; Embley and Finlay 1993; Finlay et al. 1993). Such morphological transformation was proposed to increase the surface area to volume ratio of the symbiont for an efficient uptake of hydrogen substrate

released by the hydrogenosome. The observed morphological differences in the bacteria present in the ‘funicular bodies’ and the funicular strands in the symbiotic *B. neritina* colonies could be an adaptation for successful establishment and maintenance of the mutualistic relationship and requires further investigation.

Table 4.1 *Bugula neritina* sample collection sites.

				Colony genotype and symbiont status			
				Type S		Type N	
Location	**Latitude (°)**	**Longitude (°)**	**Collection time(s)**	**Symb +**	**Symb −**	**Symb +**	**Symb −**
Morehead City Yacht Basin Marina, Morehead City, NC	34.721	-76.705	November 2014	26	3	2	1
			May 2015	48	4	–	–
			July 2015	10	0	–	–
Public docks, Oyster, VA	37.287	-75.924	July 2015	0	2	0	18
Beaufort docks, Beaufort, NC	34.716	-76.665	November 2015	44	0	–	–
Town Creek Marina, Beaufort, NC	34.726	-76.665	November 2015	34	0	–	–
Mariner's Point Marina, Salter Path, NC	34.69	-76.88	November 2015	28	2	–	–
			December 2015	–	–	3	0

Table 4.2 *Bugula neritina* genes involved in germinal vesicle breakdown (GVBD) of primary oocyte and oocyte maturation. Contigs were annotated by BLASTx search against non-redundant protein sequences. Relative expression was quantified for genes in bold.

Contig	Sequence Length (bp)	Best Match Protein	GeneBank Accession	E-value	Identity (%)
23292	**903**	**Cyclin-dependent kinase 1 (CDK1) [*Crassostrea gigas*]**	**AEJ91557**	**2E-173**	**78**
3823	**1239**	**Cyclin B2 [*Allium cepa*]**	**BAE53369**	**9E-74**	**46**
12711	981	Cyclin H [*Operophtera brumata*]	KOB67509	4E-93	48
27705	**1566**	**Anaphase promoting complex Cdc20 subunits [*Klebsormidium flaccidum*]**	**GAQ84943**	**5E-173**	**54**
25078	1995	Wee1-like kinase [*Platynereis dumerilii*]	CAA12274	2E-117	48
11412	1671	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (Myt1) [*Crassostrea gigas*]	EKC29619	1E-126	59
4502	1065	Cyclin-dependent kinase 7 (CDK7) [*Habropoda laboriosa*]	KOC69523	4E-163	66
1582	1044	CDK-activating kinase assembly factor MAT1 [*Anopheles darlingi*]	ETN62227	1E-74	45
18385	513	Calcineurin B [*Mizuhopecten yessoensis*]	BAA94543	3E-106	89
4532	1053	Serine/threonine-protein kinase mos [*Exaiptasia pallida*]	KXJ12961	9E-67	44
10547	1059	MAP kinase-activated protein kinase 2 (MAPK2) [*Crassostrea gigas*]	EKC21781	2E-174	70

Table 4.3 Primers used in the study.

Name	Sequence (5'→3')	Target	Product size (bp)	Purpose	Source
CDK1_Q1_479f	ACACACATGAGGTTGTCACG	*B. neritina CDK1* gene	201	qPCR	This study
CDK1_Q1_679r	TGTCTTCTTTTGGCGTTCCC				
CycB_Q1_89f	TGGGCAAGTCCAATGTCAAC	*B. neritina Cyclin B* gene	207	qPCR	This study
CycB_Q1_295r	TATTTCGGCTTTCGGTTGCG				
APC_Q1_812f	AAACGGGCAAATTGCTGAGG	*B. neritina APC/C* gene	255	qPCR	This study
APC_Q1_1066r	AAAAGCGTGCATCCCAAAGC				
G3P_QPCR_2f	GCCACTCAGAAATGCGTAGA	*B. neritina GAPD* gene	77	qPCR	This study
G3P_QPCR_2r	GGGATGATGTTCTGGTAGGC				
Bn18S_QPCR_f	CCGGCGACGCCTTCACTGAG	*B. neritina* 18S rRNA	154	qPCR	(Wong et al. 2010)
Bn18S_QPCR_r	CGCGCCTGCTGCAAACCTTG				

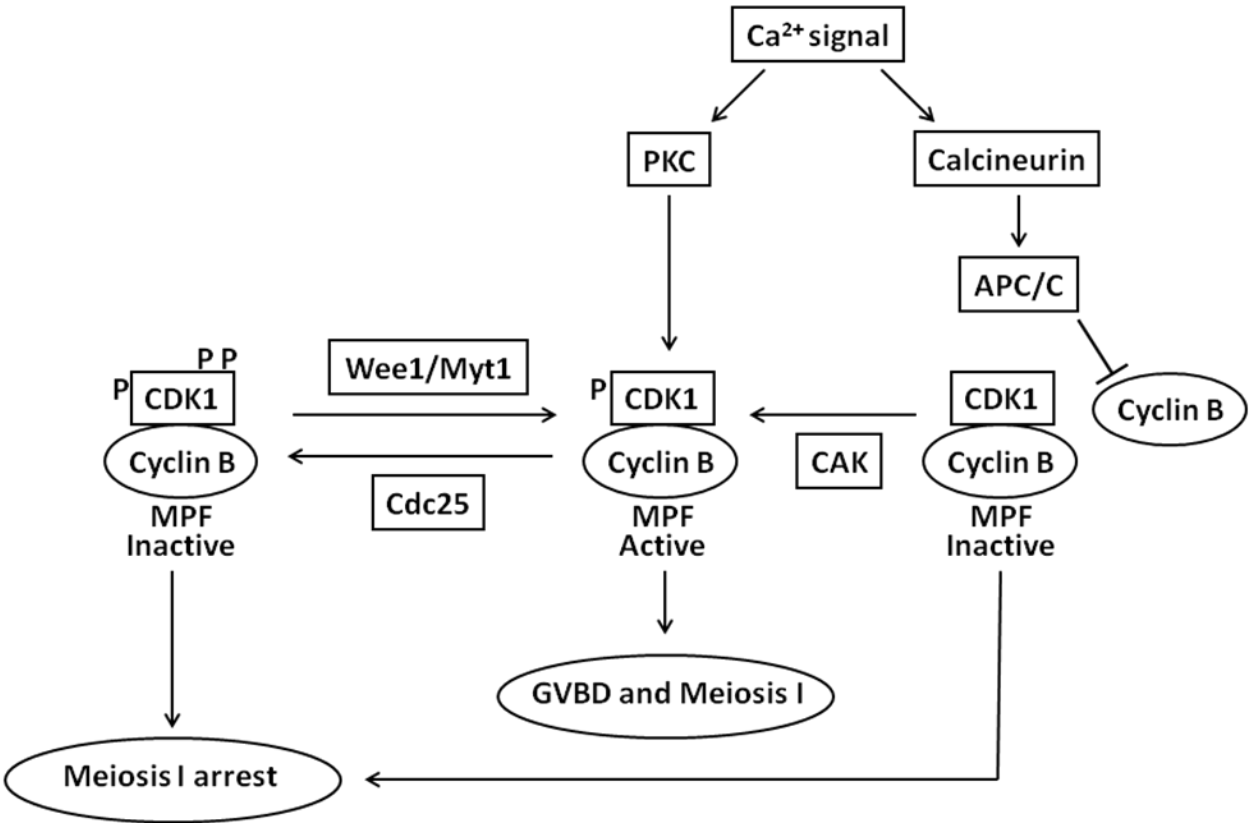

Figure 4.1 Conceptual model of initiation of oocyte maturation.
Adapted from (Coleman and Dunphy 1994; Eckberg et al. 1996; Yamashita et al. 2000; Voronina and Wessel 2003; Levasseur et al. 2013; Adhikari and Liu 2014).

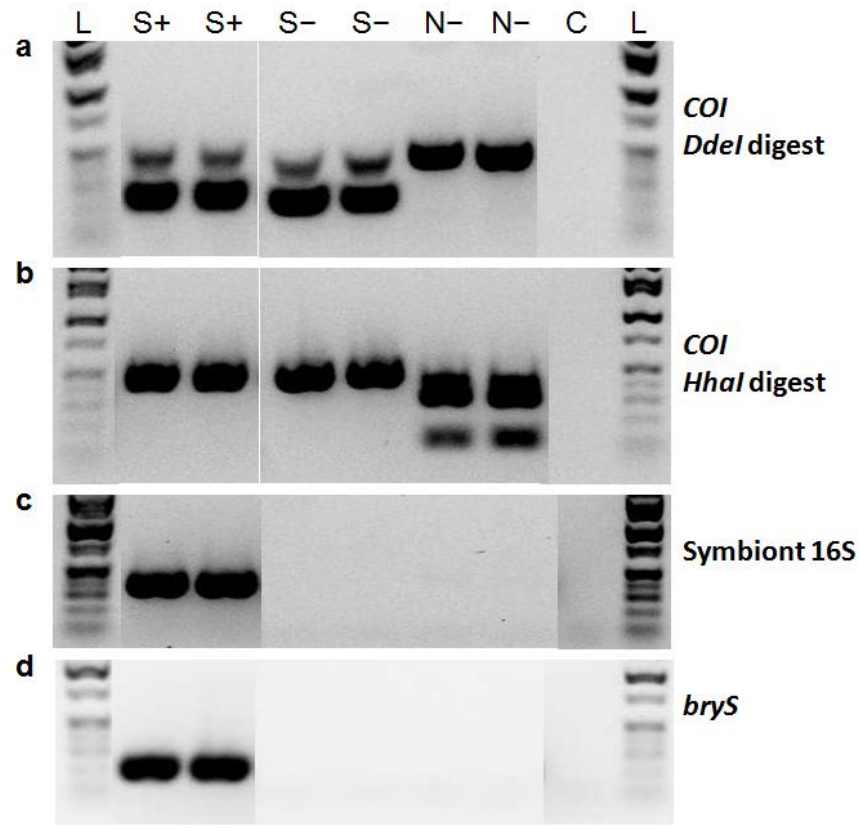

Figure 4.2 Genotyping and symbiont-screening of a subset of *Bugula neritina* colonies. Identification of *B. neritina* Type S genotype (S) by restriction digestion of *B. neritina* mitochondrial *COI* gene amplicon with *Dde1*. **b** Identification of Type N genotype (N) by restriction digestion of the *COI* gene amplicon with *Hha1*. Determination the symbiotic status of the colonies by PCR detection of **c** symbiont 16S rRNA gene, and **d** bryostatin biosynthetic gene cluster, *bryS*. (+) = symbiotic, (−) = symbiont-reduced, C = no template negative PCR control sample, and L = DNA molecular weight ladder.

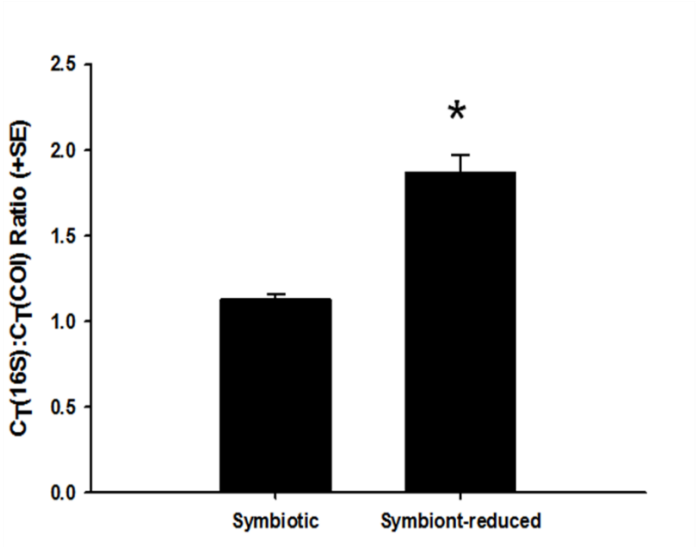

Figure 4.3 Symbiont quantification in naturally occurring *Bugula neritina* colonies. Symbiont titer in naturally occurring symbiotic and symbiont-reduced colonies estimated by qPCR analysis of symbiont 16S rRNA gene and *B. neritina COI* gene. Since C_T value is inverse to the amount of template DNA, a higher ratio of C_T (16S) to C_T (*COI*) indicates less symbiont DNA per unit of host DNA. The asterisks denote statistically significant (independent samples Student's t-Test, $P < 0.01$,) difference in the ratio of symbiont DNA normalized to host DNA. SE= standard error of the mean.

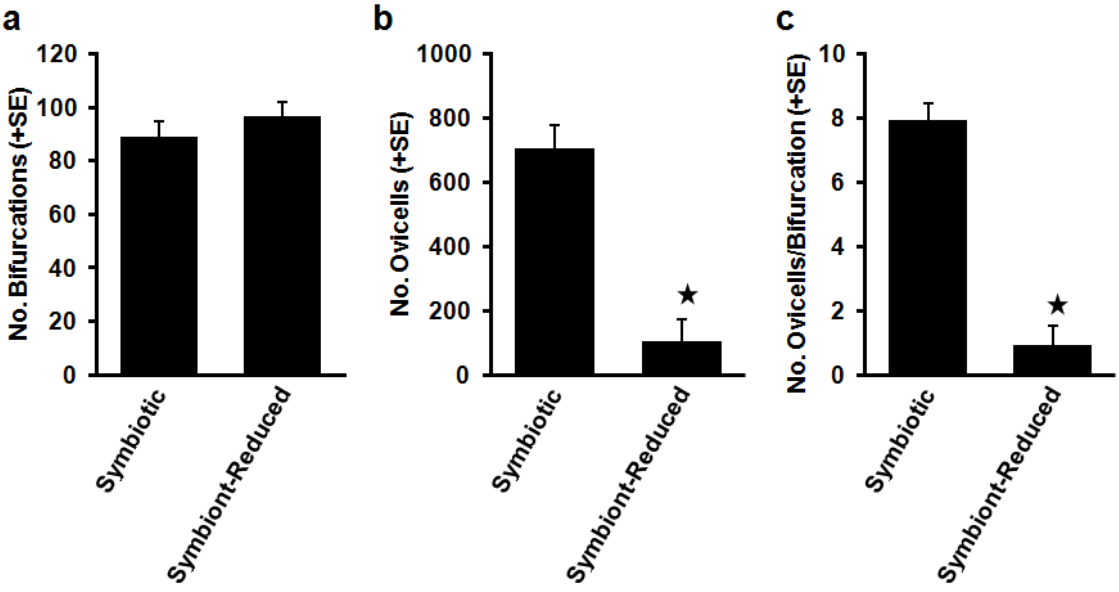

Figure 4.4 Fecundity assessment in naturally symbiont-reduced *Bugula neritina*. **a** Size as measured by the number of bifurcations, **b** fecundity as measured by the number of ovicells, and **c** the fecundity normalized to colony size of control and antibiotic treated colonies. The asterisks denote significant differences (independent samples Student's t-test, assuming equal variance, $P < 0.001$) and SE = standard error of the mean.

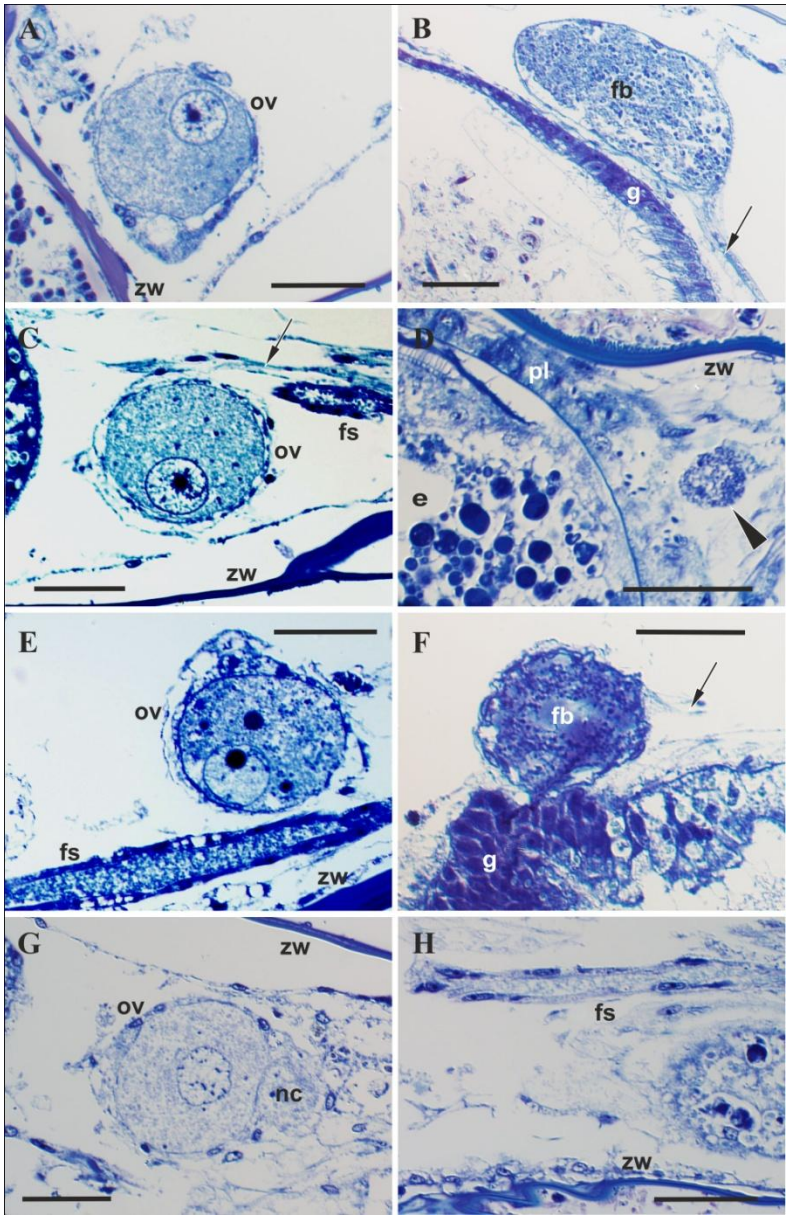

Figure 4.5 Histological sections of autozooids in reproducing colonies of *Bugula neritina*. Ovary with mature oocyte in Type S symbiotic (**A**), Type S symbiont-reduced (**C**), Type N symbiotic (**E**), and Type N symbiont-reduced (**G**) colonies. In (**C**), ovary is suspended in the visceral cavity underneath of the thin (arrow) and thick funicular strands, while in (**E**) ovary is above the thick funicular strand. 'Funicular body' on the wall of caecum in Type S symbiotic (**B**) and Type N symbiotic (**F**) colonies (cellular envelope is clearly seen). On both images the 'funicular bodies' are associated with thin funicular strand (arrows). Part of the embryo in the brood cavity, along with presumed small 'body' (arrowhead) near placental analogue in Type S symbiont-reduced colony (**D**). Thick funicular strand in zooidal cavity (cellular walls are clearly seen) in Type N symbiont-reduced colony (**H**). Abbreviations: e, embryo; g, gut wall; fb, 'funicular body'; fs, thick funicular strand; nc, nurse-cell; ov, ovary; pl, cells of placental analogue; zw, zooidal wall. Scale bars: 20 μm.

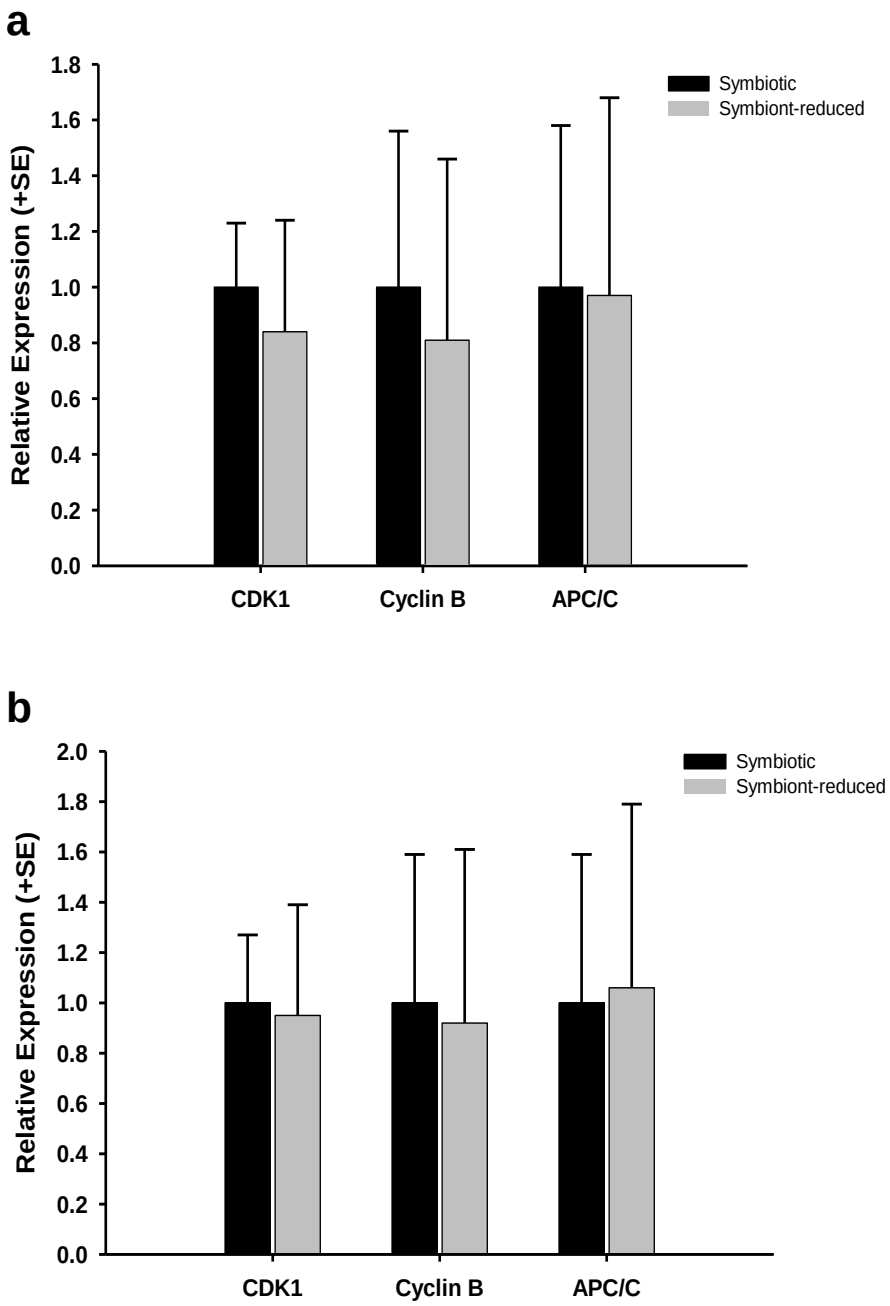

Figure 4.6 Relative expression of oocyte maturation genes in symbiotic and symbiont-reduced Type S *Bugula neritina* colonies.
Expression of the genes normalized to reference genes **a** 18S rRNA and **b** GAPDH. SE= standard error of the mean.

5 CONCLUSION

This study extends our understanding of the role of the symbiont, "*Ca.* Endobugula sertula" in the mutualistic association with the bryozoan host. Since symbiont-produced bryostatins are potent modulators of the eukaryotic signaling protein PKCs, the close association of the host and symbiont should result in interaction between the symbiont-produced bryostatins and host PKCs. Altered PKC profiles among symbiotic and symbiont-reduced *B. neritina* indicate that the bryostatins produced by the symbiont cells within the host colony potentially interact with the host PKCs to affect the host cellular processes, leading to changes in host physiology. A significant decrease in the fecundity of symbiont-depleted (gentamicin-treated) and naturally symbiont-reduced colonies suggest that the bryostatin-PKC interaction possibly influences *B. neritina* reproduction. The results suggest that *B. neritina* has adapted to the presence of symbiont-derived bryostatins in its tissues, to the extent that the bryostatins act as a signal for *B. neritina* reproduction via PKC activation. The decreased fecundity of the symbiont-reduced colonies was due to a fewer number of fertile zooids possessing an ovary in the colonies, indicating that the presence of the symbiont or symbiont-produced bryostatin potentially influence the female reproductive processes in the host. We compared the anatomy of the female reproductive system in symbiotic and naturally-occuring symbiont-reduced *B. neritina* colonies. We also assessed differential expression of genes regulating the oocyte maturation among the colonies. Interestingly, female gonads in both types of colonies were similar anatomically, as well as the reproductive process examined at molecular level, indicating that the symbiont does not affect the reproductive structures or the function of female zooids. I hypothesize an unknown role of the symbiont-produced bioactive compound in signaling the differentiation of germ cell into female sex cells and gonadogenesis, resulting in a fewer proportion of sexual

zooids in symbiont-reduced colonies. An extensive molecular and microscopical study of cells and tissues in newly budding or budded zooids in the colony would reveal the molecular mechanism governing the development of oogenic and spermatogenic tissues in fertile zooids. Furthermore, studies assessing the interaction of bryostatins with the PKCs in *B. neritina* would shed light onto the evolution of host adaptation to symbiont produced bioactive metabolites, as well as the importance of the association to holobiont fitness.

Additionally, anatomical investigation showed presence of bacteria in the funicular strands of both the types of colonies but bacteria containing structures called 'funicular bodies' were found only in symbiotic colonies. Furthermore, the bacteria found in the 'funicular bodies' and the funicular strands appeared to be morphologically different, which could possibly be an adaptation for successful mutualistic association with the bryozoan host. Identification of the bacteria within the funicular strands and the 'funicular bodies' by FISH and their morphological characterization by electron microscopy could answer the role of the bacteria localized in different tissues of the host. Another intriguing question is the mechanism of transmission of the symbiont cells from the maternal zooid to the developing larva in the ovicell; specifically the processes that allow the symbiont cells to reach placental analogue, move in between placental cells, and finally penetrate rather thick cuticle covering the cellular wall of the ooecial vesicle to enter the brood cavity. Answering all these questions requires complex approach combining microscopical and molecular methods, and our study is just the first attempt to unite them.

6 REFERENCES

Aberdam E, Dekel N (1985) Activators of protein kinase C stimulate meiotic maturation of rat oocytes. Biochem Biophys Res Commun 132: 570-574

Adhikari D, Liu K (2014) The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. Mol Cell Endocrinol 382: 480-487

Akita Y (2008) Protein kinase Cε: multiple roles in the function of, and signaling mediated by, the cytoskeleton. FEBS J 275: 3995-4004

Al Seesi S, Tiagueu YT, Zelikovsky A, Măndoiu II (2014) Bootstrap-based differential gene expression analysis for RNA-Seq data with and without replicates. BMC Genomics 15: S2

Altura MA, Heath-Heckman EAC, Gillette A, Kremer N, Krachler A-M, Brennan C, Ruby EG, Orth K, McFall-Ngai MJ (2013) The first engagement of partners in the *Euprymna scolopes-Vibrio fischeri* symbiosis is a two-step process initiated by a few environmental symbiont cells. Environ Microbiol 15: 2937-2950

Alvarez M, Schrey AW, Richards CL (2015) Ten years of transcriptomics in wild populations: what have we learned about their ecology and evolution? Mol Ecol 24: 710-725

Andrew RL, Bernatchez L, Bonin A, Buerkle CA, Carstens BC, Emerson BC, Garant D, Giraud T, Kane NC, Rogers SM (2013) A road map for molecular ecology. Mol Ecol 22: 2605-2626

Ashen JB, Goff LJ (2000) Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacterial symbioses. Applied and Environmental Microbiology 66: 3024-3030

Avazeri N, Courtot A-M, Lefevre B (2004) Regulation of spontaneous meiosis resumption in mouse oocytes by various conventional PKC isozymes depends on cellular compartmentalization. J Cell Sci 117: 4969-4978

Battaini F, Mochly-Rosen D (2007) Happy birthday protein kinase C: past, present and future of a superfamily. Pharmacological research: the official journal of the Italian Pharmacological Society 55: 461

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects Annu Rev Microbiol, pp 155-189

Bertness MD, Garrity SD, Levings SC (1981) Predation pressure and gastropod foraging: A tropical-temperate comparison. Evolution 35: 995-1007

Best MA, Thorpe JP (1985) Autoradiographic study of feeding and the colonial transport of metabolites in the marine bryozoan *Membranipora membranacea*. Mar Biol 84: 295-300

Best MA, Thorpe JP (2002) Use of radioactive labelled food to assess the role of the funicular system in the transport of metabolites in the cheilostome bryozoan *Membranipora membranacea* (L.). In: Wyse Jackson PN, Buttler CJ, Spencer Jones M (eds) Bryozoan Studies 2001. AA Balkema Publishers, Lisse/Abingdon/Exton/Tokyo, pp 29-35

Black AR, Black JD (2013) Protein kinase C signaling and cell cycle regulation. Front Immunol 3: 423

Braendle C, Miura T, Bickel R, Shingleton AW, Kambhampati S, Stern DL (2003) Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. PLoS Biol 1: 70-76

Carle KJ, Ruppert E (1983) Comparative ultrastructure of the bryozoan funiculus: a blood vessel homologue. J Zool Syst Evol Res 21: 181-193

Carroll MP, May WS (1994) Protein kinase C mediated serine phosphorylation directly activates RAF-1 in murine hematopoietic cells. J Biol Chem 269: 1249-1256

Casneuf T, Van de Peer Y, Huber W (2007) In situ analysis of cross-hybridisation on microarrays and the inference of expression correlation. BMC Bioinformatics 8: 461

Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. FEMS Microbiol Rev 34: 41-58

Clamp A, Jayson GC (2002) The clinical development of the bryostatins. Anti-Cancer Drugs 13: 673-683

Colas P, Dube F (1998) Meiotic maturation in mollusc oocytes. Semin Cell Dev Biol 9: 539-548

Coleman TR, Dunphy WG (1994) Cdc2 regulatory factors. Curr Opin Cell Biol 6: 877-882

Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szcześniak MW, Gaffney DJ, Elo LL, Zhang X (2016) A survey of best practices for RNA-seq data analysis. Genome Biol 17: 1

Costache V, McDougall A, Dumollard R (2014) Cell cycle arrest and activation of development in marine invertebrate deuterostomes. Biochem Biophys Res Commun 450: 1175-1181

Cragg GM, Newman DJ (2013) Natural products: A continuing source of novel drug leads. Biochimica Et Biophysica Acta-General Subjects 1830: 3670-3695

Crawford JM, Clardy J (2011) Bacterial symbionts and natural products. Chem Commun 47: 7559-7566

Davidson SK, Allen SW, Lim GE, Anderson CM, Haygood MG (2001) Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus* Endobugula sertula" of the bryozoan *Bugula neritina*. Appl Environ Microbiol 67: 4531-4537

Davidson SK, Haygood MG (1999) Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont "*Candidatus* Endobugula sertula". Biol Bull 196: 273-280

De Vries DJ, Herald CL, Pettit GR, Blumberg PM (1988) Demonstration of sub-nanomolar affinity of bryostatin 1 for the phorbol ester receptor in rat brain. Biochem Pharmacol 37: 4069-4073

Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, Bouletreau M (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proc Natl Acad Sci USA 98: 6247-6252

Deguchi R, Takeda N, Stricker SA (2014) Calcium signals and oocyte maturation in marine invertebrates. Int J Dev Biol 59: 271-280

Dorée M, Hunt T (2002) From Cdc2 to Cdk1: when did the cell cycle kinase join its cyclin partner? J Cell Sci 115: 2461-2464

Dube F, Golsteyn R, Dufresne L (1987) Protein kinase C and meiotic maturation of surf clam oocytes. Biochem Biophys Res Commun 142: 1072-1076

Dyrynda P, King P (1983) Gametogenesis in placental and non-placental ovicellate cheilostome Bryozoa. J Zool 200: 471-492

Dyrynda P, Ryland J (1982) Reproductive strategies and life histories in the cheilostome marine bryozoans *Chartella papyracea* and *Bugula flabellata*. Mar Biol 71: 241-256

Eckberg WR (1988) Intracellular signal transduction and amplification mechanisms in the regulation of oocyte maturation. Biol Bull 174: 95-108

Eckberg WR, Carroll AG (1987) Evidence for involvement of protein kinase C in germinal vesicle breakdown in *Chaetopterus*. Dev Growth Differ 29: 489-496

Eckberg WR, Johnson MR, Palazzo RE (1996) Regulation of maturation-promoting factor by protein kinase C in *Chaetopterus* oocytes. Invertebr Reprod Dev 30: 71-79

Eckberg WR, Szuts EZ, Carroll AG (1987) Protein kinase C activity, protein phosphorylation and germinal vesicle breakdown in *Spisula* oocytes. Dev Biol 124: 57-64

Ekblom R, Galindo J (2011) Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity 107: 1-15

Ellegren H (2008) Sequencing goes 454 and takes large-scale genomics into the wild. Mol Ecol 17: 1629-1631

Embley T, Finlay B, Brown S (1992) RNA sequence analysis shows that the symbionts in the ciliate *Metopus contortus* are polymorphs of a single methanogen species. FEMS Microbiol Lett 97: 57-61

Embley TM, Finlay BJ (1993) Systematic and morphological diversity of endosymbiotic methanogens in anaerobic ciliates. Antonie Leeuwenhoek 64: 261-271

Engelstaedter J, Hurst GDD (2009) The ecology and evolution of microbes that manipulate host reproduction. Annu Rev Ecol Evol Syst 40: 127-149

Extavour CG, Akam M (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130: 5869-5884

Fehlauer-Ale KH, Mackie JA, Lim-Fong GE, Ale E, Pie MR, Waeschenbach A (2014) Cryptic species in the cosmopolitan *Bugula neritina* complex (Bryozoa, Cheilostomata). Zool Scr 43: 193-205

Fesquet D, Labbe J, Derancourt J, Capony J, Galas S, Girard F, Lorca T, Shuttleworth J, Doree M, Cavadore J (1993) The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J 12: 3111

Finlay B, Embley T, Fenchel T (1993) A new polymorphic methanogen, closely related to *Methanocorpusculum parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema* sp. J Gen Microbiol 139: 371-378

Finlay B, Fenchel T (1991) Polymorphic bacterial symbionts in the anaerobic ciliated protozoon *Metopus*. FEMS Microbiol Lett 79: 187-190

Fisher RP, Morgan DO (1994) A novel cyclin associates with M015/CDK7 to form the CDK-activating kinase. Cell 78: 713-724

Florez LV, Biedermann PHW, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat Prod Rep 32: 904-936

Gautier J, Matsukawa T, Nurse P, Maller J (1989) Dephosphorylation and activation of Xenopus $p34^{cdc2}$ protein kinase during the cell cycle. Nature 339: 626-629

Gerwick WH, Moore BS (2012) Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. Chem Biol 19: 85-98

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29: 644-U130

Graff JM, Young TN, Johnson JD, Blackshear PJ (1989) Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. J Biol Chem 264: 21818-21823

Haine ER (2008) Symbiont-mediated protection. Proc R Soc B-Biol Sci 275: 353-361

Hansen AK, Moran NA (2011) Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. Proc Natl Acad Sci USA 108: 2849-2854

Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A (1992) MARCKS is an actin filament cross-linking protein regulated by protein kinase C and calcium calmodulin. Nature 356: 618-622

Haygood MG, Davidson SK (1997) Small-subunit rRNA genes and in situ hybridization with oligonucleotides specific for the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus* endobugula sertula". Appl Environ Microbiol 63: 4612-4616

Haygood MG, Schmidt EW, Davidson SK, Faulkner DJ (1999) Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. J Mol Microbiol Biotechnol 1: 33-43

Hillman K, Goodrich-Blair H (2016) Are you my symbiont? Microbial polymorphic toxins and antimicrobial compounds as honest signals of beneficial symbiotic defensive traits. Curr Opin Microbiol 31: 184-190

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. Science 332: 254-256

Hughes R, Manriquez P, Bishop J, Burrows M (2003) Stress promotes maleness in hermaphroditic modular animals. Proc Natl Acad Sci USA 100: 10326-10330

Iwasaki S, Kobayashi H, Furukawa J, Namikoshi M, Okuda S, Sato Z, Matsuda I, Noda T (1984) Studies on macrocyclic lactone antibiotics. VII. Structure of a phytotoxin" rhizoxin" produced by *Rhizopus chinensis*. J Antibiot 37: 354-362

Johnson CH (2010) Effects of selfing on offspring survival and reproduction in a colonial simultaneous hermaphrodite (*Bugula stolonifera*, Bryozoa). Biol Bull 219: 27-37

Joyce SA, Brachmann AO, Glazer I, Lango L, Schwaer G, Clarke DJ, Bode HB (2008) Bacterial biosynthesis of a multipotent stilbene. Angewandte Chemie-International Edition 47: 1942-1945

Justice SS, Harrison A, Becknell B, Mason KM (2014) Bacterial differentiation, development, and disease: mechanisms for survival. FEMS Microbiol Lett 360: 1-8

Kalive M, Faust JJ, Koeneman BA, Capco DG (2010) Involvement of the PKC family in regulation of early development. Mol Reprod Dev 77: 95-104

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW, Herzner G, Seger J, Strohm E (2014) Partner choice and fidelity stabilize coevolution in a Cretaceous-age defensive symbiosis. Proceedings of the National Academy of Sciences of the United States of America 111: 6359-6364 doi 10.1073/pnas.1400457111

Keough MJ (1989a) Dispersal of the bryozoan *Bugula neritina* and effects of adults on newly metamorphosed juveniles. Mar Ecol-Prog Ser 57: 163-172

Keough MJ (1989b) Variation in growth rate and reproduction of the bryozoan *Bugula neritina*. Biol Bull 177: 277-286

Koga R, Meng X-Y, Tsuchida T, Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. Proc Natl Acad Sci USA 109: E1230-E1237

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364: 249-252

Konya K, Shimidzu N, Otaki N, Yokoyama A, Adachi K, Miki W (1995) Inhibitory effect of bacterial ubiquinones on the settling of barnacle, Balanus amphitrite. Experientia 51: 153-155

Koropatnick T, Apicella MA, McFall-Ngai MJ (2002) Symbiont-induced developmental remodeling of the *Euprymna scolopes* light organ involves microbial membrane factors and host hemocyte migration. Mol Biol Cell 13: 252A

Kraft A, Reeves J, Ashendel C (1988) Differing modulation of protein kinase C by bryostatin 1 and phorbol esters in JB6 mouse epidermal cells. J Biol Chem 263: 8437-8442

Kraft AS, Smith JB, Berkow RL (1986) Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cells HL-60. Proc Natl Acad Sci USA 83: 1334-1338

Kwon HB, Lee WK (1991) Involvement of protein kinase c in the regulation of oocyte maturation in amphibians *Rana dybowskii*. J Exp Zool 257: 115-123

Labbe J, Capony J, Caput D, Cavadore J, Derancourt J, Kaghad M, Lelias J, Picard A, Doree M (1989) MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. EMBO J 8: 3053

Lackner G, Moebius N, Hertweck C (2011) Endofungal bacterium controls its host by an hrp type III secretion system. ISME J 5: 252-261

Lee PD, Sladek R, Greenwood CM, Hudson TJ (2002) Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res 12: 292-297

Levasseur M, Dumollard R, Chambon J-P, Hebras C, Sinclair M, Whitaker M, McDougall A (2013) Release from meiotic arrest in ascidian eggs requires the activity of two phosphatases but not CaMKII. Development 140: 4583-4593

Lew KK, Chritton S, Blumberg PM (1982) Biological responsiveness to the phorbol esters and specific binding of [^{3}H]phorbol 12, 13-dibutyrate in the nematode *Caenorhabditis elegans*, a manipulable genetic system. Teratog Carcinog Mutagen 2: 19-30

Li L, Mendis N, Trigui H, Oliver JD, Faucher SP (2014) The importance of the viable but non-culturable state in human bacterial pathogens. Frontiers in Microbiology 5

Lidgard S, Carter MC, Dick MH, Gordon DP, Ostrovsky AN (2012) Division of labor and recurrent evolution of polymorphisms in a group of colonial animals. Evol Ecol 26: 233-257

Lindquist N (1996) Palatability of invertebrate larvae to corals and sea anemones. Mar Biol 126: 745-755

Lindquist N, Hay ME (1996) Palatability and chemical defense of marine invertebrate larvae. Ecol Monogr 66: 431-450

Lindquist N, Hay ME, Fenical W (1992) Defense of ascidians and their conspicuous larvae: Adult vs. larval chemical defenses. Ecol Monogr: 547-568

Linneman J, Paulus D, Lim-Fong G, Lopanik NB (2014) Latitudinal variation of a defensive symbiosis in the *Bugula neritina* (Bryozoa) sibling species complex. PloS One 9: e108783

Lipp P, Reither G (2011) Protein Kinase C: the "masters" of calcium and lipid. Cold Spring Harbor Perspect Biol 3: 17

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408

Long A, Freeley M (2014) Protein kinase C: a regulator of cytoskeleton remodelling and T-cell migration. Biochem Soc Trans 42: 1490-1497

Lopanik N, Gustafson KR, Lindquist N (2004a) Structure of bryostatin 20: A symbiont-produced chemical defense for larvae of the host bryozoan, *Bugula neritina*. J Nat Prod 67: 1412-1414

Lopanik N, Lindquist N, Targett N (2004b) Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. Oecologia 139: 131-139

Lopanik NB (2014) Chemical defensive symbioses in the marine environment. Funct Ecol 28: 328-340 doi 10.1111

Lutaud G (1969) La nature des corps funiculaires des cellularines, bryozoaires chilostomes. Arch Zool Exp Gen 110: 2-30

Lutaud G (1985) Preliminary experiments on interzooidal metabolic transfer in anascan bryozoans. In: Nielsen C, Larwood GP (eds) Bryozoa: Ordovician to Recent. Olsen and Olsen, Fredensborg, pp 183-191

Mandel MJ, Schaefer AL, Brennan CA, Heath-Heckman EAC, DeLoney-Marino CR, McFall-Ngai MJ, Ruby EG (2012) Squid-derived chitin oligosaccharides are a chemotactic signal during colonization by *Vibrio fischeri*. Appl Environ Microbiol 78: 4620-4626 doi 10.1128/aem.00377-12

Mathew M, Bean KI, Temate-Tiagueu Y, Caciula A, Mandoiu II, Zelikovsky A, Lopanik NB (2016) Influence of symbiont-produced bioactive natural products on holobiont fitness in the marine bryozoan, *Bugula neritina* via protein kinase C (PKC). Mar Biol 163: 1-17

Mathew M, Lopanik NB (2014) Host differentially expressed genes during association with its defensive endosymbiont. Biol Bull 226: 152-163

Mawatari S (1951) The natural history of a common fouling bryozoan, *Bugula neritina* (Linnaeus). Misc Rep Res Inst Nat Resour 20: 47-54

McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol 10: 13-26

McFall-Ngai M (2014) Divining the essence of symbiosis: Insights from the squid-*Vibrio* model. PLoS Biol 12

McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci USA 110: 3229-3236

McGovern TM, Hellberg ME (2003) Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoan *Bugula neritina*. Mol Ecol 12: 1207-1215

McLeod HL, Murray LS, Wanders J, Setanoians A, Graham MA, Pavlidis N, Heinrich B, Huinink WWT, Wagener DJT, Aamdal S, Verweij J (1996) Multicentre phase II pharmacological evaluation of rhizoxin. Br J Cancer 74: 1944-1948

Menge BA, Lubchenco J (1981) Community organization in temperate and tropical rocky intertidal habitats: prey refuges in relation to consumer pressure gradients. Ecol Monogr 51: 429-450

Miles J, Harvell C, Griggs C, Eisner S (1995) Resource translocation in a marine bryozoan: quantification and visualization of 14C and 35S. Mar Biol 122: 439-445

Miwa J, Tabuse Y, Furusawa M, Yamasaki H (1982) Tumor promoters specifically and reversibly disturb development and behavior of *Caenorhabditis elegans*. J Cancer Res Clin Oncol 104: 81-87

Miyake Y, Yasui M, Ikeda K, Kondo T, Tsukamoto S, Hori C, Okemoto N, Mashou K, Bando R, Nakamura N (2009) Molecular cloning and expression of starfish protein kinase C Isoforms. Biosci Biotechnol Biochem 73: 1550-1560

Miyazaki S (2006) Thirty years of calcium signals at fertilization Semin Cell Dev Biol. Elsevier, pp 233-243

Mondadori RG, Neves JP, Goncalves PBD (2008) Protein kinase C (PKC) role in bovine oocyte maturation and early embryo development. Anim Reprod Sci 107: 20-29

Moosbrugger M, Schwaha T, Walzl MG, Obst M, Ostrovsky AN (2012) The placental analogue and the pattern of sexual reproduction in the cheilostome bryozoan *Bicellariella ciliata* (Gymnolaemata). Front Zool 9

Moran NA (2006) Symbiosis. Curr Biol 16: R866–R871

Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts Annu Rev Genet, pp 165-190

Morgan DO (1999) Regulation of the APC and the exit from mitosis. Nat Cell Biol 1: E47-E53

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Meth 5: 621-628

Mueller PR, Coleman TR, Kumagai A, Dunphy WG (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 270: 86-90

Murray AW, Solomon MJ, Kirschner MW (1989) The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature 339: 280-286

Mutter R, Wills M (2000) Chemistry and clinical biology of the bryostatins. Bioorg Med Chem 8: 1841-1860

Nader N, Kulkarni RP, Dib M, Machaca K (2013) How to make a good egg!: The need for remodeling of oocyte Ca2+ signaling to mediate the egg-to-embryo transition. Cell Calcium 53: 41-54

Narquizian R, Kocienski P (2000) The pederin family of antitumor agents: Structures, synthesis and biological activity. In: Mulzer J, Bohlmann R (eds) The Role of Natural Products in Drug Discovery. Springer Berlin Heidelberg, pp 25-56

Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70: 461-477

Newton AC (2001) Protein kinase C: Structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101: 2353-2364

Newton AC (2010) Protein kinase C: Poised to signal. Am J Physiol Endocrinol Metab 298: E395-E402

Nicolae M, Mangul S, Măndoiu II, Zelikovsky A (2011) Estimation of alternative splicing isoform frequencies from RNA-Seq data. Algorithms Mol Biol 6: 9

Nielsen KL, Høgh AL, Emmersen J (2006) DeepSAGE—digital transcriptomics with high sensitivity, simple experimental protocol and multiplexing of samples. Nucleic Acids Res 34: e133-e133

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308: 693-698

Nyholm SV, McFall-Ngai MJ (2004) The winnowing: Establishing the squid-*Vibrio* symbiosis. Nat Rev Microbiol 2: 632-642 doi 10.1038/nrmicro957

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ (2000) Establishment of an animal-bacterial association: Recruiting symbiotic vibrios from the environment. Proc Natl Acad Sci USA 97: 10231-10235 doi 10.1073/pnas.97.18.10231

Oke V, Long SR (1999) Bacteroid formation in the *Rhizobium*–legume symbiosis. Curr Opin Microbiol 2: 641-646

Okoniewski MJ, Miller CJ (2006) Hybridization interactions between probesets in short oligo microarrays lead to spurious correlations. BMC Bioinformatics 7: 276

Ostrovsky AN (1998) Comparative studies of ovicell anatomy and reproductive patterns in *Cribrilina annulata* and *Celleporella hyalina* (Bryozoa : Cheilostomatida). Acta Zoologica 79: 287-318

Ostrovsky AN (2013a) Evolution of sexual reproduction in marine invertebrates: Example of gymnolaemate bryozoans. Springer, Dordrecht, Heidelberg, New York, London

Ostrovsky AN (2013b) From incipient to substantial: evolution of placentotrophy in a phylum of aquatic colonial invertebrates. Evolution 67: 1368-1382

Ostrovsky AN, Gordon DP, Lidgard S (2009) Independent evolution of matrotrophy in the major classes of Bryozoa: transitions among reproductive patterns and their ecological background. Mar Ecol-Prog Ser 378: 113-124

Ostrovsky AN, Porter JS (2011) Pattern of occurrence of supraneural coelomopores and intertentacular organs in Gymnolaemata (Bryozoa) and its evolutionary implications. Zoomorphology 130: 1-15

Pannebakker BA, Loppin B, Elemans CPH, Humblot L, Vavre F (2007) Parasitic inhibition of cell death facilitates symbiosis. Proc Natl Acad Sci USA 104: 213-215

Partida-Martinez LP, Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437: 884-888

Partida-Martinez LP, Monajembashi S, Greulich KO, Hertweck C (2007) Endosymbiont-dependent host reproduction maintains bacterial-fungal mutualism. Curr Biol 17: 773-777

Pettit GR (1996) Progress in the discovery of biosynthetic anticancer drugs. J Nat Prod 59: 812-821

Pettit GR, Day JF, Hartwell JL, Wood HB (1970) Antineoplastic components of marine animals. Nature 227: 962-963

Pettit GR, Herald CL, Doubek DL, Herald DL (1982) Isolation and structure of bryostatin 1. J Am Chem Soc 104: 6846-6848

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proc Natl Acad Sci USA 99: 14002-14007

Piel J (2009) Metabolites from symbiotic bacteria. Nat Prod Rep 26: 338-362

Piel J, Hui DQ, Wen GP, Butzke D, Platzer M, Fusetani N, Matsunaga S (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. Proc Natl Acad Sci USA 101: 16222-16227

Piétu G, Mariage-Samson R, Fayein N-A, Matingou C, Eveno E, Houlgatte R, Decraene C, Vandenbrouck Y, Tahi F, Devignes M-D (1999) The Genexpress IMAGE knowledge base of the human brain transcriptome: a prototype integrated resource for functional and computational genomics. Genome Res 9: 195-209

Poli A, Mongiorgi S, Cocco L, Follo MY (2014) Protein kinase C involvement in cell cycle modulation. Biochem Soc Trans 42: 1471-1476

Poon R, Yamashita K, Adamczewski J, Hunt T, Shuttleworth J (1993) The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33cdk2 and p34cdc2. EMBO J 12: 3123

Rader BA, Nyholm SV (2012) Host/microbe interactions revealed through "Omics" in the symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri*. Biol Bull 223: 103-111

Rath CM, Janto B, Earl J, Ahmed A, Hu FZ, Hiller L, Dahlgren M, Kreft R, Yu F, Wolff JJ, Kweon HK, Christiansen MA, Hakansson K, Williams RM, Ehrlich GD, Sherman DH (2011) Meta-omic characterization of the marine invertebrate microbial consortium that produces the chemotherapeutic natural product ET-743. Chemical Biology 6: 1244-1256 doi 10.1021/cb200244t

Reed C (1991) Bryozoa. In: Giese AC PJ, Pearse VB (ed) Reprod Mar Invertebr. The Boxwood Press, Pacific Grove, CA, pp 85-245

Rinehart KL, Gloer JB, Hughes RG, Renis HE, McGovren JP, Swynenberg EB, Stringfellow DA, Kuentzel SL, Li LH (1981) Didemnins: antiviral and antitumor depsipeptides from a caribbean tunicate. Science 212: 933-935

Rose-Hellekant TA, Bavister BD (1996) Roles of protein kinase A and C in spontaneous maturation and in forskolin or 3-isobutyl-1-methylxanthine maintained meiotic arrest of bovine oocytes. Mol Reprod Dev 44: 241-249

Ruby E, Asato L (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch Microbiol 159: 160-167

Ruby EG (2008) Symbiotic conversations are revealed under genetic interrogation. Nat Rev Microbiol 6: 752-762 doi 10.1038/nrmicro1958

Rudolph J (2007) Cdc25 phosphatases: structure, specificity, and mechanism. Biochemistry 46: 3595-3604

Ryland JS (1970) Bryozoans. Hutchinson University Library, London

Sano T, Tabuse Y, Nishiwaki K, Miwa J (1995) The *tpa-1* gene of *Caenorhabditis elegans* encodes two proteins similar to Ca^{2+}-independent protein kinase Cs: Evidence by complete genomic and complementary DNA sequences of the *tpa-1* gene. J Mol Biol 251: 477-485 doi 10.1006/jmbi.1995.0449

Sarem M, Corti R (2016) Role of *Helicobacter pylori* coccoid forms in infection and recrudescence. Gastroenterología Y Hepatología 39: 28-35

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467

Scherlach K, Partida-Martinez LP, Dahse H-M, Hertweck C (2006) Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus*. J Am Chem Soc 128: 11529-11536 doi 10.1021/ja062953o

Schmidt EW (2008) Trading molecules and tracking targets in symbiotic interactions. Nat Chem Biol 4: 466-473 doi 10.1038/nchembio.101

Schmitt I, Partida-Martinez LP, Winkler R, Voigt K, Einax E, Doelz F, Telle S, Woestemeyer J, Hertweck C (2008) Evolution of host resistance in a toxin-producing bacterial-fungal alliance. ISME J 2: 632-641 doi 10.1038/ismej.2008.19

Sharp KH, Davidson SK, Haygood MG (2007) Localization of '*Candidatus* Endobugula sertula' and the bryostatins throughout the life cycle of the bryozoan *Bugula neritina*. ISME J 1: 693-702

Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407: 81-86

Shishkin AA, Giannoukos G, Kucukural A, Ciulla D, Busby M, Surka C, Chen J, Bhattacharyya RP, Rudy RF, Patel MM (2015) Simultaneous generation of many RNA-seq libraries in a single reaction. Nat Meth 12: 323-325

Silén L (1945) The main features of the development of the ovum, embryo and ooecium in the ooecioferous Bryozoa Gymnolaemata. Ark Zool 35A: 1-34

Silén L (1966) On the fertilization problem in the gymnolaematous Bryozoa. Ophelia 3: 113-140

Silén L (1977) Polymorphism. In: Woollacott RM, Zimmer, R.L. (ed) Biology of Bryozoans. Academic Press, New York, pp 184-232

Sobinoff AP, Sutherland JM, McLaughlin EA (2013) Intracellular signalling during female gametogenesis. Mol Hum Reprod 19: 265-278

Stith BJ, Maller JL (1987) Induction of meiotic maturation in *Xenopus* oocytes by 12-O-Tetradecanoylphorbol 13-acetate. Exp Cell Res 169: 514-523

Stouthamer R, Breeuwer JAJ, Hurst GDD (1999) *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53: 71-102 doi 10.1146/annurev.micro.53.1.71

Strauch O, Ehlers RU (1998) Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Appl Microbiol Biotechnol 50: 369-374

Stricker SA (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev Biol 211: 157-176

Sudek S, Lopanik NB, Waggoner LE, Hildebrand M, Anderson C, Liu H, Patel A, Sherman DH, Haygood MG (2007) Identification of the putative bryostatin polyketide synthase gene cluster from "*Candidatus* Endobugula sertula", the uncultivated microbial symbiont of the marine bryozoan *Bugula neritina*. J Nat Prod 70: 67-74

Tabuse Y, Miwa J (1983) A gene involved in action of tumor promoters is identified and mapped in *Caenorhabditis elegans*. Carcinogenesis 4: 783-786

Tabuse Y, Nishiwaki K, Miwa J (1989) Mutations in a protein kinase C homolog confer phorbol ester resistance on *Caenorhabditis elegans*. Science 243: 1713-1716

Tabuse Y, Sano T, Nishiwaki K, Miwa J (1995) Molecular evidence for the direct involvement of a protein kinase C in developmental and behavioural susceptibility to tumour-promoting phorbol esters in *Caenorhabditis elegans*. Biochem J 312: 69-74

Tamburri MN, Zimmer-Faust RK (1996) Suspension feeding: Basic mechanisms controlling recognition and ingestion of larvae. Limnol Oceanogr 41: 1188-1197

Temkin MH (1996) Comparative fertilization biology of gymnolaemate bryozoans. Mar Biol 127: 329-339 doi 10.1007/bf00942118

Thacker RW, Starnes S (2003) Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. Marine Biology 142: 643-648

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75: 291-295

Todd EV, Black MA, Gemmell NJ (2016) The power and promise of RNA-seq in ecology and evolution. Mol Ecol

Toth AL, Varala K, Newman TC, Miguez FE, Hutchison SK, Willoughby DA, Simons JF, Egholm M, Hunt JH, Hudson ME (2007) Wasp gene expression supports an evolutionary link between maternal behavior and eusociality. Science 318: 441-444

Trindade-Silva AE, Lim-Fong GE, Sharp KH, Haygood MG (2010) Bryostatins: biological context and biotechnological prospects. Curr Opin Biotechnol 21: 834-842

Tsuruo T, Oh-Hara T, Iida H, Tsukagoshi S, Sato Z, Matsuda I, Iwasaki S, Okuda S, Shimizu F, et al. (1986) Rhizoxin a macrocyclic lactone antibiotic as a new antitumor agent against human and murine tumor cells and their vincristine-resistant sublines. Cancer Res 46: 381-385

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40: e115

van Kesteren C, de Vooght MMM, Lopez-Lazaro L, Mathot RAA, Schellens JHM, Jimeno JM, Beijnen JH (2003) Yondelis (R) (trabectedin, ET-743): the development of an anticancer agent of marine origin. Anti-Cancer Drugs 14: 487-502 doi 10.1097/01.cad.0000086304.28884.93

Vermeij GJ (1978) Biogeography and adaptation: patterns of marine life. Harvard University Press, Cambridge, MA

Vijay N, Poelstra JW, Künstner A, Wolf JB (2013) Challenges and strategies in transcriptome assembly and differential gene expression quantification. A comprehensive in silico assessment of RNA-seq experiments. Mol Ecol 22: 620-634

Visick KL (2009) An intricate network of regulators controls biofilm formation and colonization by *Vibrio fischeri*. Mol Microbiol 74: 782-789 doi 10.1111/j.1365-2958.2009.06899.x

Visick KL, Foster J, Doino J, McFall-Ngai M, Ruby EG (2000) *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J Bacteriol 182: 4578-4586 doi 10.1128/jb.182.16.4578-4586.2000

Voronina E, Wessel GM (2003) The regulation of oocyte maturation. Curr Top Dev Biol 58: 53-110

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10: 57-63

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007) From parasite to mutualist: rapid evolution of Wolbachia in natural populations of Drosophila. PLoS Biol 5: e114

Wender PA, Baryza JL, Brenner SE, DeChristopher BA, Loy BA, Schrier AJ, Verma VA (2011) Design, synthesis, and evaluation of potent bryostatin analogs that modulate PKC translocation selectivity. Proc Natl Acad Sci USA 108: 6721-6726

Wender PA, Cribbs CM, Koehler KF, Sharkey NA, Herald CL, Kamano Y, Pettit GR, Blumberg PM (1988) Modeling of the bryostatins to the phorbol ester pharmacophore on protein kinase C. Proceedings of the National Academy of Sciences 85: 7197-7201

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: Master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741-751 doi 10.1038/nrmicro1969

Wilson ACC, Ashton PD, Calevro F, Charles H, Colella S, Febvay G, Jander G, Kushlan PF, Macdonald SJ, Schwartz JF, Thomas GH, Douglas AE (2010) Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. Insect Mol Biol 19: 249-258 doi 10.1111/j.1365-2583.2009.00942.x

Witczak ZJ, Rampulla RM, Jr., Bommareddy A (2012) Mycalamides, pederin and psymberin as natural carbohydrates and potential antitumor agents: Past and future perspectives. Mini-Rev Med Chem 12: 1520-1532

Wolf JB (2013) Principles of transcriptome analysis and gene expression quantification: an RNA-seq tutorial. Molecular ecology resources 13: 559-572

Wong YH, Arellano SM, Zhang HM, Ravasi T, Qian PY (2010) Dependency on *de novo* protein synthesis and proteomic changes during metamorphosis of the marine bryozoan *Bugula neritina*. Proteome Sci 8: 25

Woollacott R (1981) Association of bacteria with bryozoan larvae. Mar Biol 65: 155-158

Woollacott R, Zimmer R (1972) Origin and structure of the brood chamber in *Bugula neritina* (Bryozoa). Mar Biol 16: 165-170

Woollacott RM, Zimmer RL (1971) Attachment and metamorphosis of the cheilo-ctenostome bryozoan *Bugula neritina* (linné). J Morphol 134: 351-382

Woollacott RM, Zimmer RL (1975) A simplified placenta like system for the transport of extraembryonic nutrients during embryogenesis of *Bugula neritina* (bryozoa). J Morphol 147: 355-377

Wourms J (1987) Oogenesis. In: Giese A, Pearse, JS, Pearse, VB (ed) Reproduction of marine invertebrates - General aspects: Seeking unity in diversity. Boxwood Press, Pacific Grove, CA, pp 50-178

Yamashita M, Mita K, Yoshida N, Kondo T (2000) Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. In: Meijer L, Jezequel A, Ducommun B (eds) Progress in Cell Cycle Research. Kluwer Academic/ Plenum, New York, pp 115-129

Yang DC, Blair KM, Salama NR (2016) Staying in shape: the impact of cell shape on bacterial survival in diverse environments. Microbiol Mol Biol Rev 80: 187-203

Zchori-Fein E, Borad C, Harari AR (2006) Oogenesis in the date stone beetle, Coccotrypes dactyliperda, depends on symbiotic bacteria. Physiol Entomol 31: 164-169