The control of metamorphosis in the pollution-indicating polychaete *Capitella teleta*

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Robert Thomas Burns

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Advisor: Dr. Jan A. Pechenik

Abstract

The larvae of many marine invertebrates are planktonic and must disperse within the tides and currents of the ocean. The adults of these invertebrate species are often sessile and their planktonic larvae act as the dispersal mechanism for their species. Several biological aspects of the larvae themselves can influence potential dispersal distances and species distributions. One of the least understood aspects of larval biology that can influence potential dispersal is the time until the ability to metamorphose is gained. Another aspect of larval biology that can influence potential species distributions is the settlement cue, which stimulates larvae to settle to the benthic substrate and initiate metamorphosis. Settlement cues have not been characterized for most species.

Here, I used the salt-marsh deposit-feeding polychaete worm *Capitella teleta* as a model organism to further our knowledge about these aspects of larval biology. Larvae of *C. teleta* rapidly metamorphose in response to salt-marsh sediment; however, the chemical settlement cue in this sediment was unknown at the start of my studies. Because it would be advantageous for deposit-feeders to select substrates with adequate nutrients, I focused on determining whether vitamins could act as settlement cues. Indeed, we found that two B vitamins, nicotinamide and riboflavin rapidly stimulated larvae of *C. teleta* to metamorphose at low concentrations.

Because microbes living in salt-marsh sediments could produce these vitamins, we then isolated *Desulfovibrio oceani* from salt-marsh sediment. This bacterium rapidly stimulated larvae of *C. teleta* to metamorphose when grown as a biofilm. Furthermore, we found that menaquione-6, a form of vitamin K, was synthesized by *D. oceani* and rapidly stimulated larvae to metamorphose.

Finally, we studied the transcriptional changes that could underlie the onset of metamorphic competence. It appears that once larvae of *C. teleta* have completed development of internal structures required for juvenile life, competent larvae then up-regulate the expression of possible chemosensory proteins and neurotransmitter receptors to make the detection and transduction of a signal from external settlement cues possible.

Overall, larvae of *C. teleta* likely gain metamorphic competence once they increase expression of chemosensory receptors and other components of the signal transduction cascade leading to metamorphosis. They then could use this sensory system to assess the nutritional content of the environment around them. By metamorphosing in nutrient-rich sediments, larvae could ensure that they could successfully develop as juveniles and reach reproductive maturity.

Table of Contents

Abstract i

Table of Contents iii

Index of Tables iv

Index of Figures v

Chapter 1: General introduction 1

Chapter 2: The B vitamins nicotinamide (B_3) and riboflavin (B_2) stimulate metamorphosis in larvae of the deposit-feeding polychaete *Capitella teleta*: implications for a sensory ligand-gated ion channel 8

Chapter 3: The photodegradation of riboflavin stimulates larvae of the snail *Crepidula fornicata* and the polychaete *Capitella teleta* to metamorphose via generation of reactive oxygen species. 37

Chapter 4: Menaquinone-6 produced by *Desulfovibrio spp.* isolated from salt-marsh sediments stimulates larvae of the marine polychaete *Capitella teleta* to settle and metamorphose 59

Chapter 5: The bacterial community composition of salt-marsh sediments that rapidly stimulate larvae of the polychaete *Capitella teleta* to metamorphose 83

Chapter 6: The transcriptomic basis of metamorphic competence in the salt-marsh dwelling polychaete, *Capitella teleta*. 105

Chapter 7: Conclusions 126

Literature Cited 133

Index of Tables

Table 2.1. The lowest stimulatory concentrations of pyridines that induced larvae of *C. teleta* to metamorphose compared with the K_M of these same chemicals on the pyridine-activated ion channel of the crayfish *A. torrentium*……27

Table 3.1. Photodegradation of riboflavin caused larvae of *C. fornicata* to metamorphose…….53

Table 3.2. Photodegradation of riboflavin caused larvae of *C. teleta* to metamorphose. ……54

Table 3.3. Shell lengths of juvenile *C. fornicata* at metamorphosis, after stimulation with either riboflavin or excess KCl seawater……55

Table 4.1. Menaquinones (MK) and phylloquinone (PK) detected in mud flat sediment samples, biofilms, and TLC isolates……..79

Table 5.1. The 10 most abundant operational taxonomic units (OTUs) present within inductive salt-marsh sediment samples……..98

Table 6.1. Gene ontologies enriched in pre-competent larvae of *Capitella teleta*…..122

Table 6.2. Gene ontologies enriched in competent larvae of *C. teleta*……124

Index of Figures

Figure 2.1. Promotion of metamorphosis by nicotinamide (vitamin B_3) in larvae of *Capitella teleta*…….....…….....28

Figure 2.2. Promotion of metamorphosis by riboflavin (vitamin B_2) in larvae of *Capitella teleta*……...29

Figure 2.3. The effects of pyrazinecarboxamide and nicotinamide on metamorphosis of *C. teleta* when tested equal concentrations……30

Figure 2.4. The effect of calcium-free seawater on metamorphosis of *C. teleta* treated with nicotinamide or pyrazinecarboxamide……...31

Figure 2.5. The effect of 4-acetylpyridine on metamorphosis of *C. teleta* in the presence of nicotinamide or pyrazinecarboxamide……..33

Figure 2.6. The inhibitory dose response of 4-acetylpyridine on metamorphosis of *C. teleta* after 24 h…….…….…35

Figure 2.7. The effect of ketanserin on metamorphosis of *C. teleta* in the presence of nicotinamide or pyrazinecarboxamide…….…….36

Figure 3.1. Promotion of metamorphosis by riboflavin (vitamin B_2) in *Crepidula fornicata* larvae…….……56

Figure 3.2. The impact of salt-marsh sediment and an ethanol sediment extract on the metamorphosis of *C. fornicata*…….……57

Figure 3.3. The impact of hydrogen peroxide on the metamorphosis of *C. fornicata*. ……...….……..58

Figure 4.1. Promotion of metamorphosis by an acetone extract of Little Sippewissett salt marsh-sediment in larvae of *C. teleta*…….……..80

Figure 4.2. The absolute amounts of phylloquinone (PK) and menaquinones (MK) 4-13 contained in Little Sippewissett salt-marsh sediment……..……81

Figure 4.3. The chemical structures of menaquinone-6 (A) and methyl farnesoate (B)……....82

Figure 5.1. Sampling locations within the Little Sippewissett salt-marsh (Falmouth, MA)……...100

Figure 5.2. The mean percent organic content of sediment samples that did and did not induce larvae of *C. teleta* to metamorphose……..102

Figure 5.3. Rates of *C. teleta* metamorphosis in highly inductive salt-marsh sediments……..103

Figure 5.4. Conspicuous pink and white surface biofilms present on Little Sippewissett salt-marsh sediments……....104

Figure 6.1. Testing stage 8 and 9 larvae of *Capitella teleta* for metamorphic competence……...125

Chapter 1

General Introduction

As terrestrial animals ourselves, we are most aware of metamorphosis that takes place in terrestrial environments. Many people probably think of a caterpillar pupating and then eclosing as a butterfly when they think of metamorphosis, or tadpoles metamorphosing into frogs. However, most of the diversity of animals that must undergo metamorphosis is found in the world's oceans and estuaries. Many aspects of metamorphosis are different between terrestrial and marine animals.

In terrestrial environments, adult animals of species that metamorphose often act as the dispersal mechanism for their young. Adult lepidopterans (moths and butterflies) often exhibit remarkable choice when selecting a location to lay their fertilized eggs (Thompson & Pellmyr 1991). Often, these adult lepidopterans will lay their eggs on their own host plant species so that when their larval caterpillars hatch they can immediately begin feasting on the correct food source. It is amazing that while many of these lepidopteran species do not eat as adults, they still retain a preference for their larval food source (Thompson & Pellmyr 1991). Adults of many amphibian species will lay their eggs on plants or other surfaces that are above a pond that is a suitable habitat for their larvae to develop in (Howard 1978, Seale 1982, Warkentin 2011). After hatching, these amphibian larvae will be deposited directly into their appropriate habitat. Metamorphosis of insects and amphibians can take weeks or months to complete.

Marine invertebrates such as barnacles, bivalves, snails, urchins, sea stars, tube dwelling polychaete worms, bryozoans, and many others are either completely sessile or have low movement potential as adults. Adults of these species could not place their young in an optimal habitat to develop in even if they wanted to. Here the larvae actually act as the dispersal agents for the species. These marine invertebrates produce many

microscopic larvae that are powerless to swim against the tides and currents of the ocean and are dispersed by these physical forces. In many species, larvae do not gain the ability to metamorphose for hours or weeks after they hatch or are released from brooding structures (Pechenik 1990, Hadfield et al. 2001). This time to metamorphic competence also determines the potential distance that larvae disperse. If the time to metamorphic competence is short, the potential dispersal distance could be low; likewise, if the time to metamorphic competence is long, the potential distance of dispersal could be much higher. While these larvae are progressing towards competence in the water column they are also developing juvenile structures and organs that are essential for life after metamorphosis. By the time larvae reach competence they are essentially fully formed juveniles waiting to shed larval structures (Hadfield et al. 2001). The genetic and physiological processes underlying the onset of metamorphic competence are almost completely unknown – a process that determines the potential dispersal distances of species across the Metazoa.

As the larvae of marine invertebrates are dispersing and gain the ability to metamorphose (metamorphic competence) it is the larva's responsibility to find a suitable environment for metamorphosis that will also be suitable for juvenile development and adult persistence. Larvae settle out of the water column down to the benthos and initiate metamorphosis in response to a settlement cue, a stimulus from the environment indicating that a suitable habitat is nearby (Pawlik 1992, Hadfield & Paul 2001, Heyland et al. 2011). The cues that stimulate settlement and metamorphosis are completely unknown for the vast majority of marine invertebrates – another factor that determines the potential distributions of many species across the Metazoa. The settlement cues that

have been found to stimulate various species to settle and metamorphose have previously been reviewed by others (Pawlik 1992, Hadfield & Paul 2001, Bishop et al. 2006). Settlement cues can indicate the presence of food sources. Larvae of the urchin *Holopneustes purpurascens* will settle and metamorphose in response to low concentrations of histamine, a chemical that leaches away from their red alga food source *Delisea pulchra* (Swanson et al. 2004, 2006, 2012). Larvae of the nudibranch *Phestilla sibogae* settle and metamorphose in response to an uncharacterized chemical present in their food source, the coral *Porites compressa* (Hadfield et al. 2000). Settlement cues for gregarious settlement have also been characterized. These cues for gregarious settlement are especially advantageous for sessile organisms that must mate with other nearby members of their species. Larvae of the tube-dwelling polychaete worm *Phragmatopoma californica* settle and metamorphose in response to specific fatty acids that leach away from conspecific tube-dwelling adults (Pawlik & Faulkner 1986). Larvae of the slipper-snail *Crepidula fornicata* settle and metamorphose when treated with seawater conditioned by conspecific adults (Pechenik & Gee 1993). In their natural habitat, these larvae metamorphose in close proximity to conspecific adults and the juveniles join a mating stack where they will remain, possibly for life (Coe 1938, Pechenik & Gee 1993). Unlike terrestrial animals, settlement and metamorphosis of marine invertebrates is often completed quickly: in many cases it takes only minutes or hours (Hadfield 2000).

While the settlement cues have been characterized for some species, less is known about the mechanisms by which these cues stimulate the larvae to settle and initiate metamorphosis. Because most characterized settlement cues are chemicals, research has focused on G-protein coupled receptors (GPCRs) as these proteins commonly play roles

in chemosensation in both animals and plants. Experiments using GPCR agonists and antagonists have found that some hydrozoans, barnacles, and mollusks may be using GPCRs to sense chemical settlement cues (Heyland et al. 2011). The same type of experiments have shown that many other marine invertebrates may not be using GPCRs to sense settlement cues which is surprising given their ubiquity in the process of chemosensation (Heyland et al. 2011). Much more research must be done to determine what other types of receptors are responsible for the reception of chemical settlement cues.

After a settlement cue is perceived, what is the signal transduction cascade that leads to settlement and the initiation of metamorphosis? This signal transduction pathway is not completely understood for any marine invertebrate. However, surprising commonalities have been found in the regulation of metamorphosis across phyla. Endogenously produced nitric oxide has been shown to inhibit metamorphosis in the larvae of some ascidians, annelids, mollusks, and echinoderms (Biggers et al. 2012, Pechenik et al. 2007). Neurotransmitters such as serotonin, dopamine, GABA, and glutamine have also been shown to regulate metamorphosis (reviewed in Heyland et al. 2011). After a settlement cue is received, the rate of endogenous nitric oxide production could slow or stop; following this decrease in nitric oxide concentration, neurotransmitters could then further transduce the signal until settlement and metamorphosis are initiated (Biggers et al. 2012).

Overall, I have worked to fill in these gaps in our knowledge about the control of settlement and metamorphosis in marine invertebrates. I have worked to answer the following questions:

1. What are the chemical settlement cues that larvae could be responding to in nature?
2. What receptors are larvae using to perceive these settlement cues?
3. How is metamorphic competence regulated?

To answer these questions, I have used the polychaete annelid *Capitella teleta* (previously *Capitella* sp. I) as a model organism. The deposit-feeding juveniles and adults of *C. teleta* live in the salt-marsh and soft marine sediments that they eat (Grassle & Grassle 1976, Dubilier 1988, Blake et al. 2009). They are very easy to rear in the laboratory because they only require salt-marsh sediment and seawater to thrive. They also have a very short generation time of 4 – 6 weeks (Blake et al. 2009). An adult can produce from 50 – 200 larvae per brood (Blake et al. 2009). Their trochophore larvae do not eat and are often immediately competent to metamorphose once released from the mother's brooding structure -- the brood tube (Grassle & Grassle 1976, Dubilier 1988). It is also very easy to distinguish metamorphically competent larvae from pre-competent larvae, something that is often difficult or impossible to visually assess in larvae of other marine invertebrates. Competent larvae of *C. teleta* have a spiral gut morphology while pre-competent larvae have a straight gut morphology (Seaver et al. 2005). Because *C. teleta* has become a model organism for developmental biology studies, it has a sequenced and annotated genome as well (Simakov et al. 2014).

At the beginning of my studies we knew that the larvae of *C. teleta* rapidly settle and metamorphose in response to salt-marsh sediment; usually 90% of the larvae will metamorphose within the first 30 minutes of exposure (Dubilier 1988). If this sediment was combusted to remove all of the organic matter, the resulting ash did not stimulate any

larvae to metamorphose leading us to believe that the settlement cue contained within the salt-marsh sediment is some organic molecule (Cohen & Pechenik 1999). Also, when the sediment was passed through a 0.22 μm filter, the resulting filtrate did not stimulate any larvae to settle; the cue could be bound to sediment particles larger than 0.22 μm (Cohen & Pechenik 1999). From here I set out to determine what the actual chemical settlement cue(s) contained in this inductive salt-marsh sediment were, how larvae are sensing these chemicals, and how metamorphic competence is attained.

Chapter 2

The B vitamins nicotinamide (B_3) and riboflavin (B_2) stimulate metamorphosis in larvae of the deposit-feeding polychaete *Capitella teleta*: implications for a sensory ligand-gated ion channel

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Abstract

Marine sediments can contain B vitamins, presumably incorporated from settled, decaying phytoplankton and microorganisms associated with decomposition. Because B vitamins may be advantageous for the energetically intensive processes of metamorphosis, post-metamorphic growth, and reproduction, we tested several B vitamins to determine if they would stimulate larvae of the deposit-feeding polychaete *Capitella teleta* to settle and metamorphose. Nicotinamide and riboflavin individually stimulated larvae of *C. teleta* to settle and metamorphose, generally within 1 - 2 hours at nicotinamide concentrations as low as 3 μM and riboflavin concentrations as low as 50 μM. More than 80% of the larvae metamorphosed within 30 minutes at a nicotinamide concentration of 7 μM. The pyridine channel agonist pyrazinecarboxamide also stimulated metamorphosis at very low concentrations. In contrast, neither lumichrome, thiamine HCl, pyridoxine HCl, nor vitamin B_{12} stimulated larvae of *C. teleta* to metamorphose at concentrations as high as 500 μM. Larvae also did not metamorphose in response to either nicotinamide or pyrazinecarboxamide in calcium-free seawater or with the addition of 4-acetylpyridine, a competitive inhibitor of the pyridine receptor. Together, these results suggest that larvae of *C. teleta* are responding to nicotinamide and riboflavin via a chemosensory pyridine receptor similar to that previously reported to be present on crayfish chela and involved with food recognition. Our data are the first to implicate B vitamins as possible natural chemical settlement cues for marine invertebrate larvae.

Introduction

The larvae of many benthic marine invertebrates are planktonic and are thus 'forced to wander' the sea until they become competent to metamorphose and locate a site suitable for settlement (Pechenik 1990). Particular chemical cues then promote larval settlement and subsequent metamorphosis by competent larvae of many species (Pawlik 1992, Rittschof et al. 1998, Hadfield & Paul 2001). These chemical cues may indicate the presence of appropriate food, conspecific adults to mate with, or other environmental factors that signal the suitability of a site to live in for juveniles and adults. For example, larvae of the sea urchin *Holopneustes purpurascens* settle and metamorphose in response to histamine, which leaches away from *Delisea pulchra*, their algal food source (Swanson et al. 2004, 2006, 2012). Similarly, larvae of the tube-building polychaete worm *Phragmatopoma californica* settle and metamorphose in response to several different fatty acids that are produced by conspecific tube-dwelling adults, signaling the presence of potential mates (Pawlik & Faulkner 1986). Negative recruitment cues that prevent larvae from metamorphosing in specific areas have also been identified and can also be important in determining species distributions (Woodin 1991). However, for the vast majority of marine invertebrates, the specific chemical settlement cues remain undefined.

Capitella teleta, previously known as *Capitella* sp. I, is a small (~ 20 mm long x 1 mm wide), opportunistic, deposit-feeding marine polychaete found in salt-marsh sediments and in disturbed and polluted areas such as busy harbors, sewage outflows, and some regions affected by oil spills (Grassle & Grassle 1976, Blake et al. 2009). Its planktonic, non-feeding, metatrochophore larvae will typically metamorphose in the presence of salt-marsh sediments, making it a convenient model for studies of substrate

selection (Butman et al. 1988, Dubilier 1988, Pechenik & Cerulli 1991). Larvae of *C. teleta* metamorphose rapidly when in the presence of salt-marsh sediment; in one study 90% of the tested larvae metamorphosed within 30 minutes of treatment (Dubilier 1988). The active component of the natural settlement cue contained within the salt-marsh sediment is currently unknown; however, larvae of *C. teleta* have been shown to settle and metamorphose preferentially in response to sediments with high organic content, and those with a low carbohydrate to protein ratio (Cohen & Pechenik 1999, Thiyagarajan et al. 2005). Sediments forced through a 0.45 µm filter can still stimulate larvae to metamorphose; however, filtering the sediment to 0.22 µm removed the cue, suggesting that the cue is bound to small particulates (Cohen & Pechenik 1999). Also, combusting the sediment at 500 ºC for 6 h removed the cue from the sediment: the resulting ash did not stimulate metamorphosis, supporting the notion that the cue is organic (Cohen & Pechenik 1999).

Juvenile hormones and juvenile hormone-active chemicals are known to induce settlement and metamorphosis of *C. teleta* larvae (Biggers & Laufer 1992, 1999). Extracts prepared from marine sediments displayed juvenile hormone-activity in insect bioassays, suggesting that the chemicals in the sediments that induced settlement and metamorphosis may be similar in structure to juvenile hormones (JHs) or have juvenile hormone-activity. In addition, the induction of settlement and metamorphosis by JH was found to involve activation of protein kinase C and further activation of calcium channels (Biggers & Laufer 1999). Exposing *C. teleta* larvae to 400 nM calcium ionophore A23187, a membrane soluble chemical that shuttles calcium ions into cells, induced all of

the larvae to metamorphose in less than one hour. Whether or not the larvae could metamorphose in calcium-free seawater however was not explored.

As has been found for some other invertebrate larvae, larvae of *C. teleta* settle and metamorphose in response to serotonin and the serotonin-reuptake inhibitor fluoxetine (Ricker et al. 2011, Biggers et al. 2012), indicating that stimulation of the larval nervous system is involved in mediating the chemosensory response to chemical cues. Furthermore, different inhibitors of nitric oxide synthase such as S-methylisothiourea and aminoguanidine hemisulfate were also found to induce larval settlement and metamorphosis (Biggers et al. 2012), adding *C. teleta* to a growing list of marine invertebrates, including some gastropods (Leise et al. 2001, Pechenik et al. 2007, Bishop et al. 2008), echinoderms (Bishop & Brandhorst 2001), and ascidians (Bishop et al. 2001, Comes et al. 2007) whose metamorphosis is inhibited by the presence of endogenous nitric oxide. While we currently know much about the intermediate steps of the signal transduction cascade leading to metamorphosis of *C. teleta*, we do not know which chemicals are acting as natural settlement cues for larvae of *C. teleta* or how these chemicals actually initiate this signal transduction cascade.

Larval metamorphosis, juvenile growth, and adult reproduction are all energy-requiring processes; not surprisingly the growth and reproduction of *Capitella* are affected by diet (Gremare et al. 1988). Plant nutrients have also been shown to be quickly assimilated into the growing oocytes of *Capitella* (Marsh et al. 1990) and to supply adequate nutrition for larval development and subsequent metamorphosis. Dietary B vitamins are essential co-factors for biochemical energy production, and are known to be essential for the growth and development of some annelids (Gotthold & Koch 1974). In

this respect it should be beneficial for larvae of *C. teleta* to settle and metamorphose in locations with adequate levels of these vitamins to support post-metamorphic growth. Because B vitamins are likely to be found within the marine sediments that the deposit-feeding adults of *C. teleta* consume and are required for growth and survival, we suspected that the larvae would respond to at least some B vitamins as a natural settlement cue. To test our hypothesis, we added several B vitamins individually to seawater and monitored the metamorphosis of newly-released larvae of *C. teleta*. In addition we conducted a series of experiments using various pharmacological agents to determine how these vitamins were stimulating metamorphosis.

Results

Effects of different B vitamins

Of the 7 B vitamins tested, only riboflavin (B_2), nicotinamide (B_3), and nicotinic acid (another form of B_3) stimulated metamorphosis in *C. teleta*. Thiamine HCl (B_1), pyridoxine HCl (B_6), biotin (B_7), and cobalamin (B_{12}) did not stimulate any metamorphosis within 24 h at the tested concentration of 500 μM (data not shown). In contrast, nicotinamide stimulated metamorphosis in a nearly dose-dependent manner at concentrations as low as 3 – 8 μM, with larvae initiating metamorphosis within 30 min (Figure 1). Nicotinic acid stimulated 70.8% +/- 8.3 SEM of the treated larvae to metamorphose within 24 h. Also, riboflavin concentrations of 50 - 200 μM stimulated nearly all larvae of *C. teleta* to metamorphose within 5 hours (Figure 2). However, the larvae did not respond to the riboflavin breakdown product lumichrome even when tested for 24 h at the maximum concentration of 1 mM (data not shown).

Effects of pyridine channel activators and inhibitors

Because concentrations of nicotinamide as low as 3 – 6 μM stimulated larvae of *C. teleta* to metamorphose quickly in our experiments, we hypothesized that larvae of *C. teleta* were sensing nicotinamide via a pyridine activated ion channel, something first characterized in the walking legs of the crayfish *Austrapotamobius torrentium* (Hatt & Schmiedel-Jakob 1984, 1985, Hatt & Franke 1987). We therefore compared dose responses between nicotinamide and pyrazinecarboxamide, an agonist of the pyridine-activated ion channel, at 1 μM, 4 μM, and 8 μM. Another commonly occurring nutritive chemical, beta nicotinamide adenine dinucleotide (β-NAD), also stimulated the nicotinamide-activated ion-channel to open, with half-maximal rate of opening (K_M) at 1 mM (Hatt & Schmiedel-Jakob 1984). We therefore treated larvae of *C. teleta* with β-NAD at the concentrations of 0.5, 1, and 5 mM to determine if β-NAD also stimulated metamorphosis. As shown in Figure 3, the pyridine-activated ion channel agonist pyrazinecarboxamide was found to also stimulate larvae of *C. teleta* to metamorphose, and did so at similar concentrations as nicotinamide. Although both chemicals stimulated larvae of *C. teleta* to metamorphose in a dose-dependent fashion, pyrazinecarboxamide stimulated more individuals (about 70%) to metamorphose at 4 μM than did nicotinamide at the same concentration (Figure 3). β-NAD, however, did not stimulate any metamorphosis at 0.5, 1, or 5 mM.

Effects of calcium free seawater

To determine if an influx of extracellular calcium is required for metamorphosis to proceed when pyridine-activated ion channels open, larvae of *C. teleta* were treated with nicotinamide or pyrazinecarboxamide while bathed in either normal ASW(control) or calcium-free ASW. Pre-treating the larvae in calcium-free ASW inhibited the

metamorphic response of *C. teleta* larvae to both nicotinamide and pyrazinecarboxamide even at the high concentration of 40 μM (one-way ANOVA with Bonferroni post-hoc comparisons, $F(5,12)=234.4$, $p < 0.001$) (Figure 4). Larvae in calcium-free ASW settled to the bottom of the dish within a few minutes after the addition of nicotinamide or pyrazinecarboxamide; they then remained stationary, but did not metamorphose over the 24 h observation period. Control larvae in calcium-free ASW continued to swim normally and neither settled nor metamorphosed. At least 90% of larvae exposed to nicotinamide or pyrazinecarboxamide in ASW metamorphosed, indicating that most larvae were competent to metamorphose at the start of this experiment.

Effects of 4-acetylpyridine

4-acetylpyridine, a specific competitive antagonist of the pyridine-activated ion channel, was also tested on larvae of *C. teleta* in the presence of either nicotinamide or pyrazinecarboxamide (Hatt & Schmiedel-Jakob 1985). Treating larvae with 200 μM 4-acetylpyridine inhibited them from metamorphosing in the presence of either 40 μM nicotinamide or 40 μM pyrazinecarboxamide (one-way ANOVA with Bonferroni post-hoc comparisons, $F(5,12)=69.52$, $p < 0.001$) (Figure 5). Only about half as many larvae metamorphosed within 24 h when exposed to both 4-acetylpyridine and either nicotinamide or pyrazinecarboxamide, indicating that the inhibitory concentration allowing for 50% settlement and metamorphosis (IC_{50}) is about 200 μM. No larvae exposed to both 4-acetylpyridine and either nicotinamide or pyrazinecarboxamide metamorphosed within the first 5 h of the experiment (data not shown) and no control larvae exposed to 200 μM 4-acetylpyridine alone settled or metamorphosed within 24 h. Again, the high response of larvae in the positive controls shows that most of the larvae

were metamorphically competent when tested. Also, larvae were inhibited from metamorphosing in response to pyrazinecarboxamide in a dose-dependent fashion when tested with increasing doses of 4-acetylpyridine (one-way ANOVA with Bonferroni post-hoc comparisons, $F(7,16)=53.92$, $p < 0.001$) (Figure 6).

Effects of ketanserin

Sensory information relayed by sensory chemoreceptors such as olfactory receptor neurons usually involves sensory transduction through synapses within the central nervous system. In order to assess the involvement of the serotonergic nervous system in mediating the response of the larvae to nicotinamide and pyrazinecarboxamide, the larvae were first pre-exposed to ketanserin, an inhibitor of serotonin 5-HT_{2B} receptors, before induction by nicotinamide and pyrazinecarboxamide. Previous results have demonstrated that ketanserin blocks the response of *Capitella* larvae to natural mud sediment chemical cues, and also to the nitric oxide synthase inhibitors N-methyl-L-arginine and S-methylisothiourea (Ricker et al. 2011, Biggers et al. 2012), indicating the involvement of the serotonergic nervous system in mediating the settlement and metamorphosis of *C. teleta*. Pre-exposing *C. teleta* larvae to ketanserin inhibited most of the larvae of *C. teleta* from metamorphosing in response to both nicotinamide and pyrazinecarboxamide (one-way ANOVA with Bonferroni post-hoc comparisons, $F(5,12)=122.5$, $p < 0.001$) (Figure 7). No control larvae exposed to 2 μM ketanserin alone settled or metamorphosed within 24 h; however, all of the larvae exposed to the positive controls of nicotinamide and pyrazinecarboxamide alone metamorphosed in less than an hour, indicating that most larvae were competent when tested.

Discussion

Overall, these data support our hypothesis that at least some B vitamins may act as chemical cues for habitat selection by the larvae of *C. teleta* and may stimulate the larvae of this species to metamorphose in the field. In particular, nicotinamide (B_3), nicotinic acid (B_3), and riboflavin (B_2) stimulated larvae of *C. teleta* to metamorphose, whereas thiamine HCl, pyridoxine HCl, biotin, vitamin B_{12}, and the riboflavin breakdown product lumichrome did not. It may be advantageous for marine larvae to settle and metamorphose in response to environmental riboflavin and nicotinamide, as riboflavin is required for the synthesis of FAD, and nicotinamide serves as a building block for the synthesis of NAD; both products are important electron carriers for the electron transport chain, ATP production, and other aspects of cellular metabolism. Substantial concentrations of these vitamins may serve as an adaptive signal that the local environment is suitable for the energetically intensive processes of juvenile development and adult reproduction. In this regard, B vitamins present in ocean waters have been demonstrated to be important for the growth of phytoplankton, zooplankton, and larger marine invertebrates (Panzeca et al. 2009, Sañudo-Wilhelmy et al. 2012, Giovannoni 2012). However, even if B vitamins are contained within marine sediments, they may not always be bioavailable to deposit feeders.

Although the stimulation of settlement and metamorphosis by vitamins seems not to have been investigated in other marine invertebrate species, the larvae of several other marine invertebrates have previously been demonstrated to settle and metamorphose in response to certain vitamin derivatives (Hadfield & Paul 2001). For example, larvae of the ascidian *Halocynthia roretzi* will settle and metamorphose in response to lumichrome, a photocatalytic breakdown product of riboflavin, although they did not respond to

riboflavin itself (Tsukamoto et al. 1999). This inducer was found to be naturally biosynthesized by the larvae and adults of *Halocynthia.* Lumichrome, however, did not induce settlement and metamorphosis in other ascidian species, and also did not stimulate metamorphosis in our study, although the larvae of *C. teleta* did respond to riboflavin. In other studies, larvae of the hydroid *Coryne uchidae* were found to settle and metamorphose in response to delta-tocopherol epoxides, compounds related in structure to the alpha-tocopherol (vitamin E) produced by the brown algae *Sargassum tortile* (Kato et al. 1975).

The vitamin requirements for the growth, reproduction, and development of *C. teleta* have so far been unexplored, to our knowledge; however, as previously mentioned, we know that growth, reproduction, and development of *C. teleta* depend strongly on diet and organic content of sediments (Gremare et al. 1988, Tsutsumi et al. 1990), and that nutrients such as algal pigments, including carotenoids, are rapidly assimilated into developing oocytes (Marsh et al. 1990). As previously mentioned, larval settlement of *C. teleta* depends on the organic content of sediments, with larvae preferring sediments with a low carbohydrate to protein ratio (Cohen & Pechenik 1999, Thiyagarajan et al. 2005). Very little is known about marine annelid nutrition and vitamin requirements; however, an absolute requirement for riboflavin and nicotinamide, along with 6 other vitamins, was demonstrated using artificial culture media for the oligochaete annelid *Enchytraeus fragmentosus* (Gotthold & Koch 1974).

We do not currently know the concentrations of riboflavin and nicotinamide present naturally in the environments that larvae of *C. teleta* settle and metamorphose in. It is likely, however, that recently deposited sediments, particularly those near sewage

outflows, contain high levels of these compounds, sufficient to induce metamorphosis. Many phytoplankton species are known to contain large concentrations of B vitamins within their cells (Brown et al. 1999). Several marine microorganisms, such as the bacteria *Shewanella spp.* (Marsili et al. 2008) and *Micrococcus luteus* (Sims & O'Loughlin 1992), and the yeast *Candida* (Mitra et al. 2012), are also known to produce and release large amounts of riboflavin particularly when grown as biofilms. Remnants of settled, decomposing phytoplankton and the other microbes taking part in decomposition processes are likely to be a large component of marine sediments, which should therefore contain high concentrations of B vitamins. Indeed, biotin (B_7), thiamine (B_1), and vitamin B_{12} have been all identified in Pacific marine sediments (Ohwada & Taga 1972). Additional studies will be required to determine if riboflavin and/or nicotinamide are present in salt-marsh sediments and sediments near sewage outfalls at the low concentrations that we have found to be inductive.

Although the biochemical and sensory mechanisms through which the larvae of *C. teleta* respond to nicotinamide and riboflavin still need more investigation, our results suggest that larvae may be sensing nicotinamide with chemosensory pyridine receptors similar to those previously found in chemosensory sensilla on the walking legs of crayfish, and which are involved in the foraging behavior of those animals (Hatt & Schmiedel-Jakob 1984, 1985, Hatt & Franke 1987). The inhibitory effects of 4-acetylpyridine on chemosensation of nicotinamide support this suggestion, as do the stimulatory effects of pyrazinecarboxamide on the larvae of *C. teleta*, a powerful agonist of these pyridine-activated ion channels (Hatt & Schmiedel-Jakob 1985). In crayfish, pyrazinecarboxamide was found to be the most effective inducer, followed by

nicotinamide; we have found that this is also the case for larvae of *C. teleta* (Table 1) (Hatt & Schmiedel-Jakob 1985). Also, the IC_{50} for 4-acetylpyridine was found to be approximately 200 μM for *C. teleta* compared with a K_I of 70 μM for the crayfish *A. torrentium* (Hatt & Schmiedel-Jakob 1985). This difference in effective concentrations for 4-ACP may at least be partly due to the fact that Hatt et al. (Hatt & Schmiedel-Jakob 1985) explored the effects of 4-ACP using isolated chemosensory neurons and electrophysiological techniques, whereas the effects on *C. teleta* were explored in *in vivo* studies in which the chemical had to also penetrate the larvae. Interestingly, larvae did not metamorphose when exposed to β-NAD at concentrations as high as 5 mM. While nicotinamide, pyrazinecarboxamide, and nicotinic acid stimulated larvae to metamorphose at concentrations similar to those that stimulated action potentials in the crayfish, it may be possible that other pyridines such as β-NAD do not bind efficiently to this receptor at similar concentrations for both species. It would be interesting in this regard to also try a patch-clamp technique on the *C. teleta* larvae to demonstrate the presence of pyridine receptors.

Each year, more ligand-gated ion channels have been characterized as having roles in animal's taste reception (Pellegrino & Nakagawa 2009), although the presence of pyridine receptors used for chemodetection in other animals besides crustaceans to our knowledge has not been reported. Signal transduction carried out by these ion channels may be calcium dependent (Meunier et al. 2009). Our results indicating that calcium-free seawater inhibited metamorphosis in response to nicotinamide also therefore agree with these data. Although larvae of *C. teleta* did not metamorphose in the presence of nicotinamide or pyrazinecarboxamide in calcium-free seawater in our study, they did

settle to the bottom in those solutions within several minutes. Most likely, the nicotinamide or pyrazinecarboxamide ligand is binding to the channel and opening it; however, without calcium ions entering through the channel, the signal transduction pathway leading to metamorphosis is probably stalled shortly after initiation. These results parallel the results of Biggers and Laufer (Biggers & Laufer 1999), showing that settlement and metamorphosis of *C. teleta* can be induced by very low concentrations of the calcium ionophore A23187.

Our results using the 5-HT_{2B} receptor inhibitor ketanserin suggest that the response to nicotinamide and riboflavin requires participation of the serotonergic nervous system as well, since ketanserin inhibited this response. These data suggest that the larvae detect nicotinamide and riboflavin in the environment via chemosensory neurons likely present in the chemosensory cilia noted by Eckelbarger and Grassle (1987). Sensory depolarization of these receptors may lead to the release of serotonin either directly by the chemosensory neurons or indirectly through synaptic activation of other serotonergic neurons. Nitric oxide may play a role as a secondary messenger between the influx of ions caused by the opening of nicotinamide activated ion channels and serotonergic signaling. Larvae of *C. teleta* did not metamorphose in the presence NOS inhibitors when treated with ketanserin, a serotonin 5-HT_{2B} receptor antagonist (Biggers et al. 2012). Serotonin binding to receptors then most likely leads to settlement and metamorphosis. In this regard, the serotonin 5-HT_{2B} receptor is known to be a metabotropic receptor involving G-protein activation, secondary messengers, calcium channel activation, and downstream kinase activation such as through mitogen activated protein kinases (MAPK) involved in mediating gene expression (Launay et al. 1996).

Other chemosensory structures as reviewed by Lindsay (2009) may be also be involved in the detection of nicotinamide and riboflavin by these larvae.

Larvae of *C. teleta* may be foraging for an environment containing nutrient-rich sediment prior to metamorphosing and thereby may be exhibiting foraging behavior in choosing habitats for settlement and metamorphosis. By responding to the B vitamins nicotinamide and riboflavin as chemical settlement cues, they could be ensuring that a selected environment contains the proper vitamins to successfully grow and reproduce. As deposit feeders, *C. teleta* can utilize these vitamins only if they are contained within marine sediments. Here, larvae of *C. teleta* seem to be mirroring how adult crustaceans such as crayfish and lobsters sense nutrients within sediments via chemosensory hairs attached to their legs or antennules. The crayfish *A. torrentium* can sense nicotinamide and other pyridines while the spiny lobster *Panulirus argus* can sense ATP using their antennules (Carr et al. 1987). Interestingly, the crayfish *Astacus astacus* has been demonstrated to have specific internal oesophageal receptors for nicotinamide (Altner et al. 1986), and the external receptors on the walking legs and antennules of crustaceans may represent primitive fore-runners of internal chemoreceptors such as the oesophageal receptors (Carr et al. 1987). To our knowledge, the present paper is the first demonstration of the presence of nicotinamide or riboflavin receptors in an annelid or in invertebrate larvae.

Materials and Methods

Care and maintenance of larvae

Adults of *Capitella teleta* were provided by Dr. Judith Grassle (Rutgers University) and maintained in the lab since 2012 in 9 cm glass dishes containing 30 psu

Instant Ocean artificial seawater (hereafter called ASW) at 18°C. Sediment collected from the Little Sippewissett salt-marsh (Falmouth, MA) was sieved through a 1mm wire mesh screen, frozen for at least 24 h, aerated, and provided as food *ad libitum* (Dubilier 1988, Pechenik & Cerulli 1991). Cultures were searched for brooding females every 2-3 days; brooding individuals were then transferred individually to 6 cm glass dishes containing ASW. Dishes containing brooding females were checked daily for swimming larvae; larvae were then pipetted into a separate, clean 6 cm glass dish containing ASW. All experiments used larvae released only within the previous 24 hours. *C. teleta* larvae are usually competent to settle and metamorphose within minutes after their escape from the brood tube (Grassle & Grassle 1976, Pechenik & Cerulli 1991), so that all larvae should have been competent at the start of our experiments.

General bioassay protocol

All experiments were conducted using either 12-well tissue-culture plates or 3 cm glass dishes, with each well or bowl containing 4 mL of a vitamin solution in ASW, or a negative control (ASW). Each experiment included 3 replicates per treatment, with 8 larvae per replicate. To avoid diluting the final treatment solution, larvae were first pipetted into an intermediate bath of treatment solution (~20mL) before being pipetted into the final treatment vessel. Dishes were examined every 30 or 60 minutes under 20-50x magnification to count the number of newly metamorphosed juveniles. Larvae of *C. teleta* were considered to have metamorphosed when they elongated, lost their prototroch and telotroch cilia, and began crawling on the bottom of the vessel. All experiments were conducted at room temperature (20-23°C) under laboratory fluorescent lighting.

Testing the effects of B vitamins and other chemicals on larval metamorphosis

Thiamine HCl (B_1), riboflavin (B_2), nicotinamide (B_3), nicotinic acid (another form of B_3), pyridoxine HCl (B_6), biotin (B_7) and cobalamin (B_{12}) were obtained from Sigma Chemical Co. Thiamine HCl, nicotinic acid, pyridoxine HCl, biotin, and cobalamin were all tested on larvae of *C. teleta* at 500 μM. Nicotinic acid was also tested at 1 mM. Riboflavin was tested on larvae of *C. teleta* at 10, 50, 100, 150, and 200 μM. Nicotinamide was tested on larvae of *C. teleta* at 2, 3, 4, 5, 6, 7, and 8 μM. The concentrations tested for nicotinamide and riboflavin were based on preliminary pilot studies. The photo-degradation product of riboflavin, lumichrome, was obtained from Sigma Chemical and tested at concentrations of 100, 500, and 1000 μM. The pyridine receptor antagonists and agonists 4-acetylpyridine, pyrazinecarboxamide, and β-NAD were all purchased from Sigma Chemical Co. and tested in final concentrations as stated in results. The serotonin receptor antagonist ketanserin was purchased from Sigma Chemical Co. and prepared as a 10 mM stock solution in ethanol.

Testing the effects of 4-acetylpyridine, calcium-free ASW, and ketanserin on metamorphosis

Larvae were acclimated to ASW containing 200 μM 4-acetylpyridine for one hour and then transferred to a treatment containing either 200 μM 4-acetylpyridine + 40 μM nicotinamide, 200 μM 4-acetylpyridine + 40 μM pyrazinecarboxamide, or 200 μM 4-acetylpyridine as a negative control. Larvae that were not acclimated to 4-acetylpyradine were transferred to positive controls containing either 40 μM nicotinamide or 40 μM pyrazinecarboxamide.

Calcium-free ASW was prepared by omitting calcium chloride from the recipe given in (Baloun & Morse 1984). Larvae that were in calcium-free ASW treatments

were acclimated to calcium-free ASW for one hour and then were transferred to treatments containing either calcium-free ASW + 40 μM nicotinamide, calcium-free ASW + 40 μM pyrazinecarboxamide, or calcium-free ASW only (negative control). Larvae that were not acclimated to calcium-free ASW were transferred to positive controls containing either 40 μM nicotinamide or 40 μM pyrazinecarboxamide to assess metamorphic competence.

Larvae were acclimated to ASW containing 2 μM ketanserin for three hours and then transferred to a treatment containing either 2 μM ketanserin + 40 μM nicotinamide, 2 μM ketanserin + 40 μM pyrazinecarboxamide, or 2 μM ketanserin as a negative control. Larvae that were not acclimated to ketanserin were transferred to positive controls containing either 40 μM nicotinamide or 40 μM pyrazinecarboxamide.

Data analysis

In order to determine if calcium-free seawater, 4-acetylpyridine, or ketanserin influenced the number of *C. teleta* larvae that metamorphosed in the presence of nicotinamide or pyrazinecarboxamide, a one-way analysis of variance was conducted on the numbers of *C. teleta* that had metamorphosed by 24 h after treatment. If the results of an ANOVA were significant, Bonferroni's post-hoc test was conducted to determine which treatments were significantly different from each other. For each ANOVA, each replicate was the arcsine transformed ratio of individuals that had metamorphosed at that timepoint. It is important to note that, although the figures represented in this paper reflect the percent of *C. teleta* larvae that had metamorphosed, all statistical analyses were conducted with arcsine transformed ratio of the larvae that had metamorphosed. All statistical analyses were carried out with GraphPad Prism version 5.0.

Acknowledgements

We thank Dr. Judith Grassle (Rutgers University) for sending us a population of *C. teleta* to begin our cultures here at Tufts University. The Wilkes University Mentoring Task Force supplied funds to support this research. Wilkes University and Tufts FRAC funded the publication of this manuscript.

Tables

Table 2.1. The lowest stimulatory concentrations of pyridines that induced larvae of *C. teleta* to metamorphose compared with the K_M of these same chemicals on the pyridine-activated ion channel of the crayfish *A. torrentium.* The concentration of 4-acetylpyridine that inhibited 50% of the larvae of *C. teleta* is also compared with the IC_{50} of 4-acetylpyridine for the crayfish (adapted from Hatt and Schmiedel-Jacob, 1984).

	Species	
Chemical	*C. teleta*	*A. torrentium*
Pyrazinecarboxamide	1 μM	1.5 μM
Nicotinamide	3 μM	10 μM
Nicotinic acid	1 mM	> 1 mM
4-acetylpyridine	200 μM	70 μM

Figures

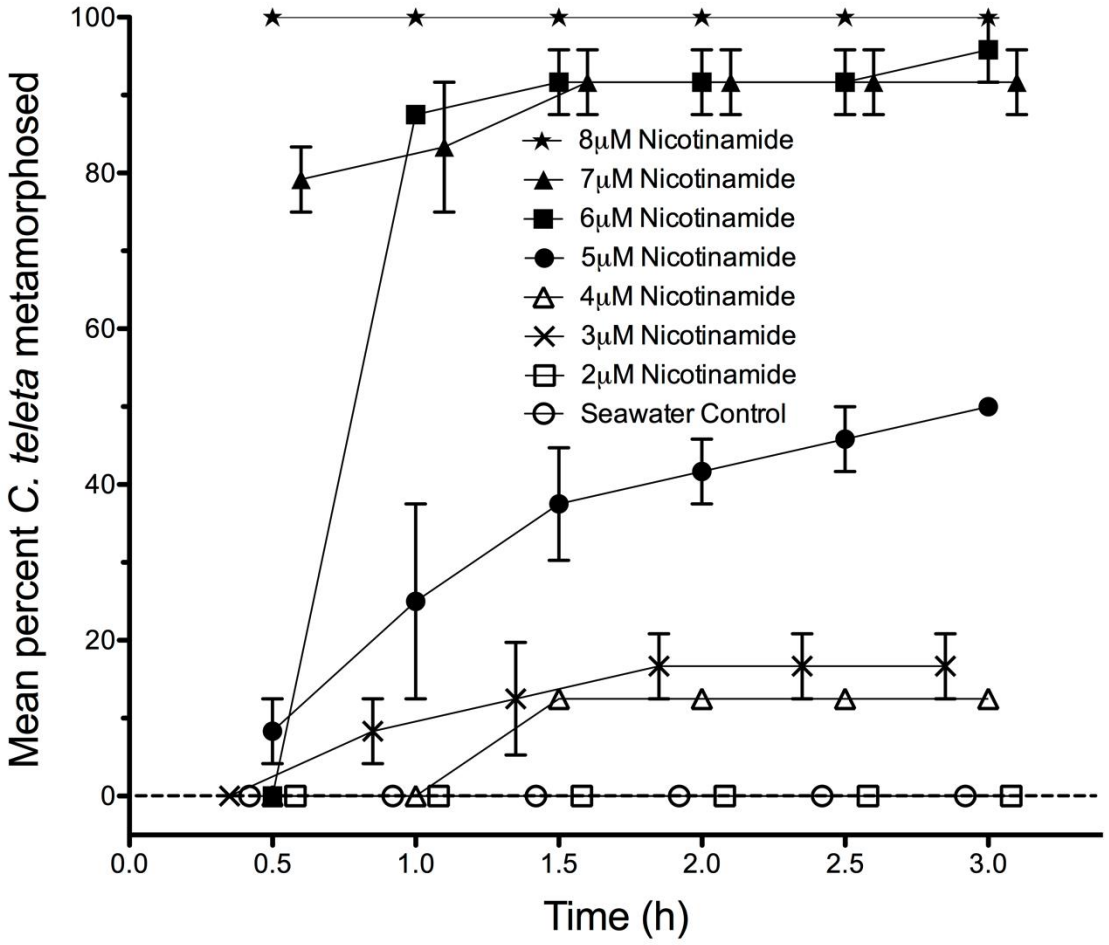

Figure 2.1. Promotion of metamorphosis by nicotinamide (vitamin B_3) in larvae of *Capitella teleta*. Each treatment consisted of 3 replicates with 8 larvae per replicate. Larvae were placed in 30 psu artificial seawater (Instant Ocean) containing the indicated final concentration of nicotinamide. Artificial seawater acted as a negative control. Error bars represent +/- 1 SEM.

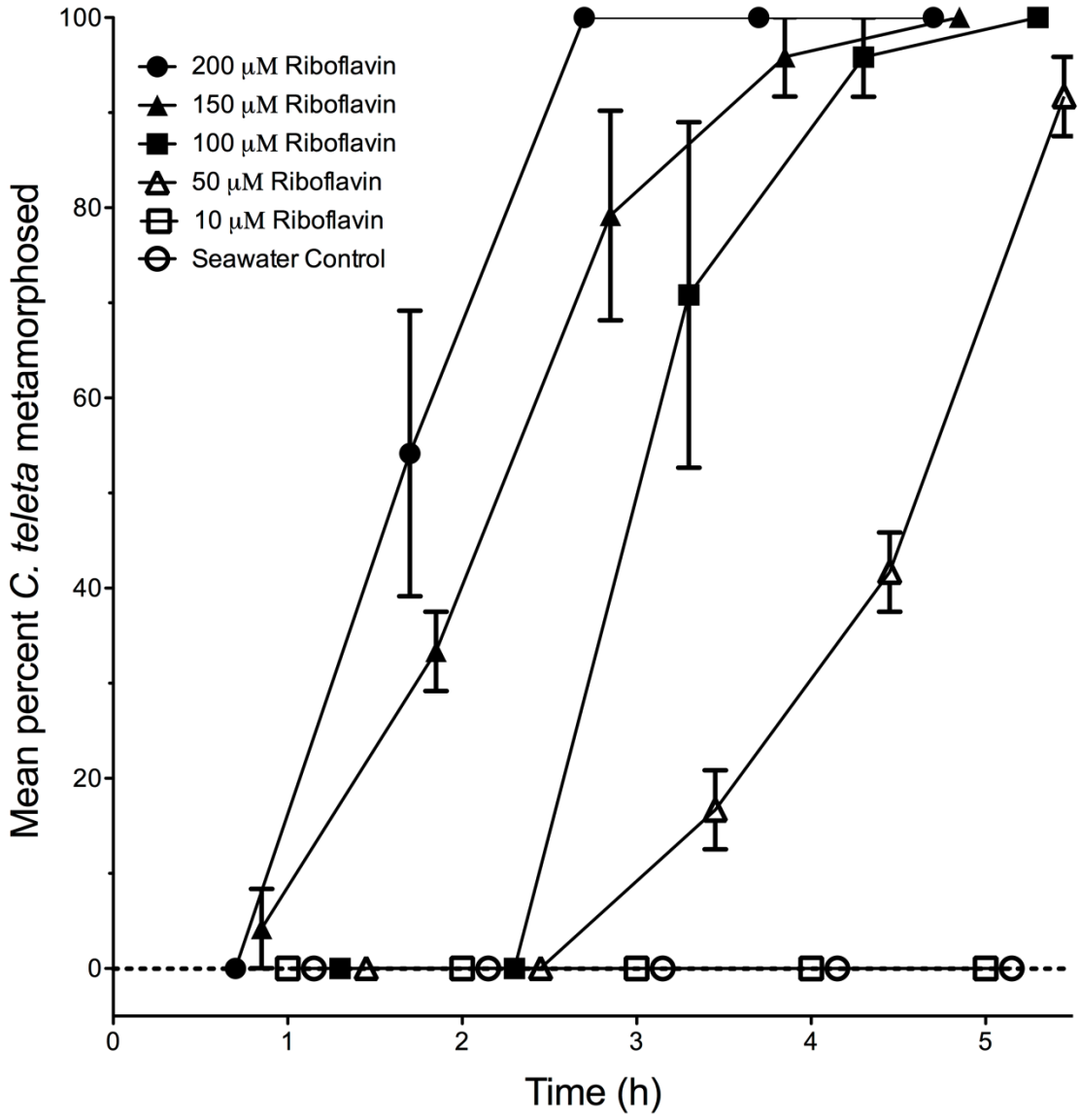

Figure 2.2. Promotion of metamorphosis by riboflavin (vitamin B_2) in larvae of *Capitella teleta*. Each treatment consisted of 3 replicates with 8 larvae per replicate. Larvae were placed in 30 psu artificial seawater (Instant Ocean) containing the indicated final concentration of riboflavin. Artificial seawater acted as a negative control. Error bars represent +/- 1 SEM.

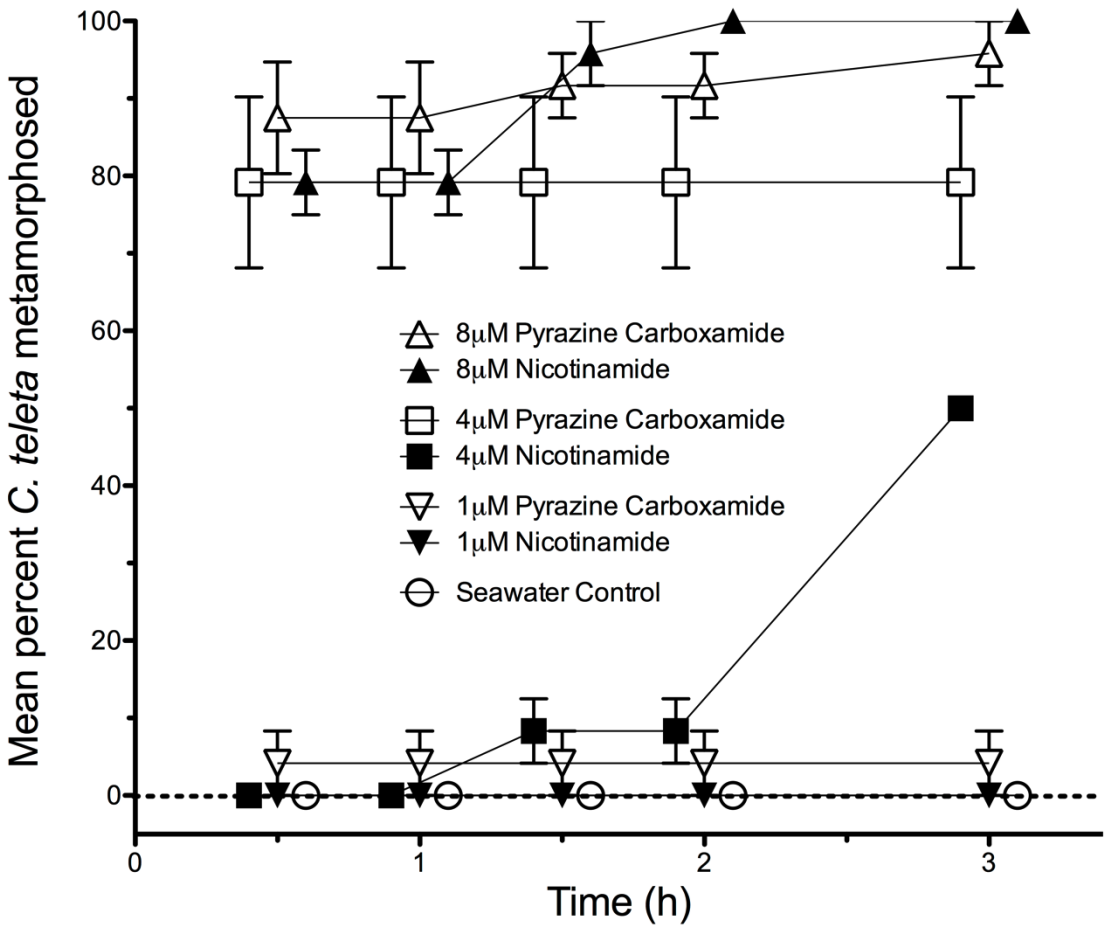

Figure 2.3. The effects of pyrazinecarboxamide and nicotinamide on metamorphosis of *C. teleta* when tested equal concentrations. Each treatment consisted of 3 replicates with 8 larvae per replicate. Artificial seawater (Instant Ocean) acted as a negative control. Error bars represent +/-1 SEM.

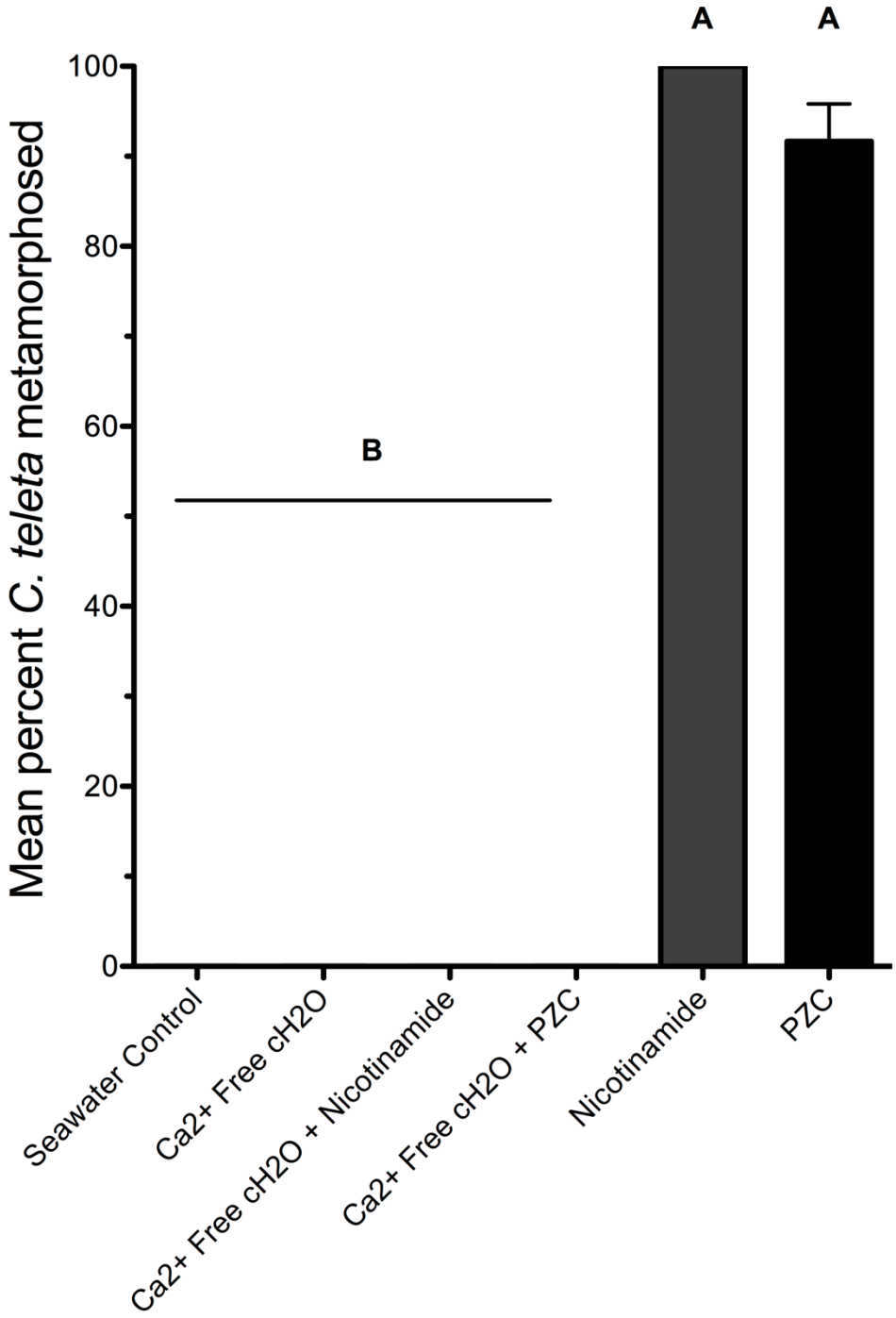

Figure 2.4. The effect of calcium-free seawater on metamorphosis of *C. teleta* treated with nicotinamide or pyrazinecarboxamide. Larvae were acclimated to calcium-free seawater for one hour before being transferred to a solution of calcium-free seawater and either nicotinamide or pyrazinecarboxamide (PZC) at 40 μM for 24 h. Each treatment consisted of 3 replicates with 8 larvae per replicate. Artificial seawater (Instant Ocean)

acted as a negative control. Letters indicate significant ($P < 0.05$) differences as determined by a Bonferroni post-hoc test. Error bars represent +1 SEM.

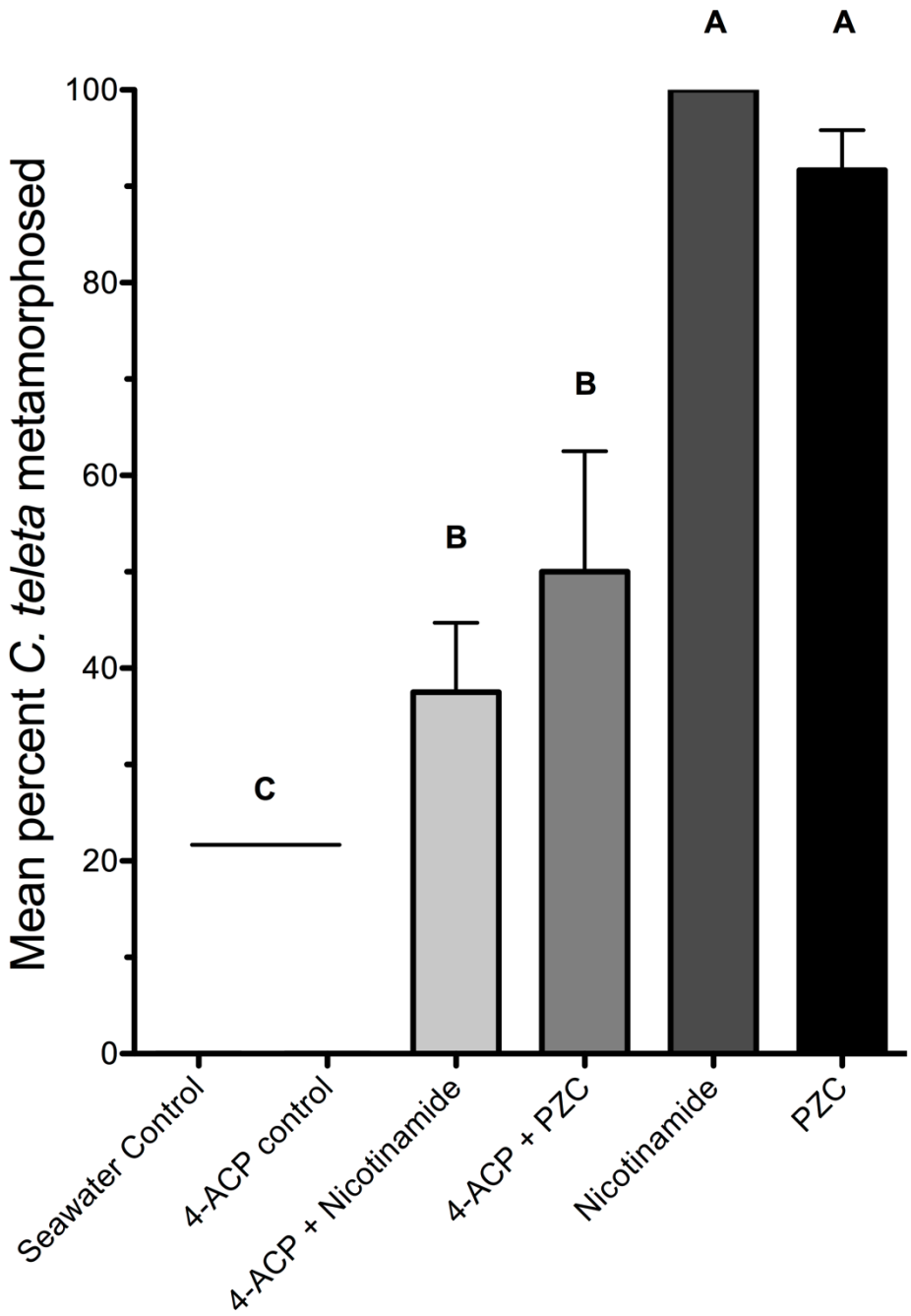

Figure 2.5. The effect of 4-acetylpyridine on metamorphosis of *C. teleta* in the presence of nicotinamide or pyrazinecarboxamide. Larvae were acclimated to 200 μM 4-acetylpyridine (4-ACP) for one hour before being transferred to a solution of 200 μM 4-acetylpyridine and either nicotinamide or pyrazinecarboxamide (PZC) at 40 μM for 24 h. Each treatment consisted of 3 replicates with 8 larvae per replicate. Artificial seawater

(Instant Ocean) acted as a negative control. Letters indicate significant ($P < 0.05$) differences as determined by a Bonferroni post-hoc test. Error bars represent + 1 SEM.

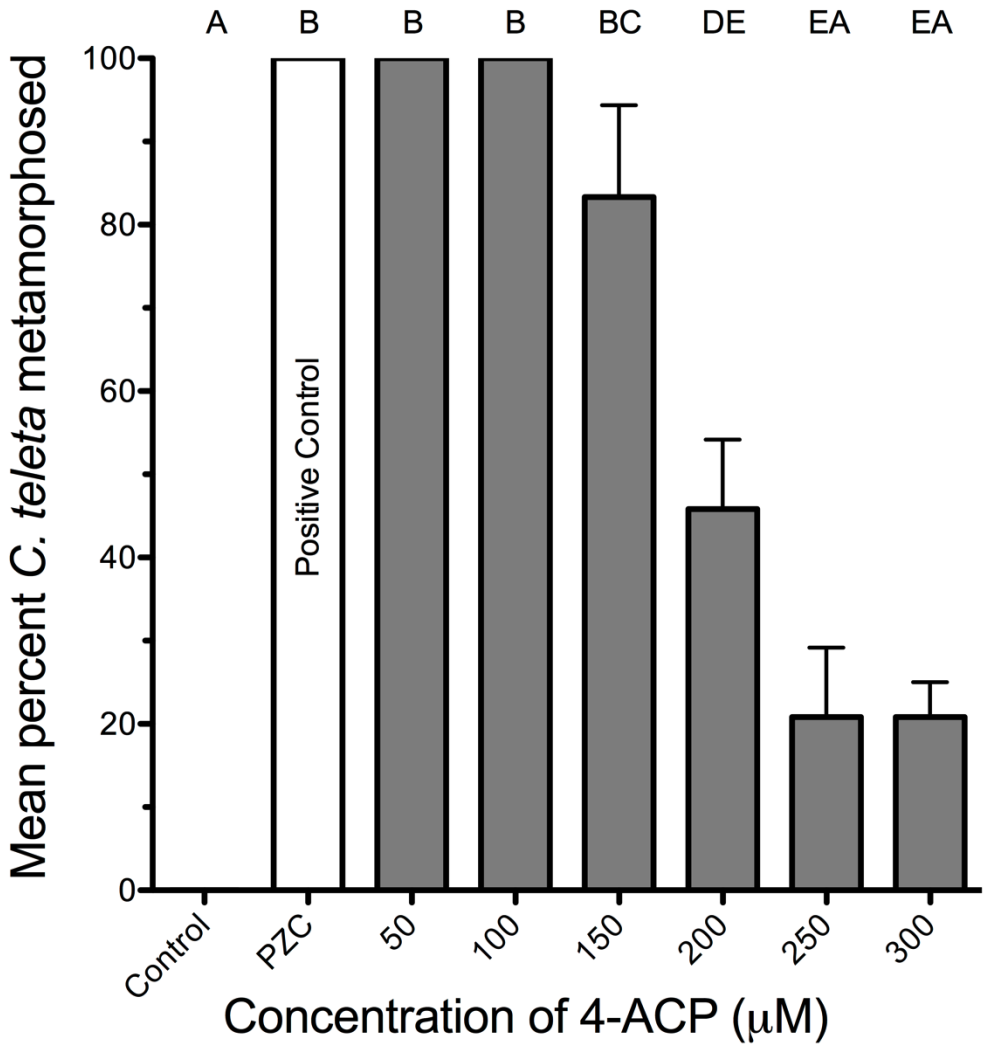

Figure 2.6. The inhibitory dose response of 4-acetylpyridine on metamorphosis of *C. teleta* after 24 h. Larvae were acclimated to the listed concentrations of 4-acetylpyridine (4-ACP) for one hour before being transferred to a solution of the same concentration of 4-acetylpyridine and pyrazinecarboxamide at 40 μM. Each treatment consisted of 3 replicates with 8 larvae per replicate. Artificial seawater (Instant Ocean) acted as a negative control. The PZC treatment acted as a positive control containing only 40 μM pyrazinecarboxamide and artificial seawater. Letters indicate significant ($P < 0.05$) differences as determined by a Bonferroni post-hoc test. Error bars represent +1 SEM.

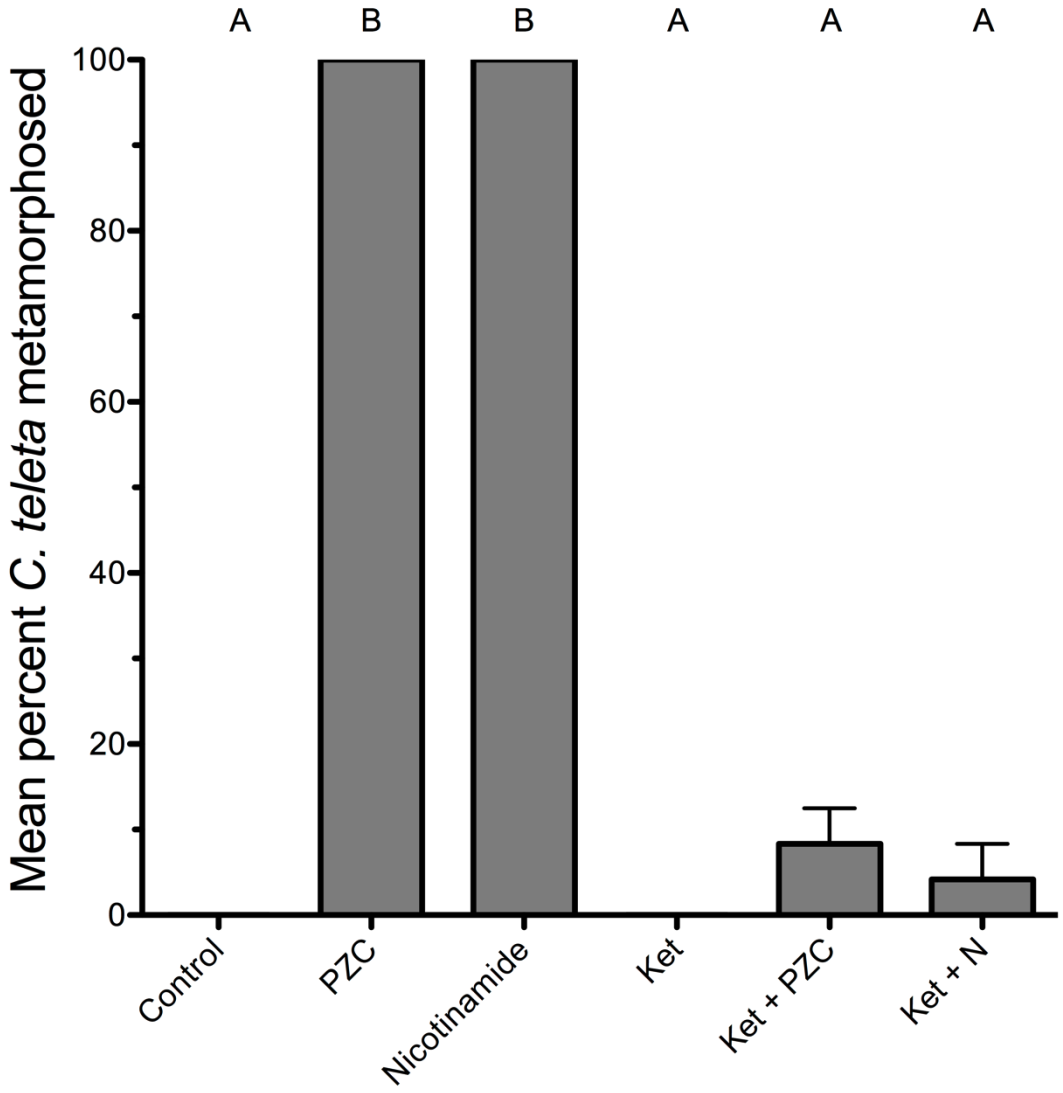

Figure 2.7. The effect of ketanserin on metamorphosis of *C. teleta* in the presence of nicotinamide or pyrazinecarboxamide. Larvae were acclimated to 2 μM ketanserin for three hours before being transferred to a solution of 2 μM ketanserin and either nicotinamide or pyrazinecarboxamide at 40 μM for 24 h. Each treatment consisted of 3 replicates with 8 larvae per replicate. Artificial seawater (Instant Ocean) acted as a negative control. Letters indicate significant ($P < 0.05$) differences as determined by a Bonferroni post-hoc test. Error bars represent + 1 SEM.

Chapter 3

The photodegradation of riboflavin stimulates larvae of the snail *Crepidula fornicata* and the polychaete *Capitella teleta* to metamorphose via generation of reactive oxygen species

Abstract

The planktonic larvae of marine invertebrates that are either sessile or have limited mobility have a very risky decision to make when choosing a site for settlement and metamorphosis. Without access to appropriate food in sufficient quantities or conspecific mates nearby, the metamorphosed larva is doomed to either starve or never reproduce as an adult. By making direct assessments of an area's nutritional composition, a larva could at least choose a site where starvation of the juvenile should not occur in the immediate future. We have previously found that larvae of the salt-marsh deposit-feeding polychaete *Capitella teleta* were rapidly stimulated to metamorphose by low concentrations of the B vitamins riboflavin and nicotinamide. Here we conducted experiments to determine if larvae of the sessile slipper-snail *Crepidula fornicata* were also stimulated to metamorphose by B vitamins. Of the 6 B vitamins tested, only riboflavin stimulated larvae of *C. fornicata* to metamorphose, and did so at concentrations as low as 50 μM. Because riboflavin stimulated larvae of both *C. fornicata* and *C. teleta* to metamorphose, we conduced experiments to determine the mechanism of riboflavin-induced metamorphosis. We found that riboflavin did not induce any larvae of either species to metamorphose in total darkness within 24 h, while the same concentration of riboflavin induced all tested larvae to metamorphose under laboratory fluorescent lighting. Because riboflavin degrades into both lumichrome and the superoxide reactive oxygen species when exposed to UV light we then tested competent larvae of both species with hydrogen peroxide (a reactive oxygen species) and lumichrome. 200 μM lumichrome did not induce larvae of either species to metamorphose within 24 h while 50 μM hydrogen peroxide induced nearly all of the

tested larvae to metamorphose within 24 h. 10 μM hydrogen peroxide, however, did not induce any larvae of either species to metamorphose within 24 h. Natural seawater concentrations of hydrogen peroxide almost never exceed 500 nM, so it is unlikely that reactive oxygen species present in the environment are acting as natural settlement cues for either *C. fornicata* or *C. teleta*.

Introduction

Most marine invertebrate phyla contain species that release dispersive planktonic larvae. The adults of these species often live in the benthos and are sessile or slow moving; their larvae are microscopic and are dispersed by the currents of the world's oceans. While adults of terrestrial animals such as amphibians (Howard 1978, Seale 1982) and lepidopterans (Thompson & Pellmyr 1991) often display remarkable behavior in distributing their eggs directly on food sources or near bodies of water protected from predators, the adults of slow moving or sessile benthic marine invertebrates cannot choose these advantageous environments for their progeny. For these benthic marine invertebrates, planktonic larvae act as the dispersal mechanism. Eventually, as these larvae are dispersing, growing, and developing, the larvae will become competent to metamorphose (Hadfield et al. 2001).

Competent larvae must then choose a benthic environment to settle onto and initiate metamorphosis. Larvae are stimulated to settle and metamorphose by a settlement cue, a specific physical stimulus from the environment (Pechenik 1990, Pawlik 1992). Often, these settlement cues are chemicals that diffuse away from potential food sources, mates, or biofilmed surfaces signaling to the larva that the surrounding area is consistently immersed by seawater (Hadfield & Paul 2001, Hadfield 2011). While many species across phyla depend on settlement cues to metamorphose in suitable locations for juvenile development and adult persistence, very few cues have been characterized.

Recently, we showed for the first time that vitamins can stimulate settlement and metamorphosis. Larvae of the deposit-feeding polychaete worm *Capitella teleta*

metamorphosed within minutes when exposed to nicotinamide (vitamin B_3) at the low concentration of 8 μM and within hours of being exposed to 50 μM riboflavin (vitamin B_2) (Burns et al. 2014). By using synthetic agonists and competitive inhibitors we have also shown that these larvae are likely sensing nicotinamide with a nicotinamide-gated ion-channel previously characterized in the freshwater crayfish *Austropotamobius torrentium* (Hatt & Schmiedel-Jakob 1984, 1985; Burns et al. 2014). Larvae of *C. teleta* were stimulated to metamorphose by the agonist of the nicotinamide-gated ion-channel pyrazine carboxamide and were inhibited from metamorphosing in the presence of nicotinamide by the competitive inhibitor of this channel 4-acetylpyridine. There is currently no known mechanism or receptor for riboflavin stimulated metamorphosis.

Because metamorphosis and juvenile development are energy intensive processes, it appears to be advantageous to metamorphose in the presence of nutritious food sources. Riboflavin and nicotinamide play especially important roles as the precursors to FAD and NAD, respectively. Other organisms could benefit from using these cues to settle in nutrient dense areas.

To determine if B vitamins can act as settlement cues in other marine invertebrates, we chose to study larvae of the slipper-shell snail *Crepidula fornicata*. Larvae of *C. fornicata* settle gregariously near adults; the juveniles will then position themselves onto a mating stack where they will likely remain for the rest of their life (Coe 1938, Pechenik & Gee 1993). These larvae are stimulated to metamorphose by adult conditioned seawater – they will cease swimming, search the substrate, and resorb their velum – the larval swimming and food collection organ (Pechenik & Heyman 1987). Larvae can also be artificially induced to metamorphose by treating them with seawater

containing a 20 mM excess of KCl (Pechenik & Heyman 1987). In this treatment the larvae will shed the velum instead of resorbing it (Pechenik & Heyman 1987). By resorbing the velum instead of shedding it, metamorphosing larvae could be reallocating velar resources to juvenile growth and development. Here, we conducted settlement assays with larvae of *C. fornicata* using the same B vitamins at the same concentrations used previously in (Burns et al. 2014) to stimulate metamorphosis of *C. teleta*. Some experiments were also conducted in parallel with larvae of *C. teleta* to characterize the mechanism of how some vitamins induce metamorphosis in these species.

Materials and Methods

Care and maintenance of larvae

Adult *Crepidula fornicata* were collected from Nahant, Massachusetts and were maintained in aerated 1 gallon glass jars containing 30 psu Instant Ocean (hereafter called ASW) at room temperature (20-23°C). Hatched larvae were collected on 120 μm nitex mesh and transferred to a 1 gallon glass jar containing 0.45 μm filtered natural seawater and kept at room temperature. Larvae were maintained on a Tahitian strain of *Isochrysis galbana* (hereafter called T-ISO) as a food source (Pechenik, 1980; Pechenik 1984). Metamorphic competence was tested by pipetting larvae into 3cm glass dishes containing 0.45 μm filtered seawater with a 20 mM excess of KCl (hereafter called excess KCl seawater) (Pechenik and Gee 1993). Larvae were used in experiments once at least 70% of the sub-sampled larvae metamorphosed the previous day (2-3 replicates of 8 larvae per replicate). Larvae were considered to have metamorphosed once the velum was shed or resorbed (Pechenik and Gee 1993).

Cultures of *C. teleta* were maintained and their larvae were collected for experiments as detailed in (Burns et al. 2014).

General bioassay protocol

All experiments were conducted using either 12-well tissue-culture plates or 3 cm diameter glass dishes, with each well or bowl containing 4 mL of an ASW vitamin solution. Each experiment included 3 replicates per treatment, with 8 larvae per replicate. To avoid diluting the final treatment solution, larvae were first pipetted into an intermediate bath of treatment solution (~20mL) before being pipetted into the final treatment vessel. In all experiments ASW served as a negative control. Excess KCl seawater served as a positive control. Dishes were examined at regular intervals under 20-50x magnification to count the number newly metamorphosed juveniles. All experiments were conducted at room temperature (20-23°C) under laboratory fluorescent lighting.

Testing the effects of B vitamins on larval metamorphosis

Thiamine HCl (B_1), riboflavin (B_2), nicotinamide (B_3), nicotinic acid (B_3), pyridoxine HCl (B_6), and vitamin B_{12} were tested on larvae of *C. fornicata*. Thiamine HCl, nicotinic acid, pyridoxine HCl, and vitamin B_{12} were tested on larvae of *C. fornicata* at concentrations 100, 200, 400, and 600 μM. Riboflavin was tested on larvae of *C. fornicata* at 10, 50, 100, 150, and 200 μM. Lumichrome, the photodegradation product of riboflavin, was also tested on larvae at the concentration of 200 μM. Nicotinamide was tested on larvae of *C. fornicata* at concentrations 25, 50, 75, and 100 μM.

Testing salt-marsh sediment and sediment extracts

To determine if larvae of *C. fornicata* would metamorphose in response to cues present within the salt-marsh sediment that triggers metamorphosis of *C. teleta* larvae in nature (Dubilier 1988, Cohen & Pechenik 1999), larvae of *C. fornicata* were exposed to an ethanol extract of the sediment. The same salt-marsh sediment used to feed *C. teleta* was air-dried in a glass dish overnight and subsequently added to reagent grade ethanol at 0.8 g dry sediment/mL. The mixture was vigorously shaken by hand and centrifuged at 8000 rpm for 2 minutes. The supernatant was then pipetted off and saved (hereafter called sediment extract). 150 μL of this sediment extract was pipetted into the bottom of 3 cm glass dishes and, once the ethanol evaporated, 4mL of ASW was added. 150 μL of plain reagent grade ethanol was evaporated in control dishes. Larvae of *C. fornicata* were added to treatment dishes with 3 replicates of 8 larvae per replicate and the number of newly metamorphosed juveniles was recorded at regular time intervals.

Larvae were also exposed to unadulterated salt-marsh sediment to determine if the ethanol extraction procedure dissolved cues that may not normally be accessible to chemosensation by larvae of *C. fornicata*. Here, 0.7 cm^3 of salt-marsh sediment was added to each well and mixed with 4 mL ASW. This sediment mixture was allowed to settle at least 8 h before larvae were added to the treatment dishes.

Determining the mechanism of riboflavin stimulated metamorphosis

We hypothesized that riboflavin was inducing the larvae to metamorphose via the production of superoxide, a photodegradation product of riboflavin. To test this we treated larvae of *C. fornicata* and *C. teleta* separately with 100 μM riboflavin using the methods above. However, immediately after pipetting larvae into the riboflavin containing dishes, half of the dishes were placed in a light-tight plastic box to prevent

photodegradation. 3 replicates of 10 larvae per light and dark treatment were used for each species. Individuals in the 'light' treatment were observed at hourly intervals for metamorphosed juveniles. Once at least 80% of animals had metamorphosed in the 'light' treatment, individuals in the 'dark' treatment were examined for metamorphosis.

Determining the metamorphic capacity of hydrogen peroxide

Because the larvae may be stimulated to metamorphose by reactive oxygen species (ROS), we also treated the larvae with hydrogen peroxide, a relatively stable ROS, to determine if it stimulated metamorphosis. Aqueous 3% hydrogen peroxide was mixed with ASW to create hydrogen peroxide concentrations of 10 and 50 μM. Competent larvae of *C. fornicata* and *C. teleta* were then to transferred treatments of 10 μM hydrogen peroxide, 50 μM hydrogen peroxide, excess KCl seawater as a positive control for larvae of *C. fornicata*, and ASW alone as a negative control for both species. Experiments using larvae of *C. fornicata* used 3 replicates of 8 larvae per treatment while experiments using larvae of *C. teleta* used 3 replicates of 10 larvae per treatment.

The effect of different metamorphic cues on juvenile growth

Because individuals of *C. fornicata* resorb their velum when they metamorphose in response to riboflavin, we hypothesized that juveniles might utilize resources from the resorbed velum to grow to a larger size than the juveniles that metamorphosed in response to KCl that had shed their velum. Here, competent larvae were treated with either 100 μM riboflavin or excess KCl seawater overnight to induce metamorphosis. The next day the resulting juveniles in each treatment were measured and transferred to 150 mL of 0.45 μm filtered seawater containing approximately 18 x 10^4 cells / mL T-ISO. The individuals that had been induced to metamorphose using riboflavin or excess

KCl seawater were maintained separately at room temperature, with their water and T-ISO food source replaced daily. Juveniles were then measured 3 and 6 days later. The longest shell axis length was measured for each individual under a dissecting microscope using an ocular micrometer at 50x magnification. The mean shell length for each treatment was compared using an unpaired t-test for each measurement day using Graphpad Prism 5.0 (Graphpad Software).

Results

Of the 6 B vitamins tested, only riboflavin stimulated larvae of *C. fornicata* to metamorphose. Thiamine HCl, pyridoxine HCl, vitamin B_{12}, nicotinamide, nicotinic acid, and lumichrome did not stimulate metamorphosis in larvae of *C. fornicata* at any tested concentration (100 μM - 600 μM) within 24 h, although the excess KCl seawater (positive control) promoted metamorphosis of all larvae within 24 h (data not shown), indicating that all larvae were competent when tested. Riboflavin concentrations of 50 - 200 μM stimulated metamorphosis of all larvae of *C. fornicata* within 24 hours (Fig. 1).

The larvae in any of the vitamin treatments besides nicotinamide and riboflavin did not appear to act any differently than those in the negative control treatment (ASW). Those larvae continued to swim throughout the 24 h treatment. However, the larvae treated with any of the nicotinamide concentrations immediately ceased swimming and sank to the bottom of the treatment wells and preceded to twitch for the duration of the treatment period. This behavior resembled what the larvae did when treated with excess KCl seawater except that they did not complete metamorphosis. Some larvae treated with nicotinamide lost their cilia but did not go on to shed or resorb their velum.

Larvae of *C. fornicata* were stimulated to metamorphose by the salt-marsh sediment extract. Metamorphosis of all larvae of *C. fornicata* took place within 24 h of exposure (Fig. 2). No larvae had metamorphosed in response to the unadulterated salt-marsh sediment within 24 h of treatment. Again, the excess KCl seawater positive control promoted metamorphosis of all larvae within 24 h; (data not shown), indicating that all larvae were competent when tested.

When larvae of *C. fornicata* began metamorphosing in response to riboflavin or the sediment extract, the velum was not conspicuously shed as is usually witnessed during excess KCl seawater exposure (Pechenik and Heyman 1987). Instead, cilia were eventually shed from the velum and the rest of the velum was then slowly absorbed by the animal, as in their response to adult conditioned seawater (Pechenik and Heyman 1987).

Larvae of *C. fornicata* did not metamorphose when treated with 100 μM riboflavin in complete darkness, although all larvae were competent to metamorphose because all of the larvae treated with excess KCl seawater had metamorphosed within 6 h of treatment (Table 1). Larvae of *C. teleta* also did not metamorphose when treated with 100 μM riboflavin in complete darkness (Table 2). Larvae of *C. teleta* were competent to metamorphose when tested because nearly all of the larvae had metamorphosed after 3 hours of treatment with riboflavin under light.

Hydrogen peroxide successfully induced larvae of *C. fornicata* to metamorphose at the concentration of 50 μM, and they metamorphosed at approximately the same rate as the larvae treated with excess KCl seawater (Fig. 3). Larvae of *C. teleta* also metamorphosed when treated with 50 μM hydrogen peroxide, with almost all larvae

having metamorphosed within 3 h (Table 2). No larvae of either species had metamorphosed at a hydrogen peroxide concentration of 10 μM within 24 h (Table 2 & Fig. 3).

Juveniles did not grow to significantly different sizes when stimulated to metamorphose with either riboflavin or KCl (Table 3). It is important to note that the riboflavin and KCl groups of juveniles were the same size when the experiment was started (day 0). The juveniles grew at an average of 97.0 μm per day.

Discussion

Larvae of *C. fornicata* only metamorphosed in response to hydrogen peroxide, potassium chloride, or riboflavin (but only in the presence of light) (Fig. 1 & Table 1). The larvae of *C. fornicata* did not metamorphose when treated with any other tested vitamin, even at high concentrations of up to 600 μM. Similarly, larvae of *C. teleta* only metamorphosed in response to riboflavin when under laboratory fluorescent lighting (Table 2). No larvae of either species metamorphosed in response to riboflavin when treated in darkness, even after having been in solution with it for 24 h (Tables 1 & 2). Curiously, while nicotinamide rapidly stimulated larvae of *C. teleta* to metamorphose at low concentrations (Burns et al. 2014), this vitamin did not stimulate complete metamorphosis in larvae of *C. fornicata*. While these nicotinamide-treated treated larvae did settle and lose cilia from their velum, they did not resorb or shed the velum to complete metamorphosis. These larvae then remained stationary until death occurred. Interestingly, larvae of *C. fornicata* metamorphosed when treated with salt-marsh sediment extract, but they did not metamorphose when treated with the sediment itself (Fig. 2). Perhaps some chemical cue is normally tightly bound to sediment particles and

not normally present in seawater. By vigorously agitating the sediment in ethanol, this cue could have separated from sediment particles and moved into suspension where larvae of *C. fornicata* could detect it, or the cue was solubilized in ethanol. Overall, salt-marsh sediment appears to act as a specific cue for larvae of *C. teleta* because larvae of *C. fornicata* did not metamorphose when treated with whole sediment, only when treated with sediment extract.

Reactive oxygen species (ROSs) could be stimulating larvae of both species to metamorphose by inhibiting endogenous nitric oxide production. Larvae of both *C. fornicata* and *C. teleta* are inhibited from metamorphosing by endogenously produced nitric oxide (Pechenik et al. 2007, Biggers et al. 2012). As riboflavin degrades under UV irradiation, it releases the ROS superoxide while transitioning into lumichrome (Sheraz et al. 2014). ROSs like superoxide and hydrogen peroxide disrupt the synthesis of nitric oxide and this process is a well-known cause of vasoconstriction in medicine (Murad 2006). Nitric oxide synthases (NOSs) require the reduced co-factors NADPH and BH4 to synthesize nitric oxide (NO); if these cofactors are oxidized or present in insufficient concentrations NOS will synthesize a superoxide anion instead of NO (Murad 2006). Here, superoxide or hydrogen peroxide can oxidize the co-factors required for NO synthesis, this both reduces the amount of NO synthesized and produces even more superoxide anions that can further reduce the amount of NO synthesized (Murad 2006). As neither high concentrations of the riboflavin breakdown product lumichome, nor the riboflavin in darkness treatment stimulated any larvae to metamorphose, ROSs are a possible mechanism behind riboflavin stimulated metamorphosis in larvae of both species. Indeed, 50 μM hydrogen peroxide induced the majority of larvae of both species

to metamorphose within 24 h. Treatment of larvae of the conch *Strombus gigas* with 50 μM hydrogen peroxide also stimulated all larvae to metamorphose within 10 h (Boettcher et al. 1997).

Could ROSs act as natural settlement cues in nature? One of the most measured ROSs in the environment is hydrogen peroxide because of its long half-life compared to other ROSs. Hydrogen peroxide concentrations in seawater can range from undetectable concentrations (<3 nM) to 500 nM depending on the sampling location, season of the year when the sample was taken, and even time of day (reviewed in Herut et al. 1998). Microbes appear to play a dominant role in the breakdown of hydrogen peroxide, with peroxidases removing 20-35% and catalases removing 65-80% of the hydrogen peroxide present (Herut et al. 1998). Hydrogen peroxide concentrations are highest in summertime mid-afternoons and lowest on winter nights. Surface water samples from Woods Hole, MA contained 18-25 nM hydrogen peroxide (Moffett & Zafiriou 1990) while water outflowing from a salt-marsh in California contained 169 nM hydrogen peroxide (Clark et al. 2010). Unfortunately we could not find any published measurements of superoxide or hydrogen peroxide in sediment samples. Marine water samples (not sediments) contain much lower concentrations of hydrogen peroxide than the 50 μM required stimulate larvae of *C. fornicata*, *C. teleta*, or even the conch *S. gigas* to metamorphose (Boettcher et al. 1997). Even the non-inductive concentration of 10 μM hydrogen peroxide is many times higher than the hydrogen peroxide concentrations contained in seawater samples. Unless the concentration of ROSs is much higher in sediment samples, it is unlikely that the larvae of these species are stimulated to metamorphose by ROSs in nature.

While it makes adaptive sense for larvae of the deposit feeding polychaete *C. teleta* to metamorphose in soft nutrient rich sediments that could potentially contain high amounts of ROSs, this does not fit with the ecology of *C. fornicata*. Larvae of *C. fornicata* settle gregariously near adults of their own species; seawater conditioned by the presence of *C. fornicata* adults has stimulated larvae of *C. fornicata* to metamorphose (Coe 1938, Pechenik & Heyman 1987). Moreover, juveniles and adults of *C. fornicata* are found in the rocky intertidal and coastal subtidal in temperate environments, these are not locations where there is much organic decomposition taking place for high concentrations of ROSs to occur. Larvae of *C. fornicata* may metamorphose in these environments if they are carried there by the current; however, their survival would likely be selected against by the soft-sediment environment.

It is surprising that the larvae of *C. fornicata* that had metamorphosed in response to riboflavin and had resorbed their velum did not grow more quickly than the larvae induced to metamorphose by KCl that had shed their velum. On day 3, the larvae that had resorbed their velum were ~ 100 μm smaller than those larvae that had shed their velum, although this difference was not statistically significant (Table 3). These results agree with those of Eyster & Pechenik (1988), where the larvae of *C. fornicata* that had metamorphosed in response to adult-conditioned seawater and resorbed their velum had juvenile growth rates equal to those individuals that metamorphosed in response to excess KCl seawater and shed their velum. Overall, it seems whether a larva's velum disintegrates or is resorbed has no impact on juvenile growth.

Overall it appears that high concentrations of ROSs themselves, or produced by photodegradation of riboflavin artificially inhibit the endogenous production of NO that

is preventing larvae of *C. fornicata* and *C. teleta* from metamorphosing. Future studies must focus on determining how natural cues stop or lower endogenous production of NO and perhaps this signaling cascade can be traced backward to a specific cue receptor protein or at least proteins playing roles earlier in the signal transduction cascade leading to metamorphosis.

Tables

Table 3.1. Photodegradation of riboflavin caused larvae of *C. fornicata* to metamorphose. Larvae were treated with 100 μM riboflavin and placed either under laboratory fluorescent lighting or in a light tight box. The seawater control only contained 30 psu Instant Ocean artificial seawater. Excess KCl seawater served as a positive control. All treatments contained 3 replicates of 10 larvae each.

Treatment	Hours of Treatment – Mean % *C. fornicata* Metamorphosed (SEM)			
	3 h	6 h	9 h	24 h
Riboflavin + Light	0	0	16.67 (16.67)	100
Riboflavin + Dark	Not Observed	Not Observed	Not Observed	0
+ 20 mM KCl	100	100	100	100
Seawater Control	0	0	0	0

Table 3.2. Photodegradation of riboflavin caused larvae of *C. teleta* to metamorphose. Larvae were treated with 100 μM riboflavin and placed either under laboratory fluorescent lighting or in a light tight box. The seawater control only contained 30 psu Instant Ocean artificial seawater. All treatments contained 3 replicates of 10 larvae each.

Treatment	Hours of Treatment – Mean % *C. teleta* Metamorphosed (SEM)			
	1 h	2 h	3 h	24 h
Riboflavin + Light	0	0	96.67 (3.333)	100
Riboflavin + Dark	Not Observed	Not Observed	0	0
10 μM H_2O_2	0	0	0	0
50 μM H_2O_2	0	60 (5.774)	100	100
Seawater Control	0	0	0	0

Table 3.3. Shell lengths of juvenile *C. fornicata* at metamorphosis, after stimulation with either riboflavin or excess KCl seawater.

	Day 0		Day 3		Day 6	
Treatment	Riboflavin	KCl	Riboflavin	KCl	Riboflavin	KCl
Mean shell length (μm)	834.6	814.9	920.5	1034	1349	1464
SEM	18.72	18.19	49.02	64.88	154.6	116.8
n	13	13	12	12	6	9
t statistic	0.7543		1.392		0.6026	
p value	0.4580		0.1777		0.5571	

Figures

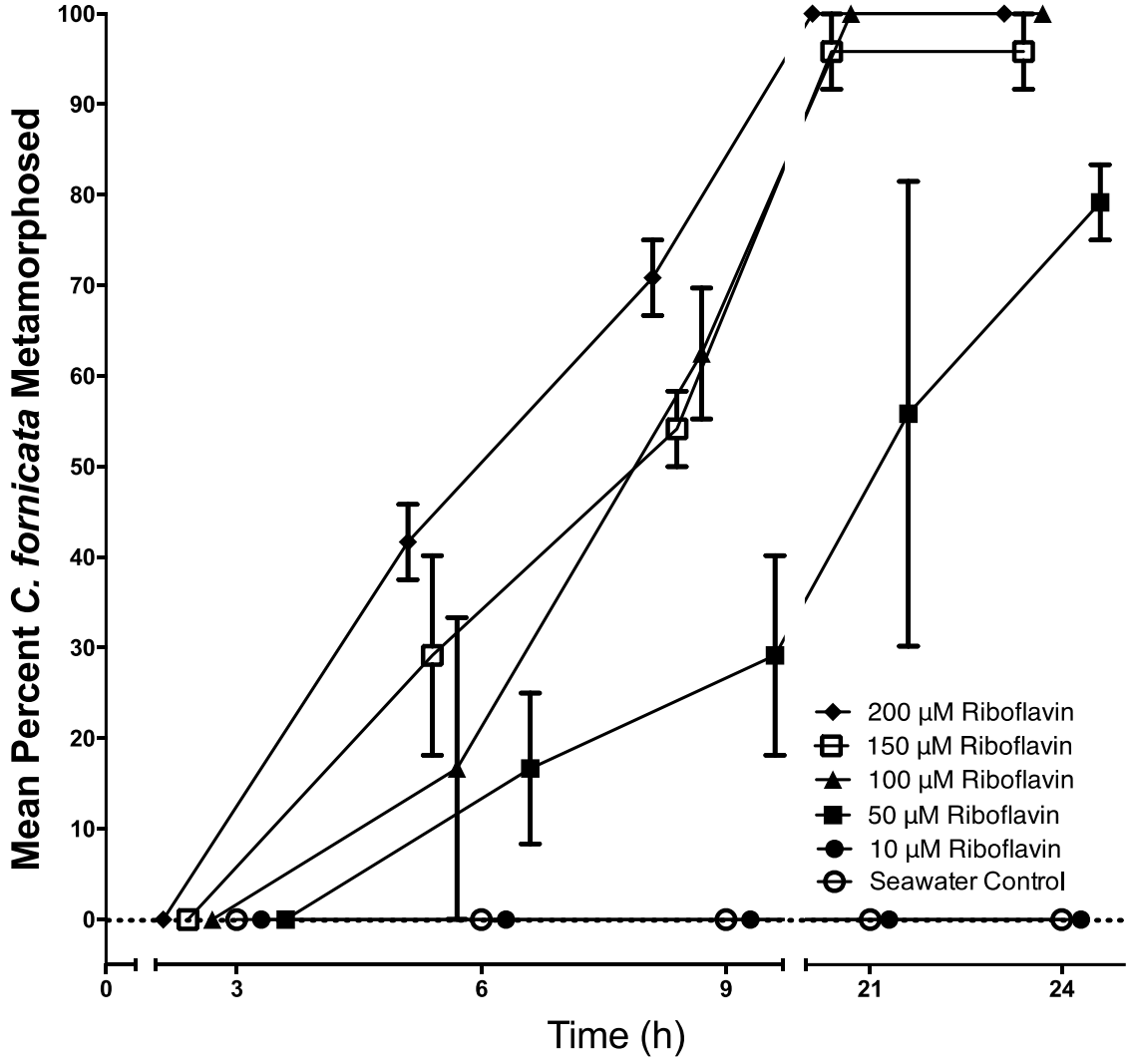

Figure 3.1. Promotion of metamorphosis by riboflavin (vitamin B_2) in *Crepidula fornicata* larvae. Each treatment consisted of 3 replicates of 8 larvae per replicate. Larvae were placed in 30 psu Instant Ocean artificial seawater containing the indicated final concentration of riboflavin. The seawater control acted as a negative control, containing only Instant Ocean. Error bars represent +/- 1 SEM.

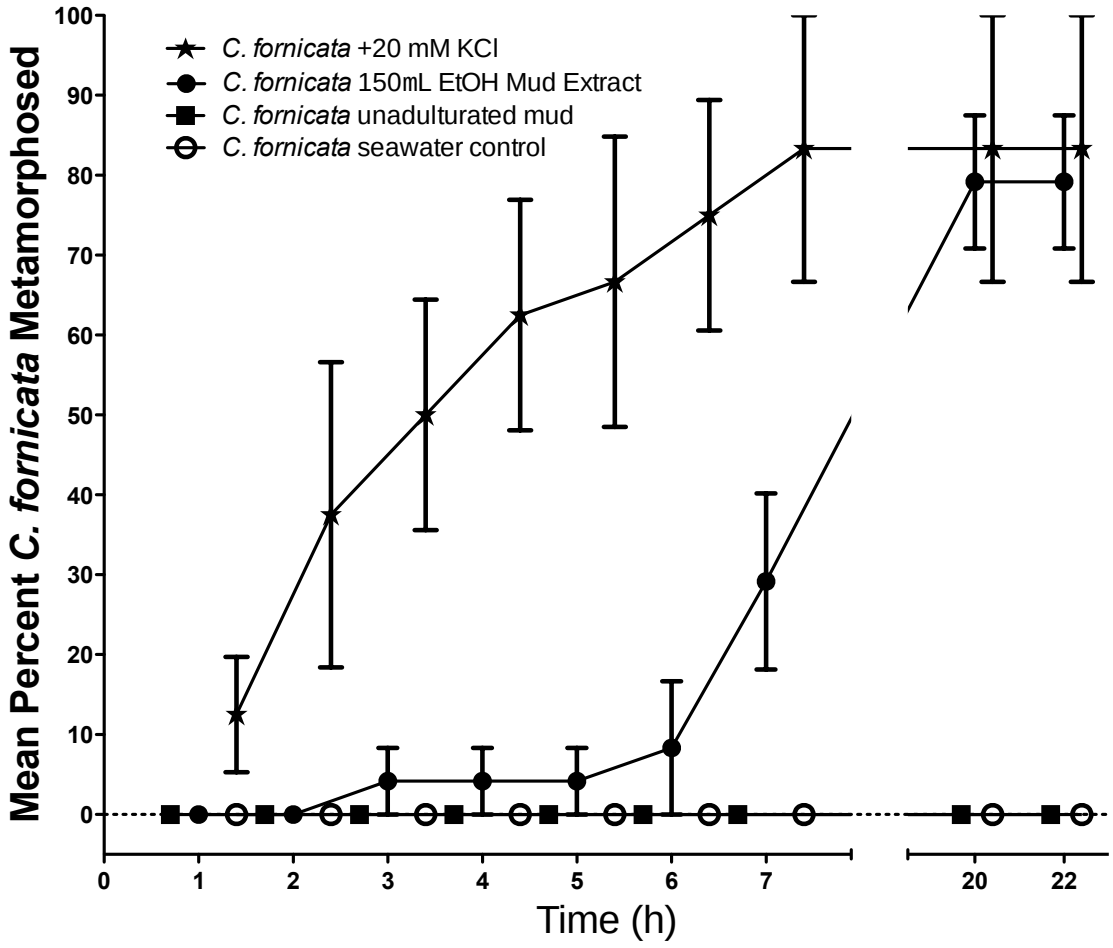

Figure 3.2. The impact of salt-marsh sediment and an ethanol sediment extract on the metamorphosis of *C. fornicata*. The ethanol salt-marsh sediment extract was evaporated in the bottom of each treatment dish and the dishes were then filled with 30 psu Instant Ocean artificial seawater. Each treatment consisted of 3 replicates of 8 larvae per replicate. The seawater control acted as a negative control, containing only Instant Ocean. The +20mM KCl treatment was a positive control. Error bars represent +/- 1 SEM.

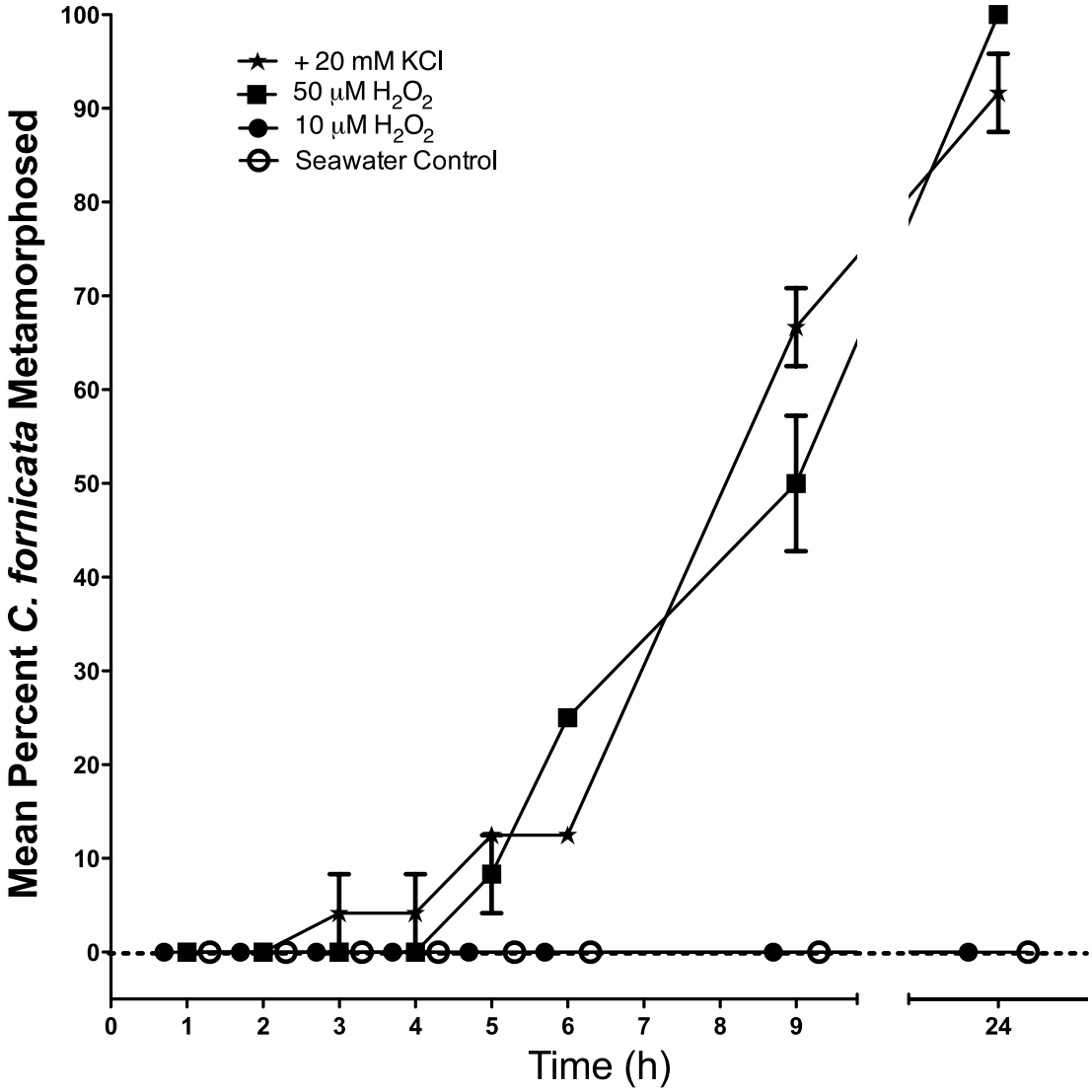

Figure 3.3. The impact of hydrogen peroxide on the metamorphosis of *C. fornicata*. Each treatment consisted of 3 replicates of 8 larvae per replicate. Larvae were placed in 30 psu Instant Ocean artificial seawater containing the indicated final concentration of hydrogen peroxide. The seawater control acted as a negative control, containing only Instant Ocean. The +20mM KCl treatment was a positive control. Error bars represent +/- 1 SEM.

Chapter 4

Menaquinone-6 produced by *Desulfovibrio spp.* isolated from salt-marsh sediments stimulates larvae of the marine polychaete *Capitella teleta* to settle and metamorphose

Abstract

Since the 1950's we have been accumulating evidence demonstrating that the planktonic larvae of marine invertebrates can be stimulated to settle and metamorphose by microbial biofilms. Although the stimulatory effect of biofilms has been shown in many species across phyla, there are few cases where the stimulatory microbe or stimulatory chemical synthesized in the biofilm has been characterized for any marine invertebrate. We have previously found that the B vitamins nicotinamide and riboflavin stimulated larvae of the salt-marsh dwelling polychaete worm *Capitella teleta* to metamorphose. Because microbes could be synthesizing these vitamins and transferring them to the salt-marsh sediment that stimulates this metamorphosis, we isolated an inductive bacterium from a salt-marsh sediment sample. 16S ribosomal subunit sequence analysis revealed that this inductive bacterium is the marine sulfur reducer *Desulfovibrio oceani*. Analytical chemical separations (TLC, HPLC/MS) of this bacterium's biofilm coupled with settlement assays revealed that the K vitamin menaquinone-6 synthesized by *D. oceani* stimulated larvae of *C. teleta* to rapidly metamorphose in less than 1 hour. High concentrations of menaquinones 4, 7, 9, phylloquinone, or ubiquinone-10 failed to stimulate any larvae to metamorphose within 24 hours. Together, these results are one of the rare instances where a stimulatory microbe and the actual stimulatory chemical they synthesize have been characterized for a marine invertebrate. Although it is not known if annelids require vitamin K, this is the third vitamin found to stimulate larvae of *C. teleta* to metamorphose. Larvae of *C. teleta* could be using menaquinone-6 as an indicator to determine if sulfur reducing bacteria are abundant, possibly indicating that sediment rich in organic matter is near.

Introduction

Adults of many terrestrial invertebrates and amphibians act as dispersal agents for their young. In these cases, the adults often display a remarkable amount of choice as to where they deposit their progeny. Insects such as butterflies and moths, for example, often lay their eggs directly on their host plant so that their caterpillar larvae can begin feeding immediately after hatching (Thompson & Pellmyr 1991). Some amphibians also lay their eggs on the undersides of leaves above sources of water, ensuring that their aquatic tadpole larvae have a suitable environment to complete metamorphosis in after they hatch (Howard 1978, Seale 1982). In contrast, the adults of many marine invertebrates are either sessile (e.g. barnacles, bivalves, etc.) or have only limited mobility (seastars, etc.). These microscopic larvae are typically dispersed over great distances before becoming capable of metamorphosis. In these species, the onus is on the larva to eventually find a suitable location for its metamorphosis, growth, survival, and eventual reproduction.

These planktonic larvae are stimulated to settle out of the water column and initiate metamorphosis by a so-called settlement cue that emanates from a favorable environment (Hadfield & Paul 2001). These settlement cues can signal that conspecific mates, food sources, or beneficial environments for post-metamorphosis development are nearby (Pechenik 1990, Pawlik 1992). While the specific chemical settlement cues for the vast majority of marine invertebrates are unknown, cues of microbial origin have been found to induce settlement in some species since the 1950's; research over the years has shown that these microbial cues can induce settlement and metamorphosis in animals from many different phyla (Wilson 1955, Hadfield 2011). Hadfield has recently reviewed

the species whose larvae have been found to settle in response to biofilms (Hadfield 2011). While Hadfield's review highlights the animals that respond to biofilms, it also points out that we do not know what inductive chemicals the microbes are producing or how the larvae sense these microbial cues. More recently, Shikuma et al. (2014) have found that the marine bacterium *Pseudoalteromonas luteoviolacea* produces phage tail-like structures that stimulate larvae of the polychaete worm *Hydroides elegans* to metamorphose (Shikuma et al. 2014). Also, Tebben et al. (2011) have found that four strains of the bacterium *Pseudoalteromonas* produced the chemical tetrabromopyrrole, which stimulated larvae of the coral *Acropora millepora* to metamorphose (Tebben et al. 2011). While we know that marine invertebrates across phyla are stimulated to settle and metamorphose in response to biofilms we still do not know whether there are common components of these biofilms that stimulate settlement, or if the cues emanating from biofilms are as diverse as the species that respond to them.

Capitella teleta, formerly *Capitella* sp. I, (Blake et al. 2009) is a small (~ 20 mm long x 1 mm wide) deposit-feeding marine polychaete found in salt-marsh sediments along the East coast of the United States (Grassle & Grassle 1976, Blake et al. 2009). *C. teleta* is an opportunistic species that is able to survive in hypoxic and high hydrogen sulfide environments such as those found in organically enriched benthic areas near marine sewage outfalls and fish aquaculture effluents (Grassle & Grassle 1976, Kunihiro et al. 2008, Blake et al. 2009). Due to its ability to survive under such stressful conditions, it is usually one of the most abundant organisms in these environments (Reish 1970, Grassle & Grassle 1976). Salt-marsh sediment contains a potent settlement cue for larvae of *C. teleta*; 90% of the larvae exposed to the sediment metamorphose within 30

minutes of treatment and some metamorphose within minutes (Dubilier 1988, Burns et al. 2014). When this sediment was combusted at 500 °C for 6 hours, the resulting ash did not induce metamorphosis, supporting the notion that the cue is an organic compound (Cohen & Pechenik 1999). Also, when the sediment was forced through a 0.22 μm filter, the filtrate did not stimulate any larvae to metamorphose; the cue could be bound to particles in the sediment larger than 0.22 μm (Cohen & Pechenik 1999). We have recently found that the B vitamins nicotinamide and riboflavin stimulated larvae of *C. teleta* to metamorphose at the low concentrations of 4 μm and 50 μm respectively, sometimes in less than 30 minutes (Burns et al. 2014).

Although we do not know whether inductive concentrations of these vitamins are present within the natural sediment, it would obviously be advantageous for deposit-feeders with limited mobility to settle in areas with adequate nutrients. Thus we hypothesized that marine microbes might be producing the settlement cues for *C. teleta* in the salt-marsh sediment. Indeed, *Shewanella* bacteria spp. actively secrete riboflavin to the external environment to aid with electron transport (Marsili et al. 2008). The bacterium *Micrococcus luteus* also produces large amounts of riboflavin when grown in the presence of pyridine (Sims & O'Loughlin 1992).

In this study, we aimed to isolate and identify inductive marine microbes from salt-marsh sediment that promotes rapid metamorphosis of *C. teleta*. After isolating these microbes we then used analytical chemical techniques to determine what chemical(s) the microbes produce that act to induce metamorphosis and in particular to determine whether the microbes might be producing inductive vitamins.

Materials and Methods

Rearing Capitella teleta

Adults of *Capitella teleta* were provided by Dr. Judith Grassle (Rutgers University) and maintained in 9 cm glass dishes containing 30 psu Instant Ocean artificial seawater (hereafter called ASW) at 18°C. Sediment collected from the Little Sippewissett salt-marsh (Falmouth, MA) was sieved through a 1mm wire mesh screen, frozen for at least 24 h, aerated, and provided as food *ad libitum* (Dubilier 1988, Pechenik & Cerulli 1991). Cultures were searched for brooding females every 2-3 days; brooding individuals were then transferred individually to 6 cm glass dishes containing ASW. Dishes containing brooding females were checked daily for swimming larvae; larvae were then pipetted into a separate, clean 6 cm glass dish containing approximately 15 mL ASW. All experiments used larvae released only within the previous 24 hours. *C. teleta* larvae are usually competent to settle and metamorphose within minutes after their escape from the brood tube (Grassle & Grassle 1976, Pechenik & Cerulli 1991), so that all larvae should have been competent at the start of our experiments.

Isolating Inductive Salt-Marsh Sediment Bacteria

Salt-marsh sediment obtained from Little Sippewissett salt-marsh (Falmouth, MA) was diluted with sterile ASW and streaked onto petri plates containing marine agar (Difco Marine Agar 2216). Anaerobic microbes were cultured in the same manner as above; however, the petri plates were incubated in BD GasPak EZ pouches in order to exclude oxygen from the microbial environment. To test the inductive capacity of each microbial colony, individual colonies were added to 60 mm glass petri dishes containing 10 ml ASW and 10 larvae. The individual colonies, representing 6 different colony morphologies including 2 orange, 3 white and 1 yellow colony, were tested in triplicate;

settlement and metamorphosis was monitored under a dissecting microscope. No aerobically grown colonies induced larvae to settle; however, an anaerobically grown colony did display a slight amount of inductive activity for settlement and metamorphosis within three hours of transfer to assay dishes (data not shown). Pure colonies were then prepared from this colony by re-streaking the plates, and this microbe was further cultured in 80 mL of broth that was a combination of Difco Marine Broth 2216 and ½ strength Difco Thioglycollate Fluid Medium in closed 100 mL glass screw top bottles. A black bacterial biofilm developed in the bottom half of the bottles after 2 to 3 days, and this biofilm was then allowed to develop for 2 weeks. To assess the inductiveness of settlement and metamorphosis by this biofilm, 1 ml aliquots of the biofilm were centrifuged at 10,000 RPM in a microfuge, and the bacterial pellets were then resuspended in 1 ml ASW and again centrifuged at 10,000 RPM. Pellets were weighed and then resuspended in 500 microliters of ASW and tested for inductive capacity by adding aliquots of up to 200 microliters to assay dishes, and settlement and metamorphosis was observed. Biofilms prepared from this colony and the ATCC culture of *Desulfovibrio desulfuricans aestuarii* were found to be inductive and were used for all subsequent studies.

Bacterial Species Identification

Gram staining and microscopy of the inductive colony revealed the bacteria to be gram-negative with a *Vibrio* morphology. Bacterial DNA was then extracted using UltraClean Microbial DNA Isolation kits following manufacturer's protocol (MoBio Laboratories Inc, Carlsbad, CA USA). Following DNA extraction, a region of the 16S rRNA gene was amplified via PCR to determine the bacteria's genus and possibly species

by characterizing sequence differences in this gene (Wintzingerode et al. 1997). This 16S region was amplified using universal eubacterial primers 8F and 1492R (Eden et al. 1991, Heuer et al. 1997). The PCR mixture contained: 5 µl of 5x Green GoTaq Reaction Buffer (Promega), 5 µl template DNA, 11.95 µl water, 1.5 µl of 25 mM MgCl2 (Promega), 0.15 µl (100 pM/µl) of each primer, 1 µl of dNTP Mix (10 mM) (Promega), and 0.25 µl GoTaq Polymerase (50 units/µl) (Promega) for a total reaction volume of 25 µl. PCR was conducted using a thermocycler (Model 2700, GeneAmp PCR System, Applied Biosystems) with the cycling parameters of 95°C for 5 min, then 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Success of the PCRs was determined via agarose gel electrophoresis, ethidium bromide staining, and visualization of the gel under UV light. The PCR products were then purified using UltraClean PCR Clean-Up Kits according to the manufacturer's protocol (MoBio Laboratories Inc, Carlsbad, CA USA). These purified PCR products were then submitted for Sanger sequencing in both the forward and reverse directions using the 8F and 1492R primers at the Tufts Core Facility (Boston, MA) on an ABI 3130XL sequencer.

Upon receipt of the DNA sequencing results, both the forward and reverse were aligned by hand and assembled into one contiguous sequence, reconstructing the entirety of the amplified 16S fragment, using Mega 5.0 (Tamura et al. 2011). In order to determine which genus and species this bacterium belonged to, this contiguous sequence was submitted as a query for a BLASTn (Altschul et al. 1997) search of the nr database on NCBI.

Preparation and testing of extracts

Sediment from the Little Sippewissett salt-marsh, which shows high inductive activity for settlement and metamorphosis of the *C. teleta* larvae (Cohen & Pechenik 1999), was air dried overnight in plastic petri plates in a fume hood. 4 g of this dried sediment was then mixed with 5 mL of pure acetone and vigorously shaken by hand until a homogeneous mixture was attained. The solids were allowed to settle and the organic supernatant was transferred into microcentrifuge tubes and then centrifuged at 10,000 x G for 2 minutes. After centrifuging, the organic supernatant was again transferred to new microcentrifuge tubes taking care not to transfer any resulting pellet with it.

Larvae of *C. teleta* were then treated with this acetone extract to test if the extract still retained an inductive capacity. In these experiments, 150 μL of the extract was pipetted into small glass dishes (3 cm diameter) and allowed to air dry onto the bottom of the dish. Negative control dishes were prepared by pipetting the same volume of pure acetone into the dish and also letting it air dry. Once dry, 4 mL ASW was transferred to the dishes and 8 larvae of *Capitella teleta* were transferred into each dish and were observed hourly under 80 – 320x magnification for newly metamorphosed juveniles. Each treatment consisted of 3 replicates containing 8 larvae per replicate.

Thin layer chromatography

Because the acetone extract of the sediment induced settlement and metamorphosis (Fig. 1), the extract was spotted onto a 2.5cm x 10cm silica TLC plates (Analtech TLC Products) to further isolate the inductive chemical(s) present in the extracts. The TLC plate was developed in a mobile phase consisted of a mixture of 20% acetone and 80% hexane (v/v). After developing, the TLC plate was removed from the

developing chamber, air-dried, observed under 380 nm UV wavelength light and fluorescent spots were outlined with a graphite pencil.

The biofilm of the inductive bacterium was also prepared for TLC analysis. A 2 mL sample of the biofilm was first mixed with 5 mL of the TLC mobile phase described previously. This biofilm/mobile phase mixture was then sonicated at 50% duty cycle for 30 seconds. The sonicated mixture was allowed to settle and the organic mobile phase was then transferred to separate microcentrifuge tubes, centrifuged at 10,000 x G for 2 minutes, and the resulting supernatant was transferred and saved in new microcentrifuge tubes taking care not to transfer any of the pellet. TLC separations of this biofilm extract were carried out exactly as detailed previously.

In order to determine if any of the fluorescent spots on the TLC plate were inductive, the spots were scraped off the TLC plates and added into 3 cm bioassay dishes each containing 8 larvae of *C. teleta* in 4 mL ASW. Negative controls consisted of silica scraped from portions of the TLC plate where no analyte traveled through and no fluorescent spots were observed. The larvae were observed hourly under 80 – 320x magnification for newly metamorphosed juveniles. The TLC spots were tested for their inductive capacity a total of 3 times to yield 3 replicates of 8 larvae per replicate for each spot. The TLC separations for each substance were carried out a total 3 times. One spot from the TLC separations, which was light yellow in color, and dark purple in color under UV light exposure, was found to be a very effective inducer of settlement and metamorphosis of the larvae.

Menaquinone Identification

Because dark purple spots observed under UV light are indicative of quinones (Dunphy et al. 1971), and menaquinones are found in high concentrations in sediments and in association with *Capitella* (Kunihiro et al. 2011) we performed analyses to determine whether or not the inductive spot was a menaquinone or combination of menaquinones using HPLC/MS (Karl et al. 2014). In these analyses, salt-marsh sediment was poured into standard plastic petri plates and allowed to air-dry overnight in a fume hood. Inductive biofilms prepared from the bacterial isolate, *Desulfovibrio desulfuricans aestuarii* ATCC culture, inductive TLC spots, and dried sediment samples were analyzed. 0.1-0.2 g of each sample was weighed, mixed into 10 mL of dH_2O, and vortexed. 15 mL of 3:2 2-propanol:hexane was then be added and the mixture was vortexed again for 2 minutes, sonicated for 1 minute, and vortexed again for 2 minutes. The samples were then centrifuged for 5 minutes at 3000RPM and afterwards the top organic layer was saved and evaporated in a Speed Vac.

Solid phase extraction (SPE) was then conducted to further purify the quinones that are present in each sample. The evaporated samples were resuspended in pure hexane. Each 500 mg SPE column was conditioned with 4 mL of 3.5% ethyl ether in hexane and then 4 mL of pure hexane. Each hexane suspended sample was then vacuum pulled through the columns and afterwards the samples adhered in the columns were washed with 4 mL hexane. Finally, each column was washed with 8 mL of 3.5% ethyl ether in hexane and the elutate was collected and evaporated via Speed Vac.

Samples were then resuspended in 30 μL methylene chloride and 170 μL of aqueous solution (2M zinc chloride, 1M acetic acid, 1M sodium chloride) for HPLC/MS. Samples were then separated by a Kinetex reversed-phase C18 column (2.6 μm, 150 x 3.0

mm) at a flow rate of 1.0 mL/min using a mobile phase of methanol (MeOH) and methylene chloride (MeCl). The mobile phase gradient consisted of 100:0 (MeOH:MeCl) for the first 2.5 min and gradually adjusted to 70:30 (MeOH:MeCl) by 10 min. The elutate running out of the HPLC was then ionized for mass spectrometry using positive atmospheric pressure chemical ionization (APCI) with a spray chamber gas temperature of 350°C and a vaporizer temperature of 400°C. Nitrogen was used as a drying gas and run at 7.0 L/min and nebulized at 45 psig. Ionization capillary voltage was 3800V with a corona current of 5 μA.

Menaquinone Dose Responses

In order to assess the relative effectiveness of different menaquinones, dose-response bioassays were conducted using purified menaquinones. Menaquinone-4, menaquinone-7, phylloquinone, and ubiquinone-10 were purchased from Sigma Chemical and Menaquinone-9 from Santa Cruz Biological. Stock solutions of these quinones were made in acetone and aliquots of the stock solutions were tested in bioassays by adding aliquots of up to 40 microliters to 10 mg of silicon dioxide powder in 3 cm dishes. The acetone was allowed to evaporate off. Once dried, 4 ml ASW was added to each dish and 8 larvae were added to the treatment. The concentrations of 10, 25, 50, and 1000 nmol / mg silicon dioxide were established as 4 separate treatments for MK4, MK6, MK9, phylloquinone, and ubiquinone-10. The negative control treatments were prepared by pipetting 40 μL of pure acetone onto 10 mg of silicon dioxide in small glass dishes and were mixed and air-dried as the other treatments were. Each treatment consisted of 3 replicate dishes each containing 8 larvae per replicate.

Results

Isolation and identification of inductive bacteria

Of the seven different bacteria (based on different colony morphologies) isolated from the Little Sippewissett salt-marsh sediments, only one bacterial isolate, which was isolated from anaerobic plates, was weakly inductive in stimulating the *C. teleta* larvae to metamorphose. Colonies of this bacterium stimulated about 10 percent of the larvae to settle and metamorphose within three hours after the addition of the individual colonies (data not shown). This bacterium was grown further in an anaerobic broth culture in closed bottles, and developed a biofilm after 2 days that was allowed to further develop for two weeks. The biofilm of this organism proved highly inductive: 50 mg of the biofilm induced 100 percent larval settlement and metamorphosis within 1 hour.

Gram staining and microscopy showed this bacterium to be gram-negative and spiral shaped. The 16S ribosomal subunit sequence that was amplified via PCR yielded one product that was ~1400 bp long. The forward and reverse Sanger sequencing reads were assembled into a contiguous sequence that was 1407 bp long. The BLASTn search of the NCBI nr database yielded a close match to *Desulfovibrio oceani* (Finster & Kjeldsen 2009). This match had an E-value of 0.0, 1402/1408 identity, and only 2 gaps were added in the alignment.

TLC separations of the salt-marsh sediment and the inductive biofilm

The acetone extract of the salt-marsh sediment was found to stimulate at more than 30% of the larvae of *C. teleta* to settle and metamorphose within only 1-2 h of treatment (Fig. 1). The rate at which larvae were metamorphosing in response to the salt-marsh sediment extract appeared to be linear, with 100% of the larvae having

metamorphosed within 3.5 h of treatment. No larvae in the negative controls treated with acetone alone metamorphosed within 24 h.

The TLC separation of the salt-marsh sediment extract yielded several fluorescent spots detected under longwave (365 nm) UV light, only one of which stimulated the larvae of *C. teleta* to metamorphose; they did so in under one hour in all 3 replicates. No larvae of *C. teleta* metamorphosed with 24 h when treated with any of the other spots. The inductive spot was a dark purple color under UV light, appeared as a light yellow spot under regular light, and exhibited a retardation factor (Rf) of 0.8950 (+/- 0.25 SEM). TLC analysis also showed the presence of five different predominant spots producing a strong pink fluorescence under UV light exposure, believed to be chlorins derived from chlorophyll breakdown. These presumed chlorins were also tested but failed to promote any settlement or metamorphosis within 24 hours.

The TLC separation of the inductive biofilm extract yielded many fewer fluorescent spots than the sediment extract TLC separation. Larvae of *C. teleta* again only metamorphosed when treated with one of these fluorescent spots and did so in under an hour in all 3 replicates. None of the other spots stimulated any larvae of *C. teleta* to metamorphose within 24 h. The inductive spot again had a dark purple color, had a retardation factor of 0.88 (+/- 0.65 SEM), and was the farthest running spot. There was no difference in the retardation factor of the inductive purples spots between the salt-marsh sediment extract TLC separation and the inductive biofilm TLC separation (t-test, t = 0.1355 P = 0.9).

Menaquinone identification

Because quinones are known to exhibit a dark purple fluorescence under UV light (Dunphy et al. 1971), and menaquinones from bacteria have been found in marine sediments in association with *C. teleta* (Kunihiro et al. 2011), the sediments and TLC spots were analyzed for the presence of menaquinones by HPLC/MS. HPLC/MS analysis of the Little Sippewissett salt-marsh sediment showed the presence of phylloquinone, along with 8 different menaquinones (Table 1). Overall, menaquinones (MK) MK6, MK7, MK8, MK9, MK10, MK12, and MK13 were extracted from the sediment. This result is in agreement with those reported by Kunihiro et al. (2011), who found these different menaquinones in marine sediments receiving fish aquaculture wastes, and indicate presence of many different bacterial species in the sediments, as expected. The inductive biofilms from the bacterial isolates (*D. oceani*) and also from the ATCC purchased bacteria, however, were found to contain MK6 alone.

When the inductive dark purple TLC spot obtained from sediment extracts was analyzed via HPLC/MS, the spot was found to contain phylloquinone, MK6, MK7, MK8, MK9, and MK10, whereas only MK6 again was contained within the inductive dark purple spot from the TLC separation of the inductive biofilms (Table 1).

When the absolute amounts of the quinones contained within the salt-marsh sediment via HPLC/MS were determined, phylloquinone was found to be the most abundant (Fig. 2). Although the HPLC/MS analysis was capable of detecting MK4 and MK5, neither of those chemicals were found within the sediment. MK6 was the third most abundant menaquinone contained within the salt-marsh sediment, with an estimated concentration of 77.5 pmol / g sediment (dry weight). MK12 and MK13 were detected, although the amounts were too low to be accurately quantified.

Responsiveness to individual quinones

No larvae of *C. teleta* metamorphosed within 24 h when treated with MK4, MK7 or MK9 at any concentration (highest concentration tested was 1 mmol/mg silica). Similarly, *C. teleta* larvae did not respond to ubiquinone-10 or phylloquinone (vitamin K_1) tested at a highest concentration of 1 mmol /mg silica, no larvae settled or metamorphosed in response to the negative control treatments on the acetone-treated silicon dioxide powder.

Discussion

Overall we have found that menaquinone-6, isolated from the bacterium *Desulfovibrio oceani,* which was in turn isolated from Little Sippewissett salt-marsh sediment, stimulated larvae of *C. teleta* to metamorphose in less than one hour. Biofilms and MK6 extracted from the ATCC purchased *Desulfovibrio desulfuricans aestuarii* also rapidly stimulated larvae of *C. teleta* to metamorphose. Both of these bacterial species produce MK6 as their only respiratory quinone. None of the other tested quinones (MKs 4, 7, 9, ubiquinone-10, and phylloquinone) stimulated any larave of *C. teleta* to metamorphose within 24 h even at the high concentration of 1 mmol/mg of silica they were adhered to. Menaquinone-6 isolated via TLC from *D. oceani* and *D. aestuarii* stimulated all tested larvae to metamorphose in less than one hour.

Menaquinones are chemicals produced by bacteria to assist with electron transport in their respiration process (Collins & Jones 1981). Structurally, they consist of a rhodoquinone ‘head’ and a ‘tail’ composed of a repeating number of isoprenoid units that vary in the number of units between bacterial species (Fig. 3A). The number of isoprenoid units in the menaquinone’s tail is given in the menaquinone’s name:

menaquinone-4 contains 4 isoprenoid units, menaquinone-12 contains 12 isoprenoid units. Menaquinones comprise the K_2 group of vitamins which have been almost exclusively studied in vertebrates (Suttie 1995). Phylloquinone (vitamin K_1) is produced by plants and is converted to MK4 in vertebrate tissues (Suttie 1995). Overall, vitamin K is required by vertebrates to aid in the calcification of a variety of tissues; animals that are deficient in vitamin K may have reduced bone density and have poor blood clotting ability (Booth 2009). However, vitamin K is among the least studied vitamins in vertebrates, and even less is known about the role it may play, if any, in invertebrates.

Experiments have found that when some invertebrates are injected with radiolabeled menaquinone precursors, menaquinone-4 is later found in their tissues. The tested invertebrates include the earthworm (*Lumbricus terrestris*), terrestrial snail (*Helix pomatia*) (Martius et al. 1965), European Green Crab (*Carcinus maenas*), the common starfish (*Asterias rubens*), the common sea urchin (*Echinus esculentus*), beadlet anemone (*Actinia equina*), and the common whelk (*Buccinum undatum*) (Burt et al. 1977). Vertebrates require MK4 in the gamma-carboxylation process of glutamate amino acids to synthesize blood-clotting zymogens; invertebrates do not use blood clotting zymogens so it is unlikely that MK4 is required for this function (Burt et al. 1977). It has been hypothesized that vertebrate blood-clotting zymogens evolved from proteolytic digestive enzymes; perhaps invertebrates require MK4 to synthesize certain proteins with digestive functions (Burt et al. 1977). It is important to note that these early studies did not use germ-free animals; their gut microbiota may have been responsible for the synthesized MK4. It is not known whether invertebrates can survive when deprived of vitamin K or

its precursors; and may be especially hard to test because their gut microbiota may be responsible for synthesizing a large portion of this vitamin requirement.

Populations of *C. teleta* thrive in locations that have high concentrations of organic matter and high nutrient inputs such as salt-marsh mud flats, sewage outfalls, and fish farms (Reish 1970, Grassle & Grassle 1976). The sediments in these areas are often rich in hydrogen sulfide and low in oxygen, creating a stressful environment that excludes many macroinvertebrate species. *C. teleta* may have evolved larvae that rapidly settle and metamorphose in response to MK6 because its presence could indicate the presence of sulfur-reducing bacteria such as *Desulfovibiro spp*. that also thrive in organic-rich, high-sulfur, low-oxygen environments. Currently there is no known menaquinone receptor so how the larvae of *C. teleta* are sensing MK6 is thus an open question. Menaquinones are lipid soluble and thus could possibly cross the larvae's cell membranes and stimulate settlement and metamorphosis intracellularly, without the need for an external chemosensory receptor (Dunphy et al. 1971).

Menaquinone-6 acting as a settlement cue for larvae of *C. teleta* also fits with data from previous *C. teleta* settlement studies. Larvae of *C. teleta* prefer to settle in sediments with high organic contents, and when the organic portion of the sediment is combusted away, the resulting ash did not stimulate larvae to metamorphose (Cohen & Pechenik 1999, Thiyagarajan et al. 2005). Also, when sediment is filtered through a 0.22 μm filter, the resulting filtrate did not stimulate larvae to settle or metamorphose (Cohen & Pechenik 1999). Because menaquinones are not water soluble, they could be tightly bound to small sediment particles – sediment samples without these small particles were not inductive. In keeping with work by Biggers and Laufer, larvae of *C. teleta* settled

and metamorphosed in response to the crustacean juvenile hormone methyl farnesoate, which is similar to MK6 because both chemicals contain farnesyl groups (Biggers & Laufer 2004). Methyl farnesoate contains one farnesyl group while the repetitive isoprenoid 'tail' of MK6 composes two farnesyl groups (Fig. 3B). Whatever enzyme was binding methyl farnesoate in earlier studies could actually have a greater binding affinity for MK6.

Menaquinone-6 may be more reliable than low-oxygen or high amounts of hydrogen sulfide as an indicator of a healthy sediment decomposition community. Hydrogen sulfide could be present in an area because of geological phenomena and oxygen could be displaced by other gasses having nothing to do with decomposition or sediment organic content. MK6, on the other hand is mainly produced by delta and epsilon proteobacteria (Kersters et al. 2006). These bacterial taxa contain marine sulfur reducing genera and other decomposers such as *Desulfobacter*, *Desulfovibrio*, *Geobacter*, *Campylobacter*, *Helicobacter*, and *Sulfurospirillum* (Kersters et al. 2006). Larvae of *C. teleta* could be using MK6 as an indicator to assess whether a sediment contains a microbial community that is supported by the same environmental conditions that *C. teleta* prefer. Indeed, microbial ecologists even use the menaquinone content of particular sediment samples to estimate the bacterial diversity that is present (Kunihiro et al. 2008). This is possible because different species of bacteria produce menaquinones of different lengths.

Is MK6 simply a settlement cue that indicates a particular sediment's suitability for *C. teleta*, or is it also a required vitamin for juveniles and adults of this species? So far, the only other other study to find vitamins stimulating metamorphosis was also

conducted on *C. teleta*; the B vitamins nicotinamide and riboflavin both stimulated substantial and rapid settlement and metamorphosis (Burns et al. 2014). It would be advantagous for larvae of deposit-feeding animals to make a direct assessment of the sediment's nutritional content before settling and metamorphosing. While B vitamins have been shown to be required by the annelid *Enchytraeus fragmentosus*, it is not known whether vitamin K is a dietary requirement (Gotthold & Koch 1974). Future studies should examine whether the larvae of other deposit-feeding animals are stimulated to settle and metamorphose by vitamins to determine if this is a widespread phenomena. Also, the role that vitamin K plays in invertebrates needs further characterization.

Acknowledgements

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Tables

Table 4.1. Menaquinones (MK) and phylloquinone (PK) detected in mud flat sediment samples, biofilms, and TLC isolates via HPLC/MS using the methods detailed in Karl et al. 2014. Y indicates that a quinone was detected in that given sample while a N indicates that a quinone was not detected for the sample. Most quinones have a lower limit of detection of 5 pmol / g. MK10 can be detected at 1 pmol / g. MK6 can be detected at 10 pmol / g. PK and MK4 can be detected at 30 pmol / g. Notice that MK6 was detected in every sample that induced larvae of *C. teleta* to metamorphose.

Sample	Quinone Detected (Y/N)?										
	MK4	MK5	PK	MK6	MK7	MK8	MK9	MK10	MK11	MK12	MK13
Mud Flat Sediment	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y
Mud Flat Inductive Spot	N	N	Y	Y	Y	Y	Y	Y	N	N	N
D. oceani Biofilm	N	N	N	Y	N	N	N	N	N	N	N
D. aestuarii Biofilm	N	N	N	Y	N	N	N	N	N	N	N
D. oceani Inductive Spot	N	N	N	Y	N	N	N	N	N	N	N
D. aestuarii Inductive Spot	N	N	N	Y	N	N	N	N	N	N	N

Figures

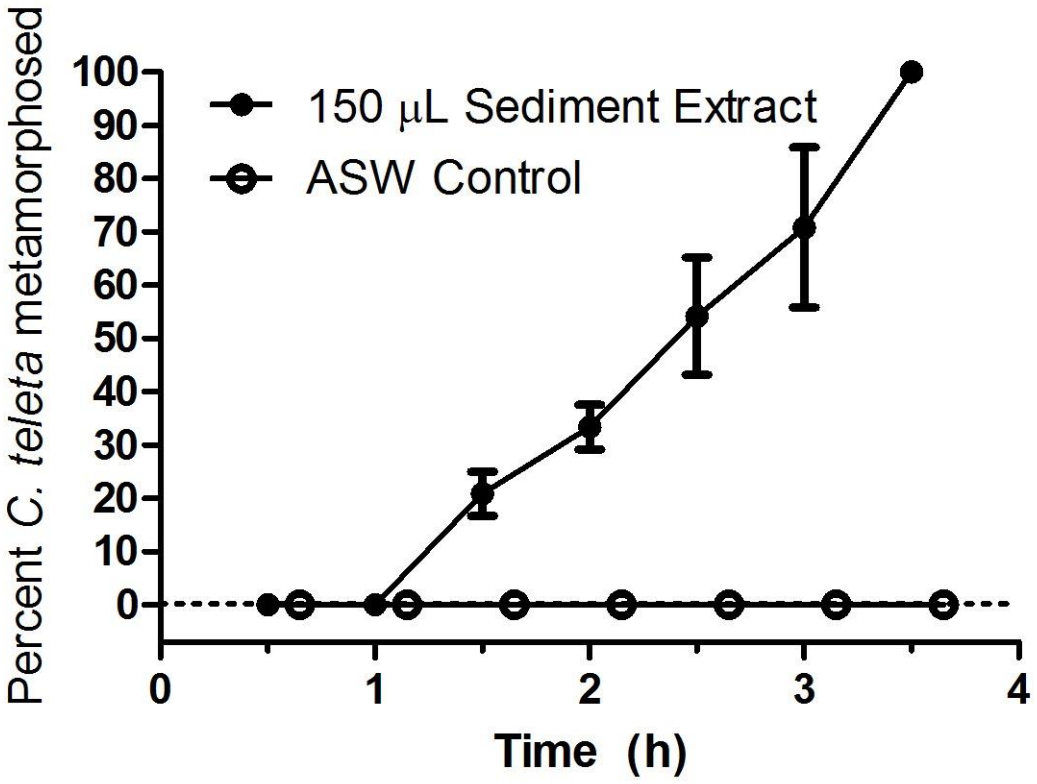


Figure 4.1. Promotion of metamorphosis by an acetone extract of Little Sippewissett salt-marsh sediment in larvae of *C. teleta*. 150 μL of this sediment extract was dried onto the bottom of each replicate's glass dish. The artificial seawater control (ASW) acted as a negative control. Each treatment consisted of 3 replicates with 8 larvae per replicate. Error bars represent +/- 1 SEM.

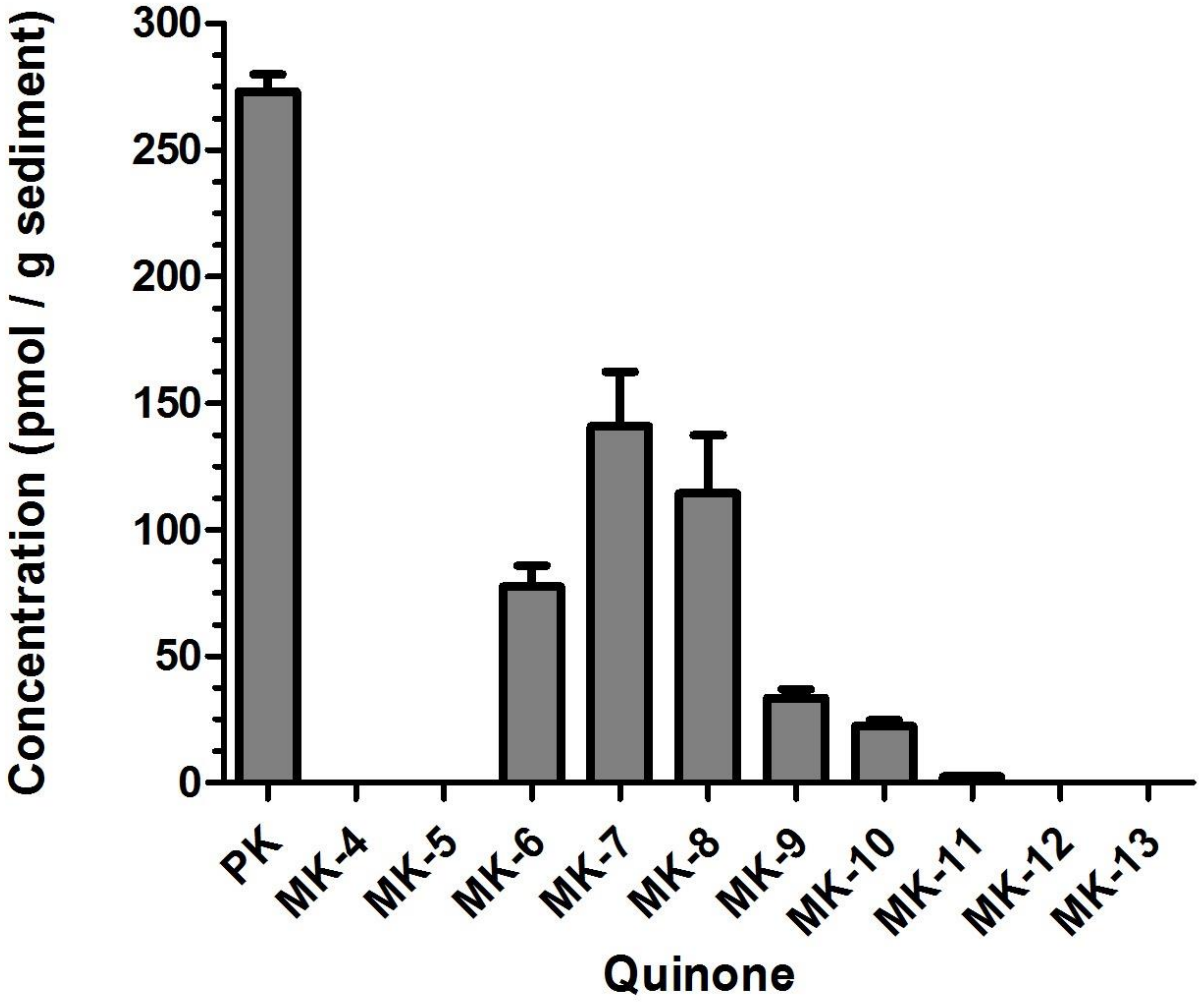
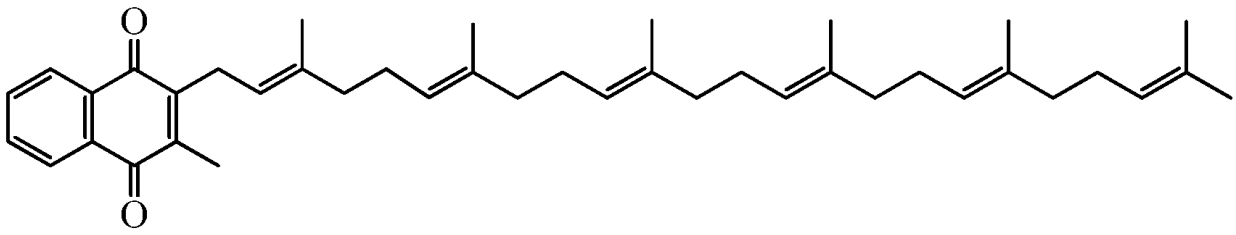


Figure 4.2. The absolute amounts of phylloquinone (PK) and menaquinones (MK) 4-13 contained in Little Sippewissett salt-marsh sediment as determined via HPLC/MS. Error bars represent + 1 SEM (n=2).

A



B

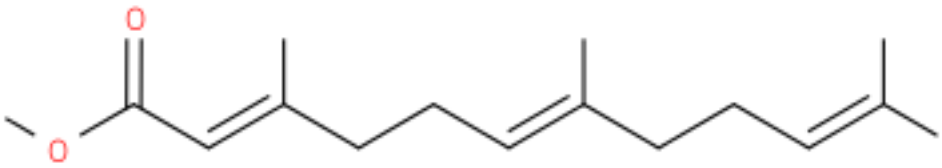


Figure 4.3. The chemical structures of menaquinone-6 (A) and methyl farnesoate (B). Notice that the repeating isoprenoid chains produce 1 farnesyl group in methyl farnesoate and 2 farnesyl groups in menaquinone-6.

Chapter 5

The bacterial community composition of salt-marsh sediments that rapidly stimulate larvae of the polychaete *Capitella teleta* to metamorphose

Abstract

Since the 1950's, it has been known that microbes associated with a benthic substrate can stimulate the larvae of marine invertebrates to settle and metamorphose. We have previously found that larvae of the salt-marsh deposit-feeding polychaete *Capitella teleta* were stimulated to settle and metamorphose when treated with menaquinone-6 synthesized by *Desulfovibrio* spp. These bacteria are found in salt-marsh sediment and this is one of the few cases where a specific bacterium and the chemical it synthesizes have been characterized as settlement cues. However, the presence of a settlement cue does not guarantee juvenile and adult success and ecological traps could exist for *C. teleta*. To better understand *C. teleta's* settlement preferences, we sampled a variety of different sediments throughout the Little Sippewissett salt-marsh. We then determined how quickly larvae settled in each sediment sample and the percent organic content of these samples. We also conducted high-throughput 16S rRNA gene sequencing to characterize the bacterial community composition of each salt-marsh site and determined if larval settlement patterns were associated with certain bacteria. Overall, the best predictor of whether larvae of *C. teleta* would settle and metamorphose in response to a salt-marsh sediment sample was the presence of a conspicuous surface biofilm on the sediment. All of the sediment samples that induced rapid settlement and metamorphosis contained this biofilm and more than 0.5% organic content, the minimum organic content required for juveniles of *C. teleta* to successfully reach reproductive maturity. High 16S rRNA abundances from *Desulfobulbaceae* and *Chromatiaceae* spp. could indicate productive bacterial communities are in these sediments that rapidly stimulated larvae of *C. teleta* to metamorphose. Larvae of *C. teleta* may settle in areas

with high abundances of *Chromatiaceae* spp. because of the high concentrations of nutrients these photosynthetic bacteria contain.

Introduction

As the microscopic larvae of marine invertebrates drift powerlessly with the tides and currents of the oceans they may pass through many different environments. Eventually, larvae of marine invertebrates will encounter a physical stimulus from an environment that is favorable for metamorphosis, juvenile development, and adult persistence (Pechenik 1990, Heyland et al. 2011). If larvae encounter this physical stimulus, called a settlement cue, the larvae will then settle out of the water column onto the benthic substrate and initiate metamorphosis. Settlement cues that have been characterized for different species of marine invertebrates are often chemicals that either signal that a potential food source or that conspecific mates are nearby (Pawlik 1992, Hadfield & Paul 2001).

Since the 1950's, it has been known that microbes associated with a benthic substrate can stimulate the larvae of marine invertebrates to settle and metamorphose (Wilson 1955). Over the years more cases have been found where biofilms, communities of microorganisms and the products they secrete, stimulate the larvae of marine invertebrates to settle and metamorphose (Hadfield 2011). However, in most cases, it is not known which microbial species or which chemicals they synthesize actually induce the larvae of many marine invertebrates to settle and metamorphose.

Previously, we have found that larvae of the salt-marsh deposit-feeding polychaete *Capitella teleta* were stimulated to settle and metamorphose when treated with the biofilm of the bacterium *Desulfovibrio oceani* (Chapter 4). This bacterium was isolated from the organic rich salt-marsh sediment that stimulates larvae of *C. teleta* to rapidly settle and metamorphose, often in less than 30 minutes (Dubilier 1988, Cohen &

Pechenik 1999). We then found that menaquinone-6, a respiratory quinone synthesized by *D. oceani*, was the chemical that stimulated larvae of *C. teleta* to rapidly settle and metamorphose.

Does the presence of a settlement cue indicate that juvenile and adult success is likely? Could ecological traps exist for *C. teleta*? Their native salt-marshes are not homogeneous habitats consisting of organic rich sediment alone. While there are organic rich mud-flat areas where *Capitellid* worms are found, much of the tidal action takes place though tidal channels that are exposed to heavy erosive forces. These tidal channels are sandy and may not have much organic matter available for deposit-feeding organisms. Juveniles of *C. teleta* require sediment with at least 0.5% organic content to successfully develop to reproductive maturity (Ramskov & Forbes 2008). Here, encountering an inductive concentration of menaquinone-6 or another cue in an area without sufficient sediment organic content would spell disaster for dispersing individuals of *C. teleta*. It would be advantageous for larvae to assess multiple cues associated with a favorable environment instead of metamorphosing in response to one possibly out of place cue.

The larvae of *C. teleta* do display remarkable choice for organic rich sediments in both still-water and flowing-water substrate choice experiments. In these experiments almost all larvae of *C. teleta* chose to settle in sediment plots instead of plots composed of glass beads regardless of the tested range of water flow rates (Butman et al. 1988, Ann Butman & Grassle 1992). However, nature is rarely this discrete with resource and habitat divisions. Larvae dispersing through a salt-marsh may encounter a gradient of

sediments with varying organic contents. How do they choose where to settle in this natural scenario?

To better understand *C. teleta's* settlement preferences, we sampled a variety of different sediments through the Little Sippewissett salt-marsh (Falmouth, MA) ranging from the very sandy tidal channels to mud-flat ooze that is known to be inductive. We then determined how quickly larvae settled in each sediment sample and the percent organic content of these samples. Finally, we conducted high-throughput 16S rRNA gene sequencing to characterize the bacterial community composition of each salt-marsh site to determine if larval settlement patterns are associated with bacteria other than *Desulfovibrio* spp.

Materials and Methods

Sediment Sample Collection and 16S rRNA Gene Sequencing

Sediment samples were collected on September 29th, 2014 from the Little Sippewissett salt-marsh in Falmouth, MA. These sediment samples were collected from a mud flat and 6 sites throughout a tidal channel at low tide that larvae of *C. teleta* could be exposed to as they drift with the tide and current (Fig. 1A). A conspicuous white surface biofilm on sandy sediment was also sampled at one site (Fig. 1A). The top 2 cm of sediment at each site was collected in sterile plastic 5 mL tubes and care was taken to collect sediment upstream of where I walked so that displaced sediment did not contaminate other collection sites. At each collection site in the tidal channel sediment samples were taken from the middle of the channel and either the side or wall of the channel just below the water line (Fig. 1B). Every sample was collected in duplicate

within 10 cm of each other. Samples were stored in a styrofoam box containing ice packs during the journey back to the lab where they were then frozen at -80 °C.

DNA was then extracted from each sediment sample using MoBio PowerSoil DNA Isolation kits using approximately 10 mg of sediment (wet weight) per extraction following the manufacturer's protocol. Bacterial amplicon libraries were then synthesized from the V4 region of the bacterial 16S rRNA gene following the methods developed by (Maurice et al. 2013). DNA samples were amplified using PCR primers that also incorporate unique identification barcodes, Illumina sequencer adapter sequence, and primer sites to amplify the V4 region of the bacterial 16S rRNA gene. Each sample was amplified in duplicate to help control for pipetting error. PCR amplification success for each reaction was determined via gel electrophoresis. PCR products were then purified using Ampure XP magnetic beads and quantified using Qubit dsDNA HS fluorometry reagents following the manufacturers protocols. All samples were then diluted to the concentration of the sample with the lowest DNA concentration and pooled together in one tube. This DNA pool was then purified and further concentrated using a Qiaquick MinElute kit following the manufacturer's protocol. This pool was separated via gel electrophoresis, the ~381 bp sized band was cut out with a sterile razor and this gel section was extracted using a Qiaquick Gel Extraction kit following the manufacturer's protocol. This pool was quantified once more using Qubit dsDNA HS fluorometry reagents and was then diluted to 10 nM. The diluted library pool was then sequenced with an Illumina HiSeq 2500 at the Harvard FAS Center for Systems Biology. 100 base pair long Illumina reads were generated.

QIIME (Quantitative Insights into Microbial Ecology) 1.9.0 was then used for the following data processing and analyses (Caporaso et al. 2010). First, the libraries were demultiplexed back into their duplicate per sediment site samples while only allowing for 1 base mismatch between the primer barcode and the sequence barcode; barcode quality scores greater than or equal to a Phred score of 20 were also required. Operational Taxonomic Units (OTUs) were then defined by clustering Illumina sequences at 97% similarity using the UCLUST algorithm with the Green Genes 16S rRNA database (DeSantis et al. 2006, Edgar 2010). The bacterial community compositions of sediment samples that induced larvae of *C. teleta* to metamorphose (described later) were then compared to the community compositions of non-inductive sediment samples and the microbial taxa that were enriched/depleted in inductive samples were identified using LEfSe (Segata et al. 2011). If an OTU of specific interest was not assigned taxonomy to at least the genus level, a secondary NCBI BLASTn search to the NCBI non-redundant database was conducted to find a more specific match (Altschul et al. 1997). Only matches with a similarity greater than 97% were considered.

Sediment Organic Content

We calculated the percent organic content of each sediment sample; juveniles of *C. teleta* cannot grow to reproductive maturity in sediments containing < 0.5 % organic matter (Ramskov & Forbes 2008). Small (~2 cm diameter) aluminum foil weigh pans were made and pre-weighed. Three replicate sediment samples per site were deposited into these pans and dried in a 50 °C incubator; drying was considered complete when samples stopped losing mass. These samples were then combusted in a muffle furnace at 500 °C for at least 12 hours and reweighed. The percent organic content was calculated

by ((dry mass – combusted mass) / (dry mass)) * 100%. The pan mass for each sample was subtracted from each mass measurement prior to calculation.

Metamorphosis Assays

Adults of *Capitella teleta* were provided by Dr. Judith Grassle (Rutgers University) and maintained in 9 cm diameter glass dishes containing 30 psu Instant Ocean artificial seawater (hereafter called ASW) at 18°C. Sediment collected from the Little Sippewissett salt-marsh (Falmouth, MA) was sieved through a 1mm wire mesh screen, frozen for at least 24 h, aerated, and provided as food *ad libitum* (Dubilier 1988, Pechenik & Cerulli 1991). Cultures were searched for brooding females every 2-3 days; brooding individuals were then transferred individually to 6 cm diameter glass dishes containing ASW. Dishes containing brooding females were checked daily for swimming larvae; larvae were then pipetted into a separate, clean 6 cm glass dish containing ASW. All experiments used larvae released only within the previous 24 hours. *C. teleta* larvae are usually competent to settle and metamorphose within minutes after their escape from the brood tube (Grassle & Grassle 1976, Pechenik & Cerulli 1991), so that all larvae should have been competent at the start of our experiments.

In order to confirm that metamorphosis actually occurred and not simply settlement, supernatents of sediment ASW mixtures were used in the metamorphosis assays so metamorphosed juveniles could be observed. 2 mL of ASW was mixed with 0.7 cm^3 of sediment and vigorously vortexed. Solids were allowed to settle for 30 minutes and the supernatant was removed from the mixture. 12 larvae were tested individually for each site in plastic 12 well plates with each well containing 600 μL of the sediment mixture supernatant. A negative control treatment only contained ASW. Wells were

observed every 30 minutes for metamorphosed juveniles under a dissecting microscope. Survival analyses were used to calculate the differences between the sediment samples' speeds of inducing metamorphosis. Median time to metamorphosis was calculated for each site and log-rank Mantel-Cox tests were used to determine whether there were significant differences between the survival curves using Prism 6.0 (Graphpad Software).

Results

Only 5 of the 17 sediment sites stimulated larvae of *C. teleta* to rapidly settle and metamorphose with a median time to metamorphosis of approximately 3 h (Figs. 2 & 3). The remaining sediment samples did stimulate all of the tested larvae to metamorphose within 24 h; however, none of these samples stimulated any larvae to metamorphose within the first 9 hours (Fig. 3). The rates of rapid metamorphosis in response to the inductive sediment samples were not significantly different (log-rank Mantel-Cox test, $\chi^2 = 6.908$, df = 4, $p = 0.1408$). However, the survival curves of the inductive sediment samples were significantly different from those of the non-inductive sediment samples (log-rank Mantel-Cox test, $\chi^2 = 204.6$, df = 17, $p < 0.001$). No larvae had metamorphosed within 24 h in a negative control treatment containing only ASW. All of the sediment samples that rapidly induced metamorphosis had a mean percent organic content greater than 0.5% (Fig. 2). All of the inductive sediment samples besides the mud flat had obvious pink or white biofilms present on the surface of the sediment before collection (Fig 4. A & B). No other sites had conspicuous surface biofilms.

Of the 34 replicate 16S amplicon libraries, the smallest library contained 101,626 100 bp reads after sequence quality control and demultiplexing. In the smallest amplicon library, 99,975 sequences were successfully assigned taxonomies from the Green Genes

16S rRNA database. To ensure even sampling and to control for different sequencing depths across samples, samples from all sites were randomly rarefied to 99,750 16S sequences with assigned taxonomy before calculating mean OTU abundances for each site. Surprisingly, no significantly enriched or depleted taxa were found between the sediments that had induced rapid metamorphosis and those that did not (Kruskal-Wallis tests conducted with LEfSe, $p > 0.05$).

The 10 most abundant OTUs present in each inductive salt-marsh site's sediment mainly belonged to the *Proteobacteria* bacterial phylum, with the most abundant classes of this phylum being the *Delta, Gamma,* and *Epsilonproteobacteria* (Table 1). Every inductive sediment sample contained high abundances of OTUs belonging to the *Chromatiaceae* family of *Gammaproteobacteria*, commonly known as purple-sulfur bacteria. Likewise, OTUs belonging to the *Desulfobulbaceae* family of *Deltaproteobacteria* were also extremely abundant within inductive sediment samples. Some of the OTUs belonging to these bacterial families resulted in high similarity BLASTn matches to 16S sequences found within 'pink berries,' photosynthetic aggregates of bacteria found in Sippewissett salt-marsh sediment (Wilbanks et al. 2014) (Table 1). All inductive sediment sites contained high abundances of 16S rRNA sequences belonging to the chloroplasts of *Stramenopiles,* a phylum containing both algae and diatoms. Unfortunately, these algal sequences could not be classified more specifically.

Discussion

Overall, the best predictor of whether larvae of *C. teleta* would rapidly settle and metamorphose in response to a salt-marsh sediment sample was the presence of a

conspicuous surface biofilm on the sediment. No other site that was sampled had obvious surface biofilms. The larvae rapidly settled and metamorphosed in response to sediments that were covered by a pink biofilm on the side of site 5 (Fig. 4A), a white biofilm between sites 4 and 5, and a biofilm covering the middle and sides of site 6 that had both pink and white components (Fig. 4B). There was no conspicuous biofilm on the surface of the mud flat sediment; if a biofilm was present, it could have been difficult to observe because of the high amount of decomposing organic content (Fig. 2).

Larvae of *C. teleta* also did not prefer to settle in sediment samples based on their percent organic content. While the larvae did settle in response to the mud flat sediment, which contained the highest percent organic content, they also settled and metamorphosed in response to sediments with much lower organic contents such as the side of site 5 (Fig. 2). Sediments from the tidal creek walls of sites 3 and 5 did not stimulate rapid metamorphosis and had higher organic contents than many of the sediments that rapidly induced larvae to settle and metamorphose (Fig. 2). These results agree with those of Cohen & Pechenik (1999) who previously established that larvae of *C. teleta* do not settle and metamorphose more quickly in sediment samples with high organic contents. All of the sediment samples that induced rapid settlement and metamorphosis did contain more than 0.5% organic content, the minimum required for juveniles of *C. teleta* to successfully grow and reach reproductive maturity (Ramskov & Forbes 2008).

Interesting trends were found in the 10 most abundant OTUs in each inductive sediment sample. Some of the most abundant OTUs belong to the *Chromatiaceae* and *Desulfobulbaceae* bacterial families (Table 1). Of particular interest were the

Chromatiaceae and *Desulfobulbaceae* OTUs that had very similar BLASTn matches to 16S sequences observed within 'pink berry' photosynthetic aggregates of microbes found in the Little and Great Sippewissett salt-marshes (Wilbanks et al. 2014). These pink berries can range from several millimeters to centimeters in diameter (see Fig. 1 in Wilbanks et al. (2014)). 16S rRNA sequencing of these pink berries has shown that they are composed of purple-sulfur bacteria in the *Halochromatium-Thiohalocapsa* genera of the *Chromatiaceae*, sulfate-reducing bacteria in the *Desulfobulbaceae* family, as well as diatoms and *Bacteroidetes* species (Wilbanks et al. 2014). Many bacteria from these families are very abundant in the sediment samples that rapidly induced larvae of *C. teleta* to metamorphose (Table 1).

Documented interactions between bacteria in Sippewissett pink berries can provide insight into other biofilms we found in the Little Sippewissett salt-marsh. Within the pink berry bacterial aggregates a photosynthetic *Chromatiaceae* sp. oxidizes sulfide into sulfate while the sulfate-reducing *Desulfobulbaceae* sp. could reduce the produced sulfate back into sulfide for the *Chromatiaceae* sp. to use (Wilbanks et al. 2014). The syntrophic interaction between these two bacteria could prevent *Chromatiaceae* sp. mortality when environmental sulfide is limiting. While the pink berry system is currently the best known case of sulfur-based syntrophy between bacteria, sulfide oxidizing bacteria have been found in abundance in marine environments with no detectable sulfur indicating that these interactions could be widespread in the ocean (Wilbanks et al. 2014).

While no pink berries were found in our sediment samples, conspicuous pink and white biofilms along with high 16S rRNA abundances from *Desulfobulbaceae* and

Chromatiaceae spp. could indicate productive bacterial communities in the sediments that rapidly stimulated larvae of *C. teleta* to metamorphose. While the pink biofilms are indicative of *Chromatiaceae* spp., the white biofilms could also indicate the presence of these same species. In low oxygen conditions, *Chromatiaceae* spp. synthesize bacteriochlorphyll and other carotenoid pigments to photosynthesize. However, when higher concentrations of oxygen are present, the synthesis of pigments required for photosynthesis are inhibited and the bacteria switch to chemolithotrophy (Imhoff 2006). In these high oxygen conditions the pigments are bleached and the biofilms become a white color. Because our sediment samples were collected at low tide, it is possible that the biofilms were exposed to water with high oxygen concentrations and were bleaching. While *Chromatiaceae* spp. are usually restricted to low-oxygen, high-sulfide environments beneath the sediment surface, if the activity of sulfate reducing bacteria such as *Desulfobulbaceae* spp. is high enough, *Chromatiaceae* spp. can rise to colonize surface sediments and develop pink/red biofilms on the sediment surface (Imhoff 2006).

Here, larvae of *C. teleta* could be stimulated to rapidly settle and metamorphose in response to substantial biofilms composed of syntrophic sulfate reducing and sulfide oxidizing bacteria. The photolithoautotrophic *Chromatiaceae* spp. could be responsible for a large amount of the nutrition that *C. teleta* derive from the sediment that they burrow through and eat. Indeed, *Chromatiaceae* spp. have been found to contain high concentrations of sulfur containing amino acids (Vrati 1984, Imhoff 2006). Fish and chicken feed have also been amended with these bacteria, which consequently increased fish survival, the number of eggs chickens laid, and the size of the chicken eggs (Kobayashi & Tchan 1973, Imhoff 2006). All of the biofilmed sediments that rapidly

stimulated larvae of *C. teleta* to metamorphose contained more than 0.5% organic matter even though the underlying sediment was quite sandy (Fig. 2).

It would be interesting to grow these two species of bacteria in the lab in the absence of sediment to determine whether *C. teleta* can survive when fed this biofilm alone. Are there other nutrients in the sediment that are required for *C. teleta* to survive that are not present in biofilms? Also, what stimulated the larvae to rapidly settle and metamorphose when treated with these biofilmed sediments? Some *Desulfobulbaceae* genera do synthesize meanquinone-6 as their respiratory quinone (Kuever 2014). The *Chromatiaceae*, however, do not synthesize menaquinone-6 (Imhoff 1984). Menaquinone-6 was previously found to rapidly stimulate larvae of *C. teleta* to metamorphose and it was also present within inductive salt-marsh sediments (Chapter 4). More work must be done to determine whether inductive concentrations of menaquione-6 are present within these inductive biofilms. Also, the long-term persistence of these biofilms must be studied. Do these inductive biofilms persist long enough to support a generation of *C. teleta* from settlement to reproductive maturity? If these biofilms persist for less than the 4-6 weeks required for one generation of *C. teleta* to complete they may be an ecological trap (Blake et al. 2009).

Acknowledgements:

We thank Dr. Ben Wolfe (Tufts University) for lending us bench space, reagents, and space on sequencing lanes for the 16S sequencing analyses. We also thank Dr. Judith Grassle (Rutgers University) for giving us individuals of *C. teleta* to begin our own cultures.

Tables

Table 5.1. The 10 most abundant operational taxonomic units (OTUs) present within inductive salt-marsh sediment samples. If an OTU was not assigned taxonomy to at least the genus level a secondary NCBI BLASTn search was conducted to determine a more specific match. % Similarity is the percent match between the OTU sequence and the best match with an assigned taxon. Blank spaces indicate that the OTU taxonomy could not be resolved further.

Site	Mean OTU abundance	Family	Genus	Species	% Similarity	NCBI accession ID
White Bio-film	6399	Chromatiaceae	Marichromatium	litoris	99	ref|NR_133046.1|
	5826.5	Stramenopiles chloroplast sequence (algae)				
	5450.5	Chromatiaceae	Thiohalocapsa	marina	100	ref|NR_115047.1|
	4298.5	Campylobacteraceae	Sulfurospirillum			
	3225	Stramenopiles chloroplast sequence (algae)				
	1420.5	Desulfobulbaceae				
	1090.5	Stramenopiles chloroplast sequence (algae)				
	964	Granulosicoccaceae	Granulosicoccus	coccoides	99	ref|NR_104509.1|
	887	Stramenopiles chloroplast sequence (algae)				
	877	Desulfobulbaceae				
Site 5 Side	24234	Chromatiaceae	Thiohalocapsa	marina	100	ref|NR_115047.1|
	4798	Flavobacteriaceae	Olleya	aquimaris	99	emb|LK022274.1|
	4197.5	Desulfobulbaceae				
	3040.5	Stramenopiles chloroplast sequence (algae)				
	1959	Chromatiaceae	Marichromatium	litoris	99	ref|NR_133046.1|
	1579	Chromatiaceae				
	1325	Stramenopiles chloroplast sequence (algae)				
	1132.5	Thiohalorhabdaceae				
	1049	Granulosicoccaceae	Granulosicoccus	coccoides	99	ref|NR_104509.1|
	937	Chromatiaceae	Pink Berry Isolate		99	gb|KF513045.1|
Site 6 Middle	4919.5	Chromatiaceae	Thiohalocapsa	marina	100	ref|NR_115047.1|
	3505	Stramenopiles chloroplast sequence (algae)				
	2298.5	Stramenopiles chloroplast sequence (algae)				
	2071.5	Chromatiaceae	Marichromatium	litoris	99	ref|NR_133046.1|
	1762	Phormidiaceae	Phormidium			
	1424	Desulfobulbaceae				
	1377.5	Helicobacteraceae	Sulfurovum		100	gb|KF465254.1|
	1354	Chromatiaceae				
	1280.5	OM60	Congregibacter	Pink Berry Isolate	100	gb|KF513041.1|
	1175.5	Campylobacteraceae	Arcobacter			
Site 6 Side	3553.5	Desulfobulbaceae	Desulfobulbus	propionicus	98	ref|NR_042971.1|
	3319.5	Stramenopiles chloroplast sequence (algae)				
	2721	Stramenopiles chloroplast sequence (algae)				
	1346.5	Chromatiaceae	Marichromatium	litoris	99	ref|NR_133046.1|
	1282.5	Campylobacteraceae	Arcobacter			
	1197	OM60	Congregibacter	Pink Berry Isolate	100	gb|KF513041.1|
	1079.5	Stramenopiles chloroplast sequence (algae)				
	762	Rhodobacteraceae	Loktanella	rosea	100	gb|HM022774.1|

	752	Desulfobacteraceae	Pink Berry Isolate		100	gb|KF513074.1|
	689	OM60	Haliea		99	gb|KF722433.1|
Mud Flat	3409.5	Chromatiaceae	Marichromatium	litoris	99	ref|NR_133046.1|
	2289	Stramenopiles chloroplast sequence (algae)				
	1659.5	Helicobacteraceae	Sulfurovum	lithotrophicum	100	gb|CP011308.1|
	1646	Stramenopiles chloroplast sequence (algae)				
	1379.5	Helicobacteraceae	Sulfurovum		100	gb|KF465254.1|
	1221.5	Helicobacteraceae				
	1176	Desulfobacteraceae			100	gb|KF513074.1|
	1122	Stramenopiles				
	1008.5	OM60	Haliea		99	gb|KF722433.1|
	871.5	Flavobacteriaceae	Robiginitalea			

Figures

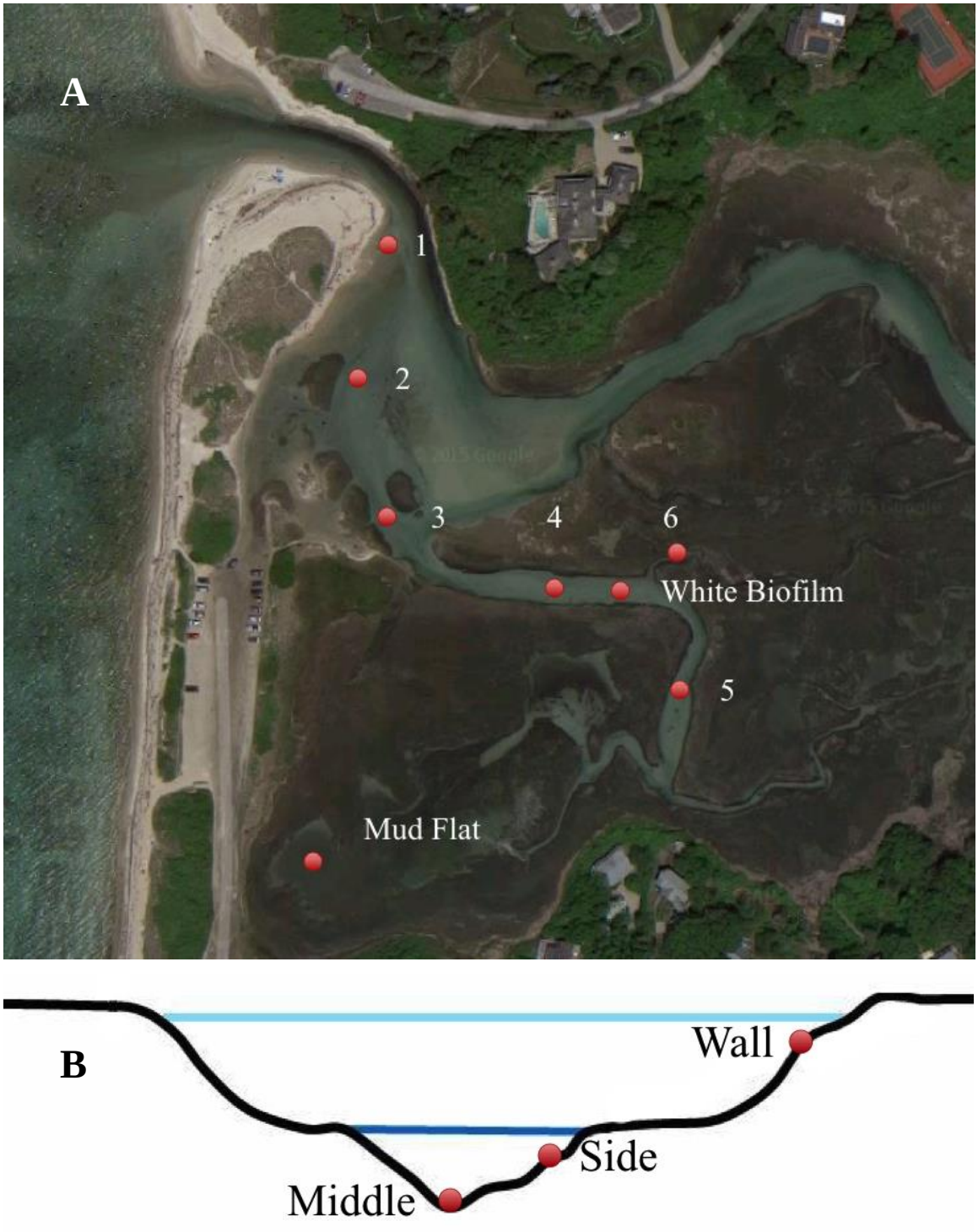


Figure 5.1. Sampling locations within the Little Sippewissett salt-marsh (Falmouth, MA). Actual sampling locations within the marsh are shown in (A). At each site samples of the top 2 cm of the middle, side, and the wall (if present) sediments were

sampled under the water level at low tide (B). The mud flat and white biofilm did not have these separate areas to sample. Image in (A) captured from Google Maps.

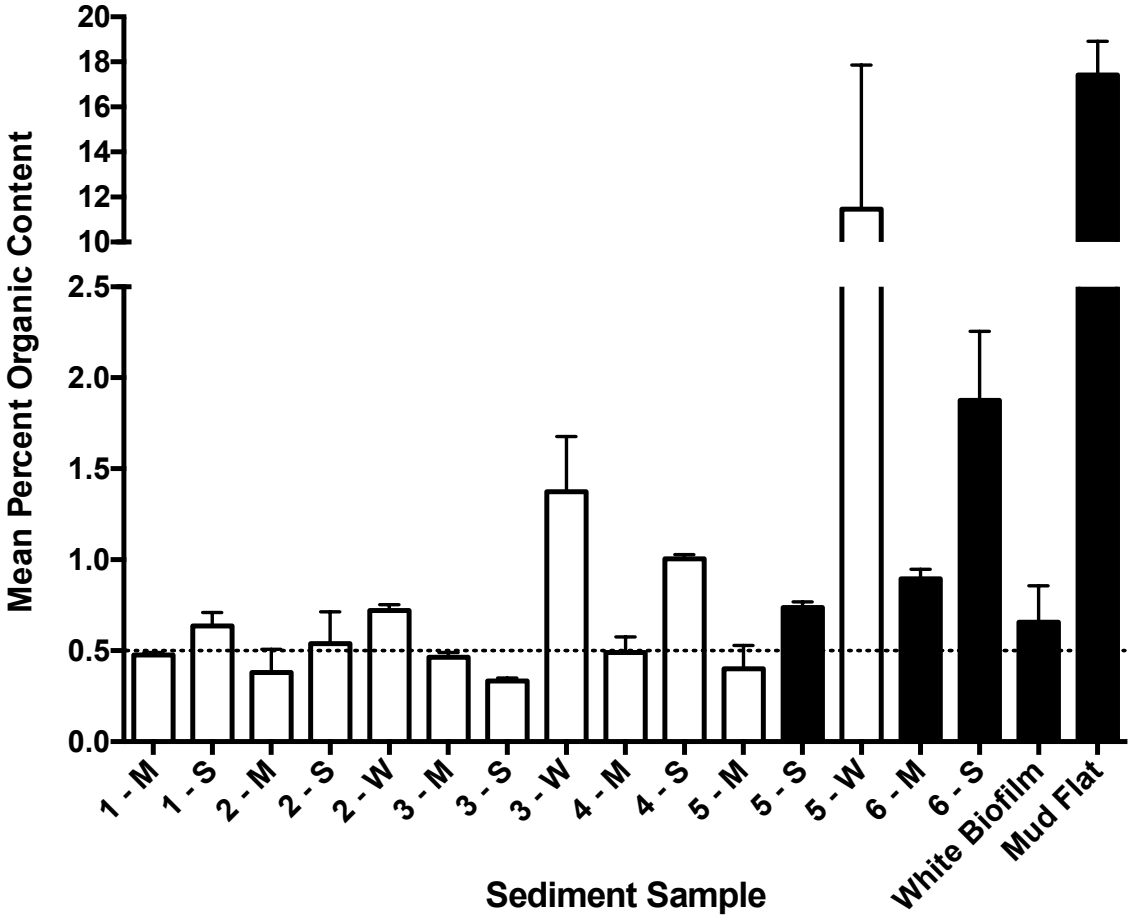


Figure 5.2. The mean percent organic content of sediment samples that did and did not induce larvae of *C. teleta* to metamorphose. 3 sediment samples from each site were dried and then combusted at 500 °C for at least 12 h and weighed to calculate the mean percent organic content of the sediment from each site. Black bars indicate sediment samples that stimulated the majority of tested larvae to metamorphose in less than 6 hours. Numbers on the x-axis indicate tidal channel site (1-6) while M, S, and W correspond to the middle, side, or channel wall. The dotted line at 0.5% indicates the percent organic content that individuals of *C. teleta* require in their diet to grow and successfully reproduce (Ramskov & Forbes 2008). Error bars indicate 1 standard deviation above the mean.

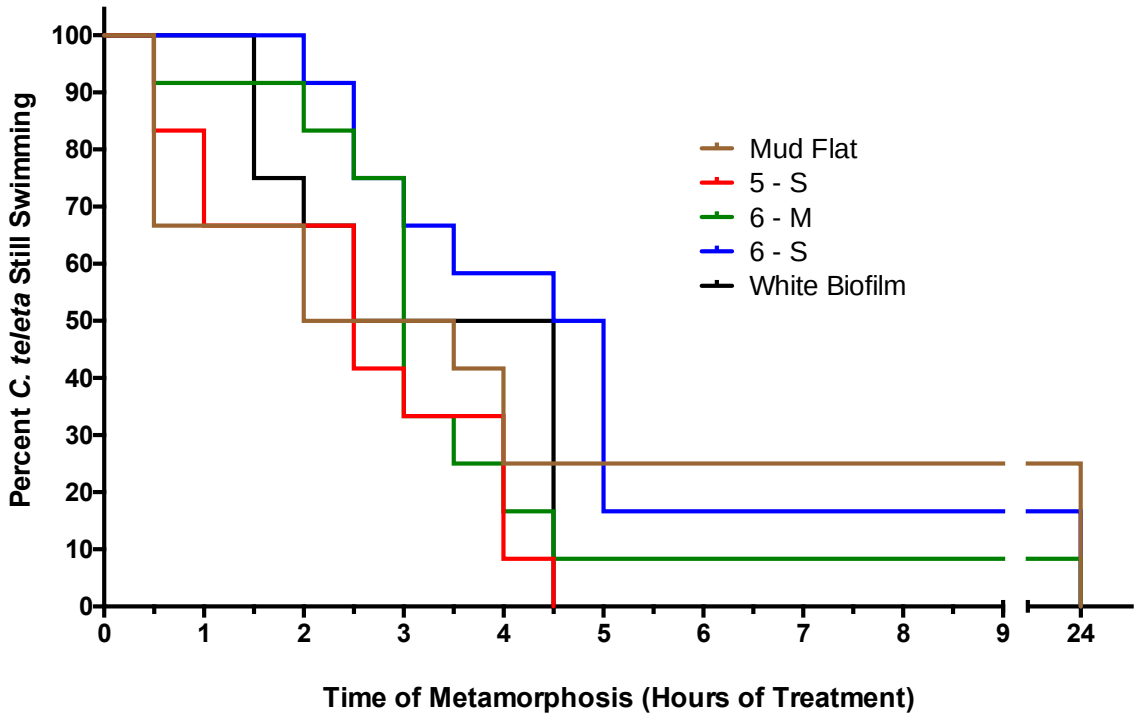


Figure 5.3. Rates of *C. teleta* metamorphosis in highly inductive salt-marsh sediments. These curves are not significantly different from each other (log-rank Mantel-Cox test, $\chi^2 = 6.908$, df = 4, $p = 0.1408$) but are significantly different from the rest of the non-inductive sediment samples (log-rank Mantel-Cox test, $\chi^2 = 204.6$, df = 17, $p < 0.001$).

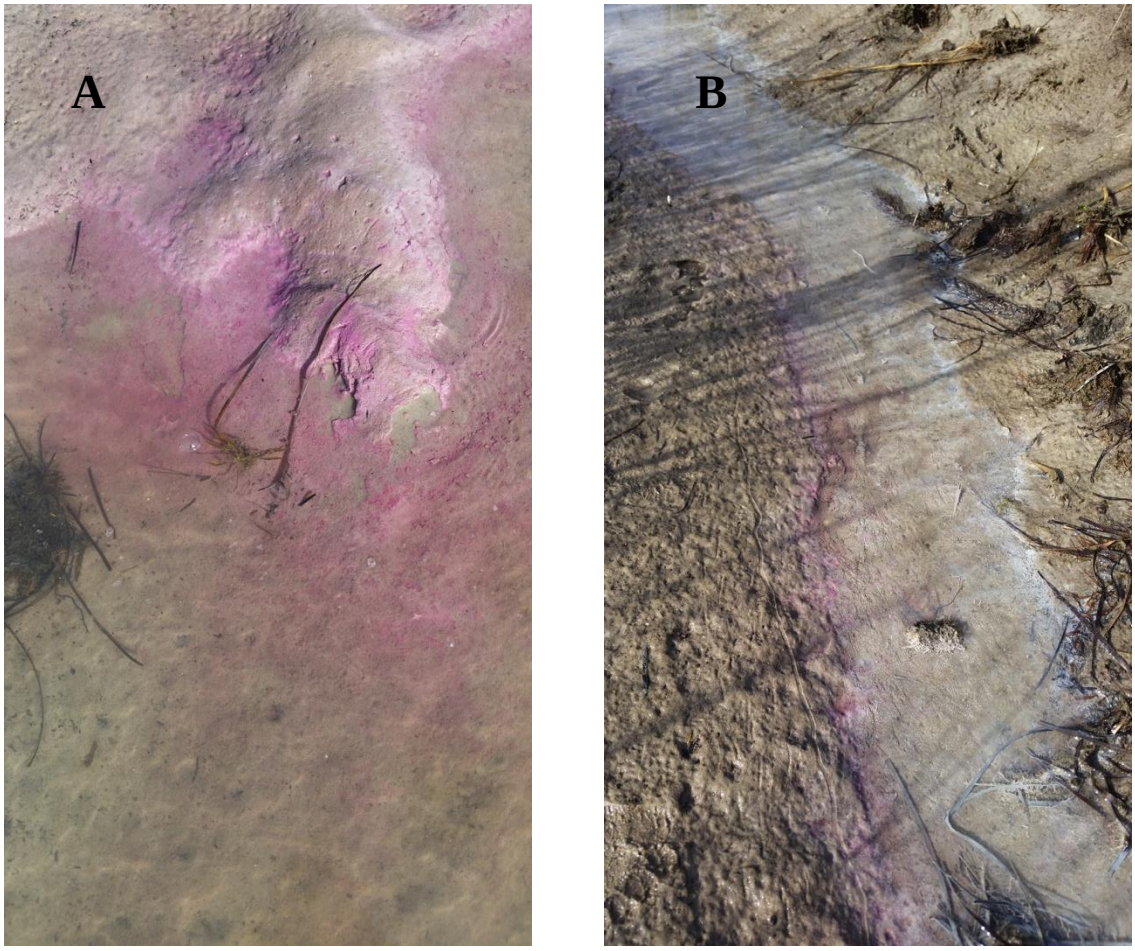


Figure 5.4. Conspicuous pink and white surface biofilms present on Little Sippewissett salt-marsh sediments. The site 5 side biofilm (A) was mostly pink while site 6 middle and side (B) was a mixture of white and pink biofilms.

Chapter 6

The transcriptomic basis of metamorphic competence in the salt-marsh dwelling polychaete, *Capitella teleta*

Abstract

Many marine invertebrates depend on their larvae as the dispersal mechanism for their species. In many of these species, the larvae are not capable of metamorphosing for hours to weeks after being released into the plankton. The mechanisms that govern this transition between pre-competent larvae which are not able to metamorphose to metamorphically competent larvae are unknown. We studied the pre-competent and competent larvae of the salt-marsh dwelling polychaete worm *Capitella teleta*, a species in which pre-competent larvae are unusually easy to distinguish from competent larvae. We sequenced the transcriptomes of the pre-competent and competent larvae of *C. teleta* to determine differences in gene expression between these two functionally different larval stages. By performing differential expression analyses, we found that 1541 genes were up-regulated in pre-competent larvae while 1063 genes were up-regulated in competent larvae. Pre-competent larvae had up-regulated the expression of genes belonging to gene ontologies having to do with growth and development while the competent larvae had up-regulated ligand-binding transmembrane channels with possible chemo and mechanosensory functions. The majority of these channels were annotated as being from the Degenerin/Epithelial sodium channel family or G-protein coupled receptor family; proteins from these families can have chemosensory functions. Serotonin and GABA receptors are among the genes that were up-regulated in competent larvae; both have been shown to induce larvae of *C. teleta* and other marine invertebrates to metamorphose and are thought to be components of the signal transduction pathway that leads to the initiation of metamorphosis. Overall, it appears that once larvae of *C. teleta* have completed development of internal structures required for juvenile life,

competent larvae then up-regulate the expression of possible chemosensory proteins and neurotransmitter receptors to make the detection and transduction of a signal from external settlement cues possible.

Introduction

Many marine invertebrate species from a wide variety of animal phyla depend on their planktonic larvae for dispersal. These microscopic larvae often spend hours to weeks in the water column before finally settling to the benthos and initiating metamorphosis (Pechenik 1990). The distance that the tides and currents of the ocean passively carry these larvae is often far greater than an adult could actively move in its entire lifetime. These larvae finally end their planktonic journey once they perceive a settlement cue – a stimulus from the environment that triggers larvae to settle to the benthos and initiate metamorphosis. However, larvae of many species must develop for hours, days, or weeks before becoming responsive to such environmental triggers (Pechenik 1990, Hadfield et al. 2001).

This time to metamorphic competence thus determines the minimum amount of time that larvae will spend dispersing in the water column before they are able to perceive a settlement cue, settle, and initiate metamorphosis. While the larvae of some species are immediately competent to metamorphose after hatching or being released from brooding, many other species spend hours to weeks drifting in the water column before they even develop the ability to settle in response to a settlement cue. While these larvae are progressing towards competence in the water column they are also developing juvenile structures and organs that are essential for life after metamorphosis. By the time larvae reach competence they are essentially fully formed juveniles waiting to shed larval structures (Hadfield et al. 2001). While some settlement cues have been characterized (Pawlik & Faulkner 1986, Tebben et al. 2011, Swanson et al. 2012, Burns et al. 2014, Shikuma et al. 2014) and various aspects of the physiological control of metamorphosis

have been determined (Leise et al. 2001, Bishop & Brandhorst 2001, Pechenik et al. 2002, 2007, Bishop et al. 2008, Biggers et al. 2012), almost nothing is known about the molecular or physiological mechanisms controlling onset of metamorphic competence – one of the key processes that determine potential distributions of marine invertebrate species.

To begin understanding the shifts in gene expression that lead to metamorphic competence in marine invertebrates we have studied the deposit-feeding salt-marsh polychaete, *Capitella teleta*. *C. teleta* is a model organism with a sequenced and annotated genome and has been used for studying developmental biology, larval settlement, and the control of metamorphosis (Blake et al. 2009). Larvae of *C. teleta* will rapidly settle and metamorphose once they contact salt-marsh sediment, often in less than 30 minutes (Dubilier 1988, Cohen & Pechenik 1999). While the larvae of many different species become competent after days to weeks of development in the plankton and at widely different times within each brood, the larvae of *C. teleta* are competent to metamorphose within minutes of release from brooding (Dubilier 1988, Cohen & Pechenik 1999).

Mated females of *C. teleta* will produce a brood tube out of mucus and sand grains and deposit their embryos on the inner walls of the tube (Blake et al. 2009). Embryos develop for approximately 8 days before they escape and swim as larvae into the water column (Seaver et al. 2005). While most larvae that are released from brood tubes are immediately competent to metamorphose, rarely, a brood of pre-competent larvae will be released (personal observations Biggers, Burns, and Pechenik). Stage 8 pre-competent larvae of *C. teleta* have a straight gut morphology while the stage 9

competent larvae have a spiral gut morphology (Seaver et al. 2005). No more than 24 h of development separate these two stages (Seaver et al. 2005). Thus *C. teleta* is especially well suited to explore the shifts in gene expression that cause larvae to become competent.

Here we took advantage of the *C. teleta* genome and easily identifiable pre-competent and competent larvae to conduct Illumina mRNA sequencing of these two larval stages to identify patterns of differential expression at the onset of competence. While we expected that some shifts in gene expression are associated with the onset of metamorphic competence, other shifts may anticipate the larva's needs during or immediately following metamorphosis. By understanding how larvae become competent to metamorphose we will piece more of the signal transduction cascade that regulates settlement and metamorphosis together. Which genes must be expressed at higher levels in competent larvae to allow metamorphosis to occur? These gene products could be present in higher concentrations in competent larvae but possibly missing or in lower concentration in pre-competent larvae. Pre-competent larvae could also be producing inhibitory substances in higher concentrations than larvae that have attained competence. Understanding these transcriptomic changes between two stages that are very close to each other in development, yet functionally very different, will aid our understanding of what is required for metamorphosis to take place.

Materials and Methods

Animal culture and collection of larvae

Dr. Judith Grassle from Rutgers University generously provided starter cultures of adult *C. teleta*. *C. teleta* were reared in 5 in diameter glass dishes containing 30 psu

Instant Ocean artificial seawater (hereafter called ASW) and maintained at 16 °C. The adults were fed sediment collected from the Little Sippewissett salt-marsh (Falmouth, MA). Salt-marsh sediment was first passed through a 1 mm sieve and frozen at -20 °C for at least 24 h before being used as food or in settlement experiments. Culture dishes were searched regularly for brood tubes, which were then transferred individually to 2 in diameter glass dishes containing only ASW. Sand grains were carefully cleared from the side of the brood tubes to allow us to observe the developing embryos. This did not disturb the developing embryos or the brooding mother as the mucus tube below the sand grains remained intact. Once embryos developed to stage 8 (straight gut) within the brood tube, the brood tube was carefully cut open and the stage 8 larvae were allowed to swim out and were collected for tissue and experiments. Stage 9 larvae (spiral gut) were collected for tissue preservation and experiments when they naturally escaped from the brood tube. All larval observations were made at 12 - 50x magnification under a dissecting microscope.

Testing larvae for metamorphic competence

To determine if stage 8 larvae were indeed pre-competent and stage 9 larvae were competent to metamorphose we placed the larvae in ASW containing salt-marsh sediment and monitored them for metamorphosis. Three replicates of 10 larvae per stage were added to 4 mL of ASW in plastic 16-well plates with each well containing 1.72 cm^3 of salt-marsh sediment. Wells were observed for metamorphosed juveniles every 30 minutes for 5 h at 50x using a dissecting microscope, with a final observation at 24 h.

Illumina mRNA sequencing library preparation

Stage 8 and 9 larvae were preserved separately after being released from their brood tubes in RNA-later solution and stored at -80 °C. Larval mRNA was later extracted by grinding the larvae in 1.5 mL microfuge tubes with a plastic pestle and then purified using Qiagen RNeasy mini kits with a final elution volume of 20 µL. We used a Nanodrop 2000 to determine that 50 larvae needed to be pooled in one extraction to yield the 1 µg of mRNA required by the Illumina TruSeq version 2.0 mRNA library preparation kit. Four replicate larval mRNA extractions were made for each larval stage and each replicate was made into an individual uniquely barcoded mRNA sequencing library using the Illumina TruSeq version 2.0 mRNA library preparation kit. The 8 individual libraries were then sent to the Tufts Core Facility where they were pooled at equal concentrations and sequenced in one Illumina HiSeq 2500 lane to yield 50 bp long single-ended reads. These libraries will be uploaded to the NCBI Short Read Archive upon this manuscript's acceptance.

Sequence quality control, alignment, and differential expression analysis

Each library was assessed for sequence quality and potential adapter or primer contamination using FastQC (Simon Andrews, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Because no large amounts of adapter or primer contamination were present and basecalls were largely greater than a PHRED quality of 30, no read trimming was performed. Each library was then aligned to the *C. teleta* genome obtained from EnsemblMetazoa (INSDC Assembly GCA_000328365.1, database version 82.1) (Simakov et al. 2014, Kersey et al. 2015) using the default settings of Bowtie 2 (v2.2.5) (Langmead & Salzberg 2012) and TopHat 2 (v2.0.14) (Trapnell et al. 2009). HTSEq-count was used to determine the number of

reads that aligned to each gene within each tissue library replicate (Anders et al. 2015). The R package EdgeR was then used to normalize libraries by scaling for library size, estimate dispersions by the quantile-adjusted conditional maximum likelihood (qCML) method, and calculate differential expression using quasi-likelihood F-tests, all of which are recommended for RNA-seq experiments with a single factor (Robinson & Smyth 2007a, b, Robinson et al. 2009, Robinson & Oshlack 2010, McCarthy et al. 2012, Chen et al. 2014, Lund et al.).

Gene ontology analysis

Genes were considered differentially expressed if they had at least a 2 fold increase or decrease of expression level between developmental stages, a p-value of < 0.05, and a false discovery rate of < 0.05. Gene ontology classifications for annotated genes were obtained from EnsemblMetazoa BioMart (Kinsella et al. 2011). A Fisher's exact test was conducted in Blast2GO to identify significantly (False Discovery Rate < 0.05) enriched and depleted gene ontology categories between pre-competent and competent larvae (Conesa et al. 2005).

Results and Discussion

Tests of metamorphic competence

All stage 9 larvae metamorphosed and burrowed within 5 h of contact with salt-marsh sediment, while no stage 8 larvae metamorphosed within this timeframe (Fig 1). However, all larvae from both developmental stages had metamorphosed within 24 h of treatment.

Sequencing and alignment results

A total of 200,064,476 50 bp reads were successfully demultiplexed into 8 separate libraries from the single Illumina HiSeq 2500 sequencing lane. The mean basecall quality of all bases in the demultiplexed reads surpassed a Phred quality of 30 (less than 1/1000 chance a base was called incorrectly). The mean library size was 25,008,060 reads; the smallest library contained 19,655,572 reads while the largest library contained 29,266,981 reads. At least 93.7 % of reads per library were successfully aligned to the *C. teleta* genome.

2604 genes were significantly differentially expressed between the stage 8 pre-competent larvae and the stage 9 competent larvae. 1541 of these genes were expressed at higher levels in the pre-competent larvae while 1063 genes were expressed at higher levels in the competent larvae.

Enriched gene ontologies for each larval stage

Overall, it appears that pre-competent larvae are continuing larval morphological and physiological development while also increasing expression of proteins that possibly inhibit metamorphosis (Table 1). In contrast, competent larvae are mainly increasing expression of genes with potential chemosensory functions and also neurotransmitter receptors that could be involved in the signal transduction cascade leading to metamorphosis (Table 2).

Up-regulated genes in pre-competent larvae

The majority of gene ontologies enriched within pre-competent larvae are associated with growth and development. These gene ontologies include categories involved with DNA replication, cell division, transcription, translation, protein folding, and other protein modifications (Table 1). These results agree with Hadfield et al. (2001)

as they argue that all juvenile structures required for post-metamorphosis juvenile life must be developed before a larva attains metamorphic competence. Through metamorphosis, larval-specific structures are shed and then because juvenile features required for benthic life are already developed, individuals can immediately proceed further with growth and development into adults. Indeed, gut development is not completed in pre-competent stage 8 larvae of *C. teleta* and circular, longitudinal, and oblique muscles are not well-developed prior to the competent stage 9 larvae of *C. teleta* (Seaver et al. 2005); these features are required for successful burrowing and deposit-feeding in juveniles of *C. teleta*. Also, embryos and larvae of *C. teleta* continue producing new segments until they reach competence at stage 9, with larvae halting further segmentation until metamorphosing, with a total of 12 - 14 segments (Seaver et al. 2005). Even development of the animal's nervous system halts once the larva reaches competent stage 9. Surprisingly, the development of *C. teleta's* nervous system could be considered complete once they reach stage 9 as the nervous system changes little between stage 9 larvae and the newly metamorphosed juveniles (Meyer et al. 2015).

Some genes responsible for the synthesis of nitric oxide and other processes that could inhibit metamorphosis are also up-regulated in the pre-competent larvae of *C. teleta*. The competent larvae of *C. teleta* along with the larvae of molluscs, ascidians, and echinoderms are inhibited from metamorphosing by endogenously produced nitric oxide (Bishop & Brandhorst 2001, Pechenik et al. 2007, Biggers et al. 2012). Not surprisingly, in many animals nitric oxide synthase requires an interaction with heat shock protein 90 to keep the essential heme cofactor bound (Bishop & Brandhorst 2003). However, when competent hydroid and gastropod larvae were stressed with increased

temperatures they metamorphosed (Kroiher et al. 1992, Gaudette et al. 2001, Bishop & Brandhorst 2003). Metamorphosis likely occurred in these larvae because HSP90 proteins were diverted from NOS to renature proteins that had denatured from thermal stress (Bishop & Brandhorst 2003). Heat shock proteins 40, 70, and 90 were up-regulated in pre-competent larvae of *C. teleta*. While these proteins also play roles in developmental processes, it is possible that increased expression of HSP90 could ensure high enough endogenous concentrations of nitric oxide to prevent metamorphosis from occurring.

Four haem peroxidases were also up-regulated in precompetent larvae. Haem peroxidases perform a variety of cellular functions including roles in innate immunity, hormone synthesis, and preventing oxidative stress in the cell. Haem peroxidases work by using hydrogen peroxide to oxidize various substrates to produce an oxidized product and water. Hydrogen peroxide has stimulated larvae from a number of marine invertebrates to either partially or completely metamorphose (Pires & Hadfield 1991, Boettcher & Targett 1998). Two superoxide dismutases, which produce either oxygen or hydrogen peroxide from the reactive oxygen species superoxide, were also up-regulated in precompetent larvae of *C. teleta*. Pre-competent larvae of *C. teleta* may be inactivating the reactive oxygen species hydrogen peroxide by catalyzing it into water. Reactive oxygen species such as hydrogen peroxide can inhibit endogenous nitric oxide production (Murad 2006) and such inhibition of the inhibitory endogenous nitric oxide in marine invertebrates could lead to metamorphosis.

Up-regulated genes in competent larvae

The most significantly enriched gene ontology categories within the competent larvae of *C. teleta* have to do with the process and function of sodium ion channels and transport. Within these two ontologies, 24 genes have been annotated as being amiloride-sensitive sodium channels. Also, 5 other genes were up-regulated in competent larvae of *C. teleta* have been annotated as having similarities to PKD proteins. Both amiloride-sensitive sodium channels and PKD channels belong to a large family of channels called the Degenerin/Epithelial sodium channel family (DEG/EnAC family) (Ben-Shahar 2011). These DEG/ENaC channels perform a variety of sensory functions in vertebrates and invertebrates including chemo- and mechanosensation. These channels are involved in the reception of salt, sour, and 'water' tastes in a variety of different organisms from humans to the fruit-fly *Drosophila melanogaster*. Mechanosensory functions of these proteins range from skin pressure reception to pain response from applied acids (Ben-Shahar 2011). Mammalian genomes only encode for 8-9 of these proteins while invertebrates such as *D. melanogaster* and the nematode *Caenorhabditis elegans'* genomes encode 30-31, making them one of the largest protein families represented among invertebrate genomes; these proteins may have evolved to perform many specialized sensory functions in invertebrates (Ben-Shahar 2011). Amiloride appeared to be weakly inductive to larvae of the hydroid *Hydractinia echinata*; a 600 µM solution of amiloride in seawater induced ~ 30% of the tested larvae to metamorphose (Berking 1988). The role that DEG/ENaC channels play in the settlement and metamorphosis of marine invertebrates has not been explored in any other species. By expressing these channels at higher levels at competence, larvae of *C. teleta* could be preparing to navigate and assess a complex pelagic landscape outside of the brood tube.

The greatest number of genes were up-regulated in competent larvae within the membrane cellular component ontology (Table 2). This category contains the sodium channels described above as well as other receptors of interest. The most abundant of these receptors were the 42 G protein-coupled receptors (GPCRs). GPCRs are a large family of transmembrane chemosensory receptors that are commonly involved in chemical sensation including taste and smell. This result is similar to GPCR transcription patterns in the demosponge *Amphimedon queenslandica* where many GPCRs were upregulated in pelagic larvae and then down regulated in benthic juveniles (Conaco et al. 2012). Here, Conaco et al. (2012) argue that GPCRs may be used by larvae to sense appropriate settlement cues and environments for settlement and metamorphosis (Conaco et al. 2012). GPCRs have often been studied as putative receptors for settlement cues in marine invertebrate larvae (Hadfield 2011). While GPCRs do not mediate settlement and metamorphosis in larvae of the polychaete *Hydriodes elegans* or the coral *Montipora capitata* (Holm et al. 1998, Tran & Hadfield 2012), metamorphosis of the larvae of the marine mollusk *Haliotis rufescens* (abalone) did appear to be modulated by a GPCR (Baxter & Morse 1987). Among these GPCRs, 35 were annotated as rhodopsin-like GPCRs, including a 5-Hydroxytryptamine 6 (5-HT_6) receptor, a glycoprotein hormone receptor, a thyrotropin-releasing hormone receptor, and a galanin receptor. The up-regulation of a galanin receptor in competent larvae of *C. teleta* is curious, as the function of the short neuropeptide galanin in invertebrates is not well understood (Liu et al. 2010). In mammalian species, galanin is involved in the regulation of food intake, pain reception, and nerve regeneration (Branchek et al. 2000). Interestingly, mammalian genomes (humans, mice, and rats) also encode 3 nicotinic-acid receptors in the A11

subfamily of rhodopsin-like GPCRs (genes *HCAR1*, *HCAR2*, and *HCAR3*). Nicotinic acid and the related B vitamin nicotinamide rapidly stimulated larvae of *C. teleta* to metamorphose (Burns et al. 2014). However, when BLASTP searches using these human protein sequences were conducted within the *C. teleta* proteome, no high quality matches were found (lowest e-value = 3.5e-14, highest percent identity = 32.6). Perhaps the ligand-binding channel that larvae of *C. teleta* use to sense nicotinamide and nicotinic acid is not a GPCR (Burns et al. 2014).

The up-regulation of the 5-HT_6 serotonin receptor in competent larvae of *C. teleta* is exciting because treatment of these larvae with serotonin or the selective serotonin reuptake inhibitor fluoxetine stimulates them to metamorphose (Biggers et al. 2012). Also, the serotonin receptor antagonist ketanserin inhibited larvae from metamorphosing in response to NOS inhibitors and nicotinamide (Biggers et al. 2012, Burns et al. 2014). Serotonin may be required in *C. teleta* to transduce the signals stimulated by the reception of a settlement cue leading to the initiation of metamorphosis. Other GPCRs included 5 GABA B receptors (4 of which were B1 while one other was only classified as B). While not a GPCR, a GABA A receptor was also up-regulated. GABA is an inhibitory amino acid neurotransmitter that has stimulated some species of marine invertebrate larvae to metamorphose (Morse et al. 1979, Pearce & Scheibling 1990, García-Lavandeira et al. 2005, Yang et al. 2015). Like serotonin, perhaps a component of the signal transduction cascade leading to metamorphosis in *C. teleta* is mediated by GABA. Other up-regulated receptors in competent larvae included 7 ionotropic glutamate receptors, a nicotinic acetylcholine-gated receptor, 2 neurotransmitter-gated ion-channels, and a gustatory receptor. Although it is not clear what ligand this gustatory receptor is

responsible for binding to, this is more evidence that competent larvae up-regulated another gene responsible for environmental chemosensation.

Overall, it appears that pre-competent larvae of *C. teleta* are completing development of structures required for survival as deposit feeding juveniles and exhibit increased expression of genes belonging to gene ontologies that reflect this status of continued growth and development (Table 1). However, once this development is completed, competent larvae had mainly increased expression of ligand-binding transmembrane channels and other potential chemosensory proteins. Although we do not know to which ligands these proteins bind, the potential for chemosensation is greater in competent larvae because of the increased expression of these genes. In combination with a potential for greater chemosensation, many neurotransmitter receptors also increased in expression in competent larvae. Here, serotonin, GABA, glutamate, acetylcholine, and other neurotransmitters may play a role in transducing the signal perceived from a settlement cue to initiate settlement and metamorphosis. Perhaps chemosensory receptors and other components of the signal transduction cascade leading to metamorphosis are highly expressed at competence as a safety mechanism. If individuals of *C. teleta* were to metamorphose before their muscles, gut, or nervous system were developed to a functional state the resulting juvenile would not be likely to survive in the benthos. Future studies should examine the role that GPCRs play in modulating settlement and metamorphosis in *C. teleta* as so many of these receptors were expressed at higher levels in competent larvae. Also, the potential for amiloride-sensitive sodium channels to act as chemosensory channels should be explored in larvae of *C. teleta* and other marine invertebrate larvae because of their higher expression level in

competent larvae of *C. teleta*. To our knowledge the possibility that these channels have roles in the control of metamorphosis in the larvae of marine invertebrates has not been thoroughly explored.

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Tables

Table 6.1. Gene ontologies enriched in pre-competent larvae of *Capitella teleta*. Type is the gene ontology category that each GO term is derived from: C = Cellular Component, F = Biological Function, and P = Biological Process. FDR is the Benjamini-Hochberg corrected p-value as calculated by a Fisher's exact test.

GO ID	GO Term	Type	FDR
GO:0006457	protein folding	P	3.50E-19
GO:0004298	threonine-type endopeptidase activity	F	1.40E-16
GO:0051082	unfolded protein binding	F	1.40E-12
GO:0005524	ATP binding	F	1.40E-09
GO:0005852	eukaryotic translation initiation factor 3 complex	C	1.70E-08
GO:0003743	translation initiation factor activity	F	1.20E-07
GO:0019773	proteasome core complex, alpha-subunit complex	C	2.40E-07
GO:0006270	DNA replication initiation	P	1.80E-05
GO:0000398	mRNA splicing, via spliceosome	P	2.00E-05
GO:0000413	protein peptidyl-prolyl isomerization	P	3.40E-04
GO:0003755	peptidyl-prolyl cis-trans isomerase activity	F	3.40E-04
GO:0004579	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	F	6.00E-04
GO:0042555	MCM complex	C	6.90E-04
GO:0006779	porphyrin-containing compound biosynthetic process	P	1.40E-03
GO:0045454	cell redox homeostasis	P	1.50E-03
GO:0003678	DNA helicase activity	F	1.70E-03
GO:0042176	regulation of protein catabolic process	P	5.70E-03
GO:0005732	small nucleolar ribonucleoprotein complex	C	5.70E-03
GO:0022624	proteasome accessory complex	C	7.40E-03
GO:0008250	oligosaccharyltransferase complex	C	7.40E-03
GO:0006950	response to stress	P	8.10E-03
GO:0006886	intracellular protein transport	P	9.30E-03
GO:0005885	Arp2/3 protein complex	C	1.20E-02
GO:0016272	prefoldin complex	C	1.20E-02
GO:0008641	small protein activating enzyme activity	F	1.40E-02
GO:0071840	cellular component organization or biogenesis	P	1.80E-02
GO:0015671	oxygen transport	P	1.90E-02
GO:0008017	microtubule binding	F	2.20E-02
GO:0006281	DNA repair	P	2.30E-02
GO:0004814	arginine-tRNA ligase activity	F	2.30E-02
GO:0006420	arginyl-tRNA aminoacylation	P	2.30E-02
GO:0006465	signal peptide processing	P	2.30E-02
GO:0008173	RNA methyltransferase activity	F	2.60E-02

GO:0000070	mitotic sister chromatid segregation	P	3.30E-02
GO:0006221	pyrimidine nucleotide biosynthetic process	P	3.30E-02
GO:0006739	NADP metabolic process	P	3.50E-02
GO:0031981	nuclear lumen	C	4.80E-02
GO:0005871	kinesin complex	C	4.90E-02
GO:0016051	carbohydrate biosynthetic process	P	4.90E-02
GO:0051087	chaperone binding	F	4.90E-02
GO:0051920	peroxiredoxin activity	F	4.90E-02
GO:0003918	DNA topoisomerase type II (ATP-hydrolyzing) activity	F	4.90E-02
GO:0006890	retrograde vesicle-mediated transport, Golgi to ER	P	4.90E-02
GO:0044877	macromolecular complex binding	F	4.90E-02
GO:0020037	heme binding	F	5.00E-02
GO:0000075	cell cycle checkpoint	P	5.00E-02
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	P	5.00E-02

Table 6.2. Gene ontologies enriched in competent larvae of *C. teleta*. Type is the gene ontology category that each GO term is derived from: C = Cellular Component, F = Biological Function, and P = Biological Process. FDR is the Benjamini-Hochberg corrected p-value as calculated by a Fisher's exact test.

GO ID	GO Term	Type	FDR
GO:0005272	sodium channel activity	F	2.90E-12
GO:0006814	sodium ion transport	P	9.60E-11
GO:0016020	membrane	C	6.80E-05
GO:0005509	calcium ion binding	F	6.40E-04
GO:0003840	gamma-glutamyltransferase activity	F	2.20E-03
GO:0006749	glutathione metabolic process	P	1.30E-02
GO:0004129	cytochrome-c oxidase activity	F	1.40E-02
GO:0080019	fatty-acyl-CoA reductase (alcohol-forming) activity	F	1.50E-02

Figures

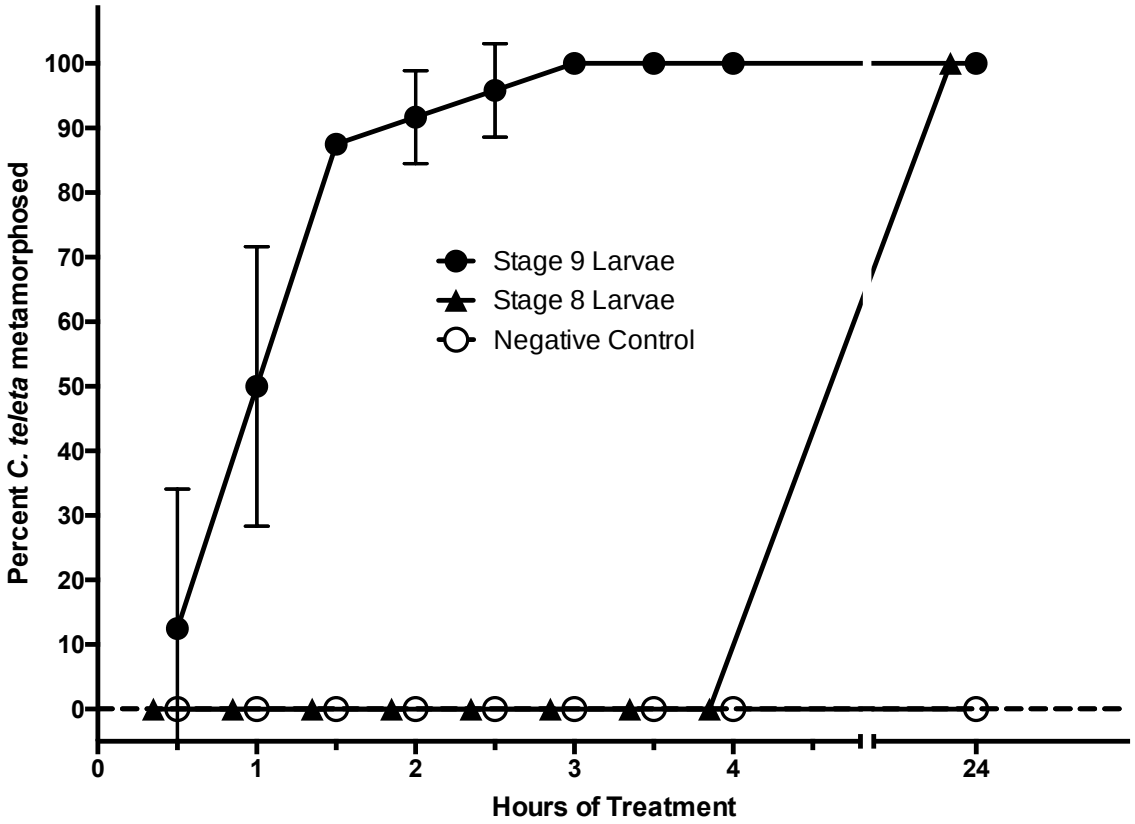

Figure 6.1. Testing stage 8 and 9 larvae of *Capitella teleta* for metamorphic competence. Each treatment consisted of 3 replicates of 10 larvae per replicate. Larvae were placed in 4 mL 30 psu Instant Ocean artificial seawater containing 1.7 cm^3 of salt-marsh sediment. The negative control contained only Instant Ocean and stage 9 larvae. Error bars represent +/- 1 SEM.

Chapter 7

Conclusions

When I first began my studies in the Pechenik lab, I set out to answer the following questions about settlement and metamorphosis using *Capitella teleta* as a model organism:

1. What are the chemical settlement cues that larvae could be responding to in nature?
2. What receptors are larvae using to perceive these settlement cues?
3. How is metamorphic competence regulated?

I have made significant progress answering these questions as detailed in the previous data chapters of my dissertation.

What are the chemical settlement cues that larvae could be responding to in nature?

We first found that two B vitamins, nicotinamide (B_3) and riboflavin (B_2), stimulated larvae of *C. teleta* to rapidly metamorphose at low concentrations (Chapter 2). These are the first results showing that vitamins can act as settlement cues (Burns et al. 2014). While it is not known if *C. teleta* require these vitamins or what concentrations of these vitamins they require for survival, the annelid *Enchytraeus fragmentosus* has been shown to have a B vitamin requirement (Gotthold & Koch 1974). These results make adaptive sense if *C. teleta* does indeed have a requirement for these B vitamins. By settling and metamorphosing in response to required nutrients the larvae can ensure that the sediments they settle in will have adequate nutrition for their future juvenile and adult life stages.

However, why do larvae of *C. teleta* not settle and metamorphose in response to B vitamins besides nicotinamide and riboflavin? The oligochaete *E. fragmentosus* was also shown to have a requirement for the B vitamins cyanocobalamine (B_{12}), pantothenic

acid (B_5), thiamine (B_1), and pyridoxine (B_6) (Gotthold & Koch 1974). Do *C. teleta* simply not require these vitamins? Or, could nicotinamide and riboflavin be limiting nutrients in *C. teleta*'s diet? If inductive concentrations of these vitamins are found in the environment, satisfactory concentrations of the other vitamins may also be present. Currently these questions cannot be answered because there are no published concentrations of B vitamins in marine sediments.

Future work should focus on determining the vitamin content of sediments that induce larvae of *C. teleta* to metamorphose and also determine a minimal essential media for *C. teleta*. By accomplishing this we could determine the nutrients that *C. teleta* have an absolute requirement for and also determine whether their settlement preferences are aligned with these requirements. The larvae of other deposit feeding invertebrates should be tested with vitamins to determine how widespread this phenomenon is.

We have also isolated the bacterium *Desulfovibrio oceani* from the salt-marsh sediment that rapidly stimulates larvae of *C. teleta* to metamorphose. Surprisingly, we found that *D. oceani* were synthesizing another potential vitamin, menaquinone-6 (vitamin K_2), that stimulated larvae of *C. teleta* to metamorphose. Furthermore, we confirmed that menaquinone-6 was present in the inductive mud-flat sediment (Chapter 4). *D. oceani* is not unique in producing menaquinone-6, many other bacteria within the *Deltaproteobacteria* also produce menaquinone-6 as their respiratory quinone (Kersters et al. 2006).

Again, because we do not know *C. teleta's* minimum dietary requirements we cannot determine whether menaquinone-6 is a vitamin or is acting as some other indicator of a suitable environment for future growth and development. We did find that

Desulfobulbaceae spp. could be present in high abundances within the salt-marsh sediments that rapidly stimulated larvae of *C. teleta* to metamorphose (Chapter 5). Some *Desulfobulbaceae* genera do produce menaquinone-6 as their respiratory quinone; however, we were unable to determine which *Desulfobulbaceae* genera were present within the salt-marsh sediment samples (Kuever 2014). These *Desulfobulbaceae* spp. could be intimately linked in a syntrophic relationship with photosynthetic *Chromatiaceae* spp. in the sediment samples and biofilms we found. Perhaps menaquinone-6 from *Desulfobulbaceae* spp. indicates that particularly nutritious *Chromatiaceae* spp. are present in the sediment (Kobayashi & Tchan 1973, Vrati 1984, Imhoff 2006).

What receptors are larvae using to perceive these settlement cues?

Much less is known about the receptors larvae use to perceive settlement cues (Hadfield 2011). While studies have focused on G protein-coupled receptors (GPCRs) because of their ubiquity in chemosensation, little evidence has been found to demonstrate that these GPCRs have widespread roles in marine invertebrate settlement cue perception (Heyland et al. 2011, Hadfield 2011). We have found that larvae of *C. teleta* may be using a nicotinamide-activated ion-channel to sense the presence of nicotinamide (Chapter 2). This channel was first discovered and characterized in freshwater crayfish (Hatt & Bauer 1980). Characterization of this channel continued through the 1980's and abruptly stopped after many agonists and inhibitors had been identified (Hatt & Bauer 1980, 1982, Hatt & Schmiedel-Jakob 1984, 1985, Hatt & Franke 1987). We currently do not know anything about this channel's protein sequence,

structure, or which ions pass through it when opened. If this channel is found to play a role sensing chemical cues in other species it should definitely be further characterized.

We have found that a receptor may not be responsible for riboflavin-induced metamorphosis (Chapter 3). When competent larvae of *C. teleta* and *Crepidula fornicata* were treated with riboflavin and kept in complete darkness no larvae had metamorphosed within 24 h. In contrast, larvae treated with riboflavin and kept under normal laboratory fluorescent lighting did metamorphose. Here it is likely that reactive oxygen species produced during the photodegradation of riboflavin inhibited the production of nitric oxide allowing metamorphosis to proceed (Chapter 3). However, a hydrogen peroxide concentration of 50 μM was required to induce any larvae to metamorphose; a 10 μM concentration of hydrogen peroxide did not induce any larvae to metamorphose. Although we do not know the concentrations of reactive oxygen species in marine sediments, the highest observed concentrations of hydrogen peroxide in seawater samples was approximately 500 nM, much lower than 10 μM hydrogen peroxide that still did not induce any larvae to metamorphose (Herut et al. 1998). Unless sediment concentrations of reactive oxygen species are high, it is not likely that riboflavin or reactive oxygen species are acting as a natural settlement cues.

How menaquinone-6 stimulated larvae of *C. teleta* to metamorphose is still unknown (Chapter 4). There is currently no known receptor for menaquinone-6, although a menaquinone-4 receptor is rumored to exist (Booth 2009). If larvae of *C. teleta* are using a receptor to sense menaquinone-6 in the environment it could be binding to chemicals containing repeating patterns of 3 isoprenoid units. Methyl-farnesoate also stimulated larvae of *C. teleta* to metamorphose; these two chemicals share a repeating

chain of isoprenoid units (Biggers & Laufer 1999). Menaquinones are lipid soluble (Saffarini et al. 2002) and could be interacting with intracellular components of the signal transduction cascade leading to settlement and the initiation of metamorphosis.

How is metamorphic competence regulated?

We then compared the transcriptomes of pre-competent larvae and competent larvae of *C. teleta* to determine the transcriptomic basis underlying metamorphic competence (Chapter 6). Pre-competent larvae had up-regulated the expression of genes belonging to gene ontologies having to do with growth and development while the competent larvae had up-regulated ligand-binding transmembrane channels with possible chemo and mechanosensory functions. Overall, it appears that while precompetent larvae are continuing the development of juvenile structures that will be needed after metamorphosis, competent larvae had up-regulated a variety of sensory receptors and neurotransmitter receptors that could be required to transduce a settlement cue's signal to initiate settlement and metamorphosis. This agrees with Hadfield et al. (2001), who argue that dispersing, pre-competent larvae are developing juvenile structures required for life after metamorphosis. Once the development of these required juvenile structures is completed, the larvae can then begin to assess their surroundings to find a suitable substrate. For example, it would not make adaptive sense for a deposit-feeder like *C. teleta* to metamorphose before its gut had completed development and begin eating sediment.

We have found that many GPCRs and Degenerin/Epithelial sodium channels were more highly expressed in the competent larvae. While GPCRs were not found to play a role in the settlement and metamorphosis of the polychaete *Hydroides elegans* (Holm et

al. 1998), it is still possible that they could have a role in regulating the metamorphosis of *C. teleta*. Our results also suggest an under-studied family of mechano and chemosensory channels to explore, the Degenerin/Epithelial sodium channels. These channels are involved in the reception of salt, sour, and 'water' tastes in a variety of different organisms from humans to flies; mechanosensory functions of these proteins range from skin pressure reception to pain response from applied acids (Ben-Shahar 2011). Future studies should focus on the roles that these receptors could play in the control of metamorphosis.

Like many scientific endeavors, we have answered some questions while generating many more. In the future we should focus on determining whether the larvae of other deposit-feeding organisms are stimulated to metamorphose by vitamins that they potentially require. We should also continue to determine which receptors larvae are using to sense settlement cues in the environment. It would be exciting to see if more commonalities in the control of metamorphosis emerge from future studies. It has already been surprising to see that nitric oxide inhibits larvae of species across phyla from metamorphosing. What other aspects of the control of metamorphosis could be conserved or evolved repeatedly?

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