THE MAINTENANCE OF GENETIC DIVERSITY IN THE NORTH ATLANTIC ISOPOD, *IDOTEA BALTHICA*

by

TINA MARIE BELL

(Under the Direction of John P. Wares)

ABSTRACT

Widely dispersing species experience a variety of abiotic or biotic variables that can affect their distribution and their population genetic differentiation. These variables can be subtle such as differences in microhabitat or community assemblage or more obvious such as continental drift or glaciation. The interaction of these variables and their ability to constrain or facilitate gene flow between populations are addressed along the North American coastline with the marine isopod, *Idotea balthica*. This species shows latitudinal population genetic differentiation in the gene, mitochondrial cytochrome oxidase I (mtCOI). The mechanisms that created and currently maintain this pattern in population genetic diversity in this species are unknown. This study addressed whether North American populations of *I. balthica* are defined by distinct feeding behaviors and whether feeding preference could be a diversity maintaining mechanism in this species. Feeding assays showed that feeding preferences did differ between North American populations. Southern populations consume *Zostera marina* at a higher rate than northern populations while northern

populations consume more *Fucus vesiculosus* than southern populations. PCR-based gut content analyses revealed that northern populations tend to be more selective in their food choices than those in southern populations. Partial reproductive barriers were found from inter-population crosses where fecundity and offspring survivorship were reduced from intra-population crosses. This finding suggests that reproductive barriers may be maintaining genetic diversity in this species and that North American populations of *I. balthica* may actually be incipient species. The historical dispersal patterns of this species between North America and Europe post-glaciation are also addressed but remain inconclusive.

INDEX WORDS: North Atlantic, *Idotea balthica*, feeding behavior, phylogeography, reproductive isolation, sexual isolation, speciation, mtCOI, gut contents, genetic diversity

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DEDICATION

To my parents and grandparents who inspired and supported my love of biology.

For LaVera and Derwood who always believed in me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The North Atlantic Intertidal

A suite of physical, and biological variables can act to shape the population genetic differentiation within species. Dispersal strategies are often a commonly used predictor of the extent of this differentiation (Vermeij et al. 1990; Thompson et al. 1999; Riginos and Victor 2001; Kinlan et al. 2005; Lester et al. 2007). However, frequently species with long-range dispersal capabilities have more complex population genetic structure than is expected (Taylor and Hellberg 2003; Rocha et al. 2005; Paulay and Meyer 2006). The effects of a species' dispersal ability and its constraint on gene flow can be difficult to assess when they are confounded by signatures of historical vicariant events such as the distributional shifts caused by glaciation and natural selection (Avise 2000). Those widely dispersed taxa that are distributed across both historically glaciated and unglaciated areas can be an ideal model to evaluate how vicariance and dispersal limitation can shape intra-specific phylogeographic patterns (Avise 2000).

Dramatic shifts in coastal topography, temperature and salinity make the North West Atlantic intertidal an useful arena for studying the complex interactions of natural selection, historical events, and gene flow and their effect on population genetic structure. Two major biogeographic provinces overlap in the North West Atlantic: the Virginian which occupies the coast from Newfoundland to Cape Cod and the Acadian which extends from Cape Cod to Cape Hatteras (Engle and Summers 1999). Biogeographic provinces are defined by the observed distributions of organisms (Lomolino and Brown 1998). The Virginian province is broadly characterized by soft bottom and seagrass communities that inhabit this region's shallow sandy

coastal shelf; while the Nova Scotian province can be broadly defined by highly productive large macroalgal species and rocky intertidal inhabitants (Engle and Summers 1999; Bertness 2007; Jenkins et al. 2008). The combination of unique community assemblages, and varied environmental conditions experienced by a species distributed across this overlap in biogeographic provinces, can potentially lead to variation in the intensity and directionality of natural selection (Slatkin 1973; 1976; Endler 1977; Jaenike and Holt 1991; Thompson 2005). One could expect that species distributed over large geographic distances such as the temperate North West Atlantic coastline might exhibit population differentiation, not only due to the geographic distance among populations, but also potentially deep population genetic subdivision because of the interaction of historical vicariance and natural selection.

Over twenty species of North Atlantic intertidal fauna, from fish to worms to crustaceans, exhibiting an equally variable assortment of dispersal strategies, show evidence of a latitudinal divergence pattern in population genetic structure along this coastline (Wares 2002; Sotka et al. 2003b; Kelly et al. 2006; Jennings et al. 2009). The general trend has been for populations residing in New England and Canada (Nova Scotian Province) to be genetically distinct from those in the southeast (Virginian Province) with a phylogeographic break occurring between New Jersey and Cape Cod (Wares 2002; Kelly et al. 2006; Jennings et al. 2009). Periodic glacial cycling has acted as a series of vicariant events which have likely played a major role in creating this pattern in population genetic divergence (Hewitt 2000; Edmands 2001; Wares and Cunningham 2001; Wares 2002; Marko 2004). Other hypotheses exist, however, that could explain the phylogeographic pattern observed along this coastline and could also then explain how divergence patterns, if initiated by glaciation, may have been maintained and then reinforced over time (Wares 2002). These major hypotheses will be addressed in more detail in the following sections as well as the potential for reproductive isolation and cryptic speciation within divergent lineages.

Ocean Currents

Ocean circulation, whether by currents or upwelling, can vary spatially and temporally in both intensity and direction (Colling 1989). Ocean circulation patterns can affect the community assemblage of a given area as well as the genetic connectivity of populations by allowing for long distance dispersal or local retention of individuals (Bucklin 1991; Rocha-Olivares and Vetter 1999; Marko 2004; Sotka et al. 2004; Hare et al. 2005; Byers and Pringle 2006; Paulay and Meyer 2006; Muhlin et al. 2008). In many instances, ocean circulation has been demonstrated in either the maintenance or creation of phylogeographic patterns and clinal genetic variation (Lessios et al. 1998; Muss et al. 2001; Sotka et al. 2004; Pringle and Wares 2007).

Two key currents (Gulf Stream and Virginia Coastal Current) reside on or near the temperate North West Atlantic coastal shelf and flow in opposing directions (Colling 1989; Townsend et al. 2006; Bertness 2007). The Gulf Stream flows in a south to north direction, but takes a strong easterly turn at Cape Hatteras, N.C. North of this point, the Gulf Stream is offshore and away from the continental shelf margin, thus having little direct influence on the dispersal of marine intertidal and coastal species (Schultz and Cowen 1994; Edwards et al. 2006). However, warm core rings or small eddies that form when Gulf Stream meanders are 'pinched off' can transport subtropical and warm temperate water to the northeast North American shelf edge (Joyce et al. 1984; Hare et al. 2002). Warm core rings have been demonstrated as a mode of northerly dispersal for plankton, larvae, and drift macroalgae and its associated fauna (Fine 1970; Cox and Wiebe 1979; Markle et al. 1980; Hare et al. 2002; Kimball et al. 2004).

The southern extension of the Labrador Current (Virginia Coastal current) is a cold water current that decreases in strength with decreasing latitude and produces a net southward flow from Greenland (Bousfield and Thomas 1975; Colling 1989; Townsend et al. 2006). This

nearshore current likely has the largest effect on the dispersal potential of many coastal and intertidal marine species north of Cape Hatteras (Townsend et al. 2006). The Virginia coastal current flows along the Nova Scotian and Maine coastlines where it finally reaches Cape Cod Bay. Flow in this area is very slow-moving allowing for the accumulation of nutrients and biota along this region of the coast (Townsend et al. 2006). In the summer, changes in wind pattern create an enclosed, re-circulating pattern in the Cape Cod Bay where retention of individuals increases (Butman 1987; Townsend et al. 2006; Jiang et al. 2007; Lentz 2008). This recirculating pattern may explain the population genetic structure shared across taxa whereby, individuals in Maine and Massachusetts are retained in their home area and have reduced dispersal potential suitable habitats farther south (Wares 2002; Jiang et al. 2007; Jennings et al. 2009). An important feature of this current system is the eventual connection of the Labrador Current with the Norwegian Current and ultimately Europe. This has been cited as a potential dispersal vector of propagules from European populations to the northeastern U. S. (Vermeij 1991; Ingólfsson 1992; Van Oppen et al. 1995; Vermeij 2005). Ocean currents offer a reasonable explanation for the observed pattern of high genetic similarity between populations along the northeastern coast of the U.S. and those in Europe and the low similarity between northeastern populations and the more closely neighboring southern populations (Wares 2001; Wares and Cunningham 2001).

Glaciation

Based on fossil and pollen records, dramatic global climate change such as glaciation has in the past caused extreme shifts in species distributions in terrestrial and marine systems (Huntly and Birks 1983; Bennett 1991; Valentine and Jablonski 1993b; Williams et al. 1998). During periods of glaciation, sheets of ice slowly crept down the coastline to as far south as Long Island Sound, where many marine intertidal species faced local extinction or were displaced into refugial populations either in southern regions or on offshore islands or

seamounts (Pratt and Schlee 1969; Shackleton et al. 1984; Ingólfsson 1992). Rocky intertidal species faced an exceptional hardship due to the lack of rocky substrate in southern areas leading to the local extinction of many of these species (Ingólfsson 1992; Cunningham and Collins 1998; Wares and Cunningham 2001). The topography of the European coastline differs from the North American coast in that there is not a dramatic shift from rocky to soft substrate and instead both habitat types are interspersed along the coast and existed in un-glaciated regions (Wares and Cunningham 2001; Maggs et al. 2008). Also dominant northward currents and the Gulf Stream ameliorated glaciation in Europe.

As the glaciers receded, these once ice-covered landscapes were re-colonized by those rocky intertidal species from European populations that were little affected by glaciation, either in refugial areas or via the Laborador current system from Europe (Ingólfsson 1992; Vermeij 2005). The re-colonization of North American habitats can be traced from Europe through the Mid-Atlantic Islands by comparing the diversity of species in intertidal and coastal habitats. Patterns in species diversity follow a stepping stone progression, whereby Europe appears to be the center of biodiversity for the North West Atlantic intertidal fauna, having the highest number of intertidal species compared to other coastal areas (Ingólfsson 1992; Maggs et al. 2008). Iceland and Faroes Islands reside along the Labrador current and have a lower percentage of the diversity in Europe while North America has an even smaller subset of that diversity (Ingólfsson 1992; Maggs et al. 2008). It is apparent from these datasets that a combination of glaciation and ocean currents have acted to create the phylogeographic pattern observed across many taxa along this coast (Wares & Cunningham 2001).

Spatial Distribution of Habitats

Undoubtedly, glacial cycling and currents played a major role in shaping the shared population genetic structure across taxa along this coast, however, there are many contemporary processes that are as equally likely to have influenced these phylogeographic

patterns. The contributions of Endler (1977), Slatkin (1973) and Antonovics (1976) initiated a myriad of both predictive and empirical studies that have routinely demonstrated the role of natural selection in creating and maintaining intra-specific gradients of genetic diversity. The North West Atlantic coastline is defined by a habitat gradient where northern areas are characterized by rocky coastlines and dominated by large macroalgal species, while southern areas have soft sediment habitats with expansive seagrass beds (Bertness 2007; Jenkins et al. 2008). Populations of wide-ranging species distributed throughout northern and southern areas along this coast are likely to experience environments that differ dramatically in both climactic variables as well as community assemblage.

It is possible that organisms, in a sense, co-evolve with their own local assemblage where within a home area one is well-adapted to a particular suite of species, but over time becomes maladapted to those assemblages in a new area (Thompson 2005). The "geographic mosaic theory of coevolution" describes the intimate interactions of competition, predation, and herbivory and how these interactions must vary in intensity across large spatial scales (Thompson 2005). This theory is often cited as a mechanism for reinforcing population subdivision and explaining the reduction in fitness for migrant individuals introduced into new habitats (Thompson et al. 2002). Although glacial cycling may explain the creation of the phylogeographic pattern shared across species along this coast, natural selection may be constraining gene flow between regions and reinforcing the effects of isolation caused by glaciation.

Maintenance of Diversity and Reproductive Barriers

An underlying issue in exploring questions regarding the maintenance of genetic diversity is that of reproductive isolation between divergent populations. The evolution of reproductive barriers generally involves the isolation of populations and subsequent natural selection or genetic drift causing populations to diverge over time (Haldane 1930). When the

original barrier to gene flow is removed populations can no longer exchange genes. These barriers can be subtle such as microhabitat or host differences or obvious such as land bridges or continental drift (Mayr 1942; Bush 1969; Via 1999; Coyne and Orr 2004; Hewitt 2004)

In marine systems, many of these obvious examples do occur, such as with the Isthmus of Panama, but seem to not be as prevalent or at least apparent (Palumbi 1994b). Allopatric speciation has historically been the dominant model for speciation in terrestrial and freshwater systems. However, for marine systems this model has been a controversial topic because of the lack of obvious physical barriers and the dispersal potential of most marine species (Mayr 1954; Palumbi 1994b; Lessios et al. 1998; Wiley 2002). The genealogical data that has accumulated for marine species over the past few decades has resulted in one unifying conclusion: we have more diversity in these systems than was ever expected (Knowlton 1983; Hilbish 1996). Although consistent intra-specific population genetic differentiation has been discovered within many coastal marine species, few studies have explored whether reproductive barriers exist between these populations (Palumbi 1994; Avise 2000; Wares and Cunningham 2001; Wares 2002). Thus, it is difficult to know if we are dealing with simply intra-specific variation, incipient speciation, or 'cryptic' species.

Focal Species: Idotea balthica

Idotea balthica is an isopod species that spans the North Atlantic Ocean from North America to Iceland to the entirety of Europe. Although isopods have direct developing offspring and thus do not undergo a planktonic dispersing larval phase, many are able to overcome this dispersal handicap by rafting on algal mats, allowing them to travel large distances (Tully and O' Ceidigh 1986, 1987; Tuomi et al. 1988; Ingólfsson 1995; Thiel and Gutow 2005). Like many other North Atlantic intertidal species, *I. balthica* exhibits population differentiation along the North American coast with strongly divergent northern and southern populations. Northern and southern populations have an estimated divergence time in the Pleistocene or late Pliocene

based on mitochondrial cytochrome oxidase I differentiation and molecular clock estimates from the snapping shrimp, *Alpheus* (Knowlton and Weigt 1998; Wares 2001).

Feeding biology has been an enigmatic feature of interest in this species. European populations, especially in the Baltic Sea, feed mainly (especially in adult life stages) on *Fucus vesiculosus* (Nicotri 1980; Salemaa 1987; Jormalainen et al. 2001). *F. vesiculosus* is a brown alga that is common in the North Atlantic Ocean; it requires hard substrates and is also known for its production of defensive anti-herbivore chemistry, making it a less favored food source for most marine herbivores. Because this algal species, along with many others that inhabit the North Atlantic intertidal, requires rocky substrate, it has a limited presence in southern soft-bottom habitats of North America (Jennings et al. 2009). *I. balthica* in North America are thought to consume a more varied diet than those in Europe (Wares 2001), but because of a lack of research in the feeding biology of North American populations, it is not known what the feeding preferences are for these populations and whether they vary on a geographic scale.

Due to *I. balthica*'s distributional range, dispersal strategy, and potential for local adaptation and specialization for food resources, it is an ideal species to address how gene flow, historical processes, and natural selection can affect population genetic structure. The following chapters present a detailed account of research conducted to address the previously outlined hypotheses for population genetic differentiation in this species, with possibly broader implications for similar fauna in this region.

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INTRASPECIFIC VARIATION IN THE NORTH ATLANTIC ISOPOD, *IDOTEA BALTHICA*: A REASSESSMENT[1]

[1]Bell, T. M. B. and J. P. Wares will be submitted to *Marine Ecology Progress Series*

INTRODUCTION

Glacial and interglacial cycles during the Pleistocene, combined with dramatic changes in sea level, have led to large latitudinal oscillations in species distributions and the restructuring of community assemblages (Southward 1991; Valentine and Jablonski 1993a; Van Oppen et al. 1995; Perry et al. 2005; Wilson et al. 2005). Paleontological records provide sound evidence of distributional changes in many terrestrial species (Huntley and Birks 1983; Pielou 1991; Tzedakis et al. 1997; Willis and Niklas 2004). However, such evidence that would ideally outline the distributions of many marine intertidal species during these time periods is difficult or impossible to access because of the rise in sea level during the Holocene (Valentine and Jablonski 1993; Marko 2004) . Therefore, our knowledge of marine intertidal species' ranges during glaciation and the location of glacial refugia based on physical evidence is lacking. Population genetic data collected from extant taxa, therefore, offer a unique opportunity to infer demographic events, distributional changes, and dispersal routes for many marine taxa where little historical information is available in the fossil record (Hewitt 1996; Cunningham and Collins 1998; Hewitt 1999, 2004; Maggs et al. 2008; Riginos and Henzler 2008).

Rocky coastlines of North America and Europe exhibit the scars of past glaciation events. Ice sheets of greater than 1 kilometer in thickness covered the coastal areas of North America as far south as Long Island Sound (LIS) and much of northern Europe (Pratt and Schlee 1969; Shackleton et al. 1984). It is the general consensus that the majority of rocky intertidal species did not persist in their current ranges during glaciation and were faced with extinction or migration to regions outside of the glacial extent (Ingólfsson 1992; Cunningham and Collins 1998; Wares and Cunningham 2001). Phylogenetic data support this hypothesis, and from these data we now know that the majority of the rocky intertidal floral and faunal communities were shifted and restructured as a consequence of glaciation (Wares and Cunningham 2001).

Glacial refugial areas for marine intertidal species can be difficult to determine due to inconsistencies in paleo-climate reconstructions, the limitations of geographic sampling, the variability around molecular clock estimates, or the confounding signal of genetic diversity created by secondary contact (Sejrup et al. 2005; Jesus et al. 2006; Maggs et al. 2008; Riginos and Henzler 2008). Southern areas of coastal North America and Europe would be available refugia and have been proven to be so for most species. But the transition from rocky coastline to soft sediments that occurs along the North American coast make the southeastern U.S. an unlikely refuge for many obligate rocky intertidal species (Maggs et al. 2008). Offshore seamounts exposed by reductions in sea level are plausible refugia; however, this is a difficult scenario to test as these areas are now submerged (Maggs et al. 2008). The Mid-Atlantic Islands (Iceland, Greenland, the Faroes Islands) and regions of the Canadian maritimes have also been cited as potential refugial areas (Holder et al. 1999; Rundgren and Ingólfsson 1999; Ægisdóttir and þórhallsdóttir 2004). Many argue the validity of these areas as potential refugia for temperate taxa because of their position in high latitudes (Ingólfsson 2006; Riginos and Henzler 2008).

Phylogeographic patterns along the North American and European coastlines have indicated that populations of North American marine coastal species are more genetically similar to some European populations than to those in closely neighboring areas (Wares and Cunningham 2001a). In general, North American populations of many marine taxa tend to have a lower genetic diversity than those in Europe (Wares and Cunningham 2001), suggesting that glaciation caused local extinction of many North American populations and the re-colonization of this region occurred from European populations (Vermeij 1991; Ingólfsson 1992; Van Oppen et al. 1995; Wares and Cunningham 2001; Vermeij 2005). However, for *Mytilus edulis* and *Arctica islandica,* dispersal from North American refugia to the European coastline via Mid-Atlantic Islands has also been proposed (Dahlgren et al. 2000; Riginos and Henzler 2008). The re-

colonization of previously glaciated areas, therefore, could have occurred by many possible scenarios and the idiosyncratic pattern of individual species makes a general pattern elusive.

Many plausible hypotheses have been proposed to explain the dispersal route across the Atlantic and to pinpoint potential source populations for the re-colonization of previously glaciated areas. Trans-Atlantic dispersal is one hypothesis; however, this probably occurred at low frequency even in species with high dispersal capabilities (Ingólfsson 1992; Colson and Hughes 2007; Riginos and Henzler 2008). High-latitude marine intertidal species have a proportionally higher incidence of brooding, or non-planktonic dispersal, than lower latitude species making the span of the North Atlantic a large obstacle (Hansen 1978; Perron and Kohn 1985). Many brooding species circumvent this obstacle by rafting on floating seaweed or other debris and it is thought possible that they could disperse in this manner across wide expanses (Ingólfsson 1995; Thiel and Gutow 2005). However, the frequency with which colonization of distant suitable habitat occurs via rafting is unknown.

Migration from neighboring refugial populations is another possibility with dispersal occurring in a 'stepping-stone' like fashion from Europe to the proposed refugial areas by way of the Faroes Islands, Iceland, and Maritime Canada (Maggs et al. 2008). Phylogenetic data from North Atlantic species yields conflicting results for the validity of these putative refugial areas to have actually harbored temperate marine taxa during a glacial maximum (Maggs et al. 2008). Those taxa that currently inhabit these islands show close genetic ties with their European counterparts although with lower diversity (Wares and Cunningham 2001). Other marine species (*Idotea balthica, Carcinus maenus, Pleuronectes platessa, Fucus serratus*, *Arctica islandica*) exhibit deeper genetic divergence between Icelandic and European populations, leading to the suggestion that there were some regions of suitable habitat in Iceland creating a refuge for these wayward populations (Dahlgren et al. 2000; Wares and Cunningham 2001; Coyer et al. 2003; Hoarau et al. 2004; Roman and Palumbi 2004). However, as geologic

records and current re-constructions of glacial extents in this region point to all parts of Iceland and the Faroes being completely encased in ice at this time period, this deep divergence between Iceland and Europe could actually be an artifact of limited geographic sampling of Northern European populations (Coyer et al. 2003; Sejrup et al. 2005; Ingólfsson 2006; Geirsdóttier et al. 2007; Norðdahl et al. 2008; Riginos and Henzler 2008).

Idotea balthica is a marine intertidal isopod species that is distributed across the North Atlantic, throughout Europe and the North American coast south to Virginia. As a species with direct developing offspring, *I. balthica* is restricted in its dispersal ability and is commonly found rafting on drifting macroalgae (Tully and O' Ceidigh 1986, 1987; Locke and Corey 1989; Ingólfsson 1995; Thiel and Gutow 2005). This type of dispersal often results in a species with a patchy distribution (Bingham 1992; Reed et al. 2000; Johnson et al. 2001). Previous studies have concluded from population genetic data that this species experienced local population extinctions in Canada and the Northeastern U.S. during glacial periods (Wares 2001). Today, northeastern U. S. populations and European populations of *I. balthica* share many mitochondrial cytochrome oxidase I (mtCOI) haplotypes suggesting that European populations re-colonized these coastal areas (Wares and Cunningham 2001). Endemic mitochondrial lineages were discovered in Iceland, Nova Scotia, and along the southern portion of the North American coastline, suggesting that these regions served as glacial refugia for this species (Wares 2001). However, many of these assertions were based on a small sample size and limited geographic sampling. The following study will address the potential dispersal routes for *I. balthica* as well as address potential glacial refugia for this species using mtCOI and increased geographic sampling on both continents.

METHODS

Collections of *I. balthica* occurred over a period of four years from 2005-2009. Isopods were collected from 15 locations in North America, four in Europe and one in the Mid-Atlantic

Islands (number of individuals collected from each population represented in Fig. 2.1). These collections were in addition to three European and Iceland populations from Wares (2001). Isopods were mainly collected using a fine mesh dip net from either seagrass beds or from detached algae masses that tend to accumulate in sheltered coves or inlets. All isopods were preserved in 95% ethanol after collection.

Molecular Analyses

Preserved collections were returned to the University of Georgia where all DNA isolations, PCR, and DNA sequencing were performed. All genetic material was isolated using PureGene DNA isolation protocol (Qiagen, Valencia, California, USA). Tissue used for DNA isolation included 2-3 pereopods from large isopods, or all pereopods from small isopods. Pereopods were ground with plastic pestles in 200μl of PureGene's Cell Lysis solution. Tissue was then allowed to digest overnight with 3 μl of Proteinase K at 37 °C. The following day, digested tissue was placed on ice for five minutes and vortexed with 66 μl of PureGene's Protein Precipitation solution. The digested tissue solution was centrifuged for five minutes in a refrigerated centrifuge at high speed. The supernatant was removed from each isolation and placed in clean 1.5 ml centrifuge tubes. Chilled 100% isopropanol was added to each supernatant (200 μl). Tubes were slowly turned back and forth (50 times) to thoroughly mix the isopropanol with the supernatant. Isolates were centrifuged for three minutes on high speeds in a refrigerated centrifuge. The supernatant was then discarded and pellets were allowed to dry in their tubes by turning the tubes upside down on clean paper towels. Pellets were washed with 100 μl of 70% ethanol, centrifuged for 1 minute, and then dried overnight in a vacuum dessicator.

Mitochondrial cytochrome oxidase I (mtCOI) primers developed by Folmer et al. (1994) were used originally. However, sequencing reactions with these primers were not consistently successful; in order to increase the success rate of sequencing reactions, *I. balthica* specific

primers were designed for mtCOI (ibF--5'-GTTGTAATAAAATTGACAGCCCCTA-3'; ibR--5'-AAGGATGATTATTCGTACGGAGTTAG-3'). Both mitochondrial gene regions were amplified using the following 20 μl recipe: 4 μl Promega GoTaq 5X Buffer (Promega, Madison, Wisconsin, USA), 0.75 mM dNTP, 3.75 mM $MgCl_2$, 0.25 μM (5 pmol) of both forward and reverse primers, 1 μg/μL Bovine Serum Albumin (NEB, Ipswich, Massachusetts, USA), 1 unit of GoTaq polymerase (Promega) and approximately 10 to 50 ng of DNA. Standard PCR conditions were used with the following cycles and temperatures: 94ºC for 60 seconds, 40ºC for 90 seconds, 72ºC for 120 seconds, for 30 cycles. Unincorporated primers and dNTPs were removed using Antarctic phosphatase and Exonuclease I (NEB) using the following protocol: in a 10 μL reaction, 7 μL of PCR product was combined with 0.1 μL of Exonuclease I (20 units/ μL), 0.5 μL of Antarctic phosphatase (5 units/ μL), and 2.4 μL of water. This reaction mixture was then incubated at 37°C for 15 minutes and 80°C for 15 minutes.

Typical sequencing reactions consisted of 8 ng of 'clean' PCR products, 3.3 pmol of reverse primer, 0.5 μl of BigDye v. 3.1 terminator (Applied Biosystems) and 2 μl of 5X sequencing buffer (Applied Biosystems) in a final reaction volume of 10 μl. All products were sequenced in the forward and reverse direction. Conditions for cycle sequencing were an initial denaturation of 96ºC for 3 minutes then 30 cycles of 96ºC for 10 seconds, 50 ºC for 5 seconds, and then 60 ºC for 4 minutes. Excess terminator dyes were removed in a precipitation reaction by the addition of 40 μl of 75% isopropanol to each cycle sequencing reaction at which point they were mixed by inversion, and allowed to sit at room temperature for 15 minutes. Isopropanol mixtures were spun in a plate centrifuge at maximum speed for 40 minutes. The mixtures are then turned upside down on a paper towel to drain off isopropanol and then spun inverted in a plate centrifuge for one minute at 500 x g. The final products are reconstituted in 10 μl of Hi-Di Formamide (ABI) and then sequenced on an ABI 3730 capillary sequencer at the University of Georgia.

Phylogenetic Analyses

Sequences were aligned and edited using CODONCODE ALIGNER v. 3.0.2 (CodonCode Corp., Dedham, Massachusetts) and the ClustalW alignment algorithm. Phylogenies were constructed using MRBAYES v. 3.1 (Ronquist and Huelsenbeck 2003) to perform four independent runs of a Markov Chain Monte Carlo (MCMC) analysis with 4 chains running for 2.5 million generations. The HKY + Γ model (α = 0.101) for sequence evolution was determined to be the best fit model according to MODELTEST v. 3.7 (Posada and Crandall 1998). Trees were sampled every 100 generations for each run (the first 5000 trees were discarded as "burnin"). Along with the posterior probabilities estimated by MRBAYES for each clade, support for clades was estimated using maximum parsimony bootstrapping (1000 replicates). Additionally, phylogenetic relationships among haplotypes were inferred with NETWORK 4.2.0.1 (Fluxus Technology Ltd., http://www.fluxus-engineering.com) using maximum parsimony and median joining methods (Bandelt et al. 1999).

The distribution of *I. balthica* was defined into three regions based on the phylogeographic comparisons (illustrated in Wares (2001)) and biogeographic provinces: 1) Southern U.S., 2) Northern U.S., Canada, and Europe, and 3) the Mid-Atlantic Islands (Faroes and Iceland). The Southern U.S. was defined as all populations < 41°N), while the Northern U.S. was defined as all populations > 41°N. The distinction of 41°N was chosen for two reasons, the first being that 41°N is considered to be the southernmost reach of glaciers during glaciation which has influenced the population genetic structure of many North American species (Bernatchez and Wilson 1998; Hewitt 2000; Sotka et al. 2003; Kelly et al. 2006). The second reason this delineation was chosen was due to the fact that it is an area of transition between species whose ranges encompass more northerly areas and those that encompass more southerly areas (Bousfield 1973; Bousfield and Thomas 1975). Individuals from the Faroes Islands and Iceland were defined in a region of their own because of their biogeographic

similarity and also because populations from these regions are genetically similar (Downes 1988; Dahlgren et al. 2000; Coyer et al. 2003; Roman and Palumbi 2004; Colson and Hughes 2007; Rundgren 2007; Riginos and Henzler 2008). Alternative group assignments involving the separation of Nova Scotia and northern North America into their own groups were originally tested; however, these alternatives resulted in higher *P*-values for Φ_{CT}.

An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) in ARLEQUIN v. 3.1.1 (Excoffier et al. 2005) was used to test the hierarchical distribution of molecular variance at different geographic scales. *F*-statistics were calculated using Tamura-Nei distances (Tamura and Nei 1993) between haplotypes and significance was assessed by 10,000 random permutations of haplotypes between each population pair. The relative contribution of molecular variance was estimated at the three following levels: (1) among regions (Φ_{CT}) (Northern North America and Europe; Mid-Atlantic Islands; Southern North America; and Nova Scotia) (2) among populations within regions (Φ_{SC}) and (3) within populations (Φ_{ST}). These were tested using 10,000 permutations of haplotypes between populations with an alpha value of 0.05. To determine if geographic patterns existed with respect to the distribution of genetic diversity, the molecular diversity indices π and θ were calculated for each population and each predefined region. In order to test for neutrality and equilibrium, Tajima's D (Tajima 1989) and Fu's F_s test (Fu 1997) were calculated using ARLEQUIN v. 3.1.1 (Excoffier et al. 2005).

RESULTS

A 491 bp fragment of the mitochondrial COI gene was recovered from 338 isopods from 19 locations spanning North America and Europe (refer to Table 2.1). Additional mtCOI sequences were included in the alignment from Wares (2001) (GenBank Accession Numbers: AF241889-AF241932). The majority of sites had base calls with PHRED scores greater than 35; however, any sites with PHRED scores less than 20 were scored as ambiguous (Ewing and Green 1998). Among the 338 individuals collected, 20 haplotypes were identified. Hierarchical

tests of population subdivision supported the regional subgroupings of 1) Northern U. S., Canada and Europe, 2) Mid-Atlantic Islands and 3) Southern U. S. The majority of the genetic diversity was found among these assigned groups (62.15%). The smallest partition of genetic diversity was among the populations within these groups (9.97%) while the remaining 28.8% of the genetic diversity was found within populations. The AMOVA showed high levels of genetic structure among these groups: Φ_{CT} = 0.62147, Φ_{SC} = 0.25811, Φ_{ST} = 0.71917 (P<0.0001 for all three levels).

The median-joining haplotype network had 4 haplogroups: 1) northeastern North America and Europe, 2) Mid-Atlantic Islands and Finland, 3) southern portions of North America, and 4) Chesapeake Bay (Fig. 2.2). This population structure was also reflected in the Bayesian majority rule consensus phylogeny where clades associated with each one of the aforementioned groups had posterior probabilities >95% and maximum parsimony bootstrap support no less than 90%. The 'Chesapeake Bay' group appears to have been derived from the 'Southern' group and is unique to North America (Fig. 2.3).

The single haplotype recovered from the Icelandic samples in Wares (2001) was also found in the Faroes Islands. This Iceland/Faroes haplotype was genetically similar to a haplotype only found in Finland. The Faroes Islands have an additional haplotype (haplotype 1) that is shared with Northern North America and all populations in Europe. Overall, there was a higher number of haplotypes for the 'Northern North America/European' group, however most of this diversity existed as singletons. Pairwise nucleotide diversity (π) was much lower in Europe than in North America. Tajima's D and Fu's F_s were not significant in any population in Europe (Table 2.1). Three populations (Chamberlain, ME; James River, VA; Nahant, MA) exhibited significantly negative Tajima's D values. A general trend in the resulting Tajima's D values was an excess of positive values on the North American coast mainly in the admixed region from LIS to Cape Cod. The results of Fu's $F_s$test revealed negative values for F_s in the Nahant, MA and

James River, VA, however there were no significant results for any population. Because of the lack of significance, Fu's F_s is not reported in Table 2.1.

The number of haplotypes steadily increases in *I. balthica* populations south of LIS. Although the number of haplotypes recovered is lower in southern North America than in northern areas and Europe, most of these haplotypes were not singletons and were shared across several populations and many individuals. Haplotype 1 was extremely widespread and exists in every population sampled (although at times at very low frequency) except for Cousin's Island, Maine and Iceland (Fig. 2.3, 2.4). Increased sampling intensity along the North American coastline reveals a clinal gradient in haplotype frequency of the two major haplotypes that inhabit this coastline (the 'northern'/European type (haplotype 1) and the 'southern'/Virginia type (haplotype 5) (Fig. 2.5). A large admixed area of the two haplotypes exists between LIS and the northern coast of Cape Cod, Massachusetts (~41°N latitude) (Fig. 2.5). The Chesapeake Bay haplogroup is at highest frequency in the Chesapeake Bay, but exists at low frequency along the coastline from the Chesapeake Bay to LIS with an anomalous population in Antigonish, Nova Scotia.

DISCUSSION

Population Structure Across the North Atlantic

Species with direct-developing offspring are often assumed to have restricted gene flow between populations and exhibit a high degree of population genetic structure. Given *I. balthica*'s lack of planktonic dispersal, it would be expected for it to show a higher level of endemism than planktonic dispersers (Behrens Yamada 1989). Although there is evidence of population differentiation in this species, it is lower than anticipated given their wide distribution (Bulnheim and Fava 1982; Wares 2001). In fact, many of the North Atlantic coastal taxa with planktonic larval development exhibit population genetic differentiation similar to *I. balthica*: North American populations are similar to those in Europe and Mid-Atlantic island populations

are either distinct from all others or related to populations in Northern Europe (Dahlgren et al. 2000; Coyer et al. 2003; Roman and Palumbi 2004; Riginos and Henzler 2008). Rafting on detached algal mats and other debris appears to substantially aid in the gene flow of this species by allowing for populations on either side of the expanse of the Atlantic Ocean to retain genetic similarity.

The molecular diversity of *I. balthica* populations can be divided into three statistically distinguishable regions: 1) the Northern U.S, Nova Scotia and Europe, 2) the Southern U. S., and 3) the Mid-Atlantic Islands. These regions are similar to what was originally described in Wares and Cunningham (2001a) for *I. balthica* and what have been described in other coastal species in the North Atlantic (Dahlgren et al. 2000; Coyer et al. 2003; Roman and Palumbi 2004; Colson and Hughes 2007; Riginos and Henzler 2008). Northern regions of North America were likely re-colonized after the last glacial maximum by individuals originally from European populations. For now, it appears that a 'stepping-stone' progression across the Mid-Atlantic Islands is not likely since North American haplotypes are more similar to southern European types than to Northern European and Icelandic types. Because of the widespread nature of the major European haplotype (haplotype 1) and the low sample sizes from both Iceland and Nova Scotia it is difficult to tell if colonization occurred by way of a 'stepping-stone' progression across the Mid-Atlantic Islands or a trans-Atlantic rafting event. Reconstruction of historical colonization routes in this species will require the use of more variable molecular markers and more thorough collections in Iceland, Europe, and Nova Scotia.

The Icelandic haplotype first identified by Wares and Cunningham (2001) was thought to be an endemic lineage that has persisted in Iceland throughout many glacial cycles. Based on the data available at the time, it was estimated that this haplotype and those of southern Europe had a 200,000 year divergence time. However, the results of this study suggest that this previously proposed endemic Icelandic haplotype is closely related to a common haplotype in

Finland. Assuming that this Finland haplotype was the source population for the Icelandic haplotype, the divergence time would thus be much less than previously estimated by Wares and Cunningham (2001). Unique Icelandic lineages have also been described in other marine intertidal to subtidal taxa (*Carcinus maenus, Arctica islandica, Pleuronectes platessa, Fucus serratus,* and *Mytilus edulis*), but with the general conclusion that these lineages dispersed to Iceland after the last glaciation from an unknown source population either in Europe or North America (Dahlgren et al. 2000; Coyer et al. 2003; Hoarau et al. 2004; Roman and Palumbi 2004; Riginos and Henzler 2008).

Although there have been a few reports of terrestrial plants and animals utilizing Iceland as a glacial refuge, geologic records suggest that the Mid-Atlantic Islands remained capped in ice during past glaciations (Holder et al. 1999; Rundgren and Ingólfsson 1999; Sejrup et al. 2005). It is currently unclear if *I. balthica* would have been able to tolerate the ocean temperatures in Iceland during this time period. In the winter months, this species all but disappears from its normal habitats in New England, suggesting a lack of cold tolerance (T. M. Bell personal observation). A lack of cold tolerance has also been proposed as an explanation for *I. balthica*'s present day distribution in Iceland where it only inhabits the southern coastline (Ingólfsson 2006). Thus, based on mtCOI haplotype data collected in this study and other geologic and ecological data it is not probable that Iceland served as a glacial refugia for *I. balthica*. Further, it is likely that today's Icelandic and Faroes Island populations originated from Northern Europe and colonization of these islands occurred after the last glaciation.

Population Structure within North America

LIS delineates an area of transition in genetic diversity for populations of *I. balthica* along the northwestern Atlantic coastline. Populations residing south of LIS served as glacial refugia as indicated by the presence of several endemic haplotypes as well as some populations containing two to four times the number of haplotypes than a northern or European population

(Table 2.1). Populations along this coast were also the only ones with significant Tajima's D values. Tajima's D statistic (Tajima 1989) is a measure of the mutation frequency spectrum between individuals within a population and is positive when there is an excess of high-frequency mutations. A positive Tajima's D is indicative of a past population bottleneck or balancing selection, while a negative D indicates selective sweeps, purifying selection, or population expansion (Tajima 1989). Positive Tajima's D values were found in the admixed region from just south of LIS in New Jersey to Cape Cod. Although these values are not statistically significant, many deviate substantially from zero (ranging from 1.78 to 2.9) pointing toward the retention of divergent lineages in these populations. This signature would be expected in populations where both major haplogroups are equally represented and offers an explanation for how this diversity is being maintained.

North American *I. balthica* had significant negative Tajima's D values in the following locations: the James River, VA, Nahant, MA, and Chamberlain, ME. Two of these locations, Nahant, MA and James River, VA had negative values for Fu's F_s, but they were not significant. Negative Tajima's D values are sometimes an indication that mtCOI may have experienced recent purifying selection or is being 'swept' along with other loci or cytoplasmic incompatibility symbionts/parasites (such as *Wolbachia* or microsporidia present in terrestrial isopods and marine amphipods respectively) (Tajima 1989; Johnstone and Hurst 1996; Hartl and Clark 1997; Rigaud et al. 1999). Given the history of glaciation along this coast, the negative values estimated for these populations are most likely a signature of population expansion that occurred following the last glacial maximum and the warming of ocean waters. Fu's F_s is suggested to be more sensitive to signatures of population expansion than Tajima's D but is also becomes unreliable at small sample sizes. Although it is not known definitively why none of the four populations significant at Tajima's D were not significant at Fu's F_s, many of the

sample sizes for this study were relatively low. This low sample size might explain the incongruence between these statistics (Ramos-Onsins and Rozas 2002).

The 'Chesapeake Bay' haplogroup is identical to the Nova Scotian clade proposed as a con-generic species (*Idotea phosphorea*) in Wares (2001). Individuals collected in this study that belong to what will be referred to as the 'Chesapeake Bay' haplogroup along with those collected in Wares (2001) have been compared at the morphological and genetic level to type specimens and field-collected individuals of *I. phosphorea* and they are clearly distinct entities (T. M. Bell unpublished data). The patchiness in the distribution of the 'Chesapeake Bay' haplogroup may imply that this group at onetime had a more widespread distribution or instead may be an artifact of the rafting dispersal strategy of this species.

Although in general the 'southern' and 'northern' haplogroups have a more continuous distribution, there is one instance with the 'southern' haplogroup that is also likely the result of a rafting dispersal strategy. *I. balthica* is in high abundance in Northern Massachusetts and southern Maine but is almost exclusively (98%) the 'northern' haplogroup at almost all populations collected in this region. One exception lies in Cousin's Island, Maine where out of the 50 individuals collected from this locality 100% belonged to the 'southern' haplogroup. Nearshore currents along the Maine coast seem to follow the path of the Gulf of Maine Coastal Current flowing in a southwest direction that would preclude the hypothesis that these individuals drifted from a more southerly population (Pettigrew et al. 2005; Muhlin et al. 2008). In general, nearshore currents are often inconsistent in their strength, directionality, and temporal longevity making them an unlikely predictor of past dispersal routes (Largier 2003). However, Locke and Corey (1989) state that amphipods and isopods often raft into the Bay of Fundy from the south during the summer. Although the exact reasoning for these anomalous populations is unknown and could always be the result of human introduction, they still are a

strong indication of how the rafting dispersal strategy may allow this species with crawl-away offspring an opportunity to disperse over large distances.

An interesting feature along the North American coastline in *I. balthica* is the cline in frequency of the 'northern' and 'southern' haplogroups (Fig. 2.5). Currently, it is unclear what has restricted the complete spatial mixing of these two groups. One explanation for this cline is of dispersal limitations, implying that either these groups are in the process of admixture, but an insufficient amount of time has transpired to allow for equal mixing of the two groups or that currents greatly restrict gene flow between the two regions. This explanation seems unlikely given that this species' has dispersed across the North Atlantic in the same time period (~20,000 ybp) and the long distance rafting events suggested here and by others (Tully and O' Ceidigh 1986; Locke and Corey 1989; Thiel and Gutow 2005). The more likely explanation is that of natural selection. Natural selection and dispersal has been posited as a mechanism for maintenance of clinal patterns in many terrestrial systems as well as in marine species (Endler 1977; Hare and Avise 1996; Hilbish 1996; Arnold 1997; Rolán-Álvarez et al. 2002; Sotka et al. 2004; Sotka and Palumbi 2006). Given the transition of biogeographic provinces and gradients in sea surface temperature that characterize this coastline, it is likely that natural selection plays a role in maintaining this cline in genetic diversity within this species. The potential for natural selection to restrict dispersal of these two haplogroups and maintain this cline in haplogroup frequency will be discussed in the subsequent chapters as well as an exploration into possible reproductive barriers between the two groups.

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Figures

Figure 2.1

Sampling locations of *Idotea balthica* in North America and Europe.
This figure shows the locations where isopods were collected during the summers of 2005-2009. Except for two locations (Reykjavik, Iceland and Galway Bay, Ireland) which were collection locations from Wares (2001). Green shading outlines glacial coverage during the LGM. Numbers in parentheses represent numbers of individuals from that location included in phylogeny.

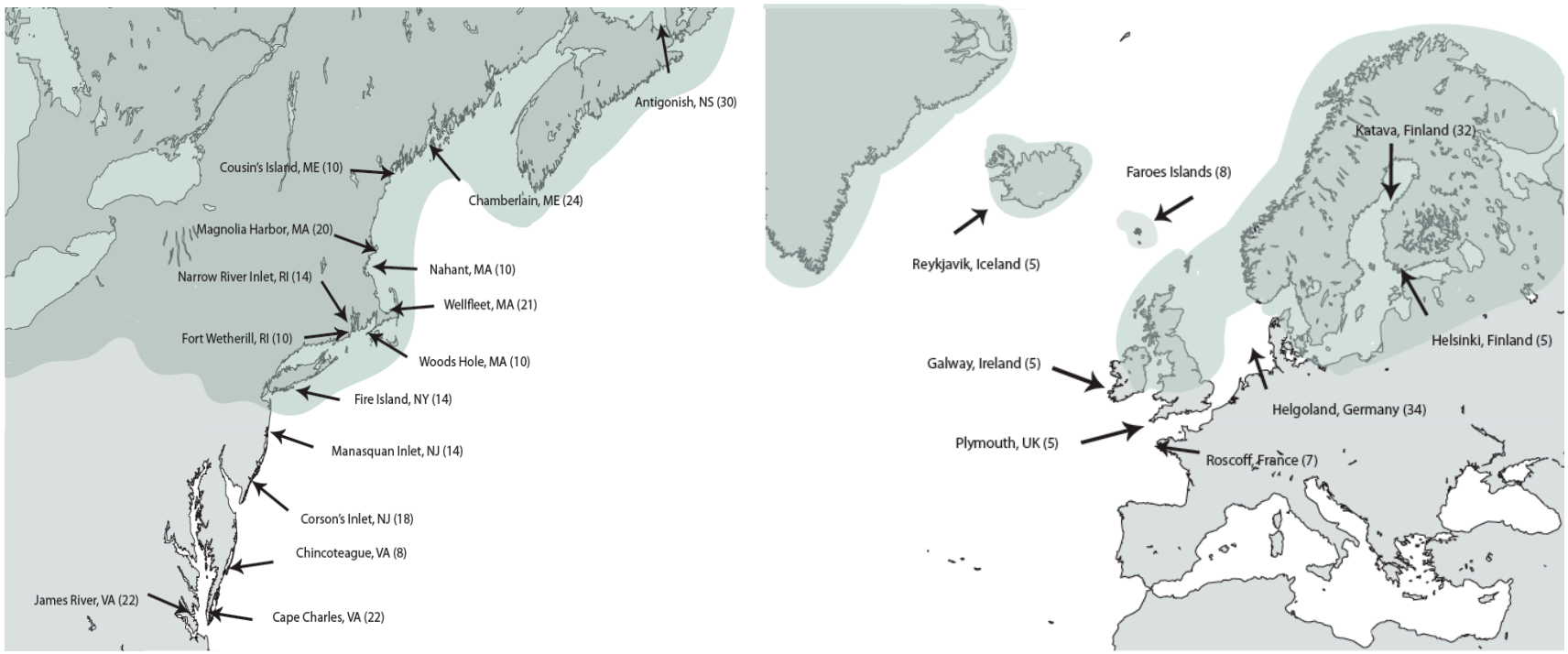

Antigonish, NS (30)
Cousin's Island, ME (10)
Chamberlain, ME (24)
Magnolia Harbor, MA (20)
Narrow River Inlet, RI (14)
Nahant, MA (10)
Wellfleet, MA (21)
Fort Wetherill, RI (10)
Woods Hole, MA (10)
Fire Island, NY (14)
Manasquan Inlet, NJ (14)
Corson's Inlet, NJ (18)
Chincoteague, VA (8)
James River, VA (22)
Cape Charles, VA (22)
Reykjavik, Iceland (5)
Faroes Islands (8)
Katava, Finland (32)
Helsinki, Finland (5)
Galway, Ireland (5)
Helgoland, Germany (34)
Plymouth, UK (5)
Roscoff, France (7)

Figure 2.2.

Median joining haplotype network for mtCOI.

This figure illustrates the mtCOI haplotype network for 338 North American and European isopods. Dashes along a given branch are mutations that have occurred along that branch. Size of haplotype circles indicates the frequency of that haplotype. Smallest circle in frequency scale = 2 individuals. Haplotype 1 represents the 'Northern' U.S./European clade first described in Wares (2001), while Haplotype 5 represents the 'Virginia' clade first described in Wares (2001).

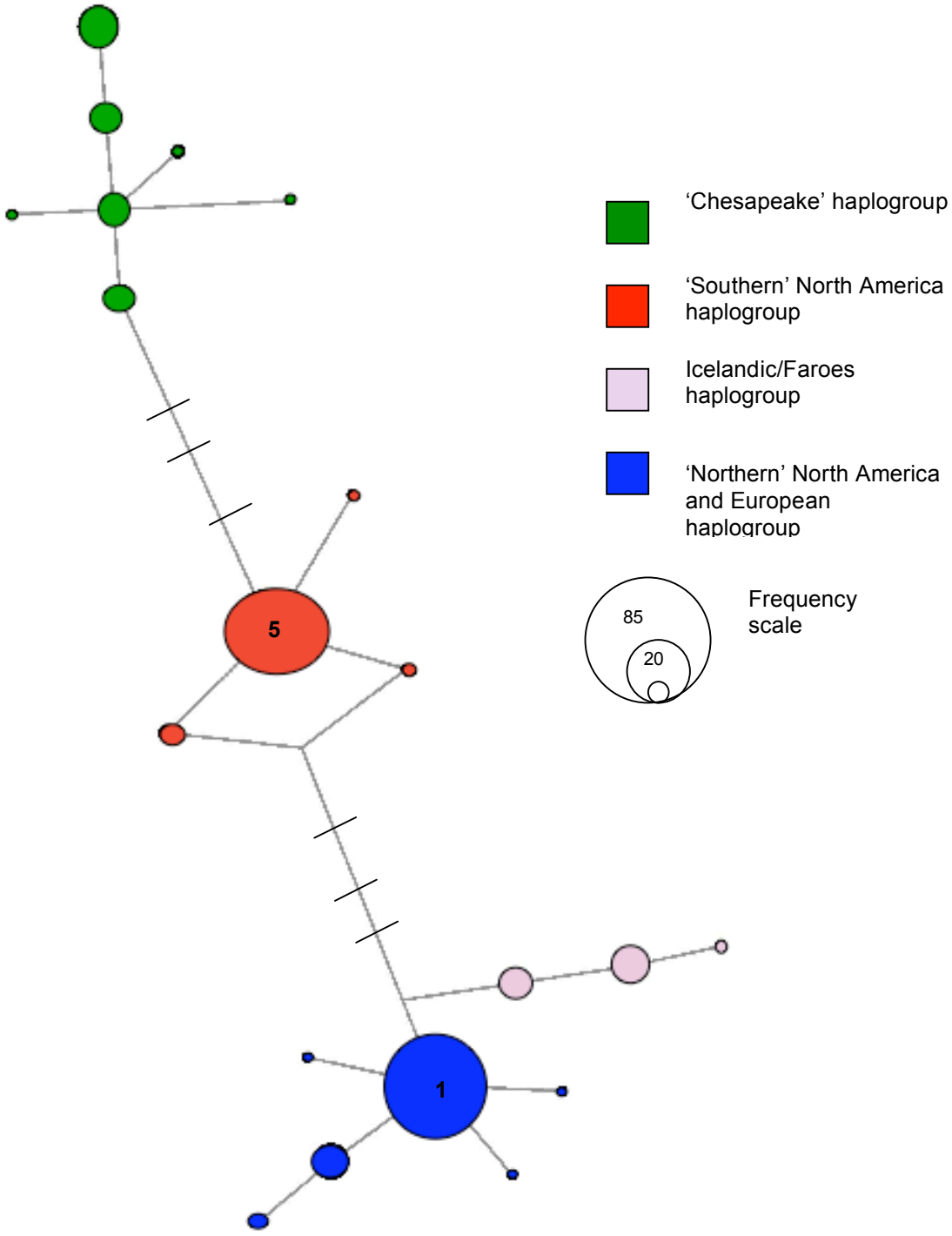

5
1
‘Chesapeake’ haplogroup
‘Southern’ North America haplogroup
Icelandic/Faroes haplogroup
‘Northern’ North America and European haplogroup
85
20
Frequency scale

Figure 2.3.

Bayesian mtCOI phylogeny across all North Atlantic populations of *Idotea balthica.*

This figure illustrates the phylogenetic relationship between North Atlantic populations. The green clade is the 'Chesapeake' clade, the blue clade is the 'Northern'/European clade, red is the 'Southern' clade while lavender is the Icelandic/Faroes/Finland clade. Localities listed next to the phylogeny are where individuals in each clade were collected. Bayesian posterior probabilities for each clade are represented above branches while bootstrap values are below branches. All probabilities and bootstrap values were above 80%. Only those values pertinent to the structure of the phylogeny and discussed in the text are depicted. Phylogeny is midpoint rooted.

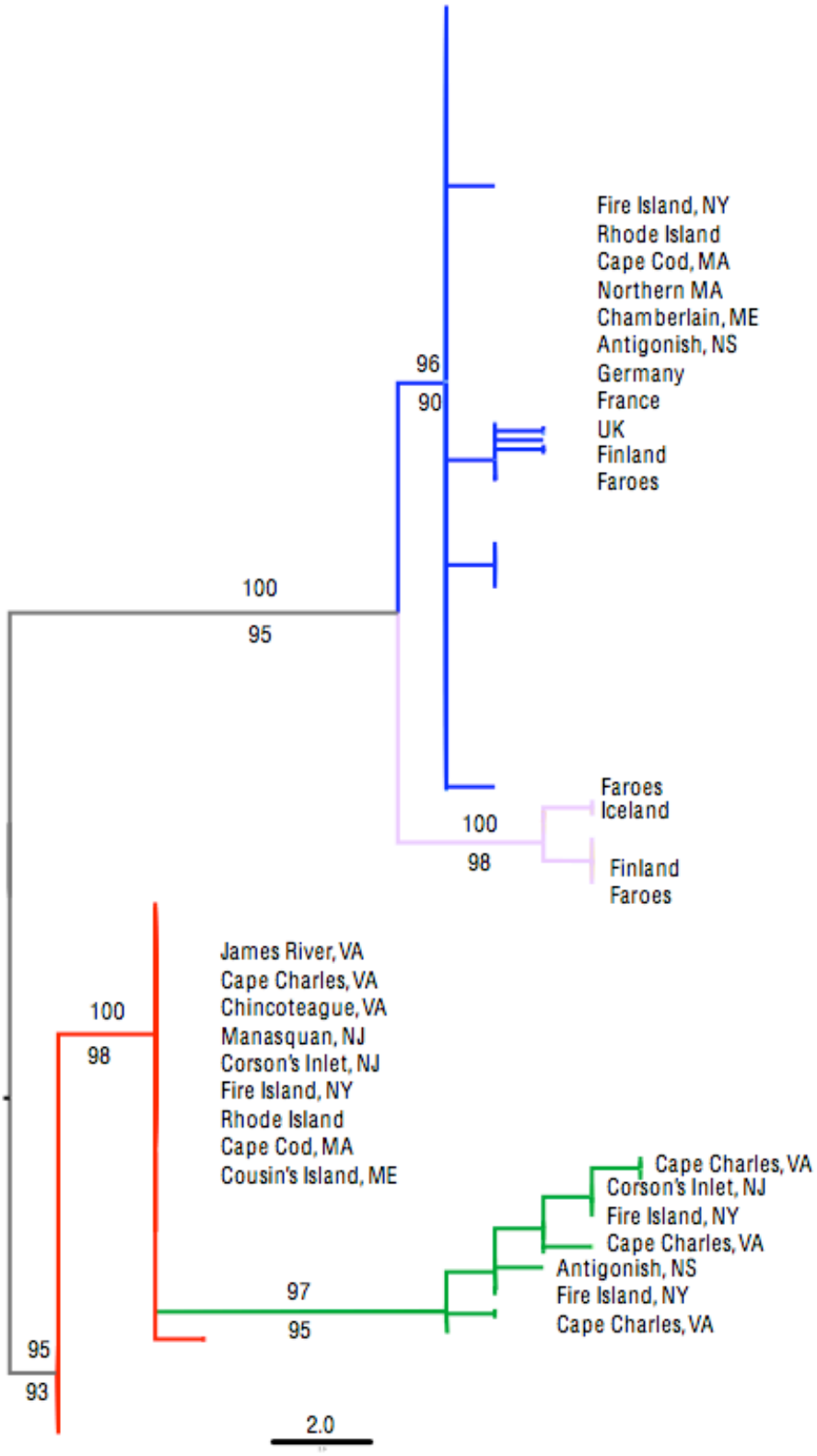

Fire Island, NY
Rhode Island
Cape Cod, MA
Northern MA
Chamberlain, ME
Antigonish, NS
Germany
France
UK
Finland
Faroes
96
90
100
95
Faroes
Iceland
100
98
Finland
Faroes
James River, VA
Cape Charles, VA
Chincoteague, VA
Manasquan, NJ
Corson's Inlet, NJ
Fire Island, NY
Rhode Island
Cape Cod, MA
Cousin's Island, ME
100
98
Cape Charles, VA
Corson's Inlet, NJ
Fire Island, NY
Cape Charles, VA
Antigonish, NS
Fire Island, NY
Cape Charles, VA
97
95
95
93
2.0

Figure 2.4.

Mitochondrial COI haplogroup frequency across the North Atlantic.

This figure illustrates the differences in haplogroup across the North Atlantic for I. balthica. Frequency is indicated by pie charts at each region. Colors on pie charts are concordant with the legend presented in Figure 2.2**.**

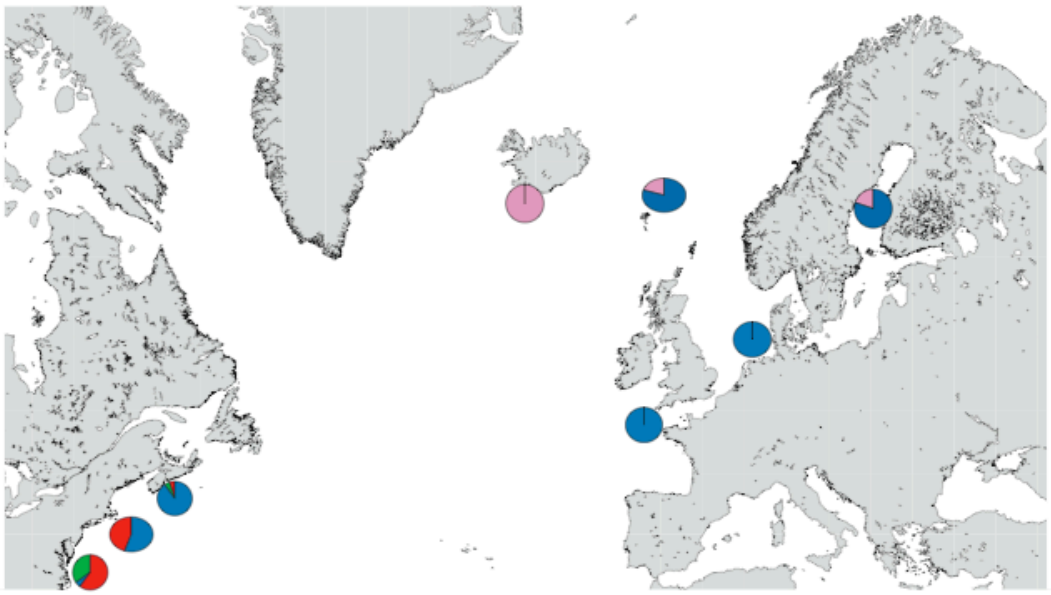
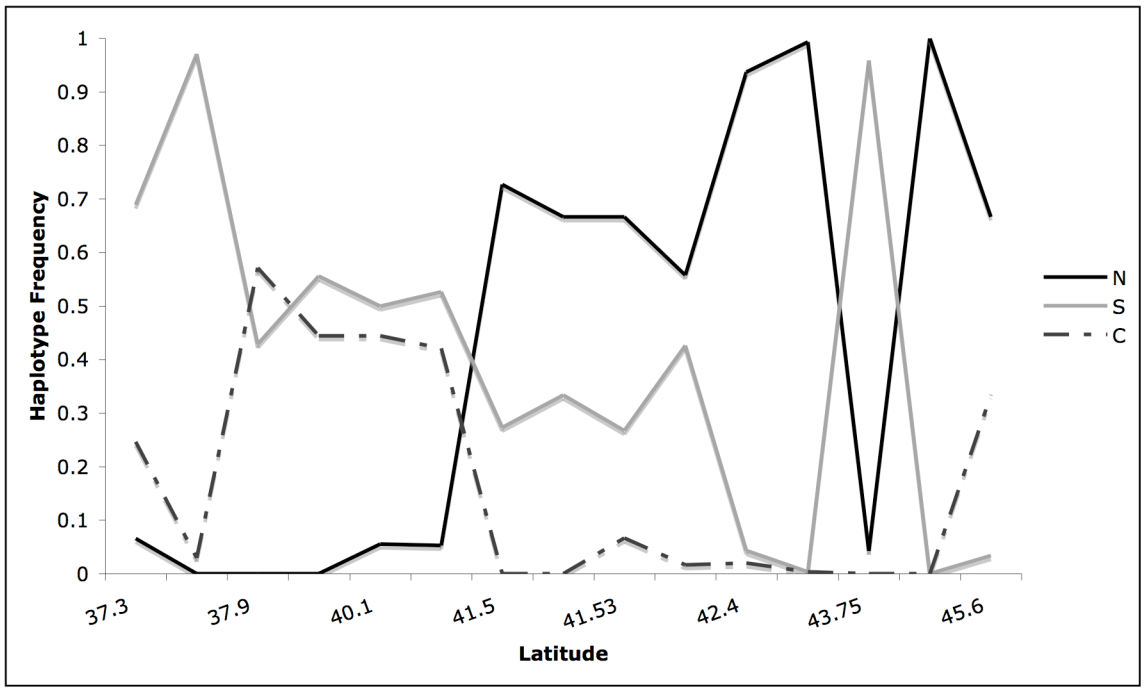


Figure 2.5.

Frequency of major North American haplogroups.

This figure represents the latitudinal variation in haplogroup frequency along the North American coastline. The 'Northern North America/European' (haplotype 1) haplogroup is denoted by 'N' in the legend; the 'Southern North America' haplogroup is denoted by 'S' (haplogroup 5); the 'Chesapeake' haplogroup is denoted by 'C.'


Haplotype Frequency
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
37.3
37.9
40.1
41.5
41.53
42.4
43.75
45.6
Latitude
N
S
C

Tables

Table 2.1

Genetic diversity by location.

SD is the standard deviation of each statistic. Asterisks indicate *P*-values <0.05 after sequential Bonferroni correction. Bold faced Tajima's D values indicate those populations that are in regions of admixture.

Population	Latitude	N	# haplotypes	θ (SD)	π(SD)	Tajima's D
Cape Charles, VA	37.3	29	9	4.838(1.824)	4.504(2.541)	-0.24
James River, VA	37.3	22	4	2.743(1.216)	0.909(0.655)	-2.28*
Chincoteague, VA	37.9	8	3	5.014(2.489)	6.321(3.355)	1.32
Corson's Inlet, NJ	39.2	21	4	3.613(1.526)	6.162(3.051)	**2.527**
Manasquan Inlet, NJ	40.1	11	5	4.779(2.209)	6.691(3.42)	**1.762**
Fire Island, NY	40.6	13	4	4.511(2.024)	7.154(3.588)	**2.43**
Fort Wetherill, RI	41.5	11	2	3.756(1.801)	3.6(1.977)	**-0.178**
Narrow River Inlet, RI	41.4	22	2	3.018(1.310)	4.571(2.334)	**1.782**
Woods Hole, MA	41.53	10	3	6.009(2.758)	7.267(3.718)	**0.973**
Wellfleet, MA	41.9	17	2	3.254(1.461)	5.824(2.929)	**2.931**
Nahant, MA	42.4	24	2	4.820(1.885)	1.500(0.936)	-2.484*
Magnolia, MA	43.6	20	2	0.282(0.282)	0.100(0.178)	-1.164
Cousin's Island, ME	43.75	6	1	0.000(0.000	0.000(0.000)	0
Chamberlain, ME	44	32	2	2.731(1.137)	0.6875(0.537)	-2.375*
Antigonish, Nova Scotia	45.6	32	2	3.973(1.525)	3.613(1.881)	-0.3039
Reykjavik, Iceland	64.1	5	1	0.0000(0.0000)	0.00(0.00)	0
Faroes Islands	61.8	5	2	1.92(1.267)	1.6(1.128)	-1.094
Plymouth, UK/ Galway Bay, Ire.	51.4	5	3	1.44(1.016)	1.6(1.128)	0.699
Helgoland, Germany	54.2	14	2	0.629(0.474)	0.879(0.654)	1.0796
Helsinki, Finland	60.18	5	2	2.4(1.513)	2(1.343)	-1.124
Katava, Finland	61.62	18	3	1.744(0.898)	2.758(1.532)	1.913
Roscoff, France	48.7	8	3	1.157(0.781)	1.393(0.951)	0.839
Regions						
Europe/Northeastern North America		224	12	3.842(1.753)	3.583(1.826)	-0.182
Southern U. S.		104	10	3.833(2.323)	5.249(2.558)	1.064
Mid-Atlantic Islands		10	2	1.414(0.993)	0.800(0.628)	-1.667

CHAPTER 3

GEOGRAPHIC VARIATION IN FEEDING BEHAVIOR OF NORTH AMERICAN POPULATIONS OF THE NORTH ATLANTIC ISOPOD, *IDOTEA BALTHICA*[2]

[2]Bell, T. M. B. and J. P. Wares will be submitted to *Oecologia*

INTRODUCTION

Organisms encounter many abiotic and biotic factors that vary in their presence and intensity on temporal and spatial scales. Widely distributed species inevitably experience a variety of environments and consequently interact with a variety of other organisms. The heterogeneity that occurs within a species' range can create opposing natural selective pressures between populations, whereby alleles favored in one region of the range may be at a disadvantage in other regions (Mayr 1963; Slatkin 1973; Antonovics 1976; Endler 1977; Jaenike and Holt 1991; Thompson 2005). Gene flow between populations can ultimately be constrained by this variation in natural selection or other extrinsic forces leading to adaptive divergence and maintenance of diversity within a species (Slatkin 1973; Antonovics 1976; Endler 1977; Jaenike and Holt 1991; Smith et al. 1997; Lu and Bernatchez 1999; Schneider et al. 1999; Briggs 2006). However, it also follows that an opposing hypothesis may also be true that widely dispersing species will have relatively high gene flow between populations constraining adaptive divergence (Jackson 1974; Gaston 1998; Storfer et al. 1999; Langerhans et al. 2003; Hendry et al. 2007).

Whether gene flow between populations in a given species will act to homogenize or diversify populations is dependent upon many factors. Effectively it is the interaction and intensity of both natural selection and gene flow that play a major role in population divergence and ultimately local adaptation. Other factors such as demography and genetic effects can also intensify and complicate the effects of this interaction (Antonovics 1976; Holt and Gomulkiewicz 1997; Holt 2003). It therefore can be extremely difficult to disentangle what combination of gene flow and natural selection (if it is present) are acting to create population genetic subdivision within a species (Rasanen and Hendry 2008). This statement is especially true for marine ecosystems where dispersal potential is likely higher than in terrestrial systems and coastal

habitats are heterogeneous both in environmental conditions and species assemblages (Palumbi 1994; Briggs 2006).

Shaped by the effects of glaciation, the coastline of the Western North Atlantic offers a rich environment in which to study the interplay between natural selection and gene flow and their roles in creating and maintaining diversity. High-energy rocky coastlines dominate northern areas; the coastal environment rapidly transitions to low energy, soft sediment from Long Island Sound southward (Bertness 2007). Patterns in algal biogeography in this region mirror this transition from hard to soft substrates (Bertness 2007; Jenkins et al. 2008). A diverse assemblage of large macroalgal species covers the rocky coastlines, while soft bottom substrates attract seagrass species that have a shallow root system anchoring them to the shifting soft sediments (Stephenson and Stephenson 1972; Raffaelli and Hawkins 1996; Bertness 2007; Jenkins et al. 2008). Changes in algal biodiversity have a cascading effect on the animals that are able to recruit and settle into these habitats because of their use of algae for food, shelter, or both (Bertness et al. 1999; Bruno and Bertness 2001; Jenkins et al. 2008).

The type and intensity of algal chemical defense against herbivores has been shown to be determined both genetically and environmentally and to be variable on both large and small geographic scales (Gaines and Lubchenco 1982; Paul and Fenical 1986; Hay and Fenical 1988; Paul and Van Alstyne 1988; Steinberg and van Altena 1992; Wright et al. 2004; Pennings et al. 2007). Small marine herbivores typically use their host alga for feeding as well as for refuge from predators and must cope with not only environmental variation but also variation in host chemistry and availability across large spatial scales. These herbivores have a variety of tactics to cope with this chemistry, from using their chemically defended host to ease the detrimental effects of predation to sequestering defensive chemistry into their own tissues (Jensen 1984; Bernays et al. 1989; Hay et al. 1990; Duffy and Hay 1994, 2000).

Increased fitness for species that closely associate with local food resources from their native area can create a barrier to gene flow between populations (Endler 1977; Jaenike and Holt 1991; Coyne and Orr 2004). Conspecifics dispersing into new areas may not be as fit as native individuals because of their inexperience with new food resources. This differential in fitness translates to a reduction in gene flow between populations and can eventually lead to population genetic subdivision (Jaenike and Holt 1991; Hawthorne and Via 2001; Jones 2001; Sotka 2005). Local adaptation to a host plant common in the native range of a species is thought to occur more often in marine species that lack a planktonic dispersal phase of their life cycle (Behrens-Yamada 1989), span over heterogeneous environments, and are small in body size relative to their host (Poore and Steinberg 2001), however, this assertion has yet to be thoroughly explored (Sotka 2005).

An example of the interaction between gene flow and selection for specialization on specific algal host along the West Atlantic coast is that of the herbivorous amphipod *Ampithoe longimana* and its host alga, *Dictyota menstrualis*. *D. menstrualis* is a chemically defended brown alga, occurring in warm temperate and subtropical waters as far north as North Carolina. Populations of *A. longimana* that coexist with *D. menstrualis* utilize this alga as both food and habitat, exploiting the defensive chemistry of the algae for refuge from omnivorous fish that are deterred by its noxious compounds (Duffy and Hay 1994, 2000). Sympatric North Carolina populations of *A. longimana* show no reduction in fitness when feeding on *D. menstrualis* and are genetically distinct from more northerly populations which suffer fitness and choice consequences (Sotka et al. 2003). This concordant pattern in genetic structure and feeding behavior suggests that natural selection for host specialization plays a major role in either creating or maintaining genetic variation in this species.

Another small direct-developing herbivorous crustacean that occurs in this same region, *Idotea balthica,* has a spatial population genetic pattern coinciding with algal biogeography.

Much is known about the feeding behavior and life history of this species in Europe, but little is known about North American populations. Although *I. balthica* is considered to be a generalist herbivore, in Europe, populations tend to have a strong preference for *Fucus vesiculosus*, a common brown alga that is chemically defended by phlorotannins and other polyphenolic secondary metabolites (Ragan and Glombitza 1986; Amsler and Fairhead 2006). Despite a reduction in its growth rate when consuming *F. vesiculosus*, European *I. balthica* still readily consume this alga (Jormalainen et al. 2001a; Jormalainen et al. 2005). *F. vesiculosus* is common along the northern rocky coastline of the West Atlantic, becoming rare in areas from Long Island Sound southward, caused by a paucity of rocky substrate (Taylor 1957). Therefore, it may be possible that *I. balthica* from southern populations will lack both tolerance and preference for this algal species because of the low likelihood of encountering it in its usual geographic range. Along the West Atlantic coast populations of *I. balthica* that reside in areas north of Cape Cod, Massachusetts, and those that reside in the region south of Cape Cod are genetically distinct (see Chapter 2; Wares (2001)). Given the distribution of algal species along this coast, regional differences in feeding preference for a particular algal species could be an underlying factor creating intraspecific diversification similar to what has been observed in *A. longimana*.

The following study has two major goals: to characterize the feeding preferences of *I. balthica* along the West Atlantic coast and to test for concordance between these preferences, population genetic structure, and algal biogeography. The results of both of these assessments will aid in the determination of the potential role of natural selection in limiting gene flow between populations within this species. The following section outlines a series of experiments specifically designed to characterize the feeding behavior of *I. balthica* at the population level in the Western North Atlantic.

MATERIALS AND METHODS

Collection of Natural Populations

I. balthica has a discontinuous distribution along the Western North Atlantic coast, where it is usually found in one of three preferred habitat types: seagrass beds, unattached drifting algal mats, and attached algae along rocky shorelines. Adult isopods were collected in the summer of 2007 and 2008 from four locations that are dominated by either seagrass or drift algae: Cape Charles, Virginia (37°16'04.70"N, 76°01'24.59"W); Wellfleet, Massachusetts (41°54'11.82"N, 70°04'50.12"W); Nahant, Massachusetts (42°25'00.11"N,70°54;38.60"W); and Magnolia, Massachusetts (42°34'32.05"N, 70°42'56.42"W).

Three hundred individuals, approximately equal numbers of males and females, were collected from each location each summer using a mesh net and then transported in aerated coolers to Northeastern University's Marine Science Center in Nahant, Massachusetts. Seven algal and one seagrass species were collected from each location (*Ulva (Enteromorpha) linza, Zostera marina, Gracilaria spp., Ulva lactuca, F. vesiculosus, Polysiphonia harveyi, Chondrus crispus*) and fed to their respective isopod population during a seven-day pre-experiment period. Seawater temperature in Cape Charles, Virginia, was 23°C at the time of collection, while seawater temperature at the Marine Science Center was 12°C. Water temperature for the Virginia populations was slowly lowered (2°C per day) in order to acclimate the isopods fully to laboratory conditions. Individuals from other populations were allowed to acclimate to laboratory conditions for seven days. During the acclimation period, all populations were fed algae collected from their source locality.

Feeding Assays

The ability for isopods to recognize different potential food resources as well as its preference to consume specific algal species was addressed using two different feeding assays. The effects of genealogical identity and geographical origin on feeding preferences were also

evaluated in these assays. Genetic identity was based on assignments to divergent mitochondrial cytochrome oxidase subunit I (mtCOI) lineages (Wares 2001a). Because these haplogroups have no obvious morphological differentiation (Wares et al. 2007), it was difficult to assign each haplogroup equally to each treatment level. To circumvent this potential problem in the experimental design, populations used in this experiment were of known haplotype frequency at mtCOI. Nahant, Massachusetts and Magnolia, Massachusetts, populations are mostly (0.93 and 0.99 respectively) of the "northern" haplogroup; the Wellfleet, Massachusetts, population is of roughly equal frequency (0.545 "northern" type, 0.455 "southern" type); while the Cape Charles, Virginia, population is mostly (0.942) the "southern" type (see Chapter 2).

A no-choice assay was conducted in the summer of 2007 to address the ability of isopods from divergent lineages and different geographic origins to recognize seldom-encountered algae as a food resource. A choice assay was conducted in the summer of 2008 to address the feeding preferences of this species along the Western Atlantic coast and to evaluate differences in preference between haplogroups when a variety of algal species was present.

No-Choice Assay

A no-choice feeding assay performed on all four laboratory-acclimated isopod populations was used to characterize *I. balthica*'s feeding behavior. All algal species chosen for this study are either species on which isopods commonly reside in the field (T. M. Bell personal observation) or species that European populations are known to prefer (Jormalainen et al. 2001a). The food choices offered in these assays were: *Ascophyllum nodosum, F. vesiculosus, U. lactuca, U. (Enteromorpha) linza, P. harveyi, G. tikvahiae*, *C. crispus* and one seagrass, *Z. marina*. All except *Gracilaria tikihaviae* and *Z. marina* were collected from No Name point in Nahant, Massachusetts (42°25'10.57"N, 70°54'11.28"W). *G. tikvahiae* was

collected from Wellfleet, Massachusetts, because it is not common in the Nahant area and *Z. marina* collections were made at the Nahant marina (42°25'00.11"N, 70°54'38.60"W).

Isopods were starved for two days prior to the feeding assay and immediately following the seven-day pre-experiment period. All epiphytes were removed from the algae using forceps prior to weighing. Algae and seagrass were blotted dry with paper towels and cut into 45-mg fragments. Algal fragments were then separately placed into small submersible floating containers (Gladware mini-round 4-oz containers, sealed with small-guage nylon mesh). Thirty individuals from each population (equal numbers of males and females) were each placed separately into a container with one type of algae. For each algal species, 10 control containers were constructed that contained only algae and no isopods.

After 72 hours, or when at least half of the algae had been consumed, all algal fragments were collected from containers, blotted dry, and reweighed. Any autogenic change in algal biomass was accounted for with the following equation:

$$T_i(C_f/C_i) - T_f,$$

where T_i and T_f are the initial and final mass, respectively, of algal/seagrass tissue offered to isopods during the experiment (Sotka and Hay 2002). C_i and C_f are the initial and final masses of the tissue in control containers. Because isopod body mass varies dramatically among populations (T. M. Bell personal observation), isopod body mass was recorded to account for potential effects of body mass on consumption rates. Following the assays, isopods were preserved in 95% ethanol.

The average consumption of each isopod was determined using the equation given above (Sotka and Hay 2002) and then scaled by body mass. Average consumption amounts were square-root transformed in order to satisfy the assumption of homoscedasticity. Even after square-root transformation the assumption of normality was not satisfied, the dataset only deviated slightly from normality and was homoscedastic (Shapiro-Wilk's W test $0.03<P<0.05$;

Bartlett's test P>0.05). However, ANOVA is considered robust to deviations from the normality assumption (Box and Anderson 1955; Lindman 1974). Consumption data were divided into northern and southern regions, Massachusetts and Virginia respectively, in order to assess whether northern populations feed differently than those further south. Regional differences in food consumption were analyzed using an ANOVA where region (Massachusetts vs. Virginia), collection site (where site was nested within region), algal/seagrass species and sex were treated as fixed effects and replicates were considered random effects nested within site and region. All potential interactions of these effects were also considered in the analyses. Effects deemed significant from this analysis were then subjected to *a posteriori* pairwise t-test to evaluate differences between specific treatment levels. There were no detectable differences between sexes in algal consumption or preference (P>0.158). The treatment effect of sex was therefore collapsed during the ANOVA and pooled with spatial effects. All statistical analyses were performed using JMP (Version 8, SAS Institute Inc., Cary, North Carolina, 1989-2008).

Choice Assay

Herbivore food preference was measured using a choice-feeding assay. This assay was performed in the summer of 2008 at the same location using the same populations described above. Food preference by population was evaluated using a similar experiment to that described previously. However, instead of offering each isopod a single food choice, an individual was offered all eight food choices simultaneously. Acclimated isopods were starved for two days prior to the experiment. One hundred and twenty containers were prepared with 25 mg of each alga and randomly assigned one isopod from one of four populations. Thirty control containers with no isopods and the eight food choices were used to control for any autogenic change in the algae/seagrass during the course of the assay. Individuals were allowed to feed on the given food choices for 72 hours or until at least half of one of the choices had been

consumed. Isopods and algae were blotted dry with a paper towel and weighed following the experiment. Isopods were then preserved in 95% ethanol for future DNA isolation.

Since each 'feeding-choice' was not independent, choice assay results were rank transformed and analyzed with a non-parametric Friedman test (Conover 1999). This analysis determines if differences in consumption exist for particular algae/seagrass species across populations within *I. balthica,* while *post hoc* pairwise Friedman tests determined which choices were the most preferred. Consumption and preference differences between regions (Massachusetts and Virginia) were compared with a nonparametric Mann-Whitney U test. An additional Mann-Whitney U test assessed differences in feeding preference based on genetic identity or haplogroup assignment. Since multiple comparisons were performed a sequential Bonferroni correction was used to give an overall alpha of 0.05 (Rice 1989b).

Population Genetic Analyses

All DNA from isopods used in the feeding assays was isolated using protocols outlined in Chapter 2. Sequences (491 bp per individual) were aligned and edited using CODONCODE ALIGNER v. 3.0.2 (CodonCode Corp., Dedham, Massachusetts) and the ClustalW alignment algorithm. The majority of sites had base calls with PHRED scores much higher than 20, however any sites with PHRED scores less than 20 were scored as ambiguous (Ewing and Green 1998a). Terminal ends (approximately 30 bp from each end) of each sequence were trimmed because of numerous ambiguous base calls and overall low PHRED scores. The divergent mitochondrial lineages described in Wares (2001) (and Chapter 2) consistently differ at 23 sites. These lineages easily sort into one of two reciprocally monophyletic clades. Haplogroup assignments were thus visually conducted on maximum parsimony phylogenies constructed in PAUP* v. 4.0b10 (Swofford 2002) that included individuals of known type.

Biodiversity Surveys

The specific suite of algal/seagrass species available to a given isopod population could greatly influence their preference for a particular food choice. Algal species richness data were therefore collected at two sites, Magnolia, MA and Cape Charles, VA. At each location, a 30 cm X 30 cm quadrat was haphazardly tossed six times in an area that *I. balthica* was known to inhabit based on previous field collections. Magnolia, Massachusetts, is a unique site in the sheer abundance of algae and isopods. At this location, unattached drift algae accumulate in large continuous mats up to 1 m in depth. Quadrat sampling of the type used at the other locations would not have given an accurate measure of the existing algal biodiversity. Instead, a 12 cm x 13 cm x 8 cm container was used to sample algae haphazardly at this location. Because the question of interest is how natural and laboratory consumption compares to resources available, this difference in sampling methods between study sites does not affect any subsequent analyses or conclusions. All algae were identified using Bohnsack (2003) and herbarium collections at Northeastern University's Marine Science Center.

Gut Content Analyses

Gut contents were analyzed from five individuals from each of the four populations. Each isopod was dissected and gut contents were removed with forceps. Forceps and scalpel blades were flame sterilized between individuals. DNA was isolated from these tissues using the PureGene (Qiagen, Valencia, California, USA) DNA isolation protocol (see Chapter 2). Gut tissues were amplified using universal algal primers for 23S chloroplast markers (Sherwood and Presting 2007) using polymerase chain reaction (PCR). The following PCR recipe was used to amplify DNA from gut tissues using the following 20μl reaction recipe: 4μl GoTaq 5x PCR Buffer (Promega, Madison, Wisconsin, USA), 0.75 mM dNTP, 3.75 mM $MgCl_2$, 0.25 μM (5 pmol) of both forward and reverse primers, 1 μg/μL Bovine Serum Albumin (NEB, Ipswich, Massachusetts, USA), 1 unit of GoTaq polymerase (Promega) and approximately 10 to 50 ng of

DNA. Standard PCR conditions were used with the following cycles and temperatures: 94ºC for 60 seconds, 40ºC for 90 seconds, 72ºC for 120 seconds, for 30 cycles.

Products from these reactions were then 'nested' or amplified in an additional PCR with the same recipe and cycling conditions to increase the quantity of products. Product bands of about ~250-300 bp in size were excised out of 2% agarose gels and purified using a Gel Extraction Kit (Qiagen, Valencia, California, USA). These products were then cloned with TopoTA vector 2.1 cloning kit (Invitrogen, Carlsbad, California, USA). One hundred clones were selected from ampicillin-positive agar plates and amplified using the following 14 μl reaction PCR recipe: 1.14 mM dNTP, 3.75 mM $MgCl_2$, 4 μl of GoTaq 5x PCR buffer (Promega, Madison, Wisconsin, USA), 0.43 μM (6 pmol) of each universal M13 forward and reverse primers, 1.0 μg/μl of Bovine Serum Albumin (NEB, Ipswich, MA, USA), and 1 U of GoTaq polymerase (Promega). PCR was conducted according to recommended conditions from the TopoTA Invitrogen vector 2.1 cloning kit. PCR products were sequenced according to protocols described in Chapter 2.

The 23S primers (Sherwood and Presting 2007) were chosen for this particular study because of their consistent amplification across a wide range of algal and angiosperm species. However, there are few algal or seagrass sequences publicly available for this particular genic region. In order to properly identify gut contents, common algae and seagrass species from various locations along the coast were collected to create a reference dataset. The reference algal set included the following 15 species (*G. tikihaviae, G. verrucosa, Punctaria latifolia, A. nodosum, Z. marina, F. vesiculosus, C. crispus, P. harveyi, Codium fragile, U. lactuca, U. intestinalis, U. linza, Palmaria palmata, Ruppia maritima, Ahnfeltia plicata*).

This reference set was amplified and sequenced with 23S primers and then aligned with gut content sequences. CODONCODE ALIGNER was used to edit and align sequence data using the same conditions described above. PAUP* v. 4.0b10 (Swofford 2002) was used to build a

neighbor-joining phylogeny with these alignments. Any sequences that could not be identified based on phylogenetic relationships with reference sequences were BLAST-ed to determine closest match. Strains of cyanobacteria were unintentionally sequenced from these techniques. Although the cyanobacterial data may be informative about the biology of this species, this information was not related to the expressed goals of this project. All cyanobacterial sequences were thus removed from the analyses.

Gut contents were compared to algal and seagrass diversity at each site in order to address whether *I. balthica* consumes the full diversity of species available to them at a site or if they choose a limited subset of that diversity. Identification to the species level is difficult using the outlined genetic analyses of gut contents. Instead, sequences from gut contents were classified into Rhodophyta (red algae), Chlorophyta (green algae), or Phaeophyceae and Heterokontophyta (brown algae and diatoms), or angiosperms (specifically the seagrass, *Zostera marina*) using phylogenetic methods. The percentages of gut contents that were either red, green, or brown algae, or angiosperms were compared to the biodiversity sampling quadrats collected at each site by a G-goodness-of-fit test, where local biodiversity percentages were the expected frequencies. Additionally, a Student's T-test was conducted on the distribution of frequencies of food types in either quadrats or gut contents. Comparisons were made across individuals within a collection site in order to distinguish significant differences between data type (gut content or biodiversity) within categories of food and differences between food categories within data types. Results were corrected for multiple comparisons using a sequential Bonferroni correction (Rice 1989).

RESULTS

No-Choice Assay

A total of 900 isopods survived the assay period leaving no less than 20 replicates per food choice per population. Field-collected isopods from Massachusetts and Virginia exhibited

similar behavior and preference when presented with only one of the eight food choices. There was a significant food choice by region interaction (P = 0.014) and overall total consumption across all food choices differed by region (P<0.0001) and collection site (P<0.0001) (Table 3.2; Fig. 3.2).

Feeding differences associated with haplogroup assignment were analyzed separately because of the unavoidable, unbalanced nature of the experimental design (haplogroup identity cannot be determined without destructive phylogenetic analyses). The frequencies of both mtCOI haplogroups are strongly correlated with latitude (R^2 = 0.627; P <0.0001, see Chapter 2). Since geography and genetics are confounded, it is difficult to distinguish regional versus haplogroup effects on consumption. Given that the main effect of region proved significant in the ANOVA, an additional ANOVA was used to determine any effect of haplogroup identity on food consumption. Wellfleet, Massachusetts was unique from other collection sites in that it has equal representation of both mtCOI haplogroups. To remove the confounding variable of region, an ANOVA was run within the Wellfleet dataset to determine if differences exist between haplogroups. No significant differences were observed between haplogroups in feeding preference when presented with only one food choice (Table 3.2; P=0.537). These results suggest that the region of origin for a population dictates the feeding behavior of that population more so than the mitochondrial haplotypes of individuals.

Choice Assay

A total of 200 individuals were recovered from the experiment where there were no less than 35 replicates per population. When isopods were offered eight food choices simultaneously, all algal/seagrass species were not consumed equally (P<0.0001; Fig. 3.1). Instead, there was a strong preference for *F. vesiculosus*, *U. (E.) linza* and *P. harveyi* across all populations according to a nonparametric Friedman Test (P<0.0001, Fig. 3.1) (Conover 1999). As in the no-choice assay, the region of origin for a population affected the overall consumption

of different algal/seagrass species (Table 3.4). Virginia populations consumed more *Z. marina* than those from Massachusetts (P<0.0001, Fig. 3.4), otherwise the two populations consumed each algal species similarly. Preferences within each haplogroup were addressed across populations using nonparametric Mann-Whitney U test. The "southern" haplogroup consumed significantly more *Z. marina* than the "northern" haplogroup (P<0.0001; Fig. 3.3) while the "northern" haplogroup consumed more *F. vesiculosus* than the "southern" type (this consumption was not significant after a sequential Bonferroni correction (P=0.0012; Fig. 3.3) (Rice 1989). Again, paired comparisons were conducted within the Wellfleet population to address haplogroup differences in preference without the confounding effects of geography. Within the Wellfleet population, there were no significant differences in preference after a Mann-Whitney U test between the two haplogroups across all food choices (Fig. 3.5).

Biodiversity Surveys and Gut Content Analysis

Biodiversity surveys revealed dramatic differences in algal/seagrass species richness between localities (Virginia species richness = 10; Massachusetts species richness = 23) (Table 3.4). The species assemblages also differed between regions (Table 3.4). Species identifications by phylogenetic relationships were often difficult in the gut content analyses. However, higher order classifications to order or kingdom could be assigned with ease, therefore gut contents were categorized into the following classification system: "*Zostera*", "brown" algae, "green" algae, "red" algae. For the purpose of comparison with gut content analyses, algal species collected in biodiversity surveys were also classified into the previous defined classifications. Available food resources at the Massachusetts and Virginia collection sites were compared using a G-test (Fig. 3.6; N=10 observations for each site, G-test: Likelihood ratio = 83.598, df=3, P<0.0001; Student's T-test: df=9, Zostera: P<0.0001; brown: P=0.927; green: P<0.0001; red: P<0.0001). The relative abundance of all food categories except for brown algae differed significantly between Massachusetts and Virginia.

From the gut content analyses, individual isopods appear to consume similar algal species and have similar preferences in wild populations as those utilized in feeding experiments. In order to ascertain whether gut contents were representative of the food choices available at each locality, comparisons within each location of survey versus gut contents were conducted using a G-test and Student's T-tests. (Figs. 3.7, 3.8). Gut contents from Virginia individuals did not differ significantly from the food resources available after sequential Bonferroni correction (Fig. 3.8, N=10 (individuals, quadrats), G-test: df=3, likelihood ratio = 13.9315, $P<0.005$; Student's T-test: *Zostera* $P<0.05$, brown $P>0.10$, green $P>0.10$, red $P>0.10$). In contrast, the gut contents from Massachusetts individuals differed significantly from the food resources available to them at all of the categories even after sequential Bonferroni correction (Fig. 3.7, N=10 (individuals, quadrats, G-test: df=3, likelihood ratio = 388.901, $P <0.0001$; Student's T-test: *Zostera* $P<0.0001$, brown $P< 0.0001$, green $P < 0.0001$; red $P < 0.0001$).

DISCUSSION

I. balthica is a generalist herbivore with specific preferences that vary in intensity between coastal regions. Although individuals for these assays were collected from distant locations and from different evolutionary lineages, they share feeding preferences. These preferences are similar to those observed in feeding assays conducted on European populations of *I. balthica* where isopods mainly prefer *F. vesiculosus* but also exhibit some preference for species of *Ulva*, *Polysiphonia*, *Pilayella,* and *Cladophora* (Jormalainen et al. 2001a; Goecker and Kall 2003; Svensson et al. 2004). Overall *F. vesiculosus* appears to be the most preferred food type between European and North American shores. Although there was no variation in the preferred food type, there was variation in the total biomass consumed or relative feeding preference among North American populations. For instance, when corrected for body mass, individuals from Massachusetts consumed significantly more *F. vesiculosus* than those from Virginia. *Z. marina* was consumed significantly more by individuals from Virginia

than those from Massachusetts. The assemblage of potential food resources in an isopod's usual geographic range is an important factor in determining their feeding preferences. Although *F. vesiculosus* does occur in southern areas, it is uncommon and not found in the area of Virginia where isopods were collected for these assays (T.M. Bell personal observation). The increased consumption of *Fucus* by individuals from Massachusetts compared to Virginia individuals may be attributed to a lack of familiarity with this algal species and its associated herbivore deterrent chemistry.

The comparison of biodiversity and gut content surveys revealed the assemblage of food resources not only affects preferences but also feeding behavior. From these data, it is apparent that *I. balthica* from Massachusetts are not consuming all available food resources at random. Instead, individuals prefer brown algal species and *Z. marina*. Although I detected no *Z. marina* beds at the location of the biodiversity survey in Magnolia, Massachusetts, it is likely that pieces of this seagrass detach and are mixed into the drift algae assemblage or that there are beds nearby. Virginia populations appear to have a consumption pattern proportional to *Z. marina* abundance in their area and similar to the overall algal biodiversity available to them. These results suggest that those isopods from seagrass habitats are less specialized than those from mixed algal habitats. This finding is in accordance with Vesakoski et al. (2008) where *I. balthica* raised on angiosperm diets expressed a more generalist behavior when later exposed to mixed algal diets than those raised solely on diets of *F. vesiculosus*.

An interesting aspect of these analyses is the difference between the observed preference for *Z. marina* in the feeding assays and the gut content results. A large portion of an individual's gut contents is *Z. marina;* however, it was one of the least preferred food choices in feeding assays. *Z. marina* has a high cellulose content in comparison to algae, which may cause slower digestion and retard assimilation of nutrients. Because of this suggested reduced digestion rate of *Zostera,* the results of the gut content analyses could be misleading whereby

increased abundance of a food type in the digestive tract is actually indicative of a slow digestion rate of that particular food type instead of preference. In the feeding assays, this reduced digestion rates could also explain the overall reduced consumption rate of *Z. marina* compared with other algal species offered during the feeding assays. Vesakoski et al. (2008) found that in Finland, isopods raised on angiosperm diets had consistently lower body size than those raised on mixed diets or *F. vesiculosus* exclusively. This difference in body size exists in North America as well, where populations of *I. balthica* from seagrass habitats have significantly lower body mass than those from mixed algal habitats (T. M. Bell unpublished data).

The gut content analyses revealed a high abundance of diatoms in the digestive tract of the collected isopods (Fig. 3.8). Although these sequences were excluded from the statistical analyses, their high abundance in the gut of these animals is noteworthy. Whether diatom consumption was targeted or merely a result of incidental intake associated with another food type was impossible to determine given logistical constraints. Isopods often have been observed consuming particulate matter from the surface of the water both in the field and in the lab (T. M. Bell personal observation). *I. balthica* often partake in a "grooming" behavior where they remove and consume particulate matter from their hind pereopods (T. M. Bell personal observation). It may be possible that the diatoms observed in these analyses were intentionally consumed and are a consistent part of isopod diet. It is also possible that the diatoms found in the digestive tract of isopods could be part of the periphyton growing on the surface of the seagrass blades (Orth and Van Montfrans 1984). Many mesograzer species (including *I. balthica*) have been known to consume materials residing on the surface of seagrasses. Some of these grazer species even selectively consume particular diatom functional groups (Jaschinski and Sommer 2008). During the feeding assays, it was noticed that *I. balthica* often consume the epidermal layer of the seagrass (epiphytes were removed prior to introduction to the isopod) instead of intentionally consuming the seagrass in its entirety, lending further

support to the idea they often ingest epiphytic and periphyton species on the surface of the seagrass blades.

Populations of *I. balthica* do not differ in their ability to recognize different algal/seagrass species as potential food resources suggesting that feeding behavior does not create an initial barrier to gene flow. However, differences in consumption of both *F. vesiculosus* and *Z. marina* indicate that regional feeding preferences may be maintaining divergence patterns within this species. Regional differences in fitness when consuming unfamiliar foods, although not addressed in this current study, could then intensify the observed population genetic differentiation (Sotka et al. 2003). Future research in this system will address fitness differences between populations as well as the mechanism of inheritance for feeding behavior and preference in this species.

Mesograzers, being small, typically difficult to identify crustaceans, are critical but often forgotten members of marine communities. During ideal environmental conditions, these species are known to aggregate into large swarms that may have drastic effects on the structure of their community, both by consuming large amounts of primary producers and by providing a substantial food resource for other carnivorous/omnivorous species (Edgar and Shaw 1995; Taylor 1998). Also, mesograzers can play a beneficial role by consuming detrimental epiphytes of algae and seagrass (Jernakoff et al. 1996; Hillebrand et al. 2000; Hughes et al. 2004; Jaschinski and Sommer 2008). Several amphipod and isopod species, including *I. balthica* in the Chesapeake Bay, increase algal/seagrass biomass by consuming these epiphytes (Williams and Ruckelshaus 1993; Hillebrand et al. 2000; Worm et al. 2000; Hughes et al. 2004; Jaschinski and Sommer 2008). However, where *I. balthica,* is abundant, it can also have detrimental effects on seagrass species by acting as a "lawnmower species,"(Duffy et al. 2003) consuming large quantities of seagrass and altering community structure. Studies of this nature are of

importance in understanding the role of often overlooked species (such as mesograzers) that influence the function of the ecosystems they inhabit.

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Figures

Figure 3.1

Results from choice feeding assay comparing feeding preferences across all populations.

This figure depicts the preferred food choices across eight algae choices without reference to population differences. Letters represent significantly different groupings ($P < 0.05$) according to a *post hoc* nonparametric Friedman test for multiple comparisons. Data were rank transformed prior to analysis.

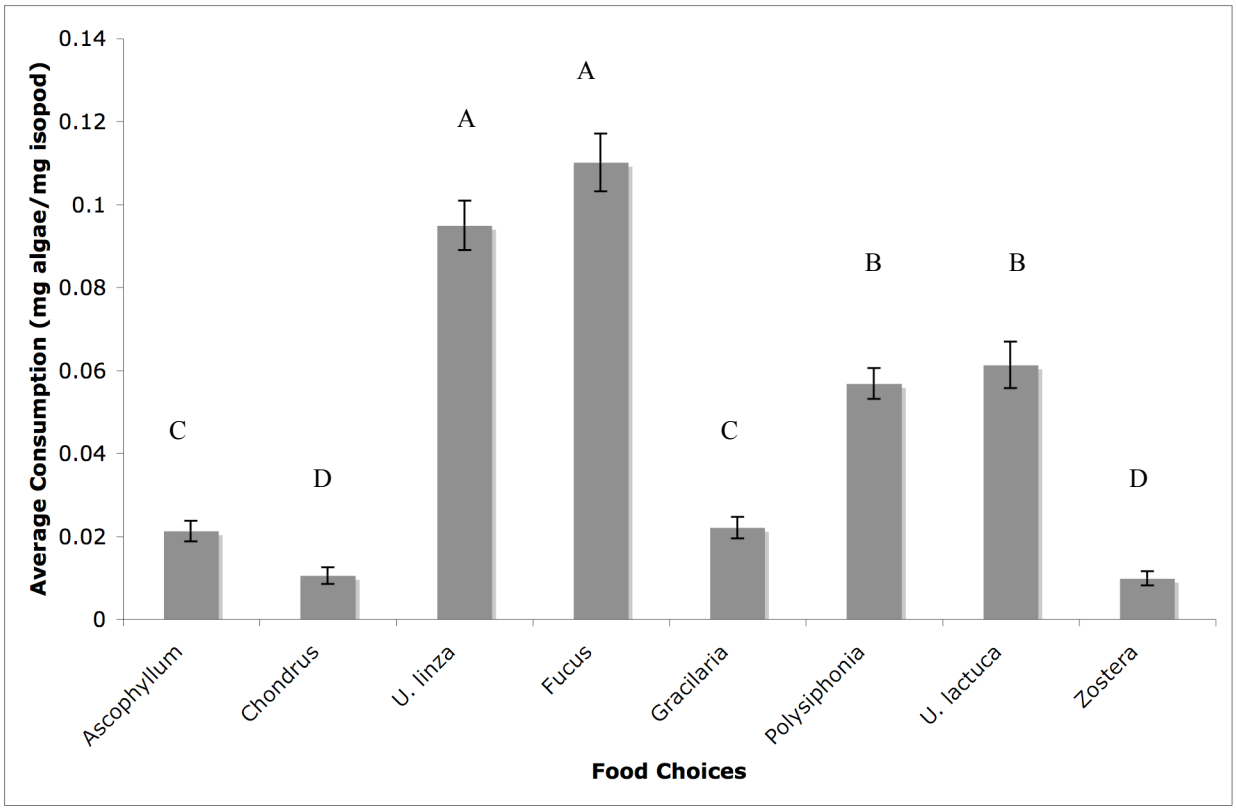

Average Consumption (mg algae/mg isopod)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
C
D
A
A
C
B
B
D
Ascophyllum
Chondrus
U. linza
Fucus
Gracilaria
Polysiphonia
U. lactuca
Zostera
Food Choices

Figure 3.2.

Regional comparison of consumption from No-choice Assay.

This figure illustrates average regional differences (pooled across region: Massachusetts and Virginia) in consumption of each food source. Asterisks represent significant differences in consumption ($P<0.05$) according to a paired t-test**.**

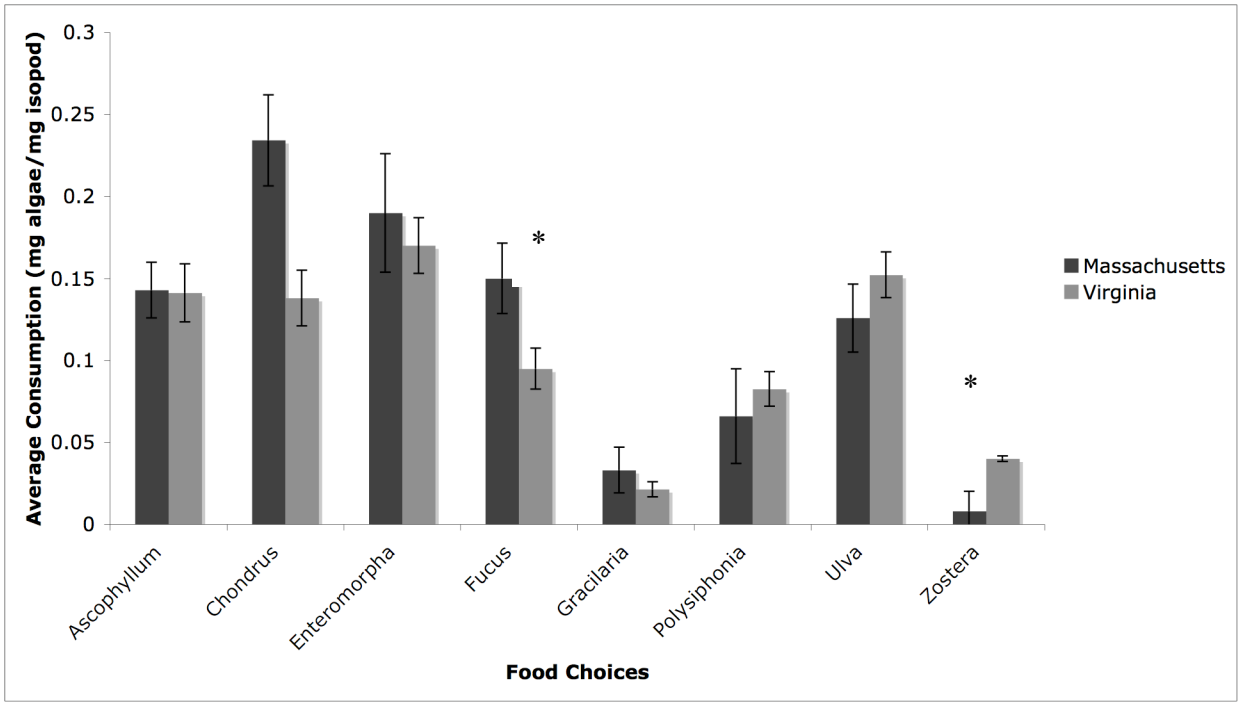

Average Consumption (mg algae/mg isopod)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
Massachusetts
Virginia
Ascophyllum
Chondrus
Enteromorpha
Fucus
Gracilaria
Polysiphonia
Ulva
Zostera
Food Choices

Figure 3.3

Haplogroup differences in preference across all populations for simultaneously available food choices.

This figure represents a comparison of the average consumption (scaled by weight) of eight algal species by different isopod haplogroups pooled across all four locactions. Dark bars represent the “northern” haplogroup, while gray bars represent the “southern” haplogroup (see Chapter 2). Asterisks represent significant differences in consumption between types according to a Mann-Whitney U Test (*Zostera*: $P<0.0001$; *Fucus* comparison was not significant after a sequential Bonferroni comparison $P=0.0012$).

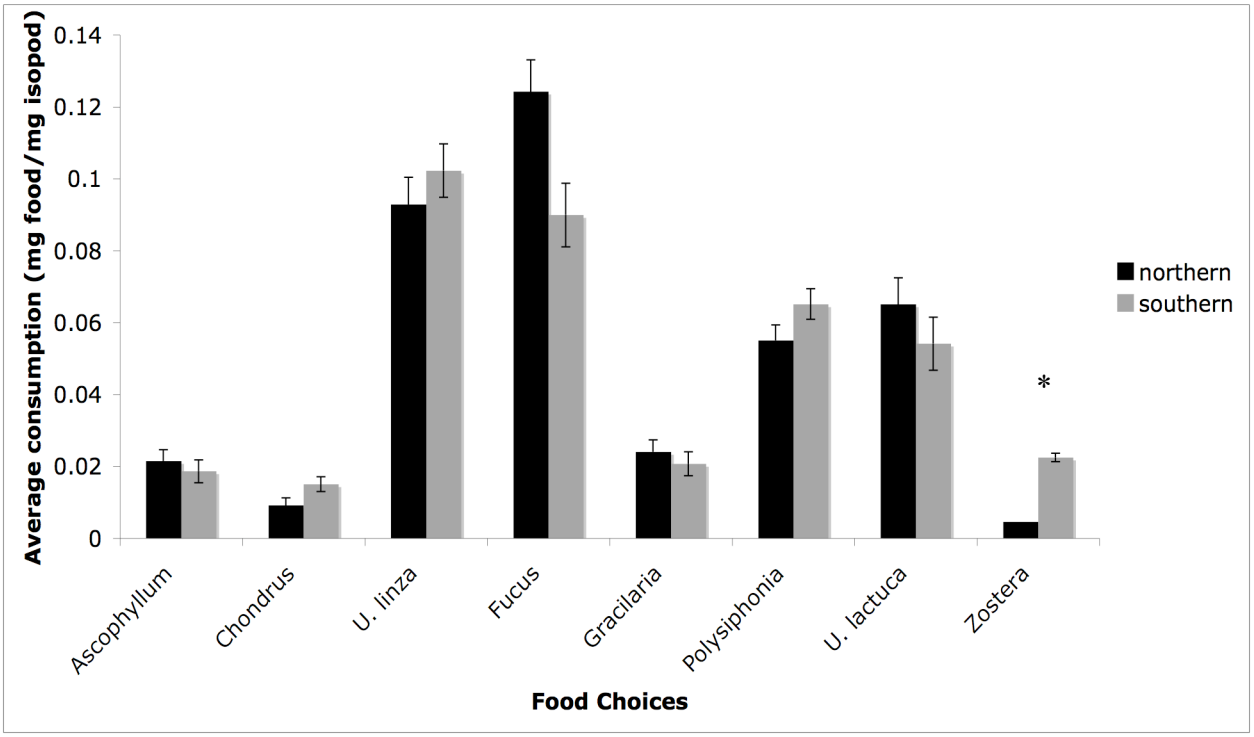

Average consumption (mg food/mg isopod)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
northern
southern
*
Ascophyllum
Chondrus
U. linza
Fucus
Gracilaria
Polysiphonia
U. lactuca
Zostera
Food Choices

Figure 3 4

Regional differences in food preference for simultaneously available food choices.

This figure is a comparison of the average consumption (scaled by weight) of eight algal species by isopods pooled across region (Massachusetts and Virginia). Asterisks indicate significantly different consumption rates according to a Mann-Whitney U Test. Dark bars represent individuals from Massachusetts while gray bars represent individuals from Virginia. Average consumption of *Zostera* significantly differs between regions ($P<0.0001$).

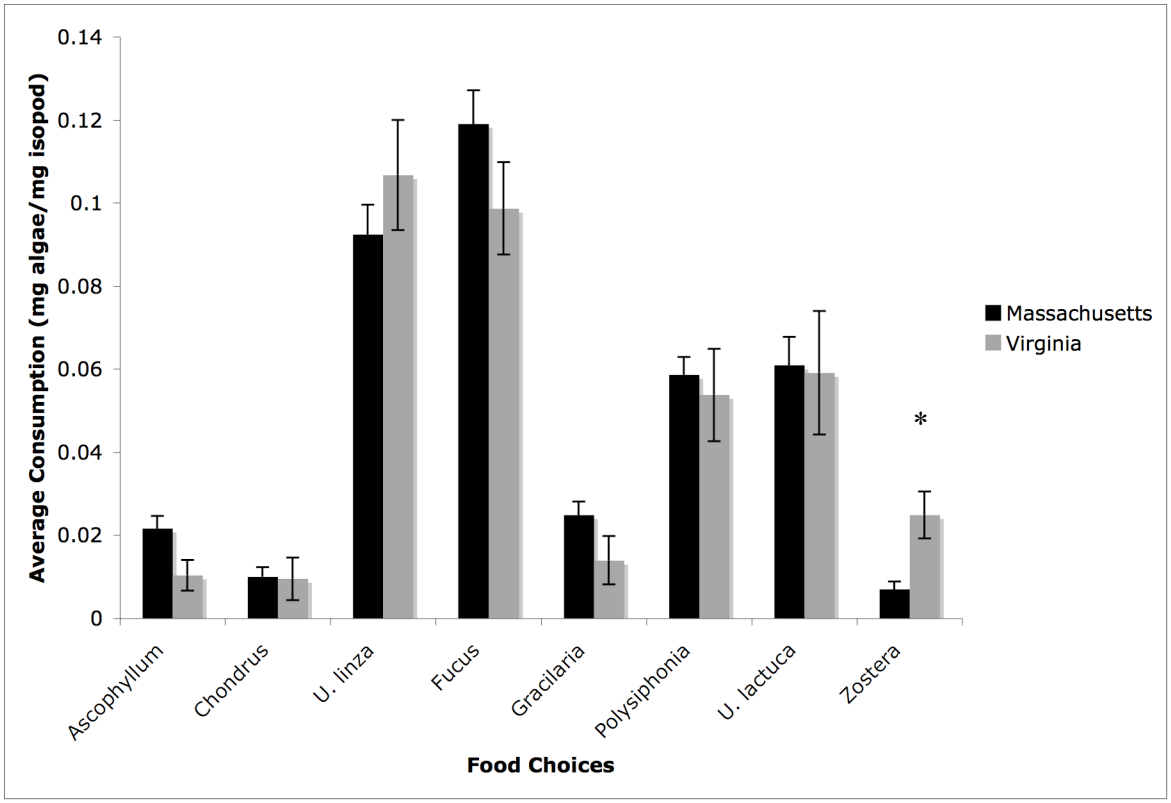

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Average Consumption (mg algae/mg isopod)
Massachusetts
Virginia
*
Ascophyllum
Chondrus
U. linza
Fucus
Gracilaria
Polysiphonia
U. lactuca
Zostera
Food Choices

Figure 3.5

Haplogroup differences in preferences among individuals collected from Wellfleet, Massachusetts.

This figure is a comparison of the average consumption (scaled by weight) of eight algal species by isopod haplogroups. Dark bars indicate "northern" haplogroups while gray bars represent "southern" haplogroups (see Chapter 2). There were no significant differences in preference between different haplogroups after a Mann-Whitney U test

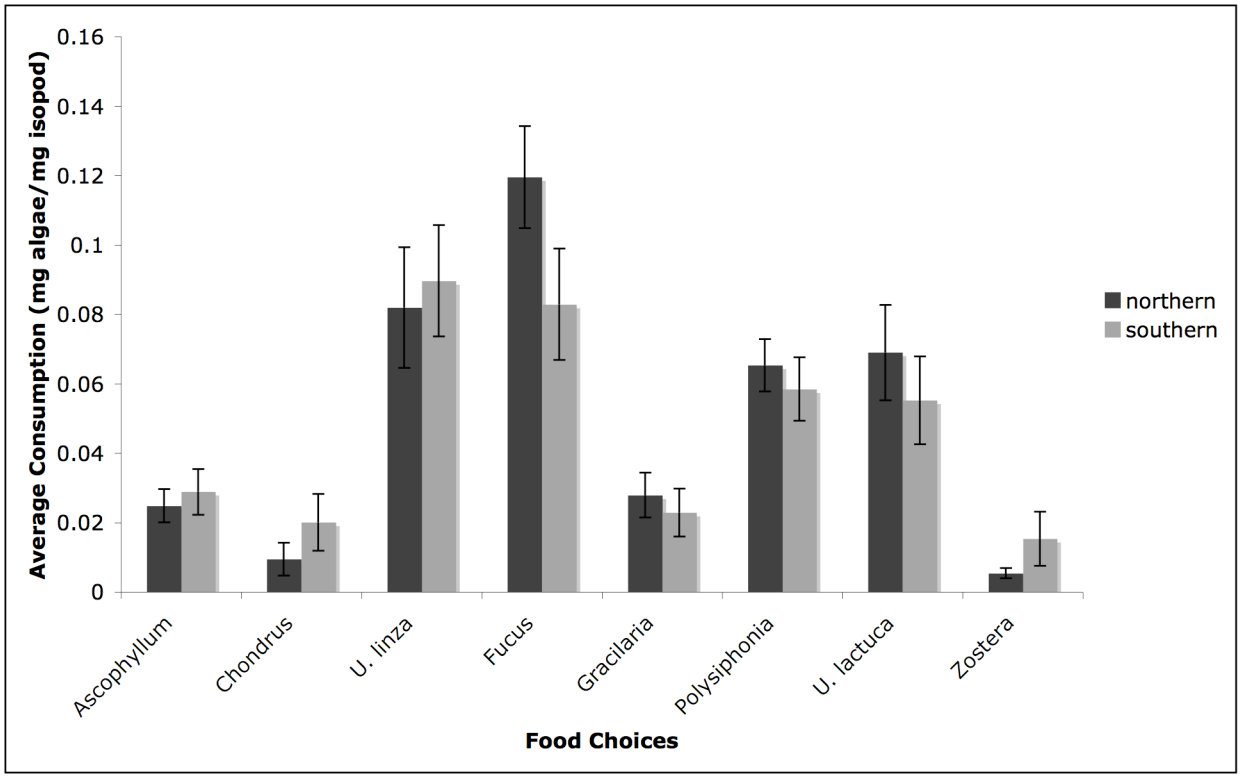

Average Consumption (mg algae/mg isopod)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
northern
southern
Ascophyllum
Chondrus
U. linza
Fucus
Gracilaria
Polysiphonia
U. lactuca
Zostera
Food Choices

Figure 3.6

Percentage of available food resources in Massachusetts and Virginia.

Available food resources were estimated from number of food categories (Brown, Red, Green, *Zostera*) present at each location. Asterisks indicate significant comparisons after a paired t-test and sequential Bonferroni correction. Dark bars represent survey data from Massachusetts while gray bars represent survey data from Virginia. P-values for algal type comparisons are as follows: *Zostera*: $P<0.0001$; Brown: $P=0.927$ Green: $P<0.0001$; Red : $P<0.0001$.

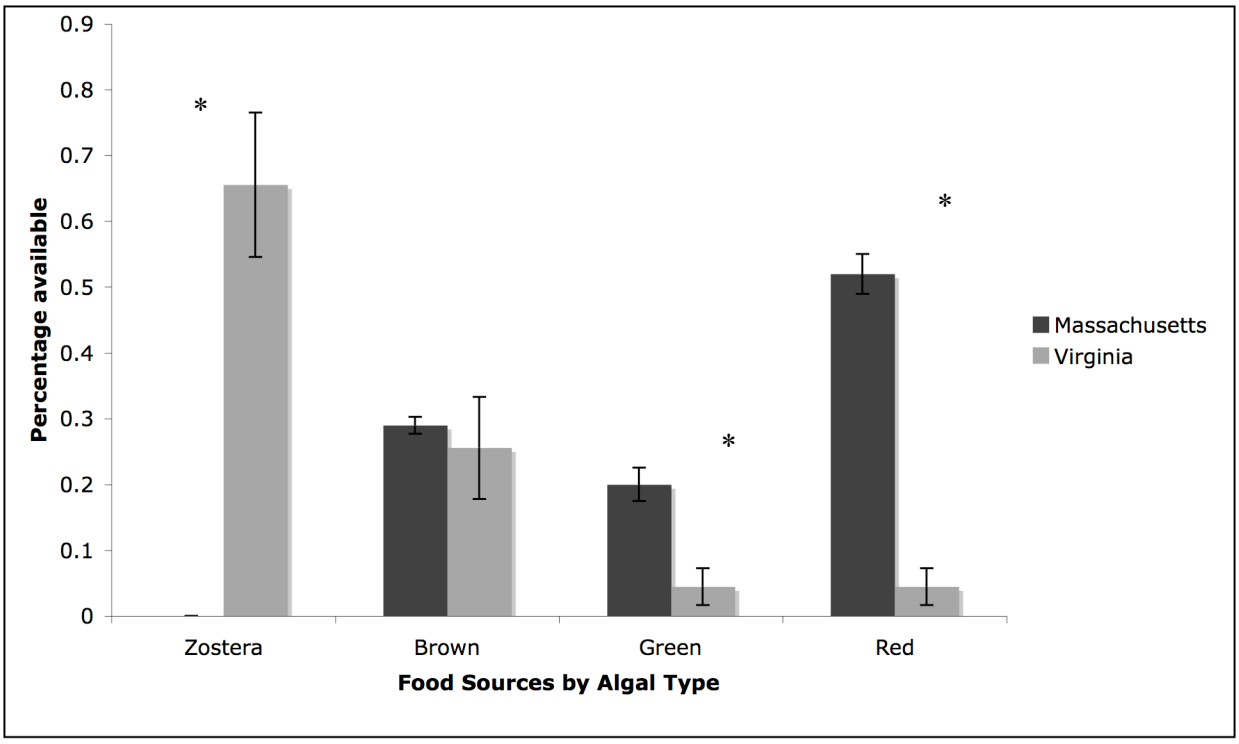
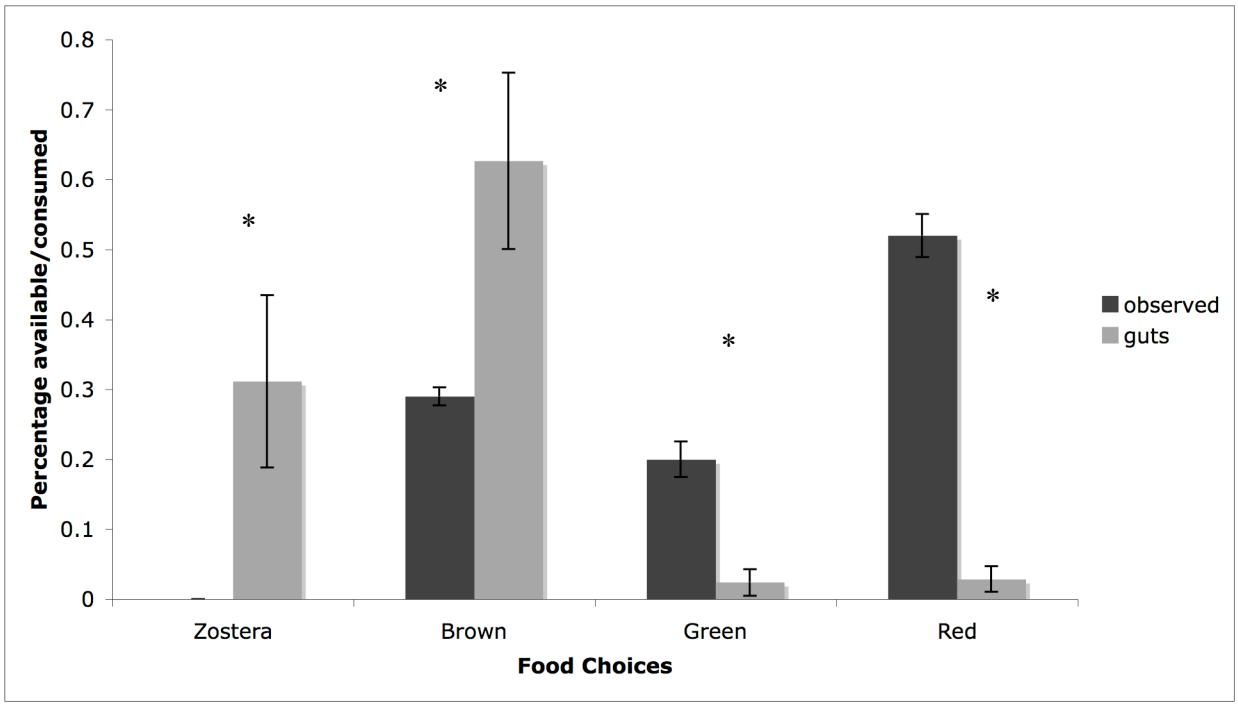

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Percentage available
*
*
*
Zostera
Brown
Green
Red
Food Sources by Algal Type
Massachusetts
Virginia

Figure 3.7

A comparison of available food resources to isopod gut contents at Magnolia, Massachusetts.

The food resources available at Magnolia, Massachusetts to the gut contents of those individuals collected from this location. Food types are based on a classification of biodiversity survey results into different algal or seagrass categories (see text). Asterisks indicate significant comparisons after a paired t-test and sequential Bonferroni corrections. Dark bars represent results from biodiversity survey while gray represents isopod gut content data. P-values for comparisons between biodiversity surveys and gut contents are as follows: *Zostera:* $P<0.0001$; Brown: $P<0.0001$ Green: $P<0.001$; Red : $P<0.0001$

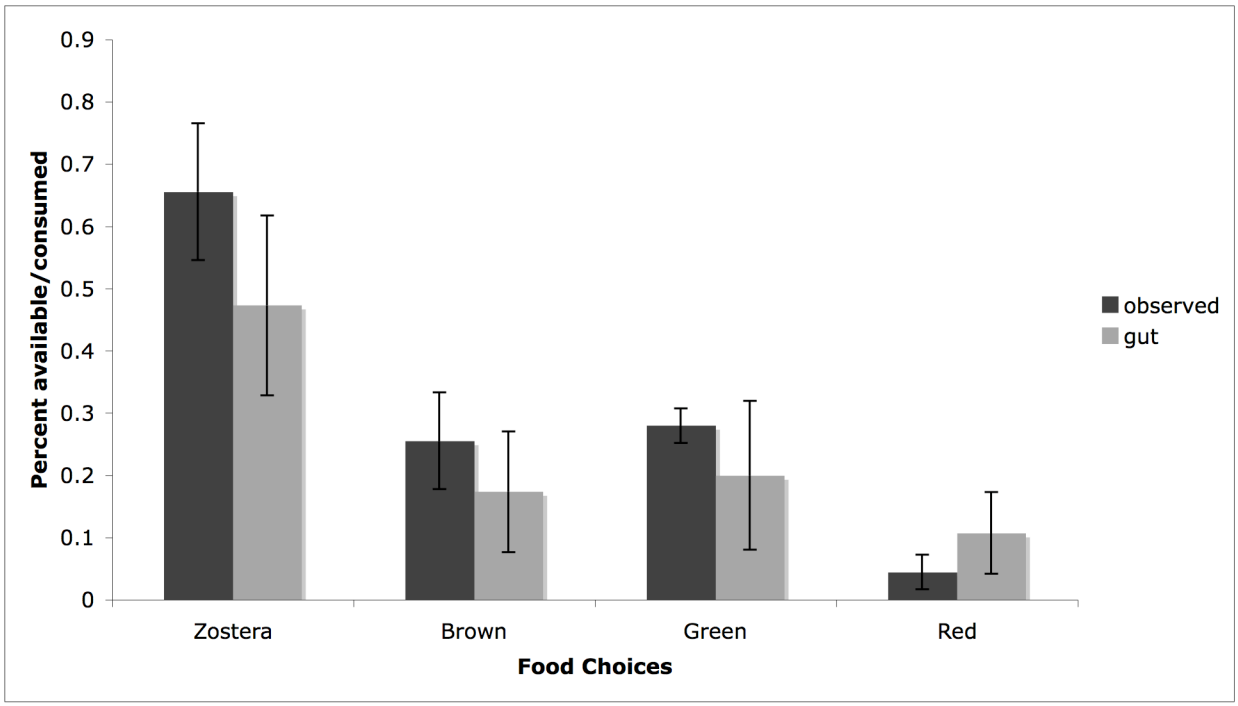

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Percentage available/consumed
*
*
*
*
observed
guts
Zostera
Brown
Green
Red
Food Choices

.

Figure 3.8

A comparison of estimated available food resources and isopod gut contents at Cape Charles, Virginia.

The food resources available at Cape Charles, Virginia to the gut contents of those individuals collected from this location. Food types are based on a classification of algal biodiversity survey results into different algal or seagrass categories (see text). Asterisks indicate significant comparisons after a paired t-test and sequential Bonferroni corrections. Dark bars represent results from biodiversity survey while gray represents isopod gut content data. P-values for comparisons between biodiversity surveys and gut contents are as follows: *Zostera*: $P<0.05$; Brown: $P>0.10$ Green: $P>0.10$; Red : $P>0.10$.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Percent available/consumed
Zostera
Brown
Green
Red
Food Choices
observed
gut

Tables

Table 3.1

No-Choice Assay ANOVA across all populations.

Degrees of freedom (DF), mean sums of squares (MS), *F*-Ratio and *P*-values are indicated. Asterisks indicate significant treatment effects. Response variable is the average consumption of a given alga corrected for body mass and autogenic change. "Site" is the location of origin for isopod individuals and regions were divided into "North" (Cape Cod, MA northward) and "South" (Cape Cod, MA southward). Data were square-root transformed prior to analysis.

Source	DF	MS	F-Ratio	Prob >F
Food Choice	7	0.26029857	34.5236	<0.0001*
Region	1	13.013233	1726.031	<0.0001*
Site [nested w/in region]	2	6.1049365	809.7381	<0.0001*
Region X Food Choice	7	0.019032142	2.5244	0.0144
Food Choice X Site	14	0.008361417	1.1091	0.3456

Table 3.2

No-choice Assay ANOVA within Wellfleet, Massachusetts.

Degrees of freedom (DF), mean sums of squares (MS), *F*-ratio and *P*-values are indicated. Asterisks indicate significant treatment effects. Response variable is the average consumption of a given alga by an isopod of a given haplogroup corrected for body mass and autogenic change. Data were square-root transformed prior to analysis.

Source	DF	MS	F-Ratio	Prob>F
Haplogroup	1	0.0070032	0.1022	0.7499
Food Choice	7	0.55726	7.8642	<0.0001*
Haplogroup X Food Choice	7	0.05928041	0.8648	0.5369

Table 3.3

Biodiversity Survey Results.

Category column represents the grouping assigned for comparison to gut content analysis.

Location	Species Richness	Species Present	Category
Cape Charles, VA	10	*Desmarestia viridis*	Brown
		Ectocarpus siliculosus	Brown
		Gracilaria verrucosa	Red
		Pilayella littoralis	Brown
		Ruppia maritima	Seagrass
		Ulva intestinalis	Green
		Ulva lactuca	Green
		Punctaria latifolia	Brown
		Ulva linza	Green
		Zostera marina	Seagrass ~ *Zostera*
Magnolia, MA	23	*Ahnfeltia plicata*	Red
		Ascophyllum nodosum	Brown
		Ceramium rubrum	Red
		Chondria dasyphylla	Red
		Chondrus crispus	Red
		Cladophora sp.	Green
		Codium fragile	Green
		Agarum clathratum	Brown
		Desmarestia aculeata	Brown
		Ectocarpus siliculosus	Brown
		Fucus distichus	Brown
		Fucus vesiculosus	Brown
		Gloiosiphonia capillaris	Red
		Cystoclonium purpureum	Red
		Laminaria saccharina	Brown
		Mastocarpus stellatus	Red
		Membranoptera alta	Red
		Palmaria palmata	Red
		Polysiphonia denudata	Red
		Polysiphonia fucoides	Red
		Polysiphonia lanosa	Red
		Ulva lactuca	Green
		Ulva linza	Green

CHAPTER 4

REPRODUCTIVE BARRIERS BETWEEN NORTH AMERICAN POPULATIONS OF *IDOTEA BALTHICA*[3]

[3]Bell, T. M. and J. P. Wares will be submitted to *Ecology Letters*

INTRODUCTION

Dobzhansky (1937) and Mayr (1942) proposed that geographical or allopatric speciation is the major mechanism for intraspecific differentiation and divergence. Speciation in allopatry involves a physical or environmental barrier that partitions a species into populations with no gene flow between them; subsequent genetic divergence (through mutation, drift, and/or selection) among isolated populations leads to the creation of new species (Coyne and Orr 2004). Researchers have historically assumed that – except in a few rare instances – the high degree of connectivity of the ocean environment, the lack of strong physical barriers to gene flow, and the long distance dispersal abilities of most marine invertebrates would prevent the degree of isolation necessary for allopatric speciation to occur (Palumbi 1994; Lessios et al. 1998; Mayr 2001; Wiley 2002).

Marine species that have high dispersal potential often show little to no population genetic differentiation even over large areas (Palumbi and Wilson 1990; Palumbi 1994; Silberman et al. 1994; Schwaninger 1999; Wares and Cunningham 2001). Species with high dispersal potential will often still face limitations by way of abiotic or biotic factors that do not act as an impermeable barrier to gene flow, but instead constrain its frequency or direction (Knowlton 1983; Palumbi 1994; Caley et al. 1996; Wares et al. 2001). Dispersal-limiting factors for these species can be subtle, such as microhabitat preference, or more obvious such as historical events like glacial cycling (Butman 1987; Pawlik 1992; Taylor and Hellberg 2003; Rocha et al. 2005). It is for this reason, quite probable that partial isolation or incomplete reproductive isolation between populations of marine species may be a common phenomenon (Palumbi 1994).

Although allopatric speciation is still the dominant speciation model, the idea of divergence in the face of gene flow has gained increasing support (Schluter 2001; Via 2001; Nosil et al. 2003). The often neglected alternative to allopatric speciation is that of parapatric

speciation, where natural selection overrides gene flow and pushes populations along separate evolutionary trajectories (Coyne and Orr 2004). This particular model of speciation may be more applicable to marine taxa because it allows for restricted gene flow between diverging populations of widely dispersing species (Rocha and Bowen 2008). It is generally accepted in the terrestrial and freshwater realms that population divergence across contrasting environments can directly lead to speciation (Lu and Bernatchez 1999a; Doebeli and Dieckmann 2000; Schluter 2001; Via 2002; Dieckmann et al. 2004; Hey 2006; Nosil 2008). Despite high dispersal potential, ecological specialization and habitat selection can occur readily and explain the spatial population genetic structure observed in marine systems (Via 2002; Gavrilets 2003; Sotka et al. 2003; Taylor and Hellberg 2003; Rocha et al. 2005).

A major component of any discussion of speciation processes must also be the evolution of barriers to genetic exchange or reproductive isolation. Reproductive isolation can become evident either upon secondary contact of previously isolated populations or in the transition area between opposing environmental conditions or habitats (Schluter 2001; Coyne and Orr 2004). In order to understand speciation and how it occurs requires two major objectives: to determine which reproductive barriers were involved in the initial reduction in gene flow and which evolutionary forces maintained them (Coyne and Orr 2004; Nosil and Crespi 2006). Due to logistical constraints, few studies of marine diversification have tested for the presence of reproductive barriers (Palumbi 1994). Thus, it is difficult to know whether the concordant phylogeographic patterns of North West Atlantic taxa are indicative of past reproductive isolation with incomplete lineage sorting or if these species are currently in the process of shifting towards reproductive isolation.

The episodic glaciation of the North American coastline has created periodic barriers to gene flow, whereby glacial coverage of intertidal habitat and reduced sea levels have forced populations into glacial refugia or to undergo local extinction (Pratt and Schlee 1969; Shackleton

et al. 1984; Ingólfsson 1992). As glaciers began to recede and sea levels rise, populations from various refugial areas recolonized coastal habitats, and populations that had previously been isolated were reunited. Over twenty species along this coast are defined by a phylogeographic pattern of a strong latitudinal population genetic divergence, where populations south of Long Island Sound are distinct from those north of Long Island Sound (Wares and Cunningham 2001b; Wares 2002; Sotka et al. 2003; Kelly et al. 2006; Jennings et al. 2009).

Natural selection may be an underlying cause for this diversity, as this region is defined by strong ecological and environmental transitions ((Engle and Summers 1999); see Chapter 1 and Wares (2002)). Divergent intraspecific lineages tend to be partitioned concordant with biogeographic realms; although this may just be the result of neutral processes, it is an indication that natural selection may be a maintaining force in this divergence (Burton 1998; Wares 2002). Since the phylogeographic pattern is shared across unrelated taxa and there is also an entire suite of environmental conditions that vary across this region, it is difficult to pinpoint one particular environmental variable that could drive this pattern (Wares 2002). This problem is exacerbated by the fact that many of these environmental variables are confounded by geography and the climatic history of this coastline.

Today, it has become commonplace to define species based on genealogical evidence alone and often using only a handful of genes (Hebert et al. 2003; Moritz and Cicero 2004; Rubinoff and Holland 2005). Although defining new species in this manner may be legitimate, it should not be the sole criterion used to define new taxa (de Queiroz 2007). The biological species concept (BSC) defines species as distinct entities that are reproductively isolated and has been the standard definition used to distinguish new species (Dobzhansky 1935, 1937; Mayr 1942). However, reproductive isolation is a very broad and ambiguous definition and it can be difficult to apply to many taxa (Coyne and Orr 2004). There has been an abundance of alternative species concepts, however, all falling into the same pitfall of the biological species

concept, in that there is one sole criterion for defining new species (i.e. reciprocal monophyly, phenotypic cohesion, genotypic cluster etc.) (de Queiroz and Donoghue 1988; Cracraft 1989; Templeton 1989; Mallet 1995; Coyne and Orr 2004a; de Queiroz 2007). These criteria are all valid in their own right since most taxa will acquire many of them during the process of speciation. However, these concepts are not broadly applicable because the sequence of criteria acquisition is widely variable (Mayden 1997; de Queiroz 2007). De Queiroz (2007) reviewed these concepts and suggests that they all have a 'unifying' theme of "separately evolving metapopulation lineages". De Queiroz (2007) regards any property of a taxon (morphological, behavioral, ecological, genealogical etc.) as useful supporting evidence for lineage separation and is also considered evidence for species delimitation.

The following study addresses these topics in a North American coastal isopod species, *Idotea balthica*. Two reciprocally monophyletic haplogroups, defined by mitochondrial loci are clinally distributed along the North West Atlantic coast, with the strongest transition between the two groups between Long Island Sound and Cape Cod (see Chapter 2). It has been suggested that these populations diverged in the Pleistocene or late Pliocene, show no population specific morphological differentiation, and are 'cryptic species' (Wares 2001; Wares et al. 2007), based solely on mitochondrial divergence (phylogenetic species concept). Here we explore what information from the "genealogical species concept" (evaluating concordance of multiple loci) and BSC suggest about the taxonomic status of these two distinct lineages. Several lines of evidence from reproductive isolation to the analysis of additional loci will be used to determine if the reciprocally monophyletic haplogroups originally described in Wares (2001) are 'cryptic' species based on the unifying species concept of De Queiroz (2007).

METHODS

In order to determine the level of population differentiation at 16S rDNA among *I. balthica* populations, twenty North American isopods were collected from eight populations

along a coastal transect from Virginia to Maine over a period of three years (2005-2008) (Fig. 4.1). Isopods were collected with a mesh net and preserved in 95% ethanol. All DNA isolation, PCR, and cycle sequencing reactions were performed according to the protocols and recipes described in Chapter 2. However, ribosomal 16S primers (Simon 1991) were used instead of species-specific mtCOI primers. 16S sequences were aligned and edited using CODONCODE ALIGNER v. 3.0.2 (CodonCode Corp., Dedham, Massachusetts) and the ClustalW alignment algorithm. The majority of sites had base calls with PHRED scores much higher than 20, however any sites with PHRED scores less than 20 were scored as ambiguous (Ewing and Green 1998b). Terminal ends (approximately 30 bp from each end) of each sequence were trimmed because of numerous ambiguous base calls and overall low PHRED scores. A haplotype network was then constructed using NETWORK 4.2.0.1 (Fluxus Technology Ltd., http://www.fluxus-engineering.com) using the median joining method (Bandelt et al. 1999a). An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) along with standard population genetic indices for this locus were calculated using ARLEQUIN v. 3.1.1 (Excoffier et al. 2005). *F*-statistics were calculated using Tamura-Nei distances (Tamura and Nei 1993) between haplotypes and significance was assessed by 10,000 random permutations of haplotypes between each population pair. Spatial groups were defined for the AMOVA using a spatial analysis of molecular variance (SAMOVA) similar to Dupanloup et al. (2002) where Φ_{CT} (i.e. the proportion of genetic variation among groups) is compared across all possible contiguous partitions of the samples. The partition with the highest Φ_{CT} is retained and used in the AMOVA.

Spatial population genetic structure was also evaluated with allozyme loci. Individuals were collected from two locations: Nahant, MA and Cape Charles, VA (Fig. 4.1). These locations were chosen because the frequencies of the two mtCOI haplogroups described in Wares (2001) are known at both of these locations: Nahant, MA has 93% northern/European haplogroup while Cape Charles, Virginia is 94.2% of the “southern” haplogroup. Twenty-four

live individuals were collected from Nahant, Massachusetts and Cape Charles, Virginia and immediately frozen in liquid nitrogen. Pereopods were removed from each individual and then sequenced at mtCOI (per methods in Chapter 2) to insure that both haplogroups were represented in the allozyme analysis. Frozen isopods were crushed with a mortar and pestle in 100μl 0.1M Tris-HCl buffer at pH 8.0. Filter paper wicks (4 x 6 mm) absorbed the extracted enzyme and buffer mixture. These enzyme-soaked wicks were stored at -80 °C for later analyses. Enzyme electrophoresis was conducted on 11% starch gels that revealed six polymorphic loci (colorimetric esterase (CE) (three loci: CE-1, CE-2, and CE-3), leucine aminopeptidase (LAP) (two loci: LAP-1 and LAP-2), and fluorescent esterase (FE)) on 34/40 Poulik Buffer (Soltis et al. 1983). The genetic basis of the allozymes was inferred by examining segregation distance between bands, while the rate of migration through the gel matrix allowed for the identification of alleles. The percentage of polymorphic loci (P), effective number of alleles (A_e), observed heterozygosity (H_o), and expected heterozygosity (H_E) were all estimated using GENALEX v. 6.1 (Peakall and Smouse 2006). Deviations from Hardy-Weinberg expectations were examined for each polymorphic locus in each population also using GENALEX v. 6.1 (Peakall and Smouse 2006). Weir and Cockerham's F_{ST} and F_{IS} were calculated using FSTAT v. 2.9.3 (Goudet 2001).

Reproductive Isolation Experiments

Approximately 100 individuals of *I. balthica* were collected from Nahant, MA and Cape Charles, VA in August of 2008. Collections were transported to the University of Georgia in aerated coolers where they were acclimated to laboratory conditions for one month prior to experimentation. Isopods were raised in plastic aerated containers at room temperature using fresh seawater collected from Tybee Island, Georgia. Lab cultures were fed a mixture of live algae (*Fucus vesiculosus*, *Polysiphonia harveyi*, and either *Ulva lactuca* or *U. linza* collected and shipped monthly from Nahant, MA), freeze-dried microcrustaceans (Cyclopeez), and HBH

Crab and Lobster Bites. Juveniles were removed from parental populations and cultured separately until their sex could be accurately determined. These juveniles were then sorted into male and female containers to insure that isopods used during the mating experiment were naïve.

I. balthica form copulatory pairs just before or soon after the female molts. One hundred females of approximately equal body mass (~20 mg) from each population were individually isolated into floating flow through containers (Gladware mini-round 4oz containers, sealed with small-gauge nylon mesh) with sufficient food and placed in a cooler with circulating 18° C seawater. Once a female molted, the molt was collected for DNA sequencing (see Chapter 2 for protocols) and the females were then paired with either a male (haphazardly chosen) from within their own population or a male (haphazardly chosen) from a different population. Individual crosses were left in an aerated container of seawater with ample food for 24 hours. After the 24-hour period the eggs residing in the brood pouches of paired females were inspected for changes in color (from white to orange) indicating successful fertilization. Females were returned to their floating containers and monitored during the approximately thirty-day gestation period. Fifty crosses each were conducted for the Massachusetts population, Virginia population, and the mixed population crosses over a period of eight months. Mortality that was not the result of cannibalism during crosses did occur on five occasions; these crosses were excluded from statistical analyses. The mtCOI haplogroup of each individual could not be determined prior to the experiment because of a lack of morphological differentiation between the haplogroups. Haplogroup frequencies were known for each site so location of collection was used as a proxy for haplogroup until DNA sequencing and phylogenetic analyses could be conducted.

Reproductive barriers were addressed based on fertilization success, mean fecundity, and offspring survival. Fertilization was classified into two categories: successful and

unsuccessful. A cross was considered successful if the female was fertilized and did not abort the brood. While a cross was determined unsuccessful if the female was not fertilized, if the female aborted the brood, or if the male consumed (cannibalized) the female. Several χ^2 contingency tests of independence (Sokal and Rohlf 1995) were conducted to address potential population differentiation in fertilization success. The first of such analyses was used to determine the effect of location of origin of a participating male or female on the frequency of fertilization success. The frequency of successful fertilization was then compared in intra- and inter-population crosses using an additional χ^2 contingency test. Within crosses deemed unsuccessful, the frequency of different potential outcomes was compared among intra- and inter-population crosses. The difference in frequency of the three potential categories of 'no fertilization', 'brood abortion', and 'cannibalism' were compared for intra- and inter-population and between population crosses using a χ^2 contingency test of independence (Sokal and Rohlf 1995). Results were corrected for multiple comparisons using a sequential Bonferroni correction (Rice 1989).

An I_{PSI} estimator by Rolán-Álvarez and Caballero (2000) was used to assess the degree of pre-mating isolation between mtCOI haplogroups of *I. balthica*. This estimator is calculated in the following manner:

$$I_{PSI} = \frac{PSI_{aa} + PSI_{bb} - PSI_{ab} - PSI_{ba}}{PSI_{aa} + PSI_{bb} + PSI_{ab} + PSI_{ba}}$$

where PSIs estimate the mate choice coefficient for each type of mating pair (intra-population: combinations aa and bb and inter-population: ab and ba). In this equation PSI estimators are calculated for every pair combination as the number of observed pair types divided by the number of expected pair types calculated from crosses. When compared to other indices used for sexual isolation and mating frequency Perez-Figuero et al. (2005) determined that I_{PSI} is the most conservative index. This index is scaled between -1 and 1, where 0 represents random

mating and -1 indicates that only intra-population or inter-population crosses were observed. Significance and *P*-values were derived by bootstrapping with 10,000 iterations. The software JMATING V. 1.0.8 (http://webs.uvigo.es/acraaj/JMsoft.htm) developed by Rolán-Álvarez and Caballero (2000) was used to estimate I_{PSI}.

The presence of post-zygotic reproductive isolation was evaluated based on female fecundity and offspring survival. Offspring were counted as they were released from the brood pouch or soon thereafter and then kept in isolated and aerated plastic containers. All offspring were observed for one week after release where survival and any phenotypic abnormalities were recorded. Differences in fecundity and offspring survival were evaluated in within and between population crosses and for the effect of location of origin of males and females using an ANOVA. All statistical analyses were performed using JMP (Version 7, SAS Institute Inc., Cary, N.C., 1989-2007).

In order to determine the effect of mitochondrial haplogroup on reproductive behavior, the mtCOI locus from the genetic material collected from molts was amplified (see Chapter 2 for protocols) from each individual used in this experiment to identify their haplogroup (either related to 'northern' clade or 'southern' clade) as defined in the Bayesian phylogeny depicted in Chapter 2). Haplogroups were determined according to a maximum parsimony phylogeny constructed using PAUP* 4vb10 (Swofford 2002). Parsimony analyses were performed using un-weighted heuristic searches, with starting trees obtained by random addition (100 replicates) holding 10 trees per replicate, and tree-bisection-reconnection branch swapping. Haplogroup identity was visually assigned based on this maximum parsimony phylogeny (with 'known' individuals characterized from Chapter 2).

RESULTS

462 characters of the mitochondrial ribosomal 16S gene region were collected from *I. balthica* from 7 collection sites along the North American coast (Fig. 4.1). The median joining

haplotype network for 16S revealed two distinct haplogroups: a northern group and a southern group which correspond to those in Chapter 2 and Wares (2001) (Fig. 4.2). From the 131 individuals sequenced at 16S, 11 haplotypes were defined with 16 segregating sites. In order to test for neutrality and demographic equilibrium among populations, nucleotide diversity (π) and Tajima's D (Tajima 1989) were calculated across all populations (Table 4.1). Fu's F_s (Fu 1997) test (Fu 1997) for neutrality was not significant for any population and is not depicted in Table 4.1. Tajima's D was significantly negative only at Nahant, MA (D = -1.844; P=0.018). As in the mtCOI dataset, for most populations residing in admixed populations Tajima's D was not significant, but was highly positive. From the SAMOVA, haplotypes were partitioned into three spatial groups: 1) Magnolia and Nahant (northern), MA; 2) Wellfleet, MA, Narrow River Inlet, RI, and Fire Island, NY (transitional); 3) Corson's Inlet, NJ and Cape Charles, VA (southern). An AMOVA was then conducted using this spatially partitioned data in ARLEQUIN to assess differences in genetic variation at the population or group level. The majority of genetic variation appears to reside within populations (51.49%) and among regions (43.82%). Hierarchical tests of population subdivision support the regional North American groupings on 'northern' and 'southern' and 'transitional' with structure between and among regions as well as population (Φ_{CT} = 0.4382, P<0.0001; Φ_{SC} = 0.0834, P<0.0001; Φ_{ST} = 0.4851, P<0.0001).

Allozymes

Six polymorphic loci were scored across the Nahant, Massachusetts (H_o = 0.444; H_E = 0.449) and Cape Charles, Virginia populations (H_o = 0.424; H_E = 0.408) (Table 4.2). A consistent pattern of homogeneity was revealed from the population genetic calculations with overall F_{ST} = 0.038 (P < 0.010) (Table 4.2). All loci were in Hardy-Weinberg equilibrium across both populations. At the nuclear level, shows some divergence exists between both the two populations and the "Northern" and "Southern" mitochondrial haplogroups (described in Chapter 2).

Reproductive Isolation

The Massachusetts individuals used in these experiments were all of the "northern" mtCOI haplogroup (74 females, 74 males), while all of the Virginia individuals were of the "southern" mtCOI haplogroup (71 females, 71 males). Fertilization success was significantly higher in intra-population crosses than inter-population crosses (70% vs. 30%) (contingency test: N=145, df =1, Fisher's exact test $P < 0.0001$; Fig. 4.3). Categories of unsuccessful fertilization (cannibalism, brood abortion, and no fertilization) did significantly differ in intra- or inter-population crosses (contingency test: N = 115, df =3, $\chi^2 = 22.080$; $P < 0.0001$; Fig. 4.3). Cannibalism as well as no fertilization occurred significantly more often in interpopulation crosses than in intrapopulation crosses ($P<0.001$ and $P<0.05$ respectively; Fig. 4.3). The sexual isolation index I_{PSI} was significant ($I_{PSI}=0.49$;SD=0.0733; $P<0.01$) indicating non-random or assortative mating. The statistic IA_{PSI} is a ratio of PSI values for inter-population crosses. An IA_{PSI} of 1 indicates symmetric (i.e. no interaction between sex and population of origin) mating frequencies between intra- and inter-population crosses. Estimations of IA_{PSI} for inter- and intra-population crosses in *I. balthica*, indicated that crosses were symmetric ($IA_{PSI}=1.2398$; SD=0.2494; P=0.2878).

An ANOVA addressed how the location of origin of individuals and cross category (inter- or intra-population) affected mean fecundity and offspring survival. Survival of offspring produced in intra-population crosses was significantly higher than those produced from inter-population crosses ($P<0.0001$) (Table 4.4, Fig. 4.4). Mean fecundity was significantly lower in mixed crosses (50.73 offspring per female; $P < 0.001$, Table 4.5, Fig. 4.4) than those from within the Massachusetts population, but not significantly different from those crosses within the Virginia population ($P > 0.10$). The population of origin of males and females had a significant effect on the mean fecundity, but no significant effect on offspring survival (P=0.0272; P=0.9799 respectively; Table 4.4, Fig. 4.5). A Student's T-Test compared mean fecundity between

populations. Mean fecundity was significantly higher from crosses comprised of individuals from Massachusetts (~83.8 offspring per female, Table 4.4, Fig. 4.4) than those from Virginia (~53.8 offspring per female; P <0.001, Table 4.4, Fig. 4.5).

DISCUSSION

A consistent but paradoxical pattern emerges from these analyses: the northern and southern lineages of *I. balthica* are strongly differentiated at mitochondrial loci, exhibit modest levels of reproductive isolation, and yet little support for this divergence is recovered in the nuclear genome (the allozyme results are corroborated by data from the nuclear ITS region and microsatellite data; T. M. Bell, unpublished). Non-concordance between allozyme and mitochondrial loci has been observed on several instances, a prime example of this phenomenon occurs within the eastern oyster, *Crassostrea virginica*. Atlantic and Gulf populations of *C. virginica* along the North American coastline are reciprocally monophyletic at mtCOI and scnDNA (Karl and Avise 1992). However, these same populations of *C. virginica* are not differentiated across eighteen allozyme loci, which Karl and Avise (1992) suggest is due to balancing selection. Similar findings in other taxa have corroborated this assertion suggesting that it is not uncommon for allozymes to be under the influence of selection and thus to be non-concordant with neutral loci (Piel and Nutt 2000; Schmidt et al. 2000; Hurwood et al. 2003; Silva and Skibinski 2009). In light of the reciprocally monophyletic clades at mitochondrial loci in *I. balthica* and the apparent reproductive barriers between populations, it is likely that the lack of regionally specific variation in the allozyme loci utilized in this study is also the result of balancing selection.

The haplotype diversity at 16S could be divided into three distinct regions: 1) northeastern U.S., 2) an admixed area (from NY to Cape Cod, MA and 3) southern U.S.. These regional designations differ from those inferred from the mtCOI in that the admixed area is its own distinct region. This difference between the two datasets is likely due to sampling. Fewer

populations and fewer individuals were sampled in 16S than at mtCOI. The Nahant, MA population again exhibited a significantly negative Tajima's D as also observed in mtCOI. A negative Tajima's D could be an indication of the occurrence of various demographic or evolutionary processes (Tajima 1989). However, given that *I. balthica* inhabits previously glaciated coastline and has likely experienced local extinction and colonization during glacial cycles, the negative Tajima's D values are most likely an indication of population expansion. As in the mtCOI data, positive Tajima's D values were found in the admixed region from just south of LIS in New Jersey to Cape Cod. Although these values are not statistically significant, many deviate substantially from zero (ranging from 1.78 to 3.23) pointing toward the retention of divergent lineages in these populations. This signature would be expected in populations where both major haplogroups are equally represented and offers an explanation for how this diversity is being maintained.

Reproductive Barriers

Reduced fertilization success and offspring survival suggest that mating between populations in the natural environment results in a reduction in individual fitness. Geographic variation in fecundity is not uncommon in other peracarids and is considered to be the result of adaptation to variation in environmental factors (Glazier 1999; Bas et al. 2007; Ituarte et al. 2007). In *I. balthica,* the mechanism behind the determination of brood size is known to at least have an environmental component, where females will experience variation in fecundity due to food quality, stress, or body size (Jormalainen 1998; Jormalainen et al. 2001a; Jormalainen et al. 2001b; Hemmi and Jormalainen 2002). Since these variables were controlled during these crosses, it is likely that the determination of brood size in this species also has a genetic component. If this is true, the Virginia allele or alleles for brood size are dominant to that of Massachusetts. This scenario would explain the reduction in fecundity that is experienced by Massachusetts females, but is not shared by those from Virginia. In a previous study, *I. balthica*

from Virginia have been crossed with individuals from Helgoland, Germany (Gutow et al. unpublished data) where a four-fold reduction in fecundity was observed in inter-population crosses. Massachusetts populations are of the same haplogroup as those individuals used in the European study. This could explain the similarity in results between these two studies as well as add further support to the existence of a genetic component underlying the determination in brood size in this species.

Offspring survivorship from mixed crosses experienced significant reduction when compared to offspring produced in intra-population crosses. Inter-population crosses often produced individuals that exhibited phenotypic abnormalities. The most common observed deformity involved torsion or twisting of the abdominal region and raised plates near the telson. Abdominal torsion usually resulted in death within the first week. Some of the other abnormalities did not seem to affect individual fitness in the laboratory. It is, however, not known if such phenotypic deformities would incur a fitness cost in natural populations. These abnormalities were never observed in offspring produced from within-population pairs, nor have they been detected in natural populations (T. M. Bell personal observation).

The cannibalistic behaviors observed during crosses occur by way of the male consuming the female, while females exhibit infrequent aggressive behavior towards males. Cannibalism is common in this and other taxa as a means for reducing the negative effects of high population density (Hunte 1984; Leonardsson 1991; Jormalainen and Shuster 1997; Jormalainen 1998; Merilaita and Jormalainen 2000; Tsai 2003; Kuroda et al. 2005; Pizzatto and Shine 2008). The results observed here are similar to those of Gutow et al. (unpublished data) where female *Idotea balthica* from Virginia, U. S. were crossed with *I. balthica* males from Helgoland, Germany. In every instance (36 crosses), males consumed females within ten minutes of copulation. How frequently this behavior occurs in natural populations is not known and it is a possibility that cannibalistic behavior is a stress-induced laboratory artifact.

Male isopods are able to distinguish females that belong to divergent haplogroups and will choose either to ignore them or to consume them significantly more often than they will choose copulation. Sexual cannibalism during copulation is a common behavior in many terrestrial insect species, though it usually involves the cannibalization of males by females after copulation (Fox 1975; Birkhead 1988; Arnqvist 1997). Cannibalism by males serving as a form of reproductive barrier and as a potential mechanism in speciation has not previously been suggested in this or other species. The comparison of success between intra- and inter-population crosses resulted in a significant I_{PSI} value, suggesting a deviation from random mating and significant sexual isolation. Mate choice or mate discrimination is considered one of the most important causes of speciation in animals and has been observed in a diverse range of taxa (Grassle and Grassle 1976; Solignac 1981; Weinberg et al. 1990; Palumbi 1994; Coyne and Orr 2004).

Conclusions

Reproductive barriers exist between mitochondrial haplogroups in the form of sexual isolation, reduced fertilization success, and reduced offspring survival. Due to this strong support for reproductive barriers between these groups, it is likely that the homogeneity of allozyme allele frequency across regions may be the result of balancing selection rather than an indication of high gene flow. The unifying species concept of De Queiroz (2007) requires that multiple independent lines of evidence be used for delimitation of new species. Although both mtCOI and 16S data suggest that these two haplogroups are "separately evolving metapopulation lineages," based on the incomplete reproductive barriers identified between these groups it would be premature to identify them as different species. The differentiation at mitochondrial loci across North American regions among *I. balthica* populations and their partial reproductive isolation however, do suggest these groups are on different evolutionary

trajectories and the differentiation between them is not simply a case of intra-specific variation but instead an indication of an incipient speciation.

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Figures

Figure 4.1

North American collection locations for *Idotea balthica*.

This figure represents collection locations where isopods were collected during the summers of 2005-2009. Areas shaded in green represent the range of glaciers during the LGM. Numbers in parentheses indicates the number of individuals from a given location included in the 16S phylogeny.

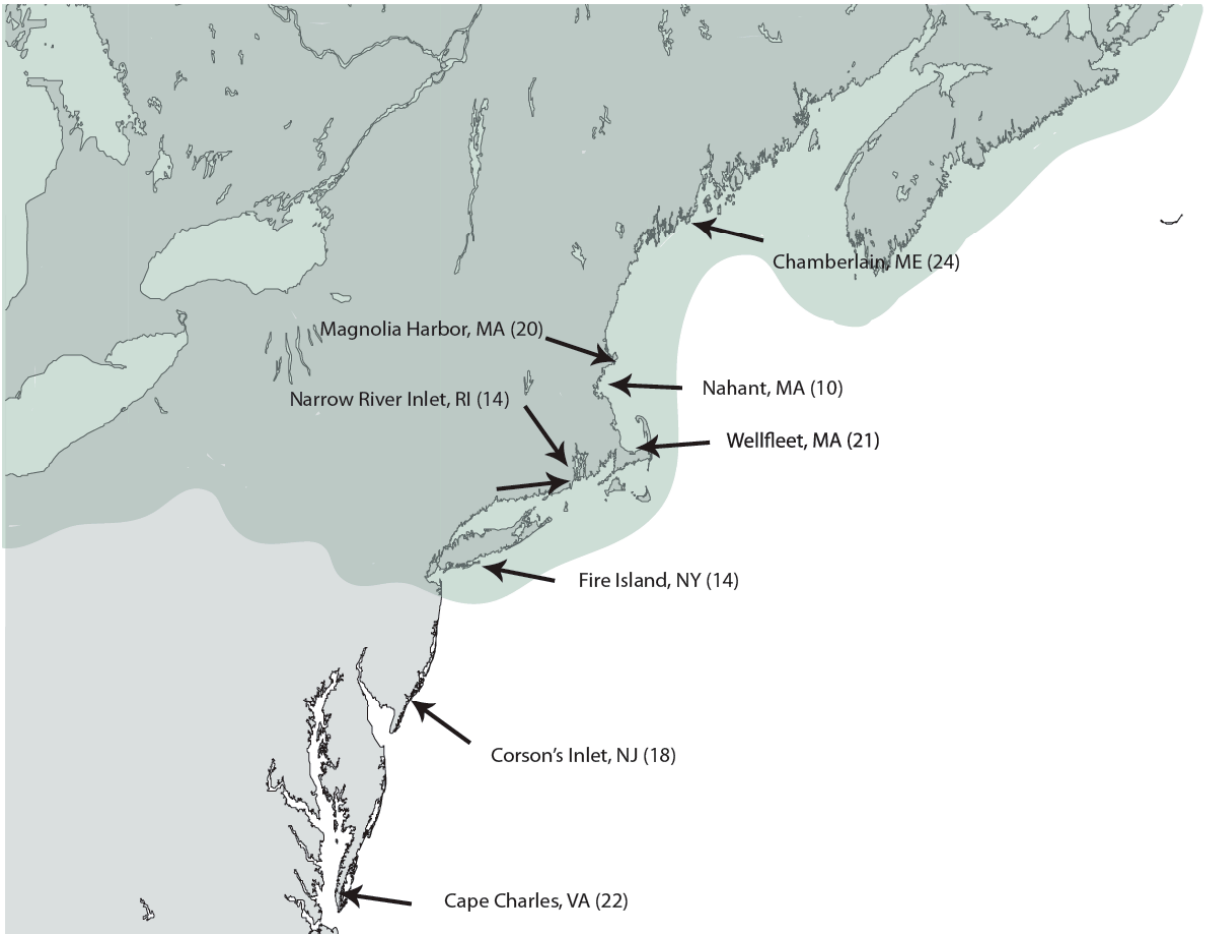

Chamberlain, ME (24)
Magnolia Harbor, MA (20)
Nahant, MA (10)
Narrow River Inlet, RI (14)
Wellfleet, MA (21)
Fire Island, NY (14)
Corson's Inlet, NJ (18)
Cape Charles, VA (22)

Figure 4.2

Median joining haplotype network for 16S.

Red indicates populations from Long Island Sound southward while blue indicates populations from Long Island Sound northward. Northern haplogroup is the northern/European clade from Wares (2001) and Chapter 2. 'Southern' haplogroup is the 'Virginia' clade from Wares (2001) or 'southern' haplogroup from Chapter 2. Blue individuals in the 'southern' group are from Cape Cod, MA and Rhode Island.

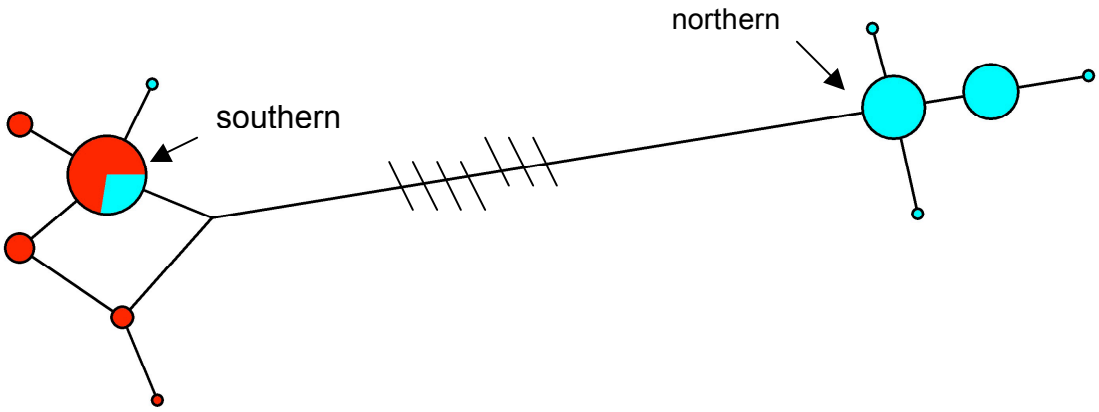

northern
southern

Figure 4.3.

Differences in fertilization success across mating category.

This figure shows the variation in fertilization success in inter- and intra-population crosses. Unsuccessful fertilization is then divided into three categories: cannibalism, brood abortion, and no fertilization. The 'mixed' category refers to interpopulation crosses. Single asterisks indicate significant differences where $P<0.05$ while double asterisks represent $P<0.001$.

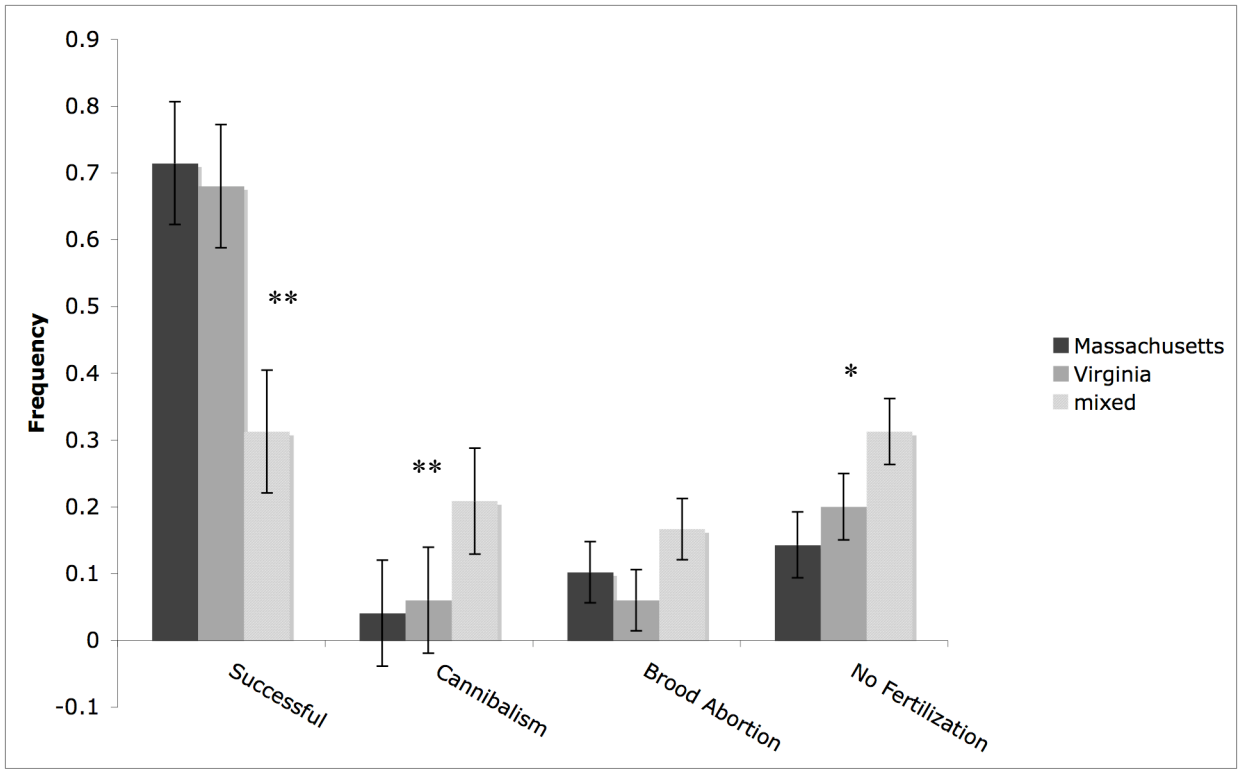

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Frequency
**
**
*
Massachusetts
Virginia
mixed
Successful
Cannibalism
Brood Abortion
No Fertilization

Figure 4.4

Mean fecundity of females from each cross category.

Mixed refers to inter-population crosses while Virginia and Massachusetts refer to intra-population crosses. Asterisks indicate $P<0.05$.

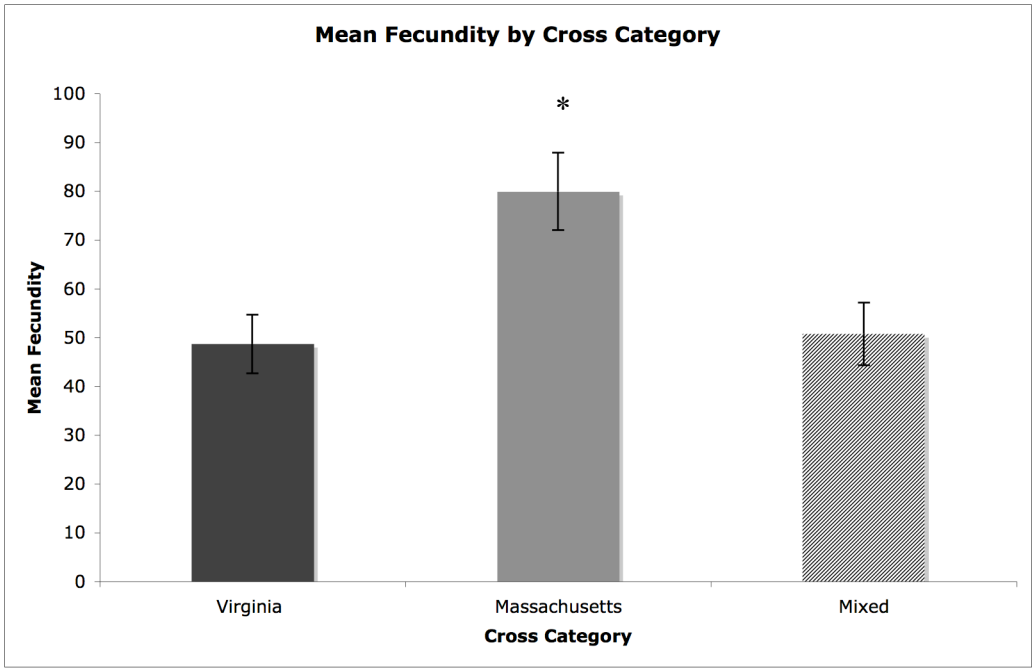
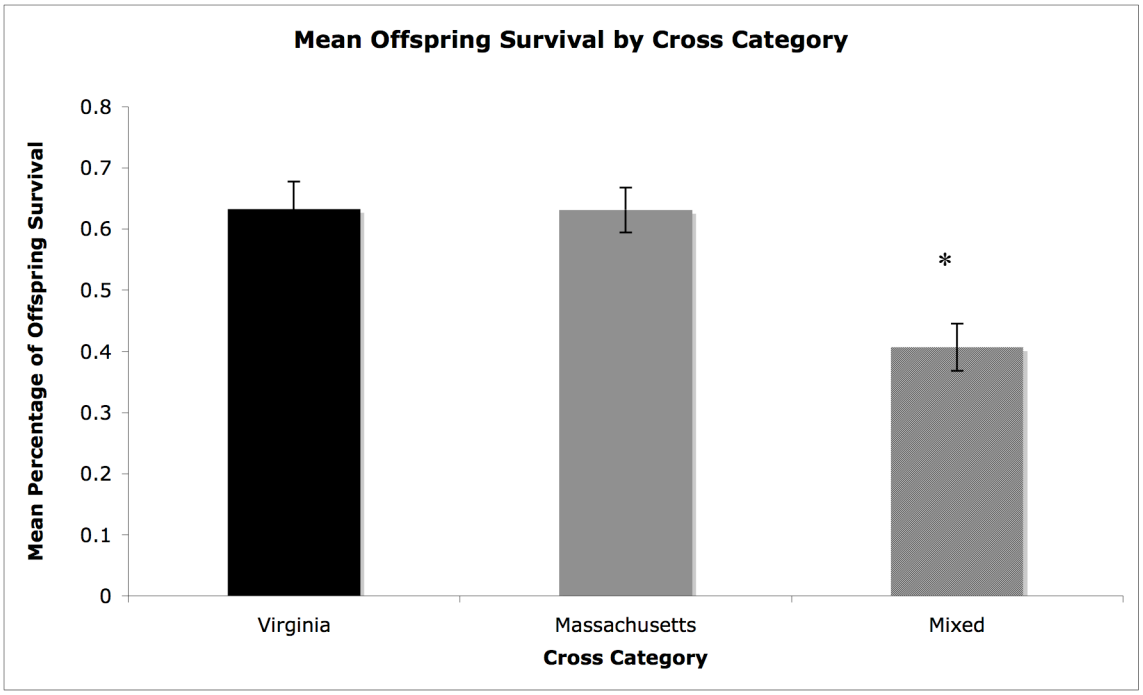

Mean Fecundity by Cross Category
100
90
80
70
60
50
40
30
20
10
0
Mean Fecundity
*
Virginia
Massachusetts
Mixed
Cross Category

Figure 4.5

Mean percent offspring survival for each cross category.

'Mixed' refers to inter-population crosses while Virginia and Massachusetts refer to intra-population crosses


Mean Offspring Survival by Cross Category
Mean Percentage of Offspring Survival
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Virginia
Massachusetts
Mixed
Cross Category

Tables

Table 4.1:

Molecular diversity indices for 16S at populations along the North American Coast.

Tajima's D values were not significant for any population. The abbreviation SD is the standard deviation for each index.

Population	# haplotypes	θ (SD)	π (SD)	Tajima's D
Cape Charles, VA	3	0.662(0.501)	0.788(0.613)	0.554
Corson's Inlet, NJ	4	1.157(0.781)	1.214(1.545)	0.204
Fire Island, NY	4	1.752(1.128)	2.067(1.338)	**0.824**
Narrow River Inlet, RI	2	4.8(2.725)	6.00(3.443)	**1.789**
Wellfleet, MA	4	2.53(0.964)	5.422(2.646)	**3.238**
Nahant, MA	2	3.057(1.335)	1.448(1.051)	-1.844*
Magnolia, MA	2	0.408(0.408)	0.571(3.992)	1.341
Chamberlain, ME	3	4.231(1.224)	3.22(1.251)	1.011

Table 4.2

Diversity indices for six allozyme loci.

The two populations are Massachusetts (MA) and Virginia (VA). The abbreviation SE is the standard error for each index. F_{ST}: P=0.010

Population	N	Loci	P	A_e	H_o (SE)	H_E (SE)	F_{IS} (SE)	Overall F_{ST}
MA	24	6	100	0.071	0.444 (0.035)	0.449 (0.021)	0.0111	0.038
VA	24	6	100	0.153	0.424 (0.063)	0.408 (0.055)	-0.0392	

Table 4.4

Results of ANOVA evaluating the effects of cross category and origin of individual on female fecundity.

Cross category includes: intra- vs. inter-population crosses. Origin includes either Massachusetts or Virginia populations. The abbreviation SS is the sums of squares for each effect. The abbreviation DF is the degrees of freedom. Asterisks indicate significant effects.

Source	SS	DF	F Ratio	Prob> F
Cross Category	7492.91	1	7.2433	0.0081*
Origin	5170.34	1	4.9981	0.0272*
Cross Category X Origin	5170.34	1	4.9981	0.0272*

Table 4.5

Results of ANOVA for the effects of cross category and population of origin on offspring survival.

Cross category includes: intra- vs. inter-population crosses. Origin includes either Massachusetts or Virginia populations. The abbreviation SS is the sums of squares for each effect. The abbreviation DF is the degrees of freedom. Asterisks indicate significant effects.

Source	SS	DF	F Ratio	Prob>F
Cross Category	1.1667	1	33.9436	<0.0001*
Origin	0.00022	1	0.0006	0.9799
Cross Category X Origin	0.00022	1	0.0006	0.9799

CHAPTER 5

SUMMARY AND CONCLUSIONS

The major goal of the presented research was to gain a better understanding of the complex interactions of gene flow, dispersal potential, and natural selection in shaping phylogeographic patterns in marine coastal species. The effects of glaciation on the present-day distributional patterns of marine species as well as the shared pattern of intra-specific population genetic differentiation has made the North Atlantic an ideal location to address these topics. The North Atlantic marine isopod, *Idotea balthica* was chosen as the focal point of this study because of its widespread distribution, its rafting dispersal habits, and its preference for chemically defended algal food resources.

Glaciation led to the extirpation of many temperate intertidal marine species due to loss of habitat and temperature change (Ingólfsson 1992; Wares and Cunningham 2001; Vermeij 2005). The source populations for species that recolonized these regions as well as their colonization route have been addressed in many studies (Ingólfsson 1992; Dahlgren et al. 2000; Wares and Cunningham 2001; Wares 2002; Coyer et al. 2003; Roman and Palumbi 2004; Vermeij 2005; Colson and Hughes 2007; Maggs et al. 2008; Riginos and Henzler 2008). *I. balthica,* along with many other species along the North American coast, have high genetic similarity with populations in southern Europe suggesting that individuals from this area were the source population for the current populations along this coast (Wares and Cunningham 2001; Wares 2002). Using the mitochondrial cytochome oxidase I gene (mtCOI) and increased sampling along the North American and European coast, potential source populations and the historical distribution patterns have been clarified (Chapter 2). This study has reaffirmed the assertion of Wares and Cunningham (2001) of a European source population for present day

populations of *I. balthica* in North America. The transatlantic dispersal of *I. balthica* could have occurred through two possible routes. One is a stepping stone progression from Europe through the Mid-Atlantic Islands into maritime Canada, while the other is a trans-Atlantic rafting event from Europe to the northeastern U.S. The exact route of dispersal for this species still remains undetermined, however, a trans-Atlantic dispersal route is most likely due to the high genetic similarity between the Mid-Atlantic islands and northern Europe (Finland) and the lack of detection of Mid-Atlantic haplotypes in North America (Chapter 2). The hypothesis that Iceland is a potential glacial refuge for this species first proposed by Wares and Cunningham (2001) has been rejected based on the genetic similarity between Icelandic and Finnish populations. Thus, it is likely that instead of Iceland serving as a glacial refuge following glaciation it was recolonized by individuals from northern Europe (Chapter 2).

In North America, a latitudinal cline in genetic diversity was discovered wherein the European haplogroup exists at high frequencies in the Northeast and Canada and an endemic southern haplogroup exists at high frequencies in the southeast. (Chapter 2) These two haplogroups are reciprocally monophyletic at mtCOI with a divergence between the two groups estimated to have occurred in the Pleistocene or late Pliocene (Wares 2001). The region between Long Island Sound and Cape Cod shows approximately equal frequencies of both types (Chapter 2). It is unclear what exactly maintains this clinal pattern, but from this study, natural selection seems to be a plausible explanation. Restricted dispersal because of patterns in ocean circulation is also plausible. However, it seems unlikely due to observations of this species rafting across large ocean expanses and population genetic data suggesting rafting events between Virginia, Maine, and Nova Scotia (Tully and O' Ceidigh 1986, 1987; Locke and Corey 1989; Thiel and Gutow 2005).

The feeding behavior of North American populations of *I. balthica* has not been previously described. Populations of this species in the northeastern U.S. are exposed to a high

diversity of large macroalgal species and inhabit either seagrass beds or floating mats of drift algae while those in the southern U.S. are generally exposed to a different suite of algal species and inhabit seagrass beds almost exclusively (Bertness 2007; Jenkins et al. 2008). Overall food preferences for each of the two populations were determined by no-choice and multi-choice feeding assays (Chapter 3). The results of these assays indicate a preference for *Fucus vesiculosus, Ulva linza*, and *Polysiphonia harveyi* across both southern and northern populations of this species (Chapter 3). European populations also have a strong preference for *F. vesiculosus* and *U. linza*. *F. vesiculosus* occurs at low frequencies in southern habitats, therefore, it is an interesting finding that southern populations prefer this algal species when they are seldom exposed to it. Individuals that belong to the southern haplogroup consume significantly more *Zostera marina* than those that belong to the northern haplogroup (Chapter 3). In general, it was found that *I. balthica* is a generalist herbivore with specific preferences. Significant differences in feeding preferences were found between regions that could affect gene flow (Chapter 3). Geographic differences in algal community assemblage were found to affect feeding behavior in *I. balthica*, where individuals from high diversity locations were more selective in their feeding preferences than those from low diversity locations (Chapter 3).

Given the divergence time between the reciprocally monophyletic haplogroups, the possibility of both haplogroups being 'cryptic species' was also explored (Chapter 4). Reproductive isolation was addressed between the different groups using no-choice mating experiments (Chapter 4). Reproductive barriers exist between these populations in the form of reduced fertilization success and offspring survivorship (Chapter 4). As reproductive isolation is not complete between the two groups (i.e. some offspring do survive from intra-population, inter-haplotype crosses), it is likely that these groups actually represent incipient species (Chapter 4).

Overall, it was found that *I. balthica* has four major haplogroups at mtCOI distributed across three geographic regions: 1) northeastern North America and Canada, 2) the Mid-

Atlantic Islands, and 3) southeastern North America. Despite *I. balthica*'s lack of planktonic dispersing larvae, its dispersal potential does not appear to be dramatically reduced. At this time, it seems plausible that a combination of natural selection and reproductive barriers may be driving the divergence pattern between these two putative species. It is not known if the results of this study can be cast as a broad generalization for the twenty other species that are phylogeographically concordant with *I. balthica* at mtCOI (Wares 2002; Sotka et al. 2003b; Kelly et al. 2006; Jennings et al. 2009) but these findings at least suggest that natural selection may play a major role in shaping observed patterns of population genetic differentiation among these species as well.

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